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RCS MEDDH - 288 ( RI )

# RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

Including

BIOCHEMISTRY, COMMUNICABLE DISEASES AND IMMUNOLOGY,  
INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY,  
PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE.

AD 863624

ANNUAL PROGRESS REPORT

1 July 1968 - 30 June 1969

## VOLUME II

WALTER REED ARMY INSTITUTE OF RESEARCH  
WALTER REED ARMY MEDICAL CENTER  
WASHINGTON, D.C. 20012

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BIOCHEMISTRY, COMMUNICABLE DISEASES AND IMMUNOLOGY,  
INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY,  
PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE

(Projects, tasks, and work units  
are listed in Table of Contents)

Annual Progress Report  
1 July 1968 - 30 June 1969

Volume II

Walter Reed Army Institute of Research  
Walter Reed Army Medical Center  
Washington, D. C. 20012

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## SUMMARY

The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigations are included on the DD Form 1498-1 introducing each work unit report, and names of investigators are given at the beginning of each report.

## FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences-National Research Council.

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**PROJECT 3A061102B71R**  
**RESEARCH IN BIOMEDICAL SCIENCES**

**Task 01**  
**Surgery**

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23. (U) 1. Factors responsible for morbidity and mortality associated with bacterial, hemorrhagic, and endotoxin shock. 2. Metabolic alterations and tissue damage produced by experimental trauma and shock, which include biochemical and histological studies. 3. Research on physiologic and biochemical problems associated with trauma. 4. Evaluation of therapeutic agents in the treatment of shock. 5. Investigate metabolic problems associated with pulmonary lipid embolism.							
24. (U) 1. Models established for production of endotoxin, hemorrhagic and bacterial shock in the dog and rat, and pulmonary embolism in the rabbit. 2. Biochemical, histological, and physiological procedures used to evaluate metabolic changes and tissue damage in shock. 3. Enzyme and isozyme patterns and substrate concentrations utilized to determine the effects of trauma on metabolic functions and tissue damage. 4. Metabolic and physiological parameter affected by intramuscular dexamethasone in the therapy of experimental peritonitis. 5. Lipase activity in pulmonary embolism.							
25. (U) 69 01 - 69 0 6. 1. Elevations of enzymatic activity and metabolite concentrations in hemorrhagic and endotoxin shock were correlatable with death or survival. 2. Enzyme and isozyme patterns indicated that tissue damage was qualitatively similar to endotoxin and bacterial shock; hemorrhagic shock produced some differences. 3. Dexamethasone produced several physiologic and metabolic changes which were correlated with its therapeutic value in experimental peritonitis. 4. Preliminary studies on the resolution of pulmonary fat embolism in the rabbit produced histological changes in the lung but no elevation of lipase activity.							
For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.							

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 091, Metabolic problems associated with injury

Investigators.

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Part I

Statement of Problems

The Department of Surgical Metabolism (functions transferred to Division of Biochemistry, WRAIR GO No. 2, 4 April 1969) conducted research on metabolic problems associated with injury, and provided professional consultation and technical support to other departments of the Division of Surgery and WRAIR. The major areas of investigation included: 1. Effects of endotoxic, hemorrhagic, and bacterial shock on metabolic processes and tissue injury. 2. Evaluation of the role of histamine in endotoxemia. 3. Effects of the corticosteroid, dexamethasone on metabolic, hemodynamic, and physiologic factors in experimental peritonitis. 4. Studies of serum enzyme levels in combat casualties. 5. Participation in the research project of other departments on: a. Resolution of experimental fat embolism, b. Evaluation of the heart pump in surgery, and c. Correlation of serum enzyme levels with the successful wound closure and healing.

Background

1. Effects of Endotoxic, Hemorrhagic, and Bacterial Shock on Metabolic Processes and Tissue Damage. Studies were continued on the endotoxic shock model, and hemorrhagic and bacterial shock models were introduced. Most metabolic processes fail or are vastly altered in the late states of shock, therefore, experiments were designed to monitor metabolic changes over the entire period from the initial insult until death or recovery. This procedure permits the correlation of the initial changes with morbidity and mortality. The approach was the measurement of selected enzymes and substrate concentrations in serum. The serum enzyme patterns were related to specific tissue damage.

2. Evaluation of the Role of Histamine in Endotoxemia. The injection of endotoxin causes the release of biologically active substances including histamine. Since many similar physiologic and hemodynamic effects are produced by endotoxin and histamine, studies were initiated to determine if intravenous injection of the substances produce the same effects on metabolic processes and tissue damage. Serum enzymes levels and isozyme patterns were used for comparison.

3. Effects of the Synthetic Corticosteroid, Dexamethasone, on Metabolic, Hemodynamic, and Physiologic Factors in Experimental Peritonitis. Dexamethasone, in pharmacological doses, provided protection to rats from bacterial shock (WRAIR Progress Report 1967 - 1968), however, the mechanism by which it effects this protection are obscure. Studies were initiated to evaluate the beneficial effects of dexamethasone in respect to selected metabolic, physiologic, and hemodynamic factors.

4. Studies on Serum Enzyme Levels in Combat Casualties. The intracellular enzymes, LDH, GOT, and GPT are found in normal serum in low concentrations. Serum levels are elevated in conditions in which cellular damage or changes in cellular permeability occur, and provide a sensitive indicator of tissue trauma. The levels and patterns of these enzymes in the serum yield information on the severity of the wound and the condition of the patient. Therefore, preliminary studies were undertaken to evaluate serum enzyme levels in combat casualties in respect to (a) wound type, (b) shock, and (c) transfusion therapy.

#### Approach to the Problem

1. Effects of Endotoxic, Hemorrhagic, and Bacterial Shock on Metabolic Processes and Tissue Damage. In all three experimental models beagle dogs were used. The dogs were anesthetized with pentobarbital sodium and placed in a supine position. Cannulae were inserted into appropriate vessels for injections, bleeding, and monitoring physiological changes. Blood samples were collected from the femoral artery prior to (control) and at selected times after the experimental procedure. The shock models were: a. Endotoxin - 1 dose, 2 mg/kg, of commercial endotoxin (Escherichia coli: 0111:B4, Boivin), which produced about 70 percent mortality within 24 hours. b. Hemorrhagic - the Wiggers technique was employed and the dogs were bled to an arterial pressure of 45 mm mercury, maintained for 2 hours, then reinfused. Preliminary studies also were performed using cardiac output instead of arterial pressure as the criteria for standardizing the hemorrhagic shock model. c. Bacterial - dogs were injected intraperitoneally with 5 ml/kg of a solution containing  $10^9$  E. coli 0111:B4 organisms and 4 g percent hemoglobin per ml. Enzyme analyses were performed using commercially available kits and isozymes were separated by gel electrophoresis.

2. Evaluation of the Role of Histamine in Endotoxemia. Twenty-four beagle dogs, 12 in each group, were randomly assigned to receive endotoxin or histamine. The dogs were anesthetized with pentobarbital sodium, and placed in dorsal recumbency. Polyethylene cannulae were inserted for injections, sampling, and monitoring arterial blood pressure (ABP). Commercial endotoxin (E. coli 0111:B4 - Boivin) in saline was injected intravenously in amounts calculated to produce 70 percent mortality. Histamine dihydrochloride, 1 mg/kg body weight, was

dissolved in 50 ml saline and given intravenously over a 15 minute interval. Baseline blood samples were collected following anesthesia and cannulation; repetitive samples were collected at selected intervals up to 72 hours after the injections. Quantitative serum enzyme analyses were performed by spectrophotometric procedures accompanying commercial kits, and isozymes were separated by gel electrophoresis and located by appropriate staining methods.

3. Effects of the Synthetic Corticosteroid, Dexamethasone, on Metabolic, Hemodynamic, and Physiologic Factors in Experimental Peritonitis. Bacterial shock was produced in 250 g male Sprague-Dawley rats by the intraperitoneal injection of a solution of *E. coli* ( $10^8$ /ml) and hemoglobin (4 g percent). Dexamethasone (8 mg/kg) or saline (control) was given intramuscularly at selected intervals prior to or following the bacterial injections. Some animals were observed up to 72 hours for lethality, others were sacrificed at selected intervals for blood and tissue analyses. Laboratory tests were serum glucose and corticosterone, blood hematocrit, adrenal corticosterone, protein, and ascorbic acid, and liver proteins.

4. Studies on Serum Enzyme Levels in Combat Casualties. All patients were American or South Vietnamese soldiers wounded less than 2 hours prior to admission to a front line hospital. Enzyme analyses were performed by colorimetric methods using commercial kits, and values were considered abnormal if the LDH exceeded 500 units, and GOT and GPT exceeded 50 units. Chi-square statistics were used, when appropriate, to compare groups.

### Results and Discussion

1. Effects of Endotoxic, Hemorrhagic, and Bacterial Shock on Metabolic Processes and Tissue Damage. Previous studies (WRAIR Progress Report 1967 - 1968) showed that the serum enzymes, lactic dehydrogenase (LDH), glutamate-oxalacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT), and isocitrate dehydrogenase (ICDH) were significantly elevated in non-surviving dogs by the third hour after receiving endotoxin. Only ICDH exceeded normal values in surviving dogs. A persistently high level of LDH, GOT, and GPT indicated a poor prognosis. The initial rise in total LDH was associated with an increase in LDH isozymes 2 and 3. (The LDH isozymes were numbered consecutively; the most anodic (heart) isozyme was designated as 1.) Persistently high levels of total LDH caused also an elevation in LDH isozymes 5 and 4. These results were correlated in the current studies. In addition, creatine phosphokinase (CPK) also was elevated in endotoxemia and usually by the third hour following the injection. The CPK isozyme associated with the increased serum level was the most anodic. This CPK isozyme occurs in tissues other than the heart or skeletal muscle. Blood lactate was usually elevated but in many instances did not correlate with morbidity and mortality. Serum glucose levels exhibited the usual biphasic pattern associated with shock.

Hemorrhagic shock also produced in non-surviving animals a dramatic increase in the activity of all the studied serum enzymes. The serum enzyme levels were increased significantly either just prior to or immediately following the reinfusion of blood. As with endotoxin the prognosis was poor when serum enzyme levels were progressively increased with time. Surviving animals showed only slight or transient elevations in serum enzyme activity. Hemorrhagic shock usually produced higher levels of serum enzyme activity than endotoxemia hence indicating greater tissue damage from induced hypovolemia. The striking increase of blood lactate levels in hemorrhagic shock also emphasizes the under perfusion and hypoxic state of the tissues. The serum glucose levels in hemorrhagic shock exhibit also a biphasic pattern.

The LDH and CPK isozyme patterns associated with hemorrhagic shock differ in some respects to those found in endotoxemia. The LDH serum isozymes corresponding to the initial increase total LDH reveal an increase in 2, 3, and 5 isozyme. In some animals LDH isozymes 2 and 3 were elevated first, then the isozyme 5; in other animals isozyme 5 was elevated first, followed by 2, and 3. This possibly indicated lung and liver damage, however, extensive intestinal damage may complicate the interpretation. LDH isozyme 1 (heart) also was elevated but usually later in the shock episode. The CPK isozymes associated with an elevation of the total CPK activity of the serum supported the finding of the LDH isozymes. The initial rise in CPK activity yielded the most anodic isozyme, this was followed by a progressive increase in the cathodic (heart or skeletal muscle) isozyme. The results would indicate tissue damage to other than the heart initially, followed by damage to the heart muscle later in hemorrhagic shock.

Difficulties were encountered in standardizing the bacterial shock model, and further studies will be required. Tissue damage, as reflected by serum enzyme levels, does not occur until near the terminal stages of shock. Preliminary studies showed that the increase in serum total LDH was associated with an increase of only LDH isozymes 2 and 3. This was similar to that found with endotoxin. GOT and CPK levels were elevated but not to the extent found in either the endotoxin or hemorrhagic models. The arterial blood pressure does not fall until the terminal stage of bacterial shock, and lactate levels are elevated only at this time.

2. Evaluation of the Role of Histamine in Endotoxemia. Eight of 12 dogs injected with endotoxin died within 48 hours. Only 2 of 12 dogs injected with histamine died, and these died within 20 and 35 minutes respectively probably because of a too rapid rate of injection. Both groups of dogs showed an immediate and similar drop in ABP. The ABP of the histamine treated animals returned to control values by 2 to 3 hours, while the endotoxin treated animals remained hypotensive (< 90 mm Hg) during the period of measurement.

The activity of serum enzymes in response to injections of endotoxin or histamine was quite different. Endotoxin causes a substantial elevation of all the studied enzymes by the third hour following injection. Results were similar to those described in Section 1, Results and Discussion, of this report. Histamine, despite the large dose administered, caused no elevation of serum LDH, ICDH, or AP. CPK was elevated over control values by the 7th and the transaminases by the 24th hour following the histamine injection. The transient elevation of transaminase activity found with histamine was similar to that noted in some animals surviving endotoxin. However, ICDH, which occurs in high concentrations in the liver, was never elevated in histamine treated animals, but was significantly elevated in all animals which survived endotoxin injections.

The serum LDH and CPK isozyme patterns indicated some heart and liver damage resulting from histamine injection. Serum LDH isozymes patterns showed transient elevations of isozyme 5 (liver), 5 to 7 hours, and frequently elevations of isozyme 1 (heart) at 24 hours following injection. In one experiment, when the dog died 35 minutes after the histamine injection, the elevation of serum total LDH was reflected by an increase in LDH isozyme 1 (heart) and liver (5). The liver damage was indicated also by a GOT to GPT ratio of less than 1. The CPK isozyme, related to the elevation of serum total CPK was the cathodic one, which is associated with heart or skeletal muscle.

The marked difference in serum enzyme levels and isozyme pattern indicate differences in tissue damage following endotoxin and histamine injections. The possibility of a synergistic effect between endotoxin and endogenous histamine has not been eliminated by this study. It is also possible that histamine injections causes the release of other biologically active substances which in turn may be responsible for the observed effects.

3. Effects of the Synthetic Corticosteroid, Dexamethasone, on Metabolic, Hemodynamic, and Physiologic Factors in Experimental Peritonitis. In those experiments, the E. coli-hemoglobin mixture produced 75 percent mortality within 24 hours. The intramuscular injection of dexamethasone reduced mortality significantly when given up to 16 hours after the inoculation of E. coli-hemoglobin.

The plasma and adrenal corticosterone, and adrenal ascorbic acid levels were determined to establish the effect of the infection on steroid release in the presence and absence of dexamethasone. Prompt increases in plasma corticosterone were found in both groups of animals. These elevations were sustained until the late stages of bacterial shock in control animals, while a more rapid return to normal was observed in dexamethasone treated animals. Similar but less dramatic changes were observed in the adrenal corticosterone levels. Definitive adrenal hypertrophy occurred only in the control animals. The functional capabilities of the adrenal were not impaired by the infection, as the injection

of exogenous ACTH late in shock produced a serum elevation of corticosteroid similar to that found early in shock. In both groups, the adrenal ascorbic acid levels were depleted initially following the infection and returned slowly to near normal values. The use of dexamethasone decreased the time for ascorbic acid recovery.

The possibility that dexamethasone exerted its beneficial effect by stabilizing glucose metabolism, was investigated by determining plasma glucose levels. The control animals exhibited a biphasic pattern in plasma glucose, that is an initial hyperglycemia followed by hypoglycemia near the terminal stages of shock. Dexamethasone did not prevent the initial hyperglycemia but did prevent the hypoglycemia late in shock. The prevention of hypoglycemia by dexamethasone, possibly through the mechanism of gluconeogenesis, could contribute to the beneficial effects of this synthetic corticosteroid. The corticosteroids as a group are reported to increase protein catabolism in most tissue and increase protein anabolism in the liver. In these studies no significant changes in the protein content of the liver or adrenals were noted.

Previously, dexamethasone was shown to increase the absorption of dye tagged protein and hence also bacteria from the peritoneal cavity. The effect of dexamethasone on fluid transport or cellular integrity was shown also in the current studies. The hemoconcentration, as revealed by hematocrit determinations, was less in the dexamethasone treated animals than in the controls. These results may then reflect improved circulation to the tissue or a preservation of the cellular and vascular integrity which resulted from dexamethasone administration.

4. Studies on Serum Enzyme Levels in Combat Casualties. Enzyme analyses were performed on the sera from 81 untreated patients upon arrival at the hospital and from 48 patients of the above group following surgical procedures. Upon admission, the percentage of patients with serum enzyme levels already elevated were: LDH 58 percent, GOT 47 percent, and GPT 19 percent. Following surgery, the percent of patients with elevated serum enzymes were: LDH 75 percent, GOT 73 percent, and GPT 31 percent. This reflected both surgical trauma and the continued release of these enzymes from damaged tissue. Only 19 of these patients were followed for up to 7 days, and in all instances the serum enzyme levels remained elevated.

The wound location had a pronounced influence on the levels of the serum enzymes. Abdominal wounds, with or without liver damage produced serum enzyme elevations in the greatest number of patients. Only in this group, and irrespective of direct liver involvement, was the GPT elevated to an appreciable extent. Blast foot injuries produced an elevation of LDH and GOT, but not GPT. The enzyme pattern was that associated with skeletal muscle. Penetrating chest wounds with lung contusion were associated with elevations of LDH and GOT, while without

lung contusion, GOT was not consistently elevated. Limb wounds without fractures also produced consistent elevations of GOT.

Shock was most prevalent in the group of patients (70 percent) with abdominal wounds. These patients also had the highest incidence of serum enzyme elevations. LDH and GOT were elevated in 83 percent of these patients and GPT in 44 percent. There was a significant increase in serum enzyme levels in patients in shock when compared to those not in shock. The elevation of the three serum enzymes studied were associated with a poor prognosis.

The effect of the volume of blood transfused on serum enzyme levels one day following injury was determined in 18 patients who had normal serum enzymes on admission. When the serum enzyme levels of these patients were compared with a similar group receiving no transfusions, only GOT was significantly elevated. There was no significant difference in enzyme levels of those patients receiving large quantities of blood (> 4000 ml) and those patients receiving lesser quantities. The results suggested that the general condition of the patient was more responsible for elevated levels of serum enzyme than the transfusion of blood.

#### Conclusions

1. Effects of Endotoxic, Hemorrhagic, and Bacterial Shock on Metabolic Processes and Tissue Damage. The study of tissue enzymes released into the serum reveal the general condition of the patient, the extent of tissue damage, and indicates the organ(s) that are affected by endotoxin, hemorrhagic, and septic shock. Enzyme analyses show that the sequence of organ damage may differ in hemorrhagic and endotoxin shock, and could provide a clue as to the appropriate therapy at different stages in the shock syndrome. This information and that gained from physiologic and hemodynamic measures provides a more precise view of patients conditions while in shock.

2. Evaluation of the Role of Histamine in Endotoxemia. The present experiment does not support a common mechanism of activity for endotoxin and histamine. The catecholamines, epinephrine and norepinephrine, are released by histamine and could contribute to the observed heart damage.

3. Effects of the Synthetic Corticosteroid, Dexamethasone, on Metabolic, Hemodynamic, and Physiologic Factors in Experimental Peritonitis. The administration of pharmacological doses of dexamethasone provided protection to rats from the high mortality of bacterial shock. Investigations into the metabolic, physiologic, and hemodynamic processes affected by dexamethasone indicated several beneficial effects of this drug: (1) it prevented the late hypoglycemia associated with bacterial shock, (2) it enhanced peritoneal absorption of bacteria, hence permitted the natural defense mechanisms of the host to be utilized more effectively and (3) it preserved the cellular integrity of the

adrenals, liver and possibly other organs, possibly by decreasing vascular resistance and increasing fluid movement. The ability of the adrenals to produce steroids were not affected by bacterial shock with or without dexamethasone, however, homeostasis of the animal was better maintained in the presence of the drug.

4. Studies on Serum Enzyme Levels in Combat Casualties. The elevation of these enzymes in the serum reflect the general condition of the patient and the severity of the wound. Wound location effects the serum enzyme patterns, and abdominal wound present the greatest problem in respect to shock. GOT elevations was the most sensitive indicator of soft tissue trauma.

#### Recommendations

1. Effects of Endotoxic, Hemorrhagic, and Bacterial Shock on Metabolic Processes and Tissue Damage. The study of endotoxin, hemorrhagic and bacterial shock have revealed differences in metabolic processes and the pattern of tissue injury related to these shock models. Future investigations will entail the modification of these basic models by drugs or surgical procedure in an attempt to isolate the metabolic process or tissue injury primarily responsible for the morbidity and mortality associated with shock. This information then will be utilized to evaluate the use of therapeutic agent in the treatment of shock.

2. Evaluation of the Role of Histamine in Endotoxemia. The use of alpha adrenergic blocking agents should assist in the clarification of an interrelationship between histamine and the catecholamines.

3. Effects of the Synthetic Corticosteroid, Dexamethasone, on Metabolic, Hemodynamic, and Physiologic Factors in Experimental Peritonitis. The physiologic, hemodynamic, and metabolic effects which were produced by pharmacological doses of dexamethasone, require further study. Each possible beneficial effect of dexamethasone needs investigation so that this drug may be used judiciously in the treatment of shock.

4. Studies on Serum Enzyme Levels in Combat Casualties. The determination of LDH, GOT and GPT and the inclusion of serum creatine phosphokinase (CPK) and LDH and CPK isozyme analyses would assist in: (a) evaluation of the severity of the wound, (b) location of tissue damage not readily apparent, (c) determining the rate of wound healing and (d) determining the best time for wound debridement and closure.

Participation in Research Project with Other Departments. The department of Surgical Metabolism provided professional consultation and technical assistance relevant to the research activities of other departments. The project in which this department participated were: a. The resolution

of experimental fat embolism with the Department of Surgical Pathology, b. Evaluation of the heart pump in surgery with the Department of Anesthesiology and Resuscitation and c. Correlation of serum enzyme levels with successful wound closure and healing with the Department of Experimental Surgery. Progress on these research activities will be reported by the designated departments.

## Part II

### Description

Studies of bacterial infections associated with traumatic wounds and other surgical problems have been concerned with identification of organisms from wounded patients, development of animal models for studying therapy and investigation of diagnostic methods.

### Progress

1. Bacteriological assay of wounded patients at Walter Reed General Hospital. Investigations were initiated to determine the relationship of normal throat and skin flora to bacterial complications of wounds suffered in Vietnam. The protocol is as follows: each patient assigned to Ward 32 is cultured (throat, skin, wound and drainage) three times at approximately weekly intervals - a total of 300 cases is planned. Results of bacteriologic studies are to be compared with medical history, treatment, antibiotic therapy and location and nature of wounds.

To date 130 patients have entered the study but only 17 percent have had the series of three cultures.

Table 1 summarizes the frequently isolated microorganisms from wound and suture sites, normal skin, throat, burns and drainage. S. epidermidis and S. aureus were quite common in wound and suture sites on normal skin and, to a certain degree, in the throat. Neisseria was predominantly isolated from the throat where streptococci were also frequently isolated. Pseudomonas was found more often at wound and suture sites and throat than on the normal skin.

The organisms recovered from the various sites were those to be expected. Pseudomonas strains and other gram negative organisms are the invaders which, because of antibiotic resistance, are the most difficult treatment problems.

Table 1. Microorganisms isolated from various anatomical loci in wounded patients.

Culture source	Number of cultures	Frequency of isolation											
		<i>S. epidermidis</i>	<i>S. aureus</i>	<i>Pseudomonas</i>	<i>Neisseria</i>	<i>S. coli</i>	Strep	Proteus	<i>Klebsiella</i>	<i>Micrococcus</i>	<i>Clostridia</i>	Others	
Wound and suture site	78	31	37	12		7	4	5	4			1	7
Normal skin	97	78	25	2		1	2		2			7	5
Throat	81	15	12	7	24	9	15	1	6	5			9
Burns	7	4	4	1	1								
Drainage	4	1						2					1

In addition to the above study, microbiologic support has been provided for the Recovery Room Ward 15, WRGH under the direction of the Department of Human Studies, Division of Surgery, WRAIR. Results of cultures of 33 patients are presented in Table 2. Again, gram negative organisms were the most common flora. The absence of positive blood cultures in this group may reflect the intensive antibiotic therapy administered to these critically ill patients.

## 2. Studies of animal models for intraperitoneal infection.

Intraperitoneal infection following abdominal wounds incurred during combat has been a significant cause of mortality and morbidity. Of 33,000 combat injuries in Vietnam from October 1965 to August 1967, 5.2 percent involved the abdomen and 18 percent of the deaths occurred in this group. Intraabdominal sepsis was a major factor in these cases. In several series of abdominal injuries, infection was a significant complication in 23 to 35 percent of the patients (LTC G. V. Aaby, personal communication).

A standard subhepatic abscess model has been established in rabbits by injecting clotted rabbit blood containing a large inoculum of Staphylococcus aureus and Escherichia coli.

A description of the technique follows: S. aureus, isolated from a surgical wound infection, was resistant to penicillin (MBC =  $10^6$  units/ml) and chloramphenicol (MBC 128 Mg/ml). E. coli, also a clinical isolate, had similar resistance to both antibiotics.

0.5 cc of a 24 hr trypticase soy broth (TSB) culture of S. aureus, approximately doubled by centrifugation and resuspension in half the volume of broth ( $10^9$ - $10^{10}$  viable bacteria/ml) is added with 0.5 cc of a 24 hr TSB culture of E. coli ( $10^9$ /ml) similarly treated, to each 9 ml aliquot of freshly collected rabbit blood. Control animals received blood containing 1.0 cc of sterile TSB instead of bacteria. After incubation for one hr at 37 C to allow clotting, the blood is then forced via syringe into the right subhepatic space of the laparotomized rabbit. Rabbits in treatment groups then receive antibiotics by various routes of inoculation or by infusion. Mortality rate is determined for the post operative period and all dead rabbits are autopsied. All rabbits surviving 10 to 14 days are sacrificed and examined for abscesses which are then excised and the exudate cultured qualitatively and quantitatively (Lindsey technique). The peritoneal cavity is cultured by sterile swab in all survivors. Unusual abscesses and associated infected tissues are preserved for pathological examination.

Table 3 shows that 95 percent of uninfected controls survived the operative procedure and had negative peritoneal cultures at 14 days.

Table 2. Frequency of isolation of microorganisms from various clinical specimens.

Source of specimen	Total no. of cultures	<i>Pseudomonas</i>	<i>Proteus</i>	<i>Klebsiella</i>	<i>Aerobacter</i>	<i>St. coli</i>	<i>St. aureus</i>	<i>St. epidermidis</i>	<i>a strep</i>	<i>B strep</i>	<i>Citrobacter</i>	Yeast	No growth
Wound	60	33	5	2	3	3	2	1	2				15
Drainage	25	10	2	1	1	4	2		2		1		2
Trachea	53	12	4	15	3	2	9	3	1			2	2
Throat	46	9	4	5	2	2	6		14	2		1	6
Sputum	20	8	2		1	1	1		1	1	1	4	
Urine	116	16	4	5	5	10		2				7	67
Stool	3	No enteric pathogens found											
Blood	70												

Table 3. Rabbit peritoneal abscess model.

	Total number	Deaths			Survivors 10-14 days	Culture results	
		During surgery	18-24 hours	2-10 days		+	-
Uninfected controls	37	1	1	0	35 95%	0	35 100%
Infected	67	2	31 46%	6	28 42%	27 97%	1
Infected-treated	55	0	24 44%	9	22 42%	20 91%	2

Infected rabbits showed a 46 percent mortality in the first day after surgery and 42 percent survived to be sacrificed at 14 days. Twenty-six of the 28 rabbits had abscesses which yielded S. aureus on culture; one other rabbit had S. aureus and E. coli isolated at 14 days. Treatment of infected animals (1 million units penicillin, 200 mg chloramphenicol in one dose immediately after closure of abdominal incision - 37 given I.P., 18 given I.M.) had no effect on the mortality or abscess development.

The early deaths are probably the result of E. coli endotoxin and E. coli did not appear to produce a chronic intraperitoneal infection. Abscesses developed on or within the omentum and occasionally were subhepatic in location. Smaller intrahepatic abscesses were noted in the latter animals. Detailed anatomic observations were not made. S. aureus was isolated from over 90 percent of these infections.

Thus, it appears that this technique has potential as a model for peritoneal abscess. However, certain problems need further study; for example, are two bacterial species necessary or will S. aureus alone cause the infection? Will E. coli alone cause the early deaths? Can abscesses be produced by gram negative organisms since these are the bacteria responsible for the human illnesses? If not, perhaps some animal other than the rabbit should be investigated. Attempts to study treatment must await more precise information on the model.

### 3. Miscellaneous diagnostic bacteriologic studies.

Bacteriologic support has been provided for a variety of projects within the Division of Surgery. For example, several experiments were performed in an attempt to quantitate bacteria in peritoneal washings by use of a Millipore filter technique. These were not successful and were discontinued. Attempts to quantitate magnitude of bacteria in experimentally infected rabbit soft tissue wounds using microscopic counting of stained tissue homogenates as well as plate counts are in progress.

### Summary and conclusions.

Organisms isolated from combat wounds reflect those present on the skin and throat of the same individuals. In the military patients gram negative organisms are frequently isolated, just as they are in civilian populations, and no unusual group of bacteria has been found. Rabbits injected with bacterial contaminated blood clots may provide a useful animal model for intraperitoneal infection since 40 percent have staphylococcal abscesses after two weeks. Further anatomic studies are necessary as are experiments with gram negative bacteria to more closely approximate the human problem.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 091, Metabolic problems associated with injury

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5. Sleeman, H. K., Simmons, R. L., and Heisterkamp, C. A.: Serum enzymes in combat casualties. *Arch. Surg.*, 98: 272, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				A. AGENCY ABBREVIATION DA OA6527	B. DATE OF REPORT 69 07 01	REPORT CONTROL CODES DD-Form (AR) 408	
1. DATE REPORTED 69 01 31	2. NAME OF CENTER D. Change	3. SECURITY CLASS. U	4. WORK STATUS U	5. RESEARCH NA	6. REPORT NUMBER RL	7. SPECIAL DATA - CONTRACTOR NUMBER <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	8. LEVEL OF EFFORT A. WORK UNIT
9. PROGRAM ELEMENT 61102A	10. PROJECT NUMBER 3A061102B71K	11. TASK AREA NUMBER 01	12. WORK UNIT NUMBER 092				
13. TITLE (Include the security classification code) (U) Intensive study and treatment of shock in man (09)							
14. SUBJECT AREA 003500 Clinical Medicine				15. FUNCTIONAL AREA 012900 Physiology			
16. ORIGIN DATE 07 65	17. SECURITY CLASSIFICATION DATE NA	18. FUNDING AGENCY DA		19. PERFORMANCE METHOD C. In-House			
20. OBJECTIVE Not applicable				21. RESEARCH ESTIMATE FISCAL YEAR 69	22. PROFESSIONAL MAN YRS 6	23. FUNDS (in thousands) 150	
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28. RESPONSIBLE ORGANIZATION Walter Reed Army Inst of Res Washington, D. C. 20012				29. PERFORMING ORGANIZATION Walter Reed Army Inst of Res Div of Surgery Washington, D. C. 20012			
30. RESPONSIBLE INDIVIDUAL NAME: Meroney, COL W. H. TELEPHONE: 202-576-3551				31. PRINCIPAL INVESTIGATOR (FUNDING AGENCY USE ONLY - include) NAME: Aaby, LTC Gene V. TELEPHONE: 202-576-3791 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
32. GENERAL USE Foreign intelligence considered				33. INTERMEDIATE INVESTIGATORS NAME: Gutierrez, MAJ V. S. NAME: McNamara, MAJ J. J. DA			
34. SUBJECT TERMS (Include the security classification code) (U) Responses to trauma; (U) Pulmonary insufficiency; (U) Coagulation Defects, (U) Serum Enzymes, (U) Screen Filtration; (U) Stress Ulcer							
35. SUMMARY STATEMENT (Include the security classification code) (U) To evaluate the physiologic responses to trauma in combat casualties.							
24. (U) All seriously wounded casualties admitted to the 24th Evacuation Hospital are studied by the surgical research team. Arterial blood gases, pulmonary function tests, and cardiac output tests evaluate the cardiopulmonary system. Serum enzymes, lactate, pyruvate, and glucose evaluate the response to shock. Coagulation studies investigate bleeding diathesis and disseminated intravascular coagulation. Gastric analysis and study of mucin Factors I, II, and III evaluate the biochemical alteration of mucin following severe shock and sepsis.							
25. (U) 69 01 - 69 06. Pulmonary insufficiency is caused by many factors: aspiration, contusion, fat embolization, intrapulmonary shunts from extremity wounds. Prolonged shock causes metabolic acidosis. However, the usual casualty develops alkalosis following resuscitation and surgery. Coagulation defects appear only after (1) multiple transfusions or (2) prolonged shock in which there is a consumptive coagulopathy. Elevations of isozymes are specific for the injured organ or tissue.							
For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.							

PII Redacted

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 092, Intensive study and treatment of shock in man

Investigators.

Principal: LTC Gene V. Aaby, MC

Associate: MAJ Donald B. Doty, MC; MAJ Roger V. Moseley, MC; MAJ Henry B. Soloway, MC; MAJ William H. Fleming, MC

1. Human Myocardial Zonal Lesions<sup>1</sup>

a. Statement of the Problem: The cause of death in hemorrhagic shock has been attributed to failure of several different organ systems. Guyton and others have documented myocardial failure as the final common pathway in death from hemorrhagic shock.

b. Background: Numerous models of hemorrhagic shock have been developed through the years and intensive investigations developed to determine the cause of death in hemorrhagic shock. Terminally, there is failure of the cardiovascular system. It has not been clearly defined whether the failure represents primary myocardial failure or failure of the peripheral vascular system or lungs and a secondary effect upon the myocardium. Myocardial zonal lesions are a consistent pathologic finding in experimental animals following hemorrhagic and endotoxic shock. Until recently this type of myocardial damage has not been demonstrated in man.

c. Approach to the Problem: A 20-year-old soldier sustained combat wounds and died of hemorrhagic shock prior to treatment. Detailed autopsy was performed five hours after death.

d. Results and Discussion: Histologic examination revealed zonal lesions located at the intercalated discs and other parts of the myocytes in the heart. These have been previously described following hemorrhagic shock in experimental animals, but lack of appropriate clinical material has failed to reveal them in man. This finding may further document the thesis that myocardial failure is the principal common pathway in demise due to hemorrhagic shock.

e. Conclusion: Myocardial zonal lesion following exsanguination in a combat casualty was demonstrated. The lesion is identical to that found in experimental models of hemorrhagic shock. Development of this lesion early in hemorrhagic shock may be the basis for subsequent myocardial failure.

f. Recommendation: Thorough autopsy examinations of casualties dying of hemorrhagic shock may unfold the confusing picture of final demise from hemorrhage.

## 2. Relationship of Crystalloid Administration during Resuscitation to Serum Solids and Development of Arterial Hypoxemia.

a. Statement of the Problem: Pulmonary insufficiency and generalized edema are commonly present after resuscitation in severely traumatized battle casualties.

b. Background: Large volumes of crystalloids are administered during resuscitation from combat wounds. Many of the casualties would have died in previous wars but have been salvaged by rapid helicopter evacuation from the battlefield to the hospital. Volume replacements using crystalloid solution have saved the patient but a few days' post-operative pulmonary insufficiency and overhydration are a common problem. The present study was designed to determine the association between total serum solids and arterial hypoxemia in a group of severely injured casualties.

c. Approach to the Problem: Sixty casualties were studied measuring blood gases, lactate, and total serum solids.

d. Results and Discussion: There was a correlation between the finding of low serum solids and arterial hypoxemia in patients that had been given large volumes of crystalloid solutions during resuscitation and surgery. Of hypoxemic patients with low serum solids 24 hours post-operatively 83% received more than 3,000 cc of Ringer's lactate. By contrast only 12.5% of the patients with normal serum solids and normal oxygen tension received this volume of fluid.

e. Conclusion: The data supports the concept that excessive hemodilution with crystalloids may result in reduction of plasma osmotic pressure and may be partially responsible for impairment of pulmonary function following severe shock and trauma.

f. Recommendation: The fluid regimens used for resuscitation of severely wounded need further study and investigation to determine the proper combination of blood and crystalloid solutions to be used during resuscitation of the severely wounded.

## 3. Obtaining a Reliable Specimen of Arterial Blood: The Problem of Venous Admixture.

a. Statement of the Problem: Investigators have documented arterial hypoxemia following severe trauma.

b. Background: Numerous investigators in the combat zone and in civilian trauma centers have documented the presence of arterial hypoxemia following nonpenetrating chest injuries, penetrating chest injuries, fractures, extremity wounds, blast injuries, fat embolization, and many other types of injuries.

c. Approach to the Problem: Four cases were documented in which arterial hypoxemia was present but repeated sampling revealed the initial low  $pO_2$  was an error and resulted from venous admixture due to aspiration of blood from a vein adjacent to the artery that was the sampling site. Because of the adjacent femoral vein and the numerous branches at the fossa ovalis the femoral artery may be a common site for venous admixture to occur during sampling. The use of long beveled needle may contribute to the error.

d. Conclusion: Errors may occur during sampling of arterial blood, permitting venous admixture and a fallacious diagnosis of arterial hypoxemia.

e. Recommendation: Adherence to careful techniques is necessary to produce valid results.

#### 4. Coagulation Disorders in Combat Casualties: Acute Changes After Wounding and Effects of Massive Transfusion<sup>2</sup>

a. Statement of the Problem: Following massive trauma requiring multiple blood transfusion, a hemorrhagic diathesis commonly occurs.

b. Background: In both military and civilian trauma large volumes of blood and other fluids are given rapidly and the patient is successfully resuscitated and undergoes surgical treatment of the wounds. During this period several thousand milliliters of blood may be given and after a prolonged period of shock, a bleeding tendency commonly occurs which has been thought to be related to a consumptive coagulopathy or dilution of the clotting factors which prevents good clot formation.

c. Approach to the Problem: The prothrombin time, partial thromboplastin time, recalcification time, platelet time, fibrinogen and presence of fibrinolysin were determined in large numbers of combat casualties admitted to surgical hospitals. Casualties were restudied after multiple blood transfusions. The clotting factors in 72 units of stored bank blood were analyzed.

d. Results and Discussion: The prothrombin time, partial thromboplastin times correlated statistically with the degree of hypotension, acidosis, and lactic acidemia. The findings were consistent with experimental observations that trauma and shock produce initial phase of hypercoagulability followed by a return to normal or hypocoagulability. The hypocoagulable state is best explained by disseminated intravascular coagulation precipitated by the presence of hemolysis, release of thromboplastin, acidosis, and hypoperfusion seen in patients with hypovolemia.

Transfusion is accompanied by dilutional coagulation defects. Platelet levels fell rapidly during massive transfusion.

e. Conclusions: Except for severe trauma resulting in disseminated intravascular coagulation or requiring large volumes of blood transfusion, stored bank blood does not result in a coagulopathy. The use of fresh whole blood will partially counteract the dilutional effect on coagulation parameters.

f. Recommendation: Additional studies to elucidate the pathogenesis of disseminated intravascular coagulation and the hypocoagulable state are needed.

### 5. Hypoxemia during the first 12 Hours after Battle Injuries.

a. Statement of the Problem: Hypoxemia and respiratory insufficiency commonly occur after severe combat injuries.

b. Background: Several investigators have demonstrated the occurrence of hypoxemia following severe injuries. The location of injuries is not necessarily related to hypoxemia. Wounds of the chest causing interference of ventilation and lung function would be expected to result in hypoxemia. However, blast injuries of the foot, extremity wounds, and severe fractures may cause hypoxemia from release of fat emboli, platelet aggregations, cellular debris, and other microemboli which interfere with pulmonary function.

c. Approach to the Problem: All casualties admitted to the hospitals having the surgical trauma team were studied and those demonstrating arterial hypoxemia with a  $pO_2$  below 80 were serially followed. Fifty-eight patients constituted this group.

d. Results and Discussion: In this group of patients the following causes of hypoxemia were identified: chest injury, 29; aspiration, 14; fat embolism, 6; overtransfusion, 4; brain injury, 5.

e. Conclusion: Hypoxemia has multiple etiologies and may be caused by any factor which influences ventilation, perfusion, and diffusion.

f. Recommendation: This multi-faceted problem needs careful investigation of the component parts.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 092, Intensive study and treatment of shock in man

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PROJECT 3A061102B71R  
RESEARCH IN BIOMEDICAL SCIENCES

Task 02  
Internal Medicine

1. DATE PROJECT STARTED 69 01 31				2. DATE PROJECT COMPLETED 69 07 01		3. PROJECT NUMBER ED 01 00 00 00 00	
4. D. Change		5. U		6. U		7. NA	
8. 61102A		9. 3A061102B71R		10. 02		11. 085	
12. 1412A(2)							
13. (U) Vascular components of cardiorespiratory disease (09)							
14. 012900 Physiology		15. 002400 Bioengineering					
16. 08 63		17. NA		18. DA		19. C. In-House	
20. NA		21. EXPIRATION:		22. 69		23. 11	
24. NA		25. & AMOUNT:		26. 70		27. 9	
28. NA		29. NA		30. 200		31. 240	
32. Walter Reed Army Institute of Research				33. Walter Reed Army Institute of Research			
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36. Meroney, COL W. H.				37. Gregg, Dr. D. E.			
38. 202-576-3551				39. 202-576-5121			
40. Foreign intelligence considered.				41. Olsson, LTC, R. A.			
				42. Elliot, Dr. E. C.			

PII Redacted

(U) Cardiovascular system; (U) circulation; (U) heart; (U) blood; (U) coronary vessels; (U) myocardium; (U) oxygen.

23. (U) Development of standardized biological preparations for long term hemodynamic and biochemical studies of the controls of the heart and circulation in the normal state and under the influence of abnormal and pathological stresses.

24. (U) Energy metabolism of the heart has been studied at sarcosome level. Regulation of regional blood flow has been studied in the unanesthetized dog with chronically implanted electromagnetic flowmeters and special tubes in the aorta, coronary sinus and coronary artery. Preparations and stresses used include the normal heart, the heart with hemorrhagic shock and with acute and chronic coronary insufficiency.

25. (U) 69 01 - 69 06. Chronically implanted intracoronary tubes for estimating coronary collateral indices have been improved. A new compound has been found which blocks ATP synthesis without affecting utilization; it should be a powerful tool in studying the mechanism of high energy transfer reactions in sarcosomes. Adenosine may be a regulator of coronary blood flow since its myocardial content rises during coronary occlusion and falls during coronary release. Intravenous calcium decreases left coronary vascular resistance. During irreversible hemorrhagic shock, systolic coronary vascular resistance is reduced during the hypotensive and postinfusion decay periods. In congenital subaortic stenosis, mean left coronary flow is reduced, flow during systole being almost entirely backflow. Experimental models have been developed to study the natural history of coronary collateral development during abrupt or gradual occlusion of a left coronary branch. Coronary collateral indices rise progressively suggesting preocclusion existence of significant dormant coronary collaterals. Intra-aortic ballooning improves these collaterals mildly. These collaterals rise more quickly following coronary reocclusion after subsequent coronary release. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 68 - 30 Jun 69.

Project 3A061102B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 085, Vascular components of cardiorespiratory disease

**Investigators.**

Principal: Donald E. Gregg, Ph.D., M.D.

Associates: LTC Ray A. Olsson, MC; Eric C. Elliot, M.D., Ph.D.; Colin M. Bloor, M.D.; Arthur S. Leon, M.D.; Lino Granata, M.D.; Andrew Huvos, M.D.; A. Pasque, M.D.; N. M. Papadopoulos, M.D.; Stanislaw Pasyk, M.D.; Darrell W. Haas, Ph.D.; Edward M. Khouri; Howard S. Lowensohn; Charles E. Cain; R. Richard Gray; William J. Mitchell; Bertram Pitt, M.D. (Johns Hopkins University School of Medicine); David E. Donald, M.D. (Surgical Research Section, Mayo Clinic); R. Lee Pyle, Ph.D. (School of Veterinary Medicine, Univ. of Pennsylvania); Frank Hastings, M.D. (Artificial Heart Program, National Heart Institute); Mary K. Gentry.

Description.

Development of standardized biological preparations for long term hemodynamic and biochemical studies of the controls of the circulation and of myocardial activity in the normal state and under the influence of abnormal and pathological stresses.

Progress and Results.

1. Development of Instruments and Methods for Cardiovascular Research.

Tubes for implantation in a coronary artery and in the aorta of the chronic dog have been improved by the substitution of silastic for the intravascular portion of the catheter.

2. Myocardial Metabolism.

Further studies on site-specific inhibitors of oxidative phosphorylation have been carried out. It has been found that it is possible to shift the site of action of some of these poisons within certain classes of compounds. The arylbiguanides, for example, show a shift from site 1 to site 2 inhibition depending on the chain length between the aromatic ring and the biguanide residue. Phenylbiguanide and its ring-substituted analogues are strong inhibitors of site 1 phosphorylations. If one or more methylene groups are introduced between the phenyl ring and the biguanide moiety, inhibition is shifted from site 1 to site 2. In the case of benzylbiguanide, substitution on the ring at the ortho position causes a shift back from site 2 to site 1. It is apparent that any group which sterically interferes with the first amine and/or the first imine of the biguanide, causes the resulting compound to react as a guanidine. This is an important observation which can lead to the

systematic separation of the phosphorylation sites for the study of the primary energy transfer mechanisms.

A further study of the carbodiimides has led to the conclusion that certain of these substances which have large aromatic substitutions on the two nitrogen residues can act competitively with ADP for high-energy phosphorylated intermediates. This should be a useful tool in the separation of the forward reaction (the oxidative synthesis of ATP) from the reverse reaction (the utilization of ATP and other phosphorylated intermediates).

### 3. Metabolic Control of Coronary Blood Flow.

The relationship between myocardial adenosine content and coronary blood flow rate was studied in anesthetized open chest dogs. The standard enzymatic spectrophotometric technique for adenine nucleoside assay was modified to give a 200-fold increase in sensitivity, thus permitting measurement of adenosine in heart muscle extracts. The myocardial content of adenosine nucleosides was found to increase during coronary artery occlusion and to fall exponentially during reactive hyperemia. Coronary blood flow rate was a function of the logarithm of myocardial adenosine content in the oxygenated heart and during myocardial reactive hyperemia. Studies of the influence of orthophosphate on the production of adenosine from AMP have been undertaken in order to explain variations in the rate of myocardial adenosine production during coronary occlusion.

### 4. Experimental Models for Study of the Coronary Circulation.

For study of the regulation of the coronary circulation under various stresses (noncoronary insufficiency) the experimental model was the trained, unanesthetized resting dog. Implantations included a tube in the ascending aorta for sampling blood pressure, a tube in the coronary sinus for estimating myocardial metabolic change, a flow transducer on the left circumflex coronary branch together with a pneumatic cuff for temporary flow zero. For study of coronary insufficiency and the collateral circulation, in addition to the preceding implantations, placement was made of a flowmeter and zeroing cuff on the left descending coronary branch, and, on the left circumflex branch, an externally adjustable hydraulic occluder for gradual (few days) or abrupt reduction of coronary flow to zero and an intracoronary tube to permit measurement of two collateral flow indices, xenon clearance, and residual coronary pressure (PCP) following temporary and/or permanent closure of the circumflex branch.

### 5. Effect of Calcium on Coronary Hemodynamics in the Unanesthetized Dog.

This project, now complete, was in collaboration with the Johns Hopkins School of Medicine. The coronary hemodynamic effects of intravenous calcium gluconate or chloride 0.5 gm (Ca) were examined in 3 trained

resting unanesthetized dogs. Ca caused initially an increase in myocardial contractility, coronary flow, cardiac output, and a decrease in late and mean coronary resistance; later as the heart rate fell, coronary resistance returned to normal or increased. In other dogs with blocked and paced hearts in which the heart rate could not change, the decrease in coronary resistance was maintained throughout. Such coronary vasodilatation following Ca is believed to be due to an initial inotropic effect followed by secondary metabolic dilatation rather than to a direct effect of Ca on the coronary vasculature.

6. Left Coronary Hemodynamics during Hemorrhagic Hypotension and Shock.

This project now complete was started some years ago in collaboration with the Institute of Human Physiology at the University of Torino, Italy. Coronary and systemic pressure-flow relations, and the distribution of the left coronary inflow within a cardiac cycle have been studied in preoperated unanesthetized dogs in irreversible hemorrhagic shock. During bleed-down, coronary resistance first increased and then decreased. However, during prolonged oligemic hypotension, coronary resistance progressively rose up to the time of blood reinfusion. With one exception, the changes of coronary resistance during postinfusion cardiovascular decay were directionally similar to those produced by hemorrhage although they occurred generally at a somewhat higher level. In the coronary blood flow pattern, the diastolic component was markedly elevated but the systolic component was markedly reduced at or near to completion of hemorrhage with evidence of a large systolic backflow. As oligemic hypotension progressed, the systolic component increased greatly exceeding the control. This increase occurred also during spontaneous hemodynamic decay, and late in this period the coronary blood flow pattern often resembled the aortic pressure pulse, the systolic flow being much higher than the diastolic flow for an equivalent period of time.

7. Coronary Artery Blood Flow in the Resting Dog with Congenital Subaortic Stenosis.

This study was undertaken for the Comparative Cardiovascular Studies Section, School of Veterinary Medicine, University of Pennsylvania, because it was felt that such an animal affords a rare opportunity to learn more about the influences of such an anomaly, and related disease states, on the coronary circulation and associated physiological and pathological conditions encountered in man. Two dogs were obtained with congenital subaortic stenosis formed by a restrictive, fibrous

band just beneath the aortic valves. Clinically, the dogs were characterized by a marked left ventricle--aorta systolic pressure gradient (118 mm Hg in one dog) and mild congestive heart failure. One dog was on a daily regimen of digoxin due to preoperative diagnosis. At autopsy, the left ventricle was grossly hypertrophied, the heart weight being 422 gm in a 34 kg dog, and 331 gm in a 25 kg dog.

Under halothane anesthesia, a pressure tube was placed in the ascending aorta and a flow transducer around the main pulmonary artery (rather than on the aorta which was massively dilated). A second flow transducer was placed on the left circumflex coronary branch together with a flow zeroing cuff. After surgery, the unanesthetized dogs were studied for approximately 4 weeks. Only the coronary flow data has been analyzed. From the second week on, when hemodynamic conditions had stabilized, the dogs had coronary flows of about 20 ml and 46 ml/100gm left ventricle/min. The coronary systolic flow was almost entirely backflow. When digoxin administration was stopped in the second dog during the third week, his coronary flow dropped from 46 ml to 30 ml/100gm left ventricle/min. These circumflex coronary flows are considerably less than that in the normal resting dog which approximates 50 ml/100gm/min.

#### 8. Regulation of the Coronary Collateral Circulation.

The natural history of development of the coronary collateral circulation has been studied in trained, unanesthetized dogs following gradual (few days) and abrupt circumflex coronary artery occlusion, and the effects thereon determined of intra-aortic ballooning, drugs, and subsequent release and reocclusion of the circumflex branch.

a. A gradual circumflex occlusion. Three successful experiments were performed bringing the total to 4 of gradual occlusion of the circumflex branch of the left coronary artery. In a previous report (Circulation Res.22:237, 1968), the effect of ameroid constriction of the circumflex on coronary flow and peripheral coronary pressure had been observed, but an estimation of the collateral flow (other than the assumption that a rise in peripheral coronary pressure indicates development of collateral flow) was lacking. It has been the purpose, therefore, in the extension of this study to obtain more evidence of the actual collateral flow. This has been attempted by xenon clearances obtained with xenon injections into the developing ischemic area of the constricted circumflex branch. It has been found to date that the xenon clearance increases as the peripheral coronary pressure rises secondary to the constriction. By the time the circumflex branch has been completely occluded (over a 5-6 day interval), the estimated collateral flow and the peripheral coronary pressure have become well-established. At autopsy the hearts had little or no infarction.

b. Abrupt circumflex occlusion. The results of 24-30 hours of abrupt circumflex branch occlusion on the coronary collateral circulation confirmed those of previous experiments. These included lack of

evidence for pain, no ventricular fibrillation, long delay in onset of ventricular tachycardia, and in K and CPK efflux into the coronary sinus, a progressive and significant rise in PCP and xenon clearance during the occlusion. Lidocaine, given intravenously gave results (increased PCP and unchanged isotope clearance) compatible with coronary collateral dilatation but the same findings with intracoronary injection into the ischemic bed could also indicate arteriolar or coronary vein constriction.

c. Diastolic assist (intra-aortic ballooning). Theoretically, redistribution of aortic blood pressure near the coronary ostia by dropping the aortic systolic pressure and raising the aortic diastolic pressure but without change in mean aortic pressure could benefit the heart in the presence of coronary insufficiency and cardiogenic shock through a decrease in cardiac work, coronary flow, and myocardial oxygen needs, and through an increase in coronary collateral function. To test this, three experiments were performed in the chronic conscious dog in which the coronary collateral indices (PCP and xenon clearance) had been increased significantly by prior abrupt or gradual (4-6 day) circumflex branch occlusion. Central aortic pressure redistribution was achieved by means of an intra-aortic balloon inserted through a femoral artery under local anesthesia and situated just below the origin of the left subclavian artery. The balloon was activated by an AVCO pump system supplied by the National Heart Institute. While too few experiments have been done to permit any conclusions, the intra-aortic assist apparatus did increase mildly the mean PCP (increased diastolic pressure with the same or lower systolic pressure) while the mean aortic pressure was essentially unchanged.

d. Regression and re-establishment of the coronary collateral circulation following coronary artery release and reocclusion. Four experiments were done in which the circumflex branch of the left coronary artery was occluded gradually (4-6 days) to zero flow. Collaterals from the other major coronary branches, notably the left descending branch, had developed and supplied adequately (without infarction) the area penalized by the occlusion as evidenced by increase in descending flow, PCP and xenon clearance. Following reopening of the circumflex branch, the collateral indices returned to normal within at least 4-5 days. A few days later, the circumflex branch was abruptly reoccluded in one step. Within less than one hour, the values of the collateral indices matched those obtained after 5 days of the initial occlusion. In a continuation of this type of study, the circumflex branch of the coronary artery was occluded abruptly and completely in 4 dogs, and the development of collateral circulation monitored for 3 to 5 days, at which time collateral indices were markedly elevated. This type of occlusion resulted in the infarction of massive areas of the bed normally supplied by the circumflex. After adequate recovery from this initial occlusion (re-establishment of sinus rhythm with heart rate back to near control) the vessel was

reopened until the PCP and other parameters were back to the pre-occlusion level. Following subsequent abrupt circumflex reocclusion, the collateral indices rose immediately and within one hour equalled those which were obtained in the initial occlusion only after one to two days.

### Conclusions

A new compound (Bis-N-N'-triphenyl methyl carbodiimide) has been found which apparently blocks the synthesis of ATP without affecting its utilization. This inhibitor could be a useful tool in the study of the mechanism of high energy transfer reactions in sarcosomes.

Adenosine has been identified in the normally oxygenated myocardium of dogs. Myocardial content of this compound rises during coronary occlusion and falls during ensuing reactive hyperemia in a way which suggests it is a primary regulator of coronary blood flow in this response.

Intravenous calcium in the unanesthetized dog decreases left coronary vascular resistance.

In irreversible hemorrhagic shock in conscious dogs, the most interesting finding is the change in systolic coronary resistance. Systolic coronary resistance increases late in bleed-down but decreases largely during prolonged hypotension and postinfusion decay, the coronary flow pattern at times resembling the aortic pressure pulse with the systolic flow being greater than the diastolic flow.

In congenital subaortic stenosis, left coronary flow during systole is largely backflow and the mean coronary flow is reduced considerably.

Observations have been made on the natural history of development of the coronary collateral circulation in the presence of abrupt or gradual (few days) closure of the left circumflex coronary artery branch. The main finding is an amazing rapidity and order of magnitude of collateral development. This indicates the opening up of built-in-pre-existing collaterals that were not previously functioning. It has not been found possible as yet to improve with drugs this natural development, with the possible exception of Lidocaine. Possibly, mild improvement follows intra-aortic ballooning.

Although these collaterals cease to function and return to normal within a few days after reopening the circumflex artery, they are immediately available if the circumflex is reoccluded some days later, and reach a high level much more quickly than following the initial occlusion.

Project 3A061102B71B, RESEARCH IN BIOMEDICAL SCIENCES  
Task 02, Internal Medicine  
Work Unit 085, Vascular components of cardiorespiratory disease

Publications

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(U) Blood and Blood Disorders (09)

00800 Life Support 002600 Biology 003500 Clinical Medicine

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NA	EXPIRATION	69	7
	ACCOUNT	70	7
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Walter Reed Army Institute of Research  
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 202-576-3551

Foreign Intelligence considered

(U) Blood; (U) Blood Transfusion; (U) Preservation;  
 (U) Bleeding; (U) Coagulation; (U) Altitude; (U) Bone Marrow; (U) Irradiation

23. (U) Basic observations of hematologic responses to disease and injury, improve the Army Transfusion Service, studies of blood, blood products and blood substitutes for the treatment of casualties and the prevention of disease.
24. (U) Studies of coagulation abnormalities in various disease and physiologic states. Studies of the mechanism of bone marrow repopulation following irradiation. Improve methods of blood banking. Quantification of the bleeding abnormalities with plasma expanders.
25. (U) 69 01 - 69 06 Studies are in progress to delineate the coagulation abnormalities in various infectious diseases and in response to heat, exercise and altitude. These seem to explain pathologic changes observed in the hemorrhagic fevers and with erythremia. Methemoglobinemia caused by usual doses of antimalarial drugs was investigated and was due to hereditary heterozygous NADH methemoglobin reductase deficiency. Basic mechanisms of hemolysis in malaria were attributed to loss of negative charge of the erythrocytic surface. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 086, Blood and blood disorders

**Investigators**

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CPT Dennis S. O'Leary, MC, M. Bernadette Garvey, M.D.,  
Charles F. Barr, Ruth G. Brennan, Donna J. Wicker and  
Harold L. Williams.

Description

Basic and clinical studies to investigate the functions and disorders of blood and blood forming organs.

Progress and Results

Clinical malaria does not occur without hemolysis and parasitemia of circulating erythrocytes. Hemolysis does not seem to be caused by antibodies or hypersplenism. Previously, we showed that hemolysis in excess of the number of observed parasitized erythrocytes in the circulation was caused by splenic pitting of parasites from infected red blood cells and the return of damaged spherocytes with a shortened lifespan to the circulation. We postulated that premature destruction of red blood cells affected by parasitemia was caused by loss of the negative charge on the surface of erythrocytes. The negative charge on the surface of red blood cells prevents physical damage to erythrocytes circulating in the blood and prevents their phagocytosis by reticuloendothelial cells.<sup>1</sup> Studies of parasitized red blood cells show changes which support this hypothesis; the infected erythrocytes migrate more slowly towards an anodal current in a cytopherometer and aggregate more rapidly than normal cells in solutions containing a surplus of positively charged compounds such as polylysine H Br. Since the negative charge on the cell surface requires N-acetylneuraminic acid, assays of this compound are being modified for measurements of malarious cells and for comparison with cells treated with neuraminidase.

The admission of soldiers to Walter Reed Hospital with a history of cyanosis in Vietnam and the possible association with the administration of conventional chemoprophylactic doses of antimalarial drugs led us to study this problem. These soldiers were shown to have a heterozygous deficiency of nicotinamide adenine dinucleotide (NADH)

methemoglobin reductase. Chloroquine, primaquine and diaminodiphenyl sulfone were each shown to provoke methemoglobinemia in enzyme deficient subjects in doses that have no measurable effect in normal persons. Further studies of the effects of antimalarial drugs upon hemoglobin and respiratory enzymes are needed in addition to information of the prevalence of NADH methemoglobin reductase deficiency. Chemical methods for measurement of assays are being automated to permit performance of large scale studies.<sup>2</sup>

Clinical studies of the effect of chemoprophylactic doses of chloroquine and primaquine are being performed to determine the effect of repeated weekly doses in Caucasians with various forms of glucose-6-phosphate dehydrogenase (G6PD) deficiency. It is known that antimalarial drugs cause decreasing hemolysis with repeated dosages in G6PD deficient Negroes because of selective destruction of aged erythrocytes. This is not believed to occur in G6PD deficient Caucasians because erythrocytes of all ages are hemolyzed by administration of oxidant drugs. Our studies seem to indicate diminishing hemolysis of cells of all ages with repeated doses of primaquine, suggesting that there is a production of cells by the bone marrow which are more susceptible than others.

Studies of a family of a patient with hemolytic anemia revealed a previously undescribed enzymatic abnormality of red blood cells. The subjects had relatively normal blood levels of pyruvic kinase, but the enzyme had an elevated Michaelis constant for its substrate, phosphoenopyruvate. Presumably, the defect results from a structural alteration of the enzyme.<sup>3</sup>

Basic investigations of red blood cell transport of cations were continued. The effect of tetraethylammonium (TEA) ion on cation transport was studied. TEA was found to inhibit potassium influx into red blood cells and competitively inhibit potassium activated sodium outflux. TEA substitutes for potassium in that it inhibits sodium influx, and it inhibits the binding of ouabain to cells when both TEA and ouabain are present simultaneously, an effect which also occurs with potassium. This provides evidence that TEA combines with a component of the cation transport system so that it is unable to perform cation transport or bind ouabain.<sup>4</sup>

Thrombosis and hemorrhage are frequent complications of many infectious diseases. Coagulation studies were performed upon blood specimens from patients and experimental animals. Humans with Korean hemorrhagic fever and meningococcemia, monkeys infected with yellow fever and guinea pigs infected with Argentinian hemorrhagic fever had multiple coagulation abnormalities suggesting the occurrence of accelerated intravascular coagulation. Similar coagulation abnormalities were found in monkeys which developed erythrocytosis in a

hypobaric environment. Coagulation changes did not occur until the erythrocytosis abated. Sections of brain and lungs from animals maintained at simulated altitudes showed both hemorrhage and thrombosis.<sup>5-8</sup>

Tracker dogs in Vietnam frequently develop a fatal hemorrhagic disease. Specific coagulation abnormalities were not found in blood specimens from animals with this disease, suggesting that the disorder is a vasculitis and not a coagulopathy.

Studies of coagulation changes were performed upon blood specimens from monkeys injected with venoms from various species of snakes. Venoms from the Crotalidae and Viperidae produced coagulation abnormalities while venoms from Elapidae and Hydrophaeidae did not. Marked thrombocytopenia was seen following the injection of certain venoms and produced changes suggestive of accelerated intravascular coagulation. Russel viper venom acted as a potent first stage procoagulant and Malayan viper venom caused a significant decrease in blood levels of factors II, V and VIII.

Changes in blood coagulation following exercise were studied. Exercise caused a marked increase in factor VIII activity and enhanced plasminogen activation. The former was prevented by the administration of propranolol before exercise but not the latter. Thus, while exercise increases factor VIII activity by beta receptor stimulation, this mechanism is not responsible for the increase in fibrinolysis.<sup>9-10</sup>

Additional coagulation studies have been performed in patients with hematologic disorders and in subjects with several disease states.<sup>11,12</sup> Standards for coagulation reagents have been established for government purchase, and materials submitted on bid are examined for conformance to standards.

The possible relationship of  $\gamma$  D immunoglobulin to malaria has been studied in Vietnamese populations. Increased serum concentrations of  $\gamma$  D globulin have been found in Montagnards and Vietnamese living in an area of high malarial endemicity (Budop), whereas levels of  $\gamma$  D globulin comparable to a North American blood donor population are found in Vietnamese living in Bien Hoa, a nonmalarial area. Serologic testing of the serum samples for malarial antibodies is being performed, and titers of antibodies will be compared to  $\gamma$  D immunoglobulin levels.

The catabolism of a low-molecular weight human protein ( $B_2$ -microglobulin) has been studied in rats. The protein has been shown to be dependent for catabolism upon functioning renal tissue. Although it is not excreted by the kidney,  $B_2$  microglobulin is extracted by the kidney. It is handled in a rapid normal fashion by rats whose ureters have been ligated, but has a prolonged half-life in anephric animals.<sup>13,14</sup>

A child with fatal mucocutaneous candidiasis was studied with the Department of Pediatrics, Walter Reed General Hospital. The child was found to have (1) absent  $\gamma$  A globulin; (2) impaired delayed hypersensitivity; (3) abnormal lymphocyte stimulation and (4) antibodies to various bovida antigens. The latter antibodies appeared to interfere with the lymphocyte stimulation.

Two crystalline myeloma globulins are under study in an attempt to obtain crystals of sufficient size to perform X-ray crystallography in conjunction with the Department of Biophysics at Johns Hopkins University.

**Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES**

**Task 02, Internal Medicine**

**Work Unit 086, Blood and blood disorders**

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION#	2. DATE OF SUMMARY	3. PORT CATALOG #
					DA OA6452	69 07 01	DD-152 (AF) 698
4. DATE PREP SUMMARY	5. KIND OF SUMMARY	6. SUMMARY ACTY	7. WORK SECURITY	8. PROGRAMING	9. DISSEM INSTR	10. SPECIFIC DATA CONTRACTOR ACCESS	11. LEVEL OF SUM
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10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	61102A	3A061102B73R	02	087			
b. CONTRIBUTING							
c. COORDINATING	1412A(2)						
11. TITLE (Precede with Security Classification Code)							
(U) GASTROINTESTINAL DISEASE (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS							
012900 Physiology		003500 Clinical Medicine					
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
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17. CONTRACT/GRANT				18. INCURRED ESTIMATE		19. PERSONNEL MAN YRS	
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20. RESPONSIBLE FOR ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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22. GENERAL USE				ASSOCIATE INVESTIGATORS			
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23. KEYWORDS (Precede EACH with Security Classification Code) (U) Intestinal Innervation; (U) Intestinal Absorption; (U) Intestinal Reactivity; (U) Intestinal Enzymes; (U) Stress Ulcer; (U) Microcirculation							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROCESSES (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code)							
23. (U) - 1. Intestinal innervation and peristaltic reflex. 2. Pharmacological reactivity of the intestinal muscles in diarrheal conditions. 3. Microcirculatory studies of intestine and liver. 4. Intestinal enzyme activity in diarrheal disease. 5. Small intestine absorption of water, electrolytes and glucose in diarrheal conditions. 6. Origin of gastric stress ulcers.							
24. (U) - 1. In vitro stimulation of peristaltic reflex and its modification by pharmacologic agents. 2. Evaluation of intestinal contractile reactivity in animals with diarrheal disease. 3. Vascular bed perfusion with silicone rubber. 4. Chemical analysis of intestinal specimens for disaccharidase activity. 5. Total small bowel perfusion with a balanced electrolyte solution. 6. Rotational stress on small animals.							
25. (U) 69 01 - 69 06. 1. In peristalsis the longitudinal and circular muscles act reciprocally. Nonadrenergic inhibitory neural mechanisms exist in the intestine. 2. Salmonella typhi endotoxin increases intestinal excitability. Infection decreases adrenergic responses. 3. Portahepatic venous shunts in cirrhosis develop from regenerating hepatic veins. 4. In the native Thai population, lactase enzyme activity is significantly less than in Caucasians. 5. Experimental salmonella infection inhibits salt and water absorption and reverses bicarbonate transport. 6. A method of reproducibly producing gastric stress ulcers has been developed. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 ~ 30 Jun 69.							

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Project 3A061102E71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 087, Gastrointestinal disease

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Leif I. Solberg, MAJ, MC; James Beardon, MAJ, MC; Tatsuo  
Hase, M.D.; Pearl R. Anderson, Ph.D.; and Betty Moss, GS-9.

Description.

The principal research activity in the Department of Gastroenterology remains focused on the pathophysiology of diarrheal diseases. Investigative emphasis is placed on gaining information concerning the effect of diarrheal disease on those intestinal mechanisms associated with the accumulation of fluid within the gut lumen and those mechanisms involved in propelling the luminal contents over the length of the intestine. A multidisciplinary approach is being followed in pursuing this program with studies being done on intestinal salt and water transport, neuromuscular interactions, mucosal disaccharidase activity, and microvascular architecture. The results of these studies are being integrated to formulate a comprehensive interpretation of the intestinal response to diarrhea producing agents.

A study of the pathogenesis of gastric stress ulcer disease has been inaugurated in the Department as a result of the presently occurring incidence and severity of the condition in seriously injured individuals in Vietnam. The scope of the study will include investigation into contributory factors, effects of medication, and potential treatment or control procedures.

Progress and Results.

1. Pathophysiology of Diarrheal Disease.

a. Intestinal Transport in Diarrheal Disease. Rat Salmonella typhimurium was the experimental model employed in these studies. The animals were challenged with S. typhimurium delivered by gastric tube and studied at two and six days. At two days, none of the animals displayed external diarrhea but all had mild mucosal changes. At six days, two patterns were observed. In approximately half of the animals there was no diarrhea but histological examination revealed moderate mucosal lesions. The remaining group of six-day animals revealed gross diarrhea plus more severe mucosal lesions on microscopic examination.

Water, electrolyte, and glucose transport was studied in jejunum, ileum, and colon by in vivo perfusion techniques. In comparison with control animals, the two-day and six-day moderately infected animals demonstrated a minimal depression of absorption in all gut levels studied.<sup>1,2</sup> Animals with severe six-day infections demonstrated identical minimal absorption changes in the jejunum and colon while the ileal segment secreted water and electrolytes. This observation is compatible with information available in the literature and suggests that one of the physiological determinants of diarrhea is intestinal secretion. Abnormalities in ileal anion transport are also evident. Despite secretion of other electrolytes, the normal secretion of bicarbonate into the ileum is inversely related to absorption. This bicarbonate absorption is not secondary to systemic acidosis and is accompanied by elevated  $pCO_2$  levels which suggests secretion of hydrogen ion into the ileal lumen.<sup>2</sup> These results are being prepared for publication. Further studies are being done on similar experimental groups in an attempt to modify the ileal secretion by the addition of glucose to the perfusion solution. The presence of glucose in the ileal perfusate inhibited the secretion noted above for this diseased segment. These results suggest that the inclusion of glucose in oral fluids for patients with similar enteric lesions might be expected to improve systemic water and electrolyte balance.

Studies are being initiated in which the perfusate bicarbonate and chloride concentrations are altered in an attempt to further evaluate the anion transport changes that have been observed in diarrheal disease. Work in several laboratories suggests that mucosal cyclic adenosine monophosphate (CAMP) levels influence membrane transport. A study is currently being developed using theophylline to alter CAMP levels in diarrheal disease and thus determine if these pharmacological mechanisms are altered in diarrhea.

b. Intestinal Neuromuscular Interactions. The previously reported observations that experimental Salmonella typhimurium infections are associated with increased intestinal motor responses to cholinergic stimuli, increased large amplitude rhythmic activity, and a loss of adrenergic responses of the gut has precipitated a study of the peristaltic reflex in diarrheal conditions. At this time the study has been limited to delineating the normal neuromuscular interactions during the peristaltic reflex. An in vitro approach is being used in which both longitudinal and circular muscle responses to co-axial electrical and pharmacologic stimuli are being recorded. The results indicate that longitudinal and circular muscles contract reciprocally and occur due to activation of separate neuronal elements. The reciprocal, inhibitory events are a result of individual inhibitory neurons serving the separate muscle layers. Through the use of the neurotoxin, tetrodotoxin, inhibitory neurons to the circular muscle have been unmasked and have been shown to not be adrenergic in nature by use of standard adrenergic blocking agents. However, the nature of the inhibitory

transmitter is undefined. This study will be continued in the experimental diarrhea model to further describe the neuromuscular changes accompanying diarrheal disease.

Preliminary studies have been started to describe the action of salmonella endotoxins on the neuromuscular matrix of the gut. Salmonella typhi endotoxin appears to increase the excitability level of Auerbach's plexus as suggested by a marked reduction in measured chronaxie values. In addition, the predominant effect of co-axial stimulation is shifted from being on longitudinal muscle to being on circular muscle. The observations may explain the observed increase in rhythmic activity and cholinergic reactivity reported previously.

In collaboration with the Department of Pharmacology, Division of Medicinal Chemistry, WRAIR, the alpha adrenergic blocking properties of WR-2823 have been reported.<sup>3</sup> The unique ability of this agent to block vascular adrenergic alpha receptors without effecting those of intestinal smooth muscle makes WR-2823 a potentially valuable drug in separating splanchnic blood flow from intestinal motor activity in in vivo animal studies.

c. Intestinal Disaccharidase Enzyme Activity. A study has been published in which the lactase activity of native Thais has been shown to be significantly lower than that of Caucasians in a nontropical environment.<sup>4</sup> The infant Thai demonstrates normal lactase activity until two to four years of age at which time it becomes markedly reduced. Since elevated dietary lactose does not lead to adaptive enzyme formation, the defect is estimated to be permanent in nature.

The intestinal disaccharidase activity of rats subjected to an experimental Kwashiorkor protein deficiency disease is being studied. In comparison with control rats fed a balanced diet and control rats fed a balanced diet but with total caloric intake matched to the experimental population, the protein deficient animals demonstrated a total decrease of disaccharidase activity. However, the activity per unit weight of mucosal protein remains relatively unchanged while total mucosal protein is reduced. The results indicate that protein deficiency leads to carbohydrate malabsorption via a proportional reduction in total mucosal enzyme content.

d. Microvascular Architecture. The silicone rubber injection technique is being used to study the development of intestinal vasculature in fetal rabbits. Early results suggests the villus vasculature develops from a vascular loop extending into the villus. The complex mucosal vasculature does not develop until very late in gestation. A paper has been published describing the methods used in these studies.<sup>5</sup> A study that used this technique to describe the development of porta-hepatic shunts in cirrhotic liver disease has also been published.<sup>6</sup>

## 2. Pathogenesis of Gastric Stress Ulcer.

A device has been fabricated which subjects small laboratory animals to a tumbling type of stress. By adjusting the rotational speed and duration of the stress it is possible to predictably produce gastric stress ulcers varying from mild focal erosions to severe hemorrhagic lesions. In collaboration with the Division of Biochemistry, selected systemic responses to stress are being analyzed. At the present time the following observations have been documented as accompanying the stress condition: liver glycogen is severely depleted; hypoglycemia is present; and blood corticosterone and catecholamine levels are increased during the early phases of stress. The hypoglycemia may be causatively related to the stress ulcer through vagal activation as suggested by an observed increase in gastric contractile tone and gastric juice secretion. Future studies will investigate the relationship between stress ulcer development and hypoglycemia, preventive measures, and nutritional factors.

### Conclusions and Recommendations.

The study of the pathophysiology of diarrheal disease in experimental animal models has yielded specific information related to changes in intestinal motor activity and intestinal transport mechanisms. Plans are currently being developed to extend these experimental studies into clinical studies of related parameters in human diarrheal disease. Along with the clinical study, animal experimentation will continue to further define the intestinal changes accompanying diarrheal disease. These animal studies will incorporate attempts to reverse the pathophysiological changes that have been defined to date.

The studies on gastric stress ulcer disease will be continued to define contributory or related systemic changes. Any such systemic responses will be manipulated in an attempt to alleviate or correct the severity of the basic condition.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 087, Gastrointestinal disease

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<b>(U) Military Nursing (09)</b>			
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Walter Reed Army Institute of Research Washington, D.C. 20012		Walter Reed Army Institute of Research Department of Nursing Washington, D.C. 20012	
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Foreign Intelligence Not Considered		Glor, MAJ Beverly A.F. Estes, CPT Zang E.	

PII Redacted

(U) Military Nursing; (U) Metabolic Cost; (U) Blood Pressure; (U) COMPSY;  
 (U) Wound Healing; (U) Oxidation

23. (U) Develop rationale underlying military nursing through the study of: current methods of application of moist heat to wounds; the effects of moist heat to wounds; the effects of moist heat on tissue; auto and photo-oxidation of phenothiazine compounds; the metabolic cost of select patient activities; the use of computers in psychiatry (COMPSY); blood pressure manometer readings recorded by nurses; and the assignments of graduates from the MNP&R Course.

24. (U) Survey of selected hospitals as to methods of moist heat application; polarographic assays of tissue respiration and respiratory enzymes; oxidative deterioration of liquid phenothiazine compounds in diluents and various light intensities; open circuit, indirect colorimetry to calculate energy expenditure; modification of tools and techniques used in COMPSY; survey of actual blood pressure measurements from synchronized sound film clips; semi-annual questionnaire sent to graduates of the MNP&R Course.

25. (U) 69 01 - 69 06. 1147 questionnaires returned regarding methods of moist heat application, data being evaluated by ADP. Testing and standardization of techniques for polarographic oxygen assay and protein quantitation. Both thioridazine and trifluoperazine appear to be more sensitive to light and equally sensitive to pH than chlorpromazine, in oxidative properties. Significant increase in minute ventilation and tidal volume demonstrated in patients with chronic diffuse lung disease, in the sitting position. (See WROH Project No. MEDDC-02B, 68-71-18) (See COMPSY Report WROH Project No. MEDDC-02P, 68-4-7) Results of blood pressure data substantiate previous findings, manuscript in progress. Graduates of MNP&R Course continue to be surveyed. For technical reports see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 68 - 30 Jun 69.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 088 Military nursing

**Investigators**

**Principal:** LTC Miriam Ginsberg Rothchild, ANC

**Associate:** LTC Rosemary T. McCarthy, ANC

MAJ Beverly A. K. Glor, ANC

CPT Zane E. Estes, ANC

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**Description.**

Military nursing is a broad field under which many aspects of nursing practice in the Army are explored. The overall aim of research under this work unit is to identify and test principles underlying nursing care and to recognize differences in military nursing from civilian practice. Eleven studies are reported.

A descriptive study concerned with the clinical manifestations and nursing care requirements observed in fifty patients with falciparum malaria, in the Republic of South Vietnam, has been completed and reported in the open literature. Since this investigation demonstrated a trend of symptomatology which might have predictive value in relapse, fifty patients were resurveyed for incidence of relapse.

An experimental study, designed to compare the ability of two fever reduction techniques to reduce elevated body temperature, was conducted using nine falciparum malaria patients who demonstrated two or more temperature spikes greater than 104° F.

Previous studies of reliability of blood pressure readings have shown no significant differences between observers when the procedure is controlled and standardized. A replication of a previous investigation was made using a broader sample of personnel, including registered nurses, Army medical technicians, and student nurses.

Another study, using film sequences of actual blood pressure measurements with electronically synchronized sound was undertaken to determine the validity of blood pressure readings.

A series of sequential studies centered about nursing aspects of the use of moist soaks has been initiated. The first part of the series is a questionnaire

survey of both military and civilian hospital personnel with regard to the various procedures employed in the application of moist soaks and the principle member(s) of the medical team making the decision as to which procedure will be used to carry out the physicians' order.

A second part of the series is an attempt to assess the respiratory activity of the skin of guinea pigs following the application of moist soaks of varying temperature and duration.

In an attempt to extend the findings of a previous study on the auto-oxidative responses of chlorpromazine hydrochloride to solution pH and light, this same property was similarly tested in two other phenothiazine-derived pharmaceuticals, thioridazine hydrochloride and tri-fluoperazine dihydrochloride.

The metabolic cost of maintaining six resting positions has been measured on 12 patients with chronic diffuse pulmonary lung disease and on six normal subjects; the method has been indirect calorimetry.

A series of small studies related to computer applications of psychiatric nursing notes has been undertaken to determine a valid and reliable tool to be used by nursing service personnel. (Reported under WRGH Project No. MEDEC-GP 68-4-7.)

A survey concerned with continuity of care was undertaken in 1961 to ascertain perceptual similarities between health nurses, administrative nurses, and hospital care nurses.

Twenty-eight graduates and five non-graduates (due to the exigencies of the Vietnam war) of a ten-month post-baccalaureate course titled Military Nursing Practice and Research were surveyed to determine career patterns.

#### Progress.

Survey findings continue to substantiate the hypothesis that relapses in patients with falciparum malaria may be predicted. Further investigations will be curtailed until such time as a nurse investigator is stationed in a malaria endemic area.

The results of fever reduction investigations have shown that the ice bag technique (one bag to each groin, axilla, center back, feet, and nape of neck) elicits a greater reduction in body temperature than does cold packs to the entire body surface. A manuscript is currently in preparation.

The results of observer reliability in blood pressure determinations substantiate the previous findings of no significant differences between observers.

The findings of the study of validity of blood pressure measurements show the accuracy of reading, within plus or minus five points, to be over 75% of the sample. However, there is some variation between observers according to age, visual and auditory acuity, and occupational factors. Manuscripts are in preparation for both of these investigations.

The results from 1147 returned moist soaks questionnaires are summarized in Table I which shows the range of variation for which any of the procedural variables must be tested.

Table II shows that, in practice, the choice of the exact techniques used for moist soaks is often (over 87%) left to the discretion of nursing personnel.

Preliminary and control studies concerning moist soak effects on skin of guinea pigs have shown no definitive results. Continued explorations are being made.

The auto-oxidative responses of two phenothiazine-derived drugs are summarized in Tables III and IV. These data show that while thioridazine does oxidize to a relatively small extent at high light intensities in acid solution, clinically significant rates are encountered only in neutral or alkaline solution for both drugs (like chlorpromazine). In all cases, the rate of auto-oxidation varies directly with pH and light intensity. The importance of these findings to the clinical nurse lies in the restrictions they impose on the pouring, storage, and dilution of these compounds in the preservation of their potency. Collectively, these may be stated as the avoidance of both neutral solution in tap water and exposure to high light intensities. (Alkaline solution has not been found to result from any of the commonly used diluents). This material is presently in manuscript, preceding publication.

Metabolic cost measurements of patients and normal subjects have been variable. One group of six patients showed a significant change in oxygen consumption, especially while in the 90° sitting position; neither the second group of patients nor the six normals showed this difference. See WRGH Project No. MEDEC-GMB 68-7-18. This project is now discontinued.

The continuity of care survey was sent to three groups of Army nurses in the grades of 0-1 to 0-5. Hospital care nurses returned only 23.1% of the 299 questionnaires; Army health nurses returned 60% of 90 questionnaires; and nurse administrators returned 46% of 136 questionnaires for an overall return of 35.4%. Analyses of these data were inconclusive. No followup of non-respondents was done. The principle investigator has retired from military service and the project is therefore discontinued.

Of the 33 graduates and non-graduates of the Military Nursing Practice and Research Course, approximately one-third have been appointed to, are attending, or have completed a graduate program at the master's level. Nine are assigned as Chief Nurses or in positions of equal or higher responsibility. An additional six are holding supervisory or equal positions, and three others have left the service. The course is being suspended for FY 70; however, the recurring survey will continue for several years.

#### Summary and Conclusion.

The eleven reported studies are independent and/or interrelated approaches towards the overall investigation of military nursing. The results of the descriptive study of symptomatology of patients with falciparum malaria and of fever reduction techniques indicate a need for extension of these studies in a malaria endemic area. Blood pressure readings have been evaluated for reliability and validity demonstrating observer reproducibility in approximately 75% of a large sample. Tissue respiration changes due to application of guinea pigs have only begun to be explored after a survey of procedures used for soaks in man. Auto-oxidative response of three tranquilizing drugs has provided guidelines for ward usage. Metabolic cost of maintaining six fixed bed-rest positions has not shown any trends. Computer usage in standardizing nursing note forms is being explored. Survey of graduates from a research methods course has shown leadership qualities being utilized in all phases of Army Nurse Corps assignments. These and other projected studies are beginning to build a foundation for nursing practice based on principles evaluated in the laboratory, rather than on tradition. As new explorations are made and fields more completely explored, a larger body of knowledge can be depended upon for nursing practice.

TABLE 1

CHARACTERISTICS OF THE PROCEDURES EMPLOYED BY MILITARY AND CIVILIAN MEDICAL PERSONNEL IN THE APPLICATION OF MOIST SOAKS

Procedural Characteristic	Per Cent Military	Per Cent Civilian
<b>Type of Solution</b>		
Saline	23.3	43.6
Water	10.0	18.2
SSBA	3.4	16.4
Neomycin	45.6	0.9
Silver Nitrate	4.4	3.7
Miscellaneous	7.7	12.7
Not Stated	5.6	4.5
<b>Duration of Soak</b>		
Continuous	20.0	44.5
15 Minutes or Less	3.3	10.7
20 Minutes	45.6	29.1
30 Minutes	27.8	7.3
1 - 2 Hours	0.0	6.3
Not Stated	3.3	2.1
<b>Temperature of Solution</b>		
Room Temperature	33.3	4.5
100 Degrees F.	12.2	12.7
105 Degrees F.	13.3	8.2
110 Degrees F.	4.4	1.0
Warm	18.9	32.7
Hot	2.2	1.8
Not Stated	15.7	39.1
<b>Frequency of Application</b>		
Continuous	20.0	44.5
QID	49.2	22.7
TID	15.6	18.2
Every 4 Hours	5.6	1.0
Every 3 Hours	3.4	0.0
Every 2 Hours	2.2	1.8
Daily	0.0	2.6
Not Stated	7.7	9.1

TABLE II

THE VERBATIM ORDER FOR MOIST SOAKS AS WRITTEN BY PHYSICIANS  
IN MILITARY AND CIVILIAN HOSPITALS

Order	Per Cent Military	Per Cent Civilian
Complete	10.0	17.3
Method Only	5.6	9.1
Method and Solution	26.7	19.1
Method and Frequency	33.3	26.4
Method and Duration	18.9	19.1
Not Stated	5.5	9.1

TABLE III

THIORIDAZINE HYDROCHLORIDE  
 PER CENT OXIDIZED PER HOUR AT INDICATED pH AND LIGHT INTENSITY

Light intensity (foot candles) pH	0	50	2500	4500
2.0	0	0	1.4	3.7
4.0	0	0	0.9	3.1
6.0	0	0.6	2.0	2.5
7.0	8.0	10.8	13.3	17.6
8.0	16.15	17.2	23.7	33.1
10.0	15.9	32.3	34.2	43.7

TABLE IV

TRIFLUOPERAZINE DIHYDROCHLORIDE  
 PER CENT OXIDIZED PER HOUR AT INDICATED pH AND LIGHT INTENSITY

Light intensity (foot candles) pH	0	50	2500	4500
2.0	0	0	0	0
4.0	0	0	0	0
6.0	0	0	0	0
7.0	4.2	6.6	6.9	8.4
8.0	8.7	9.0	11.7	15.8
10.0	12.1	15.9	23.7	22.8

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 088 Military nursing

Publications.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY	REPORT CONT. NO. (SEE 1)	
				DA 0A6464	69 07 01	DD-152 (AT) 698	
3. DATE PREP. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ACTY	6. WORK SECURITY	7. REGRADING	8A. S&P'S MOTIVE	8B. SPECIFIC DATA - CONTRACTOR ACCESS	8. LEVEL OF EFF.
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9. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	61102A	3A061102B71R	02	089			
10. CONTRIBUTOR							
11. CONTROLLER							
12. TITLE (Provide with Summary Classification Only)							
(U) BODY FLUID AND SOLUTE AND RENAL HOMEOSTASIS (09)							
13. SCIENTIFIC AND TECHNOLOGICAL AREA							
012 900 Physiology		003500 Clinical Medicine		016200 Stress Physiology			
14. START DATE	15. ESTIMATED COMPLETION DATE	16. FUNDING AGENCY		17. PERFORMANCE SERVICE			
09 54	NA	DA		C. In-House			
18. CONTRACT/GRANT				19. RESOURCES ESTIMATE	20. PERSONNEL EST. YRS	21. FUNDING CLASSIFICATION	
A. DATE/EFFECTIVE NA				B. FUNDING			
C. NUMBER				D. FUNDING			
E. TYPE				F. FUNDING			
G. KIND OF GRANT				H. FUNDING			
I. CASH AMT.				J. FUNDING			
22. RESPONSIBLE OR ORGANIZATION				23. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research Washington, D.C. 20012				NAME: Walter Reed Army Institute of Research Division of Medicine Washington, D.C. 20012			
RESPONSIBLE PERSONNEL				PRINCIPAL INVESTIGATOR (Provide name if U.S. and/or foreign)			
NAME: Maroney, COL W. H.				NAME: Teschan, COL P.E.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3529			
24. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Considered				SECURITY INVESTIGATION			
				NAME: Cirkana, LTC W. J.			
				DA			
25. RESEARCH OBJECTIVE, OR APPROXIMATE, OR RESEARCH PURPOSE (Include project identified projects identified by number, provide text of work unit summary classification (U))							
23. (U) - To investigate the renal and related mechanisms for maintaining body fluid and electrolyte homeostasis in response to disease, injury, and environmental stress, to elucidate the pathogenesis of acute renal failure, to develop and improve methods for prevention and treatment of altered fluid and solute homeostasis and acute and chronic renal failure.							
24. (U) - Conventional clearance techniques, externally monitored isotope techniques, isotope dilution, experimental models of acute renal failure, in vivo renal tubule micropuncture, in vitro renal tubule microperfusion, membrane transport, light and electron microscopy.							
25. (U) 69 01 - 69 06. Measurement of filtration rate and renal blood flow by externally monitored isotopic techniques have been applied to clinical setting in patients with acute and chronic renal failure. Clinical evaluation of the Dow Hollow Fiber dialyzer and also subcutaneous arteriovenous fistulas is underway. Morphological studies in patients with asymptomatic hematuria without proteinuria demonstrate significant pathology in most of these patients. Studies of autoregulation of renal blood flow demonstrated independence from angiotensin. Micropuncture studies in acute renal failure demonstrate normal intratubular pressure, continuing perfusion and decreased clearances. Studies of proximal tubular sodium reabsorption suggested another factor in addition to changing viscosity and glomerular filtration. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 089 - Body fluid and solute and renal homeostasis

Investigators:

Principal: COL Paul E. Teschan, MC, LTC William J. Cirksena, MC,  
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Richard L. Tannen, MC, MAJ Karl Nolph, MC, MAJ Theodore  
P. Weis, MC, CPT Richard Fein, MC\*, John Gagnon, Natalie  
Lawson, James McNeil, Amy Lee, William Kokotis, and  
James Roberson

Description: Studies have been continued in animal and human subjects directed at understanding mechanisms involved in fluid and solute homeostasis under normal conditions and in response to disease and stress. The central role of adaptive renal mechanisms and interrelationships with other body homeostatic mechanisms in the maintenance of fluid and solute balance has been emphasized to promote understanding of derangements in this area and advance their management and therapy in clinical situations. Technical competence has been achieved with 1) conventional and newer clearance methods; 2) in vivo micropuncture and microperfusion techniques; 3) in vitro cellular transport techniques; and 4) for light and electron microscopic techniques to be applied where appropriate to the study of: a) control and influences of body fluid volumes and tonicity in intact animals. b) solute and water handling by normal and diseased kidneys. c) renal hemodynamics in normal and disease states, d) acid-base homeostasis, e) production and alteration of acute renal failure and f) solute and fluid flow across biologic and dialytic membranes.

Progress:

1. Body Fluid Volume and Tonicity: The concentrating and diluting ability and alteration with varying salt intake have been studied intensively by balance techniques in 9 patients with chronic renal disease of diverse etiology. Salt depletion improved diluting ability and worsened concentrating ability in all patients. Although adaptation was slower than in normal subjects, these patients were shown to be able to maintain fluid balance except in the face of frank renal salt wasting in 2, partly by distal (aldosterone-regulated) mechanisms.<sup>1</sup> Vasopressin resistant hyposthenuria was found to be the rule rather than the exception in patients with advanced chronic renal disease.<sup>2</sup>

A patient with sustained inappropriate secretion of antidiuretic hormone was studied extensively to relate fluid and solute imbalances to intake and humoral control mechanisms. Positive water balances did not account

\* Assigned WRGH

for weight gain or hemodilution and raised questions regarding gut water transport in response to hyponatremia which require further study.

2. Renal Solute and Water Handling: Studies in dogs relating extracellular fluid volume expansion to natriuresis were concluded. Changes in calculated interstitial fluid space were correlated with a degree of natriuresis following infusions of saline. Hypo- and hypernatremia were found to modify the natriuretic response significantly! 3, 4, 5

Studies in dogs were concluded comparing renal function after closure of extensive nephrotomy wounds by suture and tissue adhesive techniques. Renal function declined in both groups both in hydropenia and after stressing wound closure by saline diuresis. No functional advantage of tissue adhesive was found.<sup>6</sup>

Micropuncture studies were undertaken to evaluate the relative contributions to natriuresis of body fluid compartment expansion and alteration in physical properties of blood. With pressure to the kidneys controlled, dogs were expanded with their own previously removed and stored blood or plasma to keep physical properties of blood constant. No effect on proximal reabsorption was noted in blood-expanded animals, while reabsorption was decreased in the plasma-expanded group. The status of renal vascular resistance at the time of expansion appears important in the response and will be investigated further.

Several studies of sodium localization and movement at the ultrastructural level using in vivo renal tubule microperfusion and electron microscopy were undertaken in the rat. Potassium pyroantimonate was used as a histochemical marker and infused before in vivo tubule fixation and infusion of sodium transport inhibitors. Studies showed:

1) ultrastructure preservation superior to other methods of tissue preservation; 2) discrete localization of electron-dense deposits along intracellular aspects of plasma membranes; 3) decreased plasma membrane deposition and increased deposits in vacuoles and other organelles after pretreatment with ouabain or production of cell injury by potassium dichromate. Electron microscopic autoradiography demonstrated that these deposits contained sodium. Studies may show the localization of sodium at cell transport sites. 7, 8

3. Renal Hemodynamics: Studies of renal blood flow (RBF) and glomerular filtration rate (GFR) in dogs by external monitoring techniques were concluded. The techniques were found to provide accurate estimates of these parameters compared with conventional clearance methods, obviate the necessity for catheterization, provide on-line data, and indicate non-steady state conditions where estimates of these parameters should be avoided.

External monitoring of renal hemodynamics was extended to human studies. Renal clearance of  $I^{125}$  iothalamate was found a reliable and simple measure of GFR in man.  $I^{131}$  iodohippurate clearance was unacceptable

as a measure of RBF in man. External monitoring of these compounds in man overestimated these parameters by 20 per cent in preliminary studies.

Autoregulation of RBF and GFR was studied during angiotensin infusion, and in dogs depleted of renin by chronic high salt diet to assess the proposal that intrarenal renin alterations control autoregulation. Studies show that autoregulation is maintained after reductions of 30 mm Hg in perfusion pressure despite either renin depletion by salt loading or suppression by angiotensin infusion.<sup>9</sup>

4. Acid-base Balance: The relationship between urine flow rate and urine pH and acid excretion was studied in seven normal men. Urine pH, net acid excretion and ammonium bicarbonate excretion increased with increases in flow rate, apparently due to unresponsiveness of mechanisms for altering urine free ammonia to flow rate. Studies emphasize importance of considering flow rate in assessing tests of urinary acidification.<sup>10</sup>

Techniques were developed to study the isolated perfused rabbit tubule in vivo and methods developed for ultra microanalysis of pH and bicarbonate to extend clearance studies of hydrogen ion secretion to definitive studies in single tubules under precise control of physiologic conditions. A pH sensitive antimony microelectrode was developed and found accurate acid feasible for use in these studies.

5. Acute Renal Failure: Results of studies utilizing varying techniques have resulted in development of a new hypothesis of pathogenesis of acute renal failure. Normal perfusion of glomeruli was seen with micropaque, while numbers of glomeruli containing red cells was markedly reduced after production of acute renal failure in the rat. Clearance studies showed reductions in GFR and RBF and filtration fraction after induction of the lesion consistent with efferent arteriolar dilatation in excess of afferent arteriolar constriction and hence redistribution of blood flow to postglomerular capillaries. Marked medullary cast formation was already present without increases in proximal intratubule pressure. Kidney weights were increased and tubule lumens dilated despite normal or low intratubule pressures. Prevention studies uncovered a variety of agents capable of preventing the lesion, not always related to an effect on urine flow while hyperoncotic albumin worsened the lesion. Micropuncture studies show failure of formation of significant amounts of filtrate at the glomerulus 24 hours after production of the lesion. Results are consistent with the hypothesis that some initiating event leads to increased afferent and decreased efferent arteriolar constriction with increased peritubular capillary flow resulting in reduced glomerular filtration, fluid movement out of peritubular capillaries into tubule lumina and interstitium and favoring the deposition of cast precursor material which at low flow rates may become obstructive.

6. Membrane Transport: The kinetics of transport and bulk flow movement of solute across lining membranes was studied in patients undergoing peritoneal dialysis. Quantitation of electrolyte and water removal by hypertonic peritoneal dialysis has shown marked patient variation in amounts removed, but ultrafiltrate always hyponatric to serum. The movement of solute by bulk solvent transport may explain these findings.<sup>11, 12, 13</sup>

Peritoneal permeability to large molecular weight solutes in patients with acute renal failure due to heat and exercise stress was found to be decreased markedly. Studies showed that impaired permeability and increased urea production rate combined to limit the effectiveness of peritoneal dialysis in such patients.<sup>14</sup>

The variation in degree of hyperglycemia in patients during peritoneal dialysis with glucose-containing solutions was found to correlate with changes in peritoneal permeability, and was more related to glucose absorption rate than to changes in serum insulin concentration.<sup>15</sup>

Recommended Further Studies:

1. Water balance studies including stool analysis to assess gut responses to hyponatremia.
2. Study of renal vascular resistance and influence of its status on the natriuretic response to volume expansion with and without control of perfusion pressure and other physical properties of blood.
3. Study of the effect of diuretic agents and other sodium transport inhibitors on sodium localization and movement across a) intact renal tubules in vivo; b) perfused renal tubules in vitro; c) other transporting epithelia.
4. Study mechanisms of renal autoregulation with both increases and decreases in perfusion pressure.
5. Study mechanisms of hydrogen ion secretion and hence acid-base homeostasis in a) intact animals with precise control of acid balance; b) intact renal tubules in vivo, and c) isolated perfused renal tubules in vitro.
6. Evaluation of the contribution of plasma protein oncotic pressure to the production of acute renal failure.
7. Studies of experimental models of hemorrhagic fever to include a) baseline hemodynamic studies; b) light and electron microscopic studies; and c) transport studies, if indicated.
8. Extension of available techniques to selected field problems as appropriate.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 089 - Body fluid and solute and renal homeostasis

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**PROJECT 3A061102B71R  
RESEARCH IN BIOMEDICAL SCIENCES**

**Task 03  
Psychiatry**

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RESPONSIBLE INDIVIDUAL NAME: <b>Meroney, COL W.H.</b> TELEPHONE: <b>202-576-3551</b>			PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic Institution) NAME: <b>Boren, J. J. Dr.</b> TELEPHONE: <b>202-576-2483</b> SOCIAL SECURITY ACCOUNT NUMBER: <b>[REDACTED]</b> <span style="border: 1px solid black; padding: 2px;">PII Redacted</span>		
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26. REVISIONS (Provide EXON unless security classification code) <b>(U) Animal and Operant Behavior; (U) Avoidance; (U) Motivation; (U) Conditioning; (U) Performance Decrement; (U) Reinforcement Learning</b>					
27. TECHNICAL OBJECTIVE, 28. APPROACH, 29. PROGRESS (Insert individual paragraphs identified by number. Precede text of each with security classification code.) 23. (U) a. To develop complex behavioral repertoires with special relevance to the neuropsychiatric area and to analyze experimentally the basic variables responsible for the behavior, b. To develop behavioral methods for producing stress and fatigue, c. To investigate the effects of stress-inducing procedures on complex behavioral repertoires. 24. (U) The approach emphasizes the application of modern behavioral technology and the techniques of the experimental analysis of operant behavior. 25. (U) 69 01 - 69 06 Continuing research on the warmup at the beginning of a stressful avoidance session indicates that the phenomenon is more akin to a reduction of an aversive state rather than to a buildup. Studies of neural structures mediating the initiation and suppression of behavior have used a stress-producing procedure based on avoidance of electric shock. In this situation destruction of the septal region of the brain resulted in avoidance performance superior to that of normals. Concurrent measures of behavioral, muscular, and autonomic changes during an emotional disturbance show that heart rate and blood pressure accelerate to above normal levels in spite of a decrease in muscle tonus and behavioral activity. Results show that monkeys can learn substantially longer response chains with a stimulus-fading procedure than with a no-fading procedure. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.					

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 03, Psychiatry

Work Unit 025, Analysis of behavior and of mediating mechanisms:  
Experimental psychological factors

Investigators.

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This report covers four types of problem areas: 1) the experimental analysis of basic variables determining behavior and the development of complex behavioral repertoires; 2) aversive control of behavior - its analysis and application to the production of stress; 3) the analysis of bodily mechanisms mediating behavior; and 4) the interdisciplinary application of behavior principles to certain medical, psychiatric and military problems. The approach to these problems emphasizes the application of modern behavior theory, reinforcement principles, and the technology of operant conditioning.

The results will be discussed in the same order as the problem areas are listed above. The first results concern the experimental analysis of basic variables determining behavior and the development of complex repertoires in animals. One set of experiments has explored several fading procedures for extending the longest response chain which a monkey could learn. With two of the fading procedures where an error enhanced the stimulus cues, monkeys could learn substantially longer chains than with a "no-fading" control procedure. With a third fading procedure which did not permit the enhancement of stimuli by errors, the results showed considerably less learning, which was only slightly better than the "no-fading" control. Current experiments are studying the effects of letting the monkey control his own stimulus cues by pressing an extra lever. To prevent the monkey from making his task too easy, counter control is exerted by increasing the work load (fixed ratio size) on the extra lever.

In any discrimination experiment, variables other than the stimuli to be discriminated affect performance. These variables may be such things as reinforcement magnitude, proportion of the time the stimuli are presented, or penalties imposed for inappropriate responses. In any given experiment, the relationship between the discriminative behavior and these non-stimulus variables can be determined, if appropriate experimental procedures are used. However, a problem then arises if one asks is there not some aspect of the performances that can be attributed only to the stimuli

being discriminated and not to some combination of stimulus and non-stimulus events. A means of separating the effects of these classes of events is suggested by the theory of signal detection which provides for two kinds of effects contributing to a given performance, sensitivity to the stimuli being studied, and factors which bias the performance. The present experiment is a three-lever procedure in which responses on the center lever turn on a clicker of one of two frequencies. Responses on one side lever are reinforced in the presence of one of the frequencies, and responses on the other lever, in the presence of the other frequency. Inappropriate responses are punished by a time out from positive reinforcement. When stable behavior has been achieved on a relatively difficult discrimination, biasing factors will be introduced to determine the operating characteristics of the animal under different conditions of bias. The first variable to be studied will be the relative probabilities of presentation of the two stimuli. Other variables to be probed will be amount of reinforcement for correct responses, and duration of time out punishment for incorrect responses. Other experimental variables such as drugs, sleep deprivation, and prolonged stress are known to affect discrimination performance; however, it is not known in most cases whether these changes are biasing effects or sensitivity effects. This experimental design will be able to make this determination.

An experiment has been designed to assess some of the interaction effects between components of a chained schedule of reinforcement. The main effects of interest here are changes produced in the performance on a FI 100 sec. schedule of reinforcement by changes in the size of the FR required to turn the FI schedule on. The experiment is currently being run about 21 hours per day, and a secondary interest is the work-rest cycles established by the animals. Recent developments in the area of psychophysics, specifically the theory of signal detection, suggest a means whereby comparable measures of discrimination can be obtained when different experimental procedures are used. These methods will be applied to the fixed-interval performances generated by the present experiment in an effort to demonstrate a continuity between the FI schedule and studies of discrimination of stimulus duration.

Three adult chimpanzees have been housed in individual cages which have access doors to a larger exercise, recreation, and social room. Locks on the doors may be controlled automatically by electrical circuits. The animals have been trained previously to work for their daily ration of food and water under a chained, intermittent schedule of delayed reward, or punishment. The chained schedule required a performance based on the passage of time. After the chimps were well trained and producing stable timing performances, the red and green cue lights were omitted and the timing performances deteriorated. The timing behavior was recovered without the red and green cue lights when the delay in food reward was reduced by requiring fewer responses on the second push-button.

Also, appropriate timing behavior was maintained without the cue lights while very gradually increasing the required number of responses back to the original 500 responses of initial training. Currently, the animals' work for food is under 24 hour surveillance to serve as baseline performances for the investigation of effects produced by opportunities for exercise, recreation, or social relations in the larger room.

The conditioned suppression procedure essentially consists of superimposing a negative Pavlovian conditioning procedure upon a positively reinforced operant baseline. The rate of the operant behavior is suppressed during the negative CS, with the magnitude of suppression monotonically related to shock intensity. However, this procedure is but one of many possible combinations of operant and Pavlovian conditioning procedures. For example, timing behavior of subjects rewarded for every response with an interresponse time greater than 30 sec. is disrupted during a positive Pavlovian stimulus. With this combination of procedures, the interresponse times decrease and the response frequency increase. Also, response rates maintained by intermittent positive reinforcement are higher during a combined positive and negative Pavlovian stimulus than during a simple negative Pavlovian stimulus. Presumably, the rate of operant responses during simultaneous presentation of positive and negative conditioned stimuli is functionally related to stimulus duration, shock intensity, and the magnitude of reinforcement. These relationships are currently being examined. In a related series of experiments, the rate of negatively reinforced operant responses is being examined during positive and negative conditioned stimuli presented singly and in combination.

The next set of results concerns the aversive control of behavior and the study of stress-inducing procedures. One research project studied total avoidance sessions as aversive events. Animals in avoidance experiments are usually observed only during the avoidance sessions, leaving us ignorant of effects the avoidance conditioning may have on pre-session or post-session behavior. To examine possible effects of the stressful avoidance conditioning on pre-session and post-session behavior, 30-minute and 1-hour sessions of food reinforced responding were inserted before and after avoidance sessions. When the stimulus conditions were identical in all food sessions, the avoidance reduced the rates of food-reinforced responding late in each food session. When stimulus conditions differed between food session that preceded and food sessions that followed the avoidance sessions, the reduction was observed primarily on those that immediately preceded the avoidance sessions. The reductions in food-responding are attributable to conditioned suppression (also known as "Conditioned Emotional Response") which is commonly produced by a warning stimulus followed by a brief aversive event (a shock). Here, the conditioned suppression occurred on an expanded time scale. The warning stimulus was food reinforcement for an hour,

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A procedure was devised in which animals' responding was produced and maintained by changes in frequency of randomly delivered shocks. This has produced some concern among avoidance theorists, since it suggests that reinforcement, usually characterized as an event that occurs at a discrete moment in time, can be based entirely on the average rate of occurrence of an event over extended periods of time, with no additional cues. A study is under way to determine the extent to which the reduction in overall rate of random shocks functions as if it occurred at a discrete moment in time. The original shock-frequency procedure is being used, but delays are imposed between responses and their consequent reductions in shock frequency. The ensuing delay-of-reinforcement function will be compared with those obtained in more conventional delay-of-reinforcement studies. An animal performing successfully on a conventional avoidance schedule is accomplishing at least two things: each response decreases the overall frequency of shock, and reduces instantaneously the probability of a shock. In conventional avoidance procedures it is impossible to assess the relative importance of these two features, either of which could reinforce the avoidance response. Procedures were devised that manipulated independently the overall shock rate relative to the animal's performance. The animal could eliminate single, impending shocks; this could result in no change in overall shock rate, or in an increase in overall shock rate. Preliminary work indicated that rats will respond to reduce the instantaneous shock probability provided that this does not increase the overall shock rate. But a recent modification of the procedure has shown that in some situations the animals will respond to eliminate the single, impending shock even when this results in a fivefold increase in the overall rate of shock. Present work is devoted to isolating the circumstances that maintain responding in the face of response-produced increases in overall shock rate.

Another research effort dealt with a technique for maintaining prolonged vigilance in rhesus monkeys. The monkeys, held in restraining chairs and housed in light-tight booths, were trained to respond within 10 seconds in the presence of a dim light to avoid a brief electrical shock. Once avoidance was well trained, a response in presence of the light avoided the shock and decreased the intensity by  $1/5$  of a log unit for the stimulus programmed on the next trial. A failure to respond in the presence of the light resulted in a brief electrical shock and increased by  $1/5$  of a log unit the stimulus programmed for the subsequent trial. Using this method we could track the visual detection threshold over long periods of time. Preliminary results indicate that when the monkeys are required to maintain a 24 hour vigil during which 2-3 signals per hour are programmed, no decrement in performance is observed.

Rhesus monkeys were trained to respond on a fixed ratio schedule in the presence of a blue light to avoid the onset of a green light.

Failure to avoid the onset of the green light required the monkeys to respond on a second fixed ratio schedule to avoid the onset of a red light paired with 3 unavoidable shocks. Once the monkeys were stabilized on this avoidance performance, the size of the fixed ratio required for avoidance was systematically increased in the presence of the green light and kept constant in the blue light. The results showed that as the ratio size was increased the number of avoidances decreased. When the ratio size correlated with the blue light was systematically increased, while that in green was kept constant, a similar inverse relationship between ratio requirement and number of avoidances completed was observed.

A third set of experiments are oriented toward the study of internal mechanisms which mediate behavior. A number of these experiments have been concerned with the septal area of the forebrain. Because organisms subjected to ablation of the septal region fail to respond normally in the presence of stress-producing stimuli which suppress responding in the intact animal, a variant of the Sidman avoidance procedure is being used to identify the role of this neural structure in mediating the behavioral response to stress. This procedure involves the parametric reduction of the response-shock interval to a value of one half or less of the shock-shock interval. Under these conditions normal rats cease to respond in terms of their previous training. This same effect, a suppression of responding, also occurs in the septal animal. Because a behavioral procedure has been found for which these brain-lesioned animals show no functional deficit it is now possible to begin isolating the basic variables which distinguish this procedure from other stress procedures showing deficits. One hypothesis suggested by the present experiment is that the septal region permits the organism to deal with concurrent contingencies when one such contingency is stress-related. Ablation of either the septal region or the hippocampus changes in the response of an organism to behavioral stress. Changes in the basic variables in these stress procedures may permit the organism to respond normally if the procedure places no functional demand on the ablated neural structure. Similarly, if the procedure does require the use of the ablated structure a deficiency in performance is observed. It is also possible to arrange a procedure in which the ablated structure would, in the intact animal, interfere with performance. In this case, the lesioned animal performs better than normal subjects. The present experiment was undertaken to account for the observed differences in behavior discussed above. The Sidman avoidance procedure was selected as one which had not been applied to animals with this type of brain lesion. It was found that septal rats can perform this task and, as anticipated, are superior to normal subjects in that they avoid more shocks while emitting fewer responses. Thus, removal of the septal region improves the organism's capacity to respond to this stress-producing procedure. A tentative hypothesis suggested by the data is that destruction of this region of the brain favors those procedures in which the organism is required to emit rather than withhold responses.

Preliminary data suggest that hippocampal lesioned animals will be deficient in this task. These results should be of significance in differentiating the roles played by these neural structures in the mediation of the organism's response to behavioral stress.

Under conditions that suppress the food-rewarded responding or normal rats, rats with lesions in the septal region of the forebrain continue to respond. Because this deficit is not motivational, septal ablation should provide useful information on the warm-up effect in avoidance. When normal rats are conditioned to avoid electric shock, they often take many shocks at the beginning of each daily experimental session even though they may avoid all shocks late in the session. This change in intrasession performance is known as the warm-up effect. Some investigators have suggested that warm-up reflects a build-up of motivation within an experimental session; recent evidence has shown that warm-up is a transient suppression of responding. If this is the case, then septal ablation may eliminate the warm-up producing a constant level of performance throughout sessions. Preoperative baselines for avoidance behavior, including the warm-up, were established in a number of rats. These were then operated and their septal nuclei bilaterally ablated. Following postoperative recovery they were re-run to compare their preoperative and postoperative performances. The performances were strikingly similar. This result questions the usual interpretation of suppression of food-reinforced responding in septal animals, and favors a different interpretation, that, rather than being unable to suppress or inhibit responding, the organism with septal lesion fails to adjust its performance to two or more concurrent contingencies. The present study was designed to examine further the interpretation that, following septal ablation, the organism fails to adjust its performance to two or more concurrent contingencies. Rats were trained to press a lever for food; then the food reinforcement was discontinued and replaced by a Sidman avoidance schedule. Finally, the food and avoidance contingencies were combined, operative on the same lever-press response. Previous work has shown that the reintroduction of food will not appreciably improve the avoidance of shock, even though the same response both avoids shock and produces food. Preoperative results confirmed this. In animals with lesions in the septal region, the reintroduction of food produced marked improvements in performance; the animals responded as though sensitive to the food contingency alone, which incidentally produced improved avoidance.

This is a further study illustrating that following septal ablation an animal can no longer adjust its performance to two or more concurrent contingencies. When normal rats are given the option of avoiding shocks either by holding a lever down or by pressing the lever repeatedly, at least once every 20 sec, they spend over 90% of their time holding the lever down. This lever holding persists even if the hungry rats are rewarded with food only for repeated pressing. Rats with bilateral septal lesions demonstrate that the same general pattern occurs in the absence of food reinforcement.

Given the option, these rats will avoid shock by lever-holding rather than by repeated lever pressing. However, when food is made contingent upon repeated pressing, these animals quickly change over to repeated pressing. With subsequent removal of the food contingency, they revert back to lever-holding. Once again, when faced with a concurrent combination of two schedules, the rats with septal lesions perform as though responding to one contingency alone.

A set of recent experiments has suggested that, in humans, the cardiovascular system may modify motor behavior. Specifically, reaction time in humans seems to be related to the amount of sinus arrhythmia, even when the arrhythmia is produced by external stimulation. We are attempting to evaluate more directly the relation between cardiovascular system and motor behavior. Monkeys were trained to release a lever quickly at the presentation of an auditory tone. With stable latencies for this response, the tone is introduced at varying points in the cardiac cycle. For example, in a given session the tone will sometimes be initiated 30 msec after an R-wave, and sometimes 175 msec after an R-wave in the monkey's electrocardiogram. The two values are alternated randomly, and response latencies are recorded separately for the two modes of presentation. The response latencies have been found to differ for different periods after the R-wave; however, the latencies come to differ only after several days of exposure to a single pair of time values. This suggests that instead of reflecting relatively direct facilitory or inhibitory effects originating in the cardiovascular system, the differences in response latency must reflect cueing functions of events in the cardiovascular system.

Adult rats were stressed by subjecting them to unpredictable inescapable electric shocks presented for 15 hrs on four successive days. Before and after each stress period the animals were injected (IP) with  $^3\text{H}$ -thymidine. Using autoradiographic techniques it was found that the autonomic ganglia of stressed rats showed increased numbers of labelled glial cells, and labelled neurons were also detected though no direct evidence of division of neurons was present. This interdisciplinary effort to identify structural changes in the nervous system in response to the functional demands of behavioral stress is continuing with emphasis being placed on determining the variables in the stress procedure which elicit in this response by the nervous system. Comparisons of signalled and unsignalled shock have been run, however, the tissue from these animals is still being processed.

Partial recovery of function following insult to the central nervous system is well-documented. However, the physical basis for this recovery remains undefined. Recently developed techniques permit the introduction of radioactive label into neurons and the mapping of neural pathways and the terminal endings via autoradiographic techniques. This technique is maximally efficient when used in the

visual system because the injection of label into the eye permits the uptake of the isotope by retinal cells. This isotope is then transported to the lateral geniculate. The availability of this technique makes the visual system an ideal model on which to test the hypothesis that axonal sprouting may be involved in recovery of function. This hypothesis suggests that destruction of neurons results in the de-nuding of synaptic sites and that recovery of function occurs when axons adjacent to these sites sprout new terminals. Previous tests of this hypothesis have had limited success because earlier methods required 16 months of postoperative recovery before sprouting could be identified. Autoradiographic techniques, however, can now be applied to this problem and allow for the possibility of longitudinal study of sprouting beginning 5 days or less following production of the neural insult. If it is possible to identify sprouting within 2 weeks to 30 days following a lesion, a stronger case could be made for implicating this phenomenon in the functional recovery of the nervous system. The lesion being used in the present study is unilateral enucleation. This procedure results in the destruction of some of the neural fibers terminating in the lateral geniculate. Consequently, synaptic sites should become available to axons from the contralateral retina. The contralateral eye was injected with labelled lucine and autoradiographs made of the terminal patterns of these fibers in the geniculate. A comparison of the terminal patterns of these subjects with those of intact animals should reveal sprouting if it has occurred. To date, tissue from subjects injected 5, 10, 30, 60, 90 and 180 days after unilateral enucleation is being processed for autoradiography.

A study of the effects of castration and injections of testosterone propionate upon second order fixed ratio avoidance has been completed. A rhesus monkey was trained to respond on a fixed ratio - 40 in the presence of a blue light to avoid the onset of a green light. Failure to avoid the onset of the green light required the monkey to respond on a second fixed ratio 40 to avoid the onset of a red light paired with 3 unavoidable shocks. The results of this training produced a stable performance in which the monkey avoided 50% of the time in the presence of the blue light and 50% of the time in the green light. The monkey was castrated after 2 weeks of this stable baseline performance. Post operation, the monkey made over 95% of his avoidances in the green stimulus and less than 5% of his avoidances in the blue stimulus. Exogenous injections of testosterone propionate increased the number of avoidances in the blue stimulus and decreased the number of avoidances completed in the green stimulus. When the monkey was injected with placebo (sesame oil), he made 95% of his avoidances in green and less than 5% of his avoidances in blue.

The presentation of a previously neutral stimulus terminated by unavoidable shock consistently reduces the rate of positively reinforced operant behavior. The present series of experiments was designed to investigate concurrent physiological events during training with this conditioned emotional response (CER) procedure. Heart

rate, diastolic blood pressure and systolic blood pressure were found to follow similar patterns throughout the acquisition of the CER. Initially, the behavioral and autonomic responses decelerated during the pre-shock stimulus. Subsequently, heart rate and blood pressure accelerated to asymptotic levels approximately 150 to 200 percent above normal while the operant behavior continued to be depressed to a near zero rate. The autonomic activity did not exhibit this biphasic development when the stimulus-shock sequence was presented during non-work periods. The collective data suggest that the initial deceleration of heart rate and blood pressure is a secondary effect due to the drastic decrease in physical activity caused by the conditioned suppression of the operant behavior, later followed by a direct conditioned acceleration of the autonomic responses. In the current investigation, the electrical activity in various peripheral muscle groups is being compared with changes in the behavioral and autonomic responses throughout the acquisition of the CER. The data to date indicate that muscular activity in all muscle groups is reliably decreased during the pre-shock stimulus, similar to the operant behavior. Simultaneously, however, heart rate and blood pressure levels accelerate to above normal levels in spite of the decrease in muscle tonus and bodily activity. Apparently, the increase in the autonomic responses are not mediated by an increase in covert muscular activity contracting upon the peripheral vasculature but are directly conditioned by the experimental procedure.

Ward 108 of WRGH is a ward for soldiers diagnosed as character disorders and is run according to operant conditioning principles. Currently the program is being changed by instituting a curriculum designed to establish some basic social, educational, work, military, and recreational behaviors in the men who pass through the ward. The approach is to use modern, instructional techniques in this curriculum which free the courses from a dependence on a specific teacher. This has several advantages. First, it means that relatively unskilled personnel can administer the courses. Second, courses will not cease to be taught when an instructor leaves the post. Third, the curriculum, when developed and revised, will constitute a package of educational material that can be transmitted for use on other wards in other hospitals. Following the course work, the program will be directed at having the patients transfer the learned behavior from the classroom to the outside world in gradual steps.

3A061102B71R 03 025, BIBLIOGRAPHY

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
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3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DES'N INSTR' <sup>a</sup>	9. SPECIFIC DATA- CONTRACTOR ACCESS	10. LEVEL OF SUB A. WORK UNIT
69 01 31	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES: <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY	61102A	3A061102B71R		03	026		
B. CONTRIBUTING							
<del>OCRELXWZKXK</del>	1412A(2)						
11. TITLE (Precede with Security Classification Code) <sup>a</sup> (U) Analysis of Behavior and of Mediating Mechanisms - Psycho-physical and Electrophysiological Data Correlation (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
012900 Physiology 013400 Psychology - Indiv							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
08 63		NA		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PREVIOUS		B. FUNDS (In thousands)	
B. NUMBER: <sup>a</sup>				FISCAL YEAR		CURRENT	
C. TYPE:				69		5	
D. KIND OF AWARD:				70		4	
E. AMOUNT:						110	
F. CUM. AMT.						125	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: <sup>a</sup> Walter Reed Army Institute of Research				NAME: <sup>a</sup> Walter Reed Army Institute of Research			
ADDRESS: <sup>a</sup> Washington, D.C. 20012				ADDRESS: <sup>a</sup> Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Meroney, COL W.H.				NAME: <sup>a</sup> Shelburne, MAJ, S.A.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-5257			
				SOCIAL SECURITY ACCOUNT NUMBER: <span style="background-color: black; color: black;">[REDACTED]</span> <span style="border: 1px solid black; padding: 2px;">PII Redacted</span>			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Considered				NAME: Sheatz, G.C., Ph.D. DA			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Perception; (U) Sensory Neurophysiology; (U) Psycho-physiological Correlates; (U) ERG; (U) EEG; (U) Visual Perception; (U) EMG							
23. TECHNICAL OBJECTIVE, <sup>a</sup> 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) This research is concerned with the perceptual determinants of behavior as these are reflected in the relationship between sensory input and neurophysiological function. Emphasis upon the visual system as a model for such psychophysiological interactions provides the basis for analyzing central and peripheral mechanisms involved in differential sensitivity to the environment and attentional factors in perceptual performance.							
24. (U) Psychophysical methods and electrophysiological recording techniques are utilized to correlate sensory input from the environment with behavioral and neurophysiological output in the analysis of mediating mechanisms in perceptual performance. Micro-electrode and gross electrode electroencephalography, electroretinography, and behavior control procedures are integrated through the application of computer programming to data analysis.							
25. (U) 69 01 - 69 06 Averaged evoked potentials to meaningful stimuli during problem solving were investigated to assess the effects of (a) the processing required by the relevant stimuli and (b) the expectancies generated by the sequential contingencies. Statistical analyses of the relation between intelligence and averaged evoked potentials were made on a group of enlisted men with some response measures showing a significant correlation with the Army Classification Battery. Data were obtained from single photoreceptors related to basic spectral mechanisms. Electrical activity of single motor units in human biceps stress by sinusoidally varying isometric loads was obtained. The effect of 5-hydroxytryptophan on a patient with trisomy 21 was studied. The effect of controlled intraocular pressure on ERG and optic nerve potentials was studied as a model of glaucoma. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 June 69.							

DD FORM 1498-1

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 66 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061102B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 03, Psychiatry

Work Unit 026, Analysis of behavior and of mediating mechanisms:  
Psychophysical and electrophysiological data  
correlation

Investigators.

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Abner B. Lall, M.S.; CPT Ted Lawwill, MC;  
CPT Peter Rosenberger, MC; Guy C. Sheatz, Ph.D;  
MAJ Samuel A. Shelburne, MC; Richard Srebro, M.D.;  
CPT Peter D. Williamson, MC

Description.

The purpose of this work unit is to investigate relations between the physical and psychological input of stimulation, both historical and contextual, and the organism output of neurophysiological functioning and behavior. It is at this interface between physiology and behavior where some of our most baffling problems occur and currently where some of our most significant advances are being made. In the end it is the behavior of the individual that is of concern, but well functioning behavior is supported and in detail mediated by physiological mechanisms and their integrative action. The elucidation of these mechanisms is fundamental to solving a wide range of problems. The principal methods are those of psychophysics, biophysics, sensory stimulation, experimental psychology, electrophysiological recording, and statistical analysis. The work is divided into two categories: (1) correlative behavioral and electrophysiological investigations, and (2) studies of central and sensory mechanisms.

Progress.

1. Correlative behavioral and electrophysiological investigations.

a. Visual evoked responses during problem solving. The series of experiments studying differences in averaged evoked potentials to meaningful and non-meaningful visual stimuli have been continued and several extensions from the basic problem have been made. It has been found that stimuli, numbers and letters, produce larger evoked responses when they are relevant to a problem solving task than when they are not. In the recently completed "midline" experiments 11 out of 11 subjects showed a statistically significant greater amplitude of evoked response

relevant stimuli than to the identical stimuli when they were irrelevant, with the brain potentials derived from vertex and occipital locations. These results were obtained with a generalized mean amplitude measure and the data are now being analyzed in finer detail with extensive measuring techniques. A great deal of computer processing has been devoted to (a) sorting the responses into categories depending on the relevance of the stimulus and prior sequence of stimuli, (b) averaging similar evoked responses from a number of runs in different sessions, (c) measuring amplitudes at certain latencies, and (d) performing statistical evaluations of response differences. Differences between the brain responses to relevant and irrelevant stimuli were obtained for both long and brief (10 micro-sec) duration light stimuli. Thus, differential scanning of the physical stimuli, for example by eye movements or by neural modulation of afferent input, is not necessary to obtain "meaningfulness" effects in the averaged evoked potential. This finding strengthens the interpretation that the AEP can reflect the neural post-perceptual processing of external stimulation and not merely the primary sensory input. Refinements permitted assessing the effects of (a) the different processing required by the relevant stimuli and (b) the expectancies generated by the sequential contingencies. The data indicate that the AEPs are sensitive not only to whether post-perceptual processes are required (relevant vs. irrelevant) but also to the nature of these processes ("storage" vs. "problem solving"). Expectancies controlled by the experimental procedure had marked effects, but differences are still found when the stimulus sequence was randomized and therefore are interpreted to be associated with post-perceptual neural processes. Thus, the AEP reflects rather subtle differences in the subject's processing of visual information, as well as differences in the physical stimuli.

In another series of experiments cerebral hemisphere dominance effects were investigated using similar experimental designs. In these "laterality" experiments 7 out of 8 demonstrated a significant difference between relevant and irrelevant stimuli at homologous parietal and occipital electrodes. In addition, 5 of 8 subjects showed clear differences in amplitude between the left and right hemispheres, the left hemisphere usually being dominant. The nature of these cerebral dominance effects appears to depend markedly on the nature of the visual stimuli. This problem is being investigated further.

Reaction time measurements are in progress with the same stimuli to study the relationships between the sensory information processing and motor responses. Computer analysis is also being performed on data obtained from subjects during the learning process. Using the same visual stimuli several subjects have performed a much more difficult problem solving task involving calculations

with the number stimuli and these results are also being analyzed. Preliminary results indicate that the AEPs are sensitive to the tasks being performed with the stimuli. Some of these findings were presented at an evoked potential conference in San Francisco and will appear in a NASA-AIBS sponsored book.

b. Brain activity and assessment of intelligence. Correlations between a large number of parameters of the visual evoked response and measurements of intelligence were made in 41 subjects. Many more subjects were run but their Form 20's were not available. Intelligence assessment was derived from the Army Classification Battery and the evoked brain responses were obtained to simple light flashes under a minimal task load condition. Several of the correlations were statistically significant and a new series of experiments is underway with some fundamental additions to the experimental procedure. This research is directed to the question whether the averaged evoked potential can be used as a culture free, non-verbal, non-motor test of intelligence.

c. Behavioral and physiological spectral mechanisms in a vertebrate. In an elucidation of basic mechanisms underlying the visual system, spectral sensitivity determinations have been made so that quantitative comparisons can be made between these behavioral data and electrophysiological and photochemical action spectra from the same organism (*Rana Catesbiana*). The behavioral testing involves a forced-choice, paired-comparisons preference procedure with test lights of controlled wavelength and energy. For some experiments a constant white light constituted the comparison stimulus. The percentage of trials that the test light was chosen increased as a function of light energy at each wavelength, except at the highest light levels. A 50% criterion was applied to these response-energy curves to obtain the relative energy required at each wavelength to "match" the constant white light. The reciprocal of these energy values yield the spectral sensitivity function. The dark-adapted curve was much narrower than the Dartnall pigment nomogram, ERG spectral sensitivity curves, or the dominator-type spectral sensitivity curves obtained from retinal ganglion cell recording. As a test of the multiple contributions to the behavioral curve and an attempt to isolate the blue mechanism, two groups were run under yellow-adapted conditions. The average yellow-adapted spectral sensitivity curve was nearly flat from 400 to 560 nm.

It has been reported (Muntz, 1962) that single units in the frog diencephalon responded more strongly to short wavelength light than to any other wavelengths. His data suggest a degree of spatial localization in the CNS of wavelength information that is without parallel in the literature. Because of the implications of this principle for understanding neural coding, electrical recording from this area has begun with the purpose of obtaining spectral sensitivity curves which can be related

to retinal spectral mechanisms, on the one hand, and behavioral spectral sensitivity curves, on the other.

d. Alpha EEG activity and eye orientation. A paper showing that visual input, not eye orientation, is a primary factor in controlling EEG alpha activity is being published in the Journal of Electroencephalograph and Clinical Neurophysiology. Mulholland and Evans' hypothesis concerning eye orientation was tested using a larger sample, fixation targets, eye movement recording, and a dark condition which eliminated the effects of differential visual input. These experiments showed that vertical elevation of the eyes had no direct influence on alpha EEG activity. This was best demonstrated by the data obtained in the dark where a significant increase with eye elevation was not found in group statistics or in individual analyses on the 35 subjects (with one exception which might be attributed to Type 1 error). Differences in alpha EEG related to eye position in the light condition were decreased when differential visual input due to eye position was decreased by the use of fixation targets. In these experiments the main variable controlling the increase in alpha activity was the reduction in visual input, caused either by closing the eyes or extinguishing the illumination. In the publication the effects of variables confounded with eye position, e.g., patterned visual input to the retina, accommodation, fixation, and effort required to maintain specified eye positions, are discussed.

e. Studies of normal development of visual and auditory evoked EEG responses. The purpose of the research is to examine and evaluate electrophysiological measures which are related to the developing sensory, perceptual and cognitive processes of children. The general method is that of recording of computer averaged evoked EEG responses to visual and auditory stimuli in normal children and in children with abnormalities of sensory and mental development. The relationship between sensory evoked potentials, electroencephalographic, behavioral and biochemical parameters of development is being examined. The applications of evoked response recording to clinical diagnosis, i.e., in patients with sensory, mental, and perceptual abnormalities, are being investigated and evaluated. The research will provide basic data on the maturation of the EEG and sensory evoked potentials of human children during the course of development and will attempt to relate data obtained from psychological and neurological testing with the neurophysiological data. It will provide normative data for comparison with that obtained from patients with sensory and neurologic handicaps.

Several investigations are in progress:

(1) Longitudinal and cross-sectional studies of the relations between evoked responses and age, behavioral state, EEG background

tracing, stimulus intensity, modality and order.

(2) Correlations between evoked response measures and scores on the Bayley Scales of Infant Development and other psychological tests; investigation of possible response differences in home-reared and institutionalized infants.

(3) Studies of response decrement with repetitive stimulation in normal versus developmentally retarded infants.

(4) Studies of evoked responses to paired stimuli as the significance or task relatedness of the stimuli are varied; these studies will be done in normal children and those with learning problems.

In the normative studies the emphasis in the past year has included completion of data gathering in several age groups and preparation of data on evoked response characteristics and EEG for computer statistical analysis. In a sample of approximately 100 infants, 1 month, 6 months, and 12 months old, we are investigating possible correlations between auditory and visual evoked response characteristics and scores on standard test of mental and motor development. Developmental patterns are being studied by Dr. A. Lodge, Childrens' Hospital, Washington, D.C. We have also examined over 350 auditory response recordings of normal hearing children, the interest here being to characterize the responses near threshold and thus help to provide normal standards by which to evaluate the EEG evoked response audiograms of hearing-impaired children.

f. EEG audiometry. The use of EEG audiometry as an aid in the diagnosis of deafness and hearing deficits in the young was studied. Clicks and pure tone stimuli were used. We are at present summarizing our experience with the first 100 children under 3 years of age referred for evaluation of possible hearing loss using the method of computer average evoked EEG audiometry. Comparison with the results of clinical audiometric methods, follow up data, and a discussion of the uses and limitations of the method are being included in this report.

g. Evoked responses in mentally retarded and brain damaged children. We have continued to investigate possible relationships between mental defect and evoked response characteristics. In a study of auditory evoked response decrement with repetitive stimulation with clicks, the findings in normal infants and infants with 21-Trisomy differ significantly. Normal infants (N=61) 6 and 12 months old show smaller amplitude averaged responses ( $p=.001$ ) to the last 25 of a series of 100 clicks than they do to the first 25 of the series. Mongoloid infants (N=42) 6 and 12 months old do not show significant response decrement. Neither normal 1 month old infants (N=22) nor 1 month old mongoloids

(N=27) show response decrement. Further studies are being carried out to find out whether the response decrement is related to habituation or the recovery cycle characteristics of the auditory system. These findings were part of a paper presented to the American Academy for Cerebral Palsy in December, 1968 and a report is being readied for publication. For paired stimuli experiments instrumentation is now being tested.

h. Ultra-violet mechanisms in primitive photoreceptors. Recording ERG's from the corneal surface of the dorsal ocellus in Limulus polyphemus has shown that the dorsal ocellus is primarily an ultra-violet receptor, with a lower sensitivity peak in the visible wavelengths. These electrophysiological data have been correlated with behavioral data. Positive phototaxis was elicited predominantly via ultra-violet radiation. The visible wavelengths do not seem to elicit phototaxis. In order to understand more fully the UV-receptor, action spectra of single visual cells in the ocellar retina have been obtained. Microelectrode techniques have been adapted for recording intracellularly. The single visual cells either give maxima action of their spectra in the near ultra violet or in the visible region of the spectrum. Thus these two classes of visual cells are proposed to be the cellular basis for the near UV and long wavelength mechanisms.

## 2. Studies of central and sensory mechanisms.

a. Glaucoma mechanisms: pathogenesis of optic nerve atrophy. Our third paper on the pathophysiology of the distal segment of the optic nerve has been accepted for publication. We are half way through a fourth study of the distal optic nerve segment using a calorimetric technique. A thermocouple probe is inserted into the optic nerve of a cat through the anterior segment of the globe. Thus far we have been able to demonstrate a change in the vasculature conductivity of the optic nerve with only a 10 mmHg change in the intraocular pressure. Our method is several times more sensitive than any previously used and has exceeded our expectations. Concurrently, we are developing a micro-oxygen electrode probe which can be inserted into the optic nerve. This study will furnish further information concerning the pathogenesis of optic nerve atrophy in chronic open angle glaucoma.

b. Evoked responses in patients with visual impairment. The visual occipital evoked response is potentially useful for testing the presence or absence of vision. The extreme difficulties of stimulus control in young subjects may limit the method for clinical diagnosis, but even at present certain inferences can be drawn from the presence or absence of responses or gross asymmetries of response. In the past 1 year period we have had the opportunity to observe 6 patients at the Childrens'

Hospital of the District of Columbia with prolonged acute cortical blindness who showed partial or full recovery of vision. We recorded visual evoked potentials during the course of recovery in an attempt to elucidate the pathologic physiology of this relatively rare condition. A report is being prepared for publication in collaboration with Drs. James Manson and Eliot Wilner of the Childrens' Hospital, Department of Neurology.

c. Organization of single motor units in human muscle under stress. A series of experiments is in progress to measure electrical activity of single motor units in human brachial biceps stressed by sinusoidally varying isometric loads. The protocol is as follows: a subject is required to produce a sinusoidally varying isometric tension by matching his tension output as displayed on an oscilloscope to a target trace displayed on the same scope. Both frequency and maximum load can be varied by the experimenter.

Data have been collected in 38 runs involving three subjects. These are in the form of polygraph records of single motor unit action potential trains recorded concurrently with tension output, subject task, and a time mark. To accomodate the low frequency response of the polygraph runs are recorded at high speed on magnetic tape and played back slowly for polygraphic display. Of interest is how firing frequency of a single motor unit, and the number of units active, vary with amplitude and frequency of the imposed load.

Currently loads up to 7.5 kg and frequencies from .05 to 1.5 Hz are under study. Preliminary results suggest marked changes in the muscle input-output relationship as frequency is increased. At the higher frequencies tension lags electrical activity by more than 90°. How this relationship holds as the muscle is stressed with greater loads is currently under study.

d. Vascular conductivity of the ocular papilla. The small vessel structure of the papilla and lamina cribrosa in man is the ciliary circulation and this circulation has a lower intravascular blood pressure than the inner retinal circulation. Elevated intraocular pressure compromises the small vessel structure of the papilla and lamina cribrosa and it has been hypothesized that this creates a functional disturbance. We have developed a clinical technique to study the dynamic relationship between the circulation of the optic nerve and its function in man. A miniature light-sensing diode has been placed in the film plane of the Zeiss fundus camera. Indocyanine green is injected intravenously and an infrared absorption curve is obtained from the optic disk as the bolus traverses the eye vasculature. This curve reflects the vascularity and rate of blood flow in the intraocular tissues. The camera facilities of the WRGH Department

of Ophthalmology are being used and the clinical study is being performed on glaucoma patients. The value of these measurements is considerable since glaucomatous visual field defects may be preceded by and are most likely the result of diminished blood flow in the distal segment of the optic nerve.

e. Serial evoked response studies in patients with Down's syndrome. Data collection for a double blind study of evoked response characteristics of infants with 21 Trisomy who are receiving the serotonin-precursor 5-hydroxytryptophan or a placebo is continuing. The children are being tested within 4-5 days of birth and at 6 months, 1,2, and 3 years.

f. EEG sleep and evoked response in patients with disorders of serotonin and catecholamine metabolism. We have recently had the opportunity to study the all night sleep patterns in a 10 year old patient before and during the administration of L-DOPA. This child had profound insomnia, gastrointestinal disturbance, and intermittent spasm of the posterior cervical musculature. Her older sister was severely afflicted with an atypical form of dystonia musculorum deformans. Sleep abnormalities have been reported in patients with dystonia but, to our knowledge, the effect of exogenously administered L-DOPA in this condition has not been reported. Dopamine has been implicated by several investigators as playing a role in the regulation of the sleep-waking cycle. This study and the mongoloid study described above are being done in collaboration with Dr. Mary Coleman. The biochemical, electrophysiological and clinical findings were reported at the American Neurological Association Annual Meeting June, 1969.

g. Effect of light damage on the retina. The investigation of retinal damage by visible light has been continued along a new track. It is currently believed that the mediating factor in light damage to the retina is chronic visual pigment bleaching. We have constructed and are now using apparatus which permits differential damage of scotopic (rod) and photopic (cone) retinal elements in the domestic cat.

h. Development of a field unit for ERG and EOG recording. A field unit capable of amplifying the electroretinogram and the electro-oculogram has been developed and is now being tested. It is a miniature integrated circuit amplifier which is attached directly to the patient. We have demonstrated that its characteristics are more than adequate and we are in the final stages of testing its field applicability and writing a report for the ophthalmic literature.

i. Operant conditioning psychophysical determination of spectral sensitivity. Using an extremely sensitive CER operant

conditioning procedure in connection with an up-and-down staircase psychophysical tracking procedure the absolute visual threshold in the white rat has been measured. The experimental procedure was extended to utilize monochromatic radiation and the relative radiance required for rod vision at the visible wavelengths of light (400 to 700 nm) was behaviorally determined.

j. Eye damage and chromium deficiency. We have consulted on a study of chromium deficiency in the white rat with Dr. Mertz, Department of Biochemistry, WRAIR. Rats deprived of chromium in their diet develop corneal vascularization and opacity. The etiology of these changes is obscure. We are studying the development and progression of the eye changes using both standard ophthalmological techniques as well as special histological methods.

k. Dark adaptation and night vision under hemoglobin saturation. The effect of a hemoglobin saturation of 85% oxygen (equivalent altitude of approximately 15,000 feet) on the proficiency of night vision and dark adaptation is awaiting apparatus.

#### Summary and Conclusions:

This work unit has been concerned with the neurophysiological mechanisms mediating between physical and psychological stimulation and behavior. The research has been divided into two categories:

- (1) Correlations between behavioral and physiological functioning;
- (2) Studies of central and sensory mechanisms.

In the first group of studies, EEG averaged evoked potentials have been found to yield a wealth of information in a wide variety of problems. Differential scanning of the physical stimuli, for example by eye movements or by neural modulation of afferent input, is not necessary to obtain "meaningfulness" effects in the averaged evoked potential. This strengthens the interpretation that the AEP can reflect the neural post-perceptual processing of external stimulation and not merely the primary sensory input. The research indicates that the AEPs are sensitive not only to whether post-perceptual processes are required (relevant vs. irrelevant) but also to the nature of these processes ("storage" vs. "problem solving"). Furthermore, expectancies controlled by the experimental procedure had marked effects. The use of the averaged evoked potential as a culture free test of intelligence has been supported by significant correlations in a group of EM and further study is in progress. Comparisons of spectral sensitivity at the electro-physiological and behavioral level for a color-detecting vertebrate eye have been obtained and are being pursued at a more central level. Alpha EEG activity has been found to depend primarily on visual input and not on eye orientation. Visual and auditory evoked EEG responses have

been obtained and evaluated as related to the developing sensory, perceptual and cognitive processes in the young. In a sample of approximately 100 infants correlations between auditory and visual AEP characteristics and scores on standard test of mental and motor development are being investigated. With repetitive click stimulation the amplitude of the evoked responses decreased in a large group of normal infants, but did not in a large group of 21-Trisomy infants. Electrical recording from single photoreceptors in a primitive photoreceptor system has revealed two classes of cells as the basis of near-UV and long-wavelength spectral mechanisms, also revealed in a behavioral study.

In the second group of studies a change in the vasculature conductivity of the optic nerve has been found with only a 10 mmHg change in the intraocular pressure. The pathologic physiology of patients with prolonged acute cortical blindness who showed recovery was studied by visual occipital evoked responses. The organization of single motor units in human muscle under stress has shown marked changes in the input-output relationship as frequency of sinusoidally varying isometric loads was increased. A clinical technique to study the vascularity and rate of blood flow in intraocular tissues has been developed using an infrared absorption curve and intravenously injected dye. A double-blind study of evoked response characteristics of 21-Trisomy infants who are receiving the serotonin-precursor 5-hydroxytryptophan or a placebo is continuing. The effect of L-DOPA and EEG sleep and evoked response in patients with disorders of serotonin and catecholamine metabolism was investigated. In studying the effect of light damage on the retina an apparatus had been constructed which permits differential damage of scotopic (rod) and photopic (cone) retinal elements. A compact unit for ERG and EOG recording has been designed and constructed. The absolute visual threshold and spectral sensitivity have been behaviorally determined in the rat by the use of a combination of operant conditioning and psychophysical tracking procedures.

3A061102B71R 03 026, BIBLIOGRAPHY

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**PROJECT 3A061102B71P**  
**BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE**

**Task 01**  
**Biochemistry**

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION NO.	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA OA6430	69 07 01	DD-RM (A1) 698
3. DATE OF SUMMARY	4. KIND OF SUMMARY	5. SUMMARY CLASSIFICATION	6. WORK SECURITY CLASSIFICATION	7. RESEARCHING AGENCY	8. REPORT NUMBER	9. CONTRACTOR ACCESS
69 01 31	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO. / CODE(S)	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	11. LEVEL OF EFFORT		
	61102A	3A061102B71P	01	A. WORK UNIT		
a. PRIMARY				070		
b. CONTRIBUTING						
c. SUPPORTING	1412A(P)					
11. TITLE (Project or Work Security Classification Code)						
(U) Biochemical Activity in Health and Disease (09)						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS						
012900 Physiology		002300 Biochemistry				
13. START DATE	14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
62 06	NA		DA		C. In-House	
17. CONTRACT/GRANT	18. EXPIRATION		19. RESEARCH ESTIMATE		a. PROFESSIONAL MAN YRS	
NA			FISCAL YEAR		b. FUNG (in Man-Yrs)	
a. DATE/EFFECTIVE	d. AMOUNT		69		9	
b. NUMBER	e. CUM. AMT.		70		140	
c. TYPE					9	
e. KIND OF AWARD					140	
19. RESPONSIBLE DSI ORGANIZATION			20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research			NAME: Walter Reed Army Institute of Research			
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			SOCIAL SECURITY ACCOUNT NUMBER: <span style="background-color: black; color: black;">[REDACTED]</span> <span style="float: right;">PII Redacted</span>			
21. GENERAL USE			ASSOCIATE INVESTIGATORS			
Foreign intelligence considered.			NAME: Mertz, W. M.D.			
			NAME: <span style="float: right;">DA</span>			
22. KEYWORDS (Project or Work Security Classification Code) (U) Nucleoproteins; (U) Protein Synthesis; (U) Fatty Acids; (U) Enzymes; (U) Bacterial Metabolism; (U) Ribonucleic Acids; (U) Enterobacteriaceae.						
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Number Individual paragraphs identified by number. Precede text of each with Security Classification Code.)						
23. (U) Basic biochemical responses to injury and in disease will be studied. Potential means of controlling infectious agents by biochemical regulatory mechanisms will be investigated.						
24. (U) The modern techniques and analytical approaches available to research biochemistry will be used to evaluate responses and to study the basic causes for disease production by infectious agents.						
25. (U) 69 01 - 69 06 During sabbatical absence in England, a crystalline fiber of TRNA TYR was obtained. X-ray diffraction analysis showed that helical regions of this TRNA are more or less parallel to the axis of the fiber rather than perpendicular. Based on this information work is continuing on obtaining a working model of TRNA TYR. Further isolation and purification of TRNA molecules involved in the regulation of RNA synthesis in bacteria is in progress. Work to assess relationships at the nucleic acid level in enterobacteria is being continued. A study of diverse strains of <u>Escherichia coli</u> reveals significantly more divergence within this species than expected. The genera <u>Shigella</u> , <u>Proteus</u> , <u>Citrobacter</u> , <u>Alkalescens-Dispar</u> group and <u>Erwinia</u> are being studied for sequences held in common with <u>E. coli</u> . These groups will be studied intensely as radiolabeled preparations are available. It has been shown that the temperate subtilis phage, SPO2 is not extensively related to its host. In addition, this phage appears to have a normal distribution of purine and pyrimidine bases about its mean base composition. Both of these findings differ from the data obtained with enterophages. It therefore appears that the extensive relationship between enterophage DNA and DNA from <u>E. coli</u> and the heterogeneous base composition within temperate enterophage DNA are not a universal requirement for establishment of the lysogenic relationship between temperate viruses and their hosts. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.						

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 070, Biochemical activity in health and disease

Investigators.

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Description:

The underlying purpose or mission of this project is to study diseases at the molecular level. Specifically, this involves the study of variations in cellular processes caused by diseases in terms of molecular biology, biochemical genetics, physico-chemical and structural studies of macromolecules. Using the disease-causing agents such as bacteria, viruses and mammalian cells, detailed studies have been pursued to understand:

- (1) The mechanism of protein synthesis.
- (2) The role of nucleic acids in protein synthesis, the mechanism of transcription, translation, and transfer of the genetic code.
- (3) The homology and heterology of nucleotide sequences in various species of DNA and its correlation to the pathogenesis of these species. This in turn will provide the basic knowledge about the host-parasite relationship at the genetic level.
- (4) The regulatory function of various macromolecules under normal and abnormal conditions of cellular growth.

Progress:

Nucleic acid relationships among species of *Neisseria enterobacteriae* and bacterial virus.

Technical aspects: Reassociated deoxyribonucleic acid (DNA) is separable from single-stranded DNA on hydroxyapatite (HA). Thermal elution of reassociated DNA from HA is time consuming because it is difficult to process more than two samples simultaneously. A method for batch

thermal elution of DNA from HA was developed. That allows simultaneous handling of up to ten samples. In this method, DNA samples are mixed with HA contained in test tubes. In each elution step the test tubes are rapidly centrifuged in a heated centrifuge and the wash solution is decanted. Fresh, prewarmed buffer is added and mixed with the HA pellet. The centrifuge method was used successfully for: a) fractionating single and double stranded DNA in reactions exhibiting high and low levels of reassociation; b) thermal elution reactions using DNA from the same or different bacteria; c) time-course reassociation studies; d) reactions carried out at different incubation temperatures. Sensitivity and reproducibility of the batch HA assay is virtually identical to that observed in the column HA assay.

Experimental aspects:

(1) DNA relationships among species of Neisseria (in collaboration with D. Kingsbury, Dept. of Microbiology Naval Medical Research Institute, Bethesda, Md.). The genus Neisseria clearly forms three groups based on the relatedness of their DNA to the DNA of N. meningitidis. It is evident that based on the ability to form interspecies DNA duplexes, the great majority of nucleotide sequences are held in common between pathogenic Neisseria. Furthermore, there is relatively little base sequence divergence as judged by the high thermal stability of these interspecies DNA duplexes relative to the stability of a homologous N. meningitidis duplex. The majority of the nonpathogenic species tested are less than 50% related to N. meningitidis and the instability of their interspecies duplexes is indicative of the extensive amounts of unpaired bases within those sequences still able to reassociate. At more stringent incubation temperatures, these organisms form 15% or less stable duplexes with N. meningitidis. N. catarrhalis is essentially unrelated to the pathogenic Neisseria. These data support the contention that pathogenic species show less divergence than nonpathogens.

(2) Nucleic acid relatedness among enterobacteria (partly in collaboration with S. Falkow, Dept. of Micro, Georgetown Univ., Washington, D. C.). The primary genetic isolating mechanism between strains of Escherichia, Escherichia and Shigella, Escherichia and Salmonella and between various Salmonella serotypes appears to be restriction enzymes. In the case of Escherichia X Shigella once restriction is overcome, the organisms exchange large sequences of genetic material at high frequency and appear to be very closely related. Even when restriction is not a factor in Escherichia X Salmonella matings, the frequency of recombination is still quite low and recombinants are largely highly unstable diploids. Mating between unrestricted Salmonella serotypes suggests that they are highly related although not identical.

With these genetic relationships in mind, the molecular relationships among many representatives of the Enterobacteriaceae were examined by DNA-DNA and DNA-RNA duplex formation in agar, in hydroxyapatite and on membrane filters. The amount of nucleic acid bound in a reassociation experiment is subject to the temperature chosen for annealing. Homologous reactions remain essentially the same regardless of the annealing temperature between 55°C and 75°C in approximately one-tenth molar salt. Hetero-specific duplex formation, however, may decrease dramatically (4 to 8 fold) when the annealing temperature is increased. This observation prompted us to examine the thermal stability of interspecific enterobacterial duplexes. The stability of E. coli - S. flexneri duplexes at both 60°C and 75°C was virtually identical to that of homologous E. coli duplexes and the degree of heteroduplex formation was minimally affected by the temperature increase (86% at 60°C, 77% at 75°C). Heteroduplexes formed at 60°C between E. coli and A. aerogenes, S. typhimurium, Serratia marcescens, Bethesda-Ballerup, Arizona, and several protei had a thermal stability some 10-13 degrees below that of reassociated E. coli duplexes. At 75°C the formation of the heteroduplexes was markedly decreased, but the stability of the DNA able to reassociate at this temperature now approximated that of reassociated homologous E. coli DNA. The degree of reassociation and the thermal stability of E. coli - S. flexneri duplexes suggest relatively little evolutionary divergence in these organisms. The other enterobacteria tested, however, have diverged to a point where less than 40% of their DNA can reanneal at 60°C and less than 8-10 percent can react at 75°C. The degree of divergence between various enterobacteria does not appear to be uniform along the DNA molecule. RNA specific sequences are conserved among most enterobacteria. An examination of DNA relatively specific for the lactose operon suggests that specific chromosomal genes may diverge more or less than the genome as a whole. Experiments in progress reveal extensive relatedness between E. coli and the alkalescens-Dispar group; enough to include these organisms in one species. Detailed current studies reveal as much as 25% divergence among strains of E. coli. Here again it appears that the clinically isolated strains have diverged from the common laboratory strains to a greater extent than the laboratory strains have diverged from one another.

(3) Lack of relatedness between Bacillus subtilis and a temperate subtilis phage. Temperate enterophage DNA's share three characteristics. They are extensively related to one another, extensively related to their enterobacterial host, and heterogeneous in nucleotide base composition. DNA from the temperate subtilis phage SPO2, was shown to lack significant base sequence heterogeneity, and to be unrelated to the DNA of its host, B. subtilis. Two virulent subtilis phages were also unrelated to SPO2. These data show that neither extensive nucleotide sequence relatedness between a temperate phage and its host, nor base sequence heterogeneity are universal requirements to form the lysogenic state.

### Biochemistry of Malaria.

Studies on isolation and characterization of nucleic acids from malarial parasites were pursued. P. knowlesi and chloroquine-sensitive and resistant strains of P. berghei were used in these studies. In collaboration with Dr. J. Wohlhieter, LTC Fitch and CPT Cook a technique was developed to separate the parasites from other DNA-containing cells in the blood. The chloroquine-resistant strains appear to propagate only on young red blood cells. The resulting DNA preparations of the sensitive strain of P. berghei and P. knowlesi were free of host DNA. All the DNA's appeared to be normal in the chemical and physical properties studied. P. knowlesi DNA had a density of 1.699 g/c.c. in CsCl and thermal denaturation temperature  $T_m$  of  $86^\circ$ , both corresponding to a base composition of 40% G:C content. Both strains of P. berghei have DNA of density 1.683 g/c.c. and  $T_m$  of  $76.5^\circ$ , corresponding to 23% G:C. A preparation of DNA from chloroquine-resistant P. berghei exhibited two additional bands upon CsCl density gradient centrifugation. These bands were deemed to be due to "contaminating" mouse DNA. No other differences were observed between DNA from the two P. berghei strains.

### Mechanism of protein synthesis.

It is known that one of the species of methionine tRNA,  $tRNA_f^{met}$ , from E. coli, has a unique function in protein synthesis: it acts as an initiator of protein synthesis. On the other hand, another species of methionine tRNA,  $tRNA_m^{met}$ , also present in E. coli, does not have this unique property. Because of this unique function of the  $tRNA_f^{met}$  detailed study of this tRNA was undertaken.

Using the combination of countercurrent distribution and column chromatography procedures it was possible to show the existence of two species of  $tRNA_f^{met}$  and two species of  $tRNA_m^{met}$  in E. coli. One each of these species of tRNA were purified and their structural and functional properties were investigated. These properties are summarized as follows:

- (1) Both  $tRNA_f^{met}$  can be charged with either yeast or E. coli synthetases, whereas  $tRNA_{m1,2}^{met}$  can be charged only with the E. coli synthetases.
- (2) Only  $tRNA_f^{met}$  can be formulated.
- (3) No difference in responses to triplet binding to ribosomes between the two species of  $tRNA_m^{met}$ .
- (4) The analysis and comparison of the PRNase and RNase T<sub>1</sub> digestion products of purified  $tRNA_f^{met}$  showed that these two  $tRNA_s^{met}$ , although having several similar biological activities, are structurally different. However, there are only minor differences between  $tRNA_{f1}^{met}$  and  $tRNA_f^{2met}$ . The same is the case with the two species of  $tRNA_m^{met}$ .

The presence of sulfur-containing nucleotides in all these species of tRNA was detected. The position of sulfur-containing nucleotide in most of the tRNA's from *E. coli* is fixed at the 8th nucleotide from the 5'-end from this and previous studies. The presence or absence of this nucleotide does not appear to matter with regard to the function of tRNA. This is now shown in the case of tRNA<sup>tyr</sup> and tRNA<sup>met</sup>, although it should be mentioned that the changes in conformation in the thionucleotide region of tRNA is observed.

Upon mild digestion of tRNA<sup>met</sup> it was possible to split the molecule at one position. Two fragments, a 3'-end containing 57 nucleotides and a 5'-end containing 19 nucleotides were separated and obtained in purified form. Competitive inhibition studies with these fragments showed that neither of these fragments interact or bind with the synthetase. When both these fragments were put together, methionine acceptor activity, (i.e., the binding with enzyme) was reestablished. At the present level of information it is firmly established that both 5'-end and 3'-end fragments are required for interaction with protein. It is also established from present studies with tRNA<sup>met</sup>, and also with tRNA<sup>tyr</sup>, that specific nucleotides of RNA may not constitute a specific protein or enzyme-interaction site. Rather it is most likely that specific conformation of a region or the entire molecule may be an enzyme-binding site. The study of the nature of protein-nucleic acid interaction on this basis is being pursued. A project is being initiated at the present time to study the nature of viral nucleic acids with their protein component.

#### Structural studies of macromolecules.

The nucleotide sequence of tRNA<sup>tyr</sup> is completed. The information obtained from these studies clearly demonstrated that the conformation of the molecule rather than any specific nucleotide sequence may contribute the enzyme interaction site. This fact is further substantiated by the studies with the fragments obtained from tRNA<sup>met</sup>. In order to study the molecular assembly of macromolecules contained in agents such as viruses, it is a prerequisite that the nature of interaction of macromolecules such as nucleic acid and proteins be understood. The facts mentioned above necessitate the study of spatial structure of tRNA to understand the relationship between its function and its structure. The attempts so far have been made by means of model building and the study of tRNA in solution. It seems unlikely that such study will solve the problem. The method of choice then is x-ray analysis of crystalline tRNA.

At first, microcrystalline forms of tRNA<sup>met</sup> were obtained. This was the first time that a nucleic acid of this nature had been obtained in crystalline form. X-ray analysis of the powder form of this microcrystalline tRNA revealed that the unit cell dimensions are: a=118, b=43.2, c=53.2 (orthorhombic) with space groups P222<sub>1</sub> or O222.

Further efforts to obtain single crystals were also successful and single crystals of tRNA<sup>met</sup>, using more favorable conditions, have been obtained. The x-ray analyses of these crystals have been pursued systematically at NIH in collaboration of Dr. David Davies of NIAMD.

One of the ways to obtain information regarding the arrangement of the helical regions of nucleic acids is to determine the orientation of the crystalline fiber by x-ray analysis using purified tRNA<sup>tyr</sup>. The x-ray analysis of this material revealed that the helices in tRNA<sup>tyr</sup> are arranged parallel to each other rather than perpendicular. This information has been confirmed by several investigators from the preliminary x-ray analysis of single crystals.

With the expectation that a systematic and detailed study of crystalline tRNA by x-ray analysis will furnish the desired information, an extensive mission has been taken. For this purpose, simplified procedures for the purification of several tRNA from a given species in large quantities have been evolved. Also it is hoped that the methods for the co-crystallization of tRNA-enzyme complex will be evolved.

In order to interpret the x-ray analysis data and elucidate the "functional sites" of the tRNA, it is essential to express the x-ray information in terms of molecular model. One of the probable forms in which the popular "clover leaf" secondary structure of tRNA can be transformed in the spatial structure of tRNA is the "H" form. With the help of Dr. Watson Fuller of King's College, London, a molecular model of tRNA<sup>tyr</sup> and nucleotides are being pursued at the present time.

The application of these studies to the studies of the nature of interaction between antigen-antibody production of interferon, enzyme-substrate interaction and protein-nucleic acid interaction in virus particles is actively being planned at the present time.

Part of the studies described were performed at (a) Medical Research Council, Laboratory of Molecular Biology, Cambridge, England, in collaboration with Drs. B.F.C. Clark *et al*, and (b) King's College, London, England in collaboration with Dr. Watson Fuller.

The regulatory function of macromolecules under normal and abnormal conditions of cellular growth. The work described in this phase of the project report was performed as a collaborative study with Dr. Alan Peterkofsky of the NIH.

It has become increasingly apparent in recent years that the function of transfer RNA (tRNA) may not be limited to directing the positioning of an amino acid into a growing peptide chain, but may include playing a role in the regulation of a number of cellular metabolic processes.

Evidence that the cell is at least equipped for a mechanism of controlling transcription at the translational level using the tRNA molecule, comes from the findings that there is extensive degeneracy in the genetic code and widespread occurrence of more than one acceptor RNA for most amino acids--many of which have been shown to respond to different codons.

In at least two situations, namely, the control of RNA synthesis and the repression of certain amino acid biosynthetic enzymes, there is much genetic and biochemical evidence implicating the involvement of aminoacylated tRNA in a regulatory role. In both instances there appears to be a primary dependence on the intracellular levels of free amino acids, and it is now clear that the amino acid in question must be activated and probably attached to its tRNA in order to assert its influence. Added support for the hypothesis that tRNA also serves as a regulator comes from several recent reports showing that certain tRNA's in different kinds of cells have been modified in response to a variety of metabolic conditions.

Examples of these modifications include: a) the appearance of chromatographically unique species of leucine tRNA in E. coli during infection with phage T2 and T4; b) alterations in the valine specific tRNA of B. subtilis during spore formation; c) changes in B. subtilis seryl-tRNA and E. coli isoleucyl- and phenylalanyl-tRNA's under different growth conditions; d) the production of new species of leucine and phenylalanine tRNA during methionine starvation of 'relaxed' mutants of E. coli; e) the appearance of a new arginyl-tRNA in mammalian cells infected with herpes virus; and f) the occurrence of differences between tyrosine acceptor tRNA's from fibroblastic cells and a number of differentiated cells from animal and human sources.

tRNA as a regulator of RNA synthesis. It has been known that the synthesis of both protein and RNA ceases when an amino acid auxotroph of E. coli is starved for the required metabolite. For several years the accepted hypothesis for this phenomenon held that the uncharged tRNA resulting from amino acid deprivation functions as a specific repressor of RNA synthesis--probably by inhibiting the enzyme RNA polymerase.

Recently, experiments appear to demonstrate conclusively that amino acid regulation of RNA synthesis is dependent upon amino acid activation, the first step in the formation of charged tRNA. Using two mutant strains, workers have shown that the inability to activate a certain amino acid analog in one case and heat inactivation of an aminoacyl-tRNA synthetase in the other, leads to a cessation of RNA synthesis.

Important to any study of this regulatory mechanism are the 'relaxed' mutants of *E. coli*. Unlike the wild type strains which exhibit a 'stringent' control over RNA synthesis, the 'relaxed' mutants continue to make RNA upon starvation for a required amino acid. A number of these mutants have been isolated thus far and all appear to result from mutations of a single genetic locus called the RC (RNA control) gene. It is of great interest to note that the relaxed mutants ( $RC^{rel}$ ) do not show a requirement for the aminoacylation of tRNA, unlike the  $RC^{str}$  strains used in the study mentioned above. The product of the RC gene is unknown. Results of experiments attempting to show differences in the stimulation of RNA polymerase in *in vitro* systems with charged and uncharged tRNA, DNA, and polymerase from both  $RC^{str}$  and  $RC^{rel}$  strains are not conclusive. There was little difference in the effect of uncharged tRNA and the acylated species and no significant difference between the effects of the stringent and relaxed components.

Because of these results and others which show no apparent correlation between the levels of uncharged tRNA in the cell and the rate of RNA synthesis, the role of tRNA in this process is not yet clear and requires further investigation. One plausible explanation of the seemingly contrary results described above is the possibility that the regulatory function in this process has been relegated to a minor species of tRNA. If, in fact, an uncharged tRNA is a repressor (or corepressor) here, then it need not be present in high concentration. Thus, a comparison of the intracellular condition of tRNA may never reflect the status of the minor species. Failure to influence the activity of RNA polymerase in the studies mentioned above should be regarded as negative evidence as it is quite possible that important elements are missing, e.g. the RC gene product, or perhaps the apparatus for protein synthesis.

If this minor tRNA species should be endowed with one or more special properties, then our proposed explanation becomes quite attractive and certainly very tenable. For example, if this species has a  $K_m$  value for its amino acid that is an order of magnitude or more larger than that of the other tRNA's, it would be very sensitive to a reduction in the amino acid pool. Another unique property might be its affinity for the corresponding synthetase. If this relationship should change from the intracellular level of amino acids, the tRNA may be selectively aminoacylated or held in the stripped form. One mechanism for this last situation has already been described, tRNA modification through methylation. It has been shown that unmethylated tRNA from  $RC^{rel}$  strains is charged at a slower rate than the methylated species and that undermethylated histidine, phenylalanine and leucine tRNAs from relaxed mutants have much lower acceptor activities than the same tRNAs after *in vitro* methylation. Also, certain methyl deficient tRNA's which have altered chromatographic and coding properties can be restored to the native state by *in vitro* methylation.

## Results

Early in the year covered by this report, we observed that amino acid starvation of a relaxed strain of E. coli for leucine results in the appearance of a chromatographically distinct minor species of leucine acceptor tRNA. This unique sub-species of tRNA is not present in the leucine-starved stringent strain nor in either cell strain when non-starved. The amount of this particular tRNA present in the cell varies with time of amino acid deprivation comprising upwards of 30% of the total leucine acceptor activity after about three hours of starvation. Evidence that this tRNA is newly synthesized by the mutant cell in response to the metabolic 'shift down' and is not the result of some reaction that alters the structure of a pre-existing tRNA comes from experiments in which cells were starved for both leucine and uracil--an RNA precursor. Under these conditions the unique leucine tRNA is not produced. Thus it appears that this tRNA which is present in the RC-cells is either the product of a gene not normally expressed or is a tRNA that has not undergone one or more of the several specific modification reactions following its synthesis. Examples of such modifications include oxidation, reduction, thiolation and methylation of specific nucleotides and the addition of certain characteristic side chains to others.

Because base methylation is known to alter both structure and function of tRNA and because methylation patterns of tRNA are known to vary remarkably with the metabolic status of the cell, experiments were performed to test the possibility that the unique species of leucine tRNA observed in this study is in fact, an under-methylated species. In vitro experiments showed the methyl acceptor activity of the bulk tRNA from leucine starved cells to be the same as that for tRNA from non-starved cells. Further co-chromatography of the 'new' leucine tRNA and methyl deficient leucine tRNA (from relaxed cells starved for methionine) failed to show any sub-species of methyl-poor leucine tRNA co-eluting with the unique species. Other tRNA modification reactions are being studied currently in an effort to determine the chemical nature of this tRNA.

Having established that leucine deprivation induces the production of a unique leucine tRNA in these regulatory mutants, the question of other distinct amino acid specific tRNA's arises. That is, when the cells are deprived of leucine, will examination of other (non-leucine) tRNA's show the presence of similarly unique subspecies? At this point we have only partially answered the question. It has been determined that leucine starvation induces the formation of a new species of histidine tRNA. A comparison of the chromatographic properties of this tRNA with that peculiar to leucine starvation shows these tRNA's to be quite distinct.

Experiments are presently in progress to determine if starvation for histidine results in new histidine and leucine tRNA's and if so, whether these species are the same as those produced by leucine deprivation. Other lines of experimentation in progress include the determination of the kinetic parameters of the unique tRNA's in the aminoacylation reaction and also their coding properties when allowed to interact with ribosomes and synthetic messenger RNA's.

If a regulatory function for these tRNAs is indicated an attempt will be made to construct an in vitro system for studying their role and elucidating the mechanism of action in the control of RNA synthesis.

#### Summary

In order to study reassociation of nucleic acids, a method for batch thermal elution of DNA from hydroxyapatite has been developed which allows simultaneous handling of as many as ten samples. This technique has been applied to evaluate relatedness between mutant strains of bacteria.

Studies of the characterization of mutant strains of P. knowlesi and P. berghei has shown differences in DNA characteristics between chloroquine-resistant and chloroquine-sensitive strains of the malarial parasite.

In the study of amino acid transfer for protein synthesis, counter-current distribution and column chromatography procedures have demonstrated two different tRNA species for methionine by E. coli. These have been purified and functional properties studied. A successful purification of tRNA of tyrosine has resulted in complete sequence definition of the amino acid structure. A crystalline preparation has allowed partial definition of the crystal structure by x-ray crystallography. A molecular model of structure is being assembled at this time.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 070, Biochemical activity in health and disease

Investigators.

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		DA					

35. (U) Enzymes; (U) Enzyme Tests; (U) Catecholamines; (U) Spinal Diseases; (U) Liver Diseases; (U) Proteins; (U) Electrophoresis; (U) Epinephrine.

23. (U) To evaluate newly reported techniques for diagnostic assistance to the physician and to develop additional concepts to be used for differential diagnosis of disease.

24. (U) Through evaluation of newly reported diagnostic biochemistry will be accomplished and in especially promising developments the techniques will be applied to programs within the Army Medical Service. Where some techniques are lacking, research will be done to develop new processes for diagnostic evaluations.

25. (U) 69 01 - 69 06 The radius cubed scale method for ultra-centrifuge data is being further evaluated. In addition to giving the sedimentation coefficient and concentration, it provides a method of determining the second moment or radius of gyration. It offers the capability of determining degree of boundary spreading, diffusion coefficients, distribution coefficients, both of sedimentation values and molecular weights. The electrophoretic method for the determination of lipoprotein patterns in serum as been published and it is now being applied to the analysis of serum samples from patients as an aid in the diagnosis of hyper- and hypo-lipoproteinemias. The early detection of abnormal lipoprotein patterns by this technique furnishes useful information for the prevention of disease by initiating appropriate treatment for the correction of the abnormality. The techniques of thin-layer chromatography, gas-liquid chromatography and agar gel electrophoresis are being used for the study of lipid lipoproteins and LDL-isoenzymes in cerebrospinal fluid with the aim of obtaining specific information for differential diagnosis of neurologic conditions. Ultraviolet methods for the determination of P, Ca, Mg alkaline phosphatase and uric acid in blood of infants are being used in order to establish normal values and furnish useful information during the first four days of life. Experimental models have been established for the production and study of shock and pulmonary embolism in animals. Biochemical histological and physiological procedures are used to evaluate metabolic changes and tissue damage in shock. Enzyme activities and isoenzyme patterns are utilized to determine the effects of trauma on metabolic functions and tissue damage. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.

P11 Redacted

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 071, Biochemical variations in abnormal health states

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**Description**

A biochemical approach is used to provide information in the study and diagnosis of disease and for the early detection of abnormal processes which may lead to disease.

**Progress**

The electrophoretic test system for the integrated analysis of iso-enzymes, proteins and lipoproteins, which was developed at WRAIR for differential diagnosis and early detection of abnormal metabolic processes, has been expanded to include additional enzymatic and clinical tests for complete and detailed biochemical information. It is being used in the analysis of blood samples from selected patients of WRGH and other military activities and in applied clinical research of specific interest to the Army.

Quantitative study of serum levels of lactate dehydrogenase (LDH) which is present in all tissues of the body provides information on abnormal metabolic processes due to disease or injury. Determination of LDH-isoenzymes provides additional information of the specific organs involved by virtue of the fact that the isoenzyme patterns are tissue specific. Determination of the enzymes creatine phosphokinase, transaminases and malic dehydrogenase provide confirmation of other enzymatic findings and expand the knowledge of cellular damage in diseased states.

Quantitative and qualitative changes of serum proteins occur during disease or injury. The electrophoretic analysis of serum proteins provides useful information, since an alteration of their patterns and concentration has been associated with pathological conditions. The determination of serum protein has been expanded to include immunoelectrophoretic and radial immunodiffusion analyses. The latter two procedures furnish specific information in disease processes: e.g., alteration of immunoglobulins G, A, and M in infectious and autoimmune diseases, and alteration of the concentration of the proteins ceruloplasmin and transferrin in diseases involving copper and iron metabolism.

The method for the determination of serum lipoprotein patterns by the same test system has been completed and published. Hyper- and hypolipoproteinemias are easily and rapidly detected by this test and very useful information is furnished to the clinicians for the diagnosis and treatment of lipid diseases.

A micro method for the determination of serum cholesterol in the presence of high bilirubin levels has been developed and is being applied to obtain accurate information of serum cholesterol values. The combined determination of serum cholesterol, triglycerides and lipoproteins provides useful information for the diagnosis of lipid abnormalities and also for the early detection of abnormal lipids and lipoprotein patterns in apparently normal individuals. Early evaluation of these observations suggests that this profile may be useful in detecting early atherosclerosis and in evaluating patients with a history of heart disease. During the past year many samples have been received from WRGH and other military activities, CONUS and OCONUS, for required special tests and differential diagnostic evaluation not furnished in the conventional clinical laboratory.

Following the development of a method for concentrating cerebrospinal fluid (CSF) by a vacuum dialysis technique using collodion membranes, the same test system of protein, lipoprotein and isoenzyme determination can now be utilized for the analysis of CSF. This test system together with the techniques of gas liquid and thin layer chromatography are now being used in the analysis of CSF samples from patients for the study and diagnosis of neurological diseases.

The long range study of lipoprotein analysis by ultracentrifugation has been concluded. Two hundred and forty two blood samples were analyzed and the results were forwarded for further consideration. Since new simple techniques for protein and lipoprotein analysis have been developed, the ultracentrifugal analysis is only used as an adjunct in special cases which require its application.

The development of ultramicrochemical techniques is continuing. The following tests have been adapted and are currently used in the analysis of blood from newborn babies in order to establish normal values and obtain information of abnormal levels in pathological conditions: uric acid, alkaline phosphatase, calcium, magnesium, and phosphorus.

Summary and Conclusions:

A diversified biochemical system has been developed for protein, enzyme, isoenzyme, lipoprotein and microliter analysis. It has been applied to the analysis of serum and cerebrospinal fluid in order to obtain information on physiological and pathological biochemical mechanisms and to provide information for the diagnosis and differentiation of disease.

The system can further be expanded to include other tests of importance in the study of diseases. It is rapid, inexpensive and relatively simple so that it can be modified to be used in the routine clinical laboratory and in field operations.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 071, Biochemical variations in abnormal health states

Investigators.

Principal: Edward C. Knoblock, COL, MSC  
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Publications

1. Papadopoulos, N.M., and Kintzios, J. Analysis of serum lipoproteins by agarose gel electrophoresis. Fed. Proc. 28: 886, 1969.
2. Papadopoulos, N. M., Bloor, C.M., and Standefer, J. Effects of exercise and training on plasma lipids and lipoprotein in the rat. J. Appl. Physiol. June, 1969.
3. Papadopoulos, N.M., and Thomas, E.R. A biochemical approach for the study of uveitis by protein and LDH-1-isoenzyme analysis. Proc. Soc. Exp. Biol. and Med. June, 1969.
4. Bloor, C.M., and Papadopoulos, N.M. Plasma LDH activity and myocardial cellular changes after the cessation of training. J. Appl. Physiol. 26: 371, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AG. ORY NUMBER	2. DATE OF ORIGIN	PROJECT CONTROL SYMBOL	
				DA 0B6437	69 07 01	DD-FORM (27) 688	
3. DATE PREP COMPLETED	4. KIND OF SUMMARY	5. SUMMARY SCOPE	6. SOURCE COUNTRY	7. RECEIVING	8. DISSEM POSITION	9. SPECIFIC DATA CONTRACTOR ACCESS	10. LEVEL OF SECURITY
69 01 31	D. Change	U	U	NA	ML	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES	PROJECT ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	61102A	3A061102B71P	01	072			
11. C. CHARACTER	1412A(2)						
11. TITLE (Provide with Security Classification Code)							
(U) Biochemical Laboratory Automation Systems (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREA							
002300 Biochemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
67 11		NA		DA		C. In-House	
17. CONTRACT/GRAANT				18. RESOURCE ESTIMATE		19. PROFESSIONAL MAN YRS	
NA				PRECEDING		FUNDING (in \$-thousands)	
A. DATES/EFFECTIVE:		EXPIRATION:		FISCAL YEAR	COUNTRY		
B. NUMBER:		C. TYPE:		69		4	100
D. KIND OF AWARD:		E. CUM. AMT.		70		4	100
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Considered				NAME: Yearby, MAJ C.			
				NAME: DA			
22. KEYWORDS (Provide with Security Classification Code)							
(U) Biochemistry; (U) Automation; (U) Clinical Chemistry.							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide technical paragraphs identified by number. Provide text of each with Security Classification Code)							
<p>23. (U) The technical objective of this work unit is to provide a system of laboratory biochemical analysis through modulization of automatic wet chemistry analysis equipment, data reduction techniques, and developmental effort.</p> <p>24. (U) Automated analytical wet chemical modules are assembled and utilized to provide a model system to accomplish biochemical analysis.</p> <p>25. (U) 69 01 - 69 06 The automated atomic absorption system was received and rendered fully operational for the determination of calcium and magnesium. Accessories permitting analyses by flame photometry have been purchased for the atomic absorption spectrophotometer. A comparative study of urine and serum sodium and potassium concentrations by atomic absorption versus flame photometry (Autoanalyser and atomic absorption spectrophotometer) is anticipated. A simultaneous method for the determination of glucose and phosphate "in vivo" has been designed on a mobile laboratory cart. Bedside monitoring of the above constituents for periods up to five (5) hours is possible. The technique does not require heparinizing the patient. The project to completely automate stool and diet analyses was continued, however, progress was impeded due to shortage of personnel. The current strength is four (4) technicians and one (1) officer. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.</p>							

PII Redacted

Project 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biochemistry

Work Unit 072, Biochemical laboratory automation systems

Investigators.

Principal: Edward C. Knoblock, COL, MSC  
Paul S. Teschan, COL, MC  
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Associate: Yin-Ying Djuh, GS-9  
Frances Wright, GS-9  
Dudley Grove, SP5  
Daniel Cronin, PFC  
Robert Fulfer, PFC

Description.

An automated clinical laboratory capability has been established which has expanded to furnish research support to investigators of the WRAIR who require repetitive analyses using standardized techniques. Special emphasis has been placed on a system using optimal methods with continuing monitoring of quality control of each procedure.

Progress.

During the period 1 January through 30 June 1969, the Automated Laboratory Facility performed 31 analytical procedures consisting of 19 procedures utilizing the AutoAnalyzer-Digitizer System, 2 "in vivo" procedures, 4 Automated Atomic Absorption methods, and 6 manual techniques. The specific methods are delineated below.

AutoAnalyzer Digitizer System

- |                         |                           |                      |
|-------------------------|---------------------------|----------------------|
| 1. Albumin              | 8. Glucose                | 14. p-Aminohippurate |
| 2. Alkaline Phosphatase | 9. Inulin                 | 15. Phosphorus       |
| 3. Ammonia              | 10. Nitrogen, Alpha-amino | 16. Potassium        |
| 4. Calcium              | 11. Nitrogen, non-protein | 17. Protein, total   |
| 5. Carbon Dioxide       | 12. Nitrogen, total       | 18. Sodium           |
| 6. Chloride             | 13. Nitrogen, urea        | 19. Uric Acid        |
| 7. Creatinine           |                           |                      |

<u>Automated Atomic Absorption</u>	<u>Manual Techniques</u>	<u>In Vivo Methods</u>
1. Calcium	1. Blood pH	1. Glucose
2. Magnesium	2. Carbon Dioxide	2. Phosphorus
3. Potassium	3. Chloride	
4. Sodium	4. Osmolality	
	5. Uric Acid (uricase)	
	6. Digestion (wet-ash)	

A total of 78,076 units (specimens, standards, and quality controls) were processed employing an average of five technicians. This compares with 47,818 units during the 1968 January-June period which employed an average of four technicians.

The quality control data pertaining to the above AutoAnalyzer-Digitizer procedures is generated in the following manner: The method's initial evaluation is based on statistical parameters obtained from a run of 30 consecutive samples of commercial quality control sera or aqueous standards. Subsequent evaluation is obtained from data generated by the same lot of quality control serum or aqueous standard included daily as the 10th or 15th sample of a routine run. The routine run is calibrated against six aqueous standards and recalibrated every 15th or 30th unknown as necessitated by baseline drift, change in sensitivity, or other variations evident during the initial evaluation. The daily quality control values are treated statistically in groups of 30 or more and the resulting statistical parameters are recorded as individual entries and as part of a running compilation. The parameters calculated are the Average, the Standard Deviation, the Standard Error, and the Coefficient of Variation. Analysis of current data indicate excellent reproducibility both within a "run" and from day to day.

The Automated Atomic Absorption System was received in January and consists of an Atomic Absorption Spectrophotometer, a 200-sample Automatic Sampling and Diluting System, a Digital Concentration Readout, a Recorder, and a Paper Tape Printer. Under the present operating conditions, simultaneous analysis is not possible; however, the rate of analysis for Magnesium, Calcium, Sodium, and Potassium is 600, 200, 144, and 90 samples per hour, respectively, when a recorder is not used. Utilizing a recorder reduces the rate for Magnesium, Calcium, and Sodium to 240 samples per hour, but does not affect the Potassium rate. Accessories permitting analyses by flame photometry have been purchased for the Atomic Absorption Spectrophotometer. These accessories are expected to increase the current precision and rates of analysis for Sodium and Potassium determinations in serum and urine. The Quality Control Program for this system is similar to that described above.

A simultaneous method for the "in vivo" determination of glucose and phosphate has been designed on a mobile laboratory cart. Bedside monitoring of the above constituents for periods up to 5 hours is possible. The technique does not require heparinizing the patient.

The AutoAnalyzer-Digitizer System which was described in the 1968 annual report continues to operate with minimum downtime. The project to completely automate stool and diet analyses was continued; however, progress was impeded due to a personnel shortage. The current strength is four technicians to one officer.

Summary and Conclusions:

An Automated Laboratory Facility has been established which has expanded to furnish research support to investigators of the WRAIR who require repetitive analyses using standardized techniques. The major system consists of AutoAnalyzers adapted to an analog-to-digital converter (Digitizer) capable of automatically calibrating its response and then detecting, printing out, and storing results of analyses produced by 12 AutoAnalyzers. Also available is an Automated Atomic Absorption System capable of multiple sample preparation and analysis and automatic identification and printouts. A mobile "in vivo" system, which provides bedside monitoring of several constituents without necessity of heparinizing the patient, is available to a limited number of researchers. Special emphasis has been placed on systems using optimal methods with continuing monitoring of quality control of each procedure.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biochemistry

Work Unit 072, Biochemical laboratory automation systems

**Investigators.**

Principal: Edward C. Knoblock, COL, MSC  
Paul S. Teschan, COL, MC  
Calvin G. Yearby, MAJ, MSC

Associate: Yin-Ying Djuh, GS-09  
Frances Wright, GS-09  
Dudley Grove, SP5  
Daniel Cronin, PFC  
Robert Fulfer, PFC

Publications.

None

**PROJECT 3A061102B71P**  
**BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE**

**Task 03**  
**Entomology**

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY	3. REPORT CONTROL SYMBOL	
				DA 0A6431	69 07 01	DD-FORM (AR) 636	
4. DATE PREP SUMMARY	5. KIND OF SUMMARY	6. SUMMARY ACTY	7. WORK SECURITY	8. REGRADING	9A. DRIP'S INSTN	9B. SPECIFIC DATA - CONTRACTOR ACCESS	9C. LEVEL OF SUMMARY
69 01 31	D. Change	U	U	NA	NL	YES <input checked="" type="checkbox"/> NO <input type="checkbox"/>	A. WORK UNIT
10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	61102A	3A061102B71P		03		035	
b. CONTRIBUTING							
c. SUPPORTING	1412A(2)						
11. TITLE (Precede with Security Classification Only)							
(U) Ecology and Control of Disease Vectors and Reservoirs (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREA							
002600 Biology 005900 Environmental Bio 010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
09 54		NA		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
Not Applicable				PREVIOUS		A. FUNDS (in thousands)	
a. DATES/EFFECTIVE				FISCAL YEAR		CURRENT	
b. NUMBER				69		3	
c. TYPE				70		3	
d. KIND OF AWARD				2. CUM. AMT.		60	
20. RESPONSIBLE DOC ORGANIZATION				21. PERFORMING ORGANIZATION			
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NAME: Meroney, COL W. H. TELEPHONE: 202 - 576-3551				NAME: Eldridge, LTC B.F. TELEPHONE: 202 - 576-3719 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Considered				NAME: Ward, Dr. R. A. NAME: Saugstad, CPT J. S. DA			
23. (U) Arbovirus; (U) Dengue; (U) Chikungunya; (U) Trypanosomes; (U) Ecology; (U) Mosquitoes; (U) Vectors; (U) Tsetse flies							
24. (U) Control of arthropod vectors of disease of military significance with emphasis on vectors of arbovirus and parasitic disease. Efforts are on incrimination of vectors, host-parasite relationships. Laboratory and field studies of vector population ecology. Final aim is the understanding of vector biology and disease transmission in order to apply control methods most effectively.							
25. (U) Mosquito collections are made for arbovirus isolations and study of mosquito ecology. Systems are developed for the study of host-pathogen-vector relationships involving mosquitoes- dengue/chikungunya and tsetse flies - Trypanosoma brucei as models.							
26. (U) 69 01 - 69 06. Study of transmission of dengue and chikungunya by Aedes aegypti is terminated with confirmation of infections by reisolation of viruses from hosts. Ecological studies of mosquito vectors of arbovirus disease on eastern shore of Maryland have begun. New laboratory colonies of Glossina morsitans have been established for transmission of new trypanosome isolates. In vitro cultures of trypanosomes remain infective for 120 hours. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

PII Redacted

Project 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03, Entomology

Work Unit 035, Ecology and control of disease vectors and reservoirs

Investigators

Principal: LTC J. E. Scanlon, MSC\*

Associate: LTC B. F. Eldridge, MSC; R. A. Ward, Ph.D.;  
CPT E. S. Saugstad, MSC; CPT M. J. Peach, MSC;  
CPT D. E. Bay, MSC; CPT L. J. Nearman, MSC\*\*  
Mr. W. Suyemoto, BS; Mr. L. H. Bell, BS;  
Mr. T. J. Neal, BS

Description

This task involves field and laboratory studies of the relationship between selected arthropods and various aspects of their natural environment, especially those aspects relating to certain pathogenic organisms, their hosts, and their reservoirs. Included are ecological and physiological studies on arthropods, studies of transmission mechanisms and the development of improved methods of control of arthropods of medical importance.

Progress

1. Transmission of arboviruses by mosquitoes

During this period, studies on the transmission of dengue-2 and chikungunya viruses by Aedes aegypti mosquitoes was terminated.

a. Experimental transmission of high mouse passaged dengue-2 virus (New Guinea C Strain). A pool of 47 Aedes aegypti which had been experimentally infected in a previous transmission attempt were ground in diluent and inoculated into suckling mice to produce seed virus. This virus had been passed through mice 32 times previously. When additional mosquitoes were infected with this virus by means of a membrane feeding technique, attempts to transmit the virus to baby hamsters by mosquito bite were successful in 7 of 69 attempts. Virus from hamsters thus infected was in turn inoculated into suckling mice to prepare additional seed virus and still another group of Aedes aegypti mosquitoes was infected by membrane feeding technique. In this instance, transmissions by mosquito bite were attempted to suckling mice at weekly intervals starting with postinfection day 7 and continuing to day 42. In all, 5 of 179 attempts were successful (2.7%). No transmission took place until day 21. The highest transmission rate was observed at day 42 when 3 of 30 attempts were successful.

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\* Until 1 March 1969

\*\* Until 1 November 1968

b. Experimental transmission of low mouse passaged dengue-2 virus (Bangkok 2358-62 Strain). In order to determine whether higher transmission rates to mice could be obtained with lower mouse passage material, *Aedes aegypti* mosquitoes were infected by membrane feeder with dengue-2 virus which had undergone 7 mouse passages. Infected mosquitoes were held at 25.6°C. Attempts to transmit virus to suckling mice by bite were made at weekly intervals beginning on post-infection day 7 and continuing until day 49. Only 2 of 209 attempts were successful. Attempts were made to increase the transmission rate by increasing the concentration of virus in the blood-virus mixture placed in the membrane feeding device. Even when sucrose was eliminated from the mixture and the concentration of virus increased four-fold, the virus titer infected mosquitoes did not increase and no successful transmission were obtained. These experiments are summarized in Table 1.

## 2. Field studies of arbovirus mosquito vectors

In late July and early August, 1968, arbovirus activity on the Delmarva Peninsula of the eastern United States manifested itself in the form of several cases of encephalitis in horses and also in domestic pheasants and partridges. These cases occurred, among other places, in the vicinity of Willards, a small town in Wicomico County, Maryland. In order to determine the species of mosquitoes which were becoming infected and presumably transmitting virus, and to complement data being collected by the Department of Virus Diseases at the Pocomoke Swamp Study Area, mosquito collections were made by light trap at two localities in Wicomico County, Maryland, three localities in Worcester County, Maryland, and at one locality in Sussex County, Delaware. These collections were made between 13 August and 12 September, 1968.

The collection sites were as follows:

A. Massey's Crossing. A secondary road crossing of the Pocomoke River at the Wicomico-Worcester County line, 3 miles southeast of Willards.

B. Mill Pond. A swamp 1½ miles south of Willards.

C. Porter's River Crossing. A secondary road crossing of the Pocomoke River, 3½ miles northeast of Snow Hill, Worcester County, Maryland.

D. Dennis Quail Farm and Woods. A farm on Maryland State Highway 354, 2 miles southwest of Willards.

E. Mattapont Landing. A landing on the Pocomoke River, Pocomoke State Forest, 5 miles southwest of Snow Hill.

F. Cypress Swamp. On a secondary road in Sussex County, Delaware, just north of the state line and 5 miles due east of Selbyville.

All of these sites were at or near fresh water swamp forests, the typical breeding habit of Culiseta melanura. Collected mosquitoes were pooled by species, frozen, and sent to the Department of Virus Diseases for isolation of virus. Three pools, all of Culiseta melanura, were positive for the virus of eastern equine encephalitis (Table 2). Two of the positive pools were from Area E (Mattapont Landing); one was from Area B (Mill Pond). The Mill Pond isolation was from mosquitoes collected on the night of 20-21 August. The two isolations from Mattapont Landing were from mosquitoes collected on nights of 10-11 and 11-12 September.

Eleven other pools of mosquitoes, including six pools of Aedes atlanticus, showed marginal cytopathologic effect in the tissue culture system used by the Department of Virus Diseases. These pools are being retested in the Department of Entomology by inoculation into suckling mice.

The 1968 epizootic on the Delmarva peninsula appeared to be one of eastern equine encephalitis occurring generally along river flood plains, and seemingly restricted to that habitat and its associated mosquito vector, Culiseta melanura. Confirmed horse cases occurred in Delaware and in the Willards area of Maryland in late July. Investigators of the National Communicable Disease Center isolated EEE from Culiseta melanura in that area 31 July to 6 August. The first isolation here reported was 21-22 August in the Willards Area. The two isolations from Mattapont Landing, 20 miles to the south, were not made until 10-12 September, although collections there had been made regularly since 21 August. Although these collections seem to indicate a north to south movement of virus activity, data for the summer of 1968 from the Pocomoke Swamp Study Area, 12 miles down river from Mattapont Landing, do not fit this pattern. EEE isolations were made from mosquitoes collected there by the Department of Virus Diseases in July, August, and September.

### 3. Studies on the effect of chemosterilants on susceptibility of mosquitoes to malarial infection

The use of agents of chemosterilization offers a dual potential of malaria control through interfering with the reproductive potential of the vector and interrupting the sporozoonous cycle of the malarial parasite. This study was designed to study the effect of Tapa (tri-(1)-aziridonyl)-phosphine oxide) in Anopheles stephensi (India) infected with the simian malarial parasite, Plasmodium cynomolgi.

Five day old mosquitoes were exposed to a chemosterilant treated surface 3 - 4 hours after an infective blood meal for a one hour period. Mosquitoes were agitated every ten minutes to assure that all individuals rested upon the treated area for maximal chemosterilant exposure. Treated females were individually transferred to glass vials for oviposition and dissections were made on the eighth day after an infective feed to observe plasmodial oocyst development.

High doses of chemosterilants are required to produce sterility. (Table 3). Residue concentrations of 20 and 40 mg/ft<sup>2</sup> produced 36 and 67 per cent sterility above that of the control. Mortality was 26 and 40 per cent above the control. There were no differences in mean oocyst counts among various dosage levels (Table 4). However, oocyst size became markedly reduced at higher residue levels. At 20 mg/ft<sup>2</sup> oocysts were one-fourth the diameter of control oocysts. It is believed that further study will indicate that these retarded oocysts will not produce infective sporozoites. Previous work performed in this laboratory suggests that exposure of mosquitoes to a treated surface for a three hour rather than a one hour period may produce a marked chemosterilant effect at lower dosage levels.

#### 4. Studies on Glossina and trypanosome transmission

The colony of Glossina austeni has been maintained in the insectary for approximately 18 months at an average level of 1800 flies. At present, 60 - 100 pupae are produced weekly which are used for colony maintenance, Trypanosoma brucei transmission studies and tissue culture investigations. The rearing procedure has been modified to produce greater adult longevity. Pupae are now incubated in large mesh cages with a layer of dry sand on the bottom rather than in moist sand in small paper cups and adults are maintained at 24°C rather than 27°C. Under this regimen, adult longevity is 8 - 16 weeks as contrasted with previous levels of 4 - 8 weeks. Effective 15 May 1969 a colony of Glossina morsitans will be started with material supplied by Dr. T.A.M. Nash, Director, Tsetse Research Laboratory, University of Bristol England to replace the G. austeni colony. G. morsitans is a more vigorous species in the laboratory than the former species and is a vector of both human and animal trypanosomiasis as contrasted to austeni which is only associated with cattle disease.

Studies on comparative infectivity of different vertebrate hosts to the flies have continued. The results are tabulated below for feeds of day old flies upon infected hosts. (Table 5). Although stumpy forms of T. brucei were observed in the peripheral circulation of rats and mice, these hosts were unsatisfactory for infecting flies.

Several new strains of T. brucei, isolated from African mammals, have been received from Dr. John Baker, London School of Hygiene and Tropical Medicine. These will be passaged in mice, frozen as stabulates, and transmission be effected with the guinea pig - tsetse system.

As mentioned in the 1968 Annual Progress Report, polymorphic trypanosomes of the T. brucei group may be cultured indefinitely on biphasic media but lost vertebrate infectivity within 24 hours. At that time it was possible to maintain virulence in T. brucei (Lugala I) for 72 hours in a medium of NCTC #135 with 5% fetal bovine serum. Experiments during the current year were designed to test various cell lines and media for maintaining vertebrate infectivity of T. brucei.

The initial studies utilized various media which were known to support the growth of other trypanosome species such as T. cruzi, T. lewisi and T. theileri (all monomorphic species). Although abundant growth of T. brucei occurred, these were all non-infective to mice with the exception of M + M medium which produced infection at 24 hours (Table 6). The addition of 5 per cent fetal bovine serum (FBS) had a marked effect on infectivity and raised it to 72 hours. A further increase in duration of infectivity was achieved by the addition of Aedes mosquito cell lines or mouse fibroblast cells (L-929) to various media. Consistently these cultures remained infective to 120 hours. Previous in vitro studies (LePage, 1967) mentioned that infectivity of cultures could regularly be maintained for 24 - 36 hours and rarely up to 70 hours.

#### Conclusions and recommendations

1. It was concluded that a mosquito transmission model for dengue using mice or similar small animals as recipient hosts and virus indicators is not feasible. All experiments were marked by low frequency of infection of mosquitoes and there was no evidence of virus multiplication in those mosquitoes which were shown to be infected by mouse inoculation. These results were not enhanced by the raising of mosquito incubation temperatures after infection, by increasing the concentration of virus in the infecting blood-virus mixture, nor by the use of low passage material.

Experimental models for the transmission of a chikungunya virus to mice or other small animals by mosquito bite have been demonstrated by other workers. Results of experiments conducted here indicate that a successful model is dependent upon the use of low mouse passage material.

2. Although it was not possible to start arborvirus studies until late in the summer of 1968 three isolates of EEE were reported from pools of Culiseta melanura collected on the Delmarva peninsula in August and September. In future studies, mosquitoes should be collected from outside the immediate vicinity of the Pocomoke Cypress Swamp to determine vector and viral movement patterns prior to the detection of virus activity within the swamp study area.

3. Chemosterilant activity of Tapa was demonstrated in an Anopheles stephensi - Plasmodium cynomolgi test system. There is a close relation between the MED and LD<sub>50</sub> of these compounds. Different methods of application and exposure of mosquitoes to these compounds should be examined to reduce the level of toxicity.

TABLE 1  
Experimental Transmission of Dengue-2 Virus to Mice by Aedes aegypti Mosquitoes

Experiment	Virus	Initial Mosquito Virus Titer*	Incubation Temperature (°C.)	No. Mosquitoes Infected/No. Mosquitoes Tested	Mean Titer No. Infected Mosquitoes*	No. Mosquitoes Transmitting/No. Transmissions Attempted
1	Ner Guinea C (3 <sup>rd</sup> Mouse Passage)	2.9	26	13/36	2.3	5/179
2	Bangkok 2358-62 (7 <sup>th</sup> Mouse Passage)	2.0	26	11/42	1.5	2/209
3	Bangkok 2358-62 (5 <sup>th</sup> Mouse Passage)	2.7	30	8/39	2.7	2/113
4	Bangkok 2358-62 (9 <sup>th</sup> Mouse Passage)	2.2	30	9/36	1.8	0/180
5	Bangkok 2358-62 (10 <sup>th</sup> Mouse Passage)	1.1	30	5/33	1.2	0/150

\*Number of Mouse Log<sub>10</sub> IC LD<sub>50</sub>'s

TABLE 2

Mosquito Collections made at six localities on the Delmarva Peninsula,  
13 August to 12 September, 1968

Species	Area						Total
	A	B	C	D	E	F	
<i>Aedes atlanticus</i>	8	10		1	284		303
<i>A. atropis</i>					1		1
<i>A. canadensis</i>	3	28		1	93		125
<i>A. cantator</i>	1	7	2		5		15
<i>A. sollicitans</i>		1	9		2		12
<i>A. taeniorhynchus</i>		2	30		3		35
<i>A. triseriatus</i>	31	20			27		78
<i>A. trivittatus</i>	1						1
<i>A. vexans</i>		1					1
<i>A. spp.</i>					1		1
<i>Anopheles crucians</i> complex	2				14	2	18
<i>An. punctipennis</i>	5		5		3	2	15
<i>An. quadrimaculatus</i>	1				1		2
<i>An. spp.</i>					1		1
<i>Culex erraticus</i>					1	2	2
<i>C. restuans</i>					1		1
<i>C. salinarius</i>					4		4
<i>Culiseta melanura</i>	2						2
<i>Culiseta melanura</i>	21	99*	11	1	315*	79	526
<i>Mansonia perturbans</i>		1			1		2
<i>Psorophora ferox</i>	18	23	10	4	155		210
<i>Uranotaenia sapphirina</i>	5	4			2		11
<b>Totals</b>	<b>98</b>	<b>196</b>	<b>67</b>	<b>7</b>	<b>913</b>	<b>85</b>	<b>1,366</b>

\*Isolation of EEE virus

TABLE 3

Effect of Tapa on fecundity, fertility and mortality in  
Anopheles stephensi

	Residue level (mg/ft <sup>2</sup> )				
	Control	5	10	20	40
% egg hatch	33	37	62	69	100
$\bar{x}$ eggs/female	61	61	68	72	72
% mortality	41	50	52	67	81
No. females	119	103	86	40	7

TABLE 4

Effect of Tapa on Plasmodium cynomolgi oocyst development in  
Anopheles stephensi

	Residue level (mg/ft <sup>2</sup> )					
	Control	1.25	2.50	5	10	20
$\bar{x}$ oocysts/ female	97	98	100	96	97	98
No. females	36	13	32	36	30	12

TABLE 5

Infection of Glossina austeni with Trypanosoma brucei (Lugala I) from different donor hosts

Host	# Feeds	# <u>Infected flies</u> # <u>Flies dissected</u>	% Flies infected
Guinea pig	20	9/106	8.5
Mouse and rat	8	0/34	0

TABLE 6

In vitro cultivation of Trypanosoma brucei (Lugala I) at 27°C in culture flasks

Medium	Maximal age of culture producing mouse infection (hours)
Tobie's	Non-infective at 24 hours
Grace's	Non-infective at 24 hours
Grace's + 5% lactoalbuminhydrolysate	Non-infective at 24 hours
GIB	Non-infective at 24 hours
M + M	24
90% Grace's + 10% M-109 (10X)	Non-infective at 24 hours
M-109 + 5% FBS	72
NCTC-135 + 5% FBS	72
50% Home's (APG) + 50% M-109 + 5% FBS	72
M-199 + 5% FBS	72
<u>Aedes albopictus</u> cells, M + M, + 5% FBS	120
<u>Aedes aegypti</u> cells, M + M + 5% FBS	120
Mouse L cells, M-109 + 5% FBS	120
Mouse L cells, M-199 + 5% FBS	120
Mouse L cells, NCTC-135 + 5% FBS	120

Project 3A061102B71P  
Task 03, Entomology  
Work Unit 035, Ecology and control of disease vectors and reservoirs

Publications

- Gould, D. J., Yuill, T. M., Moussa, M. A., Simasathien, P. and L. C. Rutledge. 1968. An insular outbreak of dengue hemorrhagic fever. III. Identification of vectors and observations on vector ecology. *Amer. J. Trop. Med. Hyg.* 17:609-618.
- Hayes, D. E. 1968. The successful induced copulation of Culiseta melanura (Coquillet). *Mosquito News* 28:109-110.
- Langer, B. W., Rutledge, L. C. and D. J. Gould. 1968. Chloroquine retention by Anopheles stephensi after ingestion in a blood meal. *Mosquito News* 28:455-458.
- Ward, R. A. 1968. The susceptibility of *Glossina austeni* to infection with Trypanosoma brucei. *Trans. Roy. Soc. Trop. Med. Hyg.* 62: 672-678.

**PROJECT 3A061102B71P**  
**BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE**

**Task 04**  
**Immunology**

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				DA 0A6432	69 07 01	DA FORM 1580-1 (7-68)	
69 01 31	D. CHANGE	U	U	RA	NL	YES	ASSET
NO. / CODES	PROGRAM ELEMENT	PROJECT NUMBER	TACTIC AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	61102A	3A061102B71P	04	015			
B. CONTRIBUTING							
C. CIPHERTEXT	1412A(2)						
11. TITLE (Precede with Security Classification Code)							
(U) Antigen-Antibody Reactions In Vivo and In Vitro (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
010100 Microbiology							
13. START DATE	14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD		
08 63	NA		DA		C. IN HOUSE		
17. CONTRACT/GRANT NA				18. RESOURCES ESTIMATE		19. FUNDS (in \$ mil)	
A. DATE/EFFECTIVE				B. MONTHS		C. PROFESSIONAL MAN YRS	
EXPIRATION				FISCAL YEAR		D. FUNDS (in \$ mil)	
A. NUMBER*				69		9	
B. TYPE				70		190	
C. KIND OF AWARD				7		160	
2. CUM. AMT.							
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME* Walter Reed Army Institute of Research Washington, D. C. 20012				NAME* Walter Reed Army Institute of Research Div of CD and I Washington, D C 20012			
ADDRESS*				ADDRESS*			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Precede SSN) (U.S. Academic Institution)			
NAME Meroney, COL W.H.				NAME* Becker, E. L.			
TELEPHONE 202-576-3551				TELEPHONE 202-576-3665			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME* Barbaro, J.F.			
				NAME:			
				DA			
22. KEYWORDS (Precede with Security Classification Code)							
(U)Allergy, (U) Enzymes, (U) Immunology, (U) Antigen, (U) Antibody, (U) Hypersensitivity							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Formal technical paragraphs identified by number. Precede text of each with Security Classification Code)							
<p>23 (U) Work under this work unit involves the study of the basic mechanisms of the immediate type allergies from the viewpoint of the enzyme systems involved. This looks to the ultimate control of such allergies by a specific inhibition of these enzymes. The development of methods for the isolation and characterization of the enzymes involved in hypersensitivity reactions.</p> <p>24 (U) Phosphonate inhibitors are being synthesized and tested in vitro for selective action against various enzymes. The distribution of blood group antibody between fluid phase and human erythrocyte is being investigated under various conditions of temperature and concentration of cells and antibody. The nature of the lymphocyte-dependent histamine release from rabbit platelets is being studied.</p> <p>25 (U) 69 01 - 69 06. Three already activated esterases capable of hydrolyzing acetyl DL phenylalanine Beta naphthyl ester exist on the rabbit peritoneal polymorphonuclear leukocytes. One of these has been shown to be the activated form of the activatable esterase of chemotaxis. Utilizing cyclo-hexyl butylphosphorofluoridate, evidence has been obtained for the involvement of an activatable esterase in the complement dependent erythrophagocytosis by peritoneal polymorphonuclear leukocytes. The lymphocyte-dependent histamine release from rabbit platelets has been divided experimentally into two distinct steps. The first involves the reaction of sensitized lymphocytes with antigen, the second is concerned with the interaction of these lymphocytes with platelets. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.</p>							

PII Redacted

Project 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

Work Unit 015 Antigen-antibody reactions in vivo and in vitro

**Investigators.**

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Schoenbechler, MD, MAJ R. Evans, MC

Description:

The purpose of this task is to study the enzymatic and other mechanisms of allergic reactions and the agglutination reactions of the human blood group.

Progress:

1. Chemotaxis of rabbit polymorphonuclear leukocytes

a. As summarized in preceding Annual reports evidence for a requirement for two serine esterases in the chemotaxis of polymorphonuclear leukocytes has been obtained using phosphonate ester inhibitors. One of them termed the "activatable esterase" exists on the cell in an inactive form and the chemotactic factor directly or indirectly transforms the esterase into an active enzyme. Evidence was also obtained suggesting that the activatable esterase was an enzyme with a particular affinity for aromatic amino acid derivatives. In attempting to directly demonstrate the activatable esterase, it was found that three already activated esterases existed on the rabbit peritoneal polymorphonuclear leukocyte that were capable of hydrolyzing the aromatic amino acid ester, acetyl DL phenylalanine  $\beta$  naphthyl ester. Esterase 1 was completely inhibited by phosphonate esters in the range of  $10^{-8}$ - $10^{-9}$ M; esterase 2 in the range of  $10^{-5}$ - $10^{-6}$ M phosphonate, and esterase 3 was not inhibited even at  $10^{-3}$ M.

b. Work over the past year on the inhibition profiles, i.e. the relationship of the structure of the phosphonates comprising a given homologous series and their ability to inhibit acetyl DL phenylalanine  $\beta$  naphthyl esterase activity using four p-nitrophenyl ethyl phenyl alkyl phosphonates, four p-nitrophenyl ethyl alkyl phosphonates, four p-nitrophenyl chloro-alkyl phosphonates demonstrated the following: The inhibition profile of esterase 1 was shown to be distinctly different from the inhibition profiles of esterase 2 and both differed markedly from the nine non-cell and cell bound esterases which have so far been studied with the same phosphonates. However, esterase 1 gave inhibition profiles with these phosphonates which were essentially identical to those given by the activatable esterase of chemotaxis whether the profiles were obtained by inhibition of chemotaxis or inhibition of "deactivation." On this basis, esterase 1 has been tentatively identified with the activated form of the activatable esterase of chemotaxis.

c. Since only inconsistent results were obtained in the search for the precursor form of the activatable esterase of chemotaxis using rabbit peritoneal neutrophils (see preceding Annual report) attention was turned to rabbit blood leukocytes. The blood leukocytes are first treated with phosphonates to destroy all activated forms of esterases 1 and 2. Using an improved assay procedure, it has been possible to show that exposure of these cells to the activated chemotactic factor leads to a small but statistically significant increase of esterase activity compared to the same lot of cells treated with either non-activated chemotactic factor, or with buffer.

## 2. Enzymatic mechanisms of phagocytosis:

a. As described in the preceding Annual report, an activated esterase distinct from the activatable esterase of chemotaxis as well as esterases 1, 2, and 3 has been demonstrated to be required in the complement dependent phagocytosis of sheep red blood cells by guinea pig peritoneal polymorphonuclear leukocytes. The exact relationship of the activated esterase of chemotaxis to the activated esterase of phagocytosis is still unknown.

b. Utilizing the p-nitrophenyl ethyl alkyl phosphonates, phenylalkyl phosphonates, or chloro-alkylphosphonates, no evidence for the involvement of an activatable esterase in phagocytosis could be obtained. However, simultaneous incubation of sensitized red cells containing complement and neutrophils with cyclohexyl butylphosphonofluoridate gave very distinct inhibition. This inhibition was not obtained if either the erythrocytes or the neutrophils were

incubated with the phosphonofluoridate alone, and then inhibitor washed out before the other cellular component was added. These results indicate that an activatable esterase might also be involved in phagocytosis. The subsequent finding that the phosphonofluoridate acts early in phagocytosis before the engulfment phase is completed is compatible with the idea that it acts on an activatable esterase.

c. We are currently attempting to obtain more conclusive evidence for the involvement of an activatable esterase in phagocytosis, and through the use of new phosphonofluoridates recently synthesized (see section 5) to relate this esterase to esterases involved in other cellular processes.

### 3. The effect of chemical modification of the antibody on its complement fixing and other biologic properties

a. As recorded in preceding Annual Reports, chemical modification of the amino groups of rabbit  $\gamma$ G antibody by amidination or carbamylation distinctly decreased the complement fixing activity for guinea pig complement. Attention was next turned to the effects of similar treatment of rabbit  $\gamma$ G antibody on its PCA activity in rabbit and guinea pig skin.

b. One preparation of rabbit  $\gamma$ G globulin, one third of which was antibody against chicken ovalbumin, was used throughout. It was prepared by repeated precipitation with one-third saturated  $(\text{NH}_4)_2\text{SO}_4$ . The PCA activity was elicited by challenge with antigen and blue dye four hours following introduction of the antibody into guinea pig skin and 72 hours after injection into rabbit skin. The PCA activity for rabbits was heat stable and sulfhydryl resistant. It was thus unlike the rabbit, heat labile, homocytotropic antibody activity discovered by Zvaifler and Becker, but rather like the rabbit, heat stable, 7S  $\gamma$ G, complement dependent, PCA activity recently described by Henson and Cochrane.

c. Carbamylation of this rabbit  $\gamma$ G globulin preparation sufficient to conjugate 80% of its free amino groups resulted in a 94% reduction in PCA activity with respect to rabbit skin, but no alteration in reactivity with respect to guinea pig skin. The loss of PCA activity in rabbit skin was attributed to the 87-98% loss of complement fixing activity associated with this degree of carbamylation.

d. Loss of PCA activity in guinea pig skin required greater than 90% carbamylation of the antibody molecule, and could be explained in terms of non-specific effects on the molecule produced by such treatment. The latter were shown by light scattering and turbidity measurements to be very great. This loss of PCA activity by 90% carbamylation of the antibody was potentiated when cross reacting, goose ovalbumin was used in place of chicken ovalbumin.

4. The latency requirements and rate of evolution of passive cutaneous anaphylactic reactions in guinea pigs produced by untreated and carbamylated rabbit  $\gamma$ G antibody.

a. The requirement for a "latent period," that is, an interval between the introduction of antibody and injection of antigen in order to obtain maximal sensitization for anaphylaxis, as well as other findings, has led to the concept that in at least certain kinds of anaphylactic reactions an actual fixation of antibody molecules to specific tissue or cellular receptor sites precedes the reaction of an antibody with antigen. The following study was carried out in an attempt to gain insight into the nature and significance of latency and the mechanisms involved in antibody fixation.

b. The same rabbit  $\gamma$ G globulin preparation, one third of which was anti-chicken ovalbumin, described in the preceding section was used as the antibody source. The "no latency" PCA reaction obtained by injecting antigen intravenously at the same time antibody was injected into the guinea pig skin was compared to the "4 hour latency" PCA reaction where there was an interval of four hours between the time the antibody was injected into the skin and the antigen injected intravenously. The minimum amount of antibody required to produce "no latency" PCA is eight times that needed for a four hour response. After a latency period of 4 hours, the injection of blue dye and antigen results in the appearance of pale bluing over the entire reaction site. This is apparent in less than two minutes after injection of the antigen dye mixture. The reaction site then progressively intensifies in color. The time of initial appearance of this response was unaffected by a thousand fold range of antibody concentration.

c. In the "zero latency" PCA, the reaction begins focally at the injection site, and then extends centrifugally with time until the maximum reaction size is attained. In this situation there is a direct relationship between the amount of antibody used, and the rate of evolution of the reaction since both the rate of diffusion of antibody and of the reaction of antigen and antibody is dependent upon local antibody concentration.

d. The differences in the pattern of evolution of the two kinds of PCA reaction depends upon the fact that in the case of the no latency reaction, diffusion of antibody is apparently rate limiting, whereas in the four hour latency response this is not the case, maximal sensitization of all sites having occurred during the 4 hour period. Thus, the above observations do not imply a fundamental distinction between the two forms of the PCA reaction.

e. Antibody with 80% of its amino groups carbamylated showed no increase in the amount of antibody required to sensitize for "no latency" PCA, nor any change in the rate of evolution of the reaction. The rate of immune aggregation is markedly diminished by the 80% carbamylated antibody. The lack of effect of this degree of carbamylation on either the zero latency or "4 hour latency" reaction indicates that they both are instituted by the formation of complexes with antigen containing one or only a few antibody molecules. The lack of effect of 80% carbamylation of the antibody on the zero latency reaction, even though there is marked depression of its complement fixing abilities indicates that, like the 4 hour latency reaction, the zero latency reaction does not require complement.

f. These resemblances between the 4 hour and zero hour latency reactions have led us to suggest that the two reactions result from the operation of the same mechanisms and that the latency requirement may be the time required for antibody on the cell surface to reassociate until an optimal configuration is attained.

#### 5. The synthesis of novel phosphonates.

Seventeen new phosphonates were synthesized. Their elementary analysis and physical properties are given in Table I and II.

#### 6. Characterization of mouse homocytotropic antibodies.

a. The previous annual report established that antisera from mice infected with Trichinella spiralis could induce passive cutaneous anaphylaxis (PCA) reactions. These antisera produced homologous PCA reactions after a sensitization period of 2 hours and 72 hours.

b. It was hypothesized that 2 hour PCA reactions were due to 7S<sub>Y1</sub> antibody while the 72 hour PCA reactions were induced by another antibody. This hypothesis was tested by absorbing mouse anti T. spiralis sera with a highly specific rabbit anti-mouse 7S<sub>Y1</sub>. This absorption completely suppressed the ability of these antisera to induce PCA reactions after a sensitization period of 2 hours but ability of the absorbed sera to induce a PCA reaction at 72 hours was essentially unchanged. These results suggest that in the mouse, 7S<sub>Y1</sub> antibody is responsible for the 2 hour PCA reaction.

c. To confirm these results and to determine the relationship of the 2 hour and 72 hour homologous (mouse) antibodies to the

antibody inducing the heterologous (rat) PCA activity the following experiments were done. Antisera from mice immunized with ovalbumin were collected at various time intervals and the ability of these antisera to produce homologous and heterologous (rat) PCA reactions were determined using a sensitization period of 2 or 72 hours. The following observations were made: 1) The homologous 2 hour PCA activity increased whereas the heterologous and homologous 72 hour PCA activities decreased during immunization. 2) The 72 hour PCA titer in mice was the same as the PCA titer in rats. 3) The 72 hour PCA activity in mice and the PCA activity in rats were both heat labile. 4) Absorption of mouse antisera with highly specific rabbit anti-mouse  $7S\gamma_1$  sera removed the 2 hour PCA activity in mice but not the mouse 72 hour PCA activity or rat PCA activity. These studies confirmed the previous results obtained with *T. Spiralis* infected mice that the 2 hour PCA activity in mice as well as the PCA activity in rats is due to a heat labile homocytotropic (reaginic) antibody.

d. These findings also suggested that the 2 hour mouse PCA activity in general could serve as an index of  $\gamma_1$  antibody activity and the heterologous rat PCA activity as a marker for heat labile homocytotropic antibody. On the assumption that these two antibodies possess different tissue affinities an attempt was made to separate the activities of these two antibodies. Normal mice were injected intravenously with mouse antiserum, containing both antibodies after 6 and 24 hour intervals these animals were bled and their sera tested for mouse 2 hour and 72 hour PCA activity. This procedure was termed biological screening of mouse PCA activity. The results indicated that by in vivo screening rat PCA activity heat labile homocytotropic antibody was completely removed within 24 hours while the mouse 2 hour ( $7S\gamma_1$ ) activity remained the same. By this technique it has been possible to show that the PCA reaction in mouse antisera obtained 8 days after immunization is produced almost entirely by heat labile homocytotropic antibody. The heat labile homocytotropic antibody present in this sera also induced a 2 hour mouse PCA reaction which is not to be confused with that produced by the  $7S\gamma_1$  antibody. As immunization continues this 2 hour heat labile, homocytotropic PCA activity is suppressed, most likely by increasing amounts of other competing antibodies. Additional data were obtained involving heated mouse antisera and specific anti-mouse  $7S\gamma_1$  sera which substantiated these findings.

#### 7. Histamine release from rabbit platelets.

a. The overall reaction of the lymphocyte-dependent release of histamine from rabbits infected with *Schistosoma mansoni* has been

divided experimentally into two distinct steps. The first involves the reaction of sensitized lymphocytes with antigen; the second is concerned with the interaction of these lymphocytes with platelets to release histamine from the latter. The reaction of antigen with sensitized lymphocytes results in a cellular change that renders the lymphocytes capable of reacting with platelets to release histamine. Cells changed in this manner were termed "activated" lymphocytes.

b. The nature of the activation process of lymphocytes was studied. It was established that once the antigen had reacted with sensitized leukocytes the excess antigen could be completely removed, leaving leukocytes capable of exerting a histamine releasing effect on platelets. Further, the leukocytes activated by antigen before the addition of rabbit platelets showed virtually no lag in comparison to controls to which leukocytes, antigen and platelets were all added simultaneously.

c. The length of time sensitized leukocytes had to be incubated with antigen at 37°C for maximal activation was determined. Maximal activation was obtained when leukocytes were pre-treated with antigen for 15 minutes. When leukocytes were pre-treated with antigen at 37°C for 180 minutes a substantial decrease in activation was observed. Control leukocytes pre-treated with Tyrode's solution at 37°C for 180 minutes showed no change in their ability to be activated.

d. Experiments were conducted to determine if the activation of leukocytes by antigen was temperature dependent. Leukocytes incubated with antigen at 0°C and 22°C showed little or no activation and optimal activation occurred at 37°C.

e. The activation of leukocytes with antigen in the cold was studied in detail. It became apparent that low temperatures had a deleterious effect on the ability of leukocytes to be activated. There is a dual effect of cold on the activation process; one, the destruction of activable sites and two, the lowering of the rate of activation.

f. The exposure of leukocytes to cold was studied to determine if the deleterious effect was on steps prior to activation or on the activated sites. The results obtained demonstrated that non-activated sites are labile to the cold if not reacted with antigen but once the activation process is completed the resultant activated state is quite stable in the cold.

8. Studies on blood group antigens and antibodies.

a. The Wurmser's have concluded on the basis of extensive work that the binding properties of the anti-B isohemagglutinins naturally occurring in the sera of normal individuals are characteristic of the genotype of the ABO blood group of the individual (Prog. Biophys. 7: 88, 1957). Because of the theoretical importance of these claims and their potential practical importance in forensic medicine and in clinical medicine (where knowledge of the homo- or heterozygosity of the father is of importance in cases of hemolytic disease of the newborn in those cases where the mother is blood group 00), the observations of the Wurmser's have been investigated.

b. In the earlier investigation the binding properties of the isohemagglutinins of 5 group 00, 2 group A<sub>1</sub>O and 4 group A<sub>2</sub> sera were tested using 500,000 to 800,000 B cells/mm<sup>3</sup> to absorb constant amounts of antiserum dilutions at both 37C and 25C. Both the log-probit assay method of Willie and Becker (J. Immunol. 74: 192, 1955) and the Wurmser assay technic were used to test the antibody free in the supernatants at equilibrium. When the reciprocal of the antibody fixed per cell was plotted against the reciprocal of the amount of antibody free in the supernatant, there were quantitative differences in the slopes and positions of the lines obtained by the two assay methods. However, in each case, the log-probit data could be converted by means of a proportionality constant to fit the Wurmser assay line. These findings have been confirmed with two additional group 00 sera and another known A<sub>1</sub>O serum. In addition, the sera from four siblings of the Cask family of known A<sub>2</sub>O genotypes have given the same results as previously found with sera from A<sub>2</sub> individuals of unknown genotypes. These findings provide further independent evidence in support of conclusions of the Wurmser's. However, to be of practical value, it is essential to demonstrate with the log-probit assay method that the results obtained with sera from homozygous A<sub>1</sub>A<sub>1</sub> persons differ in their binding properties from those found with A<sub>2</sub>O sera. An intensive search is being made to locate known A<sub>1</sub>A<sub>1</sub> individuals with no success to date. However, the mother of the Cask family has five A<sub>2</sub>O children hence it is likely that her genotype is A<sub>2</sub>A<sub>2</sub>. An investigation of her serum is contemplated as soon as her serum can be obtained.

c. The Wurmser's have reported that the ratio of the maximum number of B red cells agglutinated at 4C to the maximum number of B red cells agglutinated at 37C ( $N_4/N_{37}$  ratio) is also characteristic of the ABO genotype of the individual. However, these differences could not be demonstrated with sera heated to 56C for 60 mins. (Prog. Biophys. 7: 88, 1957). Irmgard Oeper using the Wurmser assay method and a modification of the Wilkie and Becker log-probit assay method has recently reported that the  $N_4/N_{37}$  ratios for a given genotype by the Wurmser's could not be confirmed. Humangenetik 5: 201, 1968). The  $N_4/N_{37}$  ratio for an  $A_1A_1$  serum could not be distinguished from that of an  $A_1O$  serum. Moreover, there was a wide range of variation among the eleven  $A_1$  sera, the three group OO sera and the four group  $A_2$  sera tested. Moreover, there was no difference in the  $N_4/N_{37}$  ratios of heated and unheated sera and reproducible results were not obtained with any given serum. As the determination of an  $N_4/N_{37}$  ratio is less tedious and time consuming than the determination of the binding properties of the anti-B iso-hemagglutinins, the former procedure would be of more practical value in the routine forensic medicine or clinical laboratory. In view of the fact that our laboratory has been able to confirm the Wurmser's findings of differences in the binding properties of anti-B iso-hemagglutinins of individuals of different ABO blood groups, the  $N_4/N_{37}$  ratios of the sera used in the earlier work were investigated.

d. In this investigation, the  $N_4/N_{37}$  ratios of four group OO, two  $A_1O$ , three  $A_1$  and three  $A_2$  sera were determined by the log-probit and the Wurmser assay methods. The two  $A_1O$  sera gave a ratio of 2.5 as reported by the Wurmser's but the ratios found for the group OO, the  $A_1$  and  $A_2$  sera were highly variable. In many instances the results at 37C were erratic on repeated testing. This suggests that the technic used at this temperature may be faulty. Modifications in the 37C assay are under study. The effect on the  $N_4/N_{37}$  ratio of heated and unheated sera has been studied with only one group OO serum. There was no difference in the results obtained at either temperature.

#### 9. Studies of the blood group A and B substance activities of vaccines.

a. The hemagglutination-inhibition test advocated by the Division of Biological Standards (DBS) of the N.I.H. in the 4th Revision of the Minimum Requirements for Tetanus Toxoid effective June 1, 1951 is currently being used to test vaccines for blood group A and B activity. The method is exceedingly insensitive because of the high concentration of antiserum employed (2 test doses of a serum dilution giving maximum or 4+ agglutination). Moreover,

the method is not reproducible for the following reasons: 1) The incubation period of the vaccine with antiserum is too short (10 mins) to assure completion of the reaction and 2) in the final stage of the test, the degree of agglutination is read macroscopically after centrifugation and resuspension of the packed cells by shaking. The factors contributing to the non-reproducibility of agglutination after centrifugation and shaking have been documented by Solomon, Gibbs and Bowdler (*Vox Sang.* 10: 54, 1965). To test the vaccines procured by the government for use by the Armed Forces, a highly sensitive and reproducible method is desirable. For this reason, the hemagglutination-inhibition serial dilution titration method of Springer *et al.* (*J. Lab. & Clin. Med.* 43: 532, 1954) was modified for the testing of vaccines. The modified method employs four minimum doses of antiserum giving 2+ agglutination. (One minimum dose of antiserum is the dilution giving a + reaction), a 2 hour incubation of the antiserum-vaccine mixture to assure completion of the inhibition reaction, the final agglutination stage is allowed to equilibrate for 2 hours at 25C and agglutination is read microscopically. The method has been thoroughly tested and found to be highly reproducible. It has been recommended to the Division of Biologic Standards, NIH, for consideration as their standard method.

b. The following commercial vaccines were tested for the presence of blood group A and B activity: 34 Plague, 51 Typhoid and 2 Cholera. These vaccines had no detectable A or B blood group substances.

Summary and Conclusions:

1. Three activated esterases, esterases 1, 2 and 3, exist on the rabbit peritoneal polymorphonuclear leukocytes that are capable of hydrolyzing the acetyl DL phenylalanine  $\beta$  naphthyl ester.

2. Esterase 1 was completely inhibited by phosphonates in the range of  $10^{-8}$ - $10^{-9}$ M; esterase 2 in the range of  $10^{-5}$ - $10^{-6}$ M and esterase 3 was not inhibited even at  $10^{-3}$ M.

3. Esterase 1 has essentially the same inhibition profile with three different homologous series of phosphonates as the activatable esterase of chemotaxis. On this basis, esterase 1 has been tentatively identified with the activated form of the activatable esterase of chemotaxis.

4. No evidence for the involvement of an activatable esterase in phagocytosis could be demonstrated with p-nitrophenyl ethyl alkyl, phenylalkyl or chloro-alkylphosphonates.

5. Suggestive evidence of an activatable esterase was obtained with cyclohexyl butylphosphonofluoridate.

6. Seventeen new phosphonates were synthesized.

7. Carbamylation of rabbit  $\gamma$ G globulin sufficient to conjugate 80% of its free amino groups results in a 94% reduction of PCA activity in rabbit skin, but no alteration in reactivity to guinea pig skin.

8. Loss of PCA activity in guinea pig skin could be accomplished with greater than 90% carbamylation of the antibody molecule. This was explained by non-specific effects on the antibody molecule rather than to specific inactivation of PCA sites.

9. The minimum amount of antibody required to produce "no latency" PCA reaction is eight times that needed for a "4 hour latency" response.

10. It is concluded that the "no latency" and the "four hour latency" PCA reactions result from the same mechanisms and that the latency requirement may be the time required for antibody on the cell surface to reassociate until an optimal configuration is attained.

11. Evidence has been obtained that suggest that mouse 2 hour PCA reaction is due to a 7S $\gamma$ <sub>1</sub> antibody.

12. A procedure, termed "Biological Screening", which depends on the different tissue affinities of the 2 hour and 72 hour PCA reaction was developed.

13. The lymphocyte-dependent histamine release from rabbit platelets does not occur from the production of a soluble factor resulting from the reaction of antigen and sensitized lymphocytes.

14. The effect of temperature on the activation of sensitized lymphocytes by antigen to give a cell capable of interacting with platelets is complex and probably enzymatic in nature.

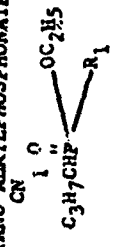
15. The Wurmser's report that the ratio of the maximum number of B red cells agglutinated at 4°C to the maximum number of cells agglutinated at 37°C is characteristic of the ABO genotype has not



been confirmed due to technical difficulties.

16. A Hemagglutination-Inhibition test for the study of blood group substances in vaccine which is highly reproducible has been developed.

TABLE I

α-CYANO ALKYLPHOSPHONATES



#	MOL. FORMULA	R <sub>1</sub>	M <sub>D</sub> <sup>25</sup>	B.P. °/mm.	C CALCD F	H CALCD F	P CALCD F	N/X CALCD F				
1	C <sub>7</sub> H <sub>13</sub> ClNO <sub>2</sub> P	Cl	1.4538	100/0.3								
2	C <sub>13</sub> H <sub>17</sub> N <sub>2</sub> O <sub>5</sub> P	-O-  -NO <sub>2</sub>	1.5189	*Xylene	500	48.8	505	5.7	9.9	9.6	9.0	8.5
3	C <sub>13</sub> H <sub>17</sub> FNO <sub>3</sub> P	-O-  -F	1.4807	127/0.07	54.7	54.4	6.0	7.0	10.9	10.6	84.9	4.8
											86.7	6.7

\* Molecular Still

TABLE II  
CYCLOHEXYL ALKYLPHOSPHONATES



MOL. FOR	R1	R2	BP O/mm	C		H		P		F/Cl		
				Calcd	F	Calcd	F	Calcd	F	Calcd	F	
C <sub>9</sub> H <sub>18</sub> ClO <sub>2</sub> P	Propyl	Cl	1.4628	79/0.05	51.9	51.6	8.7	8.8	14.9	15.0	9.1	8.6
C <sub>9</sub> H <sub>18</sub> FO <sub>2</sub> P	Propyl	F	1.4378	43/0.02	51.9	51.6	8.7	8.8	14.9	15.0	9.1	8.6
C <sub>11</sub> H <sub>22</sub> ClO <sub>2</sub> P	Pentyl	Cl	1.4703	99/0.1	55.9	55.8	9.4	9.1	13.1	13.0	8.0	8.5
C <sub>11</sub> H <sub>22</sub> FO <sub>2</sub> P	Pentyl	F	1.4408	60/0.005	55.9	55.8	9.4	9.1	13.1	13.0	8.0	8.5
C <sub>12</sub> H <sub>24</sub> ClO <sub>2</sub> P	Hexyl	Cl	1.4678	110-5/0.05	57.6	57.3	9.7	9.7	12.4	12.3	7.6	8.0
C <sub>12</sub> H <sub>24</sub> FO <sub>2</sub> P	Hexyl	F	1.4421	93/0.005	57.6	57.3	9.7	9.7	12.4	12.3	7.6	8.0
C <sub>12</sub> H <sub>16</sub> FO <sub>2</sub> P	Phenyl	Cl	1.5328	137/0.2	59.5	59.5	6.7	7.6	12.8	12.6	7.8	7.4
C <sub>12</sub> H <sub>16</sub> FO <sub>2</sub> P	Phenyl	F	1.5021	94/0.05	59.5	59.5	6.7	7.6	12.8	12.6	7.8	7.4
C <sub>13</sub> H <sub>18</sub> ClO <sub>2</sub> P	Benzyl	Cl	1.5309	*Toluene	60.9	61.0	7.1	7.3	12.1	11.7	7.4	6.7
C <sub>13</sub> H <sub>18</sub> FO <sub>2</sub> P	Benzyl	F	1.5041	96/0.025	60.9	61.0	7.1	7.3	12.1	11.7	7.4	6.7
C <sub>14</sub> H <sub>20</sub> ClO <sub>2</sub> P	Phen-ethyl	Cl	1.524 <sup>h</sup>		62.2	61.0	7.5	7.3	11.5	11.6	7.0	6.8
C <sub>14</sub> H <sub>20</sub> FO <sub>2</sub> P	Phen-ethyl	F	1.5001	113/0.02	62.2	61.0	7.5	7.3	11.5	11.6	7.0	6.8
C <sub>15</sub> H <sub>22</sub> ClO <sub>2</sub> P	Phen-propyl	Cl	1.5224	*Xylene	63.4	63.9	7.8	7.8	10.9	10.6	6.9	7.1
C <sub>15</sub> H <sub>22</sub> FO <sub>2</sub> P	Phen-propyl	F	1.4988	132/0.005	63.4	63.9	7.8	7.8	10.9	10.6	6.9	7.1

\* Molecular Still

Project 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

Work Unit 015, Antigen-antibody reactions in vivo and in vitro

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACQUISITION	2. DATE OF SUMMARY	3. REPORT CONTROL SYMBOL	
				DA 0A6433	69 07 01	DD FORM (41) 755	
4. DATE PREPARED	5. KIND OF SUMMARY	6. SUMMARY CTRY	7. WORK PRIORITY	8. REFERENCE	9. DESIGNATION	10. SPECIFIC LIMITS CONTRACTOR ACCESS	11. LEVEL OF DIS AGENCY USE
31 01 69	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. FORN USE
12. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	61102A	3A061102R71P	04	016			
B. CONTRIBUTING							
C. COUNTERMEASURE	1412A(2)						
11. TITLE (Precede with Security Classification Code)							
(U) Immunization Studies of Exotic Diseases (09)							
12. SCIENTIFIC AND TECHNOLOGICAL ARTS							
010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
08 63		NA		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATE/EFFECTIVE: NA				B. MONTHS		C. PUTES per Person/yr	
B. NUMBER				FISCAL YEAR		69	
C. TYPE				70		2	
D. KIND OF AWARD				80		90	
E. CUM. AMT.				2		90	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research ADDRESS: Washington, D. C. 20012				NAME: Walter Reed Army Institute of Research ADDRESS: Division of Veterinary Medicine Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR: (Provide NAME of U.S. Graduate including)			
NAME: Maroney, COL, W. H.				NAME: Harrison, V. R.			
TEL (PHONE): 202-576-3551				TELEPHONE: 202-576-5109			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Asbill, COL S. G.			
				NAME: Eckels, K. H.			
				DA			
13. TECHNICAL OBJECTIVE: (a) APPROX. (b) DETAILS (Precede by Security Code) (c) No classified or number property code of or with Security Classification Code)							
23. (U) Chikungunya is an arthropod-borne viral disease that is indigenous to Africa and Asia. Outbreaks of Epidemic Proportions have been reported from Africa, Southeast Asia and India. It is a seriously debilitating, though rarely fatal disease of man. The highest mortality occurs in persons under 12 years of age. This investigation is primarily concerned with the production of a vaccine offering broad spectrum protection for man.							
24. (U) Despite its widespread geographical distribution, Chikungunya virus is characterized by several closely related strains. Cognizance of these close antigenic relationships enhanced the feasibility of preparing a formalin-killed tissue culture vaccine with one well-characterized virus strain.							
25. (U) 69 01 - 69 06 Studies on the histopathology and neurovirulence of Chikungunya infection in the rhesus monkey are in progress. Forty selected tissues from each of 17 monkeys (6 vaccinated and 11 non-vaccinated) have been processed for viral isolation. Results show that virus persists in regional lymph nodes of non-vaccinated monkeys up to 7 days after inoculation. Spleen, thymus and thyroid tissues appear to be sites of extensive viral replication. Virus was not isolated from any tissues processed from vaccinated monkeys. A formalin-killed vaccine has been prepared in the human diploid WI-38 tissue culture. Potency assays of this vaccine compared favorably with those performed on vaccines prepared in the green monkey kidney tissue culture. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 68 - 30 June 69.							

PII Redacted

**Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE**

**Task 04, Immunology**

**Work Unit 016 Immunization studies of exotic diseases**

**Investigators.**

**Principal: V. R. Harrison, MS**

**Associate: K. Eckels, MS; C. Hampton, BA**

**Description.**

This task is concerned with the development, production and evaluation of either live-attenuated or formalin-inactivated vaccines against exotic viral agents.

**Progress.**

A lot of formalin-inactivated Chikungunya vaccine has been prepared in bank-frozen green monkey kidney cells, certified-free of detectable adventitious agents. This vaccine is now ready for the performance of human field trials. The constantly increasing numbers of adventitious agents being isolated from cercopithecus monkey tissues makes the continued use of this menstruum as a suitable substrate for vaccine production highly questionable. Therefore, we are investigating the potential value of the human diploid cell strain WI-38 as an alternate source of vaccine substrate. Comparative assays are being performed on vaccines prepared in WI-38 and green monkey kidney (GMK) tissue cultures to evaluate their respective immunogenicities. Also in progress, are studies to determine the efficacy of soluble antigens obtained by Tween-ether extraction of the CHIK virus, with the potential application of this technique to vaccine production. In collaboration with the Department of Veterinary Pathology, we are investigating the histopathology and neurovirulence of CHIK infection in the rhesus monkey. It is anticipated that these studies will elucidate the infectious process and the protective mechanisms of vaccine action at the cellular level.

An immunologic characterization of formalin-inactivated CHIK vaccine, Lot E-20, prepared in GMK tissue culture is presented in Table 1. In a previous report it was shown that this vaccine solidly protected rhesus monkeys against a viremia when challenged with several selected strains of the CHIK virus (Annual Report, 1968).

TABLE 1

Summary of immunologic data on CHIK vaccine, Lot E-20

Animal species and treatment	Animal No.	Serum Neut. Index	Comp. Fix. 1/CF	Hemagg. Inhib. 1/HI	Bead Neut. (mm)*
Rhesus monkeys serum, 30 days post 3-1 ml dose of vaccine given on days 0, 7, 21	679	2.86	4	40	12
	697	2.07	<4	40	13
	698	2.22	<4	20	12
	699	2.37	<4	40	15
	721	2.37	8	40	13
	905	2.43	4	80	15
Adult mouse serum, 7 days post 2-0.25 ml dose of vaccine given 1 wk apart	Group A	2.45	4	40	12
	Group B	2.00	<4	20	11
	Group C	2.65	4	20	11

\* Zone of virus neutralisation measured in mm.

Physical characterization of the CHIK virus grown in GMK and concentrated by ultracentrifugation, before and after Tween-ether (T-E) extraction, by rate zonal and equilibrium isodensity gradient centrifugation:

Sucrose rate zonal density gradients: Five to 30% (W/V) sucrose solutions were prepared in phosphate buffered saline and layered stepwise into cellulose nitrate centrifuge tubes. Concentrated CHIK virus, before and after T-E extraction, was layered on top of the gradients and centrifuged at 22,000 rpm for 1 hr in a 50L Spinco rotor. Approximately 20 fractions were collected by the drip-out method and assayed for infectivity and hemagglutinin content. Density measurements were made in Abbe refractometer. In Figure 1 are shown the infectivity levels before T-E extraction and the hemagglutinin content after T-E extraction.

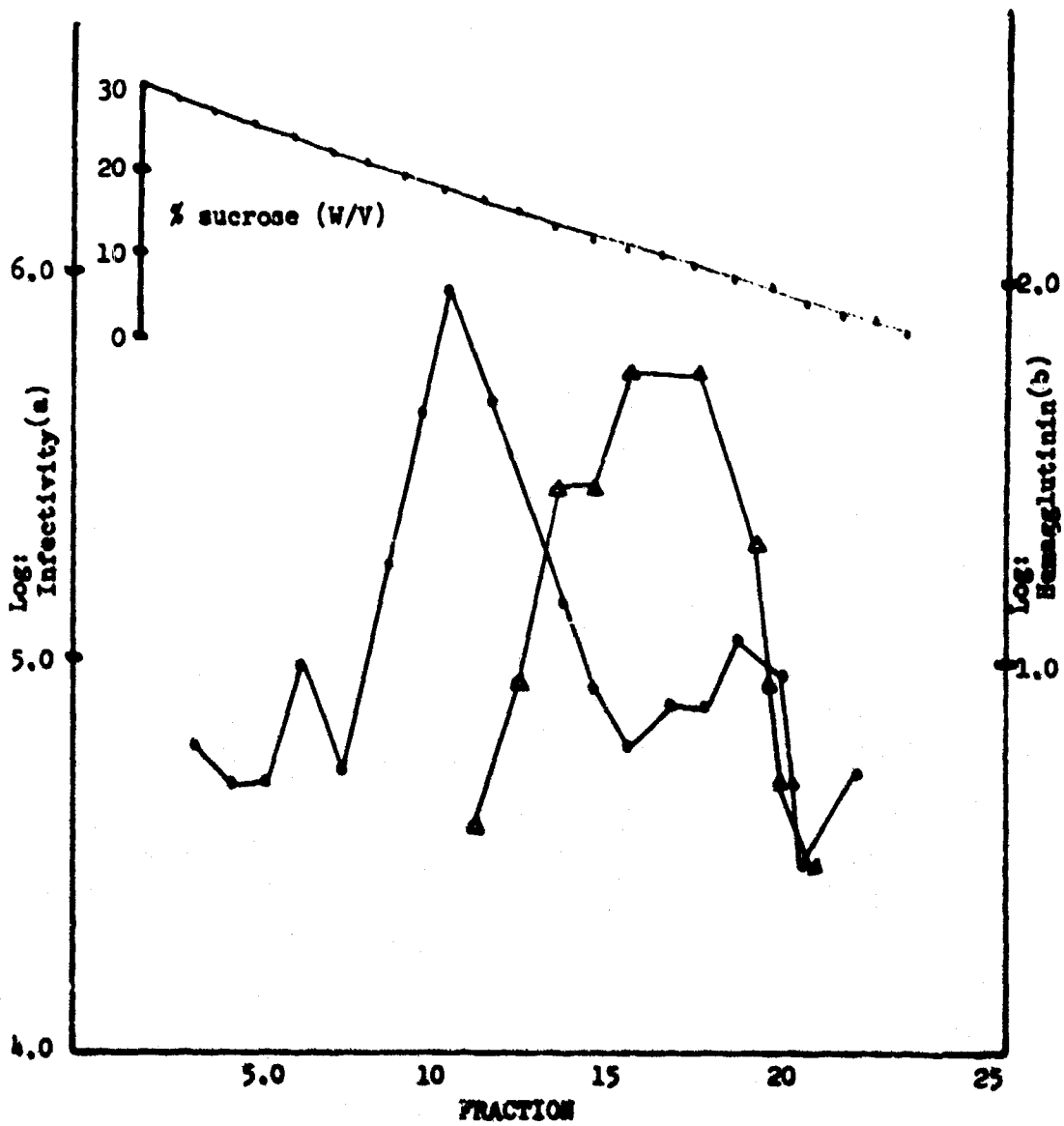
Cesium chloride equilibrium isodensity gradients: Cesium chloride solution was prepared to density of 1.25 g per ml in phosphate buffered saline. Concentrated CHIK virus, before and after T-E extraction, was mixed with the CsCl solution and centrifuged at 40,000 rpm for 18 hrs in a 50L Spinco rotor. Over 20 fractions were collected by the drip-out method and assayed for infectivity and hemagglutinin content. Refractive indices were determined in an Abbe refractometer. Figure 2 shows the infectivity level before T-E extraction and the hemagglutinin content after T-E extraction.

It is of interest to note that the buoyant density of CHIK virus increases after T-E extraction. This can be explained, in part, by the removal of lipid from the virus particle. Further studies are being made to determine the effect of this lipid removal on antigenicity. Present indications are that vaccine prepared by the T-E extraction method is less stable than vaccine prepared by the conventional method of formalin inactivation. It may be that lipid is essential to long term stability of vaccine.

A comparative study of the relative immunogenicities of vaccines prepared by the conventional formalin-inactivation method and by the Tween-ether (T-E) extraction procedure was made. Both vaccines were prepared by growing the CHIK virus in GMK and harvesting at 48 hrs and 96 hrs post-inoculation. Briefly, the T-E vaccine was prepared by shaking the viral harvest with Tween 80 (5 mg/ml) at room temperature, followed by an equal volume of diethyl ether. The ether was removed by agitation and displacement with nitrogen gas. The preparation was passed through a 0.45 u membrane filter and used as vaccine. Characteristics of the two vaccines are summarized in Table 2.

FIGURE 1

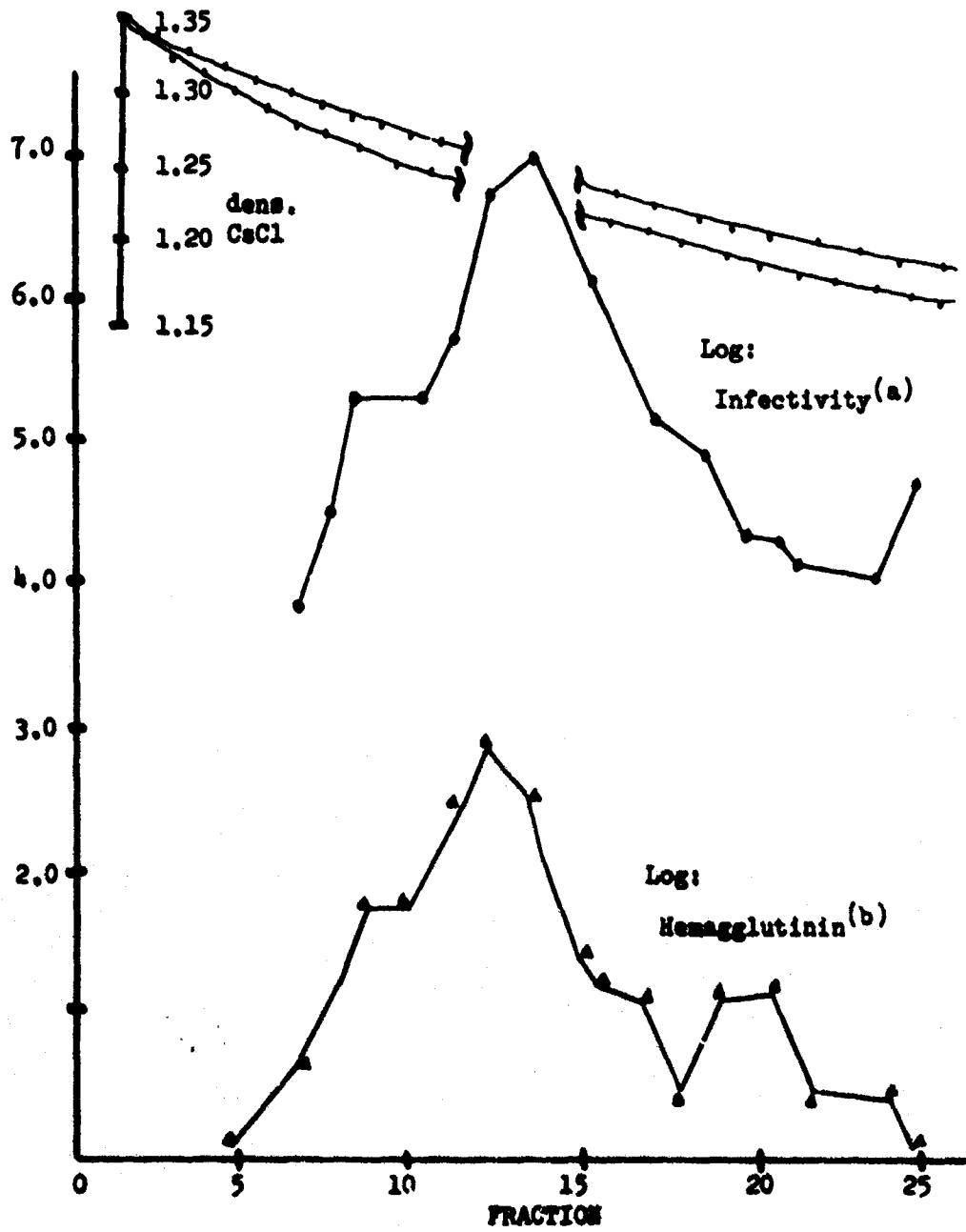
Sucrose density gradient centrifugation of CHIK virus before and after Tween-ether extraction



- (a) ●—● Infectivity (pfu/ml) before T-E extraction.
- (b) ▲—▲ Hemagglutinin (0.5 ml) after T-E extraction.

FIGURE 2

Cesium chloride equilibrium isodensity centrifugation of CHIK virus before and after T-E extraction



(a) —●— Infectivity (pfu/ml) before T-E extraction.

(b) —▲— Hemagglutinin (0.5 ml) after T-E extraction.

TABLE 2

Characteristics of Formalin-inactivated and Tween-ether extracted  
CHIK vaccines

Time of Harvest and Vaccine Type*	Infectivity Pfu/0.1 ml	Hemagg. 1/HA**	Hemagg- Inhib. 1/HI	Comp. Fix. 1/CF	ED <sub>50</sub> /ml
48 hr - FI vaccine	7.5	64	20	8	0.17
48 hr - TE vaccine	7.5	256	20	4	0.17
96 hr - FI vaccine	6.3	128	10	4	0.17
96 hr - TE vaccine	6.3	256	20	16	0.24

\* FI = Formalin-inactivated.

TE = Tween-ether extracted.

\*\* Highest dilution giving complete hemagglutination in 0.5 ml.

Although both vaccines are quite comparable in their immunogenic properties, further study is indicated to determine long term stability characteristics.

Vaccines were prepared in chicken embryo tissue cultures derived from RIF (resistance-inducing-factor) free flocks. Even though earlier studies showed chicken embryo tissue culture to be markedly inferior to GMK for vaccine substrate, it was felt that all possible substrates characterized for adventitious agents should be investigated. Although assays for the presence of interferon were not done, it may be seen from the data in Table 3 that growth curves for the incubation temperatures selected are markedly different. This is strongly suggestive of interferon activity.

TABLE 3

Characteristics of CHIK vaccines prepared in chicken embryo tissue  
cultures at selected incubation temperatures

Time of Harvest and Incubation °C	Hemagglutinin Content 1/HA	Infectivity TCLD <sub>50</sub> /0.1 ml	Vaccine ED <sub>50</sub> /ml
48 hrs - 31	≤ 2	ND <sup>a</sup>	ND
48 hrs - 35	≤ 2	ND	ND
72 hrs - 31	≤ 2	4.3	≥ 1.0
72 hrs - 35	≤ 2	2.8	≥ 1.0

TABLE 3  
(cont'd)

Time of Harvest and Incubation °C	Hemagglutinin Content 1/HA	Infectivity TCLD <sub>50</sub> /0.1 ml	Vaccine ED <sub>50</sub> /ml
96 hrs - 31	≤ 2	4.2	0.8
96 hrs - 35	≤ 2	2.6	≥ 1.0

\* ND = Not done.

Thus far adaptation of the CHIK virus to growth in the human diploid cell strain WI-38 has been carried through 18 passages. Although not as immunogenic as GMK vaccine at this passage level, vaccine prepared in the WI-38 cells is showing excellent potential. The immunologic response of mice to both types of vaccine is shown in Table 4.

TABLE 4

Immunologic response of mice<sup>(a)</sup> to CHIK vaccines prepared in GMK and WI-38 tissue cultures

Vaccine Number and Type <sup>(b)</sup>	Hemagg-Inhib. 1/HI	Comp. Fix. 1/CF	ED <sub>50</sub> /dose
#1 - WI-38	20	4	0.40
#2 - WI-38	20	4	0.28
#3 - WI-38	10	4	0.31
#1 - GMK	20	4	0.25
#2 - GMK	10	8	0.23
#3 - GMK	20	8	0.17

(a) Mice received 0.25 ml vaccine on days 0 and 7, bled out on day 14.

(b) All vaccines harvested at 84 hrs post-inoculation and formalin-inactivated with 1:1000 formaldehyde solution (37%).

Further evaluation of the immunogenic potency of vaccines prepared in WI-38 cells and GMK tissue culture was made by performing an antigen-extinction type assay of the two vaccines. These data are summarized in Table 5.

Using both types of vaccine, groups of mice were given two 0.25 ml doses by the intraperitoneal route on days 0 and 7. The vaccines were given: undilute, 1:2, 1:4, 1:8, 1:16 and 1:32. On day 14 the mice were exsanguinated and the serum from each group, by dilution, pooled and run in a serum neutralization test against the CHIK 168 virus.

TABLE 5

Serum neutralization indices of mice receiving CHIK vaccine prepared in GMK and WI-38 tissue cultures

Dilution of Vaccine Injected	Mouse Serum Neutralization Index	
	GMK Vaccine	WI-38 Vaccine
Undilute	3.0*	3.0
1:2	2.3	3.0
1:4	2.0	2.7
1:8	2.0	2.0
1:16	1.7	1.7
1:32	1.3	1.3

\* TCID<sub>50</sub>/0.1 ml. Challenge dose consisted of approximately 100 TCID<sub>50</sub>/0.1 ml.

Studies on the histopathology and neurovirulence of CHIK infection in the rhesus monkey are in progress. Forty selected tissues from each of 17 monkeys (6 vaccinated and 11 non-vaccinated) have been processed for viral isolation. Results show that virus persists in regional lymph nodes of non-vaccinated monkeys up to 7 days after inoculation. Spleen, thymus and thyroid tissues appear to be sites of extensive viral replication. This will be confirmed by fluorescence microscopy at a later date. No virus was isolated from any tissues processed from the vaccinated monkeys. Tissue sections examined by the Department of Veterinary Pathology have thus far shown no patent evidence of cellular damage attributable to viral invasion.

#### Summary and Conclusions.

A formalin-killed chikungunya vaccine has been prepared in bank-frozen green monkey kidney tissue culture, certified-free of detectable adventitious agents. The immunogenic potency of this vaccine against selected strains of the chikungunya virus has been demonstrated in rhesus monkeys. Adaptation of the chikungunya virus to growth in the human diploid cell strain WI-38 has enabled

us to prepare a vaccine in this substrate which is nearly comparable in potency to vaccine prepared in GMK tissue culture. Preliminary studies have shown that the Tween-ether extraction technique, when applied to a viral harvest, yields a delipidized subunit which is highly antigenic and possesses good vaccine potential. Further studies are in progress to evaluate the long term stability of this product. The histopathology and neurovirulence of chikungunya infection in the rhesus monkey is being investigated to determine the sites of cellular involvement and the mechanism of vaccine protection at the cellular level.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

Work Unit 016 Immunization studies of exotic diseases

Publications.

Harrison, V. R., Eckels, K., Hampton, C. and Boyer, W. Chikungunya Vaccine Project, Army Science Conference Proceedings, Office, Chief of Research and Development, Department of the Army, 18-21 June 1968.

(This paper received an Outstanding Achievement Award.)

**PROJECT 3A061102B71P**  
**BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE**

**Task 07**  
**Pharmacology**

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACRONYM	2. DATE OF ISSUANCE	3. REPORT CONTROL CLASSIFICATION	
1. DATE PREVIOUS SUMMARY	4. KIND OF SUMMARY	5. SUMMARY STATUS	6. WORK CLASSIFICATION	DA 086448	69 07 01	DD FORM (REV) 455	
28 02 69	D. Change	U	U	NA	ML	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO A. WORK UNIT	
7A. NO./CODE	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
6. PRIMARY	611102A	3A061102B71P	07	036			
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9. TITLE (Provide with Security Classification Code)							
(U) Basic Pharmacological Studies (09)							
10. SCIENTIFIC AND TECHNOLOGICAL AREA							
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07 68		NA		DA		In-House	
15. CONTINUED/START				16. RESOURCE ESTIMATE		17. PERSONNEL MAN YRS	
NA				FISCAL YEAR		FUNDING SOURCE	
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B. NUMBER				70		35	
C. TYPE				1		40	
D. KIND OF AWARD				E. CURR. AMT.			
18. RESPONSIBLE OGD ORGANIZATION				19. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D. C. 20012				ADDRESS: Division of Medicinal Chemistry Washington, D. C. 20012			
RESPONSIBLE PERSONNEL				PRINCIPAL INVESTIGATOR (Provide name if U.S. Academy providing)			
NAME: MEROONEY, COL. W. H.				NAME: HRIFFER, Dr. M. H.			
TELEPHONE: 202/576-3551				TELEPHONE: 202/576-3387			
20. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: ROZMAN, Dr. R. S.			
				NAME: DA			
21. REVIEWED BY (Provide name of reviewing official)							
(U)Pharmacology; (U)Medicinals; (U)Radioprotectants; (U)Antimalarials; (U)Snake Venoms							
22. TECHNICAL OBJECTIVE, OR APPROACH, OR FEATURES (Provide individual paragraphs if a summary is desired. Provide out of each item security classification code)							
<p>23. (U) The basic research efforts of this department are directed to investigating the pharmacology of promising medicinal agents, biological responses to radiation, drug interactions with and the nature of adrenergic receptors, the metabolism of and the interaction of radioprotectant chemicals with connective tissue. In view of the basic experience of the department some work will be continued using venoms to study the above phenomena.</p> <p>24. (U) Appropriate pharmacological, physiological, biochemical, and electrophysiological studies are conducted in vitro and in vivo. These studies encompass the acute responses to radioprotectant chemicals and their interaction with standard pharmacological and physiological agents. An outstanding feature of the capabilities of this department resides in the vast inventory of serially related and diverse chemicals which can be used in detailed and in screening studies of the nature of drug interactions with biological systems.</p> <p>25. (U) 69 02 - 69 06 The treatment of acute hemorrhagic shock with MB-2823 has been evaluated in a series of 30 dogs and 14 monkeys. Results indicate a remarkable improvement in vital parameters and survival following treatment with 90 mg/kg MB-2823, 3 hours after hemorrhage. Untreated control animals all expired. In addition, treatment with MB-2823 (90 mg/kg) appears to completely prevent the cardiovascular changes preceded by a lethal injection of E. coli endotoxin. Release of histamine as well as the usual lethal outcome is also prevented in 6 of the 6 dogs studied. Results indicate possible interference with the "trigger mechanism" of endotoxin shock. Studies to evaluate MB-2823 in the treatment of pulmonary embolism and cardiogenic shock are currently underway. The effects of certain selected snake and bee venoms on vital physiological and pharmacological parameters have now been completed. For technical reports, see Walter Reed Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.</p>							

PII Redacted

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 07, Pharmacology

Work Unit 036, Basic Pharmacological Studies

Investigators

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Robert Brockenton

Description

The basic research efforts of this department are directed to investigating the pharmacology of promising medicinal agents, biological responses to radiation, drug interactions with and the nature of adrenergic receptors, the metabolism of and the interaction of radioprotectant chemicals with connective tissue. In view of the basic experience of the department some work will be continued using venoms to study the above phenomena.

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## I Progress

### A. Studies on the Trigger Mechanism of Endotoxin Shock

The early hemodynamic changes associated with the syndrome called gram-negative bacteremic shock continue to be an elusive medical problem. Of particular concern has been the definition of the exact trigger mechanism of this form of shock which appears to set into a action series of irreversible cardiovascular alterations. These changes in vital function all appear to occur secondary to the release of some neurohumeral agent or agents into the blood stream. Substances such as histamine, serotonin, bradykinin and the catecholamines have all been implicated as being involved in these earlier stages of endotoxin shock. Reports are conflicting however, and no clear cut deleniation has been made as to which of these substances is most intimately involved in the trigger mechanism. All things considered, however, most workers have concerned themselves with the possibility of release of histamine or a histamine-like substance very early in endotoxin shock.

It is the prupose of this study, therefore, to investigate the role of histamine in endotoxin shock and to redefine its relationship to both the trigger mechanism and the lethal outcome so often associated with this form of stress.

Materials and Methods. A series of 40 adult Beagle dogs anesthetized with pentobarbitol sodium (30 mg/kg) were used in this study. Heart rate, EKG, respiratory rate and blood pressure were continuously monitored on a Sanborn polygraph. In addition, a large bore polyethylene catheter was placed in the femoral artery and advanced into the abdominal aorta to allow for the rapid sampling of arterial blood. Dogs were given graded doses of E. Coli endotoxin ranging from 0.005 mg/kg to 10 mg/kg. All injections were made directly into the femoral vein of the dog. Blood samples for histamine determination were then taken at 0, 15, 30, 45, 60 seconds and at 2, 5, and 10 minutes after endotoxin. Histamine determination were made using a flourometric technique. All animals were followed for 72 hours or until death.

Results. The effects of each dose of E. Coli endotoxin on plasma histamine levels at specific time intervals are shown in Table 1. Those doses of endotoxin which produced death at 24 hours are also indicated. It is interesting to note that ultimate lethality is usually correlated with a sharp elevation of plasma histamine at from 30 to 60 seconds post-injection.

The typical response of the anesthetized dog to a sublethal (0.05 mg/kg) injection of endotoxin is shown in Fig. 1. Changes in plasma histamine,

TABLE 1

I.V. Dose of Endotoxin (µg/kg)	Plasma Histamine Levels (µg/L)										Lethal
	control	15 sec.	30 sec.	45 sec.	1 min.	2 min.	5 min.	10 min.	30 min.	7.0	
0.1	6.4	11.3	11.1	6.0	7.9	7.9	7.1	7.0	No		
0.3	4.7	4.0	4.0	17.5	26.6	19.8	15.1	8.2	Yes		
0.5	5.3	4.4	5.9	5.9	10.2	5.2	5.5	5.2	No		
0.5	18.8	16.7	17.2	19.8	19.0	16.7	17.5	19.8	No		
0.5	5.9	4.7	4.8	4.8	4.5	5.7	6.6	6.6	No		
0.75	6.9	5.8	5.2	23.0	25.1	13.3	4.3	5.9	No		
1.0	4.7	4.1	7.8	17.8	21.4	21.0	8.1	6.8	Yes		
1.0	6.8	3.9	34.2	70.7	46.6	18.5	6.5	5.3	Yes		
1.0	4.2	3.5	3.5	3.8	4.3	4.2	3.8	3.8	No		
1.0	4.9	4.6	4.9	4.2	4.8	4.9	4.2	4.2	No		
1.0	5.9	6.1	8.8	4.7	8.8	14.3	10.8	7.8	No		
1.0	5.4	6.4	6.7	13.2	12.2	6.0	7.0	6.0	No		
1.0	6.4	5.8	6.4	14.5	36.1	28.9	--	--	Yes		
1.0	4.4	5.8	5.3	15.4	34.7	24.5	12.5	6.3	Yes		
1.0	4.7	5.6	5.5	7.1	8.3	5.3	4.9	6.2	No		
1.0	4.4	4.1	11.4	12.9	17.3	10.6	7.1	6.8	No		
1.0	5.2	5.0	6.6	5.5	5.2	5.5	5.5	5.0	No		
1.0	5.0	4.0	4.0	4.1	4.1	6.8	6.2	4.1	No		
3.0	4.7	4.3	17.7	61.7	48.5	38.5	12.8	7.5	Yes		
3.0	6.7	5.0	5.8	17.7	18.3	11.9	5.7	5.0	Yes		
4.0	5.7	10.5	13.2	18.7	18.7	16.5	9.4	6.7	Yes		
10.0	6.3	6.7	7.6	14.3	27.6	13.8	6.5	5.9	Yes		

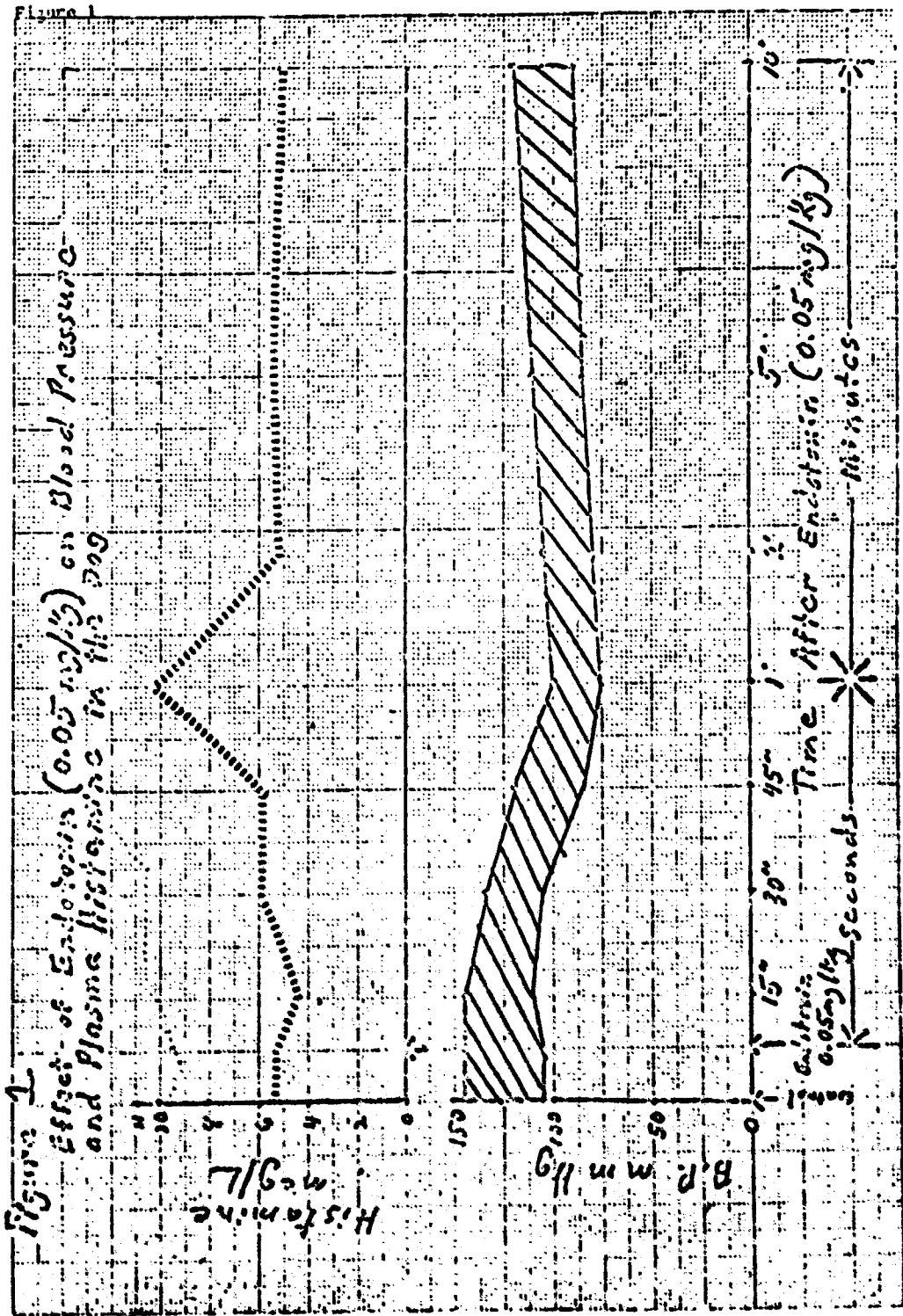


Figure 2  
 Mean Histamine Levels  $\pm$  S.E. Produced By  
 1.0 mg/Kg of *E. coli* Endotoxin

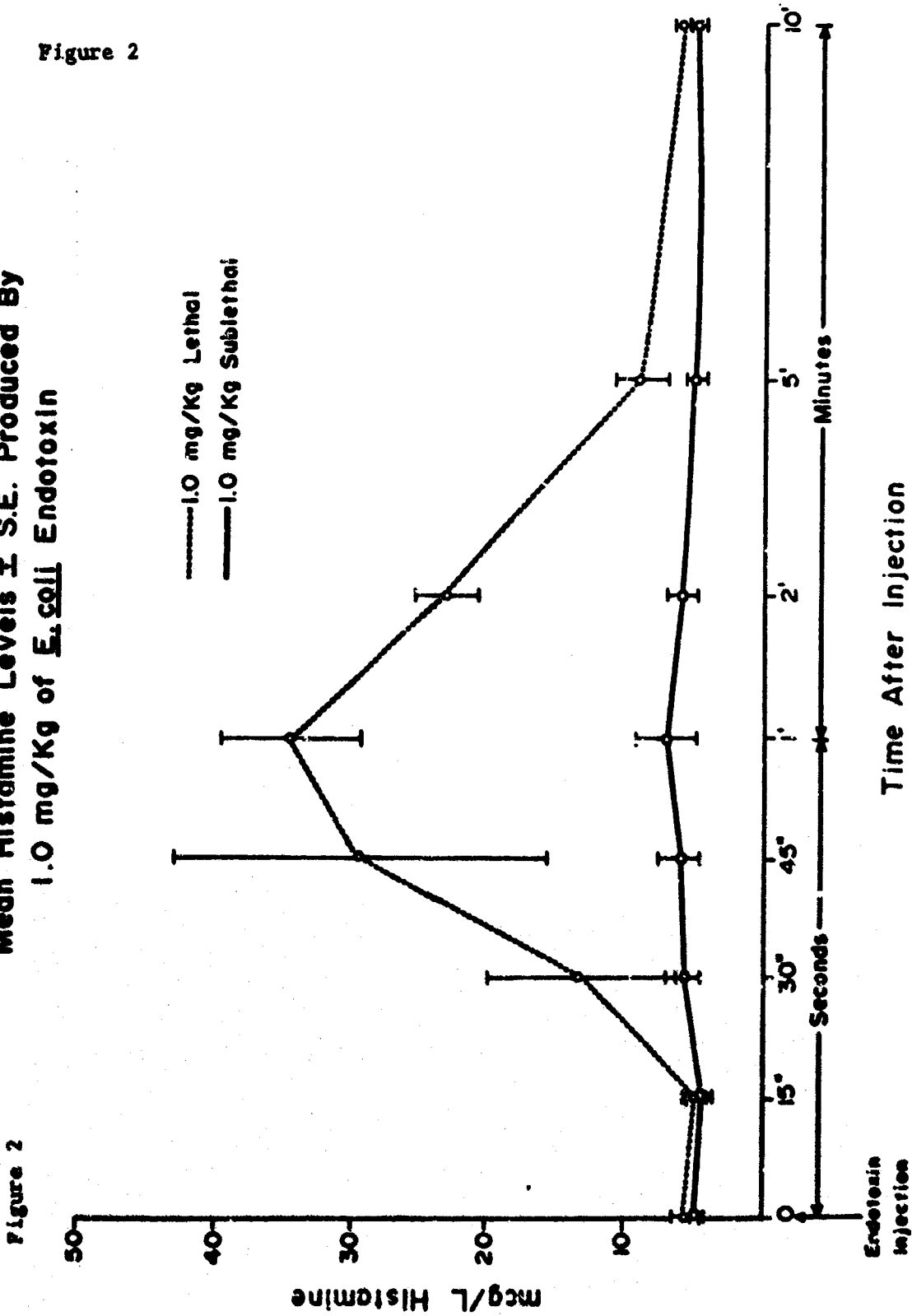


Figure 2

Figure 3  
 Mean Histamine Levels  $\pm$  S.E. Produced  
 By All Doses of E. coli Endotoxin

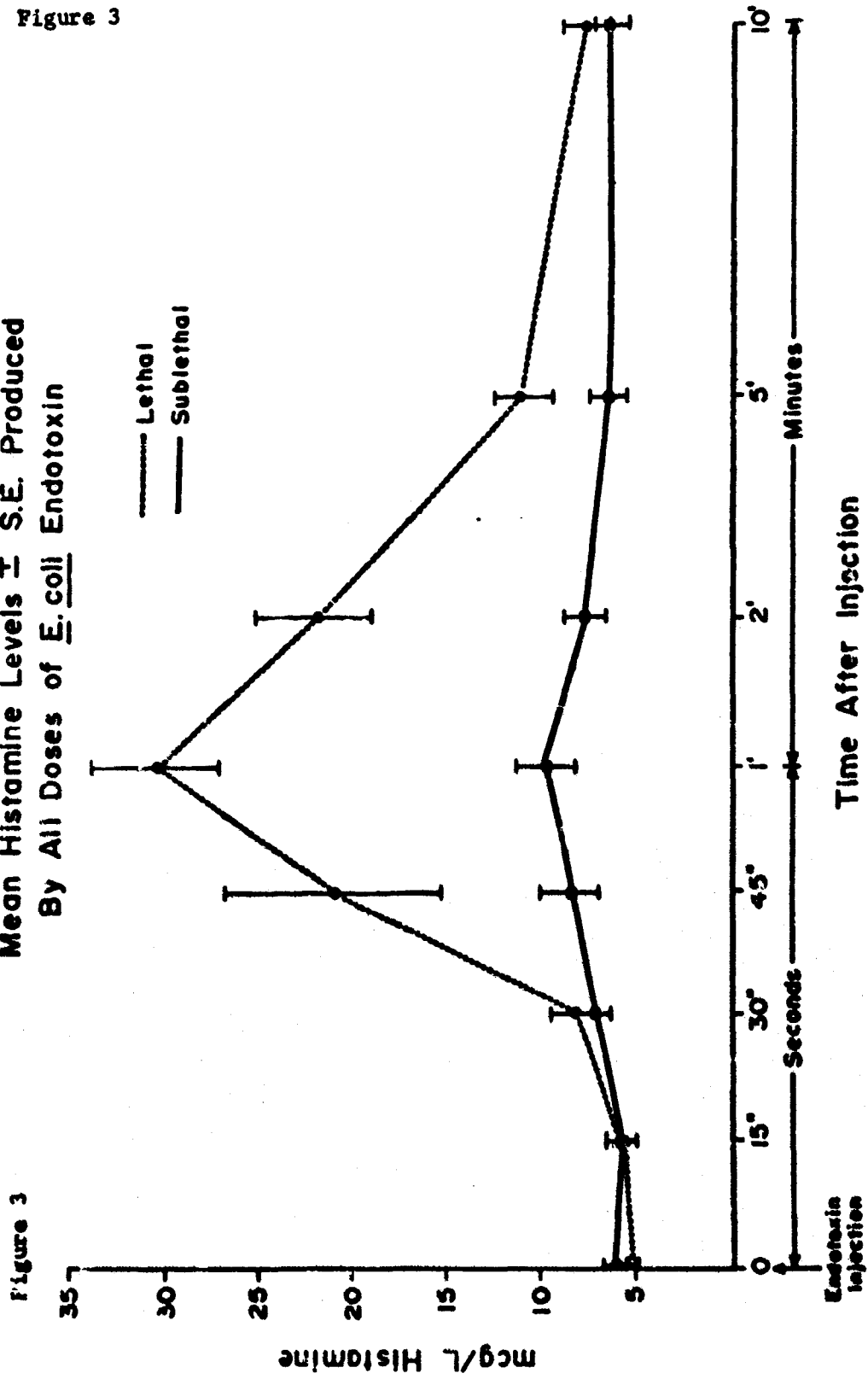


Figure 4  
 Mean Histamine Levels  $\pm$  S.E. Produced By  
 1.0 mg/Kg of E. coli Endotoxin

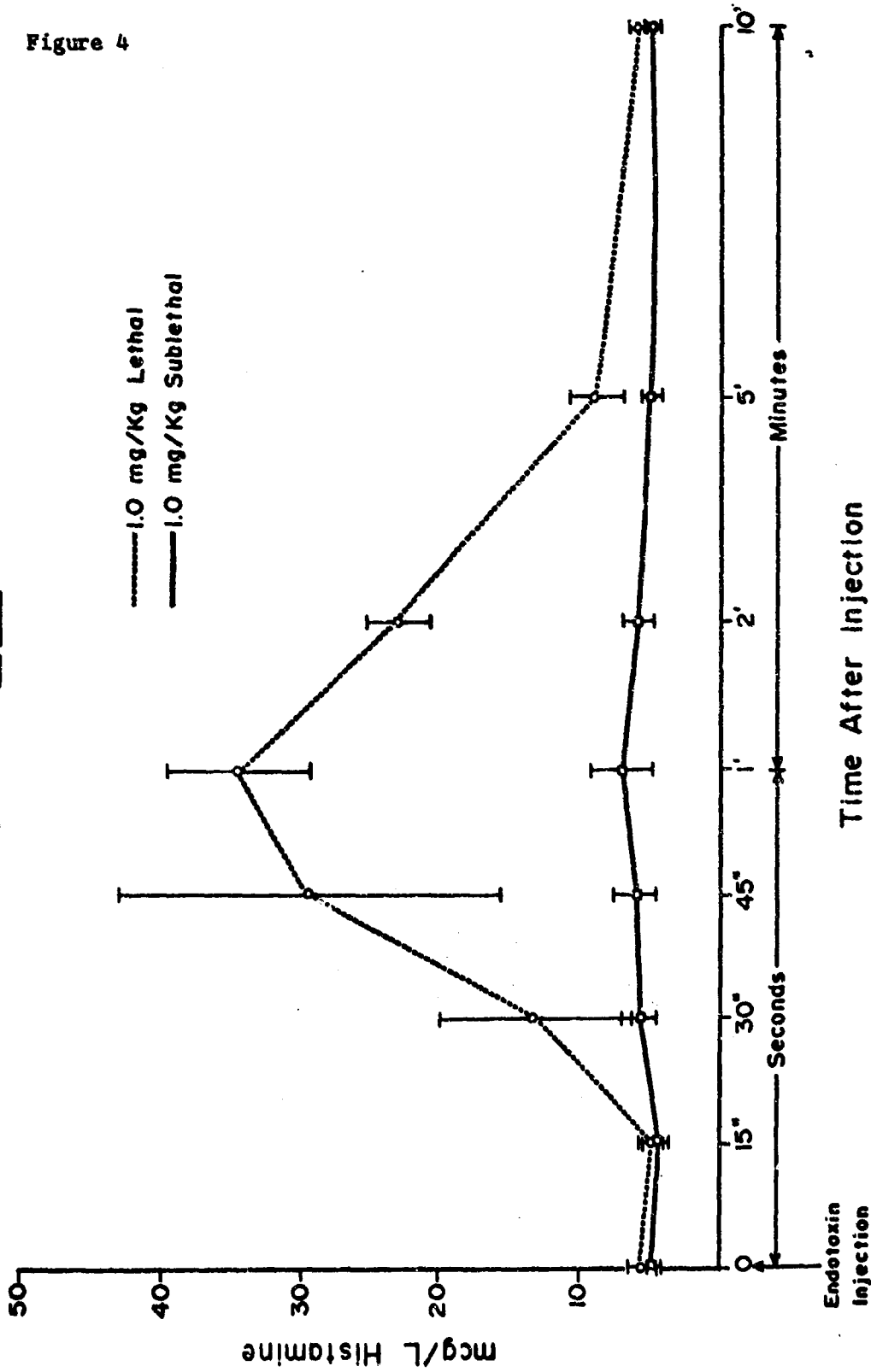


Figure 4

Figure 4

circulating platelets, and arterial blood pressure are simultaneously presented. The minimal increase in histamine, the subtle fall in platelets and the slight decrease in blood pressure is evident.

The response of the dog to a lethal (1.0 mg/kg) injection of endotoxin is shown in Fig. 2. A sharp and significant increase in plasma histamine occurs at 30 seconds which lasts for from 5 to 10 minutes. A marked decrease in platelets and a precipitous fall in arterial blood pressure occurs simultaneous and in direct proportion to the increase in histamine. As histamine levels return towards control blood pressure increases to a near pre-endotoxin level.

The average change in histamine level produced by all doses of endotoxin is shown in Fig. 3. Also shown is the standard error of the mean at each point in time. The effect of all lethal doses of endotoxin and all sublethal doses of endotoxin on plasma histamine levels are also displayed in Fig. 3. Lethal doses consistently produce elevations in histamine at from 30 to 60 seconds post endotoxin while sublethal doses do not result in significant increases at any time.

Figure 4 shows the marked differences in histamine levels between those animals which expire following 1.0 mg/kg E. Coli endotoxin and those dogs which survive after having received the same dose. In those animals which do not expire minimal increases in histamine are noted while in those animals which die significant changes are noted at from 30 to 60 seconds post-injection. Plasma histamine levels return to control or near control levels at 15 minutes.

## II In Vitro Screens

### A. Studies using the Mallard Duck Esophagus Preparation

These experiments are being conducted to investigate the effects of autonomic drugs and autocoids on the isolated Mallard Duck esophageal strips in order to determine the feasibility of using this preparation for this purpose.

Materials and Methods. Animals: Adult Mallard Ducks of both sexes were used. The ducks were anesthetized with diethylether and longitudinal strips of esophagus, between 1 and 3 cm long, were removed and placed in an isolated organ bath chamber at 37° C - 38° C in Modified in Krebs solution. Constant aeration of the chamber and reservoir was accomplished with 95% O<sub>2</sub>: 5% CO<sub>2</sub>.

All strips were allowed to equilibrate in the bath for approximately 30 minutes before drug challenge. Tension was usually adjusted to approximately 1 gram before initial drug introduction. Three levels

of esophagus from a given duck were usually used simultaneously in the bath.

Kreb's solution: The recipe used is as follows:

<u>Chemical</u>	<u>Grams/liter</u>
NaCl	6.92
KCl	0.354
CaCl <sub>2</sub>	0.282
KH <sub>2</sub> PO <sub>4</sub>	0.162
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.180
NaHCO <sub>3</sub>	2.212
glucose	2.000

**Drug:** All drugs were calculated so that final dilution in the bath was expressed in molar concentrations for direct comparison. When stock solutions were too strong, the drugs were diluted with distilled water. The drugs used were as follows: (1) Adrenergic: epinephrine hydrochloride; norepinephrine bitartrate; phenylephrine hydrochloride; isoproterenol hydrochloride; (2) Adrenergic blockers: phentolamine methanesulfonate; propranolol hydrochloride; (3) Cholinergic: acetylcholine chloride; urecholine chloride; neostigmine methyl sulfate; (4) Cholinergic blockers: atropine sulfate; scopolamine hydrobromide; (5) Ganglionic: nicotine; (6) Autocoids: histamine dihydrochloride; serotonin creatinine sulfate; (7) Autocoid blockers: diphenhydramine hydrochloride; methysergide bimaleinate.

**Results.** Preparations could readily be maintained for over 6 hours. The average experiment lasted 3 - 4 hours.

**Adrenergic:** Phenylephrine caused either constriction or no discernible effect in doses up to and including  $10^{-4}$  M. No relaxation from this drug was seen. Phentolamine  $10^{-6}$  M blocked the constriction. Propranolol  $10^{-6}$  M had no effect.

Isoproterenol caused relaxation at doses up to and including  $5 \times 10^{-6}$  M. No constriction was seen with this compound. Propranolol  $10^{-6}$  M blocked this relaxation. Phentolamine  $10^{-6}$  M had no effect.

Epinephrine caused either constriction, relaxation or constriction followed by relaxation in a given preparation. Doses were varied

from  $10^{-7}$  M to  $5 \times 10^{-5}$  M. Phentolamine ( $3 \times 10^{-7}$  M ---  $10^{-6}$  M) reversibly blocked the constriction but not relaxation. Propranolol ( $10^{-6}$  M) reversibly blocked the relaxation but not constriction. Both drugs given together blocked both responses on anequimolar basis.

Cholinergic: Acetyl choline caused constriction. This could be reversibly blocked with scopolamine.

Urecholine caused constriction. This could be blocked with atropine.

Neostigmine caused prolonged constriction.

Ganglionic: Nicotine first stimulated and then relaxed the muscle. Once a block with nicotine was established further doses produced little effect. Scopolamine did not affect the nicotine response and vice-versa.

Histamine constricted the muscle. This was blocked by diphenhydramine.

Serotonine constricted the muscle. This was blocked by methysergide.

Summary. In the isolated Mallard esophagus strip, contraction is produced by alpha adrenergic stimuli, acetylcholine and related cholinergic drugs, serotonin and histamine. The contraction can be blocked by appropriate blocking agents. Relaxation is produced by beta-adrenergic stimuli. Propranolol blocks this relaxation. The significant finding in our preliminary work with duck esophagus indicates that this preparation may be very useful in studying drugs which may possess alpha and beta adrenergic mechanisms. Smooth muscle of the intestinal tract of the many species that have been tested in the past, with the exception of the most distal section of the guinea ileum, relax to both alpha and beta-stimulating agents. These duck esophagi appear to contract to alpha adrenergic stimulating agents and relax to beta adrenergic stimulating agents, allowing a clear differentiation.

Future studies. The constricting activity of alpha adrenergic compounds will be further investigated to see if this can be used as an in vitro screen for potential alpha adrenergic blocking agents.

The cross-blocking effects of agents such as antihistamines, serotonin antagonists, and autonomic blocking drugs will be studied in more detail.

### III Drug Metabolism Studies

#### A. Metabolism of Radioactive Antimalarials

**Materials and Methods.** Animals: Adult Walter Reed strain albino mice of either sex were used. These were confined in Roth metabolism chambers with water given ad libitum. Urine and feces were collected.

Radioactive compounds: WR-40,070 (2,4-diamin-5-piperonylpyrimidine-2-<sup>14</sup>C, purity greater than 95%)

WR-27,799 (6-(3-(diethylamino)propyl)amino)-5,8-dimethoxy quinaldine-<sup>14</sup>C, purity greater than 95%)

Separation of metabolites: Thin layer chromatography on Silica Gel G, 250 u thickness, was extensively employed using a series of solvent systems.

Localization of radioactive spots was performed using a Berthold-Brinkmann two-dimensional radiochromatogram scanner.

Concentration of material using charcoal absorption with subsequent elution was tried.

Extraction of urine with organic solvents with subsequent solvent evaporation was tried, in order that a cleaner preparation be obtained.

Quantitation of radioactivity was done using standard scintillation counting techniques.

**Results.** WR-40,070-2-<sup>14</sup>C: 100 mg/kg formed two metabolites whether administered orally or intraperitoneally. This was determined chromatographically. The metabolites appear to be more water soluble than the parent compound.

The approximate urinary excretion as percent of administered dose is as follows:

	% of dose	
	oral	intraperitoneal
0 - 24 hours	64	29
24 - 48 hours	23	19
	-----	-----
TOTAL	87%	48%

WR-27,799-<sup>14</sup>C: 2.0 mg/kg was administered intraperitoneally.

A minimum of 25-30% was recovered in the 0-24 hour sample.

Thin layer chromatography indicated the possibility of a minor radioactive metabolite.

Further studies. An extensive workup on WR-40,070 is planned: Excretory patterns, including possible excretion as  $^{14}\text{CO}_2$ , will be determined in mice and possibly in rats.

Attempts will be made to characterize the structure of the urinary metabolites.

Possible fecal metabolites will be investigated.

Little further work is planned on WR-27,799- $^{14}\text{C}$ , since the radioactive compound has a low specific activity and the compound is quite toxic to animals.

#### IV Studies with Bee Venom

The venom of the honey bee (*Apis mellifera*) is a complex mixture of chemical substances. Previous studies have shown that this venom and its fractions have unique pharmacological and physiological activities (1-2). These activities are for the most part manifestations of enzymes such as phospholipase A(3), a hemolytic polypeptide melittin (4), and a polypeptide called apamin (5). There are other fractions, however, in the crude bee venom which have not as yet been identified chemically nor has their pharmacological effect been observed. It is the purpose of this study to analyze the physiological activities of the identified toxic and therapeutic fractions of bee venom by means of a polygraph and standard pharmacological preparations.

All venom used in this study was collected by C. Mraz, Middlebury, Vermont, by means of a method described by Benton, Morse and Stewart (6). 100 mg of whole venom (approx. 1000 stings) was separated into 9 fractions (Fig. 5) on a G75-40 Sephadex column. Each fraction was lyophilized and refrigerated until used. At the time of use, each fraction was reconstituted with 10 cc of normal saline and injected into the venous circulation of an adult mongrel dog anesthetized with sodium pentobarbital (30 mg/kg). Changes in arterial blood pressure, heart rate, respiration, electrocardiographic trace (ECG) and cortical electrical activity (EEG) were recorded on a Sanborn polygraph. Blood samples were drawn at hourly intervals for determination of epinephrine, norepinephrine, histamine and cortisol levels.

The fractions of bee venom are specifically identified in Figure 5. Their respective molecular weights were estimated by gel-filtration (7). Figure 5 also shows an index of the relative surfactancy of each fraction. A decrease in volume of the normal 62 drops that were

collected in each aliquot reflects a decrease in surface tension.

The weights of fractions 3, 4, 5 and 7 are 5.7, 2.5, 75 and 10.6 mg respectively. These four fractions comprise over 90% of the whole venom.

Results indicate that fractions 1, 2, 6, 8, and 9 do not produce any significant change in the physiological parameters monitored. In contrast, Fraction 3 (Phospholipase A) produced a precipitous fall in arterial blood pressure, a decrease in heart rate, a suspension of respiration and death in 10 minutes. Fraction 4 (the most surfactant fraction) produced a much less marked decrease in blood pressure and heart rate with a modest respiratory depression and no death. Fraction 5 (melittin) caused a decrease in blood pressure and a narrowing of the pulse pressure. Heart rate decreased sharply without cardiac arrhythmias. Immediately after injection of this fraction there were 1 to 3 minutes of apnea followed by recovery of respiratory function to near control values. Fraction 7 (apamin) produced similar effects and did not kill.

In addition to the described physiological changes, Fractions 5 and 7 produced sharp elevations in plasma cortisol levels (Fig. 6). It is interesting to note that the increase in cortisol following injection of Fraction 7 (apamin) was immediate in contrast to the slower more gradual increase produced by injection of Fraction 5 (melittin). The maximum increase in cortisol with melittin ultimately reached higher levels, however, than that produced by apamin. The increased levels of plasma cortisol were maintained over the entire 5 hour observation period. The effect of whole bee venom on plasma cortisol is also shown in Fig. 6.

Again, as with whole venom, Fractions 5 and 7 produce sharp increases in circulating epinephrine and norepinephrine within 15 minutes after injection (Fig 7,8). The catecholamine levels then decrease to a value significantly above control and remain such until the end of the observation period.

The effect of bee venom on plasma histamine is such that a slight decrease occurs shortly after injection; followed by a slow, sustained rise over the following 5 hours.

None of the other fractions of bee venom produced significant alteration in plasma cortisol, circulating catecholamines or histamine levels.

These studies indicate that whole bee venom is indeed a complex mixture of substances capable of producing a variety of physiological and pharmacological changes in the experimental animal. The most lethal component of the venom, Fraction 3, has been identified as

phospholipase A; it produced death by respiratory paralysis (8). Earlier work has shown that this effect on respiration is due to interference with nerve-impulse transmission at the neuro-muscular junction of the diaphragm (9). Bee-venom phospholipase A has also been shown to be similar to the phospholipase A obtained from Indian Cobra venom, an established neurotoxin (10). The observation that Fraction 5 (melittin) and Fraction 7 (apamin) produced significant and sustained increased in plasma cortisol levels is of great interest. The release of corticosteroids was previously inferred by the work of Artemov who showed increased ascorbic-acid activity after injection of bee venom (11). An increase in cortisol could explain, in part, the observation that bee venom or some of its components alleviate the symptoms of arthritic-like conditions (12-14). Numerous reports have documented the beneficial effects of cortisone and hydrocortisone on the inflammation and pain associated with arthritis and it could well be that the increased cortisol levels observed in these studies are in some way responsible for the remission of arthritic symptoms (15-18). It is also known that endogenously released steroids are far more effective therapeutically than those administered exogenously (by injection) (19-20). It could well be that apamin and melittin specifically stimulate the release of cortisol and in this way effectively control some of the symptoms of arthritis. The use of fractions of venom might also serve to circumvent the high toxicity of whole bee venom, thus allowing for larger and more effective dosage schedules.

The release of catecholamines after the injection of bee venom is not surprising since their release is often associated with the stress reaction (23). The minimal increase in histamine observed in these studies is in direct contrast to the results of other workers in the field (21-22). One explanation might be species variation.

The possibility that bee venom or its components may act in still other ways to control arthritis is by no means ruled out by this study. We have taken only one step in the understanding of an extremely complex pharmacological picture.

**Summary.** The venom of the honey bee (*Apis mellifera*) is a complex mixture of chemical substances. A deperation into its nine components of bee venom has been carried out as well as a determination of the pharmacological activity of each. Results indicate that fraction 3 (Phospholipase A) is the lethal component of venom and kills by respiratory paralysis. Fraction 5 and 7 are not lethal yet produce marked physiological and pharmacological alterations. Increases in epinephrine, norepinephrine and cortisol follow the I.V., I.P., or S.Q. administration of these components in the dog. Further studies are being carried out in the monkey to confirm these interesting observations.

Figure 5

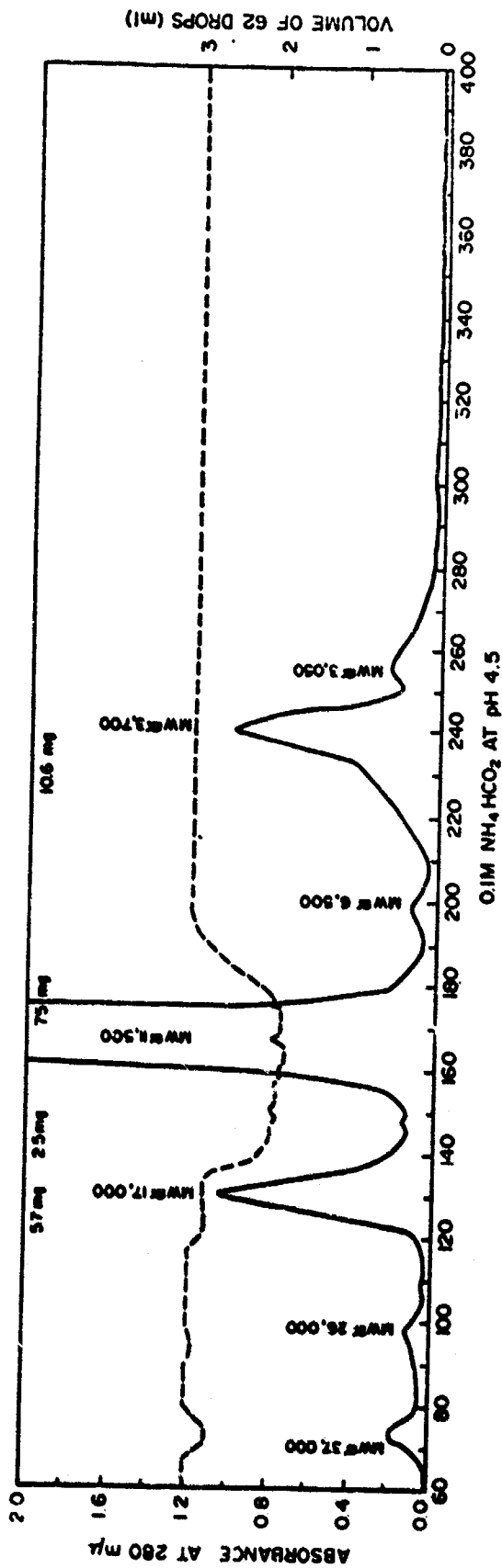


Figure 5. Separation of bee venom on G75-40 Sephadex column. The molecular weight, surfactancy and weight in mg of each fraction is indicated.

Figure 6

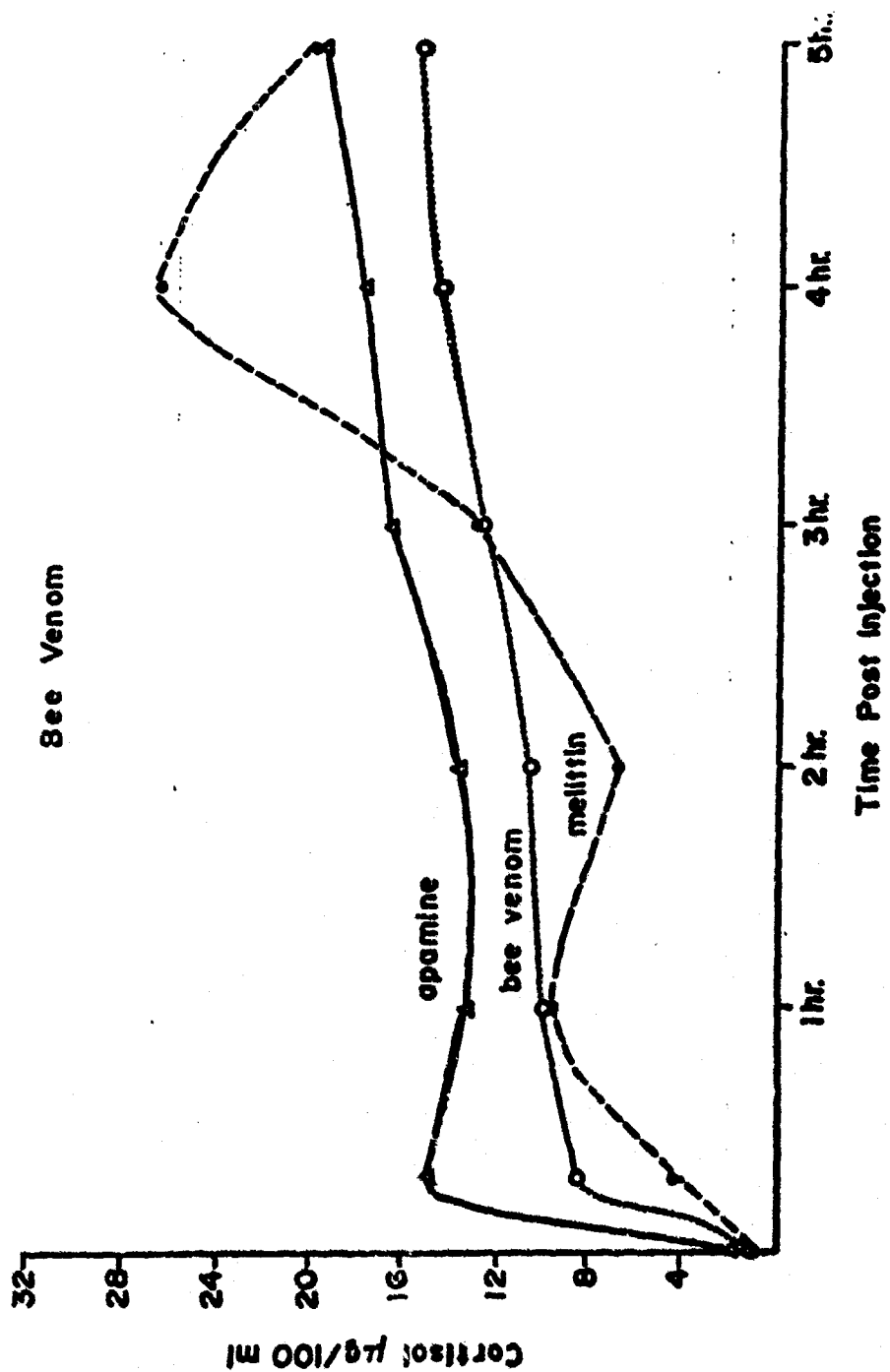


Figure 6. The effect of whole bee venom, opamine and melittin on plasma cortisol in the dog.

Figure 7

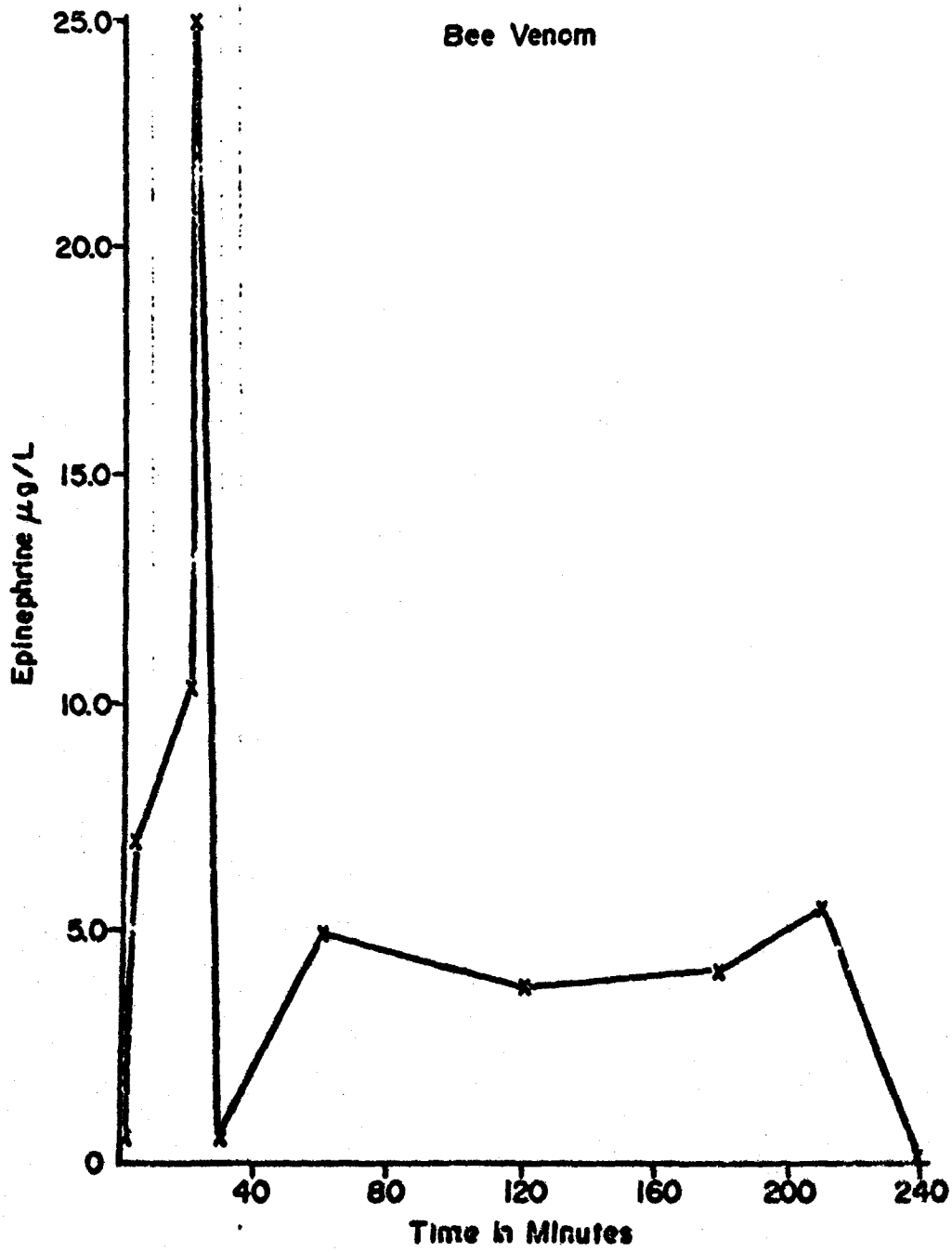


Figure 7. The effect of bee venom fractions on circulating epinephrine levels in the dog.

Figure 8

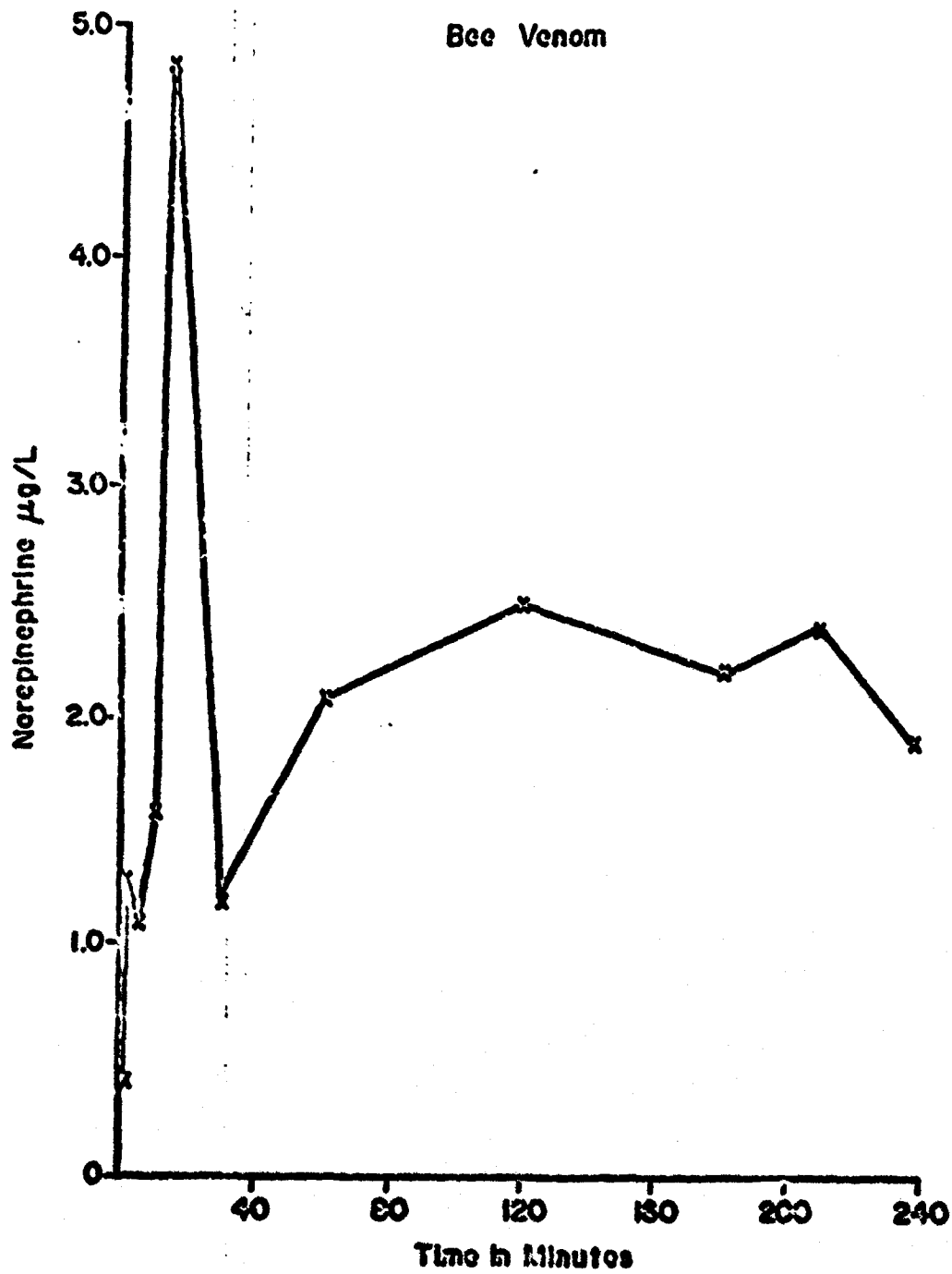


Figure 8. The effect of bee venom fractions on circulating and epinephrine levels in the dog.

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#### V The Effect of Actual Snake Envenomation on Vital Physiological Function in the Dog

The bite and the venoms of selected members of the five major families of poisonous snakes have been studied in experimental animals. Forty adult mongrel dogs weighing approximately 10 kgs were anesthetized with Na pentobarbital (30 mg/kg) and monitored for changes in arterial blood pressure, EKG, heart rate and respiration. In addition, plasma catecholamine and histamine levels were determined at hourly intervals as well as the major clotting factors. Envenomation was accomplished by grasping the head of the poisonous reptile and allowing the snake to strike the shaved exposed thigh of the dog. Estimates of venom content for each bite were made by averaging the volume of venom obtained from numerous and periodic "milking" of the same animal. Changes in vital function and blood chemistries were then recorded for 72 hours or until death.

The family and Genus species of each snake used in this study are shown in Table II. There were 13 members of the Crotalidae family of rear fanged snakes Boingidae.

Results indicate that envenomation by members of the Crotalidae family produced death at from 8 to 24 hours. Immediately following envenomation there was a slight increase in blood pressure which occurred simultaneously with an increase in plasma epinephrine and norepinephrine. Blood pressure and catecholamine levels then returned to near control readings and remained so until just prior to death. Death appeared to be due primarily to respiratory paralysis and was associated with a sharp terminal increase in epinephrine and norepinephrine. Certain snake venoms produced marked changes in blood clotting factors.

Average time to death following the bite of the Viper was 3 hours. It is interesting to note that several members of this family did not produce death; however, tissue destruction and necrosis at the site of the bite was extensive. Changes in blood pressure, heart rate, and EKG were not remarkable. Death again appeared to be due to progressive

Table II

## SNAKE BITE

CROTALIDAE	SURVIVAL TIME	APPROXIMATE DOSE OF VENOM	TISSUE DAMAGE
<i>C. rhodostoma</i>	<24 hours	50 mg	Local hemorrhage Generalized bleeding
<i>T. poepporum</i>	>72 hours	50 mg	Massive tissue damage at site of bite
<i>T. purpureomaculatus</i>	>72 hours	50 mg	Massive tissue damage at site of bite
<i>A. piscivorus</i>	< 8 hours	150 mg	
<i>A. C. Mokeson</i>	>72 hours	50 mg	Massive tissue damage at site of bite
<i>A. C. contortrix</i>	<24 hours	50 mg	Tissue damage
<i>B. atrox</i>	<14 hours	200 mg	Swelling edema
<i>C. durissus terrificus</i> S.A.	<24 hours	150 mg	No tissue damage
<i>C. horridus atricaudatus</i>	<24 hours	150 mg	No tissue damage
<i>C. horridus horridus</i>	>72 hours	150 mg	No tissue damage
<i>C. viridus oreganus</i>	>72 hours	75 mg	No tissue damage
<i>C. lepidus klauberi</i>	>72 hours	15 mg	No tissue damage
<i>L. muta</i>	<24 hours	250 mg	Swelling edema
VIPERIDAE			
<i>Bitis arietans</i>	>72 hours	200 mg	No tissue damage
<i>Vipera russelli</i>	< 3 hours	200 mg	No swelling; some local hemorrhage
ELAPIDAE			
<i>N. laje</i>	< 2 hours	100 mg	No tissue damage
<i>N. naja</i>	<1 1/2 hours	25 mg	No tissue damage
<i>N. N. Macouthia</i>	< 2 hours	200 mg	No tissue damage
<i>O. hannah</i>	28 minutes	500 mg	No tissue damage
<i>N. Nivea (Flava)</i>	12 minutes	200 mg	No tissue damage
<i>D. angusticeps</i>	36 hours	100 mg	No tissue damage
<i>D. polylepis</i>	40 minutes	75 mg	No tissue damage
HYDROPHAEIDAE			
<i>L. semifasciata</i>	>72 hours	10 mg	No tissue damage
BOIGIDAE			
<i>B. dendrophilia</i>	>72 hours	20 mg	Slight edema & hemor- rhage at site of bite
<i>D. typos</i>	24 hours	25 mg	No tissue damage

TABLE III

	<u>Crotalidae</u>	<u>Viperidae</u>	<u>Elapidae</u>	<u>Hydrophædæ</u>	<u>Boidæ</u>
Number of Bites	13	2	8	1	
Number of Survivors	6	1	0	0	
Time to Death	8-24 hours 8-24 hours	3 hours (-----)	1.7 hours (15 min-4 hr)	---	20 hours (1 hr - 36 hr)
Average Dose of Venom	105 mg (15-250)	200 mg -----	188 mg (25-500)	10 mg -----	55 mg (20-100)

and irreversible respiratory failure.

The bite of the average member of the Elapidae family produced death in 1.7 hours. Death was due to interference with nerve impulse transmission at the level of neuromuscular function of the diaphragm. No other significant changes were noted with these venoms except for the sharp initial increase in catecholamines.

The sea snake from the family Hydrophaeidae produced a death almost identical to that of the family Elapidae. A progressive decrease in both rate and amplitude of respiration followed envenomation and death occurred in 72 hours. Again no other significant changes were observed.

Rear fanged snakes kill at an average of 20 hours post envenomation. As with all of the other families of snakes death was primarily due to progressive paralysis of the respiratory mechanism.

Summary. The venoms of the 5 major families of poisonous snakes have been studied. Dried reconstituted venom and venom obtained directly from the bite of the living snake have been compared as to lethality and physiological activity. Results indicate that dried venom produces death in much the same manner as an actual envenomation. The sequence of physiological alterations from time of envenomation until death are also unmarkedly similar. Average lethal dose of each of 25 venoms has been established. Further work will be carried out to evaluate the efficacy of current therapeutic approaches to envenomation.

## VI. Research in Product Formulation

### A. Development of a Uniform Suspension Technique for Drug-Screening Laboratories

When the results from a number of drug-screening systems must be combined for subsequent analysis and evaluation, it is desirable that similar drug-handling techniques be employed in each of the several facilities.

Most testing laboratories have developed their own systems of handling large numbers of candidate drugs: some prepare suspensions via trituration in mortars, by shaking techniques, tissue grinders, or sonication while others attempt to prepare solutions, searching for a suitable solvent based on the experience of the technician. Results from such a diversity of formulations cannot be effectively compared.

It was felt that much of this problem could be eliminated by developing a simple uniform method of preparing all test drugs for administration to animals. Admittedly, any uniform method could not represent the

optimum choice in all cases, but for initial trial, uniformity was considered more important than attempts at formulation optimization.

There is usually little if any chemical or solubility data available on the candidate drugs to be prepared and tested. Aqueous vehicles are preferred to any of the several oils, usually, because of their faster rates of drug absorption, their ease of handling, and their suitability for intravenous use. If the test drug were water-soluble, then a solution would result. Since long-term stability is of no consequence in a drug-testing program, no other consideration need be made for drug solutions. If the drug is insoluble, then a suspension must be prepared. Methyl cellulose 4000 has long been used as an effective and safe suspending agent, used to add sufficient viscosity to the preparation to prevent rapid settling of the drug particles. A deflocculating agent is needed to promote wetting and to prevent the formation of agglomerates or large clumps of drug particles in the preparation. Polysorbate 80 USP has been frequently used in parenteral formulations for this purpose. The vehicle should be isotonic for satisfactory use in suspensions; there is no "general" method of correcting for osmotic disturbances that would be caused by a variety of soluble test drugs.

The solution chosen for use as a universal suspending vehicle was composed of Normal Saline to which small quantities of Polysorbate 80 and Methylcellulose 4000 had been added.

To facilitate the preparation of large numbers of test drugs in solution /suspension, the trituration step should be accomplished in the same vial from which the medication would subsequently be removed for dosing. Accordingly, vials were obtained from Brockway glass which had a maximum capacity of 10.6 mls with the rubber sleeve closure in place. The requirements of the vial were quite rigid: a finished product of 10 ml volume was desired so that w/w percentage calculations could be made mentally by the laboratory technician. Since Polysorbate 80 or some surfactant was required to promote wetting and to prevent agglomerate formation, the excess volume in the vial, above the fluid, would become filled with a very stable foam during suspension via shaking. The smaller the airspace, the less error due to foam. It was envisioned that 9.5 mls of the universal vehicle could be pre-packaged, along with an appropriate number of 1/8-inch glass beads in these vials and sterilized. At time of use, the appropriate amount of drug could be weighed out and added directly to the prepared vial and resealed. The Spex Mixer/Mill model 5000, can be used to shake the assembled vial for a specified length of time. After trituration has been accomplished, the drug formulation can be withdrawn by syringe directly from the vial, without transfer or loss of material

The major obstacle to the development of such a system was the discovery that suspensions of poor quality often resulted from the described treatment. The action of this particular model mill does not seem to be as vigorous as needed; clumps were often visible after prolonged shaking times. The use of a variety of both small and large pyrex or stainless steel balls did not resolve the problem. It is felt that the idea has merit, and alternate methods of suspension preparation are being sought.

#### B. Formulation Effects on Drug Absorption

Several of the Bunte Salts afford a degree of radio-protection when injected by any of the several routes, but are not absorbed when administered orally. A series of experiments were designed to determine the effects of certain formulation alterations on the gastrointestinal absorption of a representative member of these radio-protective agents.

The test drug selected was WR-1607, n-decylaminoethylthiosulfuric acid. This drug is one of the most effective anti-radiation compounds known in mice, providing good protection at 5 to 10 mg/kg when injected I.P. The parenteral LD<sub>50</sub> is 12 mg/kg. Orally, however, no protection had ever been shown, and the toxic dose was estimated well over 1000 mg/kg, though the typical lethargy produced by this drug had not been achieved orally at the start of these experiments.

Our screening procedure was to prepare 5% w/w suspensions of WR-1607 in various systems, intubate at 1000 mg/kg, and watch for toxic signs. Suitable solvent-system controls were run simultaneously. When promising systems were discovered, these trial formulations were run in our anti-radiation screens to determine if radioprotection was also being conferred by the additives. Two general types of formulations have been tested: suspensions containing surfactants of various types, and buffer systems of WR-1607 solutions.

Surfactant Effects. The suspensions were prepared generally through the use of a miniature waring blender, 45-ml size. These suspensions were then used to dose 5 to 10 mice, via intubation, at 1000 mg/kg; an identical system omitting the drug was also given to 5 control mice in each case. Approximately 40 vehicle systems have been prepared from the following list of surfactants and evaluated against controls. They were tried alone or in various combinations with one or more of the other surfactants, at levels ranging from 1% to 100% of the drug solvent. They were also tried in combination with water or with peanut oil. Several emulsions of water and peanut oil were also prepared and used as the suspending system for WR-1607.

Diocetyl Sodium sulfosuccinate

Tween-20 (Polyoxyethylene sorbitan monolaurate) HLB: 16.7

Tween-60 (Polyoxyethylene sorbitan monostearate) HLB: 14.9

Tween-80 (Polyoxyethylene sorbitan monooleate) HLB: 15.0

Span-85 (Sorbitan trioleate) HLB: 1.8

Span-80 (Sorbitan monooleate) HLB: 4.3

Span-20 (Sorbital monolaurate) HLB: 8.6

G-1288 (Polyoxyethylsorbitollin derivative) HLB: 16.0

Sodium lauryl sulfate HLB--40

Triethanolamine

Pluronic L-101 (Non-ionic exchange Resin)

The only surfactants that showed potential were Tween-80, when used alone as the solvent, and Diocetyl sodium sulfosuccinate, at .25%. Both of these produced initial excitation followed by pronounced lethargy. Further studies with Diocetyl sodium sulfosuccinate at higher concentrations are planned.

WR-1607 will form a milky semi-solution with Tween-80 if warmed. Repeated experiments with this combination did lead to a few instances of toxic deaths from acute respiratory depression, but no LD<sub>50</sub> has yet been established.

Five percent solutions of 1607 in Tween 80 were tested for radioprotection by Miss Grenan of the Biology Department. She found that at 1000 mg/kg, 5 out of 10 mice survived lethal (950 rad) radiation, and at 500 mg/kg, 3 out of 10 mice survived. All controls using aqueous suspensions of WR-1607 succumbed to this level of radiation. From these results we conclude that some progress has been made in promoting the oral absorption of this particular radioprotectant.

These combinations of drug and surfactant produce severe diarrhea, and would no doubt be very objectionable for human use.

#### Buffering Experiments

WR-1607 forms a sodium salt and will dissolve in water at pH 11.

When this solution was intubated to mice the drug precipitated out and no pharmacological effects were observed.

Buffer systems were tried in an attempt to keep the drug in solution long enough for some absorption to take place. Sodium acetate, 20%, was used as a solvent for 1607-Sodium, previously prepared and purified. No effects were observed in the mice at 1000 mg/kg, and at necropsy the drug was found precipitated in the stomach. Other common alkaline buffers were tried without success.

A proposal was made that WR-1607-Sodium might be absorbed from the gut if it could be passed through the stomach without being exposed to the acid pH and being reconverted to the base compound. In an attempt to protect the sodium salt from the injurious acid environment of the stomach, WR-1607-Sodium was encapsulated in large gelatin capsules and given multiple coatings of cellulose acetate phthalate, a dependable enteric film, insoluble in acid media. The reliability of the enteric coating was first confirmed by giving dogs sodium pentobarbital in these plastic enteric coated capsules (about 50 mg/kg) and observing the delay in onset of action. Uncoated capsules produced sleep in 12 to 25 minutes. Coated capsules lead to a delay of from 155 to 300 minutes, and it was presumed that the coated capsule had passed the stomach and dissolved in the intestine.

Enteric coated capsules containing either WR-1607-Sodium or a combination of WR-1607 Sodium and sodium acetate were prepared. Uncoated capsules, as described produced vomiting from 15 to 30 minutes after drug administration. Coated capsules produced anxiety, trembling, irritability, and diarrhea after about 5 to 6 hours, without vomiting, at dose levels from 103 to 208 mg/kg. No cyanosis was observed in the animals, and the effects seen could have been due to the irritation resulting from the very alkaline drug being released from the capsule.

Subsequent studies were conducted in cooperation with Major Loizeaux of the Biology Department. It had been determined earlier by Dr. Heiffer, et al., Pharmacology, that WR-1607 administered I.V. would produce a reversal of the typical depressor depression response produced by an isoproterenol challenge. This simple test was used to determine if WR-1607-Na could be absorbed by the gut when an aqueous solution was injected directly into the lumen.

Two dogs were anesthetized with Pentobarbital. A Sanborn Model 350 recorder was used to follow the blood pressure of the animals, and it was confirmed that isoproterenol doses produce hypotension. After a 17.5 mg/kg I.V. injection of WR-1607 the previously reported reversal was seen. This effect is transient lasting for two hours. A 200 mg/kg dose of WR-1607-Sodium 10% solution was injected into the lumen

of the duodenum and isoproterenol challenges were begun. After periodic challenging every 20 minutes for 3 hours no reversal had been seen. The drug was found precipitated near the site of injection, and it was concluded that no absorption had taken place.

The second dog was treated in an identical manner with the exception that the drug, 200 mg/kg was injected into the lumen of the upper colon. The results were similar to the first dog, as was the conclusion that no absorption had taken place. This further suggests that the effects observed following the enteric coated capsule ingestion were caused by local irritation to the gut rather than drug absorption. No radiation tests have yet been conducted with either enterically coated WR-1607 or intestinal injection.

#### C. Preparation of Colloidal Suspensions of Bunte Salts.

The absence of drug absorption from oral administration of the several radio-protective Bunte salts, contrasted with their remarkable efficiency when administered parenterally, has long been a problem in this drug development program.

One approach to this problem of poor gastrointestinal drug absorption is to greatly reduce the particle size of the drug, thus exposing a greater surface area to the absorbing tissues. Many of the Bunte salts are waxy or soap-like in nature, and grinding or micronization would be difficult, if not impossible by conventional means. Many topical drugs have demonstrated increased efficiency when applied as colloidal suspensions, i.g. the silver salts, complexed with peptizing proteins.

Attempts were made to prepare colloidal suspensions of the drug WR-1607 n-decylaminoethylthiosulfuric acid. While the drug is insoluble in water, it will dissolve in alkaline solutions of pH 12 or more. The sodium salt of this drug was dissolved in 0.25% Gelatin and filtered through glass wool. A Brookfield Counter-Rotating Mixer was used to provide a high rate of shear to the 1% drug solution as dilute HCl was used to neutralize and precipitate the drug as a colloid. In these initial attempts the precipitated drug quickly agglomerated and settled as soon as the mixer was turned off. Feeling that the instability was probably caused by the very high ionic concentration in the solution, a 1% solution of the drug was prepared, this time with a gelatin concentration of 0.5%. The drug was slowly precipitated by the addition dilute acetic acid with constant and rapid stirring. The precipitated product was quickly transferred to a dialysis bag and dialysed in 1000 mls of distilled water. The water was constantly stirred and frequently changed during the following 24 hours.

At the end of this time, the contents of the bag were found to have a pH of 7.5, and the product was a homogeneous suspension without visible particles. The suspension placed in bottles for settling-rate studies, showed good stability, and at the end of one week, only slight settling had occurred. The particles were not in the colloidal range, however, and starting from an average size of 5-15 microns, monoclinic crystals could be seen by the third day after preparation. By the end of the week the product was composed only of crystals, 30 to 50 microns long. Gross settling did not occur, however, for approximately 30 days.

In an effort to eliminate the ionization present in the above aqueous solutions, WR-1607 was dissolved in a quantity of hot methanol and added dropwise to a much larger volume of rapidly agitating 0.5% gelatin solution. Platelet crystals, 25 - 75 microns, immediately formed, and after two replicate experiments, this line of attack was abandoned.

Gelatin was chosen as principal peptizer because of its general effectiveness in a wide variety of colloidal systems. However, since a colloidal dispersion did not result from the above procedure, other peptizers should be examined; zein, agar, casein, pharmagel A and B, gum arabic, dextrin, albumin, and starches.

#### D. Prolonged-Release Depot-Parenteral Antimalarials

The purpose of this study was to develop a parenteral formulation of an antimalarial drug suitable for intra-muscular injection which would provide sustained blood levels sufficient to protect the individual against malaria for a prolonged period of time.

The phanthrene methanol, WR-33,063, 3-Bromo-10-[ $\alpha$ -hydroxy- $\beta$ (N,N-diheptylamine)ethyl] phanthrene hydrochloride, was chosen as the test drug because of its demonstrated efficacy as an antimalarial, and because of its resistance to detoxification mechanisms and absorption. It was felt that if this drug maintained its slow absorption when administered as a deep depot intra-muscular injection, that prolonged blood levels would result, possibly for several months, sufficiently high to be efficacious as a prophylactic.

Several trial suspensions were prepared. Microscopic analysis, conducted by Biodynamics Research Corp., Rockville, showed the material to be extremely friable. Electronmicrographs taken of dilute WR-33,063 aqueous suspensions showed the gross structure of the material to be clumps of needle crystals, which readily shattered from the weight of a coverslip. Therefore, micronization was expected to occur readily from simple trituration. Using 0.25% and 0.5% gelatin as a suspending

agent, several suspensions of 33,063 were made in concentrations ranging from 2-1/2% to 10% by weight of drug. The fluidity, viscosity, and physical stability of the products were all well within acceptable parntneral ranges. Suspension was accomplished using No. 6133 11-ml plastic vials (Spex Industries, Inc.) one to three 3/16" steel balls, and the Spex Mixer/Mill, shaking the product in the vial for 5 minutes, or until a uniform suspension was obtained.

Histopathologic toxicity studies have been started with the cooperation of the Department of Experimental Pathology. A 15% w/w aqueous suspension of WR-33,063 was prepared as described above using an autoclaved vehicle composed of 0.5% Gelatin USP added to Normal Saline Solution. The drug was assumed to be totally insoluble; accordingly no colligative correction was made to maintain isotonicity. Two mls of the above solution was injected into the left thigh (proximal) of three healthy adult rabbits. Two mls of the suspending vehicle without drug was injected similarly into the right thigh as a control. Afteh three days the animals were sacrificed, the six thighs were disarticulated and examined for histologic pathology. Early reports indicate that while no damage was caused by the vehicle alone, extensive necrosis was found around the drug injection sight. Further studies are now in progress to prepare a formulation which will produce no change in the tissues. After a safe formulation has been obtained, drug toxicity, blood levels, and efficacy studies will be initiated by this department.

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PROJECT 3A061102B71P  
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08  
Physiology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL	
				DA 0A6437	69 07 01	DD-INDL (REV) 682	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUBSIDIARY SCTY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. RECORDING <sup>e</sup>	8. DISSEM INSTR <sup>f</sup>	9. SPECIFIC DATA CONTRACTOR ACCESS	10. LEVEL OF SUMMARY
68 10 31	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODEL <sup>g</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	61102A	3A061102B71P	08	075			
b. CONTRIBUTING							
c. CONTINUOUS	1412A(2)						
11. TITLE (Precede with Security Classification Code) <sup>h</sup>							
(U) Cell Growth and Regeneration (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>i</sup>							
016200 Stress Physiology; 012900 Physiology; 002300 Biochemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
09 58		NA		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
NA				PREVIOUS		a. FUNDS (in thousands)	
a. DATE/EFFECTIVE:				b. NUMBER <sup>j</sup>		c. FISCAL YEAR	
EXPIRATION:				FISCAL YEAR		CURRENT	
b. NUMBER <sup>k</sup>				69		3	
c. TYPE:				70		3	
d. KIND OF AWARD:				3		65	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME <sup>l</sup> Walter Reed Army Institute of Research				NAME <sup>m</sup> Walter Reed Army Institute of Research			
ADDRESS <sup>n</sup> Washington, DC 20012				ADDRESS <sup>o</sup> Division of Surgery Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
				ASSOCIATE INVESTIGATORS			
				NAME: Vail, J. M., Ph.D.			
				NAME: Bartos, E. M., Ph.D.			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Wound Healing; (U) Collagen; (U) Glucosamine; (U) Cell Culture; (U) Oxygen Tension; (U) Cell Respiration; (U) Energy Metabolism							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The definition of the cellular mechanisms controlling wound healing and tissue repair following combat injury such as mechanical, thermal, or chemical trauma, infection, ionizing radiation, and shock.							
24. (U) To maximize experimental control and analytical resolution, a well defined <u>in vitro</u> system is used to study fibroblastic cell populations under normal and simulated trauma conditions through the application of a wide spectrum of biological physical chemical procedures.							
25. (U) 68 07 - 69 06: It was found that under the proper conditions fibroblasts growing <u>in vitro</u> proceed from a phase characterized by a high rate of cell division and minimal collagen synthesis to a phase characterized by minimal cell division and a high rate of collagen synthesis. The evolution of the fibroblastic cells in this <u>in vitro</u> system was thus shown to parallel the maturation of a wound in the body. In a further search for the determinants of this evolution it was demonstrated that the inhibition of cell division was preceded by a decrease of the level of the high energy compound adenosine triphosphate (ATP), an increase of the level of cellular uridine diphosphat-N-acetylglucosamine (UDPAG) and -galactosamine (UDPAGal), a marked decrease of the partial pressure of oxygen in the medium of the culture and a decrease of the cellular respiration rate. The increase of UDPAG and UDPAGal which are precursors of acid mucopolysaccharides is an additional indication of cell maturation which appears to be related to the modifications of the energy metabolism manifested by the lowering of the respiration rate and the ATP content of the cells. Uncovery of the exact nature of this relationship through further work will enhance considerably the Army's capability to control the course of wound healing in the injured soldier. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1968 - 30 June 1969.							

PII Redacted

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08, Physiology

Work Unit 075, Cell growth and regeneration

Investigators.

Principal: Andre D. Glinos, M.D.

Associate: James M. Vail, PhD; Edwin M. Bartos, PhD; Robert I. Werrlein, M.S.

The Problem.

While a great number of clinical studies in man and experimental studies in animals have revealed some of the factors influencing wound healing, the essential determinants of this process continue to elude us: we still do not know what makes fibroblasts proliferate in the early stages of a wound and what, at a later stage, causes these cells to stop dividing and to begin the synthesis of collagen which is responsible for the tensile strength of the wound. As long as this ignorance persists we will be essentially powerless to control the course of wound healing in the injured soldier.

Approach.

The reason for this failure is the great complexity of the clinical situation in man and of the experimental situation in the intact animal. To overcome this difficulty by simplifying the experimental situation, a well defined in vitro culture system is used in this department to study fibroblastic cell populations under normal and simulated trauma conditions. Details of the development of the system and of the finding that under the proper conditions fibroblasts growing in vitro proceed from a logarithmic growth phase characterized by a high rate of cell division and minimal collagen synthesis to a stationary phase characterized by minimal cell division and a high rate of collagen synthesis, have been reported previously (Walter Reed Army Institute of Research Annual Progress Reports, 30 June 1967; 30 June 1968; Glinos, A.D. in Control of Growth in the Adult Organisms, Academic Press, London, 1967).

Results.

The evolution of the fibroblastic cells in this in vitro model was thus shown to parallel the maturation of a wound in the body. In a further search for the determinants of this evolution it was found that the inhibition of cell division was preceded by a decrease of the level of the high energy compound adenosine triphosphate (ATP), an increase of the level of cellular uridine diphosphate-N-acetylglucosamine (UDPAG) and -galactosamine (UDPAGal), a marked decrease of the partial pressure of oxygen in the medium of the culture and a decrease of the cellular respiration rate.

The decrease of the ATP level originally demonstrated through the luciferin luciferase enzymatic assay method was confirmed through column anion exchange chromatography which also revealed an increased level of uridine-N-acetylamino sugars associated with the effluent peak 3 from stationary phase cells (ibid.). Removal of the acetyl group by stronger acid hydrolysis and separation on cation-exchange columns showed that the major portion of the sugar moiety was a combination of glucosamine and galactosamine, as shown in Figure 1. The volume of elutant for a particular amino sugar was compared to that required for the appearance of glucosamine, and the identity of the amino sugar determined by its ratio and compared to published values. The separation of deacetylated samples of N-acetylglucosamine and N-acetylgalactosamine yielded a ratio of 1.17 which compares favorably with the 1.20 value given by Crumpton (Biochem. J., 72: 479-486, 1959) for the separation of glucosamine from galactosamine. Analysis of the sugar moiety from peak 3 isolated from stationary phase cells showed that there were three amino sugars present. Although the baseline shifted slightly from the earlier standardization run, the ratio of 1.21 between the first and the second peak indicates that the sugars are glucosamine and galactosamine, respectively. The ratio of the small third peak to the first gave a value of 1.34, which does not correspond to values for any common amino sugar.

Integration of the curves and allowance for differences in the molar extinction coefficient of the chromagen produced by glucosamine and galactosamine showed that stationary phase cells contain approximately 62 per cent UDPAG, 31 per cent UDPAGal, and 7 per cent of the unidentified amino sugar.

A comparison of the chromatogram obtained from stationary phase cells with that of a synthetically prepared nucleotide, nucleotide-sugar mixture is shown in Figure 2. Guanosine triphosphate (GTP) was used for peak 5, in lieu of the unknown uridine compound. The uppermost elution pattern shows a stationary phase profile, obtained from a large quantity of cells, with the usual six major peaks appearing. The use of a more gradual HCl gradient permitted the resolution of the first peak into two components, labeled 1 and 1A. The occurrence of the N-acetylamino sugar chromagen under peak 3 allowed the separation and quantification of peak 3 (UDPAG/UDPAGal) from peak 4 (ATP). A linear relationship between  $A_{260}$  and  $A_{585}$  existed on the left-hand side of peak 3, due to the absence of interfering UV-absorbing substances. Because of this, the contribution of  $A_{260}$  by peak 3 on the right-hand side was graphically determined by measuring  $A_{585}$ . The difference between total  $A_{260}$  at a given point and the quantity contributed by peak 3 alone was the amount due to peak 4. The quantity of nucleotides under each peak was determined by integration of the curves and a synthetic nucleotide mixture prepared for each peak so as to imitate the upper chromatographic pattern. The nucleotide mixture was treated in the exact manner as the cell pellet from the stationary phase, and it was chromatographed the same way. A comparison of the two patterns shows that the profiles are quite similar. It appears that

adenosine monophosphate (AMP) comprises peak 1A, rather than peak 1. Adenosine diphosphate (ADP) corresponds to peak 2, UDPAG to peak 3, GTP to peak 5, and uridine triphosphate (UTP) to peak 6.

Taking into account the per cent recovery obtained and quantitatively comparing these and other relevant data, cells in the stationary phase contain the following (in umoles  $\times 10^{-9}$ /cell): UDPAG/UDPAGal = 8.31 and ATP = 2.76.

These results indicate that during the transition from logarithmic growth to the stationary phase, there is a reduction in the activity of pathways leading toward synthesis of adenine nucleotides and a corresponding increase in the synthesis of uridine-type compounds, so that uridine becomes the major component of the nucleotide pool, replacing adenine nucleotides. Although the basic cause of these changes which occur in the stationary phase is at present unknown, the increased amounts of UDPAG/UDPAGal measured is considered significant because, in addition to correlating with ATP decline, its appearance may be related to functional alterations in cellular activity.

Sugar nucleotides are known to perform two major functions in the cell: as substrates for enzymes that transform monosaccharides and as glycosyl donors in the formation of complex saccharides (Ginsburg, V. *Adv. Enzymol.*, 26: 35-88, 1964). The finding that 2/3 of the labeled UDP-N-acetylhexosamine pool of HeLa cells is eventually secreted into the medium as polymers containing amino sugars indicate that the UDPAG/UDPAGal pool in the L cell is associated primarily with the latter function. Similarly, Dingle and Webb (in: *Cells and Tissues in Culture, Methods, Biology and Physiology*, Vol. I. E.N. Willmer (ed.), p. 353. Academic Press, London, 1965) have reviewed several papers which show that fibroblasts maintained in tissue culture are able to secrete chondroitins and hyaluronic acid-mucopolysaccharides which in part arise via UDPAG and UDPAGal.

A recent study (Kornfeld, S. and Ginsburg, V, *Exp. Cell Res.*, 41: 592-600, 1966) has shown that UDPAG inhibits mammalian L-glutamine D-fructose 6 phosphate amidotransferase, the enzyme that catalyzes an initial step in the synthesis of UDPAG. This negative feedback mechanism would seem to imply that in most mammalian cells the pool size of UDPAG is well regulated. The maintenance of large quantities of UDPAG in the stationary phase compared to the logarithmic phase therefore suggests that there is an increase in the activity rather than a metabolic block in the pathway leading toward production of mucoid products.

Among the environmental factors which could possibly account for the decline of the level of cellular ATP in the stationary phase is the availability of glucose and/or oxygen to the cells. As reported earlier (Walter Reed Army Institute of Research, Annual Progress Reports 30 June 1967, 30 June 1968) the experimental results obtained eliminated glucose as a possibility and suggested that a progressive decrease of oxygen availability could indeed be involved in the induction of the

stationary phase. In order to explore this possibility further, the partial pressure of the oxygen dissolved into the medium and the rate of cellular respiration were determined daily during the progression of cultures from the logarithmic to the stationary phase.

Experimental cultures were set up in Eagle's spinner minimum essential medium (sMEM) supplemented with 10 per cent horse serum. Initial total suspension volume and population density were 200 ml and  $3.5$  to  $4.5 \times 10^5$  cells per milliliter respectively. The temperature of incubation was  $35^\circ\text{C}$  and the gas phase 5 per cent  $\text{CO}_2$  in air. The medium of the culture was changed daily. The percentage of oxygen in the gas phase was determined at the beginning and the end of each 23 hour period, by using the incubation chamber previously described (Greer and Glinos, Fed. Proc. 23: 574, 1964) in association with a magnetic oxygen analyser. A membrane type oxygen electrode was mounted on the cover of the incubation chamber and polarized to a potential of 700 millivolts by a mercury battery driving a voltage divider; the current generated by the electrode was measured with an electrometer. After standardization in the gas phase on the basis of the initial percent of oxygen determined with the magnetic oxygen analyser, the electrode was lowered into the liquid medium and the partial pressure of the dissolved oxygen measured. This procedure could be repeated as many times as desired without restandardization, since exhaustive testing has shown the response of the electrode to be linear with respect to  $p\text{O}_2$  and stable from day to day, provided the electrode is not continuously polarized.

The results obtained are shown in Figure 3, where the upper graph represents the population kinetics of a culture progressing from the logarithmic to the stationary phase. The partial pressure of oxygen dissolved into the medium is shown in the lower graph where solid lines represent the changes observed within each 23 hour incubation period and broken lines the restoration of the  $p\text{O}_2$  to 140 mm Hg upon renewal of the culture medium at the beginning of each day. The bars represent oxygen consumption in  $\text{ml} \times 10^{-10}$  / cell / minute, calculated on the basis of the difference of the oxygen present in the gas and liquid phases of the culture at the beginning and the end of each incubation period, the total number of cells present, and the time of incubation.

It can be seen that as the cell density increases the slope of the  $p\text{O}_2$  curve, within a given 23 hour incubation period, becomes progressively steeper and the final  $p\text{O}_2$  level attained lower. Thus, on day 1, with a population density of  $5.3 \times 10^5$  cells/ml, there was only a minimal decline of the  $p\text{O}_2$  at two hours after medium renewal (second point of the curve) and the lowest level reached at the end of 23 hours was 98 mm Hg. On day 12, on the other hand, with a population density of  $9.8 \times 10^6$  cells / ml, the  $p\text{O}_2$  declined from 140 to 66 mm Hg within 2 hours after medium change and to 5 mm Hg within the next 4 hours, showing only minor fluctuations for the remainder of the incubation period. In parallel with the decline of the  $p\text{O}_2$  of the medium there was also a decrease of the cellular respiration, the volume of oxygen consumed declining from

0.95 ml x 10<sup>-10</sup> / cell / minute for day 1, to 0.29 ml x 10<sup>-10</sup> / cell / minute for day 12. The decrease of the oxygen content of the gas phase, due to cell respiration, was never more than 3 per cent within any 23 hour period. Accordingly, the rapid decline of the pO<sub>2</sub> in the medium must be ascribed to the inability of the diffusion of oxygen from the gas phase to replace the oxygen consumed by the cells. This appears to lead to a decrease of the cellular respiratory rate which through a decrease in oxidative phosphorylation could in turn be held accountable for the low ATP levels characteristic of the stationary phase cultures.

#### Discussion, Conclusions and Recommendations.

If proliferation of fibroblasts and production of ground substance and collagen are considered to be the most essential part of wound healing, the problem of the mechanisms involved may be stated in the form of the following two questions:

First, what is the nature of the changes in the cellular environment which following injury induce fibroblasts to proliferate and later signal them to stop dividing and to begin the synthesis of specific macromolecules?

Second, what is the nature of the intracellular molecular interactions which occur in response to the extracellular signals and result in the early phase of DNA replication and cell division followed later by the synthesis and secretion of specific macromolecules?

1. In regard to the first question our results up to now suggest that changes in the oxygen tension of the cellular environment may be one of the signals involved, in the sense that high partial pressures of oxygen would be associated with cell proliferation and low ones with collagen and acid mucopolysaccharide formation. At this point it should be noted:

a. that, although molecular oxygen is needed for the hydroxylation of proline, a key step in the synthesis of collagen, the K<sub>m</sub> for O<sub>2</sub> of the hydroxylase involved is only 2.6 volumes per cent. If compared with the pO<sub>2</sub> recorded during the stationary phase (Figure 3) it can be seen that collagen synthesis would not be expected to be limited by lack of oxygen during this phase.

b. that the low pO<sub>2</sub> values exhibited by the model during the stationary phase when collagen is formed (cf. Figure 3) are within the range characterizing intercapillary areas in the tissues of the intact animal. In contrast, the pO<sub>2</sub> values recorded during logarithmic growth of the model cell population are found in the intact animal only directly above the capillaries, the pO<sub>2</sub> gradient between a capillary and the area adjacent to it being very steep. (Silver, I.A. in Oxygen Measurements in Blood and Tissues, J.A. Churchill Ltd, London, 1966; Med. Electr. & Biol. Engin. 3: 377, 1965)

c. that in addition to being exposed to atmospheric oxygen, a wound shows an initial profuse ingrowth of new capillaries and that later when fibroplasia begins, most of these new capillaries regress.

d. that clinically, excessive fibroplasia has been frequently associated with hypoxia.

e. that work at the University of Cambridge, England, undertaken at the suggestion of this department, has shown that in transparent chambers implanted in the ears of rabbits, fibroblast proliferation occurs only in areas of high oxygen tension associated with the advancing capillaries while deposition of collagen fibers occurs in stabilized areas where no cell division can be seen (cf. US Army Contract No. DAJA37-69-C-1169, Dr. I. A. Silver).

On the basis of the above it can be stated that the association of high oxygen tensions with fibroblast proliferation and of low oxygen tensions with synthesis of collagen suggested by experiments involving a cell culture model are consistent with clinical observations in man and experimental observations in the animal.

To test the validity of this concept further, it is proposed to subject a low density logarithmically growing culture to partial pressures of oxygen comparable to the ones recorded in Figure 3. In case oxygen tension is indeed one of the environmental factors controlling the evolution of the model, this should result in a decrease of the respiratory rate of the cells, decrease of the cellular ATP level, inhibition of cell division, increase of the cellular UDPAG and UDPAGal level and increased collagen synthesis. It is also proposed to study the possibility that other metabolites from the medium or substances released from the cells are involved in the induction of the stationary phase.

2. In regard to the second question concerning the intracellular molecular interactions responsible for the initiation of cell division in the early phase of the wound, followed later by a shift of the metabolic activity of the cells to the synthesis of specific macromolecules, our results suggest the possibility of an inverse relationship between the energy metabolism of the cells and the synthesis of collagen and acid mucopolysaccharides. At this point it should be noted that:

a. the suggested inverse relationship between energy metabolism and the synthesis of differentiated products is consistent with observations on bacteria, slime molds and fungi, where withdrawal of energy sources from the environment or blockage of a step in the Krebs cycle leads to arrest of growth and to the biosynthesis of differentiated products such as the insoluble cellulose polysaccharide complex of the cell wall. This type of molecular interaction has been shown to be mediated through the depletion or accumulation of intermediates which in addition to their role in energy metabolism are also participating in

biosynthetic reactions either as substrates or as regulators of the activity of key enzymes. (Atkinson, D.E., Science 150: 851, 1965; Wright, B. *et al.* Science 153: 830, 1966, and P.N.A.S. 60: 644, 1968; Smith, J.E. and Galbraith, J.C. New Scientist 41: 334, 1969.)

b. the data from our cell culture model suggesting an inverse relationship between energy metabolism and the synthesis of differentiated products cannot be compared with data obtained in wounds or in other cell and tissue culture experiments involving mammalian fibroblasts as this is the first time that such data were obtained. These data are, however, consistent with observations on cultures growing on glass in the sense that in both cases synthesis of collagen occurs in the stationary phase (Green, H. and Goldberg, B. Nature 200: 1097, 1963).

c. the suggested inverse relationship between energy metabolism and the synthesis of differentiated products is at the present time based on the observed sequence of appearance of a number of terminal events manifested by the cell cultures as they progress from the logarithmic to the stationary phase. These events are: decrease of cellular oxygen uptake, decrease of the cellular ATP level, decrease of mitosis, and increase of the cellular levels of hydroxyproline, UDPAG and UDPAGal. These events are considered terminal because they are the end result of changed metabolic reaction rates which in turn are controlled (1) at the level of enzyme activity (this includes substrate levels as well as effectors and inhibitors), (2) at the level of the translation of the nucleotide sequence of RNA messengers to the amino acid sequence of proteins, and (3) at the level of the transcription of the nucleotide sequence of the genetic DNA onto the RNA messengers. The suggested inverse relationship between energy metabolism and the synthesis of collagen and acid mucopolysaccharides may therefore be visualized as resulting from molecular interactions occurring at any of these three levels of control, with intermediates having multiple functions playing a key role, as described in para a, above.

Accordingly, in order to test the validity of the concept of an inverse relation between energy metabolism and the synthesis of collagen and acid mucopolysaccharides, it is proposed to undertake a systematic investigation of the metabolic reaction rates involved and their control at the three levels previously described. It is proposed to begin this investigation by using radioactive labelled precursors to study the rates of the oxidation of glucose and of oxidative phosphorylation in parallel with the rates of the synthesis of collagen, of glucosamine and galactosamine and of the adenine and uridine nucleotides, in cultures progressing from the logarithmic to the stationary phase.

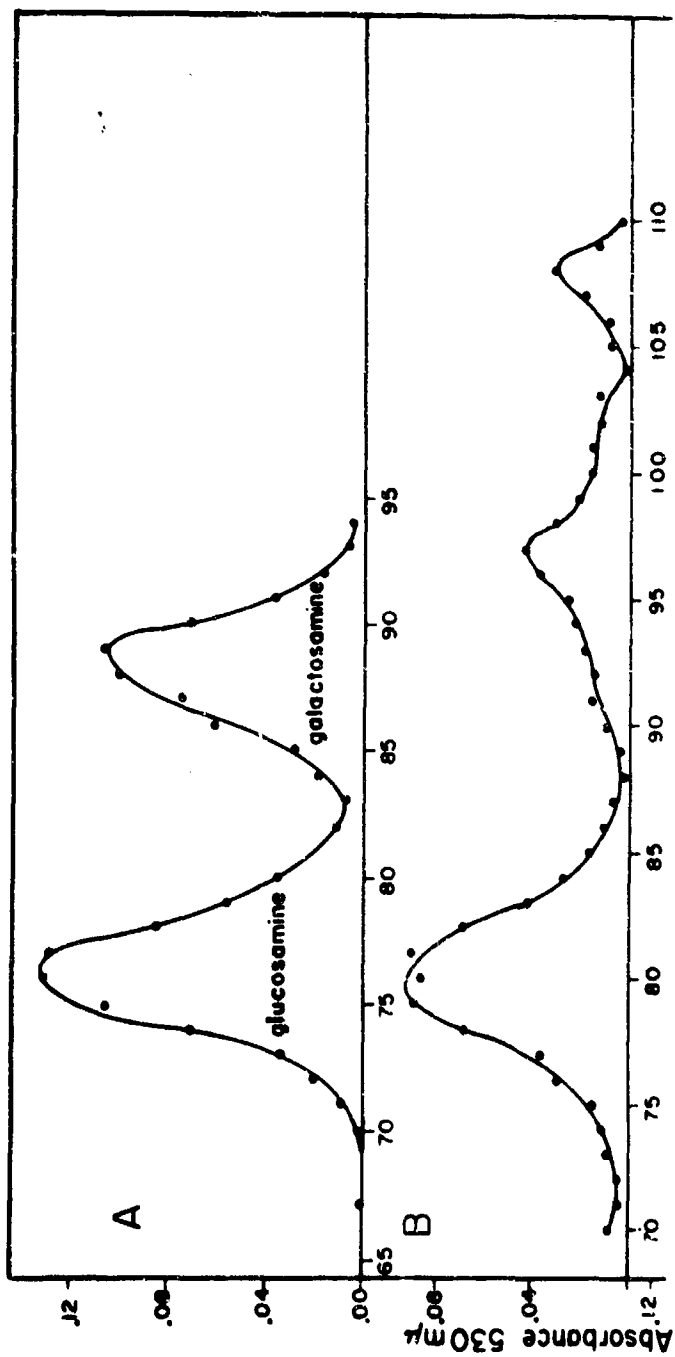


FIGURE 1

Separation of de-acetylated amino sugars. Amino sugars were obtained from N-acetyl amino sugars by acid hydrolysis and separated on standardized Dowex 50 columns as described by Crumpton (Biochem. J., 72: 479-486, 1959). A. Resolution of 200 μgrams N-acetyl-D-glucosamine and 100 μgrams N-acetyl-D-galactosamine after acid treatment. B. Separation of amino sugar moiety following de-acetylation of compounds present in ion-exchange effluent peak 3 from stationary phase cells.

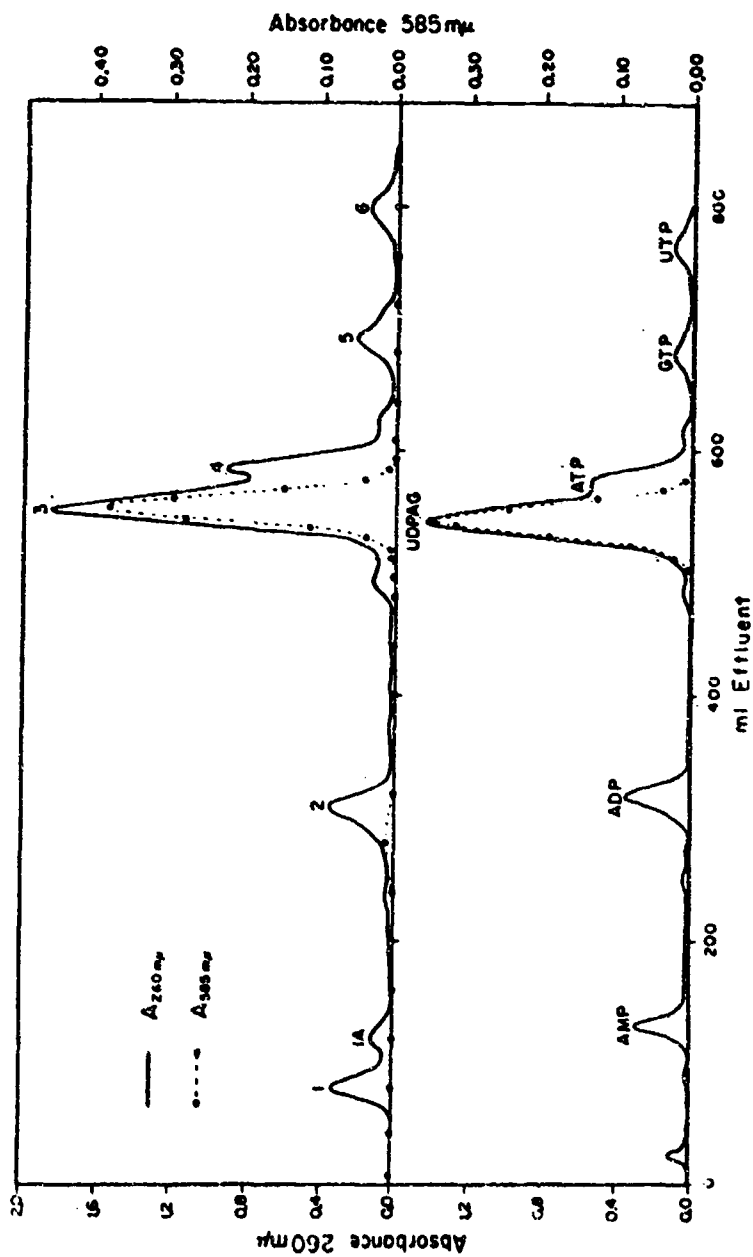


Figure 2

Ion-exchange chromatograms of cells in the stationary phase and a synthetic nucleotide, nucleotide-sugar mixture. Upper curve--Elution pattern obtained from extract from  $770 \times 10^6$  cells in the stationary phase ( $t = 24$ ), 26 hours following medium renewal. Lower curve--Chromatogram of the following mixture (in  $\mu$ moles): 0.5 ATP, 0.604 ADP, 5.16 UDPAG, 1.39 ATP, 0.51 GTP and 0.435 UTP. Cells and nucleotide mixture were extracted with 20 ml. of both 10 per cent and 5 per cent trichloroacetic acid and placed on a  $7 \times 515$  mm. column containing AG-1X-8 (100-200 mesh) in the Cl<sup>-</sup> form. Compounds were eluted by an HCl Gradient and pumped at a rate of 24 ml./hr; fractions were taken every 20 minutes and elutant measured at 260 m $\mu$ . Dashed line represents chromagen formed in test for N-acetylasino sugar as described by Reissig et al. (J. Biol. Chem., 217: 959-966, 1955).

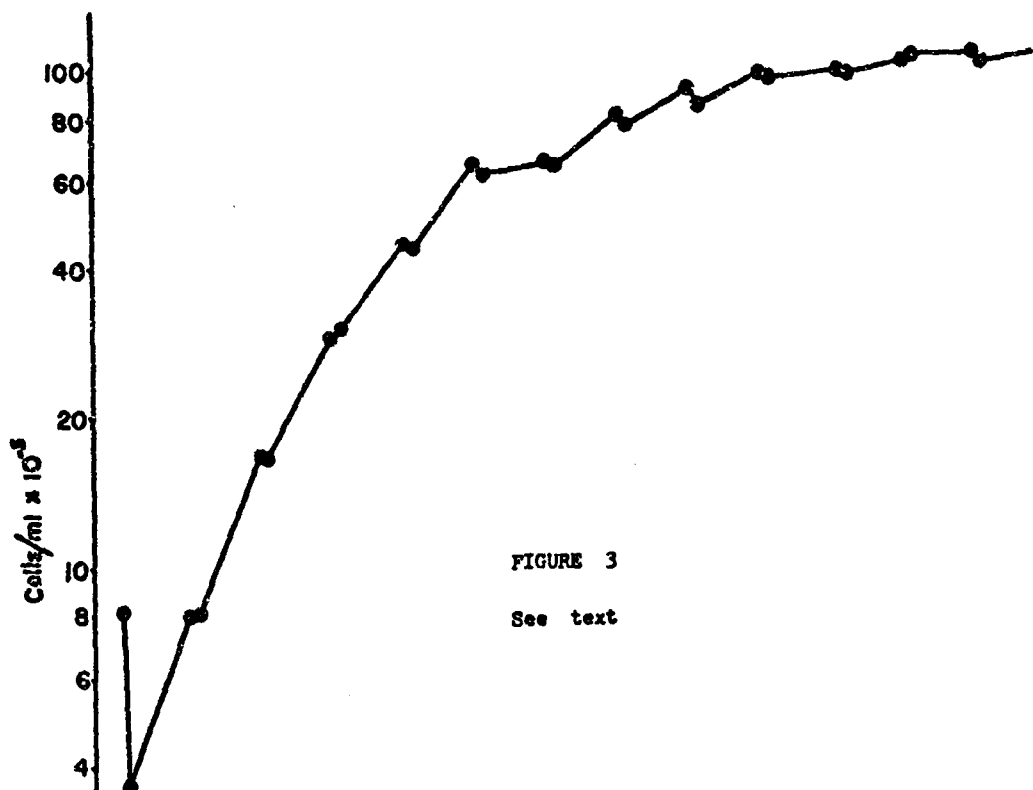
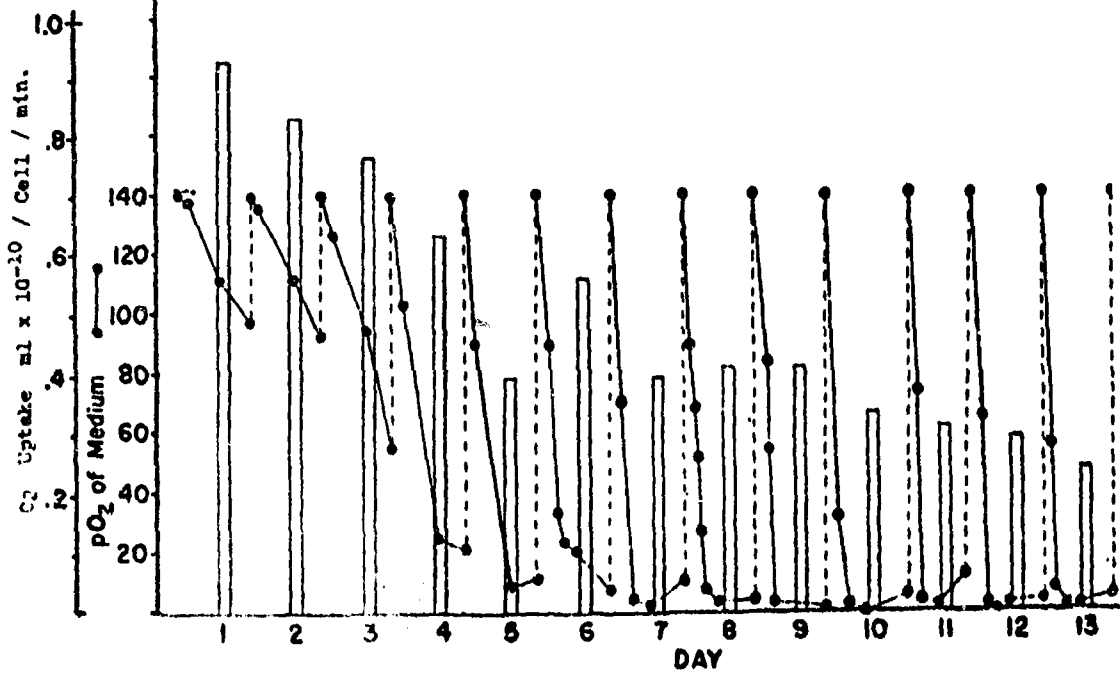


FIGURE 3

See text



Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08, Physiology

Work Unit 075, Cell growth and regeneration

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				NAME: Hodos, W. Ph.D.			
				DA			
23. (U) Autonomic Nervous System; (U) Neurophysiology; (U) Neuropsychiatry; (U) Anatomy; (U) Central Nervous System; (U) Biomedical Engineering							
23. (U) Morphological and physiological analysis of the primate and subprimate CNS directs mainly toward delineation of longitudinal systems. An analysis of structures supplied by the autonomic nervous system in regard to method of CNS control. An analysis of the effects of stress on autonomic control mechanisms.							
24. (U) Determination of CNS pathways and terminations in various primates and sub-primates by histological and histochemical techniques following appropriate surgical lesions. Use of radioautographic methods for determination of cellular activity. Recording and analysis, utilizing computer techniques, of the cardiovascular response to controlled hemorrhagic shock in awake chronic monkeys. Recording and analysis of single cell in the CNS and their relationship to other physiological events.							
25. (U) 69 01 - 69 06 New information on the organization of corticofugal systems has been obtained. Neuroanatomical correlates of stress are under study. Construction of an electron microscopic laboratory is near completion. Instrumentation for determining blood flow and cardiac output has been tested and integrated into the hemorrhagic shock studies. Detailed analysis of CNS control of physiological, endocrinological, and behavioral responses in this model continues. CNS control of liver metabolism is under study. Unsuspected visceral somatic interactions have been found in the spinal cord. Autonomic and somatic responses to increased intracranial and intraspinal pressure are being studied. Changes in reaction time during phases of the cardiac cycle have been found. Details of CNS control of bladder function are being established. An experimental method of controlling limb pain is being tested. Several special purpose electronic devices have been designed and fabricated. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.							

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Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08, Physiology

Work Unit 076, Analysis of behavior and of mediating mechanisms:  
Anatomic and electrophysiological factors

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Description.

1. The general object of this subtask is the analysis of neural mechanisms mediating behavior. The specific type of behavior under study is the response to stress. The experimental approaches which have been followed are of two types: 1) Studies utilizing the specialized techniques from the disciplines of anatomy, physiology, experimental psychology and endocrinology and 2) Studies of an interdisciplinary nature utilizing techniques from several fields simultaneously. An attempt has been made to pursue both the general and the specific aspects of behavioral mechanisms. Some aspects of the response to stress can be studied in greatest detail within one specific discipline using the classical methods of the discipline. The questions which have been asked are clearly defined, narrow in scope and well suited to solution by available techniques. However, many aspects of the response to stress do not lend themselves to study in this manner. The questions to be answered are not particularly well defined because there is not enough information available to formulate them in an ideal fashion. These aspects of behavior can be studied only when the classical approaches are extended and combined with techniques from other disciplines. Considerable effort has been put into interdisciplinary approaches to specific behavioral responses. From these studies new concepts and hypotheses have emerged which subsequently have been looked at in far greater detail with specific techniques. Thus the constant interplay between the general and the specific, the single and multidisciplinary project has formed the backbone of the approach used in this subtask.

2. It is convenient to divide the response to stress into some of its components, not only to facilitate presentation in this report but also because this breakdown allows some insights into the mechanisms

involved, and illustrates further our approach to understanding these mechanisms. We will consider four interrelated areas of study: Studies on transduction mechanisms, studies on integration mechanisms, studies on perception mechanisms and studies on response or effector mechanisms. Clearly the behavioral response to stress involves each of these mechanisms.

#### Progress.

1. Studies on Transducer Mechanisms. The first event in the complicated chain of activities leading to a response is the conversion of the energy in the stress stimulus into neural energy or neural activity. This conversion or transduction takes place in sensory receptors which subsequently relay this information to the central nervous system.

a. Physiologic identification of sensory receptors in the adrenal gland. As reported last year, three types of sensory receptors have been identified in the adrenal gland: 1) Mechanoreceptors, which respond to distentions of the gland capsule and parenchyma, 2) Baroreceptors, which respond to changes in arterial blood pressure, and 3) Chemosensitive receptors, which respond to changes in blood levels of epinephrine or norepinephrine. Additional characterization of these receptors indicates that the mechanoreceptors are highly phasic, rapidly adapting units which fire only during the stimulus. The baroreceptors are also phasic, rapidly adapting units, but since the firing threshold for many of them is exceeded by normal blood pressure levels, they fire continuously in synchrony with blood pressure changes. Severe reduction of blood pressure is followed by cessation of activity in these units. The chemosensitive receptors are tonically active, slowly firing units. Elevations of catecholamines cause a decrease or cessation in their firing.

b. Physiologic and anatomic identification of urinary bladder afferents. Identification and characterization of tension receptors in the urinary bladder has continued. Afferents have been identified in both the nerves of the pelvic plexus and in the hypogastric nerve. Conduction velocity studies indicate that the afferents are in the small myelinated and unmyelinated fiber range. Initial observations of these nerves using electron microscopy indicates an unsuspected large proportion of unmyelinated fibers. The function of these fibers is under study.

c. Distribution of afferent terminals in the spinal cord. Anatomic studies have continued in an effort to define the terminations of sensory fibers in the spinal cord. Of particular interest is the interaction of sensory afferents and nuclear groups of the

autonomic nervous system. Since we have clearly demonstrated that the binauronal arc between a sensory fiber and an autonomic efferent, as illustrated in every neuroanatomy and neurophysiology textbook, does not exist, we are trying to work out the more complicated pathways which must be present.

2. Studies on central nervous system integration. The central nervous system can be considered to contain a relatively few number of afferent fibers, a relatively few number of efferent fibers and an overwhelming number of interneurons. It is within this vast interneuronal pool that the integrative aspects of behavior take place. The following studies pertain to isolated portions of this pool.

a. Neurophysiologic studies of spinal cord integration. The electrical activity of single cells in the spinal cord is being studied with intracellular microelectrodes. Of particular interest is the discovery of a large group of cells which show marked convergence of somatic and visceral stimuli. These cells have complex receptive fields and may respond to stimulation of several viscera (i.e., gall bladder, stomach, urinary bladder, large intestine) and to stimulation of skin surfaces of one or more extremities. Combinations of excitation and inhibition from these various locations have been found. These interactions have not been previously thought to occur in the spinal cord and have been considered to be "higher" CNS functions. Of unique interest is the finding of some cells which respond both to skin stimulation and to stimulation of a viscus immediately beneath it in the abdominal cavity. Such units have been postulated, but never described, in one respected theory of referred pain.

b. Descending connections of the neocortex. The main emphasis of this anatomical study had been to determine the subcortical targets for projections from the precentral (motor), postcentral (sensory) and parietal gyri in a series of primates. Particular attention has been given to delineating pathways to the thalamus, subthalamic region, mesencephalon, basal ganglia and hypothalamus.

c. Corticofugal connections to autonomic and somatic motor cell groups in the spinal cord. A comparative study of cortico-spinal projections has been continued in several species of primates and carnivores. Of special interest has been the connections between the motor cortex and the autonomic and somatic motor neuron groups in the spinal cord.

d. Forebrain afferents from midbrain and pons. Experimental anatomical work on the important midbrain projections to the forebrain has continued. Unsuspected projections have been found from the substantia nigra to the caudate and putamen, and also from an area near the interpeduncular nucleus to the region of the cingulate cortex via the septum and around the genu of the corpus callosum.

e. Hypothalamic connections in the primate. Work on hypothalamic projections has continued. The medial hypothalamus has been found to project only to the lateral hypothalamus and to midline thalamus. The lateral hypothalamus, however, has been found to project forward to the lateral preoptic area and caudally to the midbrain tegmentum.

3. Studies on perception mechanisms. Following the transduction of a stimulus from the environment into neural activity and the integration of this activity by the central nervous system, there is a point at which this stimulus enters the organism's awareness. For integrative responses, other than simple reflex responses, the perception of the stimulus is important in determining the nature of the response to this stimulus. Using psychophysical techniques, it is possible to study the mechanisms (anatomic substrate, physiological components) of perception in both its quantitative and qualitative aspects.

a. Mechanisms of visual perception. Studies have continued on the nature of the visual pathways and visual behavior in the avian brain. These species have proven to be particularly useful because of the acutely developed visual abilities and the ease of accessibility to some anatomic structures as compared to the mammalian brain. Two distinct ascending visual systems have been found. One which goes from the retina to optic tectum, to nucleus rotundus thalami and to ectostriatum of the telencephalon. This avian pathway seems similar to the mammalian retina-superior colliculus-lat post thalamus-circumstriate cortex pathway. The second pathway goes from retina to principal optic nucleus of the thalamus to a specific zone of granule cells in the Wulst region of the telencephalon. This pathway is similar to the mammalian geniculostriate system.

Psychophysical studies have shown that lesions in the first mentioned system produce deficits in visual discrimination performance based on intensity, pattern or color. Lesions in the second system seem to produce deficits in visual discrimination performance based on minimal separable acuity and minimal detectable differences in flash rate. Lesions to both systems always produce more profound deficits than lesions to one system alone. These studies represent a unique approach to establishing structural-functional relationships in the brain.

b. Optic projections in mammals. A re-examination of mammalian visual projections following enucleation was undertaken as a result of the findings in the avian brain. Previously unreported findings have appeared such as: 1) Terminal degeneration in the upper two layers of the superior colliculus; 2) Sparse degeneration of axons in the external medullary lamina of the thalamus near the lateral geniculate nucleus; and 3) An ipsilateral projection to a layer in the middle of lamina B in the lateral geniculate, previously thought to receive only contralateral input.

c. Mechanisms of auditory perception. A parallel series of studies have been undertaken to determine auditory pathways and auditory behavior in the avian brain. The same basic approach of combining experimental anatomic and behavioral techniques is being employed to work out those structures and connections necessary for the integration of stimuli necessary for the perception of auditory information.

d. Neurophysiologic aspects of human pain perception. A neurophysiologic theory of pain mechanisms has recently been proposed. This theory postulates that in the spinal cord, large fiber afferents which mediate skin proprioceptive sensibility and small fiber efferents which are supposed to mediate pain sensibility interact. This theory suggests that large fiber activity may "gate" small fiber activity and possibly control its input to the cord and its subsequent perception. A pilot study has been carried out utilizing transcutaneous electrical stimulation of superficial nerves in patients with pain problems of the causalgia type. Since large diameter neurons are electrically excitable at lower level thresholds than the smaller diameter neurons, it is possible to selectively activate these fibers. In those few patients thus far studied, there has been significant relief of pain during and up to two or three hours following several minutes of large fiber stimulation. A more comprehensive study is being formulated now to investigate these mechanisms further and to attempt a quantitation of the apparent pain relief.

e. Dorsal Root Terminal fibers in the Substantia Gelatinosa. The substantia gelatinosa of the spinal cord has been implicated by neurophysiological experiments as a nucleus in the central pathway related to pain. This is a suggested area of large and small fiber interactions. An anatomical study has attempted to define in detail the relationships of afferent fibers in this region. Several recent modifications of anatomical techniques have been utilized in this study.

4. Studies on efferent or response mechanisms. In this series of studies, the variables being measured are functions of the response mechanisms of the organism. These response characteristics represent the end result of all the preceding types of activity and define the behavior of which the organism is capable.

a. Somatic and autonomic responses to intracranial or intraspinal pressure. Increased intracranial or intraspinal pressure evokes a cardiovascular response which elevates the blood pressure to exceed the imposed pressure. As reported last year, our studies have indicated that the thoracic spinal cord is largely responsible for these reflex adjustments. By elevating the spinal subarachnoid pressure, a response pattern is seen which closely resembles that seen in the Cushing Reflex. Besides the autonomic responses, changes in reflex excitability of the somato-motor pathways have been observed. Detailed examination of these effects using neurophysiological techniques has indicated major differences in the time course and sign of autonomic and somato-motor responses. Recent experiments have, for the first time, demonstrated synchronous firing of autonomic preganglionic fibers with Traube-Herring or Mayer waves in the blood pressure.

b. Characterization of urinary bladder efferents. Work is progressing on the characterization of efferents to the urinary bladder and the relationship of efferent activity to afferent stimulation. Efferents in both the hypogastric and pelvic nerves have been characterized and conduction velocities determined. Both tonic and phasic units have been found in normal cats. In a few animals studied with chronic spinal cord sections, an apparent absence of phasic efferent unit firing has been noted. Work is continuing in order to substantiate these findings and to examine the mechanisms operating in both the normal and chronic cord sectioned animals.

c. Autonomic responses in hemorrhagic shock. The effects of slow hemorrhage and its progression towards shock are being studied in chronic, extensively instrumented monkeys. The basic aim of this study is to determine the mechanisms and sequences of breakdown of the normal homeostatic autonomic reflexes involved in the maintenance of blood pressure and blood flow. As reported last year a reproducible model of this form of shock has been established and the natural history of the blood pressure changes has been determined. During the past year extensive redesign of the digital data acquisition system was undertaken to allow for the integration of blood flow data into the system.

It had become quite evident that blood pressure measures had to be supplemented by blood flow determinations. Our present studies are attempting to study regional as well as systemic flows and the neural mechanisms responsible for their control.

d. Biochemical concomitants of hemorrhagic shock. Blood samples taken prior to and during the hemorrhagic procedure are studied for changes in such variables as EPI, NE, 17OH and 17KS, blood sugar, insulin and growth hormone. The time course of changes in these variables, especially EPI and NE, is under study as are the possible mechanisms which stimulate their release. An additional group of determinations has been set up to study liver metabolites during hemorrhage. Recent experiments have suggested a neural role in liver metabolism and the hemorrhage model may offer some insight into this possibility.

e. Effect of physiological state on glucose metabolism. The establishment of baseline data on glucose metabolism in the liver is a prerequisite to studies on possible neural control. A technique is available, for the first time, to study liver blood flow in the conscious dog. Studies are in progress to assess the handling of the glucogenic metabolites pyruvate, lactate, glycerol and alpha amino nitrogen by the liver in the fasted, fed and post-absorptive dog. In this way, it should be possible to assess the relative importance of these glucogenic metabolites contained in the blood in relation to the normal sources of glucose produced in the liver.

f. Changes in autonomic ganglia following behavioral stress. An autoradiographic study of the superior cervical and coeliac ganglia is being performed on behaviorally stressed rats. Following exposure to unpredictable electric shock, the stressed animals show markedly increased numbers of proliferating glial cells and increased numbers of binucleated neurons in these ganglia. Of special interest is the finding of neuron nuclei labeled with  $^3\text{H}$ -Thymidine. The time course of this response is being worked out as well as the duration of stress needed to evoke such a response.

g. Changes in reaction time during the cardiac cycle. This study has been designed to determine if the motor response to an external stimulus is modified by ongoing autonomic activity such as the cardiac cycle. Monkeys have been trained to release a lever in response to a tone which is triggered at various phases of the cardiac cycle. This response has been found to take place

significantly faster during diastole than elsewhere in the cycle. Of particular interest is the observation that this facilitation effect has a time course suggestive of learning. The mechanisms responsible for such interoceptive learning are under study.

h. EEG Changes in Uremia. In conjunction with studies in the Division of Medicine, an attempt has been made to develop a simple method of quantitating EEG activity in monkeys undergoing experimental procedures producing acute and chronic uremic states. A very close correlation has been found, in the initial animals studied, between changes in electrical activity of the brain and chemical indicators of depressed kidney function.

## 5. Technical Developments.

### a. Experimental.

(1) A new laboratory of electron microscopy has been built in the Department. The electron microscope has been installed and should be completely functional in a few months. Most major support items have been installed, although some are still on order. The training of support personnel is in progress.

(2) An autoradiographic neuroanatomical tracing technique is under development. This involves the axonal transport and subsequent identification of  $^3\text{H}$ -Leucine following injection into neural tracts.

(3) An automated method has been designed and set up for the differential determination of urinary catecholamines. This procedure has increased threefold the number of determinations possible with a 50% increase in precision.

### b. Electronic.

(1) Controlled temperature bath. A servo system to maintain water at a constant temperature.

(2) Staircase generator. A device to move the horizontal beam to successively higher levels on a storage oscilloscope.

(3) Respiration monitor. A transducer to measure respirations.

(4) Paper speed control system. A programmed system to remotely control paper speed on an 8-channel Sanborn recorder.

(5) Cardio tachometer. An improved model to be used with the Offner Polygraph which has an expanded scale.

(6) Frequency and period counter. A device to measure the frequency or period of cyclic events.

(7) Cardiac pacemaker. An instrument which permits a frequency-modulated pacing of the heart.

(8) EEG preamplifier. A special purpose 8-channel EEG amplifier system which can be mounted close to subject.

(9) Tone stimulation panels. A system to present tone signals to experimental animals.

(10) EMG integrator. A device that will integrate the area under the curve of any EMG or periodic signal.

(11) Blood pressure digitizer. An instrument which determines maxima and minima of blood pressure and digitizes the mean values over time.

(12) Photo-electric pigeon key. A special photo-electric key to be used in the initial training of pigeons.

(13) Logic pattern generator. An instrument that presents a predetermined audio pattern to the experimental animal.

(14) Tape reader control unit. A control device to facilitate use of a Tally Tape reader.

(15) Pulse former. Pulse formers to be used in conjunction with SODECO parallel-entry electro-mechanical counter printers.

#### Summary and Conclusions.

The organism's response to stress has been studied using a variety of experimental approaches. Particular emphasis has been given to the role of the autonomic nervous system in mediating these responses. Morphological studies have been directed towards the delineation of longitudinal systems interrelating successive levels of central nervous system organization and control. Physiological studies have covered problems in transducer mechanisms, integration mechanisms and effector or response mechanisms. And also, interdisciplinary studies have been directed towards establishing functional properties of neuronal aggregates and their relationships to behavior, endocrine release and central control of peripheral

responses. A new definition of the autonomic nervous system from both an anatomical and a physiological viewpoint is clearly emerging from these studies. The role of this system in homeostatic reflexes and the ability for discrete as well as general actions are new concepts. The control of biochemical and endocrinological responses by the autonomic nervous system is emerging as an extremely important and unsuspected finding. Several of the listed projects were accompanied and facilitated by new developments in technical instrumentation.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ADDRESS	2. DATE OF REPORT	REPORT COVER SHEET	
				DA OA6439	69 07 01	DD FORM (AR) 608	
3. DATE PREPARED	4. KIND OF REPORT	5. SUMMARY CTRY	6. WORK SECURITY	7. RESEARCH	8. WORK UNIT	9. SPECIFIC DATA TRANSFER AGREE	10. LABEL OF THIS WORK UNIT
69 01 31	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
11. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	61102A	3A061102B71P	08	077			
b. CONTINUING							
c. TERMINATING	1412A(2)						
12. TITLE (Printed and Security Classification Code) (U) Analysis of Behavior and of Mediating Mechanisms - Neuroendocrinological Factors (09)							
13. SCIENTIFIC AND TECHNOLOGICAL AREAS 013400 Psychology /Indivi 016200 Stress Physiology 005900 Environmental Bio							
14. START DATE	15. REVISIONS COMPLETION DATE	16. PERFORMING AGENCY		17. PERFORMANCE STATUS			
08 63	NA	DA		C. In-House			
18. CONTRACT/AGREEMENT Not Applicable				19. RESOURCE ESTIMATE	20. PROFESSIONAL TIME YRS		21. FUNDING (\$)
a. DATE/EFFECTIVE				b. PERSONNEL		c. FUNDING (\$)	
d. NUMBER				e. FISCAL YEAR		f. FUNDING (\$)	
g. TYPE				70		260	
h. KIND OF AWARD				9		260	
i. CURR. AMT.							
22. RESPONSIBLE AND SUPERVISOR				23. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Print name if U.S. Armed Services)			
NAME: Meroney, COL W. H.				NAME: Mason, J. W. M.D.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3559			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
24. GENERAL USE				25. DELEGATE INVESTIGATOR			
Foreign Intelligence Considered				NAME: Ehle, CPT A. L.			
				NAME: DA			
26. KEY WORDS (Printed and Security Classification Code) (U) Stress; (U) Emotions; (U) Homeostasis; (U) Psychophysiology; (U) Neuroendocrinology; (U) Psychoendocrinology.							
27. TECHNICAL OBJECTIVE, ABSTRACT, OR PRELIMINARY FINDINGS (Printed and Security Classification Code)							
<p>23. (U) Principal objective is to study central integrating mechanisms which control and coordinate visceral and metabolic functions. Understanding such mechanisms is essential to the understanding of bodily reactions to environment and stress, both psychological and physical, and of basic concern as an objective approach in psychosomatic medicine.</p> <p>24. (U) This involves measurement of plasma and urinary hormone levels in monkeys and humans in a variety of acute and chronic stress situations, with emphasis on the concept developed by our earlier works that we must view changes in broad, overall hormonal patterns or balance, rather than in single endocrine systems as was previously customary in the stress field.</p> <p>25. (U) 69 01 - 69 06 Study of the organization of multiple endocrine responses to various physical stimuli such as heat, cold, hypoxia, infusion of organic substrates is being continued. In an effort to increase the pace of this work, negotiations are in process with COL Jones at Natick with a view to initiating collaborative research projects on heat, cold, and exercise this fall. A pilot psychoendocrine study of five extremely obese patients before, during, and after a period of marked weight reduction has been initiated in collaboration with Dr. Jules Hirsch at the Rockefeller University Medical Center. Experiments are continuing also in efforts to build an adequate series with regard to hormonal responses to electrical stimulation of selected brain areas, and hormonal responses to emotional stimuli following adrenalectomy and/or gonadectomy. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.</p>							

PII Redacted

**Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE**

**Task 08, Physiology**

**Work Unit 077, Analysis of behavior and of mediating mechanisms:  
Neuroendocrinological factors**

**Investigators.**

**Principal:** John W. Mason, M.D.; COL Joseph V. Brady, MSC  
**Associate:** CPT Albert L. Ehle, MC; CPT Marvin S. Wool, MC;  
Robert M. Rose, M.D.; Edward H. Mougey, M.S.;  
Frances E. Wherry, A.B.; David R. Collins, B.S.;  
Elizabeth D. Taylor, M.S.; Percy T. Ricketts, B.S.;  
Norman Krasnegor, M.S.

**Description.**

This program is concerned with the role of the central nervous system in the co-ordination of endocrine regulation. Instead of the conventional study of single endocrine systems in isolation, multiple endocrine systems are studied concurrently so that the overall balance between the many interdependent hormones may be investigated. In recent years we have learned that various forms of psychological and physical "stress" elicit broadly organized patterns of hormonal response involving many hormones in addition to those of the adrenal systems. A major goal is to define conclusively these distinctive "overall" hormonal response patterns for various stressful stimuli, including psychological stimuli, cold, heat, hypoxia, fasting, exercise, hemorrhage, dehydration, trauma, infection, and various nutritional changes. A substantial amount of work on the development of new and improved hormone assay procedures, as well as some basic studies of endocrine physiology, have been continued in order to provide the necessary methodological foundation for this stress research program. Some new lines of approach in the study of psychosomatic illnesses and in the study of social factors in psychoendocrine development have been explored during the past year.

**Progress.**

**1. Hormonal Balance in Emotional Stress.**

**a. Monkey Studies.**

(1) Acute Emotional Disturbances. An extensive monograph, representing a review of the current status of the psychoendocrine field along with a large body of our stress research data was published as a supplement to the September-October 1968 issue of Psychosomatic Medicine. A companion series of psychoendocrine experiments defining the pattern of organization of multiple endocrine

response to restraining chair adaptation has been completed during the past year, and this work is being prepared for publication.

Newly available radioimmunoassay methods have made it possible recently to obtain new information on psychological influences on plasma glucagon and thyrotropin (TSH) levels in monkeys. Preliminary experiments indicate that small but consistent elevations in plasma TSH and glucagon levels are associated with both conditioned avoidance and chair adaptation in the monkey. Additional experiments have been performed in the study of hormonal responses to conditioned avoidance in the adrenalectomized monkey. Preliminary indications are that the non-adrenal hormonal responses persist but may be modified in some instances, such as with the growth hormone, which appears to be exaggerated following adrenalectomy.

(2) Developmental and Social Factors. Studies on mother-infant interactions, controlled-environment developmental studies, and social hierarchy studies have been transferred within the past year largely to the Department of Psychiatry under the direction of Dr. Rose. Transfer of this work will make possible a greater emphasis on current research on physical stress within our Department.

## 2. Hormonal Balance in Physical Stress.

a. Fasting. With additional recent experiments a series has now been completed on the effects of fasting on multiple endocrine systems in the monkey. When careful measures were taken to minimize psychological reactions to the fasting situation, no 17-OHCS response was observed. Elevations were observed, however, in growth hormone, epinephrine, and thyroxine levels, with depression in the levels of norepinephrine, insulin and testosterone.

b. Heat. Effort on heat experiments in the monkey has been diminished since CPT Poe's departure and because the constant temperature chamber has been devoted to cold studies. New facilities to pursue this work in the monkey are being prepared. Pilot studies here have shown that heat is not a "stressor" to the adrenal gland if psychological factors are minimized.

c. Cold. CPT Ehle has initiated new experiments on hormonal response patterns to cold in the monkey and appears to have eliminated a ventilation problem in the constant temperature chamber which is believed to have been a problem in earlier experiments.

d. Arginine Infusion. It has been discovered this past year that arginine infusion elevates testosterone levels in the monkey, as well as insulin and growth hormone levels as was previously known. At present, a series of experiments is under way to determine other hormonal responses to amino acid infusion and other humoral stimuli.

Through COL LeeRoy Jones of the Army Environmental Medicine Research Institute at Natick, Massachusetts, plans are presently being made to initiate collaborative studies in human subjects of physical stresses such as heat, cold, exercise and hypoxia later this year.

### 3. Hormonal Balance in Medical Illness.

a. Respiratory Infection. New findings have emerged from further analysis of hormonal data from our study of basic trainees at Ft. Dix in relation to respiratory infections. Two-dimensional analyses of the frequency of extremely high or extremely low 17-OHCS, etiocholanolone, androsterone and thyroxine levels during the pre-illness period indicate a significantly higher percentage of extreme values in the pre-illness group than in a control group. While our number of subjects is relatively small, it is felt that these and other findings of hormonal changes prior to onset of respiratory infections merit publication and priority will be given to preparation of a manuscript reporting this work.

b. Obesity. A collaborative study of obese patients has been initiated with Dr. Jules Hirsch of the Rockefeller University Medical Center. "Overall" hormonal balance in five extremely obese subjects is being studied before, during, and after weight reduction on a 600-calorie diet. Preliminary findings are most interesting and provocative, particularly with regard to indications in two subjects that anabolic hormones may paradoxically rise during periods of stress, in contrast to what happens in most normal human subjects and monkeys.

It has also been observed that 17-OHCS, epinephrine, norepinephrine, and testosterone levels tend to fall during the weight reduction period. Samples are currently being collected on the fourth and final low-weight maintenance period. This study represents part of a continuing search for a disease model which may serve as a striking example of how the psychoendocrine approach linked with a broad assessment of "overall" hormonal balance may be applied to the study of certain medical illnesses.

### 4. Biochemical Methodology and Endocrine Physiology.

Substantial progress in this area has been made this year. With regard to radioimmunoassays, Mrs. Wherry has fully validated a new method for plasma TSH measurement in the monkey, and physiological studies including thyroidectomy and thyroxine injection experiments have confirmed its validity. This is the first method reported for assay of TSH in the monkey. Further studies with the plasma glucagon method also have added support to its validity in the monkey.

A semi-automated method for urinary epinephrine and norepinephrine analyses has been set up and validated fully this year under MAJ Troyer's guidance. This method more than doubles our previous output of catecholamine analyses.

Evaluation of a urinary cortisol method, using the competitive protein-binding principle indicated that this method is not reliable with only crude urinary extraction and work on this method has been discontinued.

A method for the measurement of free thyroxine has been developed by Mr. Mougey and is currently being evaluated. Indications are that the free thyroxine level may be a more sensitive and useful measurement of thyroid activity than the FBI or BEI determination.

#### Summary and Conclusions.

Further support for two major concepts in the stress field continues to emerge from our studies.

First, our studies indicate that the "stress" field must begin to move away from a preoccupation with the adrenal systems alone to a consideration of the many other hormonal changes which appear to be organized distinctively in a broad, overall manner for different psychological and physical stimuli. Further study of broad patterns of hormonal change seems likely to uncover some important basic principles of physiological co-ordination or integration.

Secondly, it appears that the entire field of "stress" theory is thrown into confusion by the new findings that the adrenal response to many "physical" stimuli is probably spurious and actually related to contaminating psychological reactions involving emotional arousal, discomfort or pain rather than the physical stimulus itself. It is imperative, therefore, that a painstaking re-evaluation of the many different "physical stresses" be carried out with close attention to minimizing and assessing possible interfering psychological factors.

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PROJECT 3A061102B71P  
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 09  
Radiobiology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION NO. DA OA6533	2. DATE OF SUMMARY 69 07 01	REPORT CONTROL NUMBER DD-RCS (AR) 696
3. DATE PREV. SUMMARY 69 01 31	4. KIND OF SUMMARY D. Change	5. SUMMARY SECTY. U	6. WORK SECURITY U	7. FEASIBILITY NA	8. AGENCY DESIGN NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: A. PRIMARY 61102A	PROGRAM ELEMENT 61102A	PROJECT NUMBER 3A061102B7IP	TASK AREA NUMBER 09	WORK UNIT NUMBER 015		
11. TITLE (Precede with security classification code) (U) Mechanisms (00)						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS 014100 Radiobiology 011500 Nuclear Weapons						
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17. CONTRACT/GRANT A. DATES/EFFECTIVE: Not Applicable B. NUMBER: C. TYPE: D. KIND OF AWARD:				18. RESOURCES ESTIMATE A. PROFESSIONAL MAN YRS B. FUND \$ (Estimate) FISCAL YEAR: 69, 70 CURRENT: 11, 7 180, 200		
19. RESPONSIBLE DOC ORGANIZATION NAME: Walter Reed Army Institute of Research Washington, DC 20012 ADDRESS: RESPONSIBLE INDIVIDUAL: Meroney, COL W.H. NAME: TELEPHONE: 202-576-3551				20. PERFORMING ORGANIZATION NAME: Walter Reed Army Inst of Res Div of Nucl Med ADDRESS: Washington D. C. 20012 PRINCIPAL INVESTIGATOR (Precede with K22. An dash preceding) NAME: Mahin, LTC D. T. TELEPHONE: 202-576-2211 SOCIAL SECURITY ACCOUNT NUMBER: [Redacted] ASSOCIATE INVESTIGATORS: Ginsberg, LTC D. M. NAME: NAME: DA		
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23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Precede individual paragraphs identified by number. Precede rest of each with security classification code.) 23. (U) Quantitative measurements of the consequences of exposure to ionizing radiation in cellular, submammalian and mammalian systems. 24. (U) Effects of radiation and radiation modifiers on DNA and influence of alterations in cell metabolism on radiation survival are being investigated in bacteria. Electron spin resonance spectrometry is used to define free radical production and the time length of action of radicals within the biological medium. Changes in genetic material within mammalian cell lines as evidenced by chromosomal breaks and translocations are quantitated with biochemical cellular constituents after radiation. Influence of stresses such as trauma and burns combined with radiation are evaluated in relation to dose response. 25. (U) 69 01-69 06 Thermoluminescent dosimetry (TLD) for ionizing radiation effects at low temperatures are being studied. Efforts are underway to correlate results of TLD with free radicals in irradiated tissues. An integrating dosimeter for microwave exposure is under development. Physico-chemical damage to DNA appears to be independent of the suspending medium, whereas biological inactivation is quite medium-dependent. This suggests that DNA damage is due primarily to direct effects, but may not be biologically significant. Evidence of protein-DNA lesions in vivo after gamma radiation was observed. A liposome system was developed for use in studies of radiation effects on lipid membranes. A feasibility study is underway to observe lymphocyte abnormalities in humans occupationally exposed to microwave radiation. Cytotoxicity studies of antisera in human lymphocytes in vitro by Cr-51 elution techniques were initiated. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.						

PII Redacted

Project 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 09, Radiobiology

Work Unit 015, Mechanisms

**Investigators.**

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Description.

Comprehensive knowledge of the biochemical and physiological basis of radiation injury in humans and other mammals is necessary for the recognition and understanding of the clinical and pathological manifestations of radiation injury, and for the development of effective prophylactic and therapeutic measures. Therefore, the objective of this work unit is to define the biochemical and physiological mechanisms by which ionizing radiation affects living organisms, submammalian and mammalian, at the levels of physicochemical, molecular and cellular, tissues, organs, and whole organisms. Research efforts under this work unit include:

1. Physicochemical studies of radiation injury.

Electron spin resonance techniques are being used in the detection, measurement, and evaluation of physicochemical parameters of radiation injury. Included in these studies are investigations of effects of various radiation response modifiers on metabolism in tissue from normal and irradiated animals.

2. Molecular and cellular biology of radiation injury.

The objective of this work is to identify and measure specific kinds of ionizing radiation damage to biomacromolecules within cells, and to determine the biochemical mechanisms involved in the induction and repair of specific cellular lesions.

3. Mammalian tissue, organ, and whole organism studies.

Immediate and long-term changes in functional integrity, chemical consistency, and genetic constitution of whole mammals after exposure to ionizing radiation are being investigated.

4. Radiation dosimetry.

The correct interpretation of biological responses to radiation exposure requires precise knowledge of the amount of radiation delivered and absorbed. This work is designed to develop and standardize accurate techniques for the measurement of ionizing and microwave radiation.

5. Human whole body radiation detection.

The Walter Reed Human Whole Body Counting Facility (HUMCO) is used in support of Health Physics and Nuclear Medical activities at the Walter Reed Army Medical Center.

Progress.

1. Physicochemical studies of radiation injury. (LTC Swartz, Dr. Copeland, Mr. Richardson, LTC Mahin).

a. Tissue studies. Previously described studies (WRAIR Annual Progress Reports, 1966-67, 1967-68) using electron spin resonance spectroscopy (ESR) to investigate the nature of free radical species in unirradiated tissue have been continued. The characteristics of such background resonance must be understood before the radiation induced changes in tissue ESR spectra can be meaningfully interpreted. Such a characterization of paramagnetic species in unirradiated tissue may also provide useful fundamental biological information.

1) Comparison of frozen and unfrozen tissue ESR spectra. In order that ESR tissue studies can be carried out in the much more convenient frozen state, it must be established that phenomena observed in living tissue slices can be equally well observed in frozen samples at 77°K. Our efforts in this regard have included the variation of pH, incubation time, incubation temperatures, addition of copper in trace amounts, and repetition of previously reported in vivo and in vitro alteration of tissue prior to ESR observation of the same specimen, both as a metabolizing tissue slice and as a frozen cylinder. Our general finding is that ESR centers of free radical origin show parallel behavior in the living and frozen states. On the other hand, ESR centers resulting from paramagnetic salts do not produce strictly parallel results in the two states. It is possible that the ligand fields influencing such ions suffer considerable transformation during the formation of an ice lattice. However, since we expect ionizing radiation to have its more significant effect on ESR centers of free radical origin, analysis of tissue samples in the frozen state by electron spin resonance seems valid.

2) Organ viability determination. Previously reported (WRAIR Annual Report, 1967-68) changes in dog liver ESR spectra as a function of the liver physiology of the donor dog have suggested ESR as a means of testing organ viability prior to transplantation. Studies designed to verify and determine the practicality of such a test have been conducted in collaboration with the Division of Surgery at WRAIR and with Dr. Starzl's group at the University of Colorado. Results to date indicate a consistent change in ESR spectra during a period of sustained hypotension followed by death. No correlation of ESR signal variation with long term success of liver transplant is possible at present.

b. Oxygen effect, freezing and protection. Consideration of the known deleterious effects of oxygen in several different circumstances including radiation, lyophilization, drying and high pressure led to a hypothesis that all of these "oxygen effects" had a common underlying mechanism. This mechanism appeared to be the reaction of oxygen with free radicals formed or uncovered by the damaging processes and by oxygen reaction with normal cell constituents to form free radicals.

Previously reported studies (WRAIR Annual Reports 1966-67; 1967-68) and work this past year have demonstrated that a freezing-oxygen effect does exist in *E. coli* on four levels. These levels are: 1) free radical formation, 2) cell membrane and/or transport damage, 3) DNA damage and 4) reduction in cell survival. These findings are consistent with a general theory of oxygen toxicity mediated via free radical reactions. The free radical reactions are a consequence of the electronic structure of the oxygen molecule, the chemical nature of cellular components and the organization of the cell.

2. Molecular and cellular biology of radiation injury. (LTC Ginsberg, CPT Hawkins, CPT Friedberg).

Previous work in this unit (WRAIR Annual Report, Ginsberg and Webster 1969) demonstrated that production of single-strand breaks in viral and bacterial DNA's by gamma radiation and modification of this effect in the presence of a radioprotectant, beta mercaptoethylamine (MEA), do not systematically correlate with radiation killing of the bacteria. Evidence from other laboratories suggests that double-strand breaks may be the significant molecular lesion in gamma-ray killing of cells and viruses. Using radiation sensitive and resistant strains of one bacterial species, *E. coli* and a common bacteriophage of *E. coli*, phage T-2, the possible lethal role of double-strand DNA breaks is being tested. Using another coliphage, T-7, studies are underway to relate the observed changes in DNA structure to the survival of virus plaque forming ability, and from this to infer the mechanisms by which radiation reproductively kills cells. The well-known UV damage repair systems in the K-12 strain of *E. coli* and the T-4 bacteriophage are being studied in an effort to understand how such repair systems may operate in the case of damage by ionizing radiation.

a. Double-strand breakage in irradiated phage T-2. Phage T-2 were grown on *E. coli* host in the presence of tritiated thymidine to label the viral DNA. The labeled phage were purified and gamma irradiated in phosphate buffer without and with MEA. After irradiation the phage were lysed and the DNA freed of protein prior to analyses by sucrose gradient ultracentrifugation. All procedures were chosen to prevent breakage of DNA during isolation and analysis.

The rate at which DNA sediments in a sucrose gradient under a high gravitational force is related to the weight of the DNA. Since the effect of breaking a DNA molecule is to produce sub-units of lower weight, changes in sedimentation patterns of the DNA can be used to interpret the degree of breakage.

The amount of double-strand breakage of phage DNA in buffer was proportional to radiation dose. The dose required to produce an average of one double-strand break per phage genome (37% unbroken) was about 120 Krads. The radiation dose that gave 37% survival ( $D_{37}$ ) of phage infectivity was 12 Krads. Thus, about 90% of the killed phage were deactivated by damage other than DNA strand breakage. In the presence of MEA, the amount of killing was significantly reduced. Under the protected condition at any given dose the ratio of double-strand breaks to killing was closer to unity. The latter observation is preliminary, but suggests that MEA prevents most of the killing attributed to damage other than strand breaks (it also appears to protect against some of the double-strand breakage). It is concluded that double-strand breaks in DNA are, indeed, lethal but that the efficiency of gamma radiation for production of double-strand DNA breaks is low compared to its efficiency in producing the other kind(s) of lethal damage. Thus, in buffer, the irradiated phage are killed by the other kind(s) of damage. This damage is prevented by MEA, and in the presence of MEA the phage are killed predominantly by double-strand breaks.

b. Double-strand breakage in irradiated Escherichia coli strain B/r. Double-strand DNA breakage in gamma-irradiated stationary phase *E. coli* B/r was measured using techniques similar to that described for bacteriophage.

While the  $D_{37}$  for killing of cells in buffer was about 12 Krads, no significant double-strand breakage of DNA was detectable at doses up to 100 Krads. The detection of double-strand breaks in the *E. coli* DNA is complicated because the bacterial genome cannot be extracted in one piece. The DNA extracts reproducibly and rather uniformly in units of 250 million molecular weight, or pieces about one-tenth the size of the intact genome. Thus, if one break occurs in the bacterial genome, it will appear in analysis in only one out of ten of the extracted units. The sucrose gradient procedure used in these analyses is not sufficiently sensitive to detect this amount of breakage. Future efforts of this kind will require either extrapolation of data from very high doses (doses at which several breaks per genome can be observed) or use of a more sensitive method of analysis, for instance, measurement of intrinsic viscosity as was used for phage T-7 studies (see following section of this unit).

c. Physical changes in DNA of irradiated phage T-7. The purpose of this research is to characterize the effect of gamma radiation on the molecular structure of the DNA from irradiated virus, and to determine the dependence of this effect on dose and on the chemical composition of the virus suspension medium. The aim is to relate the observed changes in DNA structure to the survival of virus plaque forming ability, from this relation to deduce the mechanisms of radiation killing of virus, and hence, to infer the mechanism by which radiation reproductively kills cells.

Coliphage T-7 was exposed to  $^{60}\text{Co}$  gamma radiation while suspended in phosphate buffer or in phosphate buffer plus .001 M l-histidine. Isolation of the DNA was accomplished by incubating the phage with sodium lauryl sulfate and pronase, and then extraction with cold phenol. Incubation with pronase was found necessary for clean phenolic extraction of protein from irradiated virus. This implies possible radiation induced covalent binding of protein to DNA. The intrinsic viscosity of the isolated DNA was measured in a Zimm-Carothers low-shear viscometer and is found to decrease with increasing gamma dose. This decrease is independent of the presence of l-histidine, even though plaque survival of T-7 is much greater in the presence of histidine. If random scission of monodisperse polymer is assumed, the number of double-strand breaks per virus genome, and the fraction of surviving intact virus genomes, can be calculated from the viscosity. The fraction of surviving viral genomes is found by this method to agree approximately with that estimated from the shape of the boundary formed by sedimenting the DNA in the analytical ultracentrifuge. This implies the assumed model of random double-strand breakage is substantially correct and accounts for the observed decrease in viscosity. In both solvents, the surviving fraction of intact viral genomes far exceeds the surviving fraction of plaque forming units. For T-7 irradiated in buffer alone, the double-strand breaks in DNA account for an insignificant fraction of the killing.

It is concluded that the intact protein coat of the virus protects the DNA from double-strand breakage due to attack by chemical species formed as a result of radiation induced ionization and excitation of the solvent medium. Double-strand breaks occur only as a result of ionization events in the virus particle itself; that is, they represent a direct effect of radiation. This follows from the solvent independence of DNA breakage.

The radiation-induced killing of virus is mostly due to lesions other than double-strand breakage. These other lesions may occur either in the DNA or the protein coat or may involve both. The other lesion to DNA may consist of single-strand breaks and of chemical alterations of the nucleotide residues that do not lead to chain breakage. From this work it is expected that these DNA lesions will be found independent of the suspension medium. It is expected that the solvent dependence of virus survival reflects a solvent dependence of the frequency of damage to the protein coat. A certain class of radioprotective substances, which includes histidine, is believed to protect the

virus protein by intercepting the otherwise inactivating chemical series produced by the radiation in the suspension media. The bonding of protein coat to DNA may or may not be dependent on the nature of the suspension medium. It may be possible in the future to show the existence of this protein-DNA bonding and examine its dose and solvent dependence.

d. In vitro studies of the repair of photodamage to DNA in E. coli K-12 and Phage T-4. Exposure of DNA to ultraviolet radiation (UV) results in a number of demonstrable physicochemical alterations. Of these, the most biologically significant, as gauged by in vivo studies, is the formation of pyrimidine dimers. Such studies suggest, too, that recovery from the effects of UV radiation is accompanied by enzymatic excision of dimers and repair of the DNA. They do not, however, rule out the possible existence of other, as yet unidentified, photolesions in DNA.

Current investigations in this laboratory are aimed at the identification and isolation of enzymes involved in the early stages of DNA repair in E. coli. The purpose of these studies is threefold: (a) the duplication of DNA repair in vitro; (b) an investigation of the specificity of the repair system for photodamage by examining the role of this system in the repair of ionizing radiation damage, chemical mutagenesis, and DNA damaged with radiomimetic compounds; and (c) an understanding of the mechanism of interaction of hydrolytic enzymes with DNA.

Preliminary investigations have been concerned with the effect of crude extracts of E. coli K-12 on irradiated E. coli and T-4 DNA. In all cases, unirradiated DNA has served as control. The presence of enzymatic activity in crude extracts has been detected by the release of acid-soluble radioactivity from  $^3\text{H}$ -labeled irradiated double-stranded DNA incubated with extracts; the release of acid-soluble radioactivity from  $^3\text{H}$ -labeled irradiated single-stranded DNA incubated with extracts; the presence of enzymatically-induced single-strand breaks in irradiated double-stranded DNA; and specific dimer excision from irradiated double-stranded DNA.

1) The release of acid-soluble radioactivity from  $^3\text{H}$ -labeled irradiated double-stranded DNA. Under conditions where endonuclease I activity is inhibited in crude extracts, the rate of degradation of irradiated double-stranded DNA is significantly greater than that of unirradiated DNA. The rate of degradation is UV-dose dependent and results in a curve which closely parallels the curve measuring the dose dependence of dimer concentration of the DNA. It has not yet been established whether this quantitative difference in the degradation of irradiated as opposed to unirradiated DNA also represents a qualitative difference. Degradation of DNA is demonstrable in extracts of 3 UV-sensitive mutants of E. coli at levels indistinguishable from the wild-type strain.

2) The release of acid-soluble radioactivity from  $^3\text{H}$ -labeled irradiated single-stranded DNA. In the presence of  $\text{Mg}^{++}$ , crude extracts of *E. coli* degrade unirradiated denatured DNA approximately five times faster than irradiated DNA. However, in the presence of  $\text{Mn}^{++}$ , this differential is largely eliminated. These experiments suggest that in addition to exonuclease I (an enzyme that specifically degrades unirradiated denatured DNA), there may exist in *E. coli* an exonuclease that degrades both irradiated and unirradiated DNA. Fractionation procedures are in progress in an attempt to purify such an enzyme.

3) Enzymatically-induced single-strand breaks. Examination of the sedimentation velocity of DNA in alkaline sucrose gradients reveals the presence of enzymatically-induced single-strand breaks in irradiated DNA. This effect also appears to be UV dose-dependent. This activity is present in 3 UV-sensitive mutants of *E. coli* and its significance in terms of UV repair in general and dimer excision in particular remains to be determined. This fraction is also being purified.

4) Dimer excision. Specific dimer excision has been investigated by determining the ratio of  $^3\text{H}$ -labeled thymine dimer to monomer in hydrolyzed samples of irradiated DNA. The results of experiments with unincubated DNA and DNA incubated with crude extracts of *E. coli* do not show any evidence of specific dimer excision. A large number of variables are currently being tested in order to evaluate the significance of this observation.

Studies carried out to date convincingly demonstrate the preferential degradation of UV-irradiated DNA by enzymes present in crude extracts of *E. coli*. Some of these enzymes are being purified.

A number of fundamental questions remain to be answered. Firstly, are these enzymes absolutely UV specific or do they simply degrade irradiated DNA more rapidly than unirradiated DNA? Secondly, does the inability to demonstrate specific dimer excision *in vitro* represent a technical limitation or does it have biological significance? These questions are being probed by further experimentation.

3. Mammalian tissue, organ, and whole organism studies. (Mrs. Davis, MAJ Del Favero, CPT Donati, Mrs. Hill, Dr. Jervis, LTC Johnson, COL Sprinz, LTC Stromberg).

a. Human cytogenetics. A group of 18-22 year old men who were expected to be exposed to a low level, low energy, non-ionizing electromagnetic radiation source were selected for somatic chromosome analyses. Unexposed controls were included in the group and analyses

were performed blindly. Heparinized blood samples were taken approximately 8 weeks apart before and after the exposure. Successful lymphocyte cultures were prepared from the blood of 18 of 20 individuals available for the first bleeding and from 16 of 16 individuals available for the second bleeding. Cultures were treated with colcemid and chromosome spreads were made. Dosimetry performed during the exposure proved the radiation dose to be negligible. Therefore, the results actually represent two somatic chromosome analyses performed on the same group of "normal" individuals.

No significant structural alterations of the chromosomes were found at either bleeding. There was no significant difference in the aneuploidy rate between the "exposed" and the control individuals at each bleeding (Chi square  $p = .05$ ). Not unexpectedly, there was a significant difference between the total aneuploidy rate for the first bleeding (16.3%) and that for the second bleeding (7.4%). This indicates that changes in the environment of all the individuals being studied, and/or unrecognized differences in the laboratory procedures used for preparing the chromosomes, contributed significantly to the aneuploidy rate. Such a "preparative aneuploidy" is a laboratory artifact found by all cytogenetic workers and almost certainly accounts for most of the difference in aneuploidy encountered. This is further substantiated by a closer examination of the spreads which were examined but rejected for analysis. material prepared from the first bleeding, 11,846 spreads were examined and 282 (2.3%) were accepted for analysis. In material prepared from the second bleeding, 2,983 spreads were examined and 321 (10.7%) were accepted for analysis. Rejected spreads were placed in one of five categories depending upon the reason for rejection. Distribution of rejected spreads among these categories was not essentially different in material from either bleeding.

It is felt that standardized methods of chromosome preparation and strict criteria for spread acceptance will reduce preparative aneuploidy. Such a reduction is desirable, but in its absence, the present study allows valid conclusions concerning aneuploidy rates to be drawn if control material is processed concurrently with the experimental preparations.

b. Transplantation in treatment of combined injury. A delay in wound contracture, an index of wound healing, follows whole body X-irradiation. This defect has been attributed to altered fibroblastic function. The degree of alteration in wound contracture depends on the relationship between the time of wounding and the time of irradiation, being most pronounced when wounding follows irradiation by 4 days. Partial bone marrow shielding during X-irradiation improves the wound healing pattern. This suggested that fibroblastic function and resultant wound repair may depend upon a competent bone marrow either as a direct source of fibroblastic precursors or for indirect support. Inbred rats were exposed to 675R total body X-ray, then immediately transfused with pooled donor bone marrow cells. The rats were wounded

4 days after radiation and the wound contracture curve demonstrated a partial correction toward the curve of the non-irradiated controls. Other non-irradiated and irradiated rats were transfused with bone marrow obtained from rats pretreated with rabbit anti-rat lymphocyte sera. In the irradiated animals the wound healing pattern did not revert toward the normal pattern. These data indicate that altered wound healing which follows X-irradiation is, in part, secondary to altered marrow function, and suggest that the marrow lymphocyte is necessary for wound repair.

c. Acute intestinal radiation syndrome. It has been repeatedly demonstrated in this and other laboratories that germfree animals are more resistant to irradiation with X-rays than their conventionally raised counterparts. This was recently confirmed for mice exposed to a wide range (energies) of neutrons in the mixed neutron-gamma field at the Walter Reed Research Reactor (WRRR). Germfree mice survived longer at every dose of radiation tested. The differences in survival time are greatest for doses that produce deaths resulting from damage to the hematopoietic system (where infection plays a prominent role). There are also clear-cut differences in survival times after higher doses that cause extensive damage to the gastrointestinal tract, where death is primarily the result of fluid and electrolyte losses due to diarrhea.

To determine if the longer survival of germfree mice at these doses is due to different morphologic responses of the small gut, the development of mucosal lesions was studied sequentially in germfree and conventional mice irradiated by neutrons. These experiments were conducted in collaboration with the Department of Experimental Pathology. Five month old female ICR mice, both germfree and conventional, were exposed for ten minutes in the WRRR (operated at 1 KW; dose rate 100 rads/min) receiving approximately 1000 rads (gold foil dosimetry). Five mice from each group were sacrificed two, eight and twelve hours after exposure and daily thereafter. All conventional mice developed diarrhea before the end of the fourth post-irradiation day and none survived beyond the fifth day. At this time diarrhea was observed in only a few germfree mice, but by the sixth day was noted in all of them. Deaths occurred on the sixth and seventh post-irradiation days. Germfree mice lived one to three days longer than conventional mice.

Microscopically, the initial damage in the crypts was qualitatively similar in both groups, although as early as two hours after exposure more debris and cellular disruption were seen in conventional mice. The same was true at eight hours. By twenty-four hours the difference between the two groups was more pronounced; the debris had disappeared from the crypts which by now were lined with abnormal cells in both groups. However, in the conventional these abnormal cells had already emerged from the crypts. Further examination of material obtained throughout the study demonstrated that evolution of

the mucosal lesion in the germfree mouse follows the pattern of its conventional counterpart, but more slowly. In the ileum of conventional animals, abnormal, postirradiation-formed cells reached the villus tips on the fourth day while in the germfree it took from one to two days longer. (The appearance of abnormal cells on the villus crest coincides with the onset of diarrhea). At the time of death there were no differences in the histopathologic findings of the small gut mucosa of germfree and conventional mice.

A close relationship has been established between development of acute radiation lesions and mucosal cellular dynamics. It is also known, from the work of Abrams and collaborators, that there is a lower rate of cell proliferation in the crypts of germfree mice than in those of conventional mice and, moreover, that in germfree animals the migration time is about two days longer. Since tissue sensitivity to radiation is related to mitotic activity, the initial difference in the extent of crypt damage is largely attributable to the slower cellular proliferation in the germfree small gut.

It is also suggested that the slower rate of development of the mucosal lesion is responsible for the difference in the time of onset of diarrhea and ultimately for the difference in survival time.

It is concluded that the absence of an intestinal flora in the germfree animal does not alter the sequence of pathologic changes in the intestinal mucosa, but only slows it down.

4. Radiation dosimetry. (Mr. Bass, MAJ Pitchford, Mr. Crofford, Dr. Copeland, LTC Swartz, LTC Mahin).

a. Reactor dosimetry. Several systems have been evaluated and implemented for assessing the radiation levels in the reactor and the gamma and X irradiators used by the WRAIR. Thermoluminescent materials, ion chambers, threshold foils, semiconductor diodes and chemical dosimeters are devices that are used routinely for radiation dosimetry.

A gamma-gamma coincidence counting system has been designed and partially implemented. This system will be used for assessing positron-emitting radionuclides. This system will also be used to extend the threshold-foil counting capability of the division. These foils will be used in measuring the neutron spectral distribution for the WRAIR reactor and the Diamond Ordnance Reactor Facility.

b. Thermoluminescent dosimetry. Research has been conducted to determine the thermoluminescent properties of lithium fluoride (LiF), the routinely used thermoluminescent dosimetry material, irradiated at cryogenic temperatures. These investigations have shown that the thermoluminescent response of LiF irradiated at  $-196^{\circ}\text{C}$  is reduced to approximately 40 percent of its response at  $27^{\circ}\text{C}$ . The response of LiF irradiated at  $-79^{\circ}\text{C}$  is approximately 85 percent of its response

at 27°C. In these investigations the same samples of LiF were tested for free radical concentration using the electron spin resonance (ESR) spectrometer. The ESR studies indicated a free radical concentration at -196°C that was approximately 12 percent lower than the concentration at 27°C. The concentration at -79°C was approximately 8 percent lower than the concentration at 27°C.

Investigations have been made to determine the feasibility of a throw-away dosimeter. Common NaCl, ground to a 80-120 mesh grain size, has demonstrated thermoluminescent properties that look attractive for this application. Linearity of response and precision have been adequate for routine dosimetry. Equipment procedures and sample preparation and handling are being refined to establish a basis for using this very inexpensive material as a disposable, effective dosimeter.

c. In vivo detectors. Microminiature G-M tubes have been constructed and tested for chronic implantation in the cardiovascular system of primates. These detectors will be used in conjunction with radionuclides for studying the peripheral blood flow of the heart. In addition, lithium-drifted silicon detectors have been developed and are being tested for similar applications. The G-M and silicon detectors are approximately five millimeters in diameter and three millimeters thick.

d. ESR dosimetry studies.

1) Progress on the use of ESR for in vivo dosimetry was reported at the semi-annual DASA Medical Coordination Conference, Albuquerque, New Mexico, May 1969. Previous studies (WRAIR Annual Reports 1966-67; 1967-68) indicated the feasibility of using radiation induced, long-lived tissue ESR center concentration as a measure of gamma-radiation absorbed dose. These ESR centers may or may not be free radicals. Two of the tissues in which such ESR centers are induced (bone, teeth) are not likely to be available after a human accidental radiation exposure. In previous investigations, the third candidate tissue (fingernail) was not extensively investigated. After high doses of gamma radiation in vitro, human fingernail gives an ESR spectrum very similar to that obtained from irradiated cystine, thus reflecting the high concentration of this amino acid in fingernail. Current investigations involve the use of computer averaging of ESR signals to cope with the considerable noise encountered when fingernail is gamma irradiated in vitro with low doses.

2) A comparison was made between ESR yield of F-center concentration and thermoluminescence yield on the same LiF samples irradiated at low temperatures. A linear relation was found between these two indicators of the number of electrons trapped in excited states in LiF. Approximately the same relation was found at irradiation temperatures of 77°K, 196°K and 298°K. These studies were done as a precursor to investigations of gamma-radiation dose as a function of

depth in frozen mammalian specimens. Lithium fluoride dosimeters will be implanted in rats, the animals will be frozen, and the LiF thermoluminescence dose will be compared with the ESR determined dose in adjacent tissues after gamma radiation at 77°K.

3) Work has been carried out in collaboration with Dr. Stenn of the AFIP using ESR to determine specimen dose during electron microscopic observations. Gamma-irradiated Teflon gives a characteristic ESR signal, the magnitude of which is linearly related to dose up to about  $10^6$  rads. Teflon cylinders equal in diameter to electron microscope specimens were electron bombarded in the AFIP electron microscope and the dose estimated by ESR. The intensity of the ESR signals was approximately linearly related to the time duration of exposure. It has not yet been possible to measure absolute exposure doses by this technique.

#### 5. Human whole body radiation detection. (MAJ Gardner).

a. Health physics. Routine monitoring of reactor personnel and other occupationally exposed individuals for possible gamma emitters has been continued from previous years. No established procedures for routine monitoring are available, but a standardized approach is under study.

A questionable exposure due to an exhaust fan malfunction and a legal case requiring extensive isotope identification and measurement were investigated with the Health Physics Branch, WRAMC.

b. Ward 30. Total-body potassium estimates on acromegalics and other endocrine-metabolic patients have continued. A new protocol involving experimentally induced potassium fluctuations is also underway with the counter used as an independent check on the balance studies. The constant search for more precise and accurate estimates occupies much of the counter's time.

c. Nuclear Medicine Service, WRGH (Isotope Clinic). A close working relationship has been established with the Nuclear Medicine Service, WRGH, since they are the principal users of isotopes in human subjects. The use of the counter for kinetic and distribution studies in vivo has begun with patients receiving  $^{85}\text{Sr}$  for bone Scintiscans. A screening test using the counter to pick out high activity areas for the more detailed and time consuming Scintiscans has been put into active use. Kinetic studies on several other isotopes are planned for the future. The HUMCO is also used to count other radioactivity in large volume samples obtained from patients in the clinic.

#### Summary and Conclusions.

The adaptability of ESR techniques to problems in applied and basic biology is well established. Improvements and refinements of methods for interpretation of biological and ESR data were developed, and the

validity of results using wet frozen tissue samples was evaluated. Practical application of ESR to determination of organ transplantability is of continuing interest; however, no reliable technique has been found. Examination of the fundamental processes in ionizing radiation and other physically-induced injury has led to the development of a "unifying hypothesis" about the role of oxygen in deleterious cell processes.

The nature of the initial physiochemical lesions in irradiated microorganisms and their responses to the lesions are being studied in considerable detail. Analysis of DNA damage in gamma-irradiated bacteria and viruses without and with protective compounds provided strong inferential evidence that single and double-strand breaks in DNA are not principal lethal lesions in unprotected organisms. Two newly identified potential radiation lesions were reported. In future studies, the roles of non-genomic cellular components in ionizing radiation injury should be emphasized. A new comprehensive study of enzymatic repair systems was initiated and preliminary studies have demonstrated the presence of potential repair enzyme activity in crude extracts of radioresistant bacteria.

Negative cytogenetic findings are reported from studies of humans exposed to very low doses of low energy electromagnetic radiation. The effect of bone marrow transplant on wound contracture patterns in X-irradiated rodents was examined. The results show that beneficial effects of marrow transplant on wound repair following X-irradiation are attributable to the marrow lymphocyte component.

A comparison of the small bowel histology in neutron-irradiated germfree and conventional mice indicates that the absence of an intestinal flora in the germfree animals does not alter the sequence of pathological changes in the intestinal mucosa, but only slows it down.

Reliable radiation dosimetry is a constant problem in radiobiological experiments. In collaboration with the biological investigators, the physical research staff has continued to develop and evaluate new methods of dosimetry. A promising new approach to in vivo dosimetry using ESR spectrometry is being exploited. Correlations between physical dosimeters and biological indicators of radiation absorbed dose are being developed as a means of evaluating dosimeter reliability.

Clinical support services of the Walter Reed Army Institute of Research Whole Body Counter were expanded and routine procedures are performed for the Walter Reed General Hospital Metabolic and Nuclear Medical Services.

Project 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 09, Radiobiology

Work Unit 015, Mechanisms

Publications.

1. Donati, R. M., Berman, A. R., Jervis, H. R., Stromberg, LW. R. and Sprinz, H. "Absorption of Iron Following X-Irradiation of the Exteriorized Small Intestine." Proc. Soc. Exptl. Biol. Med. 130:822, 1969.

2. Donati, R. M., McLaughlin, M. M., Levri, E. A., Berman, A. R. and Stromberg, LW. R. "The Response of Iron Metabolism to the Microbial Flora: Studies on Germfree Mice." Proc. Soc. Exptl. Biol. Med. 130(2):920, 1969.

3. Einheber, A., Wren, R. E. and Dobek, A. S. "Mortality, Morphologic Changes, and Saline Therapy After Scald Injury of Germ-free Mice and Pseudomonas-free Conventionalized Mice, With or Without Proteus mirabilis: An Inquiry into a Possible Non-Infective Role of the Microbial Flora." J. of Trauma (In press).

4. Ginsberg, D. M. and Webster, H. K. "Chemical Protection Against Single-Strand Breaks in DNA of Gamma-Irradiated E. coli." Radiation Res. (In press).

5. Hightower, D., Woodward, K. T., McLaughlin, M. M. and Hahn, F. F. "The Effect of Age, Strain and Exposure Intensity on the Mortality Response of Neutron Irradiated Mice." Radiation Res. 35:369, 1968.

6. Jervis, H. R., Donati, R. M., Stromberg, LW. R. and Sprinz, H. "Histochemical Investigation of the Mucosa of the Exteriorized Small Intestine of the Rat Exposed to X-Radiation." Strahlentherapie 137:326, 1969.

7. Johnson, M.C., Stromberg, LW. R., Del Favero, J. E. and Donati, R. M. "The Effect of Bone Marrow Transplant on Wound Contracture Patterns in the X-Irradiated Rodent." Radiation Res. Abstract (In press).

8. Krebs, A. T. and McLaughlin, M. M. "Behavior Changes in Gamma-Irradiated Ants." Radiation Res. 35:575, 1968.

9. McLaughlin, M. M., Woodward, K. T., Krebs, A. T. and Stromberg, LW. R. "Effects of the Germfree State on the Response of Mice to Mixed Neutron-Gamma Radiation." Radiation Res. 35:559, 1968.

Publications (Continued)

10. Stromberg, LW. R., McLaughlin, M. M. and Donati, R. M.  
"Combined Injury III. The Effect of Pre-Irradiation Radioprotective  
Drug Treatment." Proc. Soc. Exptl. Biol. Med. 129(1):140, 1968.

11. Swarcz, H. M. "Effect of Oxygen on Freezing Damage in E.  
coli." Ph.D. Thesis. The Georgetown University (1969).

12. Woodward, K. T., Berman, A. R., Michaelson, S. M. and  
Odland, L. T. "Plasma, Erythrocyte and Whole Blood Volume in the  
Normal Beagle." Am. J. Vet. Res. 29:1935, 1968.

**PROJECT 3A062110A806  
MILITARY PREVENTIVE MEDICINE**

**Task 00  
Military Preventive Medicine**

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY*	REPORT CONTROL DATA	
3. DATE FIRST SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SECY*	6. WORK SECURITY*	DA OA6457	69 07 01	DD-Form (AR) 501	
69 01 31	D. Change	U	U	7. IMPROVING*	8. DISSEM. SYSTEM	9. SPECIFIC DATA CONTRACTOR ACCESS	10. LEVEL OF CONTROL
11. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
12. NUMBER	62110A	3A062110A806	00				WORK UNIT NUMBER
13. CONTINUING							030
14. COMMENTS	1410B(2) (A)						

(U) Global Health Data (09)

15. SCIENTIFIC AND TECHNOLOGICAL AREA			
005100 Documentation + I, 007800 Hygiene and Sanit, 007000 Geography			
16. START DATE	17. ESTIMATED COMPLETION DATE	18. FUNDING AGENCY	
07 63	NA	DA	
19. PERFORMANCE METHOD		20. PERFORMANCE METHOD	
C. In-House		C. In-House	
21. EFFECTIVITY	22. RESOURCES ESTIMATE	23. PROFESSIONAL MAN YRS	24. FUNDS (in thousands)
25. DATES EFFECTIVE	26. FISCAL YEAR	27. FISCAL YEAR	28. FISCAL YEAR
HA	69	1	35
29. TYPE	30. AMOUNT	31. AMOUNT	32. AMOUNT
		1	35
33. KIND OF AWARD	34. CUM. AMT.	35. PERFORMING ORGANIZATION	
		NAME: Walter Reed Army Institute of Research Division of Preventive Medicine ADDRESS: Washington, DC 20012	
36. RESPONSIBLE INDIVIDUAL		37. PRINCIPAL INVESTIGATOR (FUNDING AGENCY USE ONLY - Do not fill in)	
NAME: Maroney, COL W. H. TELEPHONE: 202-576-3551		NAME: Vivona, COL S. TELEPHONE: 202-576-3553 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]	
38. GENERAL USE		39. ASSOCIATE INVESTIGATORS	
Foreign Intelligence Considered		NAME: Fred, A. C.	

PII Redacted

(U) Infectious Diseases; (U) Diseases of Animals; (U) Diseases of Nutrition and Metabolism; (U) Pub Hlth; (U) Epidemiology; (U) San Engrng; (U) Climate; (U) Geography

23. (U) Health data reports are prepared for the use of Army Medical Department officers and contain unclassified information regarding the health and sanitary conditions likely to be encountered in foreign countries to which they are deployed.
24. (U) Health data reports are unclassified reports of health and sanitary conditions in foreign countries for the use of Army Medical Department officers. They describe the geography, climate, religion, living conditions, animals and plants of medical importance, water supply, methods of waste and sewage disposal, diseases present, medical facilities, etc., of each country reported on.
25. (U) 69 01 - 69 06. Israel and Egypt, completely rewritten, and Libya, new, have been published. Jordan is in the final updating stage. Syria, Upper Volta, Uganda, North Korea, Cambodia and Ethiopia will need only minor changes before publication. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.

**Project 3A062110A806 MILITARY PREVENTIVE MEDICINE**

**Task 00 Military Preventive Medicine**

**Work Unit 030 Global Health Data**

**Investigators:**

**Principal, COL Stefano Vivona, MC  
Associate, Ann C. Fred, M.D.**

**Description.**

Health Data Reports are prepared for the use of Army Medical Service Officers and contain unclassified information regarding the health and sanitary conditions likely to be encountered in foreign countries to which they are deployed. They describe the geography, climate, religion, animals and plants of medical importance, water supplies, methods of waste and sewage disposal, diseases present, medical facilities, etc. of each country reported on.

**Progress.**

At the end of FY 1969, Health Data Reports had been completed and published on 44 countries. Updating, with complete revision of text and maps, was accomplished on Tanzania, Israel, Lebanon and Egypt. Libya was the most recent publication of a country not previously studied. Reports on North Korea, Cambodia, Ethiopia, Syria and Jordan are well on the way to completion. Afghanistan, Upper Volta, and Uganda will be the next entirely new countries offered for publication.

**PROJECT 3A062110A811  
MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA**

**Task 00  
Tropical and Subtropical Military Medical Research**

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ABBREVIATION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
				DA OB 6435	69 06 30	DD-FORM (ANS) 436	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY CTRY	6. WORK CATEGORY	7. ABBREVIATION	8. COUNTRY ORIGIN	9. SPECIFIC DATA CONTRACTOR AGENCY	10. LEVEL OF R&T
68 10 31	H. TERM	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	62110A	3A062110A811	00	301			
12. CONTINUING							
13. WORK UNIT NUMBER							
14. TITLE (Provide full primary classification code)							
(U) Military Medical Research Program, SEASIA (TH)							
15. SCIENTIFIC AND TECHNOLOGICAL AREA							
003500 Clinical Medicine							
16. START DATE	17. ESTIMATED DURATION DATE	18. FUNDING SOURCE		19. PERFORMANCE STATUS			
63 08	CONT	DA		C. IN-HOUSE			
20. CONTRACT/GRANT				21. RESOURCES ESTIMATE		22. PERSONNEL EST. YES	
23. DATE/EFFECTIVE				24. FISCAL YEAR		25. PERSONNEL EST. YES	
NA				68		6	
26. NUMBER				69		250	
27. TYPE				69		250	
28. KIND OF AWARD				69		250	
29. RESPONSIBLE ORG ORGANIZATION				30. PERSONNEL ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: USA Med Comp, SEATO Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide name of U.S. Analyst including name)			
NAME: Meroney, COL W. H.				NAME: Smith, LTC T. R.			
TELEPHONE: 202-576-3551				TELEPHONE: NA			
31. GENERAL USE				32. SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Considered				33. ASSOCIATE INVESTIGATORS			
				NAME: DA			
34. REVISIONS (Provide date and primary classification code)							
(U) Metabolism; (U) Disease							
35. TECHNICAL OBJECTIVE, 36. APPROACH, 37. PURPOSE (Provide detailed paragraphs classified to extent provided rest of each cell primary classification code)							
23 (U) To conduct the studies required to improve medical capabilities to support limited war ground combat in Southeast Asia.							
24 (U) A metabolic unit which will permit sophisticated medical procedures to be conducted on patients will be used.							
25 (U) 68 10 - 69 04 Project has been re-evaluated and a decision has been made to terminate it. Negotiations are underway with the Thai Medical Component, SEATO, and the Thai Government whereby this will be accomplished. For technical report, see Annual Progress Report, USA Med Comp., SEATO, Bangkok, Thailand.							

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACRONYM	2. DATE OF SUMMARY	REPORT NUMBER PREFIX	
				DA OA 6458	59 06 30	DD-RMB (AR) 634	
3. DATE PREPARED	4. NAME OF LABORATORY	5. BUDGETARY AGENCY	6. WORK ELEMENT	7. ORGANIZATION	8. DDD FORM NUMBER	9. SPECIFIC DATA - CONTRACTOR AGENCY	10. LEVEL OF WORK UNIT
68 10 31	H. STEEL	II	II	NA	HL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. PROGRAM ELEMENT	12. PROJECT NUMBER	13. TASK AREA NUMBER		14. WORK UNIT NUMBER			
62110A	3A06211.0A811	00		302			
15. WORK UNIT TITLE (DD FORM 1412A(2))							
(U) Military Medical Research Program, SEASIA (TH)							
16. SUBJECTS AND TERMS (DD FORM 1412A(2))							
010100 Microbiology							
17. REPORT DATE		18. REPORTS AND DOCUMENTS DATE		19. FUNDING AGENCY		20. PERFORMANCE STATUS	
63 08		CONT		DA		C. IN-HOUSE	
21. DISTRIBUTION STATEMENT				22. RESOURCE ESTIMATE		23. PERSONNEL MAN YRS	
a. DEVELOPMENTAL NA				FISCAL YEAR		b. FUNDING (in thousands)	
c. SUPPORT				68		21	
d. TEST				69		750	
e. EVALUATION				69		21	
24. PERFORMING ORGANIZATION NAME				25. PERFORMING ORGANIZATION NAME			
Walter Reed Army Institute of Research				Walter Reed Army Institute of Research			
ADDRESS				ADDRESS			
Washington, DC 20012				USA Med Comp, SEATO Washington, DC 20012			
26. RESPONSIBLE PERSONAL				27. PERSONAL INVESTIGATOR (DD FORM 1412A(2))			
NAME: Meroney, COL W. H.				NAME: Sl h, LTC T. R.			
TELEPHONE: 202-576-3551				TELEPHONE: NA			
28. GENERAL USE				29. SOCIAL SECURITY AGENCY NUMBER			
Foreign Intelligence Not Considered				DA			
30. SUMMARY OF WORK (DD FORM 1412A(2))							
(U) Virus Diseases; (U) Diarrheas; (U) Parasitic Diseases; (U) FUD							
31. Technical objective, or approach, or procedure (DD FORM 1412A(2))							
23 (U) To conduct the studies required to improve medical capabilities to support limited war ground combat in Southeast Asia.							
24 (U) A balanced laboratory staff is maintained in Bangkok, augmented by TDY personnel as required.							
25 (U) Detailed information on the research performed during the period under this work unit is being assembled for publication in Annual Progress Report, USA Med Comp, SEATO, which is not yet available. This work unit is terminated effective 69 06 30 and will be replaced by a series of new work units which will represent specific areas of research within the program of the laboratory.							

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				A. ARMY ASSIGNMENT	B. DATE OF REPORT	REPORT SECURITY CLASS.	
				DA OB 6442	69 06 30	REF ID: A649 002	
A. DATE FROM REPORT	B. NAME OF SUBJECT	C. SECURITY CLASS.	D. WORK STATUS	E. ORGANIZATION	F. REPORT NUMBER	G. REPORT TYPE	H. LEVEL OF EFFORT
68 10 31	H. TERM.	U	U	NA	HL	Y	A. USE ONLY
I. NO./CODES	J. PROGRAM ELEMENT	K. PROJECT NUMBER	L. TASK AREA NUMBER	M. WORK UNIT NUMBER			
A. PRIMARY	62110A	3A062110A811	00	303			
B. SUBVENTING							
C. REPORTING	CP06 1412A(2)						
N. TITLE (include unit security classification code)							
(U) Military Medical Research Program, SEASIA (Malaysia); (MF)							
O. OBJECTIVE AND TECHNOLOGICAL AREA							
010100 Microbiology		003500 Clinical Medicine					
P. ENTRY DATE		Q. REPORTING AND LITERATURE DATE		R. FUNDING SOURCE		S. PERFORMANCE STATUS	
63 08		CONT		DA		C. IN-HOUSE	
T. ORGANIZATION/UNIT				U. PERSONNEL ESTIMATE		V. PERSONNEL USE YTD	
A. DATE/OBJECTIVE				FISCAL YEAR		B. FUNDING CLASSIFICATION	
NA				68		1	
B. OBJECTIVE				69		0.2	
C. TYPE						1	
D. NAME OF AGENCY							
E. ORG. ABBV.							
Walter Reed Army Institute of Research				Walter Reed Army Institute of Research			
Washington, D.C. 20012				Washington, D.C. 20012			
PERSONNEL INFORMATION				PERSONNEL INFORMATION (include U.S. AND/OR FOREIGN NATIONALS)			
NAME: Meroney, COL W.H.				NAME: Raymond, COL G.P.			
TELEPHONE: 202-576-3551				TELEPHONE: NA			
OFFICIAL SECURITY AGENCY NUMBER				OFFICIAL SECURITY AGENCY NUMBER			
Foreign Intelligence Not Considered				Frick, COL L.P.			
				DA			
W. SUMMARY (include unit security classification code)							
(U) Malaria; (U) Chloroquine Refractory Malaria; (U) Survey; (U) WHO Test							
X. SUMMARY (include unit security classification code)							
23 (U) To evaluate the extent of malaria and of drug-refractory falciparum malaria in Malaysia.							
24 (U) Standard survey techniques and standard WHO drug test program will be used in cooperation with Malaysian workers.							
25 (U) 68 07 - 68 10. Through cooperation of Malaysian Government, arrangements have been made whereby surveillance for malaria can be made in a new federal resettlement area. Blood smear examinations show incidence of malaria in new settlers to be very low. Anopheline catches in human and animal biting stations are poor and all captured during the period were negative for plasmodia. Effective 31 Oct 68 project was discontinued as a separate effort. Its objectives have been transferred to the major project funded from 3A062110A811 and will be reported under DA OA 7413. For technical report, see Walter Reed Army Institute of Research, Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM; SOUTH EAST ASIA

Task 00, Military Medical Research Program, South East Asia

Work Unit 303, Military Medical Research Program, South East Asia,  
(Malaysia)

**Investigators.**

Principal: COL Garrison Rapmund, MC

**Description.**

The objective of this work unit was to evaluate the extent and foci of chloroquine-refractory falciparum malaria in Malaysia. Conventional entomological and parasitological procedures were used, including the WHO standard test for chloroquine resistance.

**Progress and Results.**

Due to the incorporation of this work into the major project conducted by USAMRU-Malaysia, only the period 1 July - 30 September 1968 is reported on here. Surveillance for malaria in a federal resettlement project was continued. Blood smears made on all incoming settlers show incidence of malaria in these persons to be very low. Anopheline catches in human and animal biting stations are also continuing to be low. Anopheles constituted only 0.1% of all mosquitoes captured. All anophelines, including A. maculatus, the principal vector in Malaysia, were negative for plasmodia by salivary gland dissection.

**Recommendations.**

Although the work conducted under this project should be continued, for reasons of administrative simplification, it was desirable to incorporate it into the larger project conducted by USAMRU-Malaysia which is reported under DA OA 7413.

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM; SOUTH EAST ASIA

Task 00, Military Medical Research Program, South East Asia

Work Unit 303, Military Medical Research Program, South East Asia,  
(Malaysia)

Publications.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	3. REPORT CONTROL SYMBOL	
				DA OA 6459	69 07 01	DD-RLE (AR) 636	
4. DATE PREV. SUMMARY	5. KIND OF SUMMARY	6. SUMMARY ACTY <sup>c</sup>	7. WORK SECURITY <sup>d</sup>	8. REGRADING <sup>e</sup>	9. DDD'S INITIALS	10. SPECIFIC DATA - CONTRACTOR ACCESS	11. LEVEL OF DUE
68 10 31	D. CHANGE	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
12. NO. JOBS <sup>f</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	62110A	3A062110A811	00	304			
13. TITLE (Provide with Security Classification Only)							
(U) Military Medical Research Program, SEASIA (VS)							
14. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>g</sup>							
010100 Microbiology 003500 Clinical Medicine							
15. START DATE		16. ESTIMATED COMPLETION DATE		17. FUNDING AGENCY		18. PERFORMANCE METHOD	
63 08		CONT		DA		C. IN-HOUSE	
19. CONTRACT/GRANT				20. RESOURCES ESTIMATE		21. PROFESSIONAL MAN YRS	
a. DATE EFFECTIVE NA				PREVIOUS			
b. NUMBER <sup>h</sup>				FISCAL YEAR		i. FUNDS (in thousands)	
c. TYPE				69		3	
d. END OF AWARD				70		4	
22. RESPONSIBLE DSO ORGANIZATION				23. PERFORMING ORGANIZATION			
NAME <sup>i</sup> Walter Reed Army Institute of Research				NAME <sup>j</sup> Walter Reed Army Institute of Research ✓			
ADDRESS <sup>k</sup> Washington, DC 20012				ADDRESS <sup>l</sup> Med Rsch Team - Vietnam Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic institution)			
NAME: Meroney, COL W. H.				NAME: Cutting, LTC Robert T.			
TELEPHONE: 202-576-3551				TELEPHONE: NA			
24. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME: DA			
25. SUBJECT (Provide with Security Classification Only)							
(U) Plague; (U) Malaria; (U) Malaria Therapy							
26. TECHNICAL OBJECTIVE <sup>m</sup> , 26. APPROACH, 26. PROGRESS (Provide individual paragraphs identified by number. Provide rest of each with Security Classification Only.)							
23 (U) To conduct the studies required to improve medical capabilities to support limited war ground combat in Southeast Asia.							
24 (U) A small staff is maintained in Saigon augmented by TDY personnel. Field units are established as required. Many studies are done in cooperation with the Pasteur Institute of Saigon.							
25 (U) 68 10 - Studies during the past year have confirmed the high incidence of <i>Wuchereria bancrofti</i> infections in indigenes resident in the Song Be area only; the high prevalence of antibiotic resistant gram negative rods associated with febrile diarrhea; search for chloroquine resistant <i>P. vivax</i> malaria has not identified such organism infecting man; Plague was studied in Na Trang to continue evaluation of flea control procedures. For technical report, see Annual Progress Report, Med Rsch Team (WRATR), Vietnam.							

<sup>a</sup> Available to contractors upon contractor's approval.

DD FORM 1498-1 (FOR ARMY USE)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACRONYM	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
				DA OA6462	69 07 01	DD-DSR (AF) 635	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. FUNDING AGENCY	6. WORK SECURITY	7. REGARDING	8. DISC'D INSTN	9. SPECIFIC DATA CONTRACTOR ACCESS	10. LEVEL OF SUMMARY
31 01 69	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./COLS.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62110A	3A062110A811	00	305			
b. CONTINUING							
c. CONTINUING	1412A(2)						
11. TITLE (Precede with Security Classification Code)							
(U) Military Medical Research Program, SEASIA (WRAIR) (Zoonoses) (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS							
010100 Microbiology							
13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD			
08 63	NA	DA		C. In-House			
17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		20. FUNDS (in thousands)	
NA		PRECEDING					
4. DATES/EFFECTIVE:	EXPIRATION:	FISCAL YEAR	CURRENT				
		69		9		300	
5. NUMBER:	6. AMOUNT:	70		7		350	
7. KIND OF AWARD:	8. CUM. AMT.	19. RESPONSIBLE DOD ORGANIZATION		20. PERFORMING ORGANIZATION			
		NAME: Walter Reed Army Institute of Research		NAME: Walter Reed Army Institute of Research			
		ADDRESS: Washington, D. C. 20012		ADDRESS: Division of Veterinary Medicine Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL		NAME:		PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
Meroney, COL, W. H.		Meroney, COL, W. H.		NAME: Asbill, COL, S. G.			
NAME:		TELEPHONE:		TELEPHONE: 202-576-5193			
202-576-3551		202-576-3551		SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
TELEPHONE:		ASSOCIATE INVESTIGATORS		NAME: Alexander, Ph.D., A. D.			
202-576-3551		NAME:		NAME:			
202-576-3551		NAME:		DA			
202-576-3551		NAME:		DA			
21. GENERAL USE							
Foreign Intelligence Considered							
22. KEYWORDS (Precede with Security Classification Code)							
(U) Zoonoses; (U) Leptospirosis; (U) Melioidosis; (U) Epidemiology; (U) Genetics; (U) Serology							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To define zoonoses that have potential military significance, to determine prevalence, sources and modes of infection, and to devise measures for diagnosis, prevention and control. Studies are coordinated with field units, emphasizing leptospirosis and melioidosis and include identification and studies of isolates obtained in epidemiological investigations, chemo and vaccine prophylaxis for leptospirosis, development of laboratory diagnostic technics for melioidosis, surveillance of these diseases in S. E. Asia.							
24. (U) Conventional cultural and serological technics are used in epidemiological studies. Genetic tool utilized for study of antibiotic resistance in melioidosis. Hamsters and dogs are used to evaluate vaccines and drugs. Major problems -- maintenance of strain virulence for experimental infections, wet-tail in hamsters, and lack of safe animal facilities for melioidosis studies.							
25. (U) 69 01 - 69 07 Sulfamylon was bacteriostatic but not bacteriocidal for pseudomonas pseudomallei at concentration of 5 mg/ml. Lower levels were not bacteriostatic. The high specificity of the HA test for melioidosis was affirmed in extensive tests for heterogeneric reactions. The CF test for melioidosis elicited reaction with different bacterial antisera, notably P. aeruginosa. Negative HA reactions in known melioidosis patients with CF antibodies could not be related to specificity of HA antigen. Observations of intraspecies growth inhibition in P. pseudomallei were extended to P. aeruginosa strains. Small molecular weight (dialysable) inhibitor was also found in P. aeruginosa cultures. The dialysable inhibitor was only active against P. pseudomallei strains but not against themselves. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 1968 - 30 June 1969.							

PII Redacted

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00 Tropical and Subtropical Military Medical Research

Work Unit 305, Military Medical Research Program, SEA, WRAIR -  
Zoonoses

**Investigators.**

Principal: COL S. G. Asbill, VC

Associate: A. D. Alexander, Ph.D.; M. Rogul, Ph.D.; L. B. Evans,  
B.S.; S. Schwarting, M.S.; V. M. Shepler, M.S.;  
A. Warner, Jr.

**Description.**

Major objectives are to evaluate real or potential military significance of selected zoonoses, to characterize etiologic agents, to define epidemiological factors and to establish methods of diagnosis, treatment, and control.

**Progress.**

1. **Leptospirosis.**

a. **Antileptospiral Drug Screening.** Drug testing for anti-leptospiral activity using a previously developed hamster-infectivity system (Annual Report 1966-1967) was limited to an examination of the anti-trypanosomal component "antrycide." Two different investigators reported this compound to be a useful prophylactic agent for leptospiras. The purported findings were not affirmed with the hamster-infectivity test system. The drug was ineffective in altering a predictable course of disease when given either parenterally or per os.

b. **Leptospirosis Surveillance.** Sera from 8 leptospirosis cases diagnosed at the 9th Army Medical Laboratory with the hemolytic test were submitted for check tests using the conventional microscopic-agglutination test. All samples were positive. On the basis of cross-agglutination reactions, seven different serotype infections occurred among the selected samples. One of the patients had predominant antibodies to hyos serotypes. This type has not previously been reported in human infections in Vietnam.

Serum samples from normal swine submitted by the USAMRU in Malaysia and the 9th Army Medical Laboratory in Vietnam were tested for the presence of leptospiral agglutinins with 18 different serotype antigens. Twenty-six (8.7%) of 297 swine from Malaysia had significant antibody titers ranging from 1:100 to 1:6400. In addition, 86 swine (29%) had doubtful reactions of 1:25. Twenty-one of 26 reactors had titers against autumnalis or pomona serotypes. The prevalence of significant antibodies in 156 swine in Vietnam was 13.5%. Titers ranged as high as 1:1600. Forty-seven swine (30%) had doubtful reactions. As is the case of Malaysia swine, titers were primarily against pomona or autumnalis serotypes. The slide test (Difco macroscopic-agglutination test) findings obtained at the 9th Army Medical Laboratory were compared with results of microscopic-agglutination tests. Approximately one-third of the samples positive on the microscopic-agglutination test were non-reactive on the slide test. Twelve sera were positive on the slide test but negative with the microscopic-agglutination test. Further comparisons were made on slide test findings of the 9th Army Medical Laboratory with slide tests done at WRAIR (Difco antigens) and the Communicable Disease Center (locally prepared antigens) on a select number of positive and negative sera. Findings are summarized in Table 1.

Table 1. Comparison of Results of Slide Tests in 3 Laboratories and Microscopic-Agglutination Findings on 33 Swine Sera from Vietnam

Microscopic Agglutination Test Results	Slide Test Results									
	9th AML		WRAIR			CDC				
	Neg.	±	+	Neg.	±	+	Neg.	±	+	
Negative			10	6	1	3	10			
Partial (±)		2	12	6	3	5	13	1		
Positive (+)	7		2	7	1	1	7	2		

Non-specific reactions apparently occurred with Difco antigens. All three slide antigens had limitations in detecting reactors in serological surveys of swine.

At the request of the Navy Medical Research Institute, serological tests for leptospirosis were done on serum samples from 78 (single) and 20 (pooled) serum samples from 80 animals trapped at Con Son, Vietnam for a plague epidemiological study. Results of microscopic-agglutination tests are shown in Table 2.

Table 2. Serologic Survey for Leptospiral Agglutinins in Animals Trapped at Con Son, Vietnam

Species	No. Tested	No. Positive	Distribution of Predominant Reactions
<u>Suncus murinus</u>	1	0	
<u>Tupaia glis dissimilis</u>	15	4*	<u>icterohemorrhagiae</u> and <u>javanica</u> 1:100 (1), <u>icterohemorrhagiae</u> 1:100 (1)*, <u>patoc</u> 1:25 (1), <u>autumnalis</u> 1:25 (1)
<u>R. rattus germaini</u>	36	9*	<u>javanica</u> 1:25 (1), <u>javanica</u> 1:100 (2), pool (1)*, <u>javanica</u> 1:400 (3), <u>grippotyphosa</u> 1:25 (1), <u>patoc</u> 1:25 (1)
<u>Rhinclophus chaseni</u>	1	0	
<u>Paradoxurus hermaphroditus</u>	4	1	<u>djasiman</u> 1:25 (1)
<u>R. rattus</u>	7	3*	<u>javanica</u> 1:400 (1), <u>javanica</u> 1:100 (1)*, <u>australis</u> 1:100 (1)
<u>R. exulans</u>	2	0	
<u>Callosciurus finlaysoni germaini</u>	1	0	
Macaque	1	0	
<u>Pteropus hypomelampus condorensis</u>	2	1	<u>australis</u> 1:100 (1)
<u>R. niviventer condorensis</u>	4	0	
Jungle cock	1	0	
<u>Cynopterus brachyotis</u>	1	1*	<u>icterohemorrhagiae</u> 1:25 (1)
<u>Herpestes sp.</u>	1	1	<u>canicola</u> and <u>icterohemorrhagiae</u> 1:25 (1)
<u>Melvgale personata</u>	1	1	<u>australis</u> 1:25 (1)

\* Includes one positive pool. Pools are treated as single specimens.

Predominant antibody titers for icterohemorrhagiae, canicola, javanica, grippotyphosa and australis were found among various trapped species. Within the limitation of number of samples tested, a high percentage of Tupaia glis dissimilis and Rattus rattus were reactors. Cultural tests on 10 frozen animals were negative.

A pathogenic leptospira (strain 267-1348) isolated from surface water in Malaysia was found to be a new serotype with serological affinities to members of the canicola, javanica, autumnalis and pyrogenes groups. This isolate has potential usefulness as a vaccine strain because of its diverse antigenic relationships.

## 2. Melioidosis.

a. Serological Studies. Support of melioidosis laboratory diagnostic services of the Armed Forces Medical Laboratories in Southeast Asia and CONUS was continued. During the period of this report, this laboratory confirmed 118 cases of melioidosis in Armed Forces personnel in Vietnam by cultural or serological or both procedures. Approximately 42 of the cases were initially diagnosed at the 9th Army Medical Laboratory during the previous fiscal year. Approximately 220 cases of melioidosis have been affirmed by this laboratory since February 1965. The sensitivity and specificity of the hemagglutination (HA) and complement-fixation (CF) tests for melioidosis was evaluated further with single or serial serum samples from 185 patients. CF titers of 1:4 and greater and HA titers of 1:40 and greater were considered to be significant. The selection of these levels of significance was based on previous test findings on sera from normal individuals with no previous history of residence in Southeast Asia. Non-specific reactions at these levels in either test was less than 5 percent. Approximately one-half of the cases were proved bacteriologically by cultural recovery of Pseudomonas pseudomallei. The remaining patients were admitted in this series if significant antibody titers were demonstrable by both HA and CF tests, or if a rise in antibody titer was demonstrable in either test in appropriately timed serum samples.

HA tests were done on sera from all 185 patients. Satisfactory CF tests were obtained on all but 10 of the patients. All but 2 of the patients had significant antibody titers on one or both tests. The two seronegative patients were fatal cases and samples were obtained during the first week of disease. Of 175 patients tested by both procedures, 168 were positive by both tests; two were CF positive only and 3 HA positive only. The correlation of HA and CF results in 175 cases was 97%; in 445 sera from these cases the CF and HA findings correlated 90% (Table 3). The upper range of HA and CF titers were 1:20,480 and 1:1020, respectively. The distribution of maximum HA and CF titers in proved cases is shown in Table 4. The geometric mean titer in HA test was 1:692; in CF test it was 1:97.

Table 3. Correlation of HA and CF Results on 425 Sera from 175 Cases of Melioidosis

Tests	Reactions			
	425 Sera		175 Patients	
HA	Negative	Positive	Negative	Positive
Negative	11	17	2*	3
Positive	26	371	2	168
Correlation	382/425 = 90%		170/175 = 97%	

\* Samples obtained during first week of disease in fatal cases.

Table 4. Distribution of Maximum\*HA and CF Titers in Sera from Proved Cases of Melioidosis

Titer	HA Test		Titer	CF Test	
	No.	%		No.	%
Negative	1	0.54	Negative	3	1.71
20	1	0.54	4	1	0.57
40	13	7.02	8	3	1.71
80	16	8.65	16	24	13.71
160	18	9.73	32	20	11.43
320	22	11.89	64	24	13.71
640	26	14.05	128	31	17.71
1280	39	21.08	256	36	20.57
2560	15	8.11	510	31	17.71
5120	18	9.73	1020	2	1.14
10240	10	5.40			
20480	6	3.24			
Total	185		Total	175	
GM titer	692		GM titer	97	

\* Maximum titers observed 1 or more weeks after disease onset

The distribution of HA and CF titers by time after disease onset is shown in Tables 5 and 6. The occurrence of high titer reactions in a high percentage of sera during the first week of disease is not surprising in view of the known manifestations of latency, recurrent attacks and prolonged prodromal periods in cases of melioidosis.

Table 5. Distribution of HA Titers of 375 Sera from 139 Cases of Melioidosis by Day of Disease

Time after Disease Onset	No. of Sera with HA Titers (Reciprocals)											Total	% Pos. 1:40 and >	Geometric Mean Titer
	Neg.	20	40	80	160	320	640	1280	2560	>2560*				
2-4 days	7	1	1	1	3		1	2		1		17	53	26
5-7 "	4	2	1	2		2		2				13	54	30
8-14 "	3	1	4	4	3	1	2	3		2		23	83	115
15-21 "			6	1	6	4	1	2	3	2		25	100	286
22-28 "		2	3	5		6		6	1	2		25	92	338
29-60 "	1	2	2	6	9	11	10	16	4	4		65	95	444
61-90 "	2	1	1	7	5	8	5	4	3	3		39	92	289
91-120"	3	2	2	1	3	5	4	11	5	4		40	87	422
121-150"			2	2	4	5	9	3	5	5		35	100	653
151-180"	3	1	2	1	5	5	2	3	6	5		28	86	410
181-365"	1	3	1	1	5	5	6	4	3	10		39	90	603
1-2 yrs.	2	3	1	1	2	3	2	3	3	1		16	69	120
> 2 yrs.			1		2	3	3	1	1			10	100	320

\* 1:5120 through 1:20,480.

Table 6. Distribution of CF Titers of 330 Sera from 134 Cases of Melioidosis by Day of Disease

Time after Disease Onset	No. of Sera with CF Titers (Reciprocals)										Total	% Pos.	Geometric Mean Titer	
	2	4	8	16	32	64	128	256	7 256*	7 256*				
2-4 days	2											8	75	23
5-7 "	2		1	1	1	1	1	1	1			7	71	16
8-14 "			1	4	3	3	2	4	4			15	100	84
15-21 "	1	1	2	5	3	3	2	2	2			22	96	51
22-28 "		1	1	2	3	5	2	2	2			21	100	81
29-60 "	4		1	9	5	4	15	9	9	11		58	88	78
61-90 "	4		2	4	3	7	8	3	3	7		39	90	54
91-120 "	3		1	3	7	6	7	5	5	6		38	92	65
121-150 "	1		2	2	5	6	7	5	5	6		34	97	87
151-180 "	1		1	7	3	4	3	5	3	3		26	96	62
181-365 "	1		2	4	5	6	3	10	6	6		37	97	86
1-2 yrs.	2		1	2	5	2	1	1	1	1		15	87	28
7-2 yrs.				1	2	1	2	3	3	1		10	100	10

\* 1:512 through 1:10,240.

After the first and second week of onset of disease, antibodies were usually detectible and reached optimum titers by 1 to 3 months. In 90% or more of the cases, HA and CF antibodies were still detectible frequently at high titer 6 months to 1 year after disease onset. The persistence of HA and CF titers one year or more after infection was not unusual.

The specificities of the HA and CF tests were further evaluated with diverse rabbit antimicrobial sera and with convalescent sera from patients with various diseases. HA tests elicited few heterogeneric reactions with the series of rabbit antisera (Table 7). The few reactions ranged in titer from 1:20 to 1:40. It was difficult to evaluate CF cross-reactions with diverse hyperimmune rabbit sera because of non-specific and anti-complementary reactions. Tests conducted on paired sera from 18 rabbits immunized with Verder serotypes of P. aeruginosa provided evidence of heterogeneric cross-reactions with the CF antigen. The CF antigen also cross-reacted with 3 of 7 sera from cystic fibrosis patients with P. aeruginosa infections. In human serum, there was a greater number of cross-reactions with CF than HA antigens (Table 8). The HA reactions were low titer and were within the range of non-specific reactions previously observed in normal human sera. The relatively strong CF reaction obtained with the serum from a human leptospirosis case in Puerto Rico poses the question of occurrence of melioidosis in this island. Melioidosis has been found sporadically in other Caribbean areas.

Sera from melioidosis cases which were seronegative on either HA or CF but not with the other respective test were studied. The negative reactions could not be attributed to strain heterogeneity. The same results were obtained when homologous strains were used as HA or CF antigens. To determine whether the lack of HA antibody was due to incomplete antibody, tests were conducted with a diluent containing 25% bovine serum albumin in lieu of the buffered salt solution that is used regularly. Incomplete antibodies incapable of agglutinating erythrocytes in saline solutions frequently cause agglutination in a colloid milieu. HA reactions of 9 sera were not significantly different in this test. A Coomb's test for incomplete antibody yielded negative results on HA-negative sera. The HA-negative but CF-positive sera were also tested in a hemagglutination test with CF antigen previously standardized. The sera were still non-reactive. Attempts to use a HA antigen in a CF procedure to test HA-positive but CF-negative sera were unsuccessful because the HA antigen was anti-complementary.

b. Intra-Strain Growth Inhibition of P. pseudomallei. Correlation had previously been made of smooth colonies of P. pseudomallei, alkaline production in agar media and the ability to inhibit

Table 7. Hemagglutination and Complement Fixation Tests for Melioidosis on Non-Melioidosis Rabbit Antisera

Type of Antibody	No. Reactions/No. Tested		Distribution of Reactions Titer (No.)
	HA	CF*	
<u>Actinobacillus lignieresii</u>	0/23	2/2	1:80 (2)
<u>Pasteurella tularensis</u>	0/1	1/1	1:16
<u>Pasteurella pestis</u>	0/2		
<u>Pasteurella pseudotuberculosis</u>	0/1	1/1	1:16
<u>Pasteurella multocida</u>	0/1	1/1	1:16
<u>Vibrio cholerae</u>	0/1	1/1	1:16, 1:32
<u>Mima sp.</u>	0/2	2/2	1:8, 1:24
<u>Leptospira sp.</u>	0/2	2/2	1:20 (3)
<u>Listeria monocytogenes</u>	3/5		1:4, 1:8, 1:16
<u>Proteus sp.</u>	0/3	3/3	1:8
<u>Brucella abortus</u>	0/1	1/1	1:16
<u>Brucella bronchiseptica</u>	0/3	1/1	
<u>Escherichia coli</u> (polyvalent)	0/2		
<u>Shigella sp.</u>	0/5		
<u>Salmonella group typing</u>	0/6		
<u>Salmonella sp.</u>	0/3	0/4	1:8 (2)
<u>Salmonella typhosa</u>	0/1	2/2	1:8 (2)
<u>Pseudomonas aeruginosa</u> (Misc.)	0/5	1/3	HA - 1:20; CF - 1:8
<u>Pseudomonas aeruginosa</u> (Parke-Davis serotypes)	0/7	2/4	1:8, 1:16
<u>Pseudomonas aeruginosa</u> (pre-immune)	2/17		HA - 1:20 (2); CF - 1:8, 1:16 (2), 1:32
<u>Pseudomonas aeruginosa</u> (post-immune)**	0/18	14/14	1:8 (2), 1:16 (8), 1:32 (2), 1:64 (2)
<u>Pseudomonas stutzeri</u>	1/5	2/2	HA - 1:40; CF - 1:8, 1:16,
<u>Pseudomonas multivorans</u>	1/3		1:20

\* Differences in numbers tested reflect anti-complementary reactions.

\*\* Seven rabbits had 4-fold or greater increase in CF titer.

Table 8. Hemagglutination and Complement Fixation Tests for Melioidosis on Convalescent Sera from Non-Melioidosis Patients

Type of Antibody	No. Reactions/No. Tested		Distribution of Reactions Titer (No.)
	HA	CF	
<u>Pasteurella tularensis</u>	0/1	0/1	
<u>Vibrio cholerae</u>	0/1	0/1	
<u>Leptospira sp.</u>	2/12*	2/8*	HA - 1:20 (2); CF - 1:4, 1:64
<u>Brucella abortus</u>	0/1	1/1	1:4
<u>Salmonella typhosa</u>	0/1	1/1	1:4
<u>Salmonella paratyphi</u>	0/1	0/1	
<u>Pseudomonas aeruginosa</u>	0/5	0/5	
<u>Pseudomonas aeruginosa (cystic fibrosis)</u>	1/9	3/7	HA - 1:20; CF - 1:8 (1), 1:128(2)
<u>Pseudomonas stutzeri</u>	1/1	1/1	HA - 1:40; CF - 1:48
<u>Influenza</u>	1/4	2/3	HA - 1:40; CF - 1:8, 1:16
<u>Syphilis</u>	0/2	0/2	
<u>Typhus</u>	3/3	0/1	1:40 (3)
ASO	0/1	AC	
C-reactive	0/1	AC	
Rheumatoid arthritis	0/4	AC	
LGV	1/6	0/5	1:20
Total	8/53	10/37	

\* Positive reactions in patients from Puerto Rico. One was positive in both tests.

all other P. pseudomallei strains (WRAIR Annual Report 1 Jul 1967 - 30 Jun 1968). Attempts to demonstrate the inhibitory substance in fluid cultures or fluid expressed from agar cultures were not successful. The use of ammonium sulfate for precipitation, dialysis and vacuum dialysis concentration on expressed agar fluids did not reveal any inhibitory substances. In order to characterize the inhibitory substance, growth was removed from inhibitor cultures. The agar surface was sterilized with chloroform vapors and covered with a dialysis membrane. Growth inhibition indicator strains (GII) were streaked at right angles to the original growth. The GII were still inhibited within 24 hours but colonies were eventually discernable in 48 to 72 hours. This indicated that (1) the inhibition was probably more static than cidal, (2) nutrient depletion was not involved and (3) the substance passed through pores of 4.8  $\mu$  diameter and was probably less than 12,000 in molecular weight.

P. pseudomallei, strain 165 (an inhibitor) and strain 7815 (non-inhibitor) were grown in Rice's protein-free broth media (Rice, C. E. et al., Canad. J. Comp. Med. Vet. Sci. 15: 284, 1951) with 0.1% ionagar. After three days of intermittent shaking at 37 C the cultures were sterilized by chloroform vapors, the cells removed by centrifugation and the supernatant fluids were chromatographed. Both cultures had two ninhydrin reacting spots in common. The inhibitor culture (strain 165) had an additional spot which migrated in a manner which was indistinguishable from lysine or arginine. This was in good agreement with the fact that both of these amino acids are diaminomono-carboxylic and could cause the alkaline conditions found in agar media. The D and L forms of these acids were purchased and incorporated into Wahba agar pH 7 and pH 8.6 (adjusted with NaOH). The concentrations of amino acids were 0, 250, 1000 and 4000  $\gamma$ /ml. Strains 165 and 7815 were grown in Wahba broth pH 7 overnight at 37 C. The cultures were diluted and spread on the plates. Although the actual number of colonies were not reduced on Wahba agar pH 8.6, the colonies did not develop in size as quickly as at pH 7. Comparative counting was obtainable between 1.5 and 5 days. All plates containing D-amino acids were compared after one week. The L configurations of lysine and arginine had no inhibitory effects in Wahba agar at either pH 7 or pH 8.6. However, at pH 8.6 inhibition by D-arginine or lysine was strikingly demonstrable at high concentrations by either reduced colony size or reduction in actual number of colonies formed (Table 9). The inhibitory culture supernate differs from the noninhibitor by one chromatography spot which is reactive with ninhydrin and has a mobility similar to arginine or lysine. Only the D configuration of these compounds are active inhibitors. It is hoped that enough of the inhibitor may be obtained so that it may be compared to arginine and lysine by infra red spectrophotometry.

Table 9. Inhibition of *P. pseudomallei* Strains by D-Arginine and D-Lysine in Wahba Agar

D-lysine µg/ml	<i>P. pseudomallei</i> 165 (Smooth)				<i>P. pseudomallei</i> 7815 (Rough)			
	pH 7.0		pH 8.6		pH 7.0		pH 8.6	
	Colony* size	Cells/ml** x 10 <sup>8</sup>	Colony size	Cells/ml x 10 <sup>8</sup>	Colony size	Cells/ml x 10 <sup>8</sup>	Colony size	Cells/ml x 10 <sup>8</sup>
0	C	1.8	C	1.3	C	4.2	C	4.1
250	C	1.6	C	1.3	C	4.0	C	4.0
1,000	C	2.4	I	1.0	C	4.6	I	4.2
4,000	C	1.9	I	1.0	C	4.3	I	1.0
D-arginine								
250	C	2.0	I	1.5	C	4.6	I	4.8
1,000	C	2.0	I	0.7	C	4.4	I	3.8
4,000	C	2.0	complete inhibition	complete inhibition	I	4.7	complete inhibition	

\* C = Equal to diameter of control colonies; I = diameter of colonies were markedly less than control colonies, i.e. inhibited.

\*\* Colony counts were normalized for comparison with control plate estimations of cells in broth inoculum.

c. The Use of Redox Dyes to Indicate the Use of Carbon Compounds by Pseudomonads. For many years the only pseudomonad to be considered an animal pathogen was Pseudomonas aeruginosa. However, our concept of this genus as primarily phytopathogens and saprophytes has slowly changed, mainly because of better laboratory diagnosis and a restructured taxonomy which have led us to realize that other pseudomonads, such as, P. pseudomallei, Actinobacillus mallei, P. multivorans, P. maltophila, P. stutzeri, and P. fluorescens are also associated with animal diseases.

The epidemiology of these organisms is still hindered by our inability to rapidly distinguish them from other gram negative aerobes, as well as other pseudomonads and biotypes within the species. Some of the difficulties are due to a lack of universally acceptable classification criteria and identification schemes using media and interpretations based on enteric bacteriology. Consequently, most clinical laboratories identify non-pigment producing pseudomonads as achromogenic P. aeruginosa or simply just pseudomonas species or even other genera.

Our attention was obliquely drawn to these considerations while investigating intra-strain inhibition within the species of P. pseudomallei. This species could be separated into two groups: 1) inhibitors which were subsequently found to be smooth and caused alkaline conditions in their agar media and 2) non-inhibitors which were colonially rough and caused acid conditions. The latter are usually mistaken for coliform on MacConkey agar. The alkalinity and inhibition seem to be due to the production of large amounts of a basic D-amino acid-like substance. It then became obvious that any diagnostic test employing pH indicators could be as much an indication of colonial dissociation as well as the by-products of carbon assimilation. Many of the media employed for gram-negative rod identification do contain pH indicators. For instance, MacConkey, SS, Brilliant green agars, phenol red and purple broth bases, OF media, triple sugar iron agar, Kligler's agar and urea broth are some of them. Except for urea, these media were designed for acid producers.

Part of Table 10 illustrates the reactions of P. pseudomallei and A. mallei in two diagnostic media containing carbohydrates. These are the oxidation-fermentation (OF) and phenol red broth base media (the OF media was not originally designed for this purpose, but is often misused to determine differential carbohydrate utilization). It is obvious that the pH varies over a period of time and even the blank tubes without added carbohydrates elicit irrelevant differential reactions. In fact, these organisms are quite capable of growth using the nitrogenous proteinaceous components of the media.

Table 10. Reactions Caused by P. pseudomallei and A. mallei in Carbohydrate Diagnostic Media Containing pH or Redox Indicators

	<u>P. pseudomallei</u> 7815			<u>P. pseudomallei</u> 7820			<u>P. pseudomallei</u> 7816			<u>A. mallei</u>		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
OF media												
glucose	A	A	A	A	A	A	A	A	A	A	A	A
sucrose	A	A	A	A	A	A	A	K	K	K	K	K
lactose	A	A	A	A	A	A	A	A	A	A	A	A
blank	A	A	A?	K	Nct	K	K	K	K	A?	A	Nct
Phenol red												
broth base												
glucose	A	A	A	A	A	A	A	A	A	A	K	K
sucrose	K	A	A	A	A	A	Nct	A	A	Nct	Nct	NC
lactose	Nct	A	A	A	NC	K	Nct	K	K	Nct	Nct	NC
blank	K	A	A	A	Nct	A	Nct	A	K	Nct	Nct	NC
DIP-liquid												
glucose	R	R	R	R	R	R	R	R	R	NC	K	R
sucrose	-	-	-	-t	-	-	-	-	-t	-	-	-
lactose	-	-	-	-t	-	-	-	-	-t	-	-	-
blank	-	-	-	-t	-	-	-	-	-t	-	-	-

Legend: R = reduced; -t = very slight turbidity.  
A = acid; K = alkaline; Nct = no change in original color but turbid; NC = no change.

The possibility existed that redox dyes might be of better aid in demonstrating substrate utilization with consequent growth and respiration than acid-base indicators. The selected dyes had electrovalent potentials which could accept  $H^+$  and electrons from NAD and cytochrome B. The dyes chosen were triphenyl tetrazolium chloride (TTC), nitroblue tetrazolium chloride (NBT), phenol-indo-2:6-dichloro-phenol (DIP) and methylene blue (MB). Reduced TTC and NBT formed precipitable insoluble formazans and had to be suspended in agar. The desired dye features were that the dye (1) would be poised enough that carry over growth of inoculum and use of trace media contaminants would not cause false conclusions, (2) not be readily affected by pH, (3) not be utilized as substrate, (4) be changed specifically by respiration, (5) not be auto-oxidized or reduced by molecular oxygen or the substrate, (6) be non-toxic, and (7) be unaffected by light or storage.

The dyes were incorporated into Stanier's minimal media (J. Gen. Microbiol. 43: 168, 1966) which contained only a single carbon source. Initially agar slants were used. At least two transfers were made in each media to dismiss the possibility of carry over growth. In general the dyes were reduced only in the butt or where there was large surface growth on the slant. All organisms tested preferred molecular  $O_2$  to the dyes, especially MB which was easily autooxidized and considered a poor choice. TTC was difficult to read and was only reduced in the vicinity of the stab and heavy surface growth. NBT was generally reduced throughout the butt. Both tetrazoliums varied in their reactions and reacted too strongly in non-carbon containing controls (carry over growth). In liquid media the presence of agar carbon contaminants were obviated. The reduced tetrazoliums precipitated and could not be easily judged. Methylene blue was too easily autooxidized. DIP reduction was easily discerned (Table 11) and discriminatory. It was picked as the redox dye of choice. Table 11 shows how a variety of strains can be differentiated by their ability to use various substrates. In a number of cases the dyes were reduced prior to any turbidity in the culture media.

The results are presented for reactions occurring after one week. Previous trials with vitamin additions have accelerated the utilization of these carbon sources but have clouded the issue by being used as substrate in some cases. The disadvantages of the DIP dye to date are that autoclaving reduces DIP to some extent and therefore is not used; the dye is preferentially reduced by some substrates, such as, inositol, xylose, ribose and tryptophan, and in general the dyes will fade noticeably at 2 months at room temperature and fluorescent lighting. Their shelf life has been increased past this time by storage in walk-in refrigerators in closed cabinets. At the present time, we are working on a diagnostic scheme which utilizes about six of these substrates in conjunction with some common laboratory media.

Table 11. Reduction of Phenol-Indo-2:6-Dichloro-Phenol (DIP)  
as an Indication of Substrate Utilization

Organism	No. of Strains Tested	Blank	Glucose	Sucrose	Lactose	Maltose	Cellulobiose	Inositol	Trehalose	Mannitol	D-Xylose	D-Fucose	M-Hydroxy Benzoate
<u>P. pseudomallei</u>	6	0	6	0	0	4	0	6	6	5	5	6	0
<u>A. mallei</u>	5	0	4	0	0	0	0	2	4	1	2	3	0
<u>P. aeruginosa</u>	8	0	6	0	0	0	0	0	0	6	8	8	0
<u>P. fluorescens</u>	2	0	2	2	0	0	0	2	1	2	2	2	0
<u>P. stutzeri</u>	3	0	3	0	0	2	0	1	2	2	3	1	0
<u>P. multivorans</u>	2	0	2	0	0	0	2	2	2	2	2	2	1
<u>P. acidovorans</u>	1	0	0	0	0	0	0	0	0	1	1	0	0

d. Chemotherapy. An in vitro drug test system was established to determine bacteriostatic and bacteriocidal levels of select antibiotics and combinations of antibiotics. Tests were conducted with six strains which varied in their susceptibility with an array of antibiotics and included strains that were sensitive and resistant to chloramphenicol. A tube dilution technique was used. Bacteriostatic activity was measured on the basis of observed growth (e.g., turbidity) in media containing appropriate dilutions of test antibiotics, and which were inoculated with a known concentration of organisms. Bacteriocidal activity was determined quantitatively using conventional cultural and agar-plate counting methods. Bacteriostatic and bacteriocidal levels were determined after 24 and 48 hours of incubation of test media. The criterion for cidal activity was 99% or greater reduction in number of organisms originally inoculated.

Two drugs were extensively tested for activity against P. pseudomallei, dapsone in view of its extensive use in troops in Vietnam for malarial prophylaxis and sulfamylon in view of occurrence of P. pseudomallei infections in burn patients treated with this compound. Dapsone was inactive for P. pseudomallei at concentrations of 25 µg/ml. At 125 µg/ml levels it was slightly bacteriostatic. The growth of P. pseudomallei was unaffected by sulfamylon in concentrations up through 2.5 mg/ml. A 5.0 mg/ml concentration was bacteriostatic but not bacteriocidal.

Preliminary tests were done on effects of combination of antibiotics. Antagonistic effects were seen with combinations of kanamycin and novobiocin with select strains but not others. With one strain, a combination of novobiocin and tetracycline appeared to have a synergistic effect.

The bacteriocidal activity of the patient's serum was tested after optimal treatment with ampicillin, gantrisin, chloramphenicol and aureomycin. Tests were conducted with the strain isolated from the patient. No significant bacteriocidal levels were detected in the 1:2 final serum dilution (after addition of culture).

#### Summary and Conclusion.

##### 1. Leptospirosis.

a. The purported prophylactic efficacy of entrycide for leptospirosis was not affirmed in a hamster-infectivity test system used for screening anti-leptospiral drug activity.

b. Microscopic-agglutination tests on serum from 8 cases in Vietnam provided presumptive evidence of 7 different serotype infections, one of which -- hyos -- has not previously been reported in this area. A high prevalence of antibodies were found in normal swine from Malaysia and Vietnam. In both countries predominant titers were against ponona and autumnalis. This contrasted with marked diversity of serotypes found in human infections in these areas. The macroscopic-agglutination (slide) test had poor sensitivity and specificity when used as a seroepidemiological tool in swine surveys. Antileptospiral antibodies for diverse serotypes were demonstrated in sera from various rodents trapped in Con Son, Vietnam. Antibodies to serotypes commonly associated with human infections in Vietnam were found in animals in this area. One of the leptospiral strains isolated from surface water in Malaysia was found to be a new serotype and has potential usefulness as a vaccine strain because of its unique antigenic affinity with diverse serological types.

## 2. Melioidosis.

a. One hundred and eighteen human cases in Armed Forces personnel were affirmed by laboratory tests. Approximately 220 cases were established at WRAIR since February 1965. The sensitivity and specificity of the hemagglutination (HA) and complement-fixation (CF) were evaluated from test results obtained on single or serial serum samples from 185 patients. All but 2 of the patients had significant antibodies on one or both tests. In the exceptional cases the sera were obtained during the acute phase of disease. Two cases were CF positive only and 3 were positive only on HA. The correlation of HA and CF results on 175 patients for which both tests were performed was 97%. The correlation of findings in 445 sera from these patients was 90%. Both CF and HA antibodies were usually present one week after onset of disease. In both tests titers reached maximum levels in 1-to 3-months and persisted in approximately 90% of the patients for more than six months. Persistence of antibody titers for 1 year or longer was not unusual. The high specificity of HA test was affirmed in extensive tests with diverse anti-microbial rabbit serum and with sera from patients with various bacterial and viral diseases. In CF tests on the same series of sera, heterogeneric reactions occurred with other Pseudomonas sp. antibodies occasionally at relatively high titers. The serological studies served to establish the HA and CF tests as excellent sero-diagnostic procedures. The relatively few seronegative or low titer reactions in HA or CF tests on sera from proved cases could not be related to strain antigenic differences or incomplete antibody.

b. The broth supernatant or expressed agar fluids of an inhibitor culture of P. pseudomallei were compared to those of a non-inhibitory strain by paper chromatography and thin layer chromatography. The inhibitor cultures contained an additional ninhydrin positive spot which had the same R<sub>x</sub> mobility as arginine or lysine. Only the D-configuration of these two compounds had inhibitory properties. Further chemical identification of the inhibitor is planned.

c. Redox dyes were incorporated into minimal media containing single sources of carbon. Growth and respiration of the pseudomonads at the expense of the carbon sources caused a concomitant reduction and color change of the dyes. Phenol-indo-2:6-dichlorophenol was considered the dye of choice.

The results obtained were a better differential of pseudomonad species than commercially available complex media containing pH indicators. At present the major difficulties involve auto-reduction of the dye by some substrates and fading on prolonged storage.

d. An in vitro drug test system was established to determine bacteriostatic and bacteriocidal levels of select antibiotics and combination of antibiotics. Concentrations of dapson and sulfamylon at levels attainable in clinical applications were not active for melioidosis organisms. The occurrence of melioidosis in sulfamylon-treated burn cases from Vietnam is consistent with in vitro test results. In preliminary tests on effects of combination of antibiotics, antagonistic effects were seen with the combination of kanomycin and novobiocin with some but not all strains. With one strain a combination of novobiocin and tetracycline appeared to give a synergistic effect. No significant bacteriocidal levels of gantrisin, tetracycline and chloramphenicol for P. pseudomallei were found in sera of a patient following drug administration.

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00 Tropical and Subtropical Military Medical Research

Work Unit 305 Military Medical Research Program, SEA, WRAIR - Zoonoses

Publications

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY	REPORT CONTAINING NUMBER	
				DA 0A6526	69 07 01	DD-128 (AR) 695	
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69 01 31	D. Change	U	U	NA	ML	<input type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODE#	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62110A	3A062110A811	00	308			
b. CONTRIBUTING							
c. CHANGING	1412A(2)						
12. TITLE (Provide with Security Classification Code)							
(U) Prophylactic Use of Gamma Globulin to Prevent Infectious Hepatitis (09)							
13. SCIENTIFIC AND TECHNOLOGICAL AREA							
003900 Clinical Medicine							
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28. RESPONSIBLE DOD ORGANIZATION		29. PERFORMER'S ORGANIZATION					
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30. GENERAL USE		31. SOCIAL SECURITY ACCOUNT NUMBER		[Redacted]			
Foreign Intelligence considered		ASSOCIATE INVESTIGATORS					
		NAME:		DA			
		NAME:					
32. KEYWORDS (Use only with Security Classification Code)							
(U) Hepatitis; (U) Gamma Globulin; (U) Passive Immunization; (U) Human Volunteer							
33. TECHNICAL OBJECTIVE, 34. APPROACH, 35. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To ascertain if the prophylactic administration of gamma globulin to troops is effective in reducing the incidence of clinical hepatitis, to determine the period of immunity and the dose required.							
24. (U) All PCS male U. S. Army personnel assigned to EUSA receive an intramuscular injection at the arial port of entry and again five months later. These injections contain either 2, 5, or 10 ml. of 16 percent gamma globulin or placebo material. The inoculations are given in a double blinded manner based upon the last digit of the soldier's serial number. Data will be analyzed based upon the incidence of documented viral hepatitis in soldiers receiving each of these materials.							
25. (U) 69 01 - 69 06 A WRAIR composite team was assigned to Korea during May 1967. This team has established an inoculation and record center at the arial port of debarkation for all U. S. Army personnel arriving in Korea. Soldiers assigned to Korea receive biologic materials upon arrival and again five months later. Scrutiny of all admissions to hospitals is maintained to insure proper documentation of hepatitis in each study patient. Ninety Six Thousand soldiers have received second injections. Gamma globulin does not protect all soldiers from the occurrence of clinical illness. Whether significant reductions in incidence or morbidity result from gamma globulin administration can not be ascertained until the study is decoded. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-Jun 69.							

PII Redacted

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00 Tropical and Subtropical Military Medical Research

Work Unit 308, Prophylactic use of gamma globulin to prevent infectious hepatitis

**Investigators**

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Associate: MAJ Bertram F. Felsher, MC, MAJ George M. Bernier, MC,  
Joseph D. Boggs, M.D., Hans F. Smetana, M.D., and  
Allen A. Young.

**Description**

Gamma globulin has been used in both civilian and military populations to prevent the occurrence of infectious hepatitis. Scrutiny of the incidence of hepatitis in troops receiving prophylactic injections of gamma globulin has failed to provide convincing evidence that it has reduced the incidence of disease. A double blinded clinical study was initiated to permit evaluation of the effectiveness of U. S. gamma globulin in the prevention of hepatitis in U. S. Forces stationed in Asia.

Despite many reports of isolation of a virus from materials obtained from patients with infectious hepatitis, there is no convincing evidence that the causative organism of infectious hepatitis has been cultivated. Studies were initiated to obtain materials from patients with infectious hepatitis and prove that they are infective so that these specimens can be distributed to various laboratories attempting to cultivate and identify the virus. It is postulated that the availability of materials from single sources and their distribution to many laboratories will provide the best opportunity to cultivate the causative organism of disease and hopefully lead to the production of a vaccine for the prevention of hepatitis.

**Progress**

During 1964 a program was initiated by the U. S. Army to immunize all military personnel stationed in the Far East and Southeast Asia with gamma globulin to reduce the incidence of infectious hepatitis. Soldiers were injected with 10 ml of 16 per cent human serum gamma globulin shortly after arrival overseas and again five months later. It was hoped that this would produce passive immunity against hepatitis and that active immunity would develop through a subclinical infection. The escalation of the conflict in Vietnam increased the number of troops stationed in Asia and markedly reduced the amount of gamma globulin available in national stockpiles. During 1965, the recommended dose

of gamma globulin was reduced to 5 ml twice during a one year tour in Asia, and during 1966 the recommendation was made that the reduced dose be administered only to soldiers assigned to units with a continued high incidence of hepatitis. Examination of the incidence of hepatitis reported from both Korea and Vietnam showed a marked and sustained reduction in the incidence of hepatitis from 1964-67 when compared to previous intervals. However, this reduction in incidence occurred several months before the initiation of the gamma globulin prophylactic program and could not be attributed to it. In addition, the incidence remained low following reduction in both the dose of gamma globulin and the number of troops included in the immunization program. This information and the relatively limited availability of gamma globulin made it desirable to ascertain the effectiveness of gamma globulin administration in a controlled study.

During May 1967 a field study was initiated in Korea in which soldiers assigned PCS to EUSA were given various doses of gamma globulin or placebo upon arrival and again five months later. Soldiers receive either: (1) 10 ml of 16 per cent human serum gamma globulin (20 per cent); (2) 5 ml of gamma globulin and 5 ml of an albumin-sucrose-potassium glutamate solution (20 per cent); (3) 2 ml of gamma globulin and 8 ml of control material (20 per cent) or (4) 10 ml of the placebo injection (40 per cent). All materials must be characterized for antibodies against known bacteria and viruses and for fragmentation and content of various gamma globulin components. Selection of soldiers for each injection is made based upon the last integer of their military serial number. The various materials used for injection are bottled in 10 ml containers marked with the ten integers, 0 through 9. Two integers are used for each material containing the various quantities of gamma globulin; (1) (2) and (3) above and four integers are utilized for control material. Each soldier receives the contents of a bottle labeled with a number matching the last number in his serial number upon arrival in the serial port of debarkation in Korea and again in medical military dispensaries throughout Korea five months later. All cases of suspected hepatitis are evacuated to one of two military hospitals in Korea where the diagnosis is evaluated by both clinical and laboratory studies. Each documented case of hepatitis is verified as a study patient by maintenance of central immunization files at the serial port of debarkation. It is believed that 100,000 man years of study will be required to document the value or limitations of gamma globulin in the prevention of infectious hepatitis.

Studies are being performed at the Illinois State Penitentiary in collaboration with Dr. Joseph D. Boggs of Northwestern University. Potentially infectious materials from patients with documented infectious hepatitis are being administered to volunteers. Each patient is hospitalized and carefully controlled clinical and laboratory tests are performed to ascertain if the volunteer develops

hepatitis. Blood, urine and feces are collected before the administration of test materials and are stored at  $-80^{\circ}$  C. until the completion of studies. Materials from subjects developing hepatitis are selected and divided into small aliquots for distribution to laboratories attempting to (1) isolate the virus of hepatitis; (2) develop antibody tests to identify the disease and (3) produce the disease in animals to obviate the need for human studies.

### Results

During the period May 1967 through May 1969, 95,000 soldiers arriving in Korea have received an injection of gamma globulin or placebo material, and 50,000 soldiers have received injections of the same biological materials five months later. Icteric hepatitis is occurring in soldiers receiving both gamma globulin and placebo material at all intervals following injection. Whether gamma globulin is providing partial protection or a reduction in morbidity of the clinical illness can not be ascertained until the code for the materials is known and subjected to statistical evaluation. The possibility that no difference will be shown has been considered and in this event it would be necessary to consider the hypothesis that the etiologic agent of hepatitis in Korea is different than that observed in the United States. The incidence of previous hepatitis for all soldiers assigned to Korea has been obtained and similar information is being obtained from each hepatitis patient; failure of soldiers with a previous history of hepatitis to develop a second infection in Korea would be suggestive of immunity. In addition, preliminary data indicates that most hepatitis patients were reared in a rural environment. This might indicate that urban U. S. populations are relatively immune to hepatitis contracted in Korea; presumably because they were exposed to this viral illness during an earlier period of life. Lifelong geographical histories are being obtained from each patient with hepatitis and from a randomly selected population of well soldiers to ascertain if this can be documented.<sup>1</sup>

Last year serum was provided by Dr. Krugman from children with hepatitis at the Willowbrook School, Long Island, New York. Oral administration of small aliquots of this serum to human adult volunteers produced clinical hepatitis thirty to forty days later. Specimens of plasma were collected from all volunteers at intervals after infection. A thirty day specimen of plasma collected from one of these patients has been fed to ten volunteers and failed to produce hepatitis. This specimen was obtained two days before laboratory tests indicative of hepatitis became abnormal. A second specimen has been selected during the interval when the transaminase was elevated but before bilirubinemia occurred and will be administered to volunteers to ascertain if it contains the causative agent of hepatitis. If so, this pool will be used for virologic isolation studies by laboratories under U. S. Army contract for this type investigation and to investigators attempting to develop an experimental animal for hepatitis studies.

### Conclusions and Recommendations

A double blinded field study was initiated in Korea by a WRAIR team to evaluate the usefulness of gamma globulin in the prevention of infectious hepatitis. This team has completed the second of three years of operations overseas. Serum pools with demonstrated infectivity for infectious hepatitis are being developed for use by investigators attempting to cultivate the etiologic agent of viral hepatitis or to develop an animal system for use in the study of this disease.

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00 Tropical and Subtropical Military Medical Research

Work Unit 308, Prophylactic use of gamma globulin to prevent  
infectious hepatitis

Bibliography

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Problems encountered with gamma globulin prophylaxis. Bull. N. Y.  
Acad. Med. 45:167, 1969.

**PROJECT 3A062110A816**  
**MILITARY MEDICAL MATERIEL**

**Task 00**  
**Military Medical Materiel**

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACQUISITION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
				DA 0A6463	69 07 01	DD-RSP (ARJ) 635	
3. DATE OF COUNTRY	4. KIND OF SUMMARY	5. SUMMARY ICY*	6. YOUR SECURITY*	7. REGRADING*	8. DISSEM INSTR*	9. SPECIFIC DATA CONTRACTOR ACCESS	10. LEVEL OF RJA
62 06	D. Change	U	U	NA	RL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62110A	34062110A016	00	203			
B. CONTRIBUTING							
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11. TITLE (Provide only Security Classification as Copy)							
(U) Military Medical Materiel (09)							
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009800 Medical and Hospital							
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21. RESPONSIBLE DSO ORGANIZATION				22. PERFORMING ORGANIZATION			
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				SOCIAL SECURITY ACCOUNT NUMBER: [Redacted]			
23. GENERAL USE				24. ASSOCIATE INVESTIGATOR			
Foreign Intelligence Considered				NAME: Kazys, L.			
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Provide in detail paragraphs if needed by number. Provide last of each with security classification code.)							
23. (U) To develop equipment and procedures to support the Army in the field which will reduce logistical requirements and improve efficiency. Greater specificity in identifications and reduction in analytical time are specific goals for toxicologic analysis.							
24. (U) The statistical evaluation of available procedures, will provide a basis for acceptance of procedures or to indicate further developments. Equipment prototypes will be developed to meet the specific requirements for support of military programs.							
25. (U) 69 01 - 69 06 A field demonstration of the dry-pack intravenous preparation and distillation system was made at Fort Bragg for the Special Forces applications for which it was designed. Evaluation of dry-pack intravenous preparation has demonstrated full feasibility for this concept under austere conditions. Further field evaluation for unrecoginized problems are continuing. The system of laboratory automation for analytical toxicology is now ready for on-line computer operation. Programming has reached the stage at which the entire system ought to be in operation as soon as analytical equipment can be interfaced to the computer. If a small laboratory computer can be acquired, this should take place within the next year. Meanwhile, arrangements are being made to provide some of the Army Area Medical Laboratories, that now have some access to computer facilities, with basic programs to process spectral data. Other laboratories are being encouraged to avail themselves of the rapid data retrieval capabilities for spectral data at the Toxicology Section, WRAIR, and requests for this service as well as information supplied are communicated by telephone. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

PII Redacted

Project 3A062110A816 MILITARY MEDICAL MATERIEL

Task 00, Military Medical Materiel

Work Unit 205, Military medical materiel

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**Associate:** Robert Permisohn, GS-09  
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John O. Brown, SP5  
Norman West, SP4  
Morris Wilkinson, SP4

**Description**

This project has included the evaluation of special items of equipment for use under field conditions for preparation of intravenous solutions and evaluation of new technical approaches for improving the operation of the medical laboratory.

**Approach to problem:**

A concept of preparation of intravenous solutions which is designed to package the desired components in a dried form in a fluorinated hydrocarbon package instead of a glass bottle was developed and has been funded by the Medical Research and Development Command. The packaged, dried reagent offers a considerable logistical advantage in several respects as well as increasing the shelf life of the product. An easily portable distillation system has been developed as a component to provide the necessary purified water for reconstitution of the intravenous solutions.

For toxicologic examinations an analytical system which uses a number of differing analytical approaches for the identification of toxic substances was considered an essential development in light of the continuing increased complexity of requirements for analysis.

**Exposures**

**System for Field Preparation of Electrolyte Solutions**

Problems of excessive weight, deterioration of solutions during shipping and storage, "casualty rate" during shipping, and other considerations of solutions packaged in glass bottles have led to production of a plastic bag of specially formulated material which now allows

packaging of dried chemical, or of highly concentrated liquid preparations, by a pharmaceutical contractor in a container which is no longer easily broken and which will withstand large extremes of temperature. The dried chemical also has another highly desirable characteristic of chemical stability, since most dry chemicals deteriorate more rapidly when placed in solution than in the dried form. Preparations made available to date include normal saline, 5 percent glucose, 10 percent glucose, bicarbonate buffer solution, and concentrated sodium lactate. These preparations allow a considerable relief of logistical burden since the dried constituent represents a maximum of 10 percent of the final weight and the plastic container is much lighter than the glass bottle. Another feature of the plastic unit is that it contains everything required for transfusion purposes as a component in the shipping package. This assures delivery of all the essential items for use in the hospital by the physician when an intravenous solution of these types are required.

The distillation unit for this assembly was made to the special requirements of use in isolated areas where transportation is very limited and where a minimum of weight to be carried is associated with success of a mission. The unit is totally contained in a suitcase-size assembly which lends itself to easy back-carry or allows air drop if necessary. Components contained in the assembly include a portable autoclave, the distillation assembly complete with quality control monitor apparatus, collection containers, and a small supply of the transfusion bags. The still has the ability to produce USP Quality Water for Injection from most naturally available water. The still may be suspended over a suitable heat source (a charcoal fire) and water is fed to both the condenser and the boiling unit from a bag or other suitable feed source. The boiling unit produces steam which passes through a condenser which is cooled by a spirally wound trough. The condensate passes through a battery-operated conductivity meter adjusted to give an audio signal in the event water purity is questionable. This "mechanical rabbit" has undergone extensive testing to establish that water produced and monitored by this device meets all requirements for intravenous applications. Selected bags containing the desired chemical are filled consecutively by a manifold connection which allows the collection of four units per pass. Four units are indicated in this situation since volume adjustment is assured by placing the bag in a calibrated can. This can also serves as a container for the terminal sterilization process which may be carried out in a portable autoclave which serves as the external container for the still components during transportation. Total weight of the assembly is approximately 30 pounds, with a production capacity of one gallon of water per hour of operation. Cleaning and maintenance are easy operations with the unit designed.

A further extension of water purification on a much larger scale (5 gallons per hour) is currently under development.

An evaluation of product produced has resulted in the following observations:

(1) It is feasible to reconstitute a packaged dried electrolyte which will comply with USP requirements for use as intravenous solutions. Intravenous solutions which were packaged by the contractor were shown to be within assay limits, sterile, and pyrogen-free for extended periods. Accelerated studies of stability by storing solutions at elevated temperatures and humidity indicate solutions may be satisfactorily stored for varying periods without any appreciable deterioration. These data are summarized in Tables I and II.

(2) The portable distillation system will produce a safe product for intravenous use even though the source water may be non-potable and heavily contaminated. Results of these examinations are listed in Table III.

(3) Suspended matter which may be contained in the dried chemicals can be satisfactorily removed from the solution by a "final filter" which has been specifically developed for this purpose. An examination of Table IV data will show that extremely small particles may be removed and that the quality of the product produced exceeds that normally contained within the glass-packaged solutions.

#### Analytical Toxicology.

Automated analysis of gas chromatography (7 units), ultraviolet spectrophotometry (Cory XV) and infra-red spectrophotometry has resulted in a highly effective analytical system which has been reported elsewhere in support of the antimalarial assay program. The addition of a mass spectrograph during this year has greatly enhanced the potential for study of medicinal metabolites and for identification of minute amounts of toxic materials. Work is continuing to add reference materials to the library of data which can be accessed by use of the off line computer facility. This type of data processing has not proven to be highly effective due to the delay in return of processed information. The system has demonstrated that for more efficient operation a dedicated laboratory computer is the best answer to this type of operation. Efforts will be continued to complete this requirement.

#### Summary and conclusions:

A system for preparation of intravenous solutions by addition of distilled water to pre-packaged dried chemicals has been evolved which has been demonstrated to be safe and feasible.

Analytical toxicology has been extended to facilitate analyses. The system has resulted in reducing analytical time and in increasing precision of examinations.

TABLE 1

One-half liter bags filled with the dry chemical, autoclaved and then reconstituted with water for injection, placed in 10 mil. H.P.P.L. overwrap and autoclaved.		PYROGENICITY TEST RESULTS																									
		Values reported are the maximum temperature rise per rabbit. Three rabbits were injected for each study and observed at 1 hour, 1-1/2 hours, 2 hours and 3 hours.																									
		Conditions under which bags were stored																									
Solution	Initial	ROOM TEMPERATURE			30°C. and 100% R.H.			15 days at 155°F., then 76 days at 30°C. and 100% R. H.			30 days at 155°F., then 179 days at 30°C. and 100% R. H.			3 days at -80°F., then 179 days at 30°C. and 100% R.H.			One day at an air pressure of 5 lbs./sq. in., then 181.5 days at 30°C. and 100% R.H.										
		3 Months	6 Months	3 Months	6 Months	3 Months	6 Months	15/15/66	8/24/66	9/12/66	12/12/66	12/28/66	1/3/67	10/3/66	10/11/66	1/12/67	11/15/66	11/16/66	12/12/66	12/28/66	1/3/67	1/12/67					
10% Dex. in H <sub>2</sub> O PL-146	5/17/66 0 -0.35 +0.05 0	8/16/66 -0.20 +0.15 +0.15	11/15/66 +0.20 +0.20 -0.15	8/24/66 +0.20 +0.05 +0.15	11/16/66 +0.20 +0.10 +0.05	3 Months +0.15 +0.05 +0.05	6 Months +0.20 +0.10 +0.05	15 days at 155°F., then 76 days at 30°C. and 100% R. H. +0.15 +0.25 +0.05	30 days at 155°F., then 179 days at 30°C. and 100% R. H. -0.10 +0.10 -0.05	3 days at -80°F., then 179 days at 30°C. and 100% R.H. +0.20 +0.10 +0.25	11/15/66 +0.05 +0.10 +0.15	11/16/66 +0.20 +0.10 +0.25	12/12/66 +0.25 +0.10 +0.40	12/28/66 +0.25 +0.20 0	1/3/67 +0.30 +0.15 +0.45	10/3/66 +0.20 +0.25 +0.05	10/11/66 +0.15 +0.15 +0.25	1/12/67 -0.05 0 +0.10 +0.15 -0.25	15 days at 155°F., then 76 days at 30°C. and 100% R. H. +0.15 +0.25 +0.05	30 days at 155°F., then 179 days at 30°C. and 100% R. H. -0.10 +0.10 -0.05	3 days at -80°F., then 179 days at 30°C. and 100% R.H. +0.20 +0.10 +0.25	11/15/66 +0.05 +0.10 +0.15	11/16/66 +0.20 +0.10 +0.25	12/12/66 +0.20 +0.10 +0.20	12/28/66 +0.20 +0.20 +0.05	1/3/67 +0.30 +0.15 +0.50	1/12/67 +0.25 +0.25 +0.10
N/6 Sodium Lactate PL-146	6/10/66 0 -0.15 +0.20	9/12/66 -0.05 +0.40 -0.05	12/12/66 +0.20 +0.15 -0.15	9/12/66 +0.10 0 +0.30	12/12/66 +0.05 -0.20 +0.15	3 Months 0 +0.10 +0.15	6 Months +0.05 -0.20 +0.15	15 days at 155°F., then 76 days at 30°C. and 100% R. H. +0.35 +0.20 +0.15	30 days at 155°F., then 179 days at 30°C. and 100% R. H. +0.25 +0.05 +0.20	3 days at -80°F., then 179 days at 30°C. and 100% R.H. +0.10 -0.10 +0.40	9/12/66 +0.05 +0.20 +0.15	12/12/66 +0.10 +0.20 +0.40	12/28/66 0 -0.10 0	12/28/66 +0.20 +0.10 +0.15	1/3/67 +0.30 +0.20 +0.10	9/26/66 +0.10 -0.10 -0.15	10/11/66 +0.15 +0.15 +0.25	1/12/67 -0.05 0 +0.10 +0.15 -0.25	15 days at 155°F., then 76 days at 30°C. and 100% R. H. +0.35 +0.20 +0.15	30 days at 155°F., then 179 days at 30°C. and 100% R. H. +0.25 +0.05 +0.20	3 days at -80°F., then 179 days at 30°C. and 100% R.H. +0.10 -0.10 +0.40	11/15/66 +0.05 +0.10 +0.15	11/16/66 +0.20 +0.10 +0.25	12/12/66 +0.20 +0.10 +0.20	12/28/66 +0.20 +0.10 +0.05	1/3/67 +0.30 +0.15 +0.50	1/12/67 +0.25 +0.25 +0.10
5% Dex. in H <sub>2</sub> O PL-146	6/27/66 +0.10 +0.10 0	9/26/66 +0.15 +0.25 +0.10	12/28/66 +0.15 +0.20 +0.15	9/26/66 +0.10 -0.10 -0.15	12/28/66 +0.25 +0.10 +0.15	3 Months +0.10 -0.10 -0.15	6 Months +0.25 +0.10 +0.15	15 days at 155°F., then 76 days at 30°C. and 100% R. H. 0 -0.10 0	30 days at 155°F., then 179 days at 30°C. and 100% R. H. +0.10 0 +0.15	3 days at -80°F., then 179 days at 30°C. and 100% R.H. +0.25 +0.20 0	9/26/66 +0.10 -0.10 -0.15	12/28/66 +0.25 +0.10 +0.15	12/28/66 +0.10 0 +0.15	12/28/66 +0.25 +0.20 0	1/3/67 +0.30 +0.20 +0.10	9/26/66 +0.10 -0.10 -0.15	10/11/66 +0.15 +0.15 +0.25	1/12/67 -0.05 0 +0.10 +0.15 -0.25	15 days at 155°F., then 76 days at 30°C. and 100% R. H. 0 -0.10 0	30 days at 155°F., then 179 days at 30°C. and 100% R. H. +0.10 0 +0.15	3 days at -80°F., then 179 days at 30°C. and 100% R.H. +0.25 +0.20 0	11/15/66 +0.05 +0.10 +0.15	11/16/66 +0.20 +0.10 +0.25	12/12/66 +0.20 +0.10 +0.20	12/28/66 +0.20 +0.10 +0.05	1/3/67 +0.30 +0.15 +0.50	1/12/67 +0.25 +0.25 +0.10
5% Dex. in N.S. in H <sub>2</sub> O PL-146	7/6/66 +0.15 -0.05 +0.15	10/3/66 -0.15 -0.05 +0.10	1/3/67 +0.15 +0.15 +0.15	10/3/66 +0.20 +0.25 +0.05	1/3/67 0 +0.05 +0.10	3 Months +0.15 +0.05 +0.05	6 Months +0.10 +0.05 +0.10	15 days at 155°F., then 76 days at 30°C. and 100% R. H. +0.05 +0.30 +0.05	30 days at 155°F., then 179 days at 30°C. and 100% R. H. +0.30 +0.20 +0.10	3 days at -80°F., then 179 days at 30°C. and 100% R.H. +0.30 +0.15 +0.45	10/3/66 +0.05 +0.30 +0.05	1/3/67 +0.30 +0.20 +0.10	10/3/66 +0.20 +0.25 +0.05	10/11/66 +0.15 +0.15 +0.25	1/12/67 +0.45 +0.10 +0.10	10/3/66 +0.05 +0.30 +0.05	10/11/66 +0.15 +0.10 +0.15	1/12/67 +0.45 +0.10 +0.10	15 days at 155°F., then 76 days at 30°C. and 100% R. H. +0.05 +0.30 +0.05	30 days at 155°F., then 179 days at 30°C. and 100% R. H. +0.30 +0.20 +0.10	3 days at -80°F., then 179 days at 30°C. and 100% R.H. +0.30 +0.15 +0.45	11/15/66 +0.05 +0.10 +0.15	11/16/66 +0.20 +0.10 +0.25	12/12/66 +0.20 +0.10 +0.20	12/28/66 +0.25 +0.20 +0.05	1/3/67 +0.30 +0.15 +0.50	1/12/67 +0.25 +0.25 +0.10
N.S. in H <sub>2</sub> O PL-146	7/12/66 0 +0.25 0	10/11/66 +0.20 -0.05 +0.20	1/12/67 +0.10 -0.10 +0.20	10/11/66 +0.15 +0.15 +0.25	1/12/67 -0.05 0 +0.10 +0.15 -0.25	3 Months +0.20 -0.05 +0.25	6 Months -0.05 0 +0.10 -0.25	15 days at 155°F., then 76 days at 30°C. and 100% R. H. +0.15 +0.10 +0.15	30 days at 155°F., then 179 days at 30°C. and 100% R. H. +0.15 +0.10 +0.15	3 days at -80°F., then 179 days at 30°C. and 100% R.H. +0.15 +0.10 +0.15	10/11/66 +0.15 +0.15 +0.25	1/12/67 +0.10 -0.10 +0.20	10/11/66 +0.15 +0.15 +0.25	1/12/67 +0.15 +0.10 +0.15	1/12/67 +0.45 +0.10 +0.10	10/11/66 +0.15 +0.10 +0.15	10/11/66 +0.15 +0.10 +0.15	1/12/67 +0.45 +0.10 +0.10	15 days at 155°F., then 76 days at 30°C. and 100% R. H. +0.15 +0.10 +0.15	30 days at 155°F., then 179 days at 30°C. and 100% R. H. +0.15 +0.10 +0.15	3 days at -80°F., then 179 days at 30°C. and 100% R.H. +0.15 +0.10 +0.15	11/15/66 +0.05 +0.10 +0.15	11/16/66 +0.20 +0.10 +0.25	12/12/66 +0.20 +0.10 +0.20	12/28/66 +0.25 +0.20 +0.05	1/3/67 +0.30 +0.15 +0.50	1/12/67 +0.25 +0.25 +0.10

TABLE 2

TABLE II

Solution	Initial	30°C. and 100% R.H.						15 days at 15°F., then 76 days at 30°C. and 100% R.H.	30 days at 15°F., then 152 days at 30°C. and 100% R.H.	3 days at -80°F., then 179 days at 30°C. and 100% R.H.	One day at an air pressure of 5 lbs/in. <sup>2</sup> , then 182 days at 30°C. and 100% R.H.
		MOON TEMPERATURE		3 Months		6 Months					
		3 Months	6 Months	3 Months	6 Months	3 Months	6 Months				
10% Dex. in H <sub>2</sub> O FL-146	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	
M/6 Sodium Lactate FL-146	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	
5% Dex. in H <sub>2</sub> O FL-146	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	
5% Dex. in 0.9% NaCl FL-146	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	
0.9% NaCl in Water FL-146	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	Passed Test	

## STABILITY TEST RESULTS

TABLE II

PREPARATION OF SOLUTIONS FROM NON-POTABLE SOURCE

SOURCE WATER: Rock Creek Park

CONDITION: Solids in excess of 15,000 ppm.  
Pyrogens - Positive - killed rabbits.  
Culture - Positive growth on Thioglycollate in 12 hours.  
Plate THIC

PRODUCT PREPARATION: 5% Dextrose - Room Temp.

Date	No. Tests	Results	
		Pyrogens	Culture
22 May 68	20	Negative	Negative
31 May 68	24	Negative	Negative
7 June 68	20	Negative	Negative
12 June 68	19	Negative	Negative
20 June 68	17	Negative	Negative

CHEMICAL TESTS - USP - Within Limits

VOLUME - Within Limits

TABLE III

TABLE IV  
EFFICIENCY OF FILTER FOR REMOVING SUSPENDED MATERIALS

Code No.	Description	Lot No.	Treatment	10.0 u	5.0 u	3.5 u	2.0 u	n
LX-347EL	Dextrose, 5%	47X1A	Unfiltered	25 ± 3.21	251 ± 42.34	1,003 ± 113.33	8,606 ± 268.08	3
			Filtered	2 ± 0	2.5 ± 0.90	4.67 ± 2.87	36.63 ± 34.95	12
			Blank	2 ± 0	3.0 ± 1.4	6 ± 0	53 ± 4.24	2
			Bottle	1 ± 1.73	10.6 ± 12.5	37 ± 44.9	210 ± 236	
LX-348EL	Dextrose, 10%	47X7	Unfiltered	18 ± 5.00	251 ± 104.01	1,404 ± 306.71	21,339 ± 11,618.08	3
			Filtered	2 ± 0	3 ± 1.34	11.83 ± 9.8	133.42 ± 118.21	12
			Blank	2 ± 0	2 ± 0	2 ± 0	26 ± 8.48	2
			Bottle	3 ± 0	12.3 ± 2.31	33 ± 2.0	201 ± 39	
LX-331EL	Sodium Chloride, 0.9%	47X0A	Unfiltered	8 ± 4.51	113 ± 6.11	443 ± 45.65	3,010 ± 636.50	3
			Filtered	2 ± 0	3 ± 1.35	4.36 ± 1.75	19.11 ± 3.48	9
			Blank	2 ± 0	4 ± 0	5 ± 1.41	23 ± 1.41	2
			Bottle	2 ± 0	3 ± 1.0	7.6 ± 2.5	49 ± 31.6	
LX-349EL	Sodium Lactate, M/6	47X6A	Unfiltered	6	40	137	929	1
			Filtered	2 ± 0	2.67 ± 1.15	5.35 ± 2.31	26.67 ± 9.02	3
			Blank	2 ± 0	4 ± 0	5 ± 1.41	23 ± 1.41	2
			Bottle	3.3 ± 1.5	15.7 ± 6.4	55.7 ± 16.2	566 ± 62	

All values are mean ± S.D. (size stated and larger)

TABLE IV

Project 3A062110A816 MILITARY MEDICAL MATERIEL

Task 00, Military Medical Materiel

Work Unit 205, Military medical materiel

**Investigators.**

**Principal:** Edward C. Knoblock, COL, MSC  
Leo Kazyak, GS-13

**Associate:** Robert Permisohn, GS-09  
Edward L. Kirk, GS-05  
John O. Brown, SP5  
Norman West, SP4  
Morris Wilkinson, SP4

**Publications:**

None.

**PROJECT 3A062110A821  
COMBAT SURGERY**

**Task 00  
Combat Surgery**

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				DA 0A6466	69 07 01	RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	
69 01 31	D. Change	U	U	NA	HL	A USE UNIT	
NO. NO./CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	CLASSIFICATION			
b. PRIMARY	62110A	3A062110A821	00				
c. CONTINUING							
d. CONTINUING	1412A(2)						
1. TITLE (Provide the security classification code)							
(U) Wound healing (09)							
2. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>b</sup>							
003500 Clinical Medicine 012900 Physiology							
3. DUTY STATION		4. EMPLOYER EMPLOYMENT CODE		5. FUNDING AGENCY		6. PERFORMANCE STATUS	
09 54		NA		DA		C. In-House	
7. CONTRACT/GRANT							
Not applicable							
8. DATES/EFFECTIVE		9. EXPIRATION		10. FISCAL YEAR		11. PERIODIC REPORTS	
b. NUMBER <sup>c</sup>		4. AMOUNT		69		5 140	
c. TYPE		2. CURR. AMT.		70		3 180	
12. RESPONSIBLE OR ORGANIZATION		13. PERFORMING ORGANIZATION		14. PERFORMING ORGANIZATION			
NAME <sup>d</sup> Walter Reed Army Inst of Res		NAME <sup>d</sup> Walter Reed Army Inst of Res		NAME <sup>d</sup> Walter Reed Army Inst of Res			
ADDRESS <sup>e</sup> Washington, D. C. 20012		ADDRESS <sup>e</sup> Washington, D. C. 20012		ADDRESS <sup>e</sup> Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL		RESPONSIBLE INDIVIDUAL		RESPONSIBLE INDIVIDUAL			
NAME Meroney, COL W. H.		NAME Aaby, LTC Gene V.		NAME Aaby, LTC Gene V.			
TELEPHONE 202-576-3551		TELEPHONE 202-576-3791		TELEPHONE 202-576-3791			
15. GENERAL USE		16. GENERAL USE		16. GENERAL USE			
Foreign intelligence considered							
17. TECHNICAL SUBJECT TERMS AND REPORT NUMBER (U) Microangiography; (U) Pulmonary microemboli; (U) Pulmonary fat emboli; (U) Pulmonary contusion; (U) Shock; (U) Local Antibiotics; (U) Debridement; (U) Surface pH							
18. TECHNICAL OBJECTIVE <sup>a</sup> OR APPROXIMATE OBJECTIVE (Provide technical objectives identified by number. Provide text of each with security classification code.)							
23. (U) 1. To evaluate the contribution of microemboli, fat emboli, pulmonary contusion causing the "shock lung" syndrome. 2. To determine effect of local antibiotics in preventing sepsis in closed spaces. 3. To correlate adequacy of debridement with surface pH.							
24. (U) Pulmonary microcirculation insulted by fat emboli, microaggregations and contusion were studied by angiography, histology, and radioactive fat. Detailed pulmonary functions were studied in animals subjected to pulmonary contusion. A model for producing subdiaphragmatic abscesses in rabbits was made. High velocity wounds of extremities were produced in animals and debrided using the surface pH electrode as a guide to adequacy of debridement.							
25. (U) 69 01 - 69 06. Pulmonary and microemboli cause pulmonary hypertension, intrapulmonary shunting, elevation of pulmonary artery pressure and arterial hypoxemia. Pulmonary contusion causes intrapulmonary shunting and aspiration of blood secretions to the normal lung with extension of the picture of "shock lung." A known quantity of bacteria has been placed in the subhepatic space to produce an intraperitoneal abscess. Good model has now been established. The surface pH of devitalized muscle is acidotic. The technique for debridement, using a surface electrode, is too cumbersome for clinical application.							
For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.							

PII Redacted

Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 120, Wound healing

**Investigators.**

**Principal:** LTC Gene V. Aaby, MC

**Associates:** LTC Teruo Matsumoto, MC; MAJ Steven R. Wyle, MC; MAJ Roger V. Moseley, MC; MAJ Irwin R. Berman, MC; CPT Paul B. Lamborn, VC; MAJ Gary M. Nemhauser, MC; MAJ John N. Henry, MC

**1. Topical Use of Mandelamine for Wound Infection**

**a. Statement of the Problem:** Infection following combat wounds continues to be a major cause of morbidity.

**b. Background:** High velocity missile wounds often result in severe infection due to gross contamination, incomplete debridement, and the host-organism relationship. Topical antibiotics, sulfamylon, have been useful in controlling burn wound sepsis.

**c. Approach to the Problem:** The wounds of 250 patients from Vietnam were cultured in Japan and the wound organisms and antibiotic sensitivity determined. Mandelamine solutions at pH 6.0 and 6.8 were tested against various strains of organisms. Toxicology of mandelamine was tested by intraperitoneal injection in mice.

**d. Results and Discussion:** *S. aureus*, *P. aeruginosa*, *E. coli* were the major causes of wound sepsis. Mandelamine was highly effective against these organisms as tested by disc sensitivity and tube dilution.

**e. Conclusion:** Mandelamine and/or other agents may be useful in controlling wound sepsis.

**f. Recommendation:** The toxic effects of Mandelamine should be investigated in depth and a clinical trial considered using Mandelamine to control wound sepsis.

**2. Enzyme Fluctuation in Wounded Soldiers during the Convalescent Period.**

**a. Statement of the Problem:** Unrecognized sepsis and residual tissue injuries are often present in the convalescent period following combat wounds.

**b. Background:** It has been found that organ specific enzymes are released following injuries to the tissues. Serum enzyme determinations

are useful in discovering occult damage to specific organs or tissues and may elucidate a diagnostic dilemma.

c. Approach to the Problem: In 205 randomly selected patients with extremity wounds, the creatine phosphokinase (CPK), lactic dehydrogenase (LDH), and serum glutamic oxaloacetic transaminase (SGOT) were measured using the method described in the Sigma Technical Bulletin.

d. Results and Discussion: The CPK was elevated in 60 of 205 patients. The LDH was elevated in 58 of 140 and 49 patients had elevated SGOT. An elevated CPK was highly specific for soft tissue injury and persistent elevation in the convalescent period suggested wound sepsis. Pseudomonas aeruginosa was cultured from the wounds of 94.1% of the patients having an elevated CPK.

e. Conclusion: Serum enzyme determinations may have clinical applications in discovering smoldering sepsis and residual tissue damage.

f. Recommendation: A clinical study using several hundred patients should be developed to further determine the specificity of serum CPK elevation in septic extremity wounds.

### 3. Serum Creatine Phosphokinase in Soft Tissue Injury: An Indicator for the Best Time for Delayed Primary Closure (DPC)

a. Statement of the Problem: The proper time for DPC of a combat wound is based on clinical findings and the experience of previous wars. An objective test of the ideal time for wound closure is needed.

b. Background: Experience had demonstrated the wisdom of debriding combat wounds and leaving the wound open for delayed closure on the 4th to 6th post-injury days. The success of DPC depends upon the adequacy of initial debridement, the presence of necrotic tissue or wound sepsis at the time of procedure.

c. Approach to the Problem: Serum enzymes were measured in 40 patients with extremity wounds to determine if the CPK correlated with a clean wound or a wound with necrotic tissue or sepsis.

d. Results and Discussion: Thirty-three patients with normal CPK values had uneventful recovery following DPC. Seven patients with elevated CPK at the time of DPC developed wound complications, either infection or dehiscence. The CPK returns to normal following adequate debridement but remains elevated in the presence of sepsis or devitalized muscle.

e. Conclusions: The CPK may be of value in determining the proper time for DPC. An elevated CPK in the presence of extremity wounds suggests that a DPC is likely to fail.

f. Recommendation: The usefulness of CPF to prognosticate success of DFC should be tested in several hundred combat casualties.

#### 4. Tissue Adhesives and Wound Healing

a. Statement of the Problem: While the use of cyanoacrylates has been effective in life threatening situations, these agents are histotoxic and interfere with wound healing.

b. Background: Animal experimental work and clinical situations have demonstrated the usefulness of tissue adhesives in providing hemostasis of solid organs or large denuded surfaces, sealing cerebrospinal fluid fistula, and reinforcing precarious vascular anastomoses. The effect of tissue adhesives used for skin closure was investigated.

c. Approach to the problem: Bilateral skin incisions were made on the backs of 120 rats. One incision was closed by conventional suturing techniques and the other by various homologues of cyanoacrylates.

d. Results and Discussions: Tissue adhesives provide greater tensile strength during the first 4 to 5 days post-wounding. Thereafter, sutured wounds had a higher tensile strength. Histologic examination demonstrated interference of collagen bridging by the cyanoacrylates.

e. Conclusions: Cyanoacrylates may add strength to precarious or weakened suture lines. However, they interfere with wound healing and decrease tensile strength during later phases of wound healing.

f. Recommendation: Tissue adhesives should be used only upon indication for life-threatening situations.

#### 5. Peripheral Nerve Grafts: Experimental Studies in Dogs and Chimpanzees to Define Homograft Limitations

a. Statement of the Problem: Nerve homografts usually fail because they engender severe host reaction.

b. Background: Military conflicts and severe civilian accidents provide large numbers of patients with nerve deficits causing severe permanent disability. Loss of nerve substance precludes primary repair; accordingly, reconstruction by homograft is required.

c. Approach to the Problem: Radial nerve injuries in dogs were repaired by (1) autografts, (2) pre-degenerated grafts, and (3) irradiated homografts, all 2 cm. in length. The nerve anastomoses were standard techniques using Silastic cuffing procedure. In six chimpanzees both radial and peroneal nerve deficits were repaired using a homograft which was degenerated and irradiated.

d. Results and Discussion: The homograft is the best means of spanning irreducible nerve gap if an appropriate autograft is not available. Irradiation, and not pre-degeneration, was the determining factor in success of homografts. In chimpanzees, homografts of 4 cm. or less were 80% successful.

e. Conclusion: Proper surgical techniques, preparation of homografts, and length of homografts are important parameters for a successful nerve graft.

f. Recommendation: Additional investigation in nerve grafts is an important military project.

#### 6. Responses of Skeletal Muscle pH to Injury: A New Technique for Determination of Tissue Viability

a. Statement of the Problem: High velocity missiles cause tissue damage beyond the grossly visible evidence. The extent of debridement is determined by clinical criteria, i.e. color, consistency, contractility, and bleeding of skeletal muscle. An objective method of determining muscle viability is needed.

b. Background: Due to dissemination of lateral energy, when a high velocity missile passes through the tissue there is damage beyond the missile tract. Recognition of devitalized tissue has been based on a clinical impression and requires extensive experience. With improper debridement sepsis and poor wound healing are likely to occur. Excessive debridement results in loss of functional tissue and increased morbidity.

c. Approach to the Problem: Tissue surface pH is logically assumed to be related to the adequacy of tissue perfusion. Crush injuries were produced in skeletal muscle of rabbits and debrided using a surface electrode to measure the pH and debridement produced until a normal pH was encountered. Burn wounds were produced and also debrided using a surface pH electrode. These techniques were compared with the tissue staining results from intravenous Patent Blue V dye to determine the margin of nonviability.

d. Results and Discussion: Following wounding there is a decrease in surface pH probably due to accumulation of acid pH metabolites. The surface pH of charred muscle did not decrease and of interest 24 hours later there was an increase in the surface pH.

e. Conclusions: A surface electrode may be of value in determining viability of crushed or charred muscle and may be a guide to the extent of debridement. The technique is laborious and precludes extensive clinical application in combat wounds.

f. Recommendation: Objective means of determining tissue viability are needed. The development of techniques to determine rapidly non-viability of tissue is a project of high priority and military importance.

**7. Demonstration of Pulmonary Microemboli by a Microradiographic Technique.**

a. **Statement of the Problem:** The histologic picture of pulmonary microemboli and their physiologic significance have been the subject of much dispute.

b. **Background:** Pulmonary embolism from massive intravascular clots or microemboli from fat, platelet aggregates, particulate cellular matter, air, and debris from infused blood is a cause of significant morbidity and mortality in both military and civilian trauma. To understand the pathophysiology, the problem requires separation into its component parts and models developed to study each component.

c. **Approach to the Problem:** Pulmonary emboli were produced by injection of 200 mg. of microspheres into the ear veins of rabbits. After embolization, heparin, 1 mg/kg., was injected intravenously and the animal immediately sacrificed. The lungs were removed and the pulmonary arterial tree infused with micro-opaque suspension.

d. **Results and Discussion:** The pulmonary arterial circulation was demonstrated to the precapillary arterial level. The gross and histologic abnormalities were related to the size of microspheres injected.

e. **Conclusion:** The use of microspheres and radiographic techniques is a useful method in studying pulmonary embolism.

f. **Recommendation:** Expansion of this method may unfold additional pathophysiologic alterations of pulmonary embolism.

**8. Cyanoacrylate Issue Adhesives: Experimental and Clinical Evaluation.** 5, 8, 12, 13, 17, 19, 21

a. **Statement of the Problem:** In extensive trauma there may be uncontrolled bleeding from denuded surfaces, weak vascular anastomoses, severe wounds of solid organs, and fistula which cannot be ideally managed by standard suture techniques.

b. **Background:** In military and civilian trauma massive tissue loss and wounds of solid organs cause extensive blood loss which is difficult to manage rapidly by standard techniques. Excessive blood loss results often in a bleeding diathesis which compounds the problem. Following wounds of some organs, fistula may result from residual devitalized tissue or closure of defects with sutures under tension. In many difficult and often precarious situations, tissue adhesives have been useful in the experimental animal model and in man.

c. **Approach to the Problem:** The cyanoacrylates have been useful in experimental models to manage difficult surgical problems. Because

of histotoxicity various homologues have been developed. To preclude the deposits of large volumes of cyanoacrylates, a spray dispenser was developed. The various homologues were tested in multiple organs to determine histotoxicity and the feasibility of using tissue adhesives as an adjunct to standard surgical techniques.

d. Results and Discussion: As the length of the alkyl chain increases, there is a decrease in histotoxicity. Alpha cyanoacrylate causes an intense polymorphonuclear leukocyte reaction whereas the longer chain radicals cause a lymphocytic reaction. A Freon propellant was developed to spray the adhesives on solid organs or denuded surfaces. Using this technique a smaller quantity of monomer was deposited in or on the tissues resulting in less tissue reaction. In the laboratory n-butyl and iso-butyl monomers using Freon propellant have been useful in controlling hemorrhage from liver, kidney, spleen, and vascular anastomosis. Because of intense fibroblastic reaction and thrombosis engendered by the monomers, their use should be confined to situations where intense scarring does not pose a residual problem. These adhesives have been used in desperate clinical situations in the combat zone and in civilian surgical emergencies in which demise is threatened by continued blood loss. The most useful application has been for control of bleeding from the surface of solid organs or large oozing areas. Even in the presence of infection, butyl cyanoacrylate spray has effectively controlled the bleeding surface of liver.

e. Conclusions: Spray application of cyanoacrylates may be life-saving where oozing from bleeding surfaces cannot be managed by standard techniques. Even in the presence of infection, cyanoacrylate spray is efficacious. The intense scarring and tissue reaction limit its usefulness in vascular surgery where intravascular thrombosis may complicate the repair.

f. Recommendations: Long-term studies and follow up of cases treated with cyanoacrylate should be continued to determine the carcinogenicity potential of the cyanoacrylates and determine the best indications for this adjunct to suture techniques.

#### 9. Topical Antibiotics for Surface Wounds<sup>2, 3, 4, 7, 9, 11</sup>

a. Statement of the Problem: Parenteral antibiotics have not solved the problem of sepsis in large surface wounds. Burn wound sepsis has been controlled by the use of Sulfamylon and similar drugs. Since sepsis is a significant cause of morbidity following combat wounds, topical antibiotics may be useful as a prophylaxis or therapeutic regimen.

b. Background: Sepsis has been the major cause of morbidity and mortality following resuscitation and surgical treatment of wounds in Vietnam. This is due to the nature of the high velocity wound which causes tissue destruction beyond the grossly visible evidence of damage and is likely to result in inadequate debridement. Land mine injuries

and booby trap wounds cause severe gross contamination and are likely to develop wound sepsis particularly if there is several hours' delay between wounding and debridement. Sepsis is related to the nature of the wound, the type of contaminating organisms, the adequacy of debridement and the host organism relationship.

c. Approach to the Problem: Soil from various troop concentrations in Vietnam was cultured for predominating organisms. The bacterial flora of 112 combat wounds was determined. A standard rabbit model was developed to simulate a combat wound which was infected and debrided by standard techniques then treated with many combinations of topical antibiotics.

d. Results and Discussion: The organisms found in infected wounds in Vietnam are similar to those found in other conflicts and in civilian wounds, i.e. Staph. aureus, pseudomonas, Aerobacter aerogenes, E. coli, and Proteus. In the experimental model and in clinical cases where there was a delay between wounding and debridement or when there was inadequate debridement several types of topical antibiotics were found to decrease the incidence of wound sepsis. Rapid helicopter evacuation in Vietnam has reduced the lag time between wounding and treatment to the shortest period in military history. Accordingly, the application of topical antibiotics in the field may have limited usefulness. When the tactical situation precludes prompt evacuation or large numbers of casualties overwhelm the treatment facility and result in a prolonged lag time between injury and debridement, the application of antibiotics directly to large extremity wounds may reduce the incidence of wound sepsis.

e. Conclusion: In the presence of contaminated wounds in which there is a delay between wounding and debridement or in which sepsis has developed following debridement, topical antibiotics may be beneficial.

f. Recommendation: Sepsis is a major cause of morbidity and mortality in military and civilian trauma. Additional investigations are warranted to untangle the problem, to discover the separate factors which are responsible for sepsis, and to determine the role of both parenteral and topical antibiotics for prophylaxis, or therapy of wound sepsis.

Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 120, Wound healing

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. ACTIVITY ACQUISITION	2. DATE OF SUMMARY	REPORTS COVERED BY THIS	
				DA 0A6467	69 07 01	DD FORM (L1) 655	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ACTY	6. WORK SECURITY	7. REGIONS	8. DA BLDG INSTN	9. SPECIFIC DATA CONTRACTOR ACCESS	10. LEVEL OF R&T WORK UNIT
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11. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
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b. CONTRIBUTING							
c. COOPERATING	1412A(2)						
11. TITLE (Provide with Security Classification Code)							
(U) Responses to trauma (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS							
003500 Clinical Medicine 012900 Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE SYSTEM	
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17. CONTRACT/GRANT Not applicable				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
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b. NUMBER				69		3	
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20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Inst of Res				NAME: Walter Reed Army Inst of Res			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence considered				ASSOCIATE INVESTIGATOR			
				NAME: Scheetz, LTC W. L.			
				NAME: Matsumoto, LTC T. DA			
22. REVIEWED (Provide with Security Classification Code) (U) Hemorrhagic shock; (U) Endotoxic shock; (U) Microcirculation; (U) Alpha Blocking Agents; (U) Venous Prosthesis; (U) Impedance as a Measure of Thoracic Volume Changes							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with security classification code.)							
23. (U) To improve the methods of diagnosis and treatment following severe injury and shock; to reduce morbidity and mortality associated with combat wounds.							
24. (U) Study of hemorrhagic shock in laboratory animals using standard model measuring cardiac output, oxygen consumption, pH, carbon dioxide pressure, oxygen pressure, lactate, pyruvate, LDH, SGOT, SGPT, isozyme pattern from heart, lung, liver, small bowel, skeletal muscle, effect of increased intracranial pressure upon surface pH of brain and upon cardiopulmonary dynamics. The intrathoracic fluid volume may be measured by electrical impedance through the thoracic cavity.							
25. (U) 69 01 - 69 06. Animal studies show tissue hypoxia is responsible for metabolic acidosis and clinical picture of shock. Severe vasoconstriction after resuscitation impedes blood flow and contributes to demise. The use of alpha blocking agents at the appropriate time with fluid replacement prevents "irreversible shock." Overhydration, overtransfusion, and pulmonary edema increase impedance and reflect intrathoracic volume changes prior to elevation of central venous pressure. Intrathoracic impedance may be a more sensitive measurement of impending pulmonary edema than central venous pressure or right ventricular pressure.							
For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.							

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Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 121, Responses to trauma

**Investigators.**

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**1. Pathologic Anatomy of Lungs following Shock and Trauma<sup>5</sup>**

**a. Statement of the Problem:** In both civilian and military trauma pulmonary complications are a major cause of morbidity and mortality.

**b. Background:** Study of morbidity and mortality statistics in Vietnam revealed that the most common cause of death following successful resuscitation and surgical treatment of combat wounds was pulmonary insufficiency. Any factor which interferes with ventilation, perfusion, or diffusion may be responsible for hypoxemia. Accordingly, airway obstruction, aspiration, retained tracheobronchial secretions causing atelectasis, pulmonary emboli, increased pulmonary vascular resistance, and myocardial failure may be responsible for hypoxemia.

**c. Approach to the Problem:** The gross and microscopic autopsy findings of 100 casualties who died following shock and/or severe trauma were carefully studied.

**d. Results and Discussion:** The incidence of pulmonary lesions was acute congestion, 46; bronchopneumonia, 39; thromboemboli, 24; pulmonary edema, 58; pleural effusion, 42; hyaline membrane, 14; chronic pneumonia, 4; hemothorax, 9; pulmonary hemorrhage, 19; tracheostomy, 49; fat emboli, 12; atelectasis, 10. The precise cause of pulmonary hyaline membranes is unknown. Clinical records of five cases demonstrated a picture of acute respiratory distress and blood gas analysis suggested an alveolar capillary block. The use of high concentrations of oxygen in the inspired air may also be a contributing factor to the formation of hyaline membranes.

**e. Conclusion:** Pulmonary insufficiency is a common problem following severe trauma and has multiple etiologies.

**f. Recommendation:** Laboratory models should be developed to study each of the contributing factors to pulmonary insufficiency.

<sup>1</sup>Commanding General, U. S. Army Medical Command, Japan

## 2. Adult Hyaline Membrane Disease: Relationship to Oxygen Therapy

a. Statement of the Problem: A diffusion block occurs in patients dying after prolonged respiratory support.

b. Background: Hyaline membrane formation is a response to pulmonary injury of various etiologies. The cause and effect relationship between oxygen therapy and hyaline membrane formation is apparent in laboratory models. Autopsy findings also show an association between prolonged oxygen therapy and hyaline membrane formation in humans.

c. Approach to the Problem: The histologic findings in the lungs of six patients dying with progressive pulmonary insufficiency due to alveolar capillary diffusion problems were studied.

d. Results and Discussion: Hyaline membranes are a conspicuous feature of prolonged oxygen therapy. The membranes may consist of fibrin, fat, hemoglobin, mucus, and proteinaceous material. Electron microscopic examination showed swelling of mitochondria in altered alveolar cells and damage to alveolar cells which produce surfactant. Once alveolar capillary block is initiated the process is self-perpetuating and requires increased concentration and pressure of inspired oxygen to maintain satisfactory arterial  $pO_2$ .

e. Conclusion: Prolonged oxygen therapy has been indicted as a cause of hyaline membrane formation and histologic examination of the lungs of patients dying in pulmonary insufficiency tends to support the indictment.

f. Recommendation: The etiology of respiratory insufficiency needs further study.

## 3. Cerebral Acidosis: Precursor to the Pressor Response to Increased Intracranial Pressure

a. Statement of the Problem: To identify alterations in cerebral perfusion which accompany elevated intracranial pressure.

b. Background: Surface pH of organs and tissues is related to the adequacy of perfusion of the tissue being studied. A relationship of cerebral perfusion to initiation of the Cushing response has been suggested but not directly identified.

c. Approach to the Problem: Sequential measurement of pH of cerebral surface and arterial blood was correlated with hemodynamic alterations and ventricular pressure. Intracranial pressure was elevated with an extradural balloon.

d. Results and Discussion: Cerebral surface acidosis regularly preceded the arterial pressor response. Fall in cerebral surface pH was greater than fall in blood pH.

e. Conclusions and Recommendation: Cerebral acidosis reflects cerebral hypoperfusion which precedes the Cushing response. Rise in arterial pressure, therefore, represents adaptation by the organism to increase flow to the ischemic brain.

#### 4. Microaggregation in Bank Blood

a. Statement of the Problem: To investigate microaggregation accompanying blood storage with acid citrate dextrose (ACD) and ACD-adenine.

b. Background: Intravascular microaggregation has been identified in combat casualties as a factor contributing to pulmonary insufficiency after trauma.

c. Approach to the Problem: Adenosine diphosphate (ADP), serotonin, and aggregation were serially determined in 10 units of bank blood preserved with either ACD or ACD-adenine.

d. Results and Discussion: Increase in aggregation occurs in blood preserved with ACD or ACD-adenine. This is associated with a rise in ADP levels. Adenosine diphosphate added to platelet concentrates increases aggregation proportionate to platelet count. Stored platelet concentrates produce aggregation of blood elements without addition of ADP.

e. Conclusion and Recommendation: Microaggregation in bank blood is the result of platelet disintegrates independent of ADP in the stored blood. Large transfusion of bank blood may, however, enhance aggregation of patient's platelets by infusion of ADP.

#### 5. Impedance Plethysmography

a. Statement of the Problem: The measurement of cardiac output and central blood volume by standard techniques has many disadvantages. Impedance plethysmography may satisfy the requirement for a simple method.

b. Background: Impedance plethysmography has been employed as a method for estimating cardiac output obviating the necessity of blood sampling or vessel cannulation. Impedance changes are due to changes in the volume of blood within the area being evaluated, i.e. the thoracic cavity. Studies by others suggest to us that the method may be of value in determining an accumulation of fluid in the lung parenchyma or pleural cavity.

Post-traumatic pulmonary problems are a major cause of death in patients with combat injuries. Some of these problems may be related to interstitial pulmonary edema that is undetectable by clinical or radiographic means. For this reason, impedance measurements are indicative of changes in intrapulmonary volume, the method could be valuable as a clinical adjunct in the study and care of patients in Vietnam.

c. Approach to the Problem: Fifteen beagle dogs were anesthetized with sodium pentobarbital and subjected to acute intravascular overload with 200 cc/kg of normal saline containing 25 gm human albumin per liter. Transthoracic impedance, blood volume and hemodynamic measurements were sequentially determined.

d. Results and Discussion: Acute intravascular overload resulted in increased cardiac output, decreased peripheral resistance, and peripheral and pulmonary arteriovenous shunting. Fall in transthoracic impedance and pulmonary edema regularly accompanied infusion of fluid. Fall in transthoracic impedance is the result of increased intrathoracic conductivity due to increased electrolyte containing fluid within the thoracic cavity.

e. Conclusion and Recommendation: Transthoracic impedance falls in a predictable manner with acute intravascular overload. Measurements of electrical impedance would appear to be as sensitive as central venous pressure measurements. The method appears to have merit as an investigative tool and as an additional means of better patient monitoring.

## 6. The Etiology of Hypoxemia during Aeromedical Evacuation

a. Statement of the Problem: Hypoxemia may occur during aeromedical evacuation.

b. Background: It has been reported that certain patients arrive in Japan from Vietnam with low total blood volume. This study was designed to evaluate this problem. Information is lacking on the physiological effects of aeromedical evacuation on battle casualties. This study was designed to assess this sphere of interest.

c. Approach to the Problem: Seventy-two patients were studied at the 24th Evacuation Hospital, Republic of Vietnam, and following evacuation at the 249th General Hospital, Japan. Cr<sup>51</sup> red cell mass (RCM) and total blood volume (TBV) were determined pre- and post-evacuation. Arterial blood gases, pH, and % saturation were determined pre-flight, in-flight and post-flight during their aeromedical evacuation from Vietnam to Japan.

d. Results and Discussion: The results of these studies indicate that the total blood volume of the majority of combat casualties evacuated from Vietnam to Japan remained unaltered. The very seriously injured (VSI) patients evacuated demonstrated the following: (1) a mean deficit of 500 cc in TBV before leaving Vietnam, and (2) no further increase in this deficit upon arrival in Japan that did not already exist in Vietnam with the following exceptions: (a) patients with tracheostomies showed a decreased plasma volume, (b) septic patients showed both a decreased RCM and decreased plasma volume, (c) fractured femurs and multiple lower extremity fractures showed a decreased RCM, (d) spinal cord and associated injuries showed a decreased RCM and decreased plasma volume, (e) extensive abdominal injuries showed both a decreased RCM and decreased plasma volume.

Arterial blood gases, pH, and % saturation were determined pre-flight, in-flight and post-flight during their aeromedical evacuation from Vietnam to Japan. The findings were a severe hypoxemia occurring in the seriously injured and very seriously injured groups. The lowest recorded  $pO_2$ s were found in the fractured femurs, multiple lower extremity fractures and the abdominal cases. Parameters to assess the status of hydration show that these patients receive ample fluid during their evacuation.

e. Conclusion and Recommendations: It is recommended that in the above-mentioned VSI group more attention to replacement of their volume deficits be accomplished prior to evacuation.

Conclusions and recommendations will be given after thorough statistical appraisal of these data.

## 7. Hyperglycemic Response to Trauma in Combat Casualties

a. Statement of the Problem: Severe trauma causes many changes in metabolic pathways.

b. Background: Hyperglycemia is a recognized metabolic response to trauma, hemorrhage and shock. Several investigators have demonstrated that acute hyperglycemia will increase cardiac output, decrease peripheral vascular resistance, and favorably influence survival rates of animals following hemorrhagic shock.

c. Approach to the problem: The pH lactate and blood glucose were measured in 67 battle casualties upon arrival in the receiving ward and prior to institution of resuscitation.

d. Results and Discussion: In casualties with severe trauma having lactates over 20 mg. % the blood glucose was elevated. Of 15 casualties with an average lactate of 43 mg. %, the blood glucose was 256 mg. %. The data support the laboratory evidence that hyperglycemia is a response to trauma in man and that the magnitude of hyperglycemia is related to the severity of trauma. This response may represent the body's attempt to increase the availability of glucose to the cell and provide adequate energy substrate to meet the increased metabolic demands that trauma imposes.

e. Conclusion: Elevation of blood glucose is a documented response to severe trauma in man.

f. Recommendation: Alterations in the metabolic pathways engendered by severe trauma should be studied in combat casualties in Vietnam.

Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 121, Responses to trauma

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RESEARCH AND TECHNOLOGY WORK SHEET SUMMARY				DA 0A6468	69 07 01	REPORT SYMBOL CODE RD-68R 250 600	
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B. CONTRIBUTING							
C. IDENTIFYING	1412A(2)						
1. TITLE (Provide full security classification code)							
(U) Experimental anesthesia (09)							
2. SCIENTIFIC AND TECHNOLOGICAL AREA							
002400 Bioengineering		012900 Physiology		008800 Life Support			
3. PART DATE		4. NUMBER OF EXPERIMENT DAYS		5. PERIODICITY		6. PERFORMANCE STATUS	
05 58		NA		DA		C. In-House	
7. CONTRACT/GRANT Not applicable				8. PERFORMED OUTSIDE			
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9. RESPONSIBLE DIS ORGANIZATION				10. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Inst of Res				NAME: Walter Reed Army Inst of Res			
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11. GENERAL USE				12. INTEREST INVESTIGATORS			
Foreign intelligence considered				NAME: Wyte, MAJ S. R.			
				DA			
13. REVISIONS (Provide full security classification code) (U) Mechanical ventilation; (U) Oxygen toxicity; (U) Pulmonary insufficiency; (U) Ventilation/perfusion inequality; (U) Pulmonary blood flow							
14. TECHNICAL OBJECTIVE, DA APPROACH, OR PROGRAM (Provide detailed paragraph identified by number, provide end of each with security classification code)							
23. (U) 1. To improve cardiopulmonary care of critically ill patients. 2. To study effects of prolonged administration of high concentrations of oxygen and mechanical ventilation on pulmonary function, and 3. to study characteristics of pulmonary arterial blood flow and pulmonary surface activity in atelectatic and re-expanded lung of the dog.							
24. (U) In conjunction with Dept. of Human Studies, Div. of Surgery, WRAIR, and Dept. of Anesthesia, WRGH, a study of pulmonary function and ventilation/perfusion inequalities in postoperative and critically ill patients is in progress. With the use of a specially designed endobronchial tube which divides the two lungs into separate units, the effects of differential oxygen tension upon the alveolar unit is being studied. Various oxygen tensions and ventilatory patterns will be investigated. Arterial blood gases, blood flow, light microscopy and surfactant activity will be monitored. Changes in function, residual capacity, compliance and resistance will be followed.							
25. (U) 69 01 - 69 06. The measuring of pulmonary artery blood flow by implanting electromagnetic flow probes on the main pulmonary artery and the left branch is progressing slowly because of lack of equipment. A number of animals expired 10 days to 3 weeks following implantation of the flow probes because of rupture of the main pulmonary artery. It is believed that this problem can be overcome by first covering the artery with teflon mesh before implanting electromagnetic flow probe. Mechanical ventilation of the lungs with double lumen endobronchial tubes to study the effect of high oxygen concentration and hypoxic mixtures of oxygen on each lung separately is in progress.							
For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.							
*Amplitude is constant over all time's covered.							

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Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 122, Experimental Anesthesia

**Investigators**

Principal: LTC Edgar O. Yhap, MC

Associate: MAJ Steven R. Wyte, MC

**1. Ventilator.**

a. Statement of the Problem: To develop a volume-cycled respirator with pressure cycling capabilities which can assist or control a patient's ventilation and satisfy the unique requirements of the Army.

b. Background: A volume-cycled respirator with capabilities of assisting or controlling a patient's ventilation was developed by Harry Diamond Laboratories and tested at the Walter Reed Army Institute of Research and in Viet Nam. Reports from testing facilities show that some modifications in the design must be made to improve performance. These modifications were:

(1) A better calibration of total volume and air-oxygen mixing volume.

(2) The large compression effect when the machine is working against severe compliance resistance load.

c. Results: The present Army volume-cycled respirator has greater overall performance capabilities as compared to the previous model. A new air-oxygen mixing valve was designed, fabricated and tested. A new trigger arrangement to reverse the function of the existing trigger was made. The purpose of this change was to reduce the internal compliance of the respirator by insuring that the bellows would be completely collapsed.

d. Conclusions: A volume-cycled ventilator is being operated in Viet Nam at present to test the new breathing valve, mixing valve and overall performance

e. Recommendation: Continued field testing of this ventilator is needed in order that proper evaluation can be made as to its capabilities and usefulness for the Army.

**2. Pulmonary Blood Flow Measurement.**

a. Statement of the Problem: Measurement of pulmonary blood flow using chronically implanted electromagnetic flow probes is a problem

because of occlusion or rupture of the pulmonary artery.

b. Background: Pulmonary artery blood flow to individual lungs following atelectasis has been indirectly measured. No one has attempted to measure pulmonary artery blood flow following re-expansion of a chronically collapsed lung. This study will attempt to measure directly the blood flow following collapse of a lung and re-expansion of a previously collapsed lung by the use of implanted electromagnetic flow probes. This study has been attempted on ten dogs.

c. Results: The electromagnetic flow probes have been implanted on the main pulmonary arteries and the left pulmonary artery of ten dogs. All the dogs died within 10 - 21 days because of rupture of the pulmonary vessels. It is believed that this problem can be solved by first wrapping the artery with teflon mesh. Several investigators who attempted to implant electromagnetic flow probes on the pulmonary arteries have reported similar results.

d. Conclusions and Recommendations: Teflon mesh will be used to wrap the vessels before implanting the electromagnetic flow probes. The experiment has been delayed pending the arrival of the teflon mesh and the electromagnetic flow meter.

### 3. Oxygen Toxicity.

a. Statement of the Problem: It is not known whether O<sub>2</sub> toxicity is a local problem caused by irritation of the pulmonary parenchyma by high O<sub>2</sub> concentration or whether it is a result of high pO<sub>2</sub>. A model to study the effect of high oxygen concentration is being devised.

b. Background: Prolonged exposure to high oxygen concentration in the lungs has resulted in pulmonary dysplasia, pulmonary insufficiency and death. What is not known is whether the pulmonary morphologic changes of O<sub>2</sub> toxicity is a local phenomenon or a result of high arterial pO<sub>2</sub>. This study will attempt to produce the classic pulmonary morphologic changes of O<sub>2</sub> toxicity in one lung.

c. Results: Results from two dogs following exposure of one lung to 100% O<sub>2</sub> for twenty-four hours and the controlled lung to room air, showed areas of atelectasis in both lungs.

d. Conclusions and Recommendations: The atelectasis seen in both lungs was probably due to prolonged ventilation. The studies have not been performed long enough for conclusions to be drawn from the results obtained. Recommend these studies be continued for much longer periods until one lung or both lungs present clinical and morphologic signs of pulmonary insufficiency.

### 4. Pulmonary Hypoxemia.

a. Statement of the Problem: Pulmonary hypoxemia causes an increased pulmonary vascular resistance and bronchiolar constriction.

b. Background: It is known that hypoxemia will cause pulmonary vasoconstriction, bronchospasm, and increase pulmonary vascular resistance. Using a double lumen canine endobronchial tube which divides the two lungs into separate units, the effects of differential oxygen tension upon the alveolar unit will be studied. The arterial oxygen tension will be kept at a normal level.

c. Results: Study to be started in August 1969.

5. Cardiopulmonary Insufficiency in the Intensive Care Unit.

a. Statement of the Problem: Respiratory inadequacy is a major problem of surgery, trauma and disease.

b. Background: In the Intensive Care Unit only two patients were studied. Both patients had low  $O_2$  tension on 50%  $O_2$  and increase shunting. One patient had an increase VD/VT ratio of .5 and required a tracheostomy and assisted ventilation. Both patients survived and were transferred to their respective wards.

c. Results: Postoperative and severely ill patients have low  $O_2$  tension when breathing room air, an increase in ventilation/perfusion ratio, and an increase in shunting.

d. Conclusions and Recommendations: Because of delay in arrival of equipment, we were unable to perform other studies. However, the results obtained have demonstrated the necessity to continue these studies.

Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 122, Experimental Anesthesia

Bibliography: None

**PROJECT 3A062110A822**  
**MILITARY INTERNAL MEDICINE**

**Task 00**  
**Military Internal Medicine**

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION# DA 0A6469	2. DATE OF SUMMARY 69 07 01	REPORT CONTROL SYMBOL DD-225 (AT) 695	
3. DATE PREV SUMMARY 31 01 69	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. RESEARCH# NA	8. DISSEM INSTR# NL	9. SPECIFIC DATA* CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES: PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY 62110A		3A032110A822		00	120		
b. CONTRIBUTING							
c. COUNTERMEASURES 1412A(2)							
11. TITLE (Precede with Security Classification Code) (U) METABOLIC RESPONSE TO DISEASE AND INJURY (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS 012900 PHYSIOLOGY      003500 CLINICAL MEDICINE      002300 BIOCHEMISTRY							
13. START DATE 08 63		14. ESTIMATED COMPLETION DATE NA		15. PERFORMING AGENCY DA		16. PERFORMANCE METHOD C. In-House	
17. CONTRACT/GRANT a. DATES/EFFECTIVE: NA      b. NUMBER: c. TYPE: d. KIND OF AWARD:				18. RESOURCES ESTIMATE a. PERSONNEL ESTIMATE b. PROFESSIONAL MAN YRS c. FUNDING (\$ thousands)		19. FISCAL YEAR	
				69		20	
				70		365	
20. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research ADDRESS: Washington, D.C. 20012				21. PERFORMING ORGANIZATION NAME: Walter Reed Army Institute of Research Division of Medicine ADDRESS: Washington, D.C. 20012			
22. RESPONSIBLE INDIVIDUAL NAME: Maroney, COL W. H. TELEPHONE: 202-576-3551				23. PRINCIPAL INVESTIGATOR (Precede with U.S. Security Classification) NAME: Teschan, COL P. E. TELEPHONE: 202-576-3529 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
24. GENERAL USE Foreign Intelligence Considered				25. ASSOCIATE INVESTIGATORS NAME: Gilliland, LTC P. F. NAME: Rosenfeld, MAJ P. E.      DA			
26. KEYWORDS (Precede with Security Classification Code) (U) Endocrine; (U) Carbohydrate; (U) Insulin; (U) Hormones; (U) Stress							
27. TECHNICAL OBJECTIVE, 28. APPROACH, 29. PROGRAM (Precede individual paragraphs identified by number, precede text of each with Security Classification Code) 23. (U) - Investigation into basic mechanisms of endocrine disease and the endocrine role in homeostasis during stress of disease and injury to provide rational approach to therapy.  24. (U) - Development and application of hormone assays (radioimmunoassay), competitive protein binding, etc. to study carbohydrate metabolism, anabolism, water and mineral metabolism in endocrine dysfunction and stress.  25. (U) - 69 01 - 69 06. Immunoassay of insulin and growth hormone approximate 2,000 assays per month. Insulin response and glucose utilization was better after oral than peritoneal glucose in uremics on dialysis. Seven of 14 normal women showed impaired glucose utilization and insulin secretion on oral contraceptives. A high molecular weight insulin has been separated from normal insulin by sephadex chromatography of serum from patients with uremia, acromegaly, obesity and from women on oral contraceptives. Biologic activity of this "Big" insulin is being evaluated. Insulin in plasma and bile have varied inversely following oral glucose and animal studies of hepatic insulin clearance are in progress. Insulin-hypoglycemia in thyrotoxic patients produced a normal rise in growth hormone, and IV glucagon did not elevate growth hormone consistently. Exercise stress in a hot climate elevated growth hormone but not 17 OHCS after physical conditioning. The cause of elevated estrogen excretion in males with lung tumors and osteoarthropathy or ectopic HCG production is under study. Evaluation of ACTH and growth hormone reserve by pseudomonas polysaccharide (Piroman <sup>®</sup> ), insulin-hypoglycemia arginine and metyropone in 27 patients showed a high incidence of divergent results. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

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Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 120, Metabolic response to disease and injury

Investigators: COL Paul E. Teschan, MC; LTC Paul F. Gilliland, MC;  
MAJ Paul S. Rosenfeld, MC; Elliot Danforth, Jr.,  
M.D.; Marcus Schaaf, M.D.; LTC Coy T. Fitch, MC;  
Joseph Bruton, Ph.D.

Development of Radio-immunoassay: Specific, sensitive assays for insulin and growth hormone have been automated, providing up to 2,000 assays per month in support of investigational work. Competitive protein binding assays for plasma thyroxine of cortisol are operational and assays for 11-deoxycortisol and testosterone are under development.

Big and Little Insulin: Insulin with higher molecular weight than normal insulin has been separated by sephadex chromatography of plasma from steroid-treated subjects and patients with acromegaly, uremia, and obesity. In vitro studies of its biologic potency are in progress.

Hepatic Regulation of Plasma Insulin: Insulin in plasma and bile (T-tube drainage) have varied inversely following oral glucose in four patients. Animal studies of hepatic clearance of insulin are in progress.

Carbohydrate Intolerance in Pheochromocytoma: Insulin secretion was normal after IV arginine but impaired after oral glucose prior to removal of a pheochromocytoma.

Carbohydrate Intolerance in Women on Oral Contraceptives: Seven of fourteen women with normal initial plasma insulin and growth hormone response to glucose and arginine showed impaired glucose tolerance with late insulin peak and increased quantities of big insulin. <sup>2,3</sup>

Glucagon Stimulation of Growth Hormone produced no consistent rise contrary to recent reports.

Growth Hormone Response to Insulin-induced Hypoglycemia in Thyrotoxicosis was normal in 10 patients contrary to published data. <sup>4</sup>

Tests of Growth Hormone and ACTH Reserve in over 27 subjects by pseudomonas polysaccharide (Piromen<sup>®</sup>), insulin-hypoglycemia, arginine, and metyrapone showed a high incidence of divergent test results. <sup>5,6</sup>

K<sup>40</sup> Counting of Total Body Potassium: Potassium changes measured by external balance and K<sup>40</sup> counting were similar in a normal subject and in a patient recovering from gastrointestinal K depletion. Serial K<sup>40</sup> counts proved helpful in detecting developing K depletion in the latter patient.

Excessive Estrogen Production in Males with Lung Tumors: High estrogen excretion was attributed to ectopic HCG production in one patient and associated with pulmonary osteoarthropathy in two patients. Studies of the source of estrogen production are in process.

Carbohydrate Intolerance in K Depletion: Plasma glucose and insulin response to glucose, arginine and glucagon before and after K depletion (quantitated by external balance and  $K^{40}$  counting) has been studied in two normal subjects. Four additional studies are scheduled.

## BIBLIOGRAPHY

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 120, Metabolic response to disease and injury

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY	REPORT COVERING PERIOD	
				DA 066445	69 07 01	DD FORM 1 (ARJ 68)	
3. DATE PREP. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ACTV.	6. WORK UNIT	7. RESEARCH	8. BUDGETARY	9. SPECIFIC DATA CONTRACTOR ACCESS	10. LEVEL OF WORK UNIT
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11. NO./CONTR.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62110A	3A062110A022	00	121			
B. CONTRIBUTING							
C. CONTRACT	1412A(2)						
12. TITLE (Print in full except Classification Code)							
(U) Pathogenesis of Enteric Disease (09)							
13. SCIENTIFIC AND TECHNOLOGICAL AREAS							
010100 Microbiology							
14. START DATE		15. ESTIMATED COMPLETION DATE		16. PERFORMING AGENCY		17. PERSONNEL CATEGORY	
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A. NUMBER		A. AMOUNT		69		4	
B. TYPE		B. CURR. AMT.		70		4	
C. KIND OF AWARD		C. CURR. AMT.				115	
21. RESPONSIBLE DOD ORGANIZATION				22. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research ADDRESS: Washington, D C 20012				NAME: Walter Reed Army Institute of Research ADDRESS: Div of CD and I Washington D C 20012			
23. RESPONSIBLE INDIVIDUAL				24. PRINCIPAL INVESTIGATOR (Print in full if U.S. Applicant Institution)			
NAME: Meroney, COL W.H. TELEPHONE: 202-576-3551				NAME: Forman, S. B. TELEPHONE: 202-576-3344 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
25. GENERAL USE				26. ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Labrac, E.H. DA			
27. KEYWORDS (Print in full except Classification Code)							
(U) Diarrhea, (U) Dysentery, (U) Bacillary, (U) Salmonellosis, (U) Immunity, (U) Immunisation							
28. TECHNICAL OBJECTIVE, OR APPLICABLE PROGRAM (Print in full except paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) To find improved procedures to control diarrheal disease. Present work involves the testing of oral vaccines against bacillary dysentery, and evaluations of the importance of intestinal immunity.							
24 (U) Attenuated dysentery strains are being developed. They are being evaluated for safety in several systems and are being treated for potency in monkeys and in man.							
25 (U) 69 01 - 69 06. The antibody response in monkeys fed a living oral attenuated dysentery vaccine is associated with the immunoglobulin M class of antibody globulins. This type of response did not change in animals studied 17 to 19 days after a course of oral immunisation. Fluorescent antibody studies of antibody forming cells in the intestinal tract of these monkeys showed the cells to be non-functional at 17 to 19 days. A chromosomal locus for penetration of epithelial cells by strains of Shigella flexneri has been found near the purine E locus. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.							

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DD FORM 1 (ARJ 68) (FOR ARMY USE)

Project 3A062110A822, MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 121, Pathogenesis of enteric disease

**Investigators.**

Principal: Samuel B. Formal, Ph.D.

Associate: Eugene H. LaBrec, Ph.D; MAJ Thomas Lawrence, MC;  
L. S. Baron, Ph.D.; Peter Gemski, Ph.D.

**Description.**

The pathogenesis of enteric disease is studied to elucidate the mechanisms by which enteric pathogens produce symptoms. By understanding the disease process improved procedures for prevention and treatment of diarrheal diseases will become evident.

**Progress.**

1. Previous experience has shown that an avirulent mutant strain of S. flexneri 2a reverted to a virulent form when fed to volunteers in doses above  $1 \times 10^8$  organisms. Last year this avirulent mutant strain was hybridized with E. coli so that the female shigella strain incorporated the xylose-rhamnose region of the E. coli chromosome into its genome. This strain protected monkeys against experimental challenge. This year the mutant hybrid strain has been tested for safety in volunteers (by Dr. Richard Hornick) and has not elicited reactions after doses of  $5 \times 10^{10}$  living organisms.

2. Because of the finding that the avirulent mutant strain of S. flexneri 2a reverted to the ability to penetrate epithelial cells and thus cause disease, an investigation was initiated last year to determine the locus on the Shigella chromosome which is responsible for epithelial cell penetration. It was found a year ago that the region lay somewhere between the lactose-galactose portion of the chromosome. However, Shigella hybrids which had incorporated this relatively large chromosomal region from E. coli invariably lost their type-specific antigen because this characteristic is controlled by genes near lactose. Because of the loss of the type-specific antigen such hybrids could not be used for oral vaccine. For this reason we have attempted during the past year, to localize more precisely the chromosomal region responsible for penetration so that hybrids might be prepared which retained the Shigella type-specific antigens but which lacked the ability to

enter epithelial cells. Such strains might then be used for oral vaccines. We believe that we have accomplished this. A chromosomal site which controls penetration (as assayed by the keratoconjunctivitis test) has been found near the purine E locus which is situated between the genes controlling lactose and galactose fermentation. By using an Hfr strain of E. coli which is purine E<sup>-</sup> one can obtain purine requiring Shigella hybrids with relatively small amounts of E. coli chromosome. These hybrids are avirulent but still retain the antigenic structure of the wild-type Shigella parent. The loss of virulence is not due to the requirement for purine, because reversions of the hybrid strains to purine-independence remain avirulent. One such hybrid strain has been tested for its ability to protect monkeys against experimental challenge. The pooled results of two experiments are presented in Table 1, and indicate a significant difference between the vaccine and control groups. However, the degree of protection which was observed was not as great as we would have wished it to be.

3. Another approach to preparing safe living, oral dysentery vaccines might be to transfer the ability to synthesize shigella antigens to E. coli. This has been accomplished using a S. flexneri 2a Hfr donor strain and an E. coli K-12 strain as a recipient. After mating and selecting for his<sup>t</sup> recombinants, a high proportion of these hybrids agglutinated in unadsorbed S. flexneri 2a antiserum. None of these hybrids expressed the type-specific II antigen. These hybrids were assumed now to possess some S. flexneri group antigens. When such a hybrid was remated with the same S. flexneri donor, selecting this time for pro<sup>t</sup> recombinants, a proportion of clones now expressed the type-specific antigen as well as the previously inherited group antigen. If such crosses were done in the reverse order i.e., pro<sup>t</sup> followed by his<sup>t</sup> selection, a different pattern of serological behavior was observed. None of the pro<sup>t</sup> hybrids possessed either type or group antigens. Subsequent mating for his<sup>t</sup> resulted in hybrids with both group and type-specific antigens. These results show that the genes controlling the synthesis of at least some of the S. flexneri group antigens (linked to the his<sup>t</sup> locus) and type-specific antigen (linked to the pro<sup>t</sup> locus) are widely separated on the chromosome. Expression of the type-specific antigen II depends also on the presence of at least some of the group antigens. Protection tests, using some of these hybrid strains as oral vaccines, will be performed in monkeys.

4. We have previously reported that the distribution of antibody forming cells in monkey organs following immunization with S. flexneri 2a vaccines was related to the route of administration. In monkeys receiving a live oral attenuated S. flexneri 2a vaccine, antibody forming cells were detected mainly in the intestinal mucosa. Occasionally antibody cells were found in the mesenteric

nodes or spleen but not in peripheral nodes such as the inguinal nodes. On the other hand, in monkeys parenterally immunized with a heat-killed *S. flexneri* 2a vaccine, antibody forming cells were detected in the inguinal nodes draining the site of injection and the spleen. Few or no cells were found in the intestinal mucosa. We reported that the serum antibody response appeared to be exclusively associated with the immunoglobulin M (IgM) class of antibody.

In the above experiments, animals were usually sacrificed at 4-8 days after the last dose of vaccine, and a few were sacrificed at 10 days. It was important to know whether this pattern of response changed if the animals were sacrificed at a later time after the last dose of vaccine. Did the animal continue to produce IgM antibodies, or did the animal begin production of immunoglobulin G (IgG) antibodies which would be indicative of a more lasting immunity?

Five monkeys were immunized at weekly intervals with 4 doses of either the oral or parenteral vaccine. The oral vaccine dose was  $2.5 \times 10^{10}$  cells in 20 ml; the parenteral dose  $10^9$  heat-killed cells. All of the animals in these groups were sacrificed 17-19 days after the last vaccine dose. An additional 5 monkeys were given two doses of vaccine and then sacrificed 14 or 15 days later. At time of sacrifice the animals were bled and portions of ileum, cecum, colon, spleen, mesenteric and inguinal nodes were removed. They were processed in the usual manner. Fluorescent antibody (FA) studies were performed to detect the presence of antibody forming cells in the specimens.

The results are presented in Table 2. The FA studies revealed that only one of the orally immunized animals had significant numbers of antibody forming cells in the intestinal tract at 17 days. In 3 other animals the antibody activity was considered marginal i.e., some antibody containing cells were found but the specific fluorescence was weak. In the parenterally immunized animals none of the animals appeared to have active antibody forming cells. In all but one of the monkeys substantial increases in antibody titers as measured by the passive hemagglutination test (HA) were found. Again, data obtained on individual serum samples by 1) gel filtration on Sephadex G-200, 2) sucrose density gradient ultracentrifugation and 3) reduction by dithiothreitol indicated that the agglutinins belonged to the IgM class of antibody globulins. We have tentatively concluded that the dose and schedule used for immunization in these experiments prolongs the production of IgM antibody, and monkeys fail to produce significant or detectable quantities of IgG which would indicate a more lasting immunity.

Indeed the FA studies showed that antibody synthesis may have ended.

Our studies on the importance of coprecipitated antibody secreted into the intestinal lumen in immunity to experimental bacillary dysentery have been hampered by the very small amounts of globulin obtained after simple washing out of the intestinal tract when the monkey was sacrificed. We have tried several perfusion experiments in an attempt to provide more intestinal secretions for study. In preliminary studies, perfusion of the large intestine proved technically difficult and only modest amounts of protein were recovered. Therefore, the small intestine was used exclusively for these studies. At the appropriate number of days after immunization the monkeys were subjected to surgery. A perfusion apparatus was attached to the small intestine and perfusion carried out in situ for 4 hours. At the end of the perfusion period the animals were sacrificed for complete studies of the immune response. Before perfusion was started the small intestine was washed out and the fluid saved for comparisons with the perfusates. When the animal was sacrificed the contents of the large intestine was also collected, homogenized, clarified and concentrated. All concentrations were done by membrane ultrafiltration at 0°C. A total of 27 monkeys were perfused, 6 normal animals, 11 orally immunized and 10 parenterally immunized. Some preliminary studies of the perfusates and intestinal washes are available. Qualitative immunodiffusion studies of the fluids using commercially prepared monospecific goat antisera to human immunoglobulin classes have been done. These antisera cross react strongly with monkey globulins. Of the perfusates examined 13 of 18 contained detectable amounts of IgA, 14 of 17 IgM, and 12 of 17 IgG. Analysis of the small intestine and large intestine washouts showed that most specimens contained detectable amounts of IgA and IgM.

However, the precipitin lines in agar suggested that the IgM determinants may exist as monomeric subunits of the molecule. Further studies are being done. It is possible that the molecule is either excreted in this form or that it is reduced to the monomeric form in the intestinal tract. The presence of IgG was variable and may be related to the presence of proteolytic enzymes present in these preparations, although the perfusates usually did not contain detectable proteolytic enzymes.

A necessary tool for the study of antibody formation, and the relationships of the various immunoglobulin classes to local and systemic immunity, is a source of monospecific antisera to the various immunoglobulin classes. The most difficult to prepare is antiserum to IgA because of technical difficulties in obtaining

pure IgA from normal serum. We have succeeded in modifying and synthesizing several methods to produce a relatively pure IgA globulin from normal monkey serum. It was prepared as follows. An appropriate amount (usually 40 ml) of serum was diluted 1:15 in distilled water and then cooled in an ice bath. The pH was lowered to 6.2 with 0.1N HCl. The resulting precipitate was removed by centrifugation. This precipitate was saved because it contains much of the IgM globulin present in serum. The supernate was cooled again and the pH lowered to 5.4 with HCl. The precipitate was removed and discarded. This precipitate contains gamma globulin and some beta globulins such as  $\beta_1C$ . The supernate containing the IgG was neutralized with NaOH and precipitated with ammonium sulfate at 2.1 molar concentration. The precipitate was dissolved in water and dialyzed against 0.01M phosphate buffer at pH 8.1. The dialyzed material was chromatographed on DEAE cellulose. Most of the remaining IgG was eluted in the first fraction. The IgA was collected by stepwise elution using increasing concentrations of NaCl in phosphate buffer. Each fraction was tested for IgA content by immunodiffusion and immunoelectrophoretic methods. The fractions containing the major portions of IgA were pooled and dialyzed against 0.01M citrate phosphate buffer at pH 5.5. This material was chromatographed on CM cellulose (Whatman CM 52). The first fraction contained most of the IgA present in the sample. This fraction was concentrated and tested for purity. No detectable IgM or IgG was found. The purified IgA was used to immunize rabbits. The rabbit antisera collected after a suitable immunization schedule precipitated strongly with the IgA antigen and the IgA present in normal monkey serum. Only a slight reaction was noted against IgG globulins.

5. Work has continued on encephalomyocarditis (EMC) infection of mice. Earlier data comparing the effect of orally administered virus with subcutaneously administered, formalin-inactivated virus in short-term experiments were confirmed in tests where the period between immunization and challenge was extended. Both preparations prevented significant viral replication in the bowel, as reflected by virus counts in faeces or in small bowel washings.

Three groups of mice were studied: control (C), parenterally immunized with formalin-inactivated virus (s.c.), and orally immunized with active infectious attenuated virus (p.o.). The attenuated virus preparation caused an asymptomatic infection with viremia and fecal excretion of virus from the second to the fourth day after administration. The three groups were studied in parallel three weeks, one month, two months, and three months after immunization.

The p.o. group was immunized with  $5 \times 10^8$  plaque forming units (PFU) of attenuated EMC (designated EMCL) in a volume of one ml. The animals were allowed to drink the EMCL after an overnight fast, individually identified, and housed five per cage. The s.c. group received 0.5 ml. of an emulsion of formalin-inactivated virulent EMC (approximately  $10^8$  PFU) mixed with an equal volume of Freund's complete adjuvant, injected subcutaneously. This injection was repeated in 14 days. At the appropriate time the three groups were fasted overnight, bled from the retroorbital plexus and challenged by being allowed to drink 1 ml. of supernatant culture fluid containing either  $2 \times 10^9$  or  $2 \times 10^8$  PFU of virulent virus.

Subsequent to challenge, fecal pellets were collected each day, homogenized in phosphate buffered saline pH 7.2, containing 2% fetal bovine serum (PBS 2% FBS), and centrifuged. The clear supernatant fluid was aspirated and frozen until used for virus analysis. The procedure was modified as follows for the three month group. A control challenge ( $2 \times 10^9$  PFU) established that nearly 100% mortality could be expected in three-month old unimmunized mice challenged with EMC (20/20 died). Consequently, control, p.o. and s.c. groups were challenged, and on successive days groups of ten each were sacrificed. Each animal was bled, and the small bowel from jejunum to ileum was removed and rinsed with two ml. of PBS 10% FBS. Serial five-fold dilutions were made of the centrifuged rinse fluid supernatant, and virus analysis was performed using 0.2 ml. per plate.

Both formalin-inactivated virus and attenuated active virus were effective in protecting mice against an otherwise lethal oral challenge with EMC. Surviving animals seemed to be protected against significant bowel infection since excretion of virus was suppressed in both experimental groups (Table 3,4). The analysis of antibody levels is in process for the later groups. In general the control mice all excreted more than five PFU per fecal pellet per day.

Antibody was recovered in small bowel washings three weeks after oral immunization. Parenteral formalin-inactivated virus did not stimulate detectable coproantibody formation. The time course of coproantibody production is illustrated in Table 5. Mice were immunized by feeding EMCL, and then sacrificed at the times indicated. Paired blood and bowel washouts were studied. Coproantibody appears between the 15th and 27th day.

The difference in coproantibody between p.o. and s.c. groups was confirmed using pooled concentrated bowel washouts. The washouts were precipitated by 50% ammonium sulphate at pH 7.6, suspended in equal volumes and dialyzed exhaustively against PBS, until no precipitate formed with  $\text{BaCl}_2$ . The active material from the p.o. bowel washout was localized to the first peak when subsequently subjected to Sephadex G-200 gel filtration. Augmentation of viral inhibition was obtained by adding anti-mouse gamma globulin to the reaction mixture after preliminary incubation of virus and washout. It thus appeared that the inhibitory material in p.o. washout was immunoglobulin.

The difference between the p.o. and s.c. groups is not conclusive because the groups could not be matched with regard to the level of serum antibody generated in response to vaccination. The failure of parenteral formalin-inactivated virus to elicit coproantibody may simply reflect its inherently poorer immunogenicity or may be related to dose. EMCL replicates in the host and there is an attendant viremia. The antigenic challenge is massive compared to that from inactivated virus. The titers from individual bleedings done 16 days after primary immunization are shown in Table 6. The s.c. group had a booster injection at day 12. The serum titers in the oral group are clearly higher than the s.c. group. The question whether parenteral non-replicating antigen can stimulate coproantibody formation is thus not resolved by these experiments. It is evident, however, that detectable coproantibody is not required to prevent significant bowel infection as measured by virus excretion.

Further studies were done on passively transferred immunity. We had previously shown that rabbit 7S hyperimmune antibodies prevented both fatal infection and viral shedding. An early bleeding from a rabbit immunized with active EMC was also tested. The antibody titer was 1:10,000 and 95% of the antibody activity was present in the 19S region by sucrose density gradient ultracentrifugation. This serum was not effective in uniformly protecting mice from virulent challenge.

The question arose whether the protective effect of the 7S antibody reflected antibody that had reached the bowel lumen. Rabbit 7S gamma globulin was isolated by DEAE cellulose chromatography at pH 8.0 conductance 25 mmhos. The isolated protein was then trace labelled with  $^{125}\text{I}$ . A single line was seen on radioimmunoelectrophoresis against anti-rat gamma globulin. The  $^{125}\text{I}$  tagged gamma globulin was diluted 1:10 in normal rabbit serum and 1 ml. was

was injected subcutaneously into mice. The animals were sacrificed by ether anesthesia at twenty-four hours and blood and bowel washings collected for the determination of radioactivity in a well-type scintillation counter. Less than .02% of blood radioactivity was found in the bowel. By radioimmuno-electrophoresis little or no activity was seen in the gamma region. These washouts were collected in the same way that washouts from p.o. animals were collected, and antibody activity was easily demonstrated in the latter. In light of these data and Keller's observation\* that newborn infants with significant levels of circulating, passively-acquired antibody had no coproantibody, it seems unlikely that the passively transferred rabbit anti-EMC antibody reached the bowel lumen.

Several facts emerge from these studies. Viral excretion is similar whether virus vaccine is given orally or parenterally, so an initial phase of gut replication is not mandatory. Oral, infectious attenuated virus produces higher antibody levels than s.c. formalin inactivated virus, and elicits demonstrable coproantibody; but the formalin treated virus, subcutaneously administered, is sufficient to establish moderately long term immunity to reinfection as measured by bowel virus count. Passively transferred 7S antibody does not reach the bowel lumen in significant quantities, but as might be predicted by the preceding is effective in preventing infection with virulent EMC, and is effective in low concentrations. Coproantibody protecting local epithelial surfaces against viral replication, seems unimportant in EMC infection in mice.

\* Pediatrics 43: 330, 1969.  
J. Immunology 101: 192, 1968.

TABLE I

Protection of monkeys against experimental challenge with *S. flexneri* 2a using a *S. flexneri* 2a-E. coli hybrid strain\* as an oral vaccine. (Expts. M116 and M118).

Group	No. of Animals	No. with Diarrhea	Dysentery	Total Ill	P
Vaccine	37	7	6	13	.02
Control	40	9	15**	24	

\* The hybrid contained the purine E region of the *E. coli* chromosome.

\*\*2 animals in the control group died.

TABLE 2

Immune response of monkeys following immunization with dysentery vaccines

Animal No.	Immunization Route	Day Sacrifice	HA Titer (Serum)		Antibody Forming Cells in Tissues
			Pre Immune	Post Immune	
M113-15*	Oral	18**	15	120	Marginal
M113-16	Oral	19	<15	30	Marginal
M113-17	Oral	17	15	960	Positive
M113-19	Parenteral	17	<15	960	Negative
M113-22	Parenteral	18	160	1280	Negative
M114-3*	Oral	15	<20	320	Marginal
M114-5	Oral	14	<20	160	Negative
M114-12	Parenteral	14	<20	5120	Negative
M114-17	Parenteral	15	<20	5120	Negative
M114-18	Parenteral	15	<20	2560	Negative

\*M113 = 4 doses of vaccine, M114 = 2 doses vaccine.

\*\* = sacrificed on day after last dose of vaccine.

TABLE 3

Mortality and virus excretion in mice challenged at three weeks, one month, or two months after oral immunization with attenuated virus (p.o) or parenteral immunization with formalin inactivated virus (s.c.).

Challenge Time	Challenge Dose	Group	Mortality	PFU excreted/fecal pellet
				0-4      5-50      > 50
3 wk	10 <sup>9</sup>	c	20/20	0
	10 <sup>8</sup>	c	15/20	2
	10 <sup>9</sup>	s.c.	0/20	19
	10 <sup>8</sup>	s.c.	0/20	20
	10 <sup>9</sup>	p.o.	2/20	18
	10 <sup>8</sup>	p.o.	2/20	19
1 month	10 <sup>9</sup>	c	20/20	3
	10 <sup>8</sup>	c	18/20	3
	10 <sup>9</sup>	s.c.	4/20	16
	10 <sup>8</sup>	s.c.	4/20	8
	10 <sup>9</sup>	p.o.	2/20	16
	10 <sup>8</sup>	p.o.	4/20	13
				4
				2
2 month	10 <sup>9</sup>	c	28/30	3 <sup>#</sup>
	10 <sup>9</sup>	s.c.	0/30	30
	10 <sup>9</sup>	p.o.	5/25	20
				10
				0
				2 <sup>+</sup>

\* no antibody titer but survived

\*\* died, no Ab titer

+ died, titer not done

++ one of two died

# two of three survived

TABLE 4

Virus titers in wash-out of small bowel after oral challenge with virulent virus three months after immunization with oral attenuated virus or parenteral formal-inactivated virus

	positive for virus			geometric mean titer	
	Day 2	3	4	2	3
Control	9/9	10/10	5/5	214	45,900
s.c.	2/10	1/10	0/5	2	0
p.o.	3/10	0/10	2/4	5	0
					229

TABLE 5

Paired blood and bowel wash-out inhibitory titers at 6, 9, 15, and 27 days after oral immunization with attenuated virus. Numbers represent  $\log_2$  of 50% plaque reduction titer.

Day	6		9		15		27	
	Blood	Bowel	Blood	Bowel	Blood	Bowel	Blood	Bowel
50% Plaque reduction $\log_2$	7	0	8	2	8	1	8	5
	6	0	9	0	7.5	0	9	3
	9	0	8	0	7.5	1	0	0
	0	0	79	0	7.5	0	8	3
	8	0	79	0	9	0	9	3

TABLE 6

Titers of serum neutralising antibody  
From mice immunized with oral attenuated virus or formation  
inactivated virus. See text.

	Log <sub>2</sub> Titer*	
P.O.		S.C.
6		0†
5		2
5		4
6		2
7		0
6		2

\* starting material was 1/20

† excreted 100 PFU when challenged

Project 3A062110A822, MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 121, Pathogenesis of enteric disease

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2. Hornick, R.B., H.L. DuPont, S. B. Formal, J.M. Snyder and E. J. Gangarosa. Progress in the development of an oral attenuated vaccine for shigellosis. Eighth Int. Cong. Trop. Med. and Malaria, Taharan, 7-15 Sept. 68, p. 476.

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4. Friedman, R., Maenza 1968. Peroral infection and resistance to reinfection with encephalomyocarditis virus in the mouse. *J. Inf. Diseases* 188: 123-134.

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6. Labrec, E.H., O. Washington, and S. B. Formal 1969. Distribution of specific antibody-forming cells in monkey organs after immunisation with *Shigella flexneri* 2a. *Bact. Proc.* 106.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY DESIGNATION	2. DATE OF REPORT	REPORT CONTROL SYMBOL
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NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Immunology Washington, DC 20012			
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Foreign Intelligence Considered				NAME: Wohlhieter, Dr. J. A.			
				NAME: DA			
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(U) Antigens; (U) Virulence; (U) Salmonella; (U) Drug Resistance							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with security classification code.)							
<p>23. (U) Definition in genetic and molecular terms of the metabolic, antigenic and pathogenic characteristics of enteric bacteria. We anticipate that it will be possible to genetically modify enteric bacteria to any desired antigenic structure and/or pathogenicity to serve as vaccine strains or as tools to study the infectious process.</p> <p>24. (U) Use of genetic recombination between strains of enteric bacteria. Where possible, the genetic results are extended to include study of the informational macromolecules involved.</p> <p>25. (U) 69 01 - 69 06. Studies on the nature of 7 lactose fermenting salmonella strain obtained from clinical sources have revealed that 6 of the strains contain transmissible extrachromosomal lac elements which promote transfer of the chromosomal genes of salmonella hosts. <i>E. coli</i> K-12 strains to which the lac elements are transferred become sensitive to male specific phage R-17. Newly initiated studies of the properties of a derepressed resistance transfer factor (RTF), which transfers resistance to 5 antibiotics, in addition to promoting host chromosome transfer, have revealed a fertile <i>S. typhimurium</i> recipient. Preliminary investigation of this salmonella strain indicates that it displays an atypical unselected marker inheritance pattern, as compared with previously studied fertile salmonella strains. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.</p>							

PII Redacted

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Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 122 Microbial Genetics and Taxonomy

**Investigators.**

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B.S.

**Description.**

The purpose of these studies is to investigate the genetic characteristics of the metabolic and antigenic changes occurring in enteric bacteria as a consequence of genetic recombination, episomic transfer and transduction.

1. Partial diploid hybrids were constructed from a Salmonella typhosa Hfr by mating with an Escherichia coli Hfr. The E. coli DNA segments contained in these hybrid Salmonella Hfr strains were mobilized and transferred to an E. coli recipient with the frequency and polarity characteristic of the E. coli Hfr. In one hybrid some cells of the population displayed the E. coli Hfr transfer polarity of the diploid segment; while others displayed the opposite transfer polarity.
2. A large number of E. coli characters, such as fermentation properties, surface antigens and susceptibility to phages, have been transferred by E. coli Hfr's to Proteus.
3. Investigation of a derepressed R-factor has revealed that it is capable of promoting intergeneric chromosome transfer and has resulted in the discovery of a uniquely fertile strain of Salmonella typhimurium.
4. A mutant of Proteus mirabilis was isolated which produces large amounts of a yellow-green fluorescent pigment.
5. Six extrachromosomal lac<sup>+</sup> elements, originally derived from clinically obtained Salmonella strains, have been shown to promote the transfer of chromosomal genes to strains of Salmonella and Escherichia coli.

## Progress.

1. Transfer of Escherichia coli Chromosomal Segments from a Partial Diploid Salmonella typhosa Hfr. In previous studies on the hybridization of Escherichia coli Hfr donor strains with Salmonella typhosa recipients, we have observed and reported the formation of partial diploid S. typhosa hybrids. In such hybrids, the transferred segment of the E. coli chromosome does not undergo recombination with, i.e., replace, the homologous S. typhosa chromosomal region. Instead, the E. coli chromosomal segment is maintained and replicated along with the resident Salmonella chromosome; so that the hybrid is diploid with regard to the genetic markers contained on that segment and haploid with regard to the remainder of its genome. This condition of the Salmonella hybrid is an unstable one and segregants may be obtained which have lost part or all of the E. coli DNA.

The manner in which the Salmonella hybrid conserves the E. coli chromosomal segment is, at present, unknown. Specifically, we do not know the nature of the chemical association between the E. coli DNA and the resident Salmonella chromosomal DNA. It is possible that the E. coli DNA is tandemly inserted into and covalently bonded with the resident Salmonella chromosomal DNA (thus producing a duplicated region within the hybrid chromosome,) and replicated as part of the continuum of a single DNA duplex. It is conceivable also that the E. coli DNA may be conserved as an independent replicon unassociated by chemical bonding with the host chromosome. As one approach to an understanding of the nature of the partial diploid state in Escherichia-Salmonella hybrids, we have constructed partial diploids from a Salmonella typhosa Hfr strain which possess various chromosomal segments derived from E. coli. These strains, in addition to their normal capacity to mobilize and transfer resident Salmonella DNA in matings with Salmonella recipients, are able to mobilize and transfer the diploid E. coli DNA segment to E. coli recipients.

The S. typhosa Hfr strain, WR4000, (which contains the integrated lac region and the sex factor, F, of E. coli) was used as a recipient in a cross with the E. coli Hfr WR2004. E. coli WR2004 transfers its chromosomal markers in the order: origin - pro (proline synthesis), ara (arabinose utilization), rha (rhamnose utilization), xyl (xylose utilization) and fuc (fucose utilization). Selection was made for those S. typhosa WR4000 hybrids which inherited the ara marker from the E. coli Hfr. Unselected marker analysis of 25 of these hybrids revealed that 22 had received the distal rha and xyl genes, and 17 of these 22 had inherited the more distal fuc gene. With regard to the extent of E. coli chromosome transfer involved in this mating, the ara marker is transferred about 12 minutes after the origin of E. coli Hfr WR2004, rha is situated 16 minutes distal to ara, xyl is 20 minutes distal to ara and fuc is 36 minutes distal to ara.

The majority of the 22 *S. typhosa* WR4000 hybrids which had received markers in addition to *ara* were unstable and readily lost the *E. coli* chromosomal segments. Four of these, however, proved stable enough for further experimentation. Two of these 4 hybrids, designated WR4000 *ara*<sup>+</sup> 1 and WR4000 *ara*<sup>+</sup> 22, contained the *ara*<sup>+</sup>, *rha*<sup>+</sup>, *xyl*<sup>+</sup> and *fuc*<sup>+</sup> markers of the *E. coli* parent. The others, designated WR4000 *ara*<sup>+</sup> 15 and WR4000 *ara*<sup>+</sup> 3, contained the *ara*<sup>+</sup>, *rha*<sup>+</sup> and *xyl*<sup>+</sup> genes, but not the *fuc*<sup>+</sup> gene.

The 4 WR4000 *ara*<sup>+</sup> hybrids were mated with the *E. coli* recipient strain WR3051. *E. coli* WR3051 does not accept transfer of *Salmonella* genes from the *S. typhosa* Hfr WR4000 (except for the lead marker *pro*, which is transferred at a low frequency). However, all of the WR4000 *ara*<sup>+</sup> hybrids were capable of transferring the diploid *E. coli* genes to *E. coli* WR3051 at frequencies characteristic of the *E. coli* Hfr WR2004. Moreover, with 3 of the 4 hybrid donors, a transmission gradient similar to that of *E. coli* Hfr WR2004 was observed (See Table 1), strongly suggesting that the *E. coli* diploid material in these hybrids is a continuous DNA segment. The WR4000 *ara*<sup>+</sup> 15 hybrid, however, displayed nearly identical frequencies for *pro*<sup>+</sup> and *xyl*<sup>+</sup> transfer.

The WR4000 *ara*<sup>+</sup> 15 hybrid was further examined in matings with *E. coli* WR3051, in which selections were made for *xyl*<sup>+</sup>, *pro*<sup>+</sup> and *ara*<sup>+</sup>, (arginine biosynthesis). The *arg* gene is situated between *ara* and *xyl*. The transfer frequencies of *E. coli* genes from the WR4000 *ara*<sup>+</sup> 15 hybrid were as follows: *ara*<sup>+</sup>, 1.5 x 10<sup>-3</sup>; *pro*<sup>+</sup>, 7 x 10<sup>-4</sup>; *arg*<sup>+</sup>, 7 x 10<sup>-4</sup>; *xyl*<sup>+</sup>, 1.4 x 10<sup>-3</sup>. These frequencies suggest that the *E. coli pro* to *xyl* segment in the WR4000 *ara*<sup>+</sup> 15 hybrid is transferred with a polarity similar to that of *E. coli* WR2004 by some of the cells, while others transfer it with the opposite polarity. This notion was supported by the examination of selected recombinants from the cross between WR4000 *ara*<sup>+</sup> 15 and *E. coli* WR3051. Some of the *xyl*<sup>+</sup> WR3051 hybrids had inherited either *pro*<sup>+</sup> or *arg*<sup>+</sup> markers, suggesting that these genes were not transferred proximal to *xyl*<sup>+</sup>. Since *E. coli* WR3051 is resistant to streptomycin, 50 of the *xyl*<sup>+</sup> hybrids were examined for their response to this antibiotic and 33 were found to be streptomycin sensitive. Thus, they had inherited the *E. coli* streptomycin sensitivity (*str*) gene, present on the diploid segment of WR4000 *ara*<sup>+</sup> 15, at a frequency which suggests that this marker was transferred prior to *xyl*<sup>+</sup>. This finding also indicates that the *xyl*<sup>+</sup> selected recombinants are those which have received *xyl*<sup>+</sup> as the result of a transfer polarity in which *pro* is transferred as a lead marker, followed by *xyl*<sup>+</sup>, and thus, opposite to that of *E. coli* Hfr WR2004. Examination of 50 *ara*<sup>+</sup> recombinants from the WR4000 *ara*<sup>+</sup> 15 x WR3051 cross indicated that the *ara*<sup>+</sup> selected cells are derived from those donor cells which transfer the *E. coli* segment with the WR2004 polarity; 26 received the *pro*<sup>+</sup> marker, indicating the transfer proximal to *ara*<sup>+</sup>, whereas none received either *arg*<sup>+</sup> or *xyl*<sup>+</sup>.

Table 1. Transfer of Escherichia coli Genes by Partial Diploids  
 Constructed from the Salmonella typhosa Hfr WR4000\*

<u>Donor</u>	<u>Selected Marker</u>		
	<u>pro</u> <sup>+</sup>	<u>xyl</u> <sup>+</sup>	<u>fuc</u> <sup>+</sup>
WR4000 <u>ara</u> <sup>+</sup> 1	10 <sup>-2</sup>	4 x 10 <sup>-4</sup>	2.5 x 10 <sup>-5</sup>
WR4000 <u>ara</u> <sup>+</sup> 3	4 x 10 <sup>-2</sup>	8 x 10 <sup>-4</sup>	—
WR4000 <u>ara</u> <sup>+</sup> 15	2 x 10 <sup>-3</sup>	2.5 x 10 <sup>-3</sup>	—
WR4000 <u>ara</u> <sup>+</sup> 22	10 <sup>-2</sup>	3 x 10 <sup>-4</sup>	1.5 x 10 <sup>-5</sup>
<u>E. coli</u> Hfr WR2004	9 x 10 <sup>-3</sup>	3 x 10 <sup>-4</sup>	10 <sup>-5</sup>

\* The recipient strain was E. coli WR3051. Transfer frequencies are expressed as the number of recombinants per donor cell.

One of the WR4000 ara<sup>+</sup> hybrids, WR4000 ara<sup>+</sup> 3, was tested for its ability to promote transfer of its resident Salmonella chromosome to an S. typhimurium recipient. Since the S. typhimurium strain does not accept transfer of E. coli DNA, we were able to determine whether the WR4000 ara<sup>+</sup> 3 hybrid would still function in the manner of the S. typhosa Hfr WR4000 from which it was derived. Transfer of the Salmonella genes arg<sup>+</sup> and his<sup>+</sup> (histidine biosynthesis) from the WR4000 ara<sup>+</sup> 3 donor occurred at frequencies of  $8 \times 10^{-6}$  and  $7 \times 10^{-8}$  respectively. By comparison, the S. typhosa Hfr WR4000 transferred arg<sup>+</sup> and his<sup>+</sup> at frequencies of  $10^{-4}$  and  $7 \times 10^{-7}$  respectively. Thus, the WR4000 ara<sup>+</sup> 3 hybrid donor still displays its ability to promote transfer of its resident chromosome, although the frequency at which this transfer is accomplished is reduced approximately 10-fold.

2. Chromosomal Transfer from Escherichia coli to Proteus. It has been possible to transfer extensive portions of the E. coli chromosome to Proteus mirabilis by mating with E. coli Hfr's. The hybrids formed as partial diploids which are unstable as evidenced by the occasional segregation of E. coli characters with the reappearance of the original Proteus phenotype. Genetic markers from about half of the E. coli chromosome have been detected in the Proteus hybrids.

Lactose fermentation was the first character to be transferred by E. coli Hfr's to Proteus. Two different E. coli Hfr's, W1895 and HfrH, have been used to produce lac<sup>+</sup> diploids. These diploids differ from an F-lac episomal infection of Proteus in that F characteristics cannot be detected in these Proteus diploid strains. For instance, E. coli characters are not transferred out. Male specific phages do not propagate on them. No F pili can be observed in the electron microscope. Moreover, the Proteus diploid can be remated with E. coli Hfr's and additional E. coli characteristics can be added so that larger diploids can be constructed. DNA from Proteus has a 39% GC composition while E. coli has a 50% GC composition. Due to this difference in base composition, and hence density, mixtures of these DNA's band at separate positions in the CsCl density gradient. When DNA is extracted from the Proteus diploids and examined in a CsCl density gradient it is possible to observe and quantify the E. coli DNA in hybrids. It has been shown that as the number of E. coli characters increases there is an increase in the amount of E. coli DNA added to the Proteus cell. The satellite band of the lac<sup>+</sup> Proteus diploid is 6% of the total DNA; the lac<sup>+</sup> and ara<sup>+</sup> Proteus diploid is 14%. (See Table 2.)

Other unselected markers besides the fermentation characters have been detected in Proteus. Among these are E. coli type I pili and the cell-surface Ti receptor site.

Table 2. Correlation of E. coli Characters  
and DNA in Proteus Hybrids

<u>Hybrid Characters</u>	<u>% DNA in Satellite Band</u>
<u>lac</u> <sup>+</sup>	6
<u>lac</u> <sup>+</sup> <u>ara</u> <sup>+</sup>	14
<u>lac</u> <sup>+</sup> <u>ara</u> <sup>-</sup> segregant	6
<u>lac</u> <sup>-</sup> <u>ara</u> <sup>-</sup> segregant	0
<u>lac</u> <sup>+</sup> <u>gal</u> <sup>+</sup>	16
<u>lac</u> <sup>+</sup> <u>ara</u> <sup>+</sup> <u>gal</u> <sup>+</sup>	26
<u>lac</u> <sup>+</sup> <u>mel</u> <sup>+</sup>	9
<u>lac</u> <sup>+</sup> <u>ara</u> <sup>+</sup> <u>mel</u> <sup>+</sup>	19
<u>lac</u> <sup>+</sup> <u>ara</u> <sup>+</sup> <u>mal</u> <sup>+</sup> <u>mtl</u> <sup>+</sup> <u>tna</u> <sup>+</sup> <u>strS</u>	32
<u>lac</u> <sup>+</sup> <u>ara</u> <sup>+</sup> <u>mtl</u> <sup>+</sup>	20
<u>mal</u> <sup>+</sup> <u>mtl</u> <sup>+</sup> <u>tna</u> <sup>+</sup> <u>strS</u>	18
<u>mal</u> <sup>+</sup> <u>mtl</u> <sup>+</sup>	15

lac<sup>+</sup> = lactose utilization

ara<sup>+</sup> = arabinose utilization

gal<sup>+</sup> = galactose utilization

mel<sup>+</sup> = melibiose utilization

mal<sup>+</sup> = maltose utilization

mtl<sup>+</sup> = mannitol utilization

tna<sup>+</sup> = tryptophanase production

strS = streptomycin sensitivity

The presence of E. coli type I pili was scored by slide agglutination tests employing antiserum prepared against purified E. coli type I pili. The presence of coliphage T1 receptor was scored by spotting a high multiplicity of T1 phage on faint lawns of the Proteus diploids. After about 4 hours of incubation diploids possessing the T1 receptor were partially lysed, presumably a killing phenomenon. Although we have been unable to plaque T1 on such hybrids, the validity of this test is supported by adsorption experiments and experiments showing inhibition of growth by high input ratios of phage T1.

The largest Proteus diploids that have been constructed were made by crossing a lac<sup>+</sup> ara<sup>+</sup> diploid with the Hfr AB2297, which has its origin near the mal A (maltose utilization) region and the streptomycin sensitivity locus. From this cross we have obtained a lac<sup>+</sup> ara<sup>+</sup> mannitol<sup>+</sup> maltose<sup>+</sup> diploid. When DNA is extracted from this diploid, 32% of the total DNA is E. coli DNA. (See Table 2.)

With the Hfr AB2297 the first marker transferred is streptomycin sensitivity which is dominant to streptomycin resistance of the host Proteus chromosome. We have been particularly interested in this mating because adjacent to the mal A region is the lambda receptor site. These maltose<sup>+</sup> Proteus diploids adsorb lambda phage, but no plaques are observed. Loss of the mal A region of the diploid leads to loss of the ability to adsorb lambda phage. Attempts have been made to isolate lambda mutants that would plaque on Proteus, but these have been unsuccessful. This class of Proteus hybrid appears to differ from others we have studied in that propagation tests for male phage indicate that this hybrid is sensitive to the male phage R-17. Attempts to transfer the mannitol-maltose segments out of Proteus, however, have been negative. It appears that the mannitol, maltose hybrid may be an F<sup>-</sup>merogone or at least harbors a free F factor.

It has been possible to transfer segments of E. coli chromosome from about half of the chromosome to Proteus. Not all the characters are transferred at one time, but it is possible by repeated matings to build up the number of E. coli genes in Proteus. The presence of all the markers in any one diploid may also be difficult to achieve since the segregation of existing E. coli segments in Proteus diploids is not easy to control. In spite of these difficulties a large portion of the E. coli chromosome has been transferred to Proteus and it has been possible to examine E. coli fermentation characters and demonstrate E. coli surface antigens and susceptibility to E. coli phages in Proteus.

3. Studies on a Derepressed R-factor. Most multiple drug resistance transfer factors (RTF) are subject to a repression-derepression control of their genetic transfer from one host cell to another. Recently, mutant derepressed R-factors have been isolated. Unlike the wild type R-factor, such mutant plasmids are genetically altered to remain in a constant derepressed state. Hence, host cells with a derepressed R-factor are always, regardless of cell age, etc., able to serve as efficient genetic donors of their antibiotic resistance genes. Most of the information on derepressed R-factors is based on recent studies within the E. coli mating systems. Since very little information is available on the behavior of derepressed R-factors in intergeneric matings, we have initiated studies of this nature on one such derepressed plasmid which confers on host cells resistance to chloramphenicol, streptomycin, kanamycin, ampicillin and sulfadiazone. Our results from intergeneric matings, in which E. coli K-12 with this R-factor was employed as donor and in which Salmonella typhosa, S. typhimurium and Proteus mirabilis were used as recipients, indicate that this type of R-factor is 100 to 1,000 times more efficiently transferred to different genera classified in the Enterobacteriaceae than a wild type R-factor. Furthermore, preliminary data indicates that some of the drug resistance genes conferred by this plasmid can segregate independently. Most frequently, cells that have received the R-factor are resistant to all 5 antibiotics. On the basis of 69 S. typhimurium clones analyzed for their pattern of resistance to kanamycin, chloramphenicol and ampicillin, however, we have found 1 clone which is kanamycin resistant and sensitive to ampicillin and chloramphenicol, and 7 different clones which are sensitive to kanamycin but resistant to ampicillin and chloramphenicol. Although the data are preliminary, they suggest that kanamycin genes for resistance may in some hosts exist independent of the other resistance markers.

Our investigations on this derepressed R-factor have also revealed that it is capable of promoting chromosome transfer on the intergeneric level. This finding was made possible by the fortuitous employment of a uniquely fertile S. typhimurium LT2 recipient line in these studies. This strain, termed THAX, was found to be a very efficient recipient of the R-factor. Furthermore, during R-factor matings between E. coli donors and the THAX strain, the transfer of chromosomal genes controlling the biosynthesis of threonine was detected at frequencies of about  $10^{-2}$  per input male. Since efforts to transfer other chromosomal genes by R-factor mediated crosses have failed, we suspect that the THAX strain may harbor genes linked to the threonine region which determine the fate of chromosomally transferred E. coli segments. Since R-factor mediated chromosome transfer is not as efficient as Hfr chromosome transfer, we have employed the THAX fertile recipient line in matings with E. coli K-12 Hfr donors to further characterize its recipient

ability for chromosomal markers. The results of preliminary matings support our original notion of the importance of the threonine chromosomal region, since the only chromosomal transfer readily detected is for the threonine marker. Furthermore, we have recovered at low levels hybrids selected for lactose utilization. Of 5 such hybrids presently available, all were found to have inherited as an unselected marker the threonine chromosomal segment suggesting again that this chromosomal segment in some way determines the fate of conjugally transferred DNA and, hence, the extent of chromosomal hybridization. Further study of this strain may provide information on the genetic and molecular mechanisms controlling the intergeneric hybridization of the Enterobacteriaceae.

4. Production of a Green Fluorescent Pigment by a Proteus mirabilis Mutant. During the course of experiments designed to produce auxotrophic mutants of Proteus mirabilis strain WR11 with the mutagen N-methyl-N'-nitro-N-nitrosoguanidine, we encountered a strain producing large amounts of a green pigment which diffused into the media. To eliminate any possibility of a contaminant, the presumed green mutant of P. mirabilis was compared with the original Proteus strain, WR11. The results of tests for DNA base composition, agglutination by P. mirabilis antibody, susceptibility to a Proteus phage specific for WR11, production of urease and presence of known genetic markers characteristic of WR11 indicated that, aside from the production of the green pigment, the mutant and its WR11 parent are identical.

Since the color of the pigment of the mutant strain closely resembled that of fluorescein, a fluorescent dye produced by a number of species of Pseudomonas, the Proteus strains were examined under long-wave ultraviolet blacklight for possible fluorescence. Plates of both the WR11 parent and the pigment-producing mutant were found to exhibit fluorescence under a source of blacklight. The plate of the WR11 parent strain showed a definite blue fluorescence whereas its pigment-producing mutant had a yellow-green fluorescence. The fluorescence of the supernatant fluid from both the WR11 parent and the mutant intensified at alkaline pH. When the supernatant fluid was acidified, however, the fluorescence of these pigments was lost. After neutralization with NaOH, the fluorescence of the pigments reappeared. The pigments were concentrated from cultures grown in minimal media by repeated extraction with large volumes of n-butanol. After extraction of the pigment material from the medium with butanol, the addition of a small volume of water to the butanol resulted in removal of the pigments from the butanol into the water. This procedure produced both a purification and a concentration of the pigments.

The extracted pigments were next examined in a spectrophotofluorometer and the spectrum of the yellow pigment showed adsorption and emission maxima very similar to those of the fluorescent dye, fluorescein.

Adsorption and emission maxima of the unknown blue fluorescent pigment found in both the WR11 parent and the green mutant were different from those of fluorescein. This blue pigment as well as trace or larger amounts of the yellow fluorescein-like pigment have been found to be present in all P. mirabilis, P. vulgaris, P. rettgeri and P.morganii cultures tested.

Paper chromatograms of these pigments were prepared using a butanol: ammonium hydroxide:water:ethanol solvent mixture. The results of these chromatograms indicated that the yellow fluorescent pigment produced by the mutant had approximately the same Rf value as did authentic fluorescein, whereas the Rf value of the unknown blue pigment was considerably lower.

We have also noticed the presence of 2 similar pigments produced in small amount in cultures of E. coli K-12 grown in minimal broth. After concentration of a 2 liter volume of supernatant fluid from K-12 cultures by lyophilization, 2 distinct fluorescent bands were observed when this concentrated material was being desalted on G15 Sephadex columns. The leading band exhibited a blue fluorescence with the second band showing a yellow fluorescence. These materials behaved as though they were adsorbed to the Sephadex column, but they could be removed from the column in 2 separate fractions which appeared to have similar characteristics to the pigments observed in Proteus.

Despite a number of attempts, we have not been able to isolate a mutant of E. coli K-12 producing large amounts of the fluorescein-like pigment nor have we been able to isolate additional mutants in Proteus.

These results indicate that Proteus, E. coli and very likely other enteric species have small amounts of the fluorescent pigments produced in larger amounts by the P. mirabilis mutant described here. These fluorescent pigments appear to be very similar to those made by many Pseudomonas species.

5. Promotion of Chromosome Transfer by Extrachromosomal lac Elements of Salmonella Strains Obtained from Clinical Sources. We have continued to investigate 6 lactose fermenting Salmonella strains obtained from clinical sources which harbor transmissible extrachromosomal lac elements (see Annual Report, WRAIR, 1968). Six S. typhimurium WR5000 strains, each harboring one of the lac elements, were examined for their ability to promote transfer of S. typhimurium chromosomal determinants. The recipient used in each cross was S. typhosa WR4204. Selection was made for the ara<sup>+</sup> and fuc<sup>+</sup> markers of S. typhimurium WR5000. At the same time selection was also made for transfer of the extrachromosomal lac elements. Transfer of ara<sup>+</sup> chromosomal determinants was detected at

low frequency (ca.  $10^{-7}$ ) in all cases; transfer of fuc<sup>+</sup> occurred at the same frequency in all but one of the crosses. In each of the crosses, transfer of the extrachromosomal lac elements occurred at high frequency ( $10^{-3}$ ).

We were concerned also with establishing whether the lac elements would promote transfer of E. coli chromosomal markers. Moreover, it was desired in this instance, to obtain a lac donor against which streptomycin counterselection could be employed. The E. coli strain WR3010 was chosen for this purpose. E. coli WR3010 behaved rather poorly as a recipient of the lac elements but we were able to transfer to it a lac element, designated lac-32. E. coli WR3010 containing lac-32 was crossed with the E. coli recipient WR3050, and selections were made for the chromosomal markers pro<sup>+</sup>, ara<sup>+</sup>, arg<sup>+</sup>, xyl<sup>+</sup>, fuc<sup>+</sup> and his<sup>+</sup>. With streptomycin counterselection (as well as the methionine requirement) against the donor, low frequency transfer ( $1-5 \times 10^{-6}$ ) was observed for all markers. With regard to the transfer of the extrachromosomal lac elements, E. coli WR3050 is, like E. coli WR3010, a poor recipient. However, in a simultaneous experiment, the E. coli WR3010 containing the lac-32 element transferred it to E. coli WR3000 at a frequency of  $6 \times 10^{-3}$ .

#### Summary and Conclusions.

1. Partial diploids were constructed from the Salmonella typhosa Hfr WR4000 by using this Hfr as a recipient in matings with the Escherichia coli Hfr WR2004. Four relatively stable S. typhosa WR4000 hybrids were obtained, 2 of which possessed E. coli DNA segments bearing the pro<sup>+</sup>, arg<sup>+</sup>, his<sup>+</sup>, xyl<sup>+</sup> and fuc<sup>+</sup> markers and 2 of which contained the E. coli markers arg<sup>+</sup>, ara<sup>+</sup>, rha<sup>+</sup> and xyl<sup>+</sup> but not fuc<sup>+</sup>. All 4 of these partial diploid hybrids were capable of mobilizing and transferring the E. coli genes to the E. coli recipient WR3051. Three of the hybrids transferred the diploid E. coli genetic material with a transmission gradient similar to that of the E. coli Hfr WR2004, suggesting that the E. coli DNA segment contained in these hybrids is continuous. The remaining hybrid displayed the characteristics of a population in which some cells transferred the E. coli diploid segment with the polarity of the E. coli Hfr WR2004, while other cells transferred this segment with the opposite polarity.

2. It was possible by conjugation with various E. coli K-12 Hfr donors to transfer at low frequency extensive portions of the E. coli genome to Proteus mirabilis. Examination of DNA from such hybrids by CsCl density gradient ultracentrifugation showed that in addition to the

Proteus DNA, another component was present which was identical to the E. coli donor DNA. By mating Proteus hybrids with different E. coli Hfr strains, other genetic characters could be added so that diploid Proteus hybrids have now been constructed containing up to half of the E. coli genome. Corresponding amounts of E. coli DNA were found when such Proteus hybrids were examined in density gradients. The extent of the E. coli genetic material in these unstable Proteus diploid hybrids included segments with the following selected markers: gal, lac, ara, mel, mtl and mal A. Unselected markers known to map throughout this region of the chromosome also were detected in these hybrids. Among the markers expressed in Proteus hybrids with the mal A region was the receptor site for coliphage lambda. Although plaques were not seen, lambda was adsorbed by these Proteus hybrids. Thus, a large number of E. coli characteristics such as fermentation properties, surface antigens and susceptibility to phages have been transferred by E. coli Hfr's to Proteus.

3. We have investigated the properties of a derepressed resistance transfer factor (RTF) which, in addition to transferring at high frequencies resistances to chloramphenicol, kanamycin, ampicillin, streptomycin and sulfadiazine, also promotes transfer of the chromosome of its E. coli host. In the course of investigation we have discovered a uniquely fertile Salmonella typhimurium recipient strain derived from a normally sterile cell line, which accepts RTF promoted transfer of E. coli chromosomal markers. Preliminary investigations of this Salmonella strain in matings with E. coli Hfr donors indicate that it displays an atypical unselected marker inheritance pattern as compared with previously studied fertile Salmonella strains.

4. In an experiment to obtain auxotrophs of P. mirabilis with N-methyl-N'-nitro-N-nitrosoguanidine, we encountered a mutant producing large amounts of a green pigment which diffused into the medium. Possibility of a contaminant was eliminated by characterizing the mutant biochemically and serologically. This strain remained sensitive also to a virulent Proteus phage and had the DNA GC content of P. mirabilis. The green pigment fluoresced under long-wave UV light at alkaline pH. Fluorescence of the pigment was lost on acidification but reappeared after neutralization with NaOH. The pigment was concentrated from cultures grown in minimal media by extraction with butanol. Addition of a small volume of H<sub>2</sub>O removed the pigment from the butanol. Spectrophotofluorometric analysis at alkaline pH showed maximum adsorption and emission to be very similar to the green fluorescent dye fluorescein. At acid pH these maxima were different from those of authentic fluorescein, suggesting that the pigment is conjugated to another compound. Wild-type P. mirabilis in minimal media produced trace amounts of the green fluorescent pigment as did all P. vulgaris, P. rettgeri and P. morganii cultures tested. It is therefore likely that the mutant synthesizes an excess of the pigment found in trace amounts in wild-type Proteus cultures.

5. Six lac<sup>+</sup> Salmonella strains isolated from clinical sources and containing transmissible lac elements were examined for the mobilization of chromosomal genes. These lac elements when contained in S. typhimurium WR5000 promoted transfer of the chromosomal gene ara, and all but one promoted the transfer of fuc to a Salmonella recipient. One element contained in E. coli K-12 promoted transfer of the chromosomal genes pro, ara, arg, xyI, fuc and his to an E. coli recipient. Transfer frequencies were low for all markers.

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 122 Microbial Genetics and Taxonomy (CD&I)

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DEFENSE AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
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11. TITLE (preceded by Security Classification Code)*							
(U) Histopathologic Manifestations of Diarrheal Disease (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREA*							
00 26 00 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
08 63		NA		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
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e. CUM. AMT.						100	
20. R. RESPONSIBLE LOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2677			
31. ORIGINAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATOR'S			
				NAME: DA			
32. TECHNICAL OBJECTIVE, 33. APPROACH, 34. PROGRESS (Precede individual paragraphs 12-14 with 10. Priority list of each 10. Security Classification Code.)							
23. (U) Pathology and Pathogenesis of various conditions of the gastro-intestinal tract of man and experimental animals is studied by multidisciplinary approaches with emphasis on morphology. These investigations are considered essential parameters for a comprehension and scientifically based therapy of diarrheal diseases and radiation injury to the intestine.							
24. (U) Principally morphologic, including light, fluorescent and electron microscopic examinations. Kinetic Studies using tritium-labelled thymidine and histochemical investigations are also employed.							
25 (U) 69 01 - 69 06 - The manuscript on benign idiopathic cholestasis has been revised. The study of intestinal globule leucocytes appeared in the Anatomical Record. The two studies of the effects of x-radiation on morphology and function of small intestines have been published in Proc. Soc. and Strahlen therapy respectively. Work on intestinal spirochetosis, cholera and in radiation biology is continuing. Three papers were presented before the Fed. Am. Soc. Exp. Biol., one before the Assoc. for Gnotobiotics, one before the Am. Gastroenterological Assoc. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

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Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 123, Histopathologic manifestations of diarrheal diseases

Investigators:

Principal: Colonel Helmuth Sprinz

Associates: Akio Takeuchi, M.D.; Helen R. Jervis, Dr. Sc.; Major Michael R. Zimmerman; Major Daniel G. Sheahan; Thomas G. Merrill, Ph.D.

Description

Studies of the pathogenesis and the evolution of histologic lesions of various infectious forms of enteritis were continued or started.

Progress

The progress made in the area of this work unit was summarized by Col. Sprinz in a review article (see bibliography).

1. With the arrival of Major Sheahan, an expert in the histochemistry of intestinal mucins, we returned to the study of the acute effects of a non-lethal dose of staphylococcal enterotoxin on the mucosa of the gastrointestinal tract. Applying the latest techniques, the distribution and the sequential alterations in the amounts of neutral, sialo- and sulfo-mucins were determined. The findings were correlated with morphologic alterations and with the enzymatic histochemical changes previously reported from this department. A manuscript is in preparation. Major Sheahan is applying identical techniques to the study of intestinal mucins in experimental cholera. In a related field he has completed a study of the distribution of tissue blood group glycoproteins in human intestinal mucosa in health and disease.

2. Major Zimmerman is the principal Associate Pathologist who together with the Chief of the Department handles consultations. He is also engaged in a collaborative study with Dr. S.B. Formal on the effects of bacterial hybrids on the gastrointestinal tract of guinea pigs. As his personal project he has selected a multidisciplinary study of malabsorption in rats. Malabsorption is induced by diverting the bile into the urinary bladder, and replacing the endogenous bile with exogenous bile acids.

3. Dr. Takeuchi has concentrated on the study of intestinal spirochetosis in man and monkeys. He is applying multiple morphologic techniques, such as light microscopy, transmission and scanning electronmicroscopy, freeze-etching and negative staining electronmicroscopy. Intestinal spirochetosis had been previously described but the entity was lost sight of during the past decade. Dr. Takeuchi's rediscovery and application of the latest techniques have kindled a great interest. The work was presented at the Federation Meeting (Takeuchi, A., and Sprinz, H. Spirochetes in the colon of the rhesus monkey. Fed. Proc. 28: (2) 513, 1969), and a manuscript is in preparation. As an outgrowth of his lecture trip to Japan (see Annual Report 1968) he was invited to submit a monograph in Japanese on the mechanism of penetration of enteric pathogens. This paper consisting of 90 pages and 35 figures will appear in the Proceedings of the 49th Meeting of Japanese Society for Microbiology held 1968 in Tokyo. The paper entitled "Electron microscope observations on enteric infections by pathogenic bacteria will be part of the to be published Symposium on "Invasion of the Gut Mucosa by Pathogens". A shorter version of this work of approximately 25 typescript pages plus 20 text figures will appear in the Current Topics in Pathology Series (Springer Verlag, New York) by invitation. An unrelated study on the effect of pilocarpine on intestinal mucus led to an investigation of the globule leucocyte (see bibliography).

4. Dr. Jervis has been the principal associate collaborating with members of the Department of Radiobiology, Division of Nuclear Medicine. She further explored the effect of radiation and radiomimetic drugs on the small intestine of mice and rats (Jervis, Helen R. Effect of Irradiation with 2000 R X-rays on the exteriorized small intestine of the rat. A communication to the meeting on "A Study of the Metabolic Aspects of Therapy of Radiation Injury in the Soldier." of Sept. 24-25, 1968). A study of the effects of neutron irradiation on the small intestine of conventional and germfree mice is near completion. Preliminary results of these studies were presented at the Federation Meetings in April 1969 (Jervis, H.R. and M.M. McLaughlin. A reappraisal of the phenomenon of intestinal denudation in case of radiation and radiomimetic drug injury. Fed. Proc. 28: (2) 514, 1969), and at the 8th Annual Meeting of the Association of Gnotobiotics, in June 1969 (Jervis, H.R. and M.M. McLaughlin Acute intestinal radiation syndrome: Comparison of the small bowel histology in germfree and conventional mice following neutron irradiation - A communication to the 8th Annual Meeting of the Association for Gnotobiotics. Oak Ridge, Tenn. June 10-13, 1969). She completed a manuscript of work started by Dr. K. Madi, a Brazilian Fellow, on the effects of protein depletion in the rat.

5. Dr. Merrill completed a manuscript on the ultrastructural changes in experimental cholera and is continuing work on electron histochemical tracers in cholera. A preliminary report was presented on this work at the Federation Meeting (Electronhistochemical demonstration of increased vascular permeability in cholera. T. G. Merrill, Fed. Proc. 28: (2) 1059, 1969).

#### Summary and Conclusion

Captains Kasdon and Maenza were succeeded by Majors Sheshan and Zimmerman. Dr. Merrill left in early 1969. In our research effort we have been trying, to the extent that we are permitted or that material is made available to us, to study human conditions or experimental models applicable to man. Great emphasis was placed on multidisciplinary and collaborative research.

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(1 April 1968 through 30 June 1969)

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 123, Histopathologic manifestations of diarrheal diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION NO.	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
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B. CONTRIBUTING							
C. OPERATIONS	1412A(2)						
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(U) Absorption and Loss of Radioisotopes by the Gut (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>c</sup>							
014100 Radiobiology		012900 Physiology					
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D. CUM. AMT.						85	
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24. GENERAL USE				25. SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence considered				26. ASSOCIATE INVESTIGATORS			
				NAME: Schade, MAJ S. G. DA			
				NAME:			
27. REFERENCES (Precede each with Security Classification Code)							
(U) Absorption; (U) Intestine; (U) Iron; (U) Heme; (U) Hemoglobin; (U) Polymer							
28. TECHNICAL OBJECTIVE, 29. APPROACH, 30. PROGRESS (Provide individual paragraphs identified by number. Precede each with Security Classification Code)							
23. (U) Studies of mechanisms regulating the absorption, excretion and body content of trace elements.							
24. (U) Studies of intraluminal, mucosal and corporeal factors influencing the absorption of organic and porphyrin iron.							
25. (U) 69 01 - 69 06 Iron deficiency is a major nutritional problem throughout the world. Collaborative undertakings with other laboratories have provided improved methods for quantifying this problem and establishing world standards. Basic studies of iron absorption delineated the mechanisms responsible for the better absorption of iron from meat than vegetable diets. Studies of intraluminal factors revealed the role of hydrochloric acid in iron absorption. Absorption of many trace metals was shown to occur through common absorptive pathways in the intestinal mucosa. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.							

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<sup>a</sup> Available to contractor upon contractor's request.

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 125, Absorption and loss of radioisotopes by the gut

**Investigators**

Principal: COL Marcel E. Conrad, MC

Associate: MAJ Stanley G. Schade, MC, MAJ George M. Bernier, MC,  
and Harold L. Williams

Description

Studies of the mechanisms regulating the body content of trace metals by investigation of both absorption and excretion of radioactive labeled compounds.

Progress and Results

Iron deficiency is a major nutritional problem throughout much of the world. It reduces the capability of affected persons to remain self sufficient and maintain sustained productive work. The incidence of iron deficiency in populations has been estimated in population studies by the frequency of anemia. Since there are many other causes of anemia and mild iron deficiency is not accompanied by significant anemia, it was believed that better methods for quantification of iron deficiency were needed. Measurements of the serum iron concentration and the total iron binding capacity provided better methods of determining the incidence of iron deficiency than the hemoglobin concentration. However, there are no established standards for these tests and the methodology is moderately complex, time consuming and requires standardization before reliable results which permit comparison between laboratories are obtained. Under the auspices of the International Committee for Standardization in Hematology and in collaboration with the World Health Organization improved methodology for both standardization and practical use are being developed. Serum specimens and artificial standards containing measured amounts of purified transferrin and albumin are prepared and distributed to eight collaborating internationally recognized laboratories with competence in the field of iron metabolism. Laboratory results obtained in these laboratories with various methods are compared. Methods are improved based upon comparisons of results and retested. These analyses have resulted in significant modifications of currently accepted chemical methods. A method for autoanalysis of methodology is being developed for large nutritional studies. Although serum iron methods are reasonably standardized at present, marked variations in measurements of transferrin continue to be a problem which requires additional scrutiny. The effects of storage of standards for intervals under various conditions of temperature and humidity and in wet and lyophilized states are being investigated.

Iron deficiency occurs in many geographic areas because of an iron depleted diet. However, the problem is more complex because iron deficiency anemia is commonplace in many populations who consume an iron replete diet. Intestinal parasites, chronic infection and frequent pregnancy contribute significantly to the severity of iron deficiency in poorer populations. However, the common denominator seems to be the consumption of a protein deficient diet. Marked differences in the quantity of iron absorbed from various radio-labeled foods attest to the importance of the chemical composition of the diet. Iron is absorbed efficiently from meat and relatively poorly from most vegetable foods such as rice and corn. This difference can partially be explained by the absorption of heme from the diet into intestinal mucosal cells as a metalloporphyrin. However, iron is poorly absorbed as heme when compared to test doses of hemoglobin-iron indicating that amino acids from globin are important facilitators of iron absorption. Amino acids prevent polymerization of heme into macromolecules which cannot be absorbed by the gut. Similarly, amino acids and certain sugars improve the absorption of inorganic iron, and vegetable iron is absorbed in greater quantities when meat is added to the diet. Depolymerization of heme occurs at the alkaline pH encountered in the duodenum. However, similar reactions with inorganic iron occur only at an acid pH. This latter observation provides a role for hydrochloric acid in the stomach. Acidification of inorganic iron in the diet in the presence of amino acids, sugars, amides or ascorbic acid permits the formation of complexes which remain soluble and available for absorption after they pass into the alkaline duodenum. This observation explains the poor absorption of iron by achlorhydric subjects and the high incidence of iron deficiency after gastrectomy.<sup>1-5</sup>

Other studies of intraluminal factors were continued. Previously, we showed that endotoxin markedly depressed iron absorption. Investigation of drugs that ameliorated the effects of endotoxin such as antihistaminics and corticosteroids had no effect upon iron absorption. However, certain drugs affecting the autonomic nervous system increased iron absorption. Studies of the alpha adrenergic blocking agents showed that their primary effect was slowing of intestinal transit time to permit more prolonged contact of iron with absorptive cells, and that it was unrelated to the endotoxin effect.

Studies were initiated to investigate the interrelationships of nonferrous metals upon iron absorption. Cobalt, nickel and manganese each compete for the same absorptive sites in the intestinal mucosa as iron. Absorption of these metals is significantly altered in the various states of iron repletion and by conditions which affect iron absorption. Additional scrutiny of these observations may provide a better understanding of basic factors affecting mucosal uptake of trace metals from the gut.

### Conclusions and Recommendations

Iron deficiency is a major nutritional problem throughout the world. Collaborative undertakings with other laboratories have provided improved methods for quantifying this problem and establishing world standards. Basic studies of iron absorption delineated the mechanisms responsible for the better absorption of iron from meat than vegetable diets. Studies of intraluminal factors revealed the role of hydrochloric acid in iron absorption. Absorption of many trace metals was shown to occur through common absorptive pathways in the intestinal mucosa.

**Project 3A062110A822 MILITARY INTERNAL MEDICINE**

**Task 00 Military Internal Medicine**

**Work Unit 125, Absorption and loss of radioisotopes by the gut**

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PROJECT 3A062110A823  
MILITARY PSYCHIATRY

Task 00  
Military Psychiatry

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
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11. TITLE (Precede with Security Classification Code)							
(U) Social and Preventive Psychiatry (09)							
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002500 Clinical Medicine 013400 Psychology							
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23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with security classification code.)							
(U) Community Psychiatry; (U) Cross-cultural Communication; (U) Aggressive Behavior; (U) Psychiatry; (U) Stress Performance; (U) Endocrine Response							
23. (U) To investigate developmental and sociocultural factors in primates and man influencing production of maladaptive psychophysiological and group behavior, using results to maximize individual adaptive behavior in military units, e.g., reduction of man days lost due to psychophysiological complaints, AMOL, basic training stress, and improvement of the ability of the soldier to communicate with members of other cultures.							
24. (U) To study influence of individual and sociocultural factors in clinical and field circumstances in the US Army, SE Asia and certain portions of American civilian society on human behavior. The techniques employed are individual psychiatric interviews, questionnaires, social anthropologic investigations, semantic analysis, operant conditioning, analysis of formal and informal structure of organizations, and measurements of psychoendocrine reactivity. Since these studies are carried out using humans operating in varying environments, the major technical problems arise from unexpected environmental changes, e.g., intensification of the struggle in Vietnam.							
25. (U) 68 10 - 69 06 Investigations continue into origins and maintenance of alcoholism at Ft. Meade, Md. Followup has been completed demonstrating the effectiveness of operant conditioning in a therapeutic environment on restoration to duty. Work continues correlating individual personality characteristics, field performance, and endocrine activity at Aberdeen Proving Ground. Longitudinal studies of aggressive and dominant behavior in primates have been initiated in collaboration with Yerkes Regional Primate Center. A new laboratory to measure testosterone activity in primates, rats, and humans is being completed. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul68-30 Jun 69.							

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Project 3A062110A823 MILITARY PSYCHIATRY

Task 00, Military Psychiatry

Work Unit 030, Social and preventive psychiatry

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SP4 Lester Karns

I. INTRODUCTION

Leadership potential and the ability for sustained motivation or drive are two related and crucial determinants of effective performance. These characteristics are the product of many influences, for example, past personal history, education or training of the individual alone or in groups, as well as the role of various biological factors. The ability to assume command and pursue a task vigorously may be viewed as an appropriate and channeled expression of aggressive behavior. This is in contrast to the inappropriate and impulsive expression of aggression seen in individuals with a chronic history of anti-social or delinquent acts. This ability also contrasts markedly with individuals who show the absence of drive or motivation, those who show a diminished capacity for accomplishment, those with inadequate aggressive behaviors. In the past year the Department of Psychiatry has undertaken a new program to study the relevance of endocrine variables on the expression of aggression, and on the ability for sustained motivation and leadership. This new area of investigation into the psychoendocrine basis of aggression complements the field studies underway on alcoholism, repetitive AWOL behavior, group influences on behavior and the follow-up of patients with behavioral disorders treated on Ward 108 in 1967, 1968.

II. PSYCHOENDOCRINE BASIS OF AGGRESSION

A. Background and Goals

In the past several years the crucial influence of testosterone on aggressive behavior in animals has been documented. Male animals deprived of testosterone during a specific period early in life failed to respond with normal aggression later in adult life. Female monkeys treated with androgens early in life

exhibit male patterns of aggression and play behavior during adolescence and adulthood. The precise influence of testosterone on human aggressive behavior is unclear. Castrates and hypogonadal males are reported anecdotally to show decreased libido and aggression. The effect of absent testosterone may also be appreciated from the work done with genetic males who recently have been shown to have an enzymatic defect in utilizing the normal amount of testosterone they produce. These individuals have the external appearance of females and behave like females despite their male XY chromosome complement ("testicular feminization syndrome").

The precise influence of an excess of testosterone on aggressive behavior is also unclear, but there are some clues to its importance. There exists the clinical lore of administered testosterone increasing drive and some relatively uncontrolled studies in the 1940's reporting salutary effects of administered androgens on underperforming adolescents. Recent work by Klaiber and associates at the Worcester Foundation for Experimental Biology and Medicine demonstrates that infused testosterone improves performance in mental arithmetic during stress. There has been a flurry of interest recently about males with an XYY chromosome complement and the possible relationship of the extra Y chromosome to increased impulsive, aggressive behavior. There are some reports in the literature that these men excrete increased amounts of gonadotrophins in the urine and some are reported to have unusually high levels of plasma testosterone.

A recent study completed in our department in collaboration with the Department of Neuroendocrinology has shown that some men in Basic Combat Training at Ft. Dix, New Jersey and other men at a Special Forces "A" Team in Vietnam had diminished excretion of testosterone and the two major 17-ketosteroids. Lacking longitudinal data, it is not certain that this was in response to the training or combat environment, but supports the view that stress potentially alters testosterone secretion.

This brief background review provokes the question of what the relationship is between an individual's stable and stressed level of testosterone secretion and the ability to behave aggressively, to respond with appropriate determination and drive to accomplish his mission.

It is hoped that studies designed to clarify testosterone-behavioral interactions will have the following three relevant goals:

- 1) To provide a more objective and reliable basis for selecting men with high leadership potentials.
- 2) To potentially screen men whose response to stress endocrinologically tends to diminish their performance effectiveness.

- 3) To help discriminate among the group of men with a history of delinquent, antisocial behavior, those individuals who by virtue of their endocrine-behavioral set, are less rehabilitable and show less potential for change.

## B. Progress

The department is in process of pursuing the issue of testosterone-behavioral interactions in rats, primates and humans. During the past year a new laboratory was set up with the help of recently assigned Biochemist (MSC) and Enlisted Chemical Laboratory Specialist to measure the extremely low level of testosterone in the plasma and urine of animals and man as well as the capacity to measure secretion rates of testosterone using tracer doses of radioisotopes. Permission to administer radioisotopes to volunteers being studied in collaboration with the Behavioral Research Laboratory at the Aberdeen Proving Grounds has been obtained from the AEC and from the Secretary of the Army. Principal Investigator has attended the radioisotope user's course given by Health Physics, WRAMC to qualify him as a principal user and enable him to administer radioisotopes to humans as well as to animals.

### 1. Animal Studies

Studies have been initiated in rats, relating variables of developmental history, the appearance of aggressive behavior in adulthood and testosterone activity. Rats differ with respect to their susceptibility to stress, and this has been shown to relate to handling experience, group contact, etc. As stress appears to inhibit testosterone in rats, studies are in progress to correlate the degree of stress response, the decrement of performance during stress and testosterone levels. Do animals survive longer who show less inhibition of testosterone during stressful experiences? In addition, brain stimulation studies are in progress to elucidate the areas responsible for testosterone activation and inhibition in rats.

Studies in primates are being conducted in collaboration with the Department of Neuroendocrinology, WRAIR, Dr. Levine, Behavioral Research Laboratory, Aberdeen Proving Grounds, and with Dr. Bernstein, Yerkes Regional Primate Center, Emory University, Atlanta, Georgia. During this past year four compounds and associated observation areas have been constructed at the Yerkes Field Station outside Atlanta. These provide for the unhampered observation of groups of rhesus monkeys in as nearly natural surroundings as is feasible.

Rhesus macaques establish a strict dominance hierarchy maintained by a great deal of aggressive interactions. One group of 40 males will be established this summer and, after the group has stabilized,

the most and least dominant animals will be removed for study of testosterone and cortisol responses during stress. Two groups of animals with a normal age and sex distribution will be established to provide infants whose genetic and behavioral history will be precisely known. In collaboration with the Behavioral Research Laboratory, Aberdeen, it has been shown that the animal's endocrine response to certain stresses is related to his previous dominance status and to certain aggressive propensities. The exact relationship between the animal's adequacy of performance during stress, his aggressive and dominance characteristics and his testosterone and cortisol responses may be clarified in studying these groups of animals. Dominant male monkeys have been observed to undergo a very dramatic shift in behavior, reminiscent of that seen in human depression following expulsion from group leadership. Experimental manipulation of the group structure at Yerkes will also provide an opportunity to study the endocrine correlates of this behavioral state following shift in leadership roles. The dominant male rhesus is highly motivated to maintain his leadership and usually exhibits well directed and specific aggressive behaviors in preserving his dominance status. He and his low status, subordinate counterpart present an excellent model for the study of the influence of testosterone on goal-directed aggressive behavior.

## 2. Human Studies

### a. Aberdeen Proving Grounds.

More specific studies of the relationship between performance, endocrine function and stress in humans were started this year in collaboration with the Behavioral Research Laboratory and the Small Weapons Branch, Human Engineering Laboratory, Aberdeen Proving Grounds. A barracks was modified to provide maximum control and observation of the group of 12 men by cadre living continuously with them. The first pilot group of 12 subjects was selected from graduates of AIT, Ft. Dix, and brought to Aberdeen for two and a half months. Intensive psychological testing was performed, daily behavior observations were made, urine and multiple plasma samples were collected continuously for the entire period. Performance was measured on rapid rifle fire with the contingency of the subject being shot at by a BB machine gun should he fail to adequately engage in the target. Men were adequately protected so as to prevent any serious injury. Analysis of more than 60 days of urine collection and the numerous plasma samples is not yet complete. Initial results indicate the usefulness of urinary 17-OHCS as a sensitive measure of stress. Each man maintained an extraordinary constant level of excretion of corticosteroids during this prolonged period of study. Most men adapted rapidly to this environment, initially thought to be fairly stressful. There were some preliminary correlations between performance and estimates of personality styles, which are serving as a guide for selection of subjects

for our next study to begin this summer. Testosterone values have not yet been determined, and correlation with aggressive behavior awaits completion of laboratory and subsequent sample analysis.

b. Patuxent Institute for Defective Delinquents.

Preliminary work has begun on the study of prisoners serving indeterminate sentences at the Patuxent Institute, Jessup, Maryland. These men have a history of violent crimes and many show presumably uncontrollable outbursts of undirected, impulsive aggressive behavior. In a pilot study, several men are being followed over a six to eight week period with frequent blood samples drawn for analysis of testosterone and cortisol content to be correlated with outbursts of aggressive behavior. This study will help in the design of a proposed longitudinal study of men in the stockade at Ft. Meade, to be selected on the basis of persistent delinquent behavior and to include men with an XYY chromosome complement.

c. Depressed Patients.

In order to test the hypothesis that individuals may inhibit their own secretion of testosterone during psychological stress, clinically depressed male patients are being studied. It is possible that some of the symptoms of apathy, withdrawal from the environment, and general lack of interest may be maintained or intensified by a fall in testosterone secretion. A pilot study of six male patients before and after ECT therapy has begun. Plasma and urine samples are being collected for testosterone and cortisol analysis, with cortisol being used to monitor the intensity of the stress associated with depression. It is possible that the depressed patient may represent the extreme lower end of the motivation-aggression continuum and, in addition, study of these patients permits a more accurate investigation of the dynamic range of testosterone inhibition-activation with the individual being studied serving as his own control.

d. Stockade and XYY Study.

If there is a genetic predisposition to more aggressive or violent behavior, it is possible that the phenotypic expression is mediated through testosterone's action on the brain. Although the prevalence of the XYY chromosome abnormality in the general population has not yet been established, studies on individuals in penal institutions and mental hospitals yield an incidence of 1:100 to 1:250. A recent Danish study reports that individuals with the normal male XY complement but with a presumably larger than normal Y chromosome also have a tendency for aggressive, criminal behavior. There are no studies to date correlating psychiatric and psychological observations with testosterone activity. There is some evidence that these individuals may exhibit

an acute or chronic abnormality in testosterone secretion, and they may constitute the extreme end of the high testosterone-impulsive behavior continuum.

The Genetic Analysis Laboratory at Andrews Air Force Base Hospital has expressed the wish to collaborate with our department, providing genetic analyses of suspect XYY males at the stockade at Ft. Meade. We propose to follow men who have been shown to be XYY over a 4 to 6-week period, with frequent blood and urine samples to be correlated with the behavioral observations. This project is still in the planning stage and requires support at the field level. It provides an excellent opportunity to investigate in detail the relationship between anti-social behavior, testosterone activity and possible hereditary disposition to respond with inappropriate aggression to demands from the environment.

### III. FIELD STUDIES

#### A. Ft. Meade

##### 1. Alcoholism.

The Walter Reed NP Field Research Team at Ft. Meade has continued work this year into investigation of the factors that contribute to and maintain chronic alcoholism. CPT Sklar, MC and his associates have collected a great deal of data this year on 600 patients and controls, 150 alcoholic patients and their spouses, 150 patients with other diagnoses from the MHCD, 150 men from the Stockade and a control group of 150 individuals. They have obtained historical data on the age and extent of loss of either parent of the subject during his childhood, administered various psychological tests including the MMPI, and administered a questionnaire to assess style of interpersonal interaction. This data is being processed for computer analysis and results are incomplete. Preliminary findings indicate that the alcoholic patients and those men with a history of repetitive AWOL behavior have experienced a more severe and prolonged disruption of their family during childhood compared to the control group. Group treatment of alcoholic patients has continued. Present goals are to prepare a plan to optimize placement of dependents when separated from the head of household due to active duty commitments. We are also preparing recommendations for the optimum placement of individuals with a history of alcoholism so as to minimize the interpersonal difficulties they experience.

##### 2. AWOL Offenders.

CPT Hartnagel and associates have completed the first stage of a study of repetitive AWOL offenders. The first group of

men studied were from the Stockade at Ft. Meade and 100 individuals had an extensive structured interview lasting one and one-half hours, and took several psychological tests. They will be compared to men diagnosed as behavioral and character disorders admitted to the NP Ward at Walter Reed General Hospital. As many of the men on the NP Wards with this diagnosis have a history of chronic AWOL behavior, they will serve as a comparison group to the men studied from the stockade. Individuals from both groups will be followed for 6 to 12 months to determine the outcome of their career in the Army. The men will be compared in an effort to determine what personality characteristics lead to admission to the hospital rather than confinement in the stockade. Another goal will be to examine the hypothesis that being confined in the stockade by itself may tend to prejudice the future performance of the individual and actually contribute to any tendency for repeat AWOL behavior. In addition, it is hoped that this study will be coordinated with the XY-endocrine project that has been proposed.

#### B. Thailand-Group Relationships.

The field research involved in this study was carried out in the period April 1965 to June 1968 as a project of the U.S. Army Medical Component, SEATO Medical Research Project. This research was concerned with the comparative analysis of the determinants of systems of medical care and the analysis of how health and illness behavior alter group relationships. The data were collected through the use of standard anthropological participant observation interviewing two villages in northern Thailand. Six hundred households were covered by the surveys and data were collected on medical, social and economical phenomena. The past year's work has involved the organization, reduction and analysis of data acquired during the previous three years.

#### C. Alaska.

Field studies in Barrow, Alaska, and in a village in the Canadian Northwest Territory were completed in September 1967. The studies focused on small isolated communities which manifested a high degree of interdependence among the members. These studies have helped clarify the changes in group structures that occurred following isolation and intensification of interdependence among the members. These studies are relevant to the individuals who are running the DEW line, those aboard oceanographic vessels, lightkeeping and loran stations. Another finding that has emerged from these studies is the discovery of an excess consumption of alcohol in the two communities studied.

#### IV. Clinical Studies

##### A. Hypertension.

In collaboration with the Department of Experimental Psychophysiology and the Cardiology Unit at WRGH a study was begun in the operant conditioning of hypertension. There have been preliminary reports in the literature that individuals with both cardiac arrhythmias and essential hypertension have responded to the use of operant techniques in the control of these cardiac disorders. In order to more systematically evaluate this and potentially apply it to patients treated at WRGH, a study was begun on normal subjects as controls and a pilot study. The aim is to explore more systematically the parameters used in establishing operant control of cardiac function, as well as obtain base lines for studies in patients.

##### B. Short Term Psychotherapy.

A study was initiated to investigate the usefulness of short-term psychotherapy in treating individuals with various acute psychiatric emergencies. It is hoped that this study will yield a more systematic and reproducible approach to short-term psychotherapy, which may be applied in MHCD at various Army installations. The project involves the video taping of twelve one hour psychotherapy sessions in which independent ratings of diagnosis and treatment techniques will be made. Follow-up of the individuals will be made at a 6 and 12 month period to provide more systematic data for comparison of therapeutic effectiveness. In addition, a home interview at the onset as well as at the termination of therapy will be conducted by a Sociologist to provide collaborative data to that which is obtained in the clinical interview. The project also involves the participation of one or two residents in psychiatry at WRGH and potentially may develop into a teaching seminar on the application of operant principles in psychotherapy.

##### C. Ward 108.

The follow-up study of patients admitted to Ward 108 and matched controls admitted to WRGH from January 1967 through April 1968 has been completed. The experimental group has an overall success rate of 62 percent compared to the success rate of only 28 percent of the control group. Success was defined as either still on active duty and functioning successfully or honorably discharged. Failure was defined as having received an administrative or dishonorable discharge or on AWOL or in a stockade. The crucial test of any therapy program is to show that the specific procedure used rather than a "charismatic" leader, is the important independent variable in its effectiveness. In order for a treatment program to become a routine operation in psychiatric wards and hospitals, it must be demonstrated that the skills required from the staff are

easily transferable to the usual personnel available. To test these variables, a regular Army psychiatrist and psychologist, previously untrained in the application of operant techniques to a clinical population were trained for ten two-hour sessions over a three month period in operant ward management principles and procedures by members of the NP Division staff. In July 1968 these men took over as leaders of the ward with the former Principal Investigators as consultants. The treatment program has continued to function well and although follow-up on patients treated since July 1968 is still incomplete, indications to date are that the results will be comparable. In addition, an extensive manual was written describing the therapy and principles, as well as the techniques involved in establishing a controlled therapeutic environment.

#### D. Neurological Studies.

A recently assigned Neurologist has taken over the behavioral studies in aphasia initiated previously. The plans include continuation and extension of the method of testing various forms of aphasia; matching individual choices, oral naming, and written naming are used as a response form for sample stimuli presented in visual tactile and auditory modes. Individual patients showing new, previously undiscovered deficit to profiles will be studied more intensely. This Neurologist also consults on the neurology wards at WRGH once a week in resident training. In addition patients with temporal lobe disorders are being selected for potential study of temporal lobe epilepsy and changes in testosterone function. This work is based on stimulation studies done in the rat and in primates demonstrating the importance of the temporal lobe in regulating neuroendocrine secretion.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
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016200 Stress Physiology 009400 Man-Machine Relat 013400 Psychology							
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f. CUM. AMT.							
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Foreign Intelligence Considered				NAME:			
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24. REVISIONS (Precede with Security Classification Code) (U) Psychophysiology; (U) Human Volunteer; (U) Stress; (U) Mental Hygiene; (U) Human Performance; (U) Learning; (U) Memory							
25. TECHNICAL DESCRIPTIVE, 26. APPROACH, 27. PROCESSES (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) This research focuses upon analysis of principles underlying behavior environment relationships leading to performance decrement and associated physiological responses. Emphasis is placed upon development of models which are predictive of performance at different levels of task complexity. Research content includes vigilance degradation, human data processing, behavioral stress, concept formation, memory, psychological fatigue, programmed instruction, and physiological conditioning processes.							
24. (U) Laboratory experimentation with human volunteer subjects is performed to define underlying processes which consistently lead to stress and fatigue phenomena. Psychophysiological measurements are used to assess physiological cost associated with exposures to experimentally defined environments. Performance limits are experimentally defined under a variety of normal and unusual environmental conditions.							
25. (U) 69 01 - 69 06 Research has been extended to study effects of 72-hour sleep deprivation on complex vigilance performance. A total of six test subjects have been tested over continuous 14-day periods each. A second project performed to study normal oscillations in panel monitoring performance over long (22-hour) periods of continuous monitoring. Each subject was given three of these 22-hour sessions, and the resulting data were analyzed using spectral analyses. Current efforts include the study of rapid shifts in work-rest schedules on complex vigilance performance. Two 14-day investigations in this study have been completed, each involving a four-hour shift. Longer shifts will be studied in the near future. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 June 69.							

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Project 3A062110A823, MILITARY PSYCHIATRY

Task 00, Military Psychiatry

Work Unit 031, Analysis of behavior and of mediating mechanisms:  
Measurement of performance and decrement of performance

Investigators.

Principal: Thomas W. Frazier, Ph.D.

Associate: CPT James P. Flanders, MSC; CPT James R. Gentile, MSC,  
Harold Lawson, M.A.

Description.

This work unit studies influences of social and environmental variables upon human performance. It includes psychophysiological measurements to determine physiological costs associated with maintaining demanding task performance in aversive laboratory and natural settings. Research content includes decrements in human vigilance performance, behavioral stress, verbal learning and retention, interpretation of psychological tests and measurements, and group behavior.

Progress.

1. Rhythmicity in baseline vigilance performance.

The vigilance research program has been extended from basic methodological investigations to identify and characterize factors leading to performance decrement. One of the completed studies in this program was performed to investigate oscillations in vigilance work and efficiency performances within the range from 1-hr. to 24-hr. variation. While previous studies had demonstrated strong 24-hour (circadian) rhythms, both in visual scanning rates and in measures of signal detectability, this study was concerned with the possibility of fluctuations at higher frequencies. The main focus of concern was directed toward investigating whether a period of 90 - 100 minutes could be detected through spectral analysis. This 90 - 100 minute cycle was associated with past findings that the electroencephalogram routinely reveals similar fluctuations during undisturbed sleep. Others have suggested that these fluctuations occur throughout the day, as a basic biological rhythm which might be manifested in vigilance measurements.

A total of six test subjects were tested for three 22-hour sessions each, and were given only 15 minute breaks at 2 1/2 intervals in the course of a daily session. The task employed for the investigation consisted of detecting pointer deflections on three test meters, each programmed to present signals according

to qualitatively different schedules. The results are as follows: On a group basis, power density spectral analyses revealed four consistent sources of cyclic variations in efficiency and work level data. The primary spectral intensity observed was associated with a period of about 24 hours (the circadian rhythm). Another intensity, which was even larger in the case of several measures than the 24-hour rhythm was associated with the 2 1/2 hour work-rest schedule. A third spectral intensity appeared at around 9 hr. The source of this rhythmicity has not yet been identified. A small intensity was consistently observed at around 100 minutes, and it was most pronounced in the signal detectability measures. Rectal temperature measurements revealed only the 24 hour rhythm. Heart rate was highly similar in spectral configuration to visual scanning rate data, with rhythmicities noted at all of the points described above. Day-night differences in spectral configuration were observed in each of the signal detectability measures, with enhancement of the rhythms described above during daytime hours. Additional analyses, including cross-spectral analysis, coherence analysis, and phase spectral analysis supported the conclusions described above, in showing that the rhythms detected were shared across the different estimates of vigilance performance.

## 2. Effects of sleep deprivation of complex vigilance performance.

A second area of experimental emphasis concerning performance decrement has been the investigation of how sleep deprivation induces decrement in human vigilance performance. A series of three continuous 17-day studies have been completed to study how 72-hour sleep deprivation alters normal performance rhythmicity, and how quickly individuals deprived of sleep for this period recover to original baseline levels. Two test subjects were employed in each 17-day test. Each individual was tested for six 45-minute sessions per day, following one day of initial practice on the visual monitoring task. A subject was given two additional test sessions on sleep deprivation days during nighttime hours. Subjects were also monitored during sleep during baseline days to study heart rate and rectal temperature at those times. During the 72 hours of sleep deprivation, subjects were always accompanied by assistants to insure that they stayed awake. The results are as follows:

a. Heart rate, and rectal temperature. As sleep deprivation progressed there was a marked decline in heart rate, with a suggestion of stabilization during the third day of deprivation. Rectal temperature also declined continuously. Superimposed upon these declines was a 24-hour rhythm, which was most pronounced in rectal temperature. Heart rate showed evidence of more complexity, associated with other sources of rhythmicity.

b. Signal detectability. The group data revealed a pattern

of decreasing efficiency as sleep deprivation progressed for all of the signal detection measurements. The individual trends were highly similar from one measurement to another. The extent of this change by the third day was in the range of 50% baseline levels.

c. Visual scanning rates (observing rate). The rate of scanning individual meters showed highly different trends, as a result of the different schedules of pointer deflections employed. The first meter, programmed according to a fixed-interval schedule, showed first an increase in rate of responding followed by a subsequent decrease on the third day of deprivation, reflecting an initial attempt to compensate for increasing difficulty in signal detection, followed by a weakening of effort levels. The second meter (variable-interval schedule) revealed a continuous decline in effort as time progressed, which was highly similar to the trends observed for heart rate and signal detectability measures. The third meter (differential reinforcement of low rates schedule) showed simply increasing variability in scanning as time progressed. This variability was attributed to increasing periods of no responding, and the development of bursts of observing, as attempts to compensate for the pauses in responding.

d. Variability in scanning rates. Minute-to-minute variability in scanning rates varied from one schedule to another. For the fixed-interval schedule, strong circadian fluctuations were observed during deprivation, superimposed upon a slightly increasing trend. For the variable-interval schedule, variability increased continuously. For the differential reinforcement of low rates schedule, variability increased continuously, with increasing amounts of session to session fluctuation as deprivation progressed.

e. Recovery. After only about 8 hours of sleep following the deprivation period, performance levels approached pre-deprivation baseline levels.

### 3. Effects of work-rest schedule alterations on complex vigilance performance.

The current efforts in the vigilance performance program concern studies of performance decrement associated with sudden shifts in work-rest schedules. Two of these studies have been completed. Both of these studies involve shifting the normal activity cycle forward by four-hour increment following determination of baseline performance levels. Test subjects are given the same basic complex vigilance performance task used in the previous investigations. After one week of baseline performance, the work-rest schedule is shifted to the new schedule for the second (final) week. In the two studies which remain to be done, the shift will be a backward shift, so that after 7 days of baseline measurements,

the new schedule will be set back by a 4-hr value. Analyses will be performed to assess degree of performance decrement and the amount of time required before performance changes reflect the phase shift of the circadian rhythm to the new activity cycle. This work will be subsequently extended to include assessing shifts in excess of four hours.

#### 4. Effects of behavioral stress on tracking performance.

Research has been continued to study effects of punishment avoidance contingencies upon compensatory tracking performance. The goal of this study has been to distinguish factors which lead to increased effectiveness in tracking performance from those which induce performance degradation. Work up to the present time includes (a) study of contingent punishment on tracking performance alone; (b) study of how avoidance contingencies based upon a secondary (choice reaction time) task influence combined tracking and secondary task performances; (c) how avoidance contingencies based upon the tracking task influence combined tracking and secondary task performance; and (d) how avoidance contingencies based upon the secondary task influence performance when it is performed alone.

Experimental hypotheses suggested that these four conditions might lead to performance enhancement in the case of shock associated tasks and performance decrement in the case of the same tasks when the contingency was transferred to the other concurrent task. The results obtained are as follows:

a. When the tracking task was performed alone and shock avoidance was made contingent upon minimizing error levels, performance efficiency increased in the presence of the contingency.

b. When the choice reaction time task was used alone the results were highly similar. Reaction times consistently decreased in the presence of the avoidance contingency.

c. When both tasks were performed together and the contingency was based upon the reaction time task, performance on the reaction time task consistently improved. Number of control commands increased during availability of shock. The error levels in tracking performance increased during availability of punishment, however, revealing decrement in tracking accuracy.

d. Analysis of how the contingency based upon the tracking task influences reaction time measurements on the secondary task is not yet complete, but the results thus far suggest that decrements in this situation occur for reaction time performance.

#### 5. Effects of group discussion and real threat upon risk-taking

## decisions.

An extensive literature on risk-taking indicates that individuals advocate riskier solutions to risk-taking problems after discussing the problems with a group than the solutions advocated alone or before group discussion. Thus far, this literature is based upon hypothetical problems and inconsequential consequences of the decisions which are made. The relevance of the "risky shift" to group decisions in situations involving actual threat has been alleged but never investigated. One experiment is near completion which had the following objectives: (a) to examine the generality of the risky shift phenomenon by employing different group tasks, one with real aversive consequences, one with real trivial consequences, and one with hypothetical consequences; (b) to relate heart rate to group decision dependent variables, and (c) to assess the risky shift using other tasks in addition to the much-used Stoner dilemmas with the same subjects.

College volunteer males came to the laboratory and were formed into three-man groups. Each member of a control group made decisions on a series of 12 equal-expected-value two-choice problems and the 12 Stoner dilemmas on three occasions: individual pretest, group-consensus test, and individual posttest. Payoffs for the two-choice problems were poker chips, and payoffs for the Stoner dilemmas were hypothetical, as usual. Subjects in the experimental group received identical treatment except that in the two-choice problem on group and posttest occasions the payoffs were electric shocks rather than chips. Probability preferences on the two-choice task and level of risk chosen on the Stoner dilemmas constituted indices of risk, while heart rate was monitored throughout the experiment. Data acquisition has been completed and analyses are being performed at this time.

## 6. Stress-associated forgetting of verbal materials.

Gluckensberg and King (Science), 1967, 158, 517-519) reported that significantly greater forgetting in paired-associates could be obtained by shocking words which were implicitly associated (from word association norms) with the originally learned words. Their materials were an A-B list, composed of a nonsense syllable and a common word (e.g., CEF stem); a C list composed of associates of the B words (e.g., flower); and a D list, composed of word associates of C but not of B (e.g., smell). Subjects first learned the A-B list without shock, then received electric shocks on some words on the D list. Although the C list was never presented, significantly greater forgetting occurred on shock-associated B words than on non-shock associated B words.

Gentile replicated and extended these results, giving half the subjects D words and half C words, some of which were shocked. It was argued that if forgetting occurs in B words when associated D words are shocked, even more forgetting should occur in B words when associated C words are shocked. When original learning of the A-B list was controlled, there were no differences in forgetting attributable either to the C or D word lists or to the shock-no shock conditions.

Since these findings contradicted the Glucksberg-King results, as well as a more recent replication of those results, a further extension was completed by Gentile and Lawson. The same basic procedure was used, except that a Galvanic Skin Response (GSR) measure was obtained simultaneously with the recall measure on the A-B list. Then the C and D lists were presented to obtain further GSR data. Significantly greater amplitude GSRs were obtained on the shocked than on the non-shocked D words, demonstrating that the shock had its expected aversive effect. However, the GSRs on the other lists were reversed: the nonshock-associated words yielded higher amplitude responses than shock-associated words ( $p < .05$  on A-B list, nonsignificant on C list). In addition, there was no significant difference in recall of the B words, although the difference was in the predicted direction. These results are interpreted as indicating that the hypothesis of motivated forgetting mediated by implicit verbal chains is, if not incorrect, of limited importance for the prediction and understanding of forgetting.

#### 7. The content of the manifest anxiety scale (MAS).

Flanders and Gentile replicated and extended a study by Kimble and Posnick (*J. Pers. Soc. Psychol.*, 1967, 7, 108-110) which tested the hypothesis that subjects respond not so much to the anxiety-related content of the MAS as they respond to the way in which the statements are phrased. In one study, words judged to be most anxiety related in the items (such as nervous in the item "I am about as nervous as other people") were deleted, and subjects solved the deleted content MAS (DMAS), followed by the actual MAS items. In another study, subjects filled in the missing word or words in the same DMAS items.

The following results were obtained: (1) The DMAS and MAS correlated  $+ .79$  thus sharing over 60% of their variation. (2) The more content deleted, the more subjects answered in the anxiety-keyed direction. (3) The more content deleted in a given item, the less variance that item shared with the total scale scores.

These results were interpreted as follows. The correlation between scales remains high despite deleted content, apparently because a large number of nonanxiety related responses can be

substituted into the deleted space. These substituted responses allow the subject to respond in the same direction they would were the anxiety content not deleted. The particular item phrasing is probably responsible for this directional response tendency (response set). However, this does not imply that specific content is unimportant. In fact, as more key content words are deleted, the variance shared by an item with total scale scores (and with the original item) drops to virtually zero, even though item phraseology remains the same.

Thus, while the present results suggest that it is doubtful that what is measured by the MAS is any very specialized or limited trait, they do demonstrate that Kimble and Posnick overstated the case for the importance of item phraseology. Content does make a difference.

#### 8. Analogy Item Solutions.

A program of research, in cooperation with Delores K. Kessler and Robert Seibel of The Pennsylvania State University, has produced this year three publications describing (1) a rating scale measure of word relatedness, (2) the process of solving analogy items, and (3) sociocultural differences in the solution of analogy items. The first area of research presents the Word Relatedness Rating Scales (WRRS) which (a) yields relatedness measures on an ordinal scale for individuals as well as groups, (b) forces a measure on each group of words, (c) is easily administered and scored, and (d) has shown a high degree of replicability and concurrent validity.

The second area of this research presents the results of four studies, designed to identify the processes used in solving analogy items. The results of Experiment I suggested that an associative relatedness process was being used to generate and evaluate the relationships assumed to be important in the solution of the items. Experiments II and IV used the WRRS in a rank order correlational technique to predict the judged degree of correctness of each item's alternative choices from ratings of the associative relatedness of the words comprising the alternative choices. Experiment III used a transfer design to determine experimentally the relation between primed associates of the analogy stem word pairs and the solutions to the items. The results suggest that associative relatedness among the item's words may account for from 28% to 50% of the variance in the solutions.

The third area of this research program has attempted to answer the question why do lower sociocultural status groups obtain fewer correct answers on standardized analogy test than do higher

sociocultural groups? Deficiencies in definitions of the words comprising an item appear to be part of the reason for the poorer performance of students of lower sociocultural standing. However, thus far the results indicate that definition deficiencies are only a minor part of the problem. The major reason appears to be that the different sociocultural groups have differences in associates to the words comprising the items. This hypothesis is presently being tested.

#### 9. Measuring Mediational Processes.

The typical study using a mediation chaining paradigm (A-B, B-C, A-C) compares the rate of learning of the A-C list by the experimental group with various controls. If facilitation occurs in the experimental group vs. controls, mediation is said to occur. Nowhere in this process is any measure obtained of the assumed changes in strength of the newly learned associations.

Gentile and Sorensen, in three studies using CVC nonsense syllables, have attempted to obtain new sensitive measures of the associative hierarchy following learning the lists. In each study subjects first learned the original lists (e.g., A-B, B-C) to a criterion of five consecutive perfect trials. In Study I subjects then rated all possible pairs of CVCs on a 9-point rating scale from high to low association. In Study II, subjects then received all possible pairs of items and were asked to recognize whether each had been paired together in the lists. In Study III, subjects were then asked to associate to each A, B, and C term, giving the first response they think of from the list CVCs.

In the first two studies the distribution of associative relatedness and recall of the pairs was ordered approximately as expected. However, there appeared to be some restriction in range and no difference was found between the facilitation and interference pairs. In the third study the results are not completely clear, but suggest that the facilitation items are more available as responses than the interference items.

Follow-up studies are planned to replicate the finding and demonstrate the strength of associates due to the mediation paradigm using words as well as nonsense syllables.

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PROJECT 3A062110A824  
IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00  
Ionizing Radiation Injury, Prevention and Treatment

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL	
				DA OAG471	69 07 01	DD-RSE (AR) 836	
3. DATE FILED SUMMARY	4. KIND OF SUMMARY	5. AUXILIARY CTRY	6. WORK SECURITY	7. REGRADING <sup>3</sup>	8A. DISC'D DISTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	8C. LEVEL OF SUB
68 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>4</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62110A	3A062110A824	00	055			
b. CONTRIBUTING							
c. COUNTERCHANGE	1212B(21)						
11. TITLE (Precede with Security Classification Code) <sup>5</sup>							
(U) Chemical Protection Against Irradiation (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>6</sup>							
014000 Radio and Radiation							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
05 59		NA		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING		b. FUNDS (\$ in thousands)	
b. NUMBER: NA				FISCAL YEAR		69	
c. TYPE:				CURRENCY		6	
d. KIND OF AWARD:				e. AMOUNT:		200	
e. CUM. AMT.				70		6	
20. RESPONSIBLE DOD ORGANIZATION				23. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D.C. 20012				Division of Medicinal Chemistry			
				ADDRESS: Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Precede SSAN if U.S. Academic institution)			
NAME: Meroney, COL W. H.				NAME: Jacobus, Dr. D. P.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2280			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Sweeney, Dr. T. R.			
				NAME: DA			
22. KEY WORDS (Precede with Security Classification Code)							
(U) Activity; (U) Chemical; (U) Compound; (U) Dose; (U) Protection; (U) Radiation Injury; (U) Human Volunteers							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Precede in individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The objective of this research is to develop a militarily useful pill to protect personnel against the lethal effects of ionizing radiation. In addition to a strictly tactical military use an efficient antiradiation compound would be useful to the Army from the clinical standpoint.</p> <p>24. (U) Approach to the objectives is through accepted drug development protocols. Synthesis and testing of potential agents is being carried out. Test results are analyzed for structure activity relationships and fed back into the synthesis program. Promising compounds are carried forward to testing in large animals and the pharmacology of these compounds investigated. In addition chronic toxicity studies, dose reduction factor studies and drug antagonism studies are being carried out. The development of efficient methods of handling chemical and biological information which can be applied to the program are being developed.</p> <p>25. (U) 68 07 - 69 06 Areas of most interest in the synthesis program during the year were the thiazolidines, to enhance oral absorption, terpenoid amidinium thio-sulfates, basically substituted aryl ethers, thiophosphoramides and diamine phosphorothioates. Emphasis on these areas continues along with the functionalizing of the carbon backbone in the 3-aminopropanethiol series. Compound WR-113191 has been tested in dogs and clinical studies have been continuing on WR-638. Two other compounds are scheduled for the clinic. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.</p>							

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Project 3A062110A824 IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00 Ionizing Radiation Injury Prevention and Treatment

Work Unit 055, Chemical protection against irradiation

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## Progress

### I. General

There has been continuing support of a program to develop a chemical prophylaxis drug to offset the effect of ionizing radiation on exposed troops. The major technical problems confronting this study required the separation of the antiradiation activity of the drugs from their chemical toxicity and the development of drugs which are tolerated in man. The approach to these objectives is through accepted drug development protocols. Synthesis and testing of potential agents are being carried out. Test results are analyzed on a continuing and cumulative basis and fed back into the synthesis program.

Promising agents are then moved forward to testing in large mammals; then submitted for preclinical and pharmacological evaluation. After these careful considerations, the most promising chemicals are studied for human tolerance. Findings with a small number of agents in man indicate that sufficient success has been achieved to support the research approach and to recommend an acceleration of this program.

## Chemistry

### II. Contract Synthesis Program

As of the end of FY 69, there are six active synthesis contracts; one of these with the University of Cincinnati, is new to the program. One contract, University of Southern Mississippi, was terminated during the fiscal year. In addition, there are two preparations laboratory contracts for the scale-up of compounds of interest. One of these with Aerojet-General Corporation, is new to the program. Finally, there is one contract with the New England Nuclear Corporation for the synthesis of radiolabeled compounds.

During FY 69 there were 232 target compounds submitted that were synthesized in the contract program. Fifteen new compounds were requested from the preparations laboratories during the year. Eleven compounds have been received; three of these were requested the previous year.

A meeting of all contractors, including biology and pharmacology, was held on 27 and 28 January to update them on the program, to exchange information and to elicit new ideas that would be worthy of pursuit. Each contractor gave a presentation of his work.

The growing realization of the importance of sulfur-covering groups in the aminothiols type of antirad with respect to toxicity and activity has given impetus to research in this area. Classes of compounds illustrating sulfur-covering would include polysulfides, hydrosulfides, sulfenamides, dithiosulfates, dithiophosphates, sulfenylisothioureas, acyl

polysulfides, hemimercaptals, mercaptoles, unsymmetrical disulfides, and perthiocarbamates. This list is illustrative rather than exhaustive.

Problems with oral absorption of the thiosulfates have not been surmounted. In an attempt to increase absorption a number of the best compounds have been synthesized with the mercaptoethylamino moiety tied up as a thiazolidine. In general, oral activity has been increased by this device compared to the Bunte salts but results have been erratic.

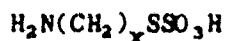
Heteroaryloxyalkyl moieties substituted on the N atom of mercaptoethylamine or derivatives continued to enjoy high priority during the past year. The 5-halopyridines are the most promising compounds in this area in that the thiazolidines have the highest oral protective index. Because of the poor absorption characteristics of the Bunte salts emphasis has shifted to the thiols and phosphorothioates.

The amidines have assumed increased importance during the year because of outstanding activity uncovered in the terpenoid and adamantane series. The oral activity of WR-109342 is particularly noteworthy.

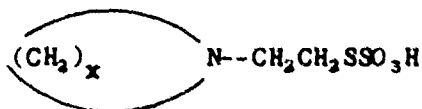
Although the aminopropanethiol series has proven to be inferior to the aminoethanethiol series in the past, the introduction of functionality on the propyl backbone increases activity tremendously and work in this area has been started during the year.

### III. Organic Chemistry Laboratory

Work on a series of primary aminoalkanethiosulfuric acids (I), prepared by the reaction of bromoalkylamine hydrobromide salts with sodium thiosulfate, was completed. These Bunte salts, having carbon



I



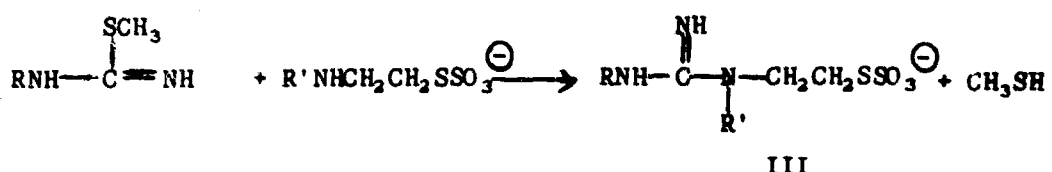
II

chains consisting of 4, 5, 6, 8 and 10 atoms, were tested for potential radioprotective activity and found to be inactive in mice.

Other aminoalkanethiosulfuric acids prepared include the methyleneiminoethanethiosulfuric acids, II ( $x=4, 5, 6$ ).

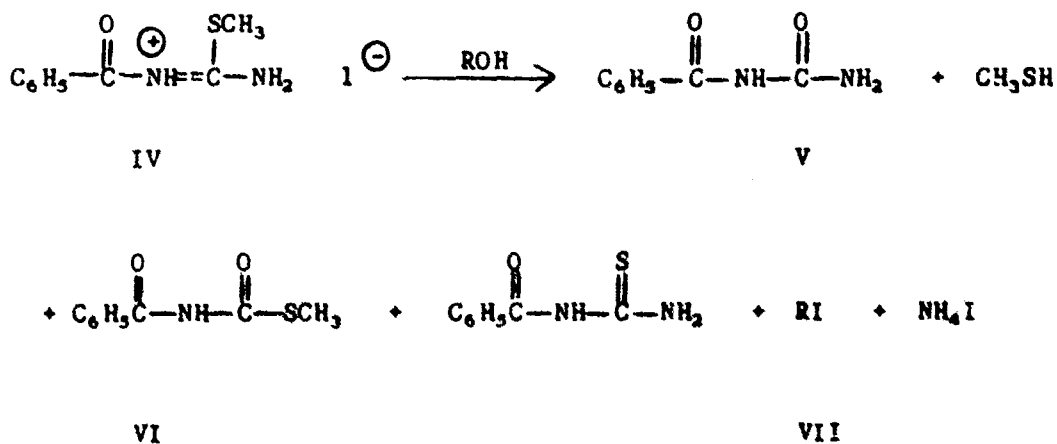
The chemistry of the S-methyl derivatives of thioureas has continued to be of high interest to this laboratory. Such compounds have been used in a variety of reactions designed to yield potential antiradiation agents.

The guanidinoalkanethiosulfuric acids (III) synthesized by the reaction of an S-methyl derivative of a thiopseudourea with the sodium salt of an aminoalkanethiosulfuric acid were tested for antiradiation activity



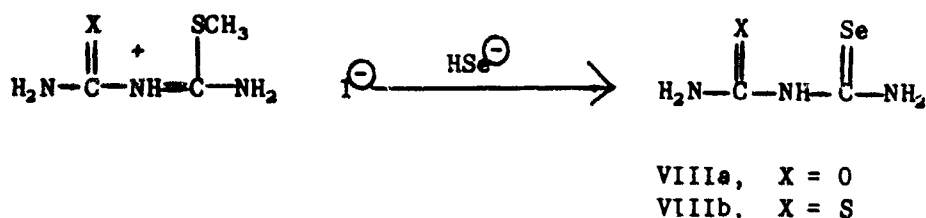
as a function of R and R'. Progress of the reaction was followed by the evolution of methyl mercaptan. Of the compounds thus prepared and tested, best radioprotective activity was observed for the parent compound, 2-guanidinoethanethiosulfuric acid (III, R=R'=H).

Previous work on the reactions of the S-methyl derivative of 1-benzoylthiourea (IV) with hydroxylic solvents has been extended and completed.



A procedure for the separation and quantitation of products V, VI, and VII has been developed. In addition, an improved synthesis of VII (from which IV is prepared by addition of methyl iodide) has been achieved.

New selenourea derivatives have been prepared through the nucleophilic displacement of methyl mercaptan from thiopseudoureas by the hydroselenide ion at pH 8. The previously unknown 4-selenobiuret (VIIIa) and 2-seleno-4-thiobiuret (VIIIb) are included among the compounds prepared.

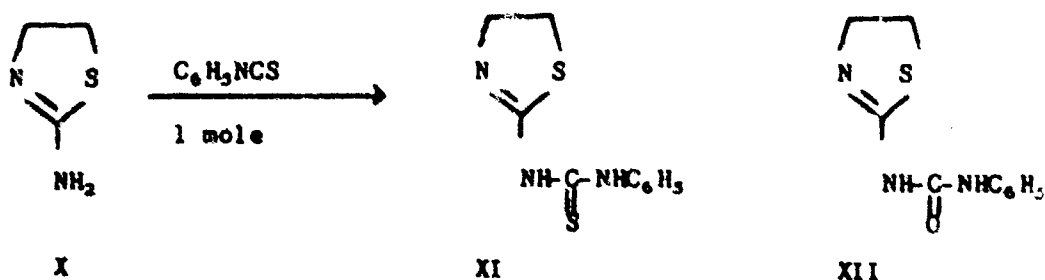


in this manner. By adjusting the pH at which the displacement is carried out (to approximately pH = 5), an amine function is eliminated, giving selenothiocarbamic esters (IX).

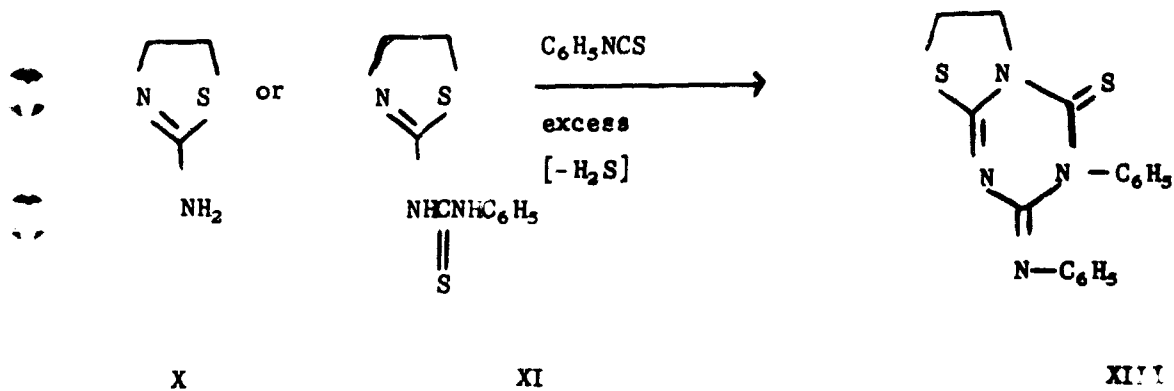


IX

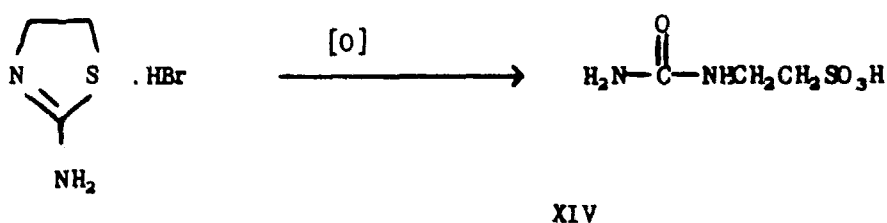
The reaction of 2-amino-2-thiazoline (X) with a molar equivalent of phenylisothiocyanate has been shown to give, contrary to an early literature report, only 1-phenyl-3-(2-thiazolin-2-yl)-2-thiourea (XI), regardless of the reaction temperature. An analogous adduct (XII) was obtained using phenylisocyanate reagent. Structures of the complex products formed in the reactions of X, XI, and XII



with excess phenylisocyanate and phenylisothiocyanate were also determined. When X or XI was heated with an excess of phenylisothiocyanate, H<sub>2</sub>S was evolved and 2-phenylimino-3-phenyl-4-thioxo-thiazolo[3.2-a]tetrahydro-s-triazine (XII) formed.



Attempts to prepare sulfoxide or sulfone derivatives by oxidation of 2-amino-2-thiazoline hydrobromide yielded instead 2-ureidoethane sulfonic acid (XIV).



The S-methyl derivatives of thiobiuret, dithiobiuret, and guanylthiourea were prepared. Further reactions of these systems are being studied.

#### IV. Rodent Testing Program

The primary screening test in which new chemical compounds are evaluated for antiradiation activity is the mouse system. Animals are lethally irradiated following administration of the test compound, and 30 day survival is recorded to evaluate the protective activity. Primary screening was performed in two laboratories: Woodard Research Corporation under the direction of Dr. Henry Horn and the Walter Reed Army Institute of Research. The Walter Reed test facility screens new compounds specifically synthesized for antiradiation testing and performs secondary tests on those compounds showing activity in the Woodard Laboratories. As much data as possible regarding the acute toxicity and pharmacology of the agent is collected and recorded at the time of the primary test. The following table summarizes the testing performed in each of the screens during the past fiscal year. Compounds listed in this table were administered intraperitoneally at 15 or 30 minutes prior to irradiation.

Table I

Facility	No. Tested	Activity		
		Good (100-50%)	Fair (49-20%)	Poor (19-0%)
Woodard Laboratories	2064	530 *		
Walter Reed	530	69	51	410

\*Compounds showing 33% survival are listed as active.

Active compounds are further evaluated at Walter Reed for effectiveness by oral administration, effectiveness at reduced drug levels, and duration of action. A summary of the activity of drugs given orally follows: 107 drugs were tested of which 26 showed good activity, 29 fair activity, and 52 poor activity. In addition, 31 compounds were tested for duration of action at time periods greater than 30 minutes prior to irradiation. Twenty-three drugs were active at 60 minutes and eight were active for periods longer than 60 minutes pre-irradiation.

The "Astro" (ICR/CR - Caesarean originated, barrier-sustained) strain of Charles River Mouse Farms, Inc. was used for all drug screening at the Woodard Laboratories and the ICR/CR strain of the Walter Reed Army Medical Center at Forest Glen was used in the Walter Reed Screen.

A limited number of agents were further evaluated in mice for radioprotective effect under contract at the University of Chicago. During the past FY, 28 compounds from previous screening programs which were consistently radioprotective were evaluated for their dose reduction factor (DRF) values alone, in combination with mercaptoethylamine (MEA), paraminopropiophenone (PAPP), and with both MEA and PAPP. Eleven compounds gave DRF values of over 1.30 when tested alone, the highest value being 1.69 for bis(diglyme) sodium hexacarbonyl vanadate. Twenty-four compounds were tested in combination with MEA and 19 of these had a DRF value greater than 1.50. Five had values greater than 1.88 and the highest value was 2.2 for p-aminophenol trifluoromethyl ether. Of 22 combinations of these compounds with PAPP, 9 had DRF values greater than 1.73. All of the rest had DRF values of 1.49 or better. Triple combinations of the screening compounds and MEA and PAPP were of particular interest with DRF values ranging from 3.27 down to a minimum of 1.67. The triple combination of 2822, MEA and PAPP which gave the highest value was evaluated further to ascertain the type of protection afforded by it and at which x-ray dosage level either the intestinal or hematopoietic protection diminished. To study this aspect of protection cholinesterase measurements and spleen weight determinations were conducted. Fourteen special compounds from WRAIR were studied further. Their toxicities were accurately determined alone, after pre-treatment with phenobarbital to induce the hepatic microsomal enzymes, and after pre-treat-

ment with EPN to inhibit hydrolysis. Preliminary enzyme studies were conducted with all fourteen of these compounds to study the metabolic aspects of their radioprotective activity and toxicity. DRF values were obtained for these compounds alone, after phenobarbital pre-treatment and after EPN pre-treatment. Cholinesterase studies and spleen weight determinations were conducted on both the screening compounds and the special WRAIR compounds to differentiate between the two kinds of protection (intestinal and hematopoietic). Compounds will be combined on the basis of the results of these determinations for further study of drug specificity and potentiation.

#### V. Radiation Protection in Dogs

Chemical agents exhibiting significant radioprotective activity in the mouse screen at Walter Reed were selected for evaluation in dogs. Those compounds that protected mice at dose levels well below the maximum tolerated dose were of particular interest. Compounds exhibiting radioprotection in mice only at near toxic levels have frequently failed to show protective activity in dogs. A preliminary toxicity study was performed with each compound prior to use for radiation studies. The initial radiation study was generally performed using the maximum tolerated dose. Compounds are routinely injected intravenously to dogs but a few compounds having exceptional oral efficacy in mice are given orally to dogs.

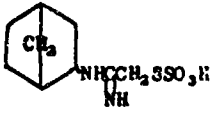
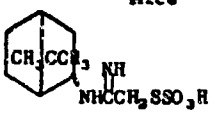
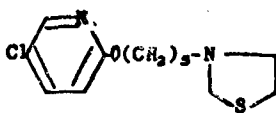
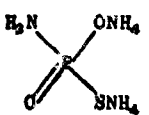
Radiation exposures were performed using a Triga Mark F. Nuclear Reactor. Exposures were made possible through the cooperation of the Diamond Ordnance Fuse Laboratory reactor staff under the direction of Mr. Walter Giesler. Animals were exposed in the wood-lined exposure room adjacent to the reactor tank. The room is 20 ft. by 20 ft. in diameter with a 10 ft. ceiling. Animals were confined during irradiation in rectangular lucite exposure cages arranged in a four cage array parallel to the gamma isodose curve produced by the reactor flux. The individual cages are 12 inches by 12 inches by 20 inches. The mid-line of the cages was 130 centimeters distant from the tank wall. By maintaining a thickness of 70 centimeters of tank water between the core of the reactor and the exposure room neutrons were selectively reduced. In this configuration the gamma radiation contributes over 98% of the dose in Rads delivered to the animals. Of the less than 2% neutron contribution to total dose, energies were predominantly thermal. The total distance between the reactor core and the cage mid-line was 2 meters. The measured radiation dose varied by less than 5% within the exposure cages. The gamma dose rate with the reactor operated at 250 kilowatt power steady state was 100 to 108 R per minute measured at the mid-line in air using a tissue equivalent ionizing chamber.

The results of all the drugs tested in dogs in the fiscal year of 1969 are listed in Table II. A comparison to the results in mice is also listed.

Table II

Structure	NR No.	Test Dose mg/kg	Route	Minutes Pre- Radiation	Percent Survival	
$\begin{array}{c} \text{O} \\ \parallel \\ \text{NH}_2\text{C}(\text{CH}_2)_2\text{NH}-\text{CHSH} \\ \text{CH}_3 \end{array}$	2861					
		Dogs	200	IV	30	0% (0/9)
		Mice	600	IP	30	53%
$\begin{array}{c} \text{OH} \\   \\ \text{NH}_2\text{CH}_2-\text{CH}-\text{CH}_2\text{SPO}_2\text{H}_2 \end{array}$	77913					
		Dogs	680	IV	30	88% (6/7)
			720	IV	30	88% (8/9)
			720	IV	30	0% (0/3) Toxic
		Mice	1000	IP	30	100%
			800	IP	30	100%
			600	IP	30	87%
			400	IP	30	100%
			300	IP	30	87%
			250	IP	30	67%
			150	IP	30	0%
		$\text{NH}_2(\text{CH}_2)_2\text{NH}(\text{CH}_2)_3\text{SPO}_2\text{H}_2$	74172			
Dogs	400			IV	30	50% (1/2) 5/7 toxic
	300			IV	30	0% (0/9)
	200			IV	30	0% (0/2) 1/3 toxic
Mice	1000			IP	30	100%
	800			IP	30	80%
	500			IP	30	100%
	400			IP	30	93%
	200			IP	30	13%
	100			IP	30	0%

Table II (continued)

Structure	WR No.	Test Dose mg/kg	Route	Minute Pre- Irrad.	Percent Survival	
	113,191	Dogs	25	IV	30	0% (0/6) Tox.
		Mice	30	IP	30	80%
	92973	Dogs	15	IV	30	0% (0/8) 1/9 Toxic
		Mice	15	IP	30	100%
	85562	Dogs	200	PO	45	0% (0/8)
			30	IV	30	0% (0/9)
		Mice	300	PO	30	73%
			80	IP	30	93%
	77912	Dogs	3	IV	30	0% (0/8) 1/9 Toxic
		Mice	15	IP	15	80%
			7.5	IP	15	53%
			2.0	IP	15	7%

Under the above conditions the radiation LD<sub>50</sub>30 day was found to be 432 R (Figure 1). A lethal dose of 650 R was used for drug evaluation studies. Of 33 control dogs radiated at this exposure level during this fiscal year, there were no survivors. The death peak (Figure 2) occurred at 10.0 to 11.0 day and the mean at 10.05 days.

One combination study was done in fiscal year 1969. Results are shown in Table III.

Table III

Structure	WR No.	Test Dose mg/kg	Route	Percent Surv.
$\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{SPO}_3\text{H}_2$	44923	100	IV	56% (5/9)
$\begin{array}{c} \text{OH} \\   \\ \text{NH}_2\text{CH}_2\text{CHCH}_2\text{SPO}_3\text{H}_2 \end{array}$	77913	300	IV	30

Results seem to indicate some synergistic action between these two compounds but additional runs must be made before definite conclusions can be drawn. As a general rule, the alkyl thiophosphates have afforded the best protection in dogs, followed by thiosulfates, thiols and thiozolidines.

#### Partial Body Irradiation

During the past year a number of experiments have been conducted with the Pharmacology Department involving the use of high dose partial body irradiation. These experiments are presently being continued.

#### VI. Liver Perfusions

Nitrogen mustard mimics the effect of radiation on cells and has been used as a substitute for radiation therapy in certain neoplastic conditions. Several of the drugs effective in protecting against radiation effects are also protective against the action of nitrogen mustard.

Hepatic neoplasia is generally considered an inoperable condition. Classic treatment has been perfusion of the organ with nitrogen mustard, a technique which produces gross insult to the normal parenchyma as well as the neoplastic cell. Breedis and Young (Am. J. Path., 30, P. 969-977, 1954) demonstrated that malignant neoplasms growing in the liver tend to acquire an exclusively arterial blood supply, regardless of the route by which tumor emboli reached the liver. In contrast, normal parenchyma received

Figure 1

RADIATION LETHALITY STUDY  
DORF REACTOR-GAMMA  
DOGS

Dose (Rads)	Animals Exposed	Animals Dead	% Mortality
292	12	2	17
356	12	4	33
432	12	6	50
518	8	6	75
650	33	33	100

Probit Graph

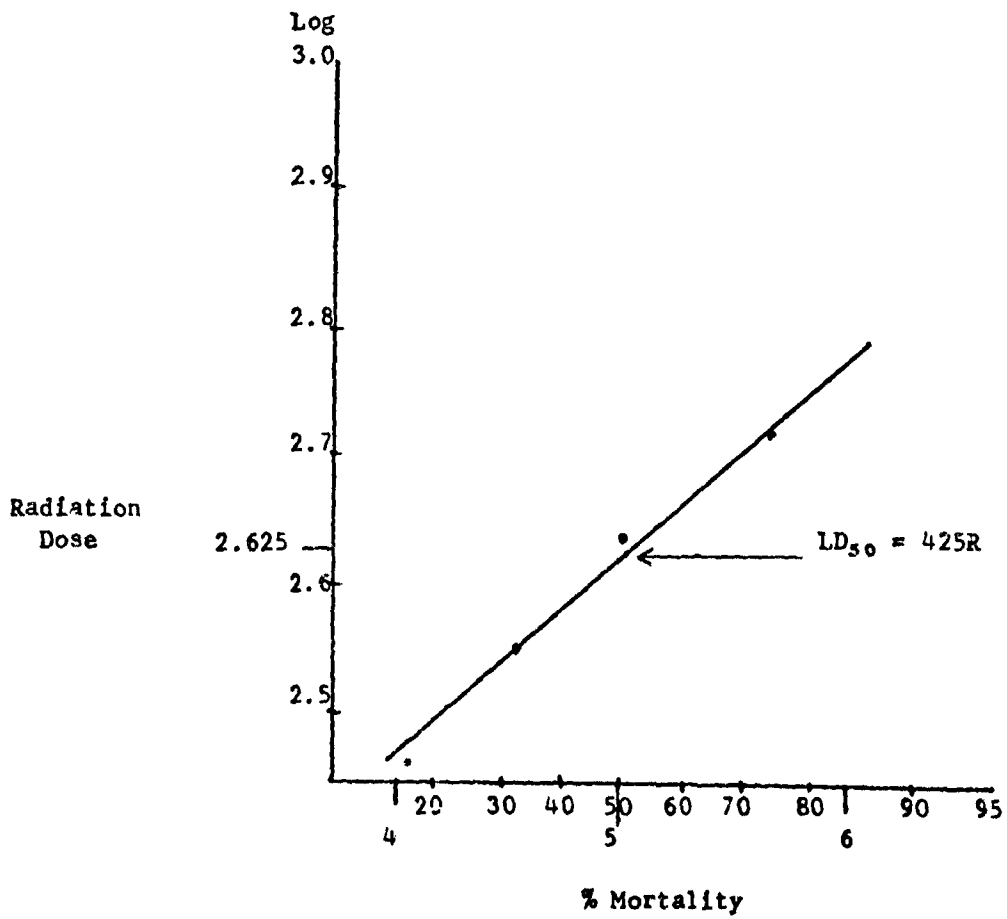
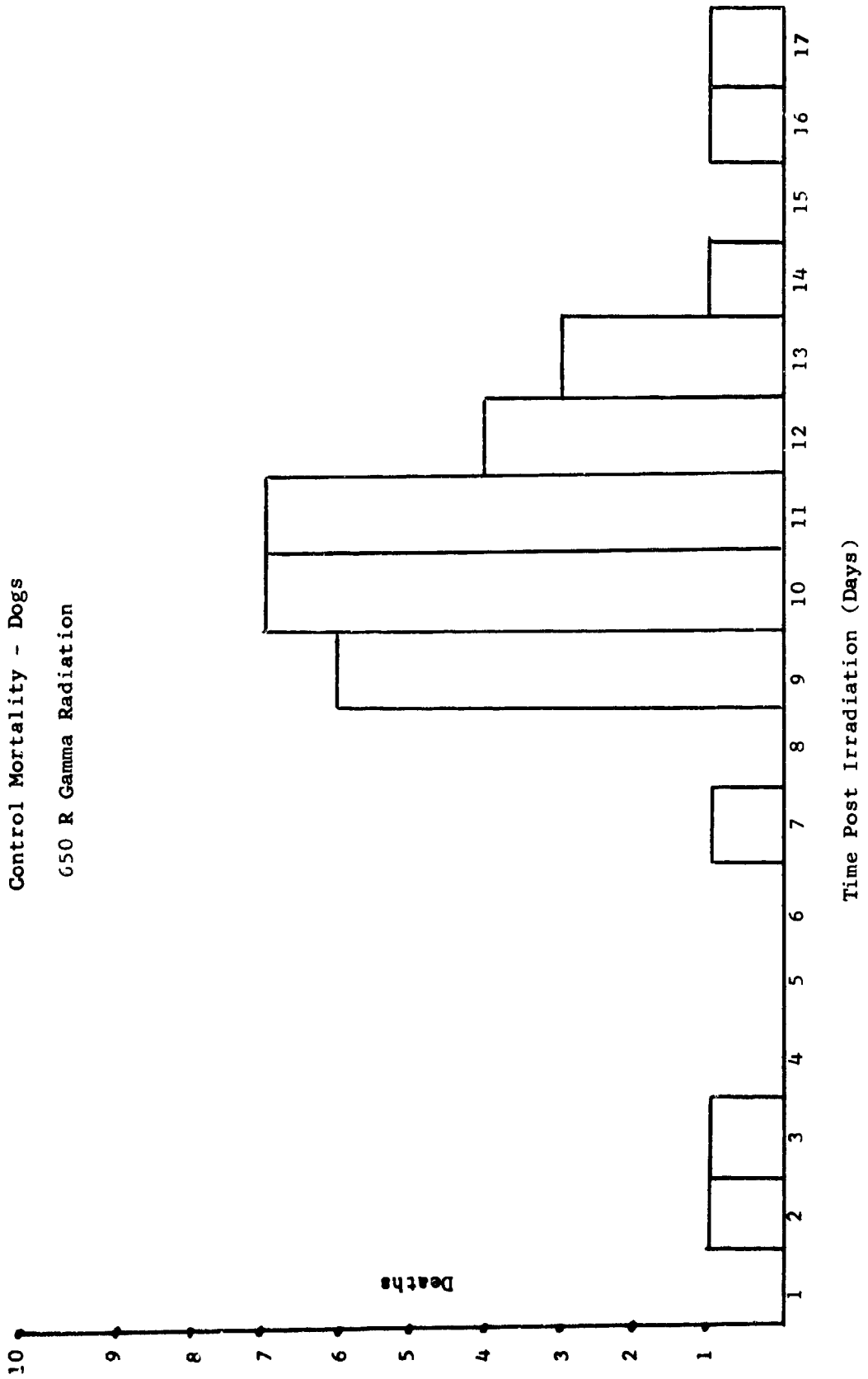


Figure 2  
Control Mortality - Dogs  
650 R Gamma Radiation



blood primarily via the portal circulation. The two distinctive circulatory patterns of the neoplastic liver enable one to be selective in perfusion of the organ. Nitrogen mustard introduced via the arterial route should go primarily to the neoplasm and hepatic tissues adjacent to them. Introduction via the venous route of a nitrogen mustard antagonist should protect the normal parenchyma. It was the purpose of this study to see if the normal parenchyma could, in fact, be protected against known lethal doses of nitrogen mustard.

During the past year, the operative procedure and the nitrogen mustard antagonist have remained unchanged. A nitrogen mustard antagonist is a drug combination of WR-348 at 400 mg/kg and WR-2347 at 300 mg/kg. The nitrogen mustard administered to the liver remains at 2.0 mg/kg. The mustard antagonist was successful in protecting four of eight dogs. The dogs were sacrificed at days 56, 63, 65, 66. The number of survivors is not felt to be the significant point, but rather that normal cells can be protected sufficiently in order that they may recover.

The initial work has been sufficiently encouraging that support from the veterinary pathology for both gross and microscopic post mortem examination has been obtained. In addition, facilities have been set up to measure blood BUN, alkaline phosphatase, SGOT, SPGT and glucose.

At the present time it is felt that the toxic side effects of the mustard antagonist is the cause of most of the fatalities which have resulted from this procedure. The definitive answer to this question is presently being sought.

#### VII. Primate Antiallergy Drug Study

##### Description

The transfer of allergic antibody (reagin) from man to other primates has shown to sensitize the skin of the recipient primates. Challenge with an appropriate antigen will produce a reaction in the sensitized animal. The allergic reaction can be localized for study purposes by injecting the reagins sera intradermally to produce a wheal or erythema. Intravenous injection of antigen and Evans blue dye produces a circumscribed blue area due to extravasation of dye at the sensitized site. Since reagin has been shown to be thiol sensitive, it should be sensitive to certain thiol containing compounds. In this procedure .1 ml of reaginic sera from clinical patients was injected intradermally into mature, healthy, *Macaca mulatta* monkeys at three sites per serum dilution. Undiluted serum plus serum dilutions of 1:2, 1:5, 1:20 and 1:125 were used in each monkey. Drug injections were given intravenously

immediately after serum injections. Another dose of drug was given 24 hours later followed thirty minutes later by an intravenous injection of a mixture of 0.2 ml of commercially prepared antigen and 2 ml of 1% Evans blue dye. Injection sites were checked after 30 minutes for color changes resulting from the extravasation of the dye. Drug effectiveness was based on a comparison of the reaction in treated animals as compared to the controls. An absence of an allergic reaction at all serum dilutions was noted with injections of WR-377 at 500 mg/kg; WR-638 at 250 mg/kg; WR-771 at 5 mg/kg; WR-1616 at 350 mg/kg; and WR-2347 at 450 mg/kg. WR-2529 gave only a slight reduction in severity at 500 mg/kg. Repeat studies are being run to confirm these results. No indication of the exact site of drug action has been derived from these studies.

An attempt to produce an allergic reaction by an intradermal injection of antigen followed by intravenous injection of reaginic serum and Evans blue dye showed little promise for studying passive cutaneous anaphylaxis in monkeys.

#### VIII. Fetal Lathyrism Studies

A series of earlier experiments conducted by M. M. Grenan, et al, of this Division, demonstrated that mercaptoethylamine (MEA) and certain other radioprotective drugs are capable of producing osteolathyrism in the laboratory mouse. Osteolathyrism evolves from defects in the connective tissue (skeleton, blood vessels and skin) and is usually manifested by aortic aneurysm, skeletal defects and/or decrease in skin tensile-strength. This drug-induced condition represents a side effect that could negate the usefulness of an otherwise efficient antiradiation compound.

Miss Grenan's experiments were based upon detection of skeletal deformities in mice after they had consumed a drug-containing diet for an extended period of time. Such a test system is workable in a limited sense, but paired-feeding experiments which continue for three to eight weeks are seldom adaptable to the screening of a large number of compounds. With this limitation in sight, it seemed important to investigate a less cumbersome screening technic for the detection of chemical lathyrogens.

H. M. McCallum (1) reports that fetal mice develop lathyrism if the mother is given lathyrogenic substances by mouth or by injection during the third trimester of pregnancy. His pilot studies were confined to the use of B-aminopropionitrile (BAPN) at rather high doses. Examination of the fetuses revealed such lathyric changes as abnormal aortic laminae and one or more sharp upper thoracic kyphoses. When this fetal assay was employed

to test a series of nitriles, semicarbazide and MEA, McCallum was lead to conclude that the only difference between lathyrism in mice and in rats is the dose response; and that a fetal assay system can be more efficient than the usual paired feeding experiments. His inability to demonstrate fetal lathyrism after the administration of well known antiradiation agent (MEA) is somewhat discouraging. However, Ferm (2) found osteolathyrogenic effects in fetal rats from mothers who had received cystamine, the disulfide of MEA. The reported skeletal changes consisted of thoracic kyphoses and abnormal rib development. The lesions were easily demonstrated in the cleared and Alizarin red stained fetuses, and seemed to be dose dependent. The above reports were controversial enough to support interest in evaluating a fetal assay system for detecting chemical lathyrogens of the aminoalkylthiol class.

#### Materials and Methods

WRAIR albino mice, ten weeks of age, were used for these pilot experiments. Females were housed with males overnight and pregnancy determined by the presence of a vaginal plug. Day-one of pregnancy was recorded as the day of appearance of the plug. The two known lathyrogenic agents used, BAPN and MEA, were dissolved in water and administered to the pregnant mice daily via stomach tube. Two dose-schedules were followed: (1) days 10, 11 and 12 for teratogenic effects and (2), days 14, 15, 16 and 17 of pregnancy for osteolathyrogenic effects. The fetuses were harvested on day 18, fixed in 95 percent alcohol, cleared in 1 percent KOH and stained with Alizarin red. Examination with a dissecting microscope was made for teratology and for skeletal changes indicative of osteolathyrism. At the time the fetuses were harvested, the number of implantation sites were recorded, and dead fetuses were noted. Suitable control animals were handled in a parallel fashion.

#### Results and Discussion

The initial results are shown in Table 1. Administration of the drugs during the period of major organ formation (gestation days 10-12) did not result in teratogenicity. However, fetal toxicity was manifested by BAPN through an increase in resorbed and dead fetuses and a decrease in the average fetal weight. MEA at a moderate dose tended to decrease fetal weight but was otherwise non-toxic. The fetal lathyrism studies (administration on gestation days 14-17) were more informative. As expected, BAPN caused skeletal abnormalities in all litters. The predominant lesions were sharp thoracic kyphosis and defects in rib structure. The posterior ribs were most severely affected and appeared twisted

Table 1  
Effects of BAPN and MEA on the Developing Mouse Fetus

Agent	Oral Dose (mpk/day)	Days Of Adm.	No. Of Litters	Percentage Resorption	Fetuses			Structural Abnormalities	
					Total (No.)	Dead (No.)	Live (No.) (Av. Wt.)		
BAPN	750	10-12	5	66	20	0	20	1.25 g	0
BAPN	750	14-17	4	11	28	15	13	1.17 g	Kyphosis & rib defects
BAPN	375	14-17	5	0	55	17	38	0.97	Kyphosis & rib defects
CONTROL	0	- - -	5	0	54	0	54	1.48	0
MEA	300	10-12	5	2.9	69	0	69	1.00	0
MEA	300	14-17	4	0	49	1	48	0.97	0

and enlarged in the medullary region. An impression was gained that maximum damage occurred in the region of diaphragm-rib attachments. Kyphosis was easily seen in the fresh fetus and caused an upward thrust of the interscapular brown fat organs and a hump-backed head-to-tail contour, much like the silhouette of an American bison. These changes were not noted in fetuses from females receiving MEA. One MEA fetus had unilateral enlargement of ribs 9 through 13. This change suggests that larger doses of MEA may also be productive of significant skeletal abnormalities.

#### Conclusion

The above studies confirm the findings of McCallum for the fetal lathyrogenicity of BAPN and the utility of this quick method for demonstrating the lathyrogenic potency of small quantities of chemicals. Larger doses of MEA (500 mg/kg/day) will have to be used before a decision can be made concerning the adaptability of such a test for screening potential radioprotective aminoalkylthiols. The teratogenicity studies emphasize one problem of drug development, i.e., well tolerated doses of BAPN for adult mice are extremely toxic for the developing fetus. Fortunately, this increased toxicity is not seen with equimolar doses of MEA and may not be a hazard should aminoalkylthiols be administered to the general population. Our preliminary findings are encouraging enough to support additional experiments in the field of fetal osteolathyrism.

#### References

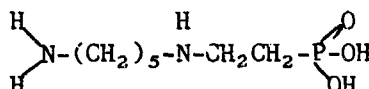
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## IX Pharmacology of Radioprotectants

### A. Studies on Adrenergic Mechanisms

#### 1. On the Specificity of the Alpha Adrenergic Blocking Properties of WR-2823.

During the past year, the pharmacology department investigated the specificity of the alpha-adrenergic blockade exhibited by WR-2823 whose structure is as follows:



S-2(5-aminopentylamino)ethyl phosphorothioic acid

Since WR-2823 is a straight chain aliphatic amine differing in structure to all other known alpha adrenergic blocking compounds, we set forth the following rigid criteria that the compound must fulfill before one could call it a specific alpha adrenergic blocking drug: (1) The chemical must depress the pressor action of epinephrine; (2) The compound must depress the pressor response to norepinephrine and this depression of the pressor response of norepinephrine must be dose (WR-2823) related; (3) The epinephrine reversal must not be due to a decrease in Cardiac output; (4) The agent must not antagonize epinephrine tachycardia; (5) The pressor response to epinephrine must be restored by a beta-adrenergic blocking agent; (6) The antagonism of the pressor response to epinephrine and norepinephrine must not be due to the stimulation of the beta-adrenergic receptor sites; and (7) The pressor response of various doses of angiotensin must not be antagonized by WR-2823.

#### Methods

Beagles dogs were anesthetized with 30 mg/kgm of pentobarbital sodium (given intravenously). The right femoral vein was catheterized for intravenous injections of test drugs. A catheter was introduced into the abdominal aorta through the right femoral artery and was connected to a Sanborn-267-B pressure transducer for the purpose of measuring arterial pressure. All the animals were heparinized so that accurate observations of the pulse pressure could be made. In those experiments where cardiac output was to be measured, an electromagnetic flow meter probe (transducer) was placed around the ascending aorta and connected to a sine wave electromagnetic flow meter. The instrument enabled us to record both the cardiac output, the maximum velocity of flow through the ascending aorta and a function of stroke

volume. Heart rate was measured by means of a cardiometer using a beat to beat mode where the height of the tracing obtained was proportional to the interval between each (and every) beat. For the purpose of measuring air exchange, the dogs were intubated orally and the tracheal catheter was attached to a Sanborn pneumotachograph head which in turn was connected to a Sanborn 270 differential gas pressure transducer activated by a Sanborn 350-1100 carrier pre-amplifier.

All of the animals were bilaterally vagotomized. Isoproterenol was used a puer beta adrenergic receptor site stimulating drug. Propanolol was used as the beta-adrenergic blocking agent. Dose responses of all agonists (epinephrine, norepinephrine, isoproterenol and angiotensin) before and after one and only one of various doses of WR-2823 (16, 25, 31.25, 43.75, 50, and 63 mg/kgm.)

### Results

WR-2823 in lower doses, 16 and 25 mg/kgm, depressed the pressor response to epinephrine and in the higher doses caused epinephrine reversal. The pressor response to norepinephrine was uniformly depressed and this depression was dose related. WR-2823 did not cause a decrease in cardiac output during the epinephrine reversal and in fact caused an increase cardiac output during epinephrine reversal. Classical alpha-adrenergic blocking drugs bearly meet this criteria at the doses most commonly used and in fact phenoxybenzamine (dibenzylamine) will cause a decrease in cardiac output during epinephrine reversal even in as low as 0.5 mg/kgm when administered too rapidly to the dog. The epinephrine reversal seen after WR-2823 was primarily due to a decrease in diastolic pressure with little or no decrease in systolic pressure. WR-2823 always caused a widening of the pulse pressure (pulse pressure changes from 45 mmHg to close to 90 mmHg were observed). The compound did not antagonize epinephrine tachycardia in spite of the fact there was always a bradycardia following an intravenous injection of WR-2823. The pressor response to epinephrine was without exception restored by propanolol. The results indicated that WR-2823 was able to block more alpha adrenergic receptors without causing any cardiotoxicity than phenoxybenzamine. The dose response to isoproterenol was unaltered by any dose of WR-2823 employed in this study indicating that the alpha adrenergic receptor site antagonism was not due to stimulation of the beta-adrenergic receptors. The pressor response of angiotensin was not inhibited or antagonized by WR-2823 indicating the blood pressure could respond (increase) to an agent which causes a pressor response through some other mechanism. Our previous work demonstrated that the depressor response to acetylcholine, 5-hydroxytryptamine and histamine were unaltered by WR-2823 (all of these responses are

modified by phenoxybenzamine). This data coupled with present study strongly indicated that WR-2823 is a specific and a rather selective alpha adrenergic blocking agent.

## 2. Further studies on the Inhibitory Properties of WR-2823 Against Pressor Amine Induced Contractions in Isolated Aortic Strips.

These studies were carried out in collaboration with the Department of Gastroenterology of the Division of Medicine.

Spiral strips of rabbit aorta were suspended in a 37° C water bath containing Krebs-henslait solution under one gram of tension and were allowed to equilibrate for 30 minutes. Isometric contractions were recorded electronically. Concentration response curves were obtained for norepinephrine, phenylephrine, epinephrine, and 5-hydroxytryptamine. The sequence of these agonists and their respective doses were randomized. The arteries were allowed to fully recover between each and every challenge. WR-2823 in a concentration of  $2.3 \times 10^{-4}$  Molar was introduced into the bath and allowed to remain in contact with the arteries for one hour at which time the agent was washed out of the bath and the concentration response curves were repeated. The accompanying figure is a graphic representation of the results of this experiment where the ordinate represents the tension developed by the arteries and the abscissa represents the concentration, (in gms/ml) of the various agonists used. The bars represent + or - one standard error. The results show that WR-2823 antagonizes the norepinephrine, phenylephrine, and epinephrine induced contractions of the isolated rabbit aortic strips but does not antagonize those contractions induced by 5-hydroxytryptamine.

## 3. Onset of Autonomic Effects in the Anesthetized Mouse Following Intraperitoneal Injections of WR-2823.

**Abstract.** A mixture of chemicals consisting of 75% 5-aminopentyl-aminoethyl phosphorothioic acid monohydrate, 12% inorganic phosphate, and 12% bis 5-amino pentylaminoethyl sulfide was injected intraperitoneally at a dose of 25 mg/kg into anesthetized mice. The cardiovascular responses to 1 mg/kg of epinephrine were recorded at varying intervals before and after the injection of the drug mixture to assess the onset of -adrenergic blockade. There was an immediate potentiation (both magnitude and duration) of the pressor effects of epinephrine followed by a progressive blockade of the pressor response to epinephrine. The inhibition began at about 15 minutes and progressed for at least 60 minutes. The disulfide contaminant was tested similarly and produced comparable effects but appeared to be slower in onset and had more potentiating effect with weaker blocking potency.

Introduction. Heiffer et al., have shown that a chemical mixture of 75% 5-aminopentylaminoethyl phosphorothioic acid monohydrate with inorganic phosphate (12%) and bis-5-aminopentylaminoethyl sulfide produces an effective blockade of the -adrenergic cardiovascular effects of catecholamineo in several species. They reported that the onset of this blockade was delayed but the time of the onset could not be assessed because of the direct cardiovascular effects from the intravenous administration of this drug mixture.

The present experiment was conducted to evaluate the time course of the onset of adrenergic blockade in the mouse following intraperitoneal injection of the drug mixture where the direct cardiovascular effects of the drug were minimal. The design also included a test for sex difference. Some animals were pretreated with phenobarbital to test the effect of induction of drug metabolizing enzymes on the onset of adrenergic blockade.

A second experiment was used to examine the pharmacodynamic effects of the disulfide contaminant in the drug mixture to assess its role in the pharmacodynamics of the mixture.

The results of these experiments confirm the blocking properties of the mixture known as WR-2823. There was no evidence of a sex difference in the reactivity of the mixture nor did pretreatment with phenobarbital affect the reactivity of the mixture. The disulfide contaminant appeared qualitatively to be comparable to the mixture.

Experimental. The general design of the experiment involved the comparison of the cardiovascular effects of an injected bolus of epinephrine (1 mg/kg) before and after the intraperitoneal injection of the test drug mixtures to characterize the onset of the modification of the cardiovascular effects of epinephrine.

Materials. The WR-2823 used in these experiments has been characterized by Lim (1969) as a mixture of 5-aminopentylamino ethyl phosphorothioic acid monohydrate (75%), inorganic phosphate (12%), and bis-5-aminopentylamino ethyl sulfide (12%). This mixture was dissolved in distilled water at the rate of 56 mg/ml (approximately isometric).

The disulfide contaminant was prepared by dissolving 520 mg of 5-amino pentylamino ethyl thiol in distilled water. The pH of this solution was adjusted to 8.5 by the addition of NaOH. Room air was bubbled through this solution until no positive test could be obtained with nitroprusside reagent. The final concentration was adjusted so that each ml contained 50 mg of material.

Walter Reed mice of the ICR strain, of either sex, aged 6-26 weeks

were used. They were anesthetized by intraperitoneal injection of a mixture of pentobarbital sodium (60 mg/kg) plus phenobarbital (80 mg/kg) injected at a dose of 10 mg/kg. Each animal was cannulated for recording arterial pressure and for intravenous injections of epinephrine. Whenever technically possible, a control animal was paired with an animal of the same sex that had been pretreated with phenobarbital (50 mg/kg twice daily for 3 days) to induce drug metabolizing enzymes.

At least four reproducible responses to an intravenous bolus of epinephrine were obtained during the control period by introducing a solution of epinephrine into the venous cannula at the rate of 0.1 ml/gm (1 mg/kg) and flushed into the circulation with an injection of 30 µg of 0.9% sodium chloride solution delivered in one second. The mixture of WR-2823 or the disulfide was injected intraperitoneally (25 mg/kg) and the epinephrine challenges were repeated at varying time intervals subsequently for periods up to 60 minutes after the drug injection.

Results. The mean responses to 1 mg/kg of epinephrine after WR-2823 or the disulfide contaminant are shown in figures 1 and 2. There was no evidence of a sex difference nor did pretreatment of the animals with phenobarbital modify the reactivity of the drug mixture.

Qualitatively, there was no difference in the effects of the two drugs; however, the disulfide contaminant appeared to exert its effect more slowly and appeared to be less potent as an adrenergic blocker than the mixture called WR-2823.

Generally, these two drug preparations showed no consistent effect on the cardiovascular parameters of the anesthetized mouse when administered intraperitoneally at a dose of 25 mg/kg. Frequently, but not always, there was a moderate bradycardia and a mild hypertension, both being transient. There was an immediate increase in the magnitude and duration of the pressor effect and chronotropic effect of epinephrine which reached a maximum at about 3 minutes in the case of WR-2823 and about 9 minutes after the disulfide. In both cases this potentiation decayed rapidly and was converted to an inhibition at about 15 minutes following WR-2823 and at about 45 minutes after the disulfide. This depression continued to progress for the duration of the experiment (60 minutes) and longer in some cases.

Frequently, there was an increase in the pulse pressure following the administration of the drugs; however, this appeared after epinephrine had also been administered.

Discussion. These experiments were not designed to define the mechanisms of action of WR-2823 or the disulfide contaminant. The data provide only a description of the effects of these agents to modify the cardiovascular effects of epinephrine. The initial potentiation of the pressor response of epinephrine, both in magnitude and duration, is prompt and probably represents a direct effect of the drugs to impede the inactivation mechanisms for the catecholamines. The secondary inhibition of the magnitude of the pressor effects of epinephrine is delayed and may represent the effect of a common metabolite of these drugs. Heiffer *et al.*, (1959) have presented data indicating that the inhibition of the pressor effects of catecholamines is due to the  $\alpha$ -adrenergic blocking properties of WR-2823. The present data are consistent with this hypothesis.

#### 4. The Effect of Intraperitoneal Injection of WR-2823.

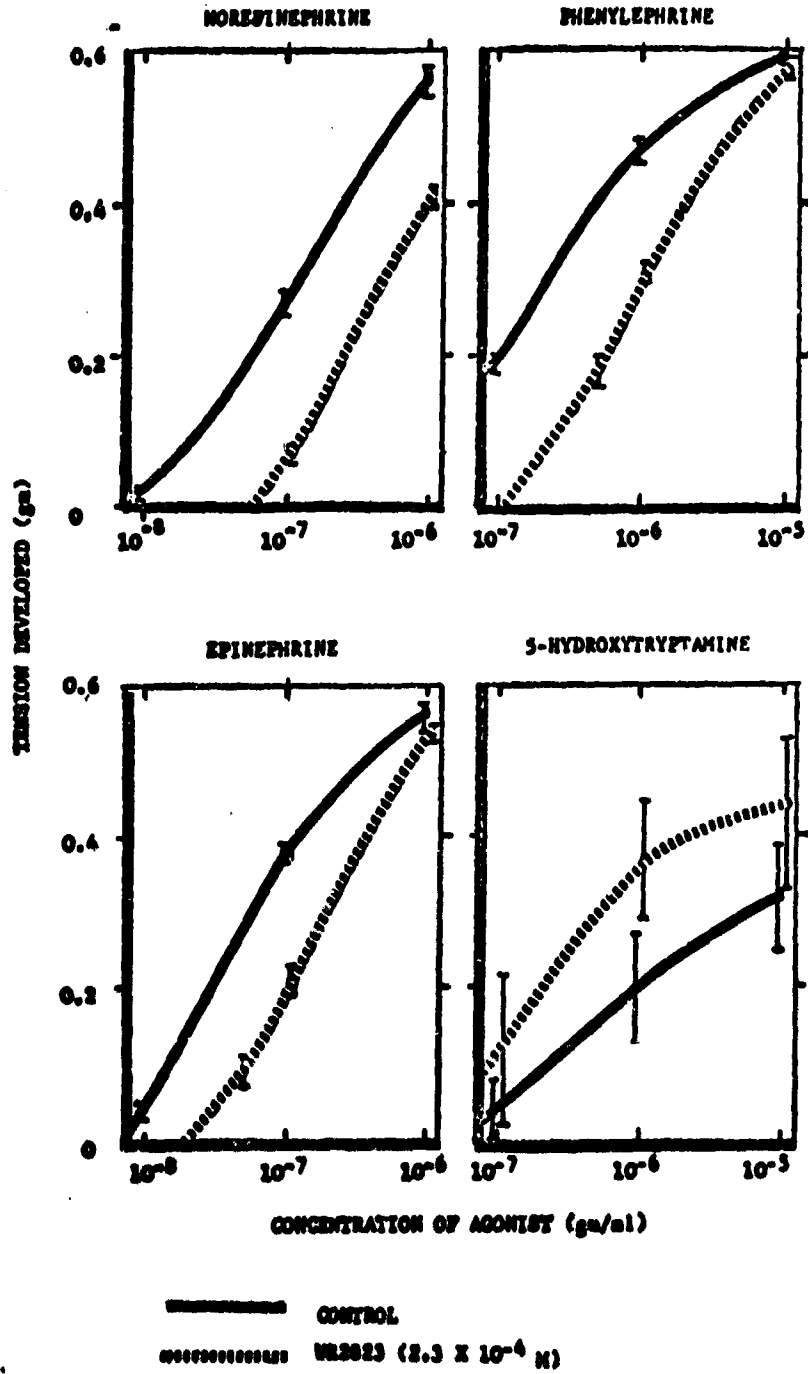
Detail chemical analysis revealed that the WR-2823 lot B (the lot used in all of our studies) contained 11% water (this was expected, since it is a monohydrate form), 7% monobasic sodium phosphate, 7% bis-5-aminopentylaminoethyl sulfide and 75% of the parent compound (WR-2823). The 7% disulfide was of some concern to us since the WR-2823 we used (intravenously administered) produced an immediate but fleeting hypotension and bradycardia. Therefore, dogs were given the compound intraperitoneally. When the agent is administered in this manner, no hypotension is observed and bradycardia was minimal. A probable explanation is that when the drug passes through the portal circulation before going to the heart, the disulfide portion is reduced to the corresponding thiol when before it reaches the heart. The disulfide form is a potential histamine liberator since it contains a primary amine group at both ends. Diamines are in general potent histamine releasers. Therefore, during the synthesis of this compound every possible effort will be employed to avoid disulfide formation and it is recommended that the compound not be exposed to air light re-heat until it is ready to be used in the laboratory or in the clinic.

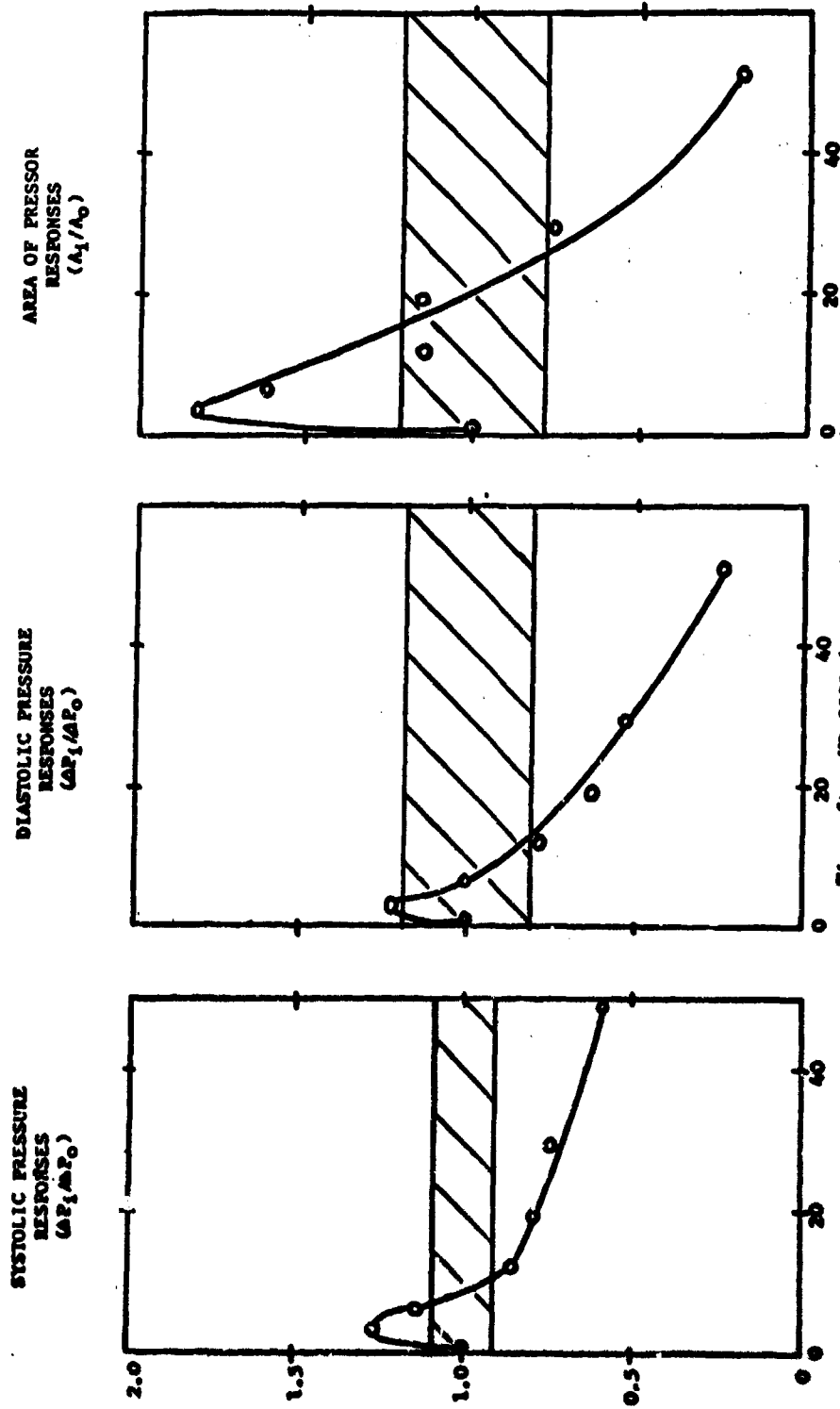
#### B. In Vitro Screens

##### 1. Screen for Antiadrenergic Properties and Myocardial Toxicity of Antiradiation Chemicals.

The use of the isolated, spontaneously beating atrium of the guinea pig continues to provide insight into the pharmacophonic requirements for these two biological effects. The close correlation of these two responses raises interesting questions concerning the mechanisms of these effects, which are being pursued at this time. The

EFFECT OF WR2823 ON THE RESPONSES OF ISOLATED RABBIT AORTIC STRIPS





Time after VR 2823 (minutes)  
Figure 1

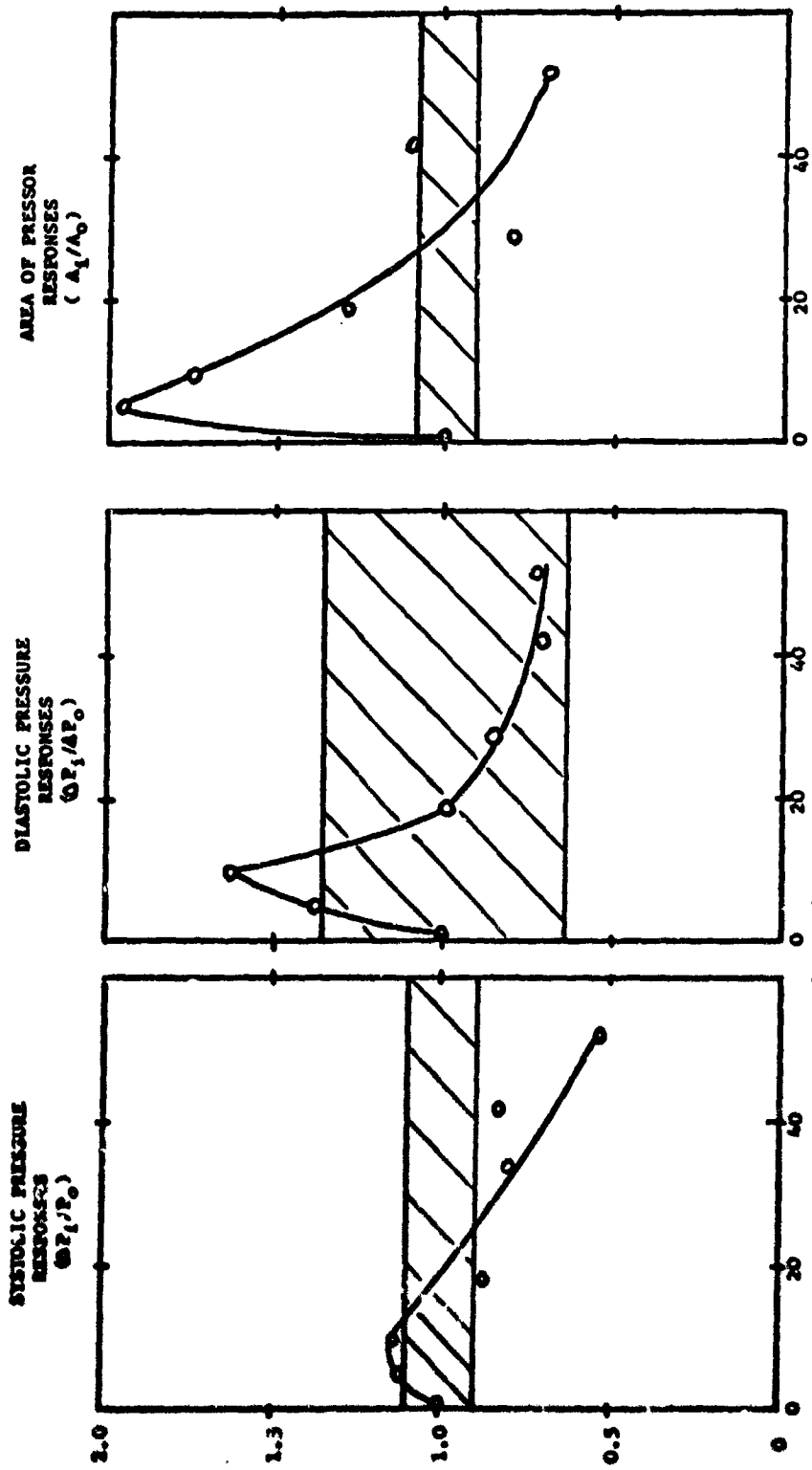


Figure 2

Legend for Figure 2

Figure 2. The effects of the disulfide contaminant of WR-2823 on the pressor responses to epinephrine in the anesthetized mouse.

This figure represents the effects of the disulfide contaminant of WR-2823 (25 mg/kg, I.P.) on the pressor responses to epinephrine (1 mg/kg, I.V.) as a function of time in the anesthetized mouse, expressed as a ratio of the response at time,  $t_1$  to the average response during the control period (time,  $t_0$ ). Systolic and diastolic pressure responses are expressed as the change in blood pressure (DP). The area of the pressor response (A) is the total positive area under the response curve.

The shaded area represents the band which contains 95% of the estimates of the mean response for  $n=6$  during the control period. Each circle represents the mean response ( $n=6$ ) following the administration of the disulfide contaminant of WR-2823.

observation that high concentrations of Calcium in the bath can reverse the depression suggests that the mechanism of myocardial depression by alkyl aminoethylthiosulfuric acids may be related to an antagonism of the fluxes of this ion. This hypothesis will be tested.

The productivity of this test system has been hampered by a physical move, and minor technical difficulties and delays in obtaining replacement parts. These problems have been overcome, and by design modification, the output of this system has been quadrupled.

#### C. Metabolism of Non-Radioactive Radioprotectants.

Materials and Methods. Animals: Adult Walter Reed strain albino mice of either sex were used. Urine was collected in glass metabolism cages.

The compounds investigated were as follows: (1) WR-2823 (S-2-(5-aminopentylamino)ethyl phosphorothioic acid dihydrate); and (2) WR-1729 (N-(2-mercaptoethyl)-1,5-pentanediamine dihydrochloride)

Separation of metabolites: Thin layer chromatography on Silica Gel G, 250 u thickness, was used. Distilled water or 10% methanol were the usual solvents.

Visualization of amine groups by means of ninhydrin spray was successful.

Visualization of sulfhydryl groups by means of cyanide-nitroprusside sprays were unsuccessful.

Results. WR-2823, 150 mg/kg intraperitoneally, appears to have at least two metabolites containing accessible amine group.

WR-1729, 50 mg/kg intraperitoneally, appears to have at least two metabolites containing accessible amine groups.

One metabolite seem common to both compounds. However, the other appears to chromatograph a little differently.

Future studies. Since both compounds are to be synthesized with radioactive sulfur, little further metabolism work on the non-radioactive material is planned.

Studies on absorption and excretion of the radioactive compounds will be made.

Urinary metabolites of the radioactive compounds will be isolated, and attempts made to characterize them.

D. Cardio-Vascular Effects of WR-2529, WR-2721, and WR-638.

1. WR-2529

A dose of 200 mg/kg I.V. caused immediate fall in arterial blood pressure of 125 mmHg and a narrowing of pulse pressure. Blood pressure gradually increased to near control values in about 1 hour. Respiration increased in rate initially but then returned to near control in about 10 minutes. WR-2529 caused bradycardia which persisted for 30 minutes. There was a progressive shortening of the QRS complex after drug administration lasting for several hours.

2. WR-2721

Dogs given 200 mg/kg WR-2721 I.V. experienced a slow gradual increase in arterial blood of 120 mmHg in the first 30 minutes. After the initial rise blood pressure decreased by 130 mmHg below control levels by 50 minutes post injection. Pulse pressure narrowed as pressure decreased. Respiration rate increased slightly post drug but returned to control values in 10 minutes. Bradycardia was observed initially but returned to control values in 20 minutes. An I.V. dose of 800 mg/kg of WR-2721 resulted in a respiratory - cardiovascular death.

3. WR-638

Blood pressure was decreased by 60 mmHg following an I.V. injection of 300 mg/kg WR-638. Blood pressure returned to near control values in 15-20 minutes. Respiration rate increased initially and remained faster than control values. Slight tachycardia was observed initially but returned to near control values in 5 minutes.

A dose of 1,000 mg/kg was found to produce a respiratory - cardiovascular death in all animals tested.

E. Studies on Treatment of Hemorrhagic Shock with WR-2823

1. Treatment of Hemorrhagic Shock with a New Alpha Adrenergic Blocking Agent.

The continuing controversy concerning the most effective means of treating shock has produced a number of seemingly conflicting reports. (1-3) Like other forms of shock, acute hypovolemic hypotension has been treated with both vasopressors and vasodilator agents. (4-6) To add to this dilemma, some investigators have proposed the use of beta adrenergic stimulants in the treatment of hemorrhagic shock. (7,8) All things considered, however, this form of shock has responded.

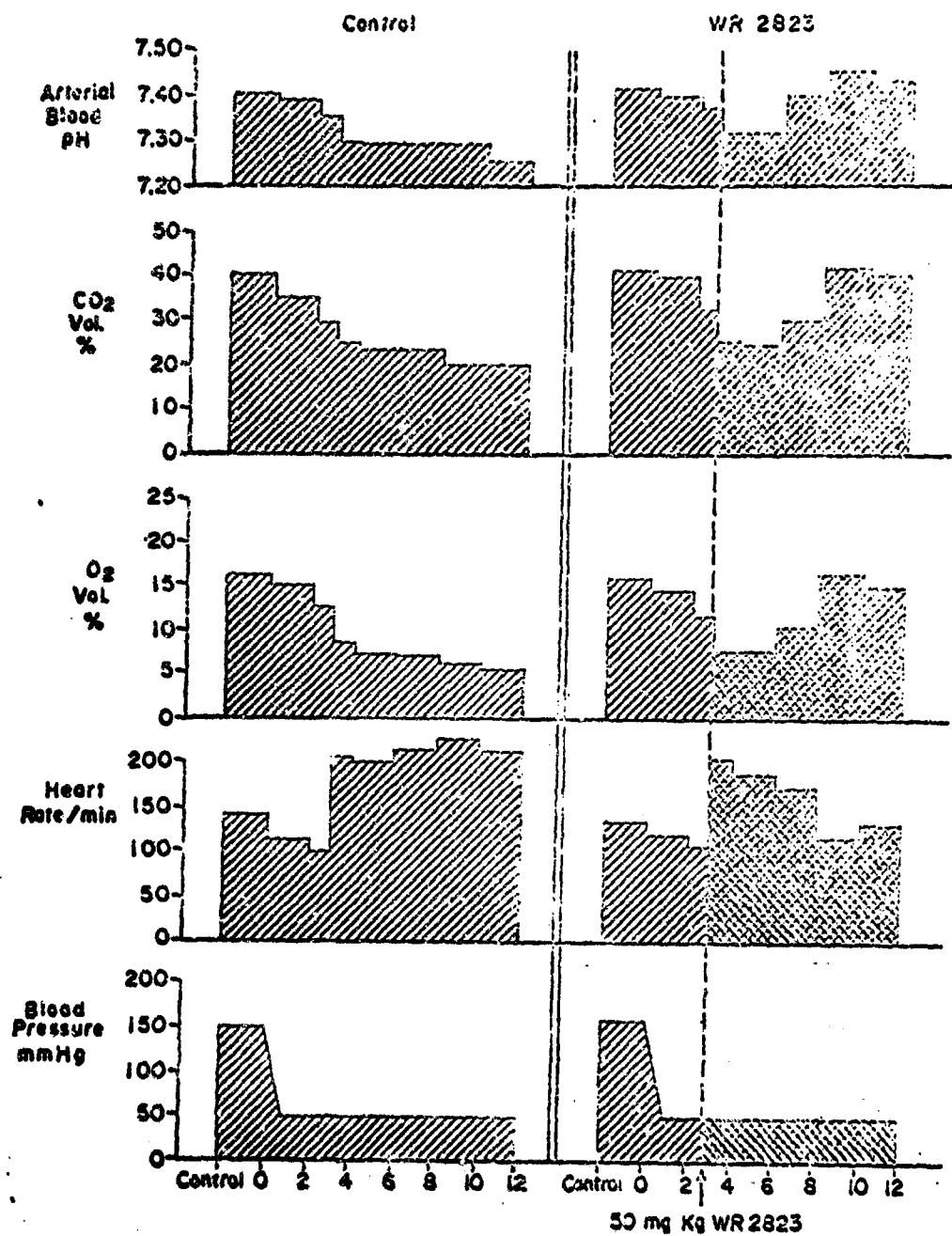
most favorable to agents which reduce total peripheral resistance rather than to those which cause arteriolar vasoconstriction.(9, 10) S-2-(5-aminopentylamino)ethyl phosphorothioic acid (WR-2823), a radioprotectant compound appears to have alpha adrenergic blocking qualities and can effectively decrease resistance to flow in the peripheral vasculature.(11, 12) It is with these actions in mind, therefore, that the present study was initiated to evaluate WR-2823 in the post-treatment of acute hypovolemic (hemorrhagic) shock. Twenty-two adult mongrel dogs and 14 adult Rhesus monkeys were anesthetized with pentobarbital sodium (30 mg/kg, I.V.). Changes in arterial and venous blood pressures, EKG, heart rate, and respiratory rate were continuously recorded on a Sanborn polygraph. In addition, hourly samples of arterial and venous blood were drawn for determination of pH, O<sub>2</sub>, and CO<sub>2</sub> content. Samples were analyzed on a Beckman Physiological Gas Analyzer. All animals were bled to a mean arterial pressure of 50 mmHg and maintained at that level for 8 hours. This method of producing hemorrhagic shock had previously been described by both Lamson and Fine (13, 14). Three hours after initial hemorrhage, paired animals were divided at random into either a control or a treatment group. Control animals received no therapy while the treated group received 50 mg/kg of WR-2823 intravenously. Figure 1 presents the actual data obtained from these studies. All animals showed a progressive decrease in arterial pH from a control of 7.42±.03 to 7.30±.08 during the first three hours after hemorrhage. Oxygen content in the venous blood fell from 15.6±2.1 vol % to 8.7±3.8 vol %, while arterial carbon dioxide decreased from 42.0±4.1 vol % to 25.3±4.4 vol %. Average heart rate increased from 140/min to 204/min at 3 hours post-hemorrhage. Control animals continued to deteriorate during the entire observation period. At 8 hours venous O<sub>2</sub> had decreased to 6.0±2.0 vol %, arterial CO<sub>2</sub> to 18 vol ± 3.0 %, pH to 7.24±.08. Heart rate increased to 240/min. Animals treated with WR-2823 at 3 hours showed slow but progressive recovery of pH; venous O<sub>2</sub> and arterial CO<sub>2</sub> towards pre-hemorrhage values. At 8 hours arterial pH stabilized at 7.45±.03, venous O<sub>2</sub> at 15.5±2.3, vol % and arterial CO<sub>2</sub> at 44.8±5.1 vol %. Heart rate decreased from 204 beats/min to 120 beats/min during this same time period. Seven of the eleven dogs treated with WR-2823 permanently survived the hemorrhagic shock syndrome. Seven of the seven monkeys treated in the same manner likewise survived. None of the control dogs or monkeys were alive at 24 hours. Results of this study indicate that WR-2823 effectively prevents "irreversible" hemorrhagic hypotension in both dogs and monkeys. The mechanism by which this drug produces this beneficial effect may be related to its known alpha adrenergic blocking properties and/or to its direct vasodilating action on the peripheral vasculature. The decrease in heart rate consistently noted after administration of this compound, coupled with the vasodilation, may serve to increase both stroke volume and cardiac output

and in this way re-establish blood flow to hypoxic tissues. Our survival data strongly support this concept.

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2. The Treatment of Canine Hemorrhagic Shock with a New Alpha Adrenergic Blocking Agent, WR-2823; Hemodynamic Observations.



Hemorrhage normally evokes a sympathetic response which, if prolonged, may be detrimental to the ultimate survival of the organism. Since 1948 when Wiggers successfully pretreated dogs with dibenamine, numerous reports have appeared on the beneficial effects of pre- and post-treatment of experimental hemorrhagic shock with classical alpha-adrenergic blocking agents. An alpha-adrenergic blocking drug with a simple and unique structure has recently been discovered. This drug, WR-2823 (S-2-(5-aminopentylamino)ethyl phosphorothioic acid) has been shown to increase survival when given to dogs subjected to hemorrhage. We have investigated the hemodynamic events associated with treatment of hemorrhagic shock with WR-2823 in order to understand the mechanism of its beneficial action. The experimental design used avoids the problem of variable patterns of blood loss and reuptake in the treated and control animals by retransfusion of all shed blood in both groups at the time of drug administration.

Materials and Methods. Twelve beagles 9-13.6 kg of either sex were anesthetized with pentobarbital (25 mg/kg), anticoagulated with 10,000 u heparin and hemorrhaged from the femoral artery into a glass reservoir placed at a height to maintain a constant pressure of 50 mmHg. The pressure of 50 mmHg was reached within 10 minutes. Three hours after the onset of hemorrhage all shed blood remaining in the reservoir was retransfused into the external jugular vein in 30 minutes. Six dogs were randomly selected to receive 50 mg/kg of WR-2823 dissolved in 10 ml of normal saline. The drug was given during a two minute period at the midpoint of the transfusion. The remaining six dogs received only the transfusion. No further treatment was given to either group. All animals were monitored for six hours from the beginning of hemorrhage and observed for 48 hours or until death.

Aortic and right atrial pressures were measured continuously with Sanborn 267 BC transducers and were recorded along with the electrocardiogram and heart rate on a Sanborn 7868 A recorder. Cardiac outputs were determined intermittently using indocyanine green dye, a Gilford 103 IR densitometer, and a Texas Instruments Rectiriter recorder. The area under the dye curve was electronically integrated with a Gilford 104 dye curve computer which also was used to compute the mean transit time. Total peripheral resistance was calculated by (mean arterial pressure, mmHg)-(mean right atrial pressure, mmHg)/cardiac output, L/min. Central blood volume was calculated by (mean transit time, sec) x (cardiac output, ml/sec).

Results. There are no significant differences in the baseline measurements of the two group of animals as listed in Table 1. The results of the measured and calculated variables during hemorrhage,

transfusion and WR-2823 treatment are shown graphically in figures 1 and 2. Significant differences in the two groups occurred only after treatment with WR-2823 and are indicated in the figures. These differences existed at some time in the mean arterial pressure, heart rate, cardiac output and peripheral resistance. No significant differences existed at any time in the volume of blood in the reservoir or the central blood volume. A suggestive (p .07) difference existed in the stroke volume at 6 hours.

In the WR-2823 treated group there was no overlap in the values of cardiac output, stroke volume and central blood volume for the survivors and non-survivors (Table 2). The other hemodynamic variables were similar within the drug treated group no matter what the outcome.

In this small study 4/6 animals treated with drug survived in contrast to 1/6 animals in the control group. This survival data is not statistically significant.

Discussion. Alpha adrenergic blockade seems to have salutary effects on ultimate survival in canine hemorrhagic shock. Nonetheless very few investigators have reported the hemodynamic consequences of such treatment when compared to a group where the degree and pattern of hemorrhage are strictly controlled. We have studied a new alpha adrenergic blocking agent, WR-2823, which has been shown previously to improve survival in a standard hemorrhagic shock model. Our present model differs in that both control and drug treated groups received all remaining shed blood after three hours of hypotension. In this way, both groups of animals had the same degree of hemorrhage at all times (see Fig. 1), and all measured differences can be attributed solely to the effects of the WR-2823.

A major difference in the two groups is a lack of vasoconstriction following retransfusion in the treated group. Peripheral resistance returned to and remained at baseline levels while the control group had a progressive increase in peripheral resistance after transfusion. The cardiac output increased with transfusion in both groups of animals but the WR-2823 treated group maintained a higher output which was significant at six hours. As a consequence of the lack of vasoconstriction, arterial pressure was significantly lower in the WR-2823 treated group. WR-2823 treatment decreased heart rate immediately after administration but this effect was transient. Although stroke volumes were not significantly different by the usual criteria (p .05) there were suggestive differences (p .07) at 3-1/2 and 6 hours which would require larger groups to evaluate. Survival was not significantly improved in this study which was designed with a small number of animals to evaluate primarily the hemodynamic consequences of treatment with WR-2823.

TABLE 1

Baseline Comparisons of Animals  $\pm$  SEM

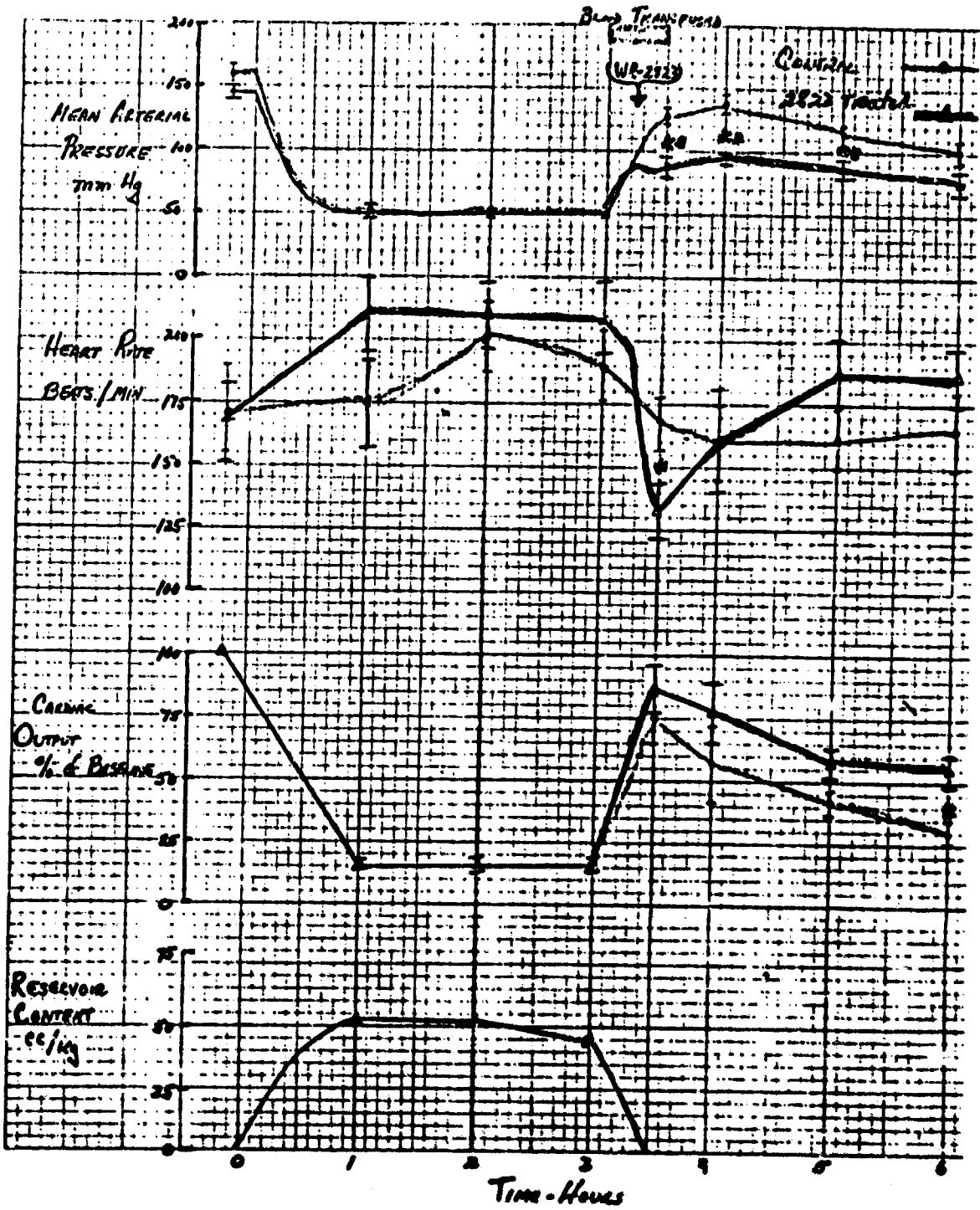
	<u>Control</u>	<u>Treated</u>
Number	6	6
Weight (kg)	10.8 $\pm$ 0.4	12.2 $\pm$ 0.5
Mean Arterial Pressure (mmHg)	159 $\pm$ 8	144 $\pm$ 5
Heart Rate (beats/min)	171 $\pm$ 19	168 $\pm$ 12
Cardiac Output (L/min)	3.31 $\pm$ 0.42	3.65 $\pm$ 0.42
Pharipheral Vascular Resistance ( $\frac{\text{mmHg}}{\text{L/min}}$ )	53.3 $\pm$ 3.2	41.1 $\pm$ 4.1
Stroke Volume (cc)	20.4 $\pm$ 0.6	21.7 $\pm$ 1.5
Central Blood Volume (cc)	214 $\pm$ 19	236 $\pm$ 15

TABLE 2

Range of Values at 6 Hours for WR-2823 Treated Animals

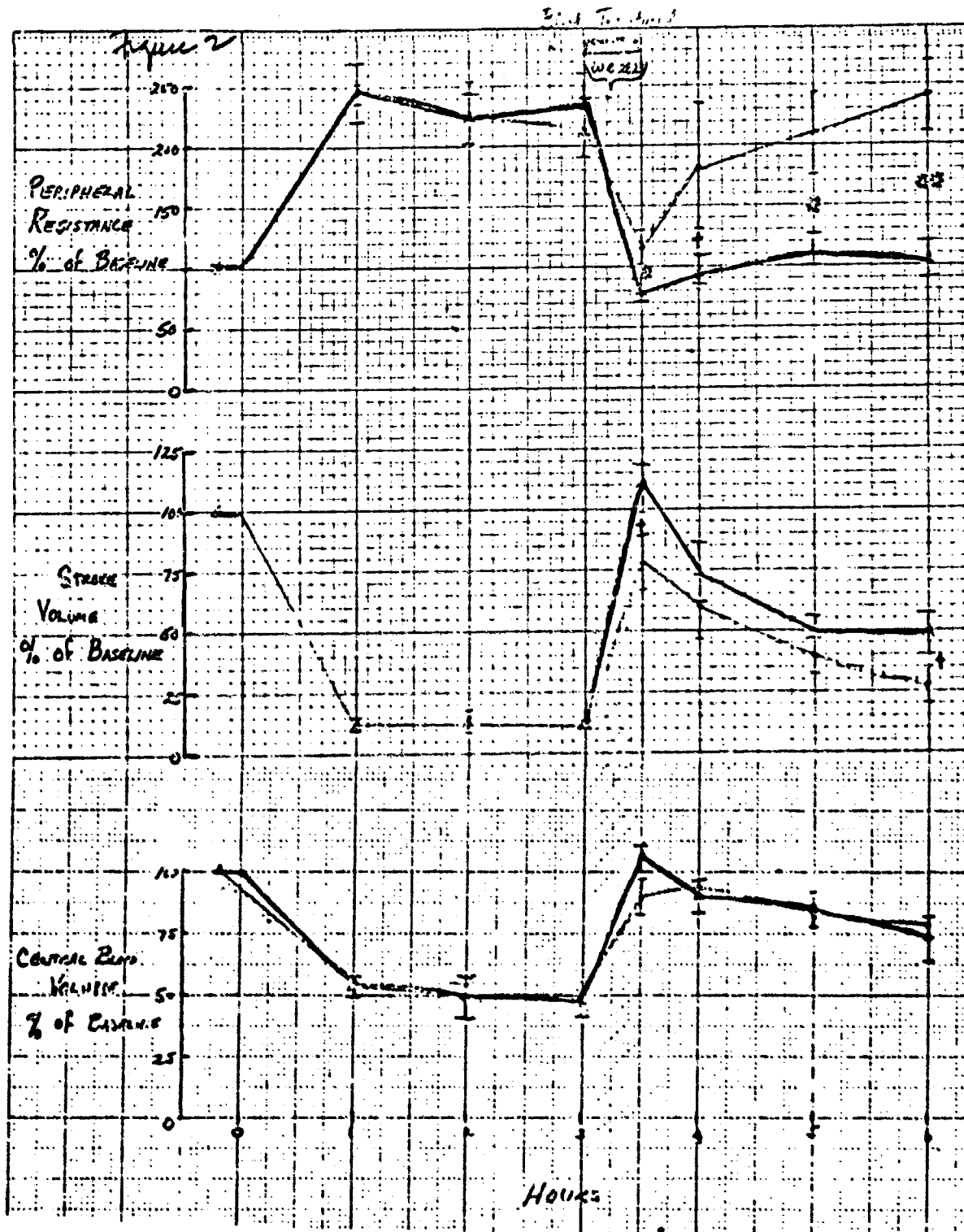
	<u>Survivors</u>	<u>Non-Survivors</u>
Cardiac Output (% of Baseline)	42-81	31-36
Stroke Volume (% of Baseline)	42-72	26-32
Central Blood Volume (% of Baseline)	67-105	40-66
Mean Arterial Pressure (mmHg)	55-110	50-75
Heart Rate (Beats/min)	155-210	160-220
Total Peripheral Resistance (% of Baseline)	59-115	105-155

FIG. 1



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The low cardiac output and central blood volume in the presence of a normal total peripheral resistance after treatment with WR-2823 suggest that optimal therapy would have to include in addition to drug, further intravascular volume expansion to maintain cardiac output. This may be critical to survival as indicated by the fact that the two drug treated animals which died had the lowest cardiac outputs, stroke volumes and central blood volumes at 6 hours post-hemorrhage than any of the four surviving animals.

Thus the effect of alpha adrenergic blockade with WR-2823 on the course of hemorrhagic shock is primarily a return of total peripheral resistance to normal. However, survival also seems to depend on the maintenance of cardiac output which may require supplemental therapy with plasma volume expanders.

#### F. Studies with E. Coli Endotoxin

##### 1. Prevention of Endotoxin Shock and Death by WR-2823 Pretreatment.

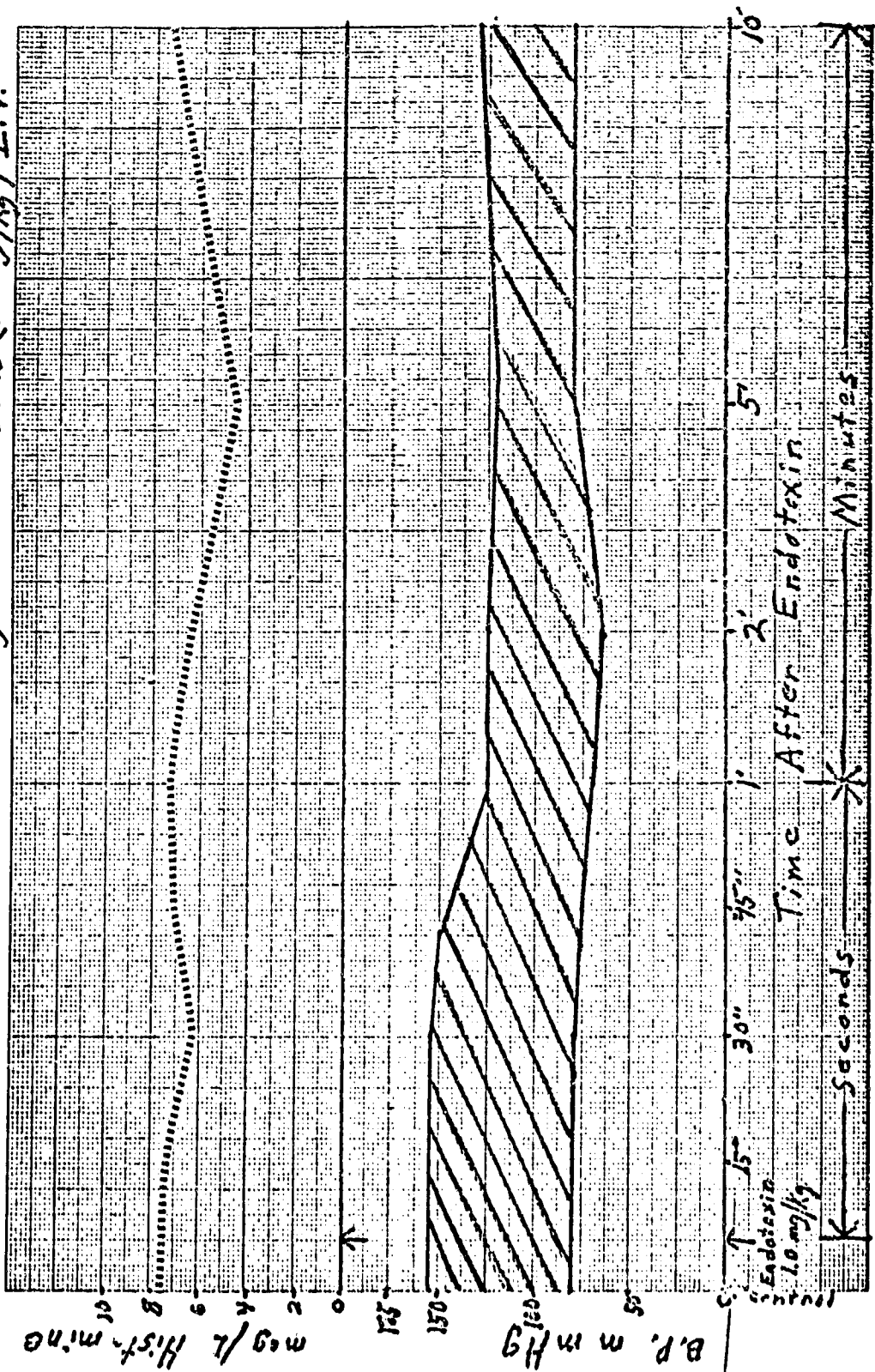
The possible interaction of WR-2823 and E. Coli endotoxin has been studied. Ten adult mongrel dogs anesthetized with sodium pentobarbital, 30 mg/kg were given 50 mg/kg WR-2823 I.V. and followed for changes in heart rate, EKG, respiration, and blood pressure. Arterial blood samples were drawn for histamine determination at 0, 15, 30, 45, and 60 seconds and at 5, 15, 30, and 60 minutes after WR-2823 a previously established lethal dose of E. Coli endotoxin (1.0 mg/kg) was injected into the femoral vein. A second set of arterial blood samples were also drawn after endotoxin for analysis of histamine levels in the blood.

Results indicate that WR-2823 causes the release of histamine into the circulation of the dog at from 5 to 30 minutes after intravenous injection. This elevation of plasma histamine returns to control level at 60 minutes. Arterial blood pressure decreases during the period of elevated plasma histamine and likewise returns towards control as the histamine level decreases. The most remarkable phenomenon observed in these studies was the prevention of the precipitations fall in arterial blood pressure that is usually observed in any of the ten animals used in this study. No increase in plasma histamine was detected nor was there any significant decrease in circulating platelets. Some indication of an interference with the "trigger mechanisms" of endotoxin shock is revealed by these results. Survival data supports this concept in that 10 of the 10 dogs given the lethal dose of endotoxin permanently survived.

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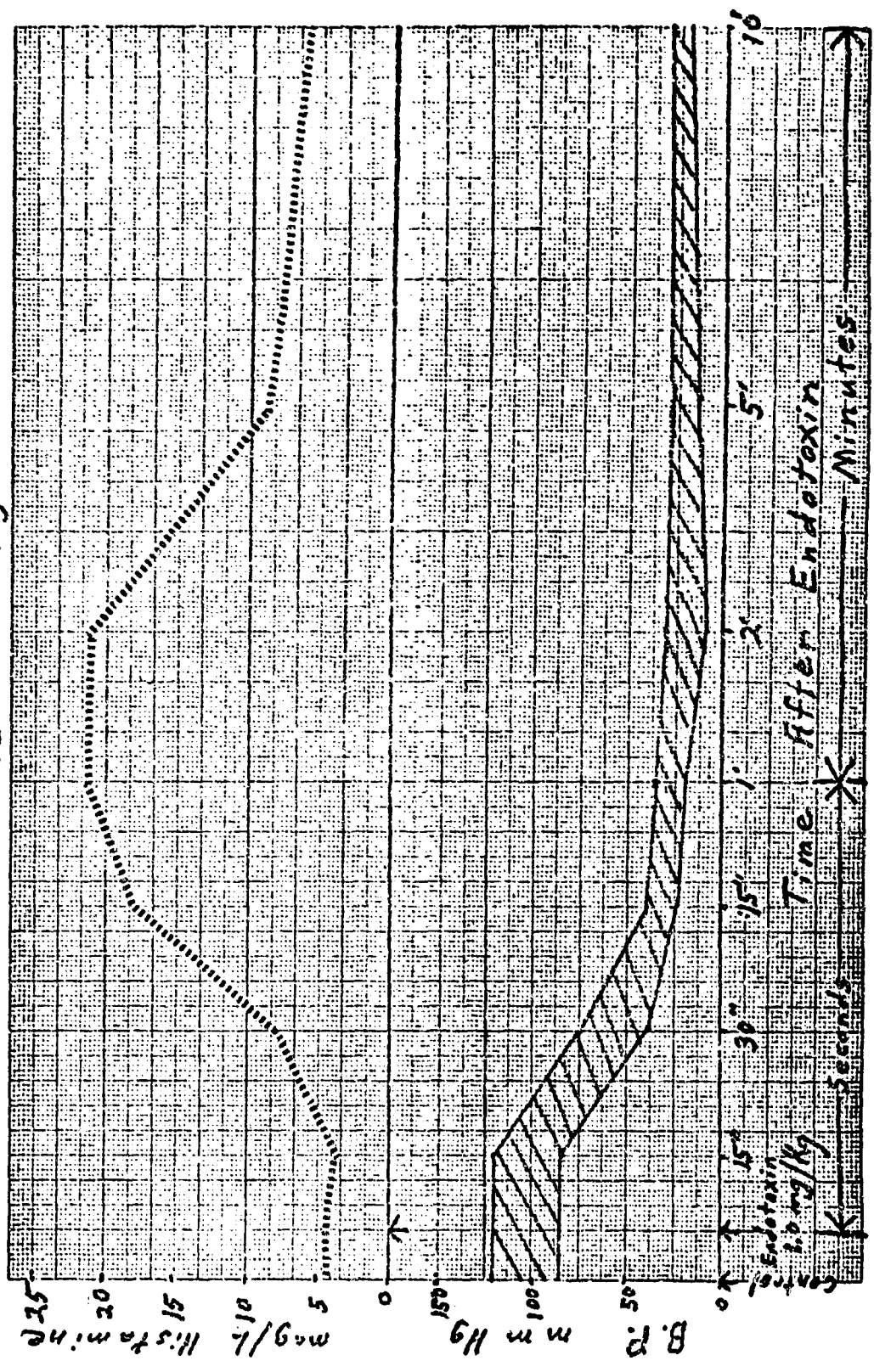
Effect of Endotoxin on Blood Pressure  
and Plasma Histamine in the Dog After NR 2823 (50 mg/kg) I.V.



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# Effect of Endotoxin on Blood Pressure and Plasma Histamine in the Dog



## X. Summary and Conclusion

Areas of most interest in the synthesis program during the year were the thiazolidines, to enhance oral absorption, and terpenoid amidinium thiosulfates, basically substituted aryl ethers, thio-phosphoramides and diamine phosphorothioates. Emphasis on these areas continues along with the functionalizing of the carbon backbone in the 3-aminopropanethiol series. Additional compounds have been tested in dogs and clinical studies have been continuing with WR-638. Two other compounds are being formulated and have been scheduled for clinical evaluation.

The in-house supporting research has progressed to the point that additional medicinal uses have been demonstrated for the antiradiation agents. Aside from arthritis, promising potential in the fields of allergy and hemorrhagic shock has been unmasked. Especially important, at this time, are the findings with WR-2823 in hemorrhagic, traumatic and endotoxin shock. All concerned are sure this drug will be ready for clinical trials within the next few months. These additional uses for antiradiation agents have already broadened interest in this class of compounds to the extent that many outside investigators are requesting to participate in the program. Increased interest makes it possible for a large number of specialists to apply their specialties and to eventually produce enough data to understand the wide spectrum of biological activity of the sulfhydryl drugs. Such understanding is a necessary basis for the design of efficient and well tolerated antiradiation agents.

Project 3A062110A824

Task 00

Work Unit 055

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11. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Radiation protective agents; (U) Drug tolerance; (U) Chemoprophylaxis							
12. TECHNICAL OBJECTIVE <sup>k</sup> 24. APPROACH, 25. PROGRAM (Provide individual paragraphs identified by number, precede text of each with Security Classification Code)							
23. (U) The definition and quantitation of chemical, biochemical and physiological changes occurring after administration of chemical compounds that show reduction in mammalian mortality after exposure to ionizing and neutron radiation.							
24. (U) Basic biochemical mechanisms of radioprotective drug action at the cellular, organ, and whole body levels are being explored in bacteria, mammalian cell cultures, and small mammals. An integrated multidisciplinary effort involves chemistry, biochemistry, physiology and pharmacology leading to definition of the mechanism of action aminothiols in cellular and mammalian systems. Radioisotopic techniques for distribution, retention and excretion of radiation modifiers are employed where applicable.							
25. (U) 69 01-69 06 Structure-function studies of high purity aminothiol radioprotectors continue. Twelve compounds were prepared and rigorously analyzed for purity and free thiol content. Eight compounds were tested for radioprotection in E. coli at 1 degree C and -196 degree C and for effects on free radical parameters. Preliminary results are being evaluated. Crystal structure of the basic compound, beta-mercaptoethylamine, has been elucidated. Preliminary crystallography of 3-carbon analogs is in progress. Certain aminothiols in radioprotective dosage were found to predispose animals to lethal effects of certain forms of shock-inducing trauma. This adverse influence can be modified without negating radioprotection. Effects of radioprotectants on tolerance for other stressors and measures for abolishing lowered shock-resistance while allowing radioprotection are being explored. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.							

PII Redacted

Project 3A062110A824, IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00 Ionizing Radiation Injury, Prevention and Treatment

Work Unit 056, Protective effect of aminothiols against ionizing and neutron radiation

**Investigators.**

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Description.

The overall objectives of this work unit are (1) to determine the mechanism of radioprotective action of aminothiols through performance of studies utilizing a broad variety of methods; (2) to study absorption, fate, excretion, and duration of effect of selected compounds with a view toward their possible future use in humans; (3) to measure the effects of treatment with these drugs, either alone or in combination with radiation, on animal response to other types of trauma; and (4) to utilize the available drugs of this class to explore the basic mechanisms by which ionizing radiation damages biological systems. This work unit overlaps Work Unit 015, Mechanisms. Studies under this work unit are subdivided as follows:

1. Animal studies.

The purpose of this subdivision is to measure and define some aspects of the interactions of protective drugs with recipient animals, but does not include pharmacologic effects which are the subject of study in the Division of Medicinal Chemistry. Studies have been designed to investigate the alteration of lethality induced by these drugs when their administration is combined with other forms of sublethal injury, as well as the interdependence of individual drugs in producing radioprotection.

2. Structure-function studies in bacteria.

In previous WRAIR Annual Reports studies were reported which established a close relationship between radioprotection and the free radical production of ionizing radiation. These studies have been

designed to examine in detail the relationship between chemical structure and radioprotective activity. A number of compounds have been synthesized and are currently being used in these studies.

### 3. Tissue culture studies.

The effects of radioprotectant chemicals on the radiation responses and physiology of mammalian cells in vitro are being studied in terms of various models of radiation injury and protection.

#### Progress.

1. Whole animal studies. (Dr. Einheber, Mr. Wren, LTC Mahin, LTC Johnson, Mrs. Hill, Miss Berman, Miss Grenan, Mrs. Davis).

a. Effects of aminothiols on trauma resistance. Three years ago the effect of the radioprotective chemical, WR 2823B, on the response of the mouse to Noble-Collip Drum (tumbling) trauma (NCDT) was tested. Lethality of NCDT was increased markedly by pre-treatment with the radioprotective dose of 300 mg/kg body weight. Recently material from the same batch of compound was found to be more toxic by weight. On chemical assay it was found to be of composition different from that three years ago. For this reason, and because WR 2823 has been found to possess alpha-adrenergic blocking activity, which can have beneficial effects on shock, this chemical's influence on the mouse's ability to survive tumbling trauma was reevaluated. In these studies the following agents were given intraperitoneally and were found to protect mice against the lethal effects of NCDT: a) three-year old WR 2823B, at a non-radioprotective dose of 50 mg/kg, given 15 minutes before initiation of NCDT, and b) dibenzylamine, an alpha-adrenergic blocking agent, at a dose of 5 mg/kg given 15 minutes or 60 minutes before NCDT. In contrast, propranolol, a beta-adrenergic blocking agent, given intraperitoneally to mice at a dose of 0.5 mg/kg 30 minutes before NCDT was found to increase mortality greatly. However, when this dose of propranolol was followed 15 minutes later by a dose of three-year old WR 2823B, mortality was slightly less than that of the water-injected controls, but slightly more than that of mice receiving WR 2823B alone. These results suggest that a) alpha-adrenergic blockade is beneficial and beta-adrenergic blockade is detrimental to survival during NCDT, and b) the anti-shock action of three-year old WR 2823B may be due either in whole or in part to its alpha-adrenergic blocking activity.

These and other studies measured the influence of certain radioprotective chemicals on the mouse's response to stressful procedures that involve direct tissue injury, such as tourniquet injury and NCDT. It was desired to contrast such results with those obtained when the direct-injury factor during exposure to stress was minimized. Therefore, a reciprocating up-and-down shaking device (2 inch stroke, 9 cycles/second) was designed and tested. This device subjects 20 mice simultaneously to severe acceleration and deceleration stress while they are simultaneously held in snug, foam rubber-padded compartments.

Preliminary mortality among mice treated with MEA (150 mg/kg), hexobarbital, or both, prior to this stress are similar to results obtained previously for stress in the NCDT experiments. This device should prove useful in detecting subtle differences in the physiological actions of various chemotherapeutic agents.

b. Tissue distribution of WR 2721 in mice. Of the aminothiols tested in mice, the compound S-2-(3 amino propylamino) ethyl phosphorothioic acid (WR 2721) produces the longest duration of radioprotection. An oral dose of 300 mg/kg provides radioprotection up to 5 hours. The free sulfhydryl form of this drug is a much poorer radioprotectant. Therefore it appears that covering the sulfhydryl residue with phosphate enhances protection and might somehow be responsible for prolonged duration of effect. The present study was designed to determine the tissue concentrations of the individual sulfur and phosphate moieties of WR 2721 at various periods of time after oral or intraperitoneal administration.

Two batches of the agent were synthesized under contract: one batch was labelled with  $^{35}\text{S}$ ; the other with  $^{32}\text{P}$ . The tagged compound, as delivered, was found to be contaminated by visible amounts of colored material. In any case, the compound was utilized for this study.

The radioactive compound was diluted with cold WR 2721 and solubilized shortly before use. Mice were injected intraperitoneally or per os with an agent dose of 300 mg/kg body weight and sacrificed at various intervals thereafter. Various body tissues and fluids were assayed for  $^{35}\text{S}$  and  $^{32}\text{P}$  by liquid scintillation counting.

The highest concentrations of both isotopes were found in the gut about 30 minutes after dosage by either route. In all tissues, including red blood cells and plasma, there was a very early divergence of  $^{32}\text{P}$  and  $^{35}\text{S}$  concentrations. Sulfur-35 concentrations rose to a peak at about 30 minutes after intraperitoneal injection and about 60 minutes after oral injection. After 8 hours, little  $^{35}\text{S}$  remained in most tissues. By contrast the  $^{32}\text{P}$  concentrations in most tissues reached a plateau after 2-4 hours and remained stable for 24 hours.

The rapid appearance of disproportion between  $^{35}\text{S}$  and  $^{32}\text{P}$  indicates that the phosphate group is rapidly removed from the agent in vivo. The plateau of tissue  $^{32}\text{P}$  concentrations suggests that this portion of the molecule was distributed into the body phosphate pool. These studies suggest, but do not prove, that following injection and after the agent is freed of the phosphate moiety, the radioprotective form of the compound is the free sulfhydryl.

These results suggest that the phosphate covering function per se is not responsible for radioprotection or duration of effect. Such factors as rate of synthesis and absorption rates occurring

in vivo may regulate the distribution and concentration of this agent in body organs. These factors would favor a model for radioprotection in which the presence of a phosphate covering function enhances initial transport of the agent to critical organs.

c. Effects of WR 2721 on radioiodine retention and distribution. Mice given 300 mg/kg of the phosphorylated thiol S-2-(3-amino-propyl-amino) ethyl phosphorothioic acid (WR 2721) up to several hours before exposure survive levels of X or gamma radiation that are lethal (LD<sub>100/30</sub>) to control mice. To determine whether WR 2721 caused biochemical or physiological changes, routine tests were performed for evaluation of thyroidal status: whole body retention, excretion, total plasma activity, thyroidal uptake and conversion ratio of radioiodine in plasma. These measurements were made 4, 24 and 48 hours after the isotope (<sup>131</sup>I) was given.

WR 2721 changed whole body retention values; treated mice retained approximately twice as much radioiodine as their controls. Drug effect was apparent when there was a 5-minute interval between treatment with the agent and administration of the isotope (e.g., 24-hour retention values: treated - 72%, control - 37%). It was still evident when there was a 4-hour delay (61%, 29%), but was no longer detectable when 24 hours elapsed between the drug and the radioiodine tests (37%, 35%). Excretion of the isotope in treated animals reflected increased retention. The greatest decrease occurred during the first 4 hours and was associated with oliguria; by the end of 48 hours total excretion approached control values. Elevated plasma levels of radioiodine were found in treated mice; however, conversion ratio values, which indicate rate of hormone synthesis and release, were not different from control values. Thyroidal uptake (with the exception of the 24-hour interval) was always higher in the treated mouse than in the control. Differences were greatest when there was a 3 or 4 hour delay before the isotope was given (50% vs 25%). In almost every instance, extra-thyroidal concentrations (carcass, viscera and kidneys) were higher in treated mice.

Although treatment with WR 2721 caused increased whole body retention, elevated plasma levels and high thyroidal uptake, the rate of appearance of labeled components or newly synthesized hormone remained unaffected as shown by the unchanged conversion ratios. The high retention values, and inversely, the low excretion values, indicate that there was delayed <sup>131</sup>I excretion related to renal and/or circulatory changes. As a result of this delay in excretion, more of the isotope is available to the thyroid gland for accumulation over a prolonged period of time and the uptake is increased. High uptake values are thus secondary to alterations in excretion rates which follow administration of the agent and do not indicate a direct drug effect on thyroid function.

These data show that 1) temporary renal and circulatory changes that follow administration of 300 mg/kg of WR 2721 affect excretion rate of iodine, 2) these changes influence the absolute amount of

radioiodine concentrated by the thyroid, but 3) the drug does not appear to affect the physiological function (the rate of hormone synthesis and release) of the thyroid gland.

d. Effect of aminothiols on pertussin-induced lymphocyte mobilization. Stephen Morse first described the lymphocyte mobilization induced by pertussis vaccine in mice. Peripheral lymphocyte, and to a lesser extent granulocyte, counts in peripheral blood were elevated and a maximum total peripheral leukocyte count of  $2 \times 10^5/\text{mm}^3$  was observed 4 days after intravenous pertussis vaccine injection. In previous studies at WRAIR, gamma irradiated mice were injected with pertussis vaccine 3 days postirradiation and blood counts were made on the seventh day. The results showed that mobilized peripheral leukocytes were reduced with near-linear radiation dose-response relationships. The mobilized leukocyte count is reduced by a factor of about two after whole body irradiation with 100 rads. By contrast, only a slight reduction is observed in the normal peripheral leukocyte count in similarly irradiated mice that were not stimulated to mobilize leukocytes. This "biological amplification" suggests that the system might be suitable for sensitive detection of biological effects of irradiation in the low dose range of 0-100 rads, a range where such detection is otherwise difficult.

The model was used in an attempt to demonstrate a radioprotective effect of WR 2721. The experiment was modified as follows: on day one, mice were injected with pertussis vaccine; on day three, they were administered various doses of WR 2721 and irradiated 30 minutes later; on day four, peripheral leukocyte counts were made. The radiation dose was 100 rads  $^{60}\text{Co}$ . The doses of WR 2721 were 0, 1.5, 3, 4.6, 6 and 600 mg/kg. Control mice were injected with WR 2721 and not irradiated. The results were as follows: irradiation markedly reduced mobilized peripheral leukocyte counts in comparison to unirradiated controls; peripheral leukocyte counts were markedly elevated by the preirradiation treatment with pertussis vaccine. Peripheral leukocyte counts were not significantly altered in either irradiated or unirradiated animals by treatment with any of the above doses of WR 2721. Thus, (1) the system is sensitive to radiation in the 100-rad dose range, (2) WR 2721 does not affect leukocyte mobilization patterns in non-irradiated mice and, (3) the absence of a dose-response relationship between WR 2721 dose and leukocyte count indicates that protection against radiation death cannot be predicted by use of this system (a dose of 600 mg/kg is highly protective).

2. Structure-function studies. (Dr. Copeland, Dr. Heller, CPT Jandacek, Dr. Lofberg, Mr. Richardson, LTC Swartz).

a. ESR studies in bacterial systems. In an effort to elucidate the molecular basis of radiation damage in living systems, an attempt has been made to correlate the structure of radioprotective compounds with their protective capacity. The selection of compounds for study is based on systematic variations of beta mercaptoethylamine (MEA), including chain length variations and substitutions on the nitrogen and

sulfur residues. The basic biological system is *E. coli* B/r which can be irradiated either at 1°C or at 77°K after equilibration with nitrogen or oxygen. With such a system it is possible to measure not only survival fraction but also, by employing electron spin resonance (ESR) spectrometry, the type and quantity of free radicals associated with the bacteria-protectant system. Reactions undergone by these radiation-induced radicals in the presence and absence of protective compounds can be controlled by temperature variation and observed with ESR. Coordination of data on chemical structure and free radical reactions with bacterial survival thus provides information on molecular radiation damage.

When members of the series of compounds were found to be radioprotective they also selectively reacted with certain bacterial free radicals, accelerating their transformation and decay. In oxygen-equilibrated samples, effective radioprotectants reduced the number of peroxy (or hydroperoxy) radicals which otherwise form when molecular oxygen couples to radiation-induced radicals. In nitrogen-equilibrated samples, effective protectants seem to reduce the concentration of a particularly damaging bacterial radical, converting it to a less deleterious form.

Results of studies on effects of structure variation indicate that the thiol or disulfide group is critical for protection. When the sulfhydryl function is covered by forming the Bunte salt, protection falls markedly. The amino group appears to play a secondary role. Protection remains when amino hydrogens are replaced by methyl groups, but is lost when the nitrogen is incorporated into the bulky piperidine ring. When both the amino and thiol ends of the molecule are covered (piperidine-MEA-Bunte), radio-sensitization results. When the thiol group is removed as in ethylamine, radiosensitization is also found. When the carbon atom chain length was increased by one carbon (mercapto-propylamine), there was no change in radioprotection. Alterations of the amino and thiol ends of this 3-carbon derivative produced the same effects as found for similar analogs of MEA.

b. Macromolecular studies. Molecular mixtures of radioprotective aminothiols and the enzymes glyceraldehyde-3-phosphate dehydrogenase and invertase were prepared by lyophilization of weight-ratio series solutions of the two compounds. These solid-state preparations were irradiated in vacuo at 77°K. The concentration of free radicals formed was determined by ESR spectrometry. Radical reactions which result in transfer of radiation energy from the enzymes to the protectant were evaluated by observing decomposition of the ESR spectrum. Studies conducted with MEA demonstrated marked energy transfer to the radioprotectant. Future studies are expected to show correlations between energy transfer to the agent and increased protection of enzymatic activity from radiation damage. Such results would corroborate the findings obtained with frozen bacterial systems.

c. Amino-thiol crystal structure. The crystal structure of  $\beta$ -mercaptoethylamine (MEA) hydrochloride was determined by X-ray diffractometry of the single crystal. The space group is monoclinic,  $P2_1/c$  with cell parameters  $a = 7.760$ ,  $b = 8.584$ ,  $c = 8.728 \text{ \AA}$ ,  $\beta = 101.28$  degrees, and  $Z = 4$  molecules per unit cell. The conformation of MEA is that of a distorted "U" with the sulfur about  $3.2 \text{ \AA}$  from the nitrogen. The nitrogen atom is approximately  $3.2 \text{ \AA}$  from three chloride ions. The intramolecular bond lengths are S-C =  $1.86$ , C-C =  $1.50$ , and C-N =  $1.46 \text{ \AA}$ . The R value is 11.9% for 347 observed reflections.

The crystal structure of the three-carbon analog, 3-mercapto-propylamine is being undertaken.

### 3. Tissue culture studies. (Mrs. Davis, MAJ Del Favero, LTC Johnson).

Effect of WR 2721 on in vitro radiation response of lymphocytes. Proliferation of phytohemagglutinin-stimulated lymphocytes was measured after exposure to radioprotective agent WR 2721 with and without subsequent exposure to 800 rads  $^{60}\text{Co}$  radiation. Indices of lymphocyte proliferation 96 hours after initiation of the culture were: 1) change in cell concentration, and 2) rate of radioactive thymidine incorporation. In unirradiated cultures, final cell counts were reduced to 90% of control by a 10-minute exposure to 2 or 4 mg/ml of WR 2721. Thymidine incorporation was reduced to 60% of control culture values by this treatment. In irradiated cultures, cell counts were reduced to 25% of the control values and thymidine incorporation to 10%. Preirradiation exposure of cells to 2-8 mg/ml WR 2721 for 10 minutes did not result in cell-count or thymidine-incorporation values significantly different from values found after irradiation alone.

### Summary and Conclusions.

The effects of aminothiols on the radiation responses of biological systems have been studied at several levels of biological complexity. The three-dimensional molecular conformation of one of the most fundamental radioprotectants, mercaptoethylamine, has been determined. Preliminary studies on the energy transfer from irradiated enzymes to aminothiols in the solid state are reported. An extensive structure-function study of aminothiols and their effects on bacterial responses to irradiation was outlined and discussed in detail. Whereas initial studies (WRAIR Annual Report 1967-68) showed no effect of WR 2721 on in vitro lymphocyte radiation damage, the system holds promise and exploratory efforts have continued. Whole animals have been used to evaluate the effect of two aminothiols, WR 2721 and WR 2823B, on various parameters of mammalian radiation response. Tissue distribution studies with WR 2721 doubly labeled with  $^{32}\text{P}$  and  $^{35}\text{S}$  suggest that the radioprotective form of the molecule does not contain the phosphate moiety. It also appears that the phosphate covering function favorably influences in vivo rates of oxidation-reduction and of absorption. WR 2721 was found to increase whole body retention of

radioiodine by inducing temporary renal and circulatory changes. This aminothiols was found not to have any effect on the physiological function of the thyroid gland. Three-year old WR 2823B was found to have an anti-shock action perhaps related to its alpha-adrenergic blocking activity. A system which produces stress without concomitant direct tissue damage has been used to demonstrate that MEA sensitizes mice to stress and that this sensitization can be in part alleviated by hexobarbital administration.

Project 3A062110A324 IONIZING RADIATION INJURY, PREVENTION AND  
TREATMENT

Task 00 Ionizing Radiation Injury, Prevention and Treatment

Work Unit 056 Protective effect of aminothiols against ionizing and neutron  
radiation

Publications

None.

PROJECT 3A663713D829  
MALARIA PROPHYLAXIS

Task 00  
Malaria Investigations

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL NUMBER	
				DA OA6517	69 07 01	DD-222 (AR) 634	
3. DATE PREP. COUNTRY	4. KIND OF SUMMARY	5. SUMMARY SCY*	6. WORK SECURITY*	7. REGRADING*	8. DISSEM INSTR*	9. SPECIFIC DATA-CONTRACTOR ACCESS	10. LEVEL OF RISK
31 01 69	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
E. PRIMARY	63713A	3A663713D829	00	106			
D. CONTINUING							
C. CONCURRENT	1412A(2)						
11. TITLE (precede with Security Classification Code)*							
(U) Antigenic Fractionation, Serology of Malaria (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
07 65		NA		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
NA				FISCAL YEAR		20. FUNDS (in thousands)	
A. DATES/EFFECTIVE:		B. EXPIRATION:		69		3	
C. NUMBER:		D. AMOUNT:		CURRENT		100	
E. TYPE:		F. CUM. AMT.		70		3	
G. KIND OF AWARD:		H. CUM. AMT.				100	
21. RESPONSIBLE DOD ORGANIZATION				22. PERFORMING ORGANIZATION			
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24. REVISIONS (Precede each with Security Classification Code)							
(U) Malaria; (U) Plasmodium; (U) Immunity; (U) Erythrophagocytosis; (U) Autoimmunity; (U) Diagnosis							
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23(U) To isolate and purify various protein antigens from plasmodia. To characterize these substances immunochemically, to relate immunochemical characteristics to biologic activities, such as protective immunity, diagnostic specificity, cross reactions with normal host tissue components, etc.							
24(U) Separate parasite proteins by physical and chemical means. Determine the presence and activity of metabolic antigens in the plasma of acutely infected animals. Analyze the fractionated proteins with analytical ultra-centrifugation, polyacrylamide gel, electrophoresis, immunoelectrophoresis, complement fixation, hemagglutination, and fluorescent antibody tests. Study erythrophagocytosis in spleen smears of infected and control animals.							
25(U) 69 01 - 69 06 Nineteen volunteers were infected with sporozoite or blood induced falciparum or vivax malaria. Antibodies were detected by the use of the SAFA and the IHA tests using infected erythrocyte lysate antigen. Both tests provide a specific and sensitive method for following the course of antibody development in either falciparum or vivax malaria. Antibodies were detected at about the time the infections became patent. Maximum antibody titers were reached early in the infection and were correlated with maximum parasite counts. Malaria antibodies were still detected several months after parasites were last seen in the peripheral blood. Antibody titers observed in P. falciparum infections were usually higher and persisted longer than in P. vivax infections. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

PII Redacted

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 106, Antigenic fractionation, serology of malaria

Investigators

Principal: Elvio H. Sadun, Sc.D., Lib. Doc.

Associate: David F. Clyde, M.D., Ph.D.; William A. Colgate; Rufus W. Gore; Maurice J. Schoenbechler; CPT Daniel J. Stechschulte, MC; and Bruce T. Wellde

1. An indirect hemagglutination test for malaria using an antigen from the lysate of parasitized erythrocytes.

An indirect hemagglutination (IHA) test for malaria using as antigen a crude saline extract from *Plasmodium berghei* parasites adsorbed onto tanned formalinized sheep erythrocytes was described by Desowitz and Stein. Antigens from *P. cynomologi*, *P. vivax* and *P. coatneyi* prepared by similar methods were used in subsequent studies. The specificity of the test was improved by removing leucocytes from *P. berghei* parasitized blood before extraction of the crude parasite antigens and by using non-formalinized erythrocytes. Mahoney systematically extracted antigens from crude *P. knowlesi* and *P. falciparum* suspensions and reported a sensitive and specific IHA test. Recently Rogers et al. reported a microhemagglutination test using an antigen extracted from *P. knowlesi* parasites by methods similar to those described by Mahoney.

Antigen preparations used in the IHA and most of the other tests described for the detection of malarial antibodies using soluble antigens involved lysis of parasitized cells and subsequent extraction of antigens from the "freed" parasites. The initial lysate of the infected cells in these procedures was usually discarded and therefore has received relatively little attention. However, Davis found that hemolysates of *P. knowlesi* infected RBC's contained complement fixing antigens which could be purified to some degree by ammonium sulfate precipitation. He suggested that these antigens may exist in part outside the parasite and that they may be similar to the serum antigens first demonstrated by Eaton in the serum of infected monkeys. Serum antigens have also been described recently by Todorovic and his coworkers. Serological tests employing soluble antigens extracted from plasmodia have not been widely used due in part to the scarcity of antigens available and to the relative instability of these materials.

With these limitations in mind, studies were initiated to determine whether a sensitive, specific and reproducible IHA test could be developed utilizing lysates of parasitized erythrocytes as antigen. Initial testing of lysates of *P. berghei* infected erythrocytes indicated that parasite antigens were present and would react with homologous antisera. The

preparation, partial purification, and utilization of lysate antigens obtained from parasitized RBC's in rodent and human malaria is described in the following studies.

### Parasites

The NYU-2 strain of P. berghei maintained in ICR mice for over 4 years and in young WRCF rats for over 3 years in our laboratory was used in these studies. The Camp. strain of P. falciparum was maintained in splenectomized chimpanzees for over 4 years.

### Preparation of Lysates

P. berghei antigen was obtained from infected mouse blood harvested the fourth day after I.P. inoculation of  $2 \times 10^7$  P. berghei parasitized mouse cells. The blood from 20-50 infected mice was collected in chilled saline citrate and centrifuged at 1500 rpm for 20 minutes after which plasma was decanted. The cells were then washed 3 times in physiological salt solution. After the third wash the cells were reconstituted to a 50 percent concentration, shell frozen in a CO<sub>2</sub>-ethanol bath, and subsequently thawed at room temperature. The freeze-thaw procedure was repeated 3 times. The resulting lysate was then centrifuged at 27,000 x g for 30 minutes. The supernatant fluid was decanted and centrifuged as before. Microscopic examination of the sediment indicated that both erythrocytes and parasites were disrupted by the process of repeated freezing and thawing. The nonturbid, claret red supernatant fluid was used for fractionation procedures. Plasmodium falciparum lysates were prepared in a similar manner except that initially 200 to 400 ml of infected chimpanzee blood were obtained by femoral puncture and collected into acid citrate dextrose solution. Control "antigens" were prepared from non-parasitized mouse and chimpanzee blood following the above procedures. Lysates were stored at -70°C, and were usually fractionated soon after their preparation, although one P. berghei lysate which had been stored for 2 months provided satisfactory antigen after fractionation.

### Fractionation of Lysates

Initial fractionation was performed by mixing 6 volumes of saturated ammonium sulfate solution and 4 volumes of lysate at room temperature. The suspension was then centrifuged at 1500 rpm for 20 minutes. The precipitate was washed successively with decreasing concentrations of ammonium sulfate. After each wash the supernatant fluid was separated by centrifugation, decanted, and extensively dialyzed against phosphate buffered saline (pH 5.6) at 4°C until barium chloride produced no reaction in the dialysate. Each of these eluates was then tested for its ability to sensitize tanned sheep RBC's by agglutination with hyper-immune P. berghei antisera. The supernatant fluid from the initial precipitation was treated with 70 percent and 75 percent concentrations of ammonium sulfate and the resulting precipitates were reconstituted in PBS (pH 5.6), dialyzed, and tested as above.

Fractionation by gel filtration was performed by applying 4 to 5 ml of lysate to a Sephadex G-200 column (2.5 x 100 cm) and eluting with 0.15 M phosphate buffered saline (pH 7.2). The eluate was monitored by O.D. readings at 2800 angstroms and collected over a period of 6-8 hours. Four pools were made arbitrarily and each was concentrated by pressure dialysis at 4°C to the original volume and then each was tested for its ability to sensitize tanned sheep erythrocytes.

The lysates were also fractionated by ion-exchange chromatography. Five ml of lysate were added to 2.5 x 45 cm columns packed with DEAE Sephadex A-25. The material was eluted with a series of phosphate buffers of decreasing pH and increasing salt concentration. The eluate was monitored and processed as above.

Antigens obtained from P. berghei lysates were prepared by all 3 fractionation methods described above, while P. falciparum antigens were prepared by ion exchange chromatography only. Protein concentrations were determined by the method of Lowry.

#### Detection of Antigens and Antibodies

Initially the indirect hemagglutination test was used to identify lysate antigens by utilizing antisera presumed to contain antibodies against the homologous parasite. After the antigenicity of the lysate fractions had been established, the IHA test was then used to detect antibodies in various test sera. The general IHA procedure described by Campbell et al. was followed and adapted to the micromethod described by Sever. Sheep erythrocytes collected in Alsever's solution were stored at 4°C and were used within 30 days. These cells were tanned in a 1:10,000 dilution of reagent grade tannic acid and sensitized with antigen for 30 minutes at room temperature. After washing, the sensitized cells were resuspended in 1:100 absorbed rabbit serum in saline. All sera were inactivated at 56°C for 30 minutes and absorbed with an equal volume of packed sheep erythrocytes for 10 minutes at room temperature before testing. Then 0.05 ml of the sensitized cell suspension was added to 0.05 ml of serum diluted in the normal rabbit sera diluent. Hemagglutination patterns were developed at room temperature for 3 hours, then at 4°C for 2 hours. Serum titers were expressed as the reciprocal of the highest serum dilution giving a 2+ reaction.

Optimal tannic acid concentration was 1:10,000 and there was a decrease in sensitivity as the acid was diluted. Attempts to sensitize untanned sheep erythrocytes with lysate antigens failed, as evidenced by the absence of agglutination with specific antisera at any dilution. Cells sensitized at pH 5.6 were the most reliable and the 1:100 normal rabbit serum diluent was used at pH 6.4. Best results were obtained when there was little or no hemolysis in sera and diluent. In one experiment, sheep cells fixed in gluteraldehyde were used for approximately one year.

The fluorescent antibody (IFA) test employing blood smears of P. berghei infected mouse cells was performed as described by Voller. The soluble antigen fluorescent antibody (SAFA) test was performed according to the method of Sadun and Gore.

#### Sources of Sera Tested

WRCF rats inoculated with P. berghei parasitized rat cells were bled from the tail and the serum was separated and stored at -20°C. Other P. berghei antisera were obtained from rats given multiple inoculations of P. berghei infected rat RBC's and this serum was used to establish the IHA test. Control rat serum was obtained from noninfected WRCF rats and from rats which had received injections of normal rat blood.

Tests were conducted with 431 human sera. These included 155 from parasitologically proven malaria infections in New Guinea and 16 from malaria-infected volunteers. The specificity of the reaction was determined with 121 sera from patients with infections other than malaria and with 141 sera from healthy individuals. The healthy individuals included applicants for entrance to a military academy, the volunteers before they were infected and individuals undergoing routine physical examinations.

#### Solubility of Antigen in Neutral Salt Solutions

Various concentrations of ammonium sulfate in distilled water were used to determine the solubility of the antigens and to partially separate them from contaminating materials. As seen in Table 1, the antigens were precipitated in a 60 percent solution of ammonium sulfate, but they could be eluted from the precipitate in solutions containing 50 to 33 percent salt. Most of the hemoglobin was soluble in the 60 percent salt solution and remained in the supernatant fluid. No reactive substance could be extracted from this supernatant solution.

#### G-200 Chromatography

Specific antigenic activity was found in fraction No. 3 eluted before the hemoglobin peak (fraction 4). The cells sensitized with fractions 1 and 2 often agglutinated in normal serum and in the 1:100 normal rabbit serum diluent.

#### Ion Exchange Chromatography

Lysates were eluted from DEAE Sephadex columns. Fraction No. 1 from these columns contained most of the hemoglobin and had no IHA activity. Although specific activity was found in fractions 2, 3 and 4, the greatest activity was observed in fraction 3, which was clear and nearly colorless. The same elution pattern and location of sensitizing activity was observed when P. falciparum lysate was

fractionated and tested by IHA using human sera from parasitologically proven malaria infections. Nonparasitized lysates chromatographed in the same manner showed an elution pattern similar to that of Figure 2. However, none of the 4 fractions contained material that would sensitize sheep erythrocytes for agglutination in antimalarial sera. This method of separation appeared to be the most efficient in terms of antigen yield, and, therefore, P. falciparum or P. berghei fraction No. 3 from ion exchange chromatography was used in all further work. These antigens were stable at -70°C for at least 9 months.

Table 1

Solubility of P. berghei Antigens in Ammonium Sulfate Solutions

Precipitate		
Salt Concentration (percent)	Protein Concentration* (mg/ml)	Titer
50	6.00	2560
45	.31	2560
37	1.24	2560
33	.98	5120
Below 33 (25-5)**	2.68 -	1280 <20
Supernatant		
70%	21.48	<20
75%	19.68	<20

\*All fractions adjusted to approximately .313 mg/ml before testing.

\*\*In two experiments no reactive materials could be eluted with salt concentrations of 25% or less.

Effect of Antigen Concentration and Sensitization Time on IHA Titers

Studies were conducted with P. falciparum lysate fraction No. 3 to determine the optimal conditions for sensitizing tanned erythrocytes. The amount of antigen per ml that was used to sensitize tanned cells had a marked effect on the IHA titer (Table 2). Cells sensitized with a 50 percent antigen solution were reactive at the greatest antiserum dilutions, but 25 percent and 12.5 percent antigen solutions provided cells which reacted adequately and thereby conserved antigen.

The time and temperature of incubation of antigen with tanned cells also affected the sensitivity of the reactions (Table 3). Thirty minute incubations afforded significantly higher titers without sacrificing specificity. Incubation for 2 hours at 4 appeared to give better results than the 10 minute room temperature incubation.

Table 2

## Effect of Antigen Concentration on Sensitization of Erythrocytes

Antigen Concentration (percent)	mg Protein per ml	IHA Titers				
		<u>P. falciparum</u> Antisera			Normal Sera	
		(T.M.)**	(L.D.)**	(R.P.)**	(T.K.)**	(W.P.)**
50	.385	40,960	20,480	20,480	5,120	<20
25	.205	10,240	5,120	5,120	5,120	<20
12.5	.090	2,560	2,560	5,120	5,120	<20
1.2	*	<20	<20	<20	<20	<20
.1	*	<20	<20	<20	<20	<20

\*Concentration too low for determination.

\*\* ( ) patient's initials.

#### Reproducibility

Table 4 shows the general reproducibility of the test from day to day using erythrocytes from 4 different sheep. The results of testing 4 immune and 3 normal sera on different days with varying aliquots of erythrocytes from the same and different sheep are tabulated. Normal serum gave negative results except in one instance. The reactions with P. berghei and P. falciparum antisera were reproducible and no negative reactions were recorded with any of the antisera on any day.

#### Specificity of P. falciparum Antigen

The specificity of the IHA test antigens fractionated from P. falciparum lysates and tested against a variety of sera from infected individuals is shown in Table 5. Positive reactions were obtained at dilutions of 1:20 or greater in 93 of 94 sera from P. falciparum infections, and most of them reacted at a relatively high titer. Six of 51 P. vivax antisera and 2 of 26 P. malariae antisera were considered non-reactive at 1:20. Weak reactions were present in all 8 of these samples which gave partial agglutination of sensitized cells to titers of 1:640. Only 11 of 141 sera from apparently healthy individuals reacted and these were at low titers. No reactions were observed in tests of 32 syphilitic sera. The relatively few sera from other infections which reacted may be due to prior or concurrent malaria infections since some of these sera were obtained in areas where malaria is endemic. The two filarial sera reacting at relatively high titers were obtained in Nigeria from patients infected with Onchocerca volvulus.

Table 3  
 Effect of Time and Temperature on Sensitization\*\* of Erythrocytes

Sensitization Conditions	IHA Titers**							
	<u>P. falciparum</u> Antisera			Normal Serum				
	(T.S.)*	(K.P.)*	(V.K.)*	(L.D.)*	(P.L.)*	(P.B.)*	(B.K.)*	(M.S.)*
10 min. Room Temp.	80	160	<20	<20	80	40	640	<20
30 min. Room Temp.	5120	1280	640	5120	640	1280	5120	<20
24 Hrs. 4°C	80	320	<20	640	160	160	1280	<20

\*( ) patient's initials.

\*\*25% concentration of P. falciparum antigen.

Table 4  
 R productivity of the JHA Test with Single Sera Run on  
 Different Days with Different Aliquots of Sensitized\*\* Sheep Erythrocytes

Serum Source	No. Times Tested	No. of Times Given Titer Obtained									
		<20	20	160	320	640	1280	2560	5120	10240	20480
<u>P. berghei</u> infected rats (pool)	22	0	0	0	0	1	9	10	2	0	0
Uninfected rats (pool)	22	22	0	0	0	0	0	0	0	0	0
<u>P. falciparum</u> Infected humans (U.K.)	4	0	0	0	0	0	2	1	2	3	1
(B.K.)	12	0	0	0	0	3	2	1	2	3	1
(L.D.)	19	0	0	0	1	0	4	7	5	2	0
Uninfected humans (M.S.)	25	25	0	0	0	0	0	0	0	0	0
(W.P.)	19	18	1	0	0	0	0	0	0	0	0

\*\*Antigen concentration - P. berghei 10%, P. falciparum 25%.

Table 5  
Sensitivity and Specificity of the IHA Test\* with Human Sera

Serum	Number Tested	Number Reacting at Titer												
		<20	20	40	80	160	320	640	1280	2560	5120	10240	20480	40960
Falci <sup>o</sup> parum	94	1	1	2	4	9	11	17	15	14	8	9	2	1
Vivax	51	0	2	10	7	9	6	3	3	3	0	0	0	2
Malariae	26	2	3	4	1	2	2	4	4	2	1	1	0	0
Healthy controls	141	130	9	1	1	0	0	0	0	0	0	0	0	0
Filariasis	18	16	0	0	0	0	1	0	1	0	0	0	0	0
Schistosomiasis	18	13	3	1	1	0	0	0	0	0	0	0	0	0
Amebiasis	11	9	2	0	0	0	0	0	0	0	0	0	0	0
Cutaneous Leishmaniasis	22	22	0	0	0	0	0	0	0	0	0	0	0	0
Syphilis	32	32	0	0	0	0	0	0	0	0	0	0	0	0
Histoplasmosis	10	10	0	0	0	0	0	0	0	0	0	0	0	0
Coccidioidomycosis	10	10	0	0	0	0	0	0	0	0	0	0	0	0

\*Tanned cells sensitized with P. falciparum antigen at 25% concentration.

### Inhibition of Titers

Incubating *P. falciparum* antigen with homologous antisera before adding sensitized cells significantly reduced its reactivity. This was demonstrated when 0.5 ml of antigen was added to aliquots of 16 known positive sera. These were incubated for 30 minutes at room temperature and then tested by the addition of sensitized cells. For controls, 0.05 ml saline was added to other aliquots of the same sera and incubated in the same manner. The resulting titers of pre- and post-absorption sera along with saline controls appear in Table 6.

Table 6

#### IHA Titers of Antisera Before and After Absorption with *P. falciparum* Antigen

Treatment	Titer	
	Geometric mean	Range
Pre-absorption	4,070	1280 - 10,280
Saline absorption	3,322	640 - 10,280
Antigen absorption	410	<20 - 5,120

### Time-Course Development of Antibodies

To test the time-course development of antibodies in rats infected with *P. berghei*, male rats weighing 150-200 gm were injected intraperitoneally with  $1 \times 10^8$  *P. berghei* parasitized rat RBC's. Serum titers were measured for 150 days using the indirect fluorescent antibody test (IFA) in 5 rats, the soluble antigen fluorescent antibody test (SAFA) in 4 rats and the indirect hemagglutination (IHA) test in 4 rats. The IFA and SAFA titers paralleled each other closely; both rose rapidly to a peak during the second week of infection and decreased slowly until the end of the experiment. On the other hand, the IHA titers followed a bimodal curve. They reached a peak on day 7, then decreased consecutively on days 10, 13 and 16 and reacted at a titer of <10 by day 19. Antibodies at increasing titers were detected again through days 26 and 34 and reached a second peak on days 111 and 150.

### Comparison of Antibody Responses in Primary and Secondary Infections

Ten rats previously infected with *P. berghei* and 3 uninfected controls were bled and tested on day 314 by IHA. Eight animals were positive by IHA at relatively low titers, ranging from 1:20 to 1:2560 with a geometric mean of 1:58 while the 3 control animals were negative at 1:20. The previously infected animals along with 3 original noninfected controls

were then challenged on the same day with  $1 \times 10^8$  P. berghei parasitized rat cells and were bled at 7-8 day intervals for the next 50 days. Eight days after the rechallenge all 10 of the previously infected animals had developed high titers ranging from 1:640 to 1:10,240 with a geometric mean of 1:2,560. These titers gradually declined and ranged from 1:320 to 1:2,560 (GM 1:686) 50 days later. Titers of the 3 control rats rose at a slower rate than those of the previously infected animals and did not show the distinct fluctuating pattern observed in young rats on primary challenge. No patent parasitemia appeared in the previously infected animals, although the control animals developed low parasitemias.

#### Long Term Preservation of Erythrocytes

Glutaraldehyde fixed sheep RBC's which were tanned and sensitized 6 months and one year after their preparation reacted specifically with malaria antisera yielding titers similar to those obtained when the same sera were tested with fresh cells. Both P. falciparum and P. berghei antisera gave specific reactions when tested against homologous antigens at the 1 year period. P. berghei also gave specific reactions at the 6 month interval.

Davis reported the presence of complement fixing substances in the lysate of P. knowlesi infected erythrocytes when most parasites appeared intact, leading to the supposition that these antigens occur in part outside the organism. The lysate antigens described in this paper may be composed of both parasite and extraparasite material since the freeze-thaw method employed disrupted both the erythrocytes and parasites. The effects of soluble elements of other blood components (WBC's, platelets, etc.) present in the crude lysates are not known. Since the lability of the crude antigenic fractions reported by other authors, was not evident in the course of these experiments, it is possible that the proteolytic enzymes responsible for rapid degeneration of other preparations might have been destroyed by the repeated freeze-thawing.

The specifically reacting substances could be separated from the major host hemoglobin component by all 3 methods investigated, and although no thorough analysis of the reactive substances has been made as yet, a few general characteristics are evident. The solubility of the antigen in ammonium sulfate solutions appeared to be different from that of hemoglobin, and since it was eluted before hemoglobin on Sephadex G-200 columns, its molecular size is probably somewhat larger than hemoglobin. The most distinct separation of the fraction with specific antigenic activity was provided by the use of DEAE Sephadex chromatography. The antigens appear to be negatively charged molecules in 0.01 M pH 7.5 phosphate buffers since they adhered to the DEAE column until 0.1 M pH 6.5 phosphate buffer was added. Antigenic activity of both P. falciparum and P. berghei lysates was found in the corresponding fractions from the DEAE columns, indicating that similar physicochemical properties could be expected in antigens recovered from both parasites.

Sera from individuals with P. vivax or P. malariae infections reacted with the P. falciparum antigen at titers lower than those observed in P. falciparum infected patients. This is in agreement with previous observations, indicating that cross reactivity among the species of human malaria may not be complete and that higher titers will occur in the homologous antigen-antibody system. Further extraction and purification of lysate antigens may provide a system whereby species specificity of antigenic materials could be better defined.

The bimodal curve of IHA titer observed in young rats, coinciding with the time of crisis, may be due to the depletion of hemagglutinating antibody by large amounts of antigen released during this period. It is also possible, however, that the initial rise in titer may be due to antibody belonging to a class of immunoglobulins which is produced initially against the infection but which disappears or is present in reduced concentration as the infection progresses. Antisera obtained from rats early in the course of infection contained 19S antibody and this was not found in hyperimmune serum. A shift of antibody production from 19S to 7S type could explain the bimodal IHA titers noted during the course of infection. This fluctuation of titer was not observed in older rats with less severe infections, in rats challenged a second time, or in human volunteers whose infections were controlled by antimalarial drugs. Desowitz et al. observed IHA antibody fluctuations after chloroquine therapy and splenectomy in Rhesus monkeys infected with P. cynomolgi. Antibody fluctuations in human patients as measured by the indirect fluorescent antibody test have been previously reported.

The use of gluteraldehyde fixed erythrocytes may provide a stable cell population which can be used for long periods of time, thus reducing some of the variation encountered in different aliquots of RBC's. By using human type O negative cells, the time consuming absorption of human test sera that is required when other cells are used is eliminated. Further purification of malarial antigens coupled with recently developed sensitization procedures may also be useful in the development of the IHA test.

The small amount of both antigen and sera needed to perform the IHA test in addition to the large numbers of sera which may be titrated in a short period of time makes the test a practical tool for studying the seroepidemiology of malaria.

2. A comparison of the soluble antigen fluorescent antibody and the indirect hemagglutination tests using Plasmodium falciparum-parasitized erythrocyte lysates as antigen.

A soluble antigen fluorescent antibody (SAFA) test for the serologic diagnosis of human malaria was described recently. A stable antigen fractionated by ion exchange chromatography conferred to this test a high degree of sensitivity, specificity and reproducibility. Subsequent observations indicated that this lysate antigen could also be employed in the indirect hemagglutination test (IHA) with consistently satisfactory results when sera from infected men and lower animals were used.

The primary objective of the present investigation was to determine and compare the time-course development of fluorescent and hemagglutinating antibodies in human volunteers after sporozoite-induced or blood-induced infections with Plasmodium vivax or P. falciparum.

Nineteen white male volunteers ages 21 to 44 were examined and found to be in excellent physical condition before being allowed to participate in the project. They were divided into four groups. Four volunteers (K.W., J.J., J.E., R.F.) were given sporozoites of P. falciparum obtained from infected mosquitoes. Five others (L.H., J.C., S.L., J.W., D.R.) were inoculated with blood from infected donors. One of these (J.C.) eight months earlier had been given blood from a volunteer infected with P. vivax, and another (S.L.) five months earlier had been given blood from a volunteer infected with P. falciparum. No patent infection had resulted from either of these two exposures. Conversely, a patent parasitemia was observed in J.W. who had been inoculated with P. falciparum 10 months before entering this project. Five others (R.P., D.D., J.D., J.C., R.B.) were given sporozoites of P. vivax obtained from infected mosquitoes and the remaining five (R.S., R.D., W.H., W.P., D.R.) were given blood from P. vivax infected volunteers.

Blood smears were made daily before and during patency. White blood cell counts were used to calculate the number of parasitized cells per mm. Intermittent antimalarial chemotherapy was given to control the infection. The infected volunteers were under close observation for intervals varying from 37 to 315 days after exposure to infection.

Serum specimens for antibody determinations were obtained at approximately 1 week intervals and stored at -20°C until tested. The serologic tests were conducted with strict adherence to the published methods. Sera with titers of less than 10 in the SAFA test and less than 20 in the IHA test were considered non-reactive. Preinfection serum specimens from each volunteer were used as normal controls.

Infections with either P. falciparum or P. vivax produced antibodies in amounts sufficient to be detected by SAFA and IHA tests. Both tests indicated that antibody production closely followed the appearance of parasites in the peripheral circulation (Tables 7 and 8). All specimens were non-reactive at the time of infection except for volunteers S.L. and J.W. who had been exposed to malaria infection previously. Since serum samples for antibody determinations were not taken at daily intervals, antibodies might have been detectable a few days earlier than indicated. The titers increased rapidly, and they reached a peak soon after the onset of patency.

The SAFA and IHA antibody curves followed almost parallel lines. In general, antibody levels increased rapidly during the first few weeks of infection and were sustained for several months. Antibody titers observed in P. falciparum infections were usually higher and of longer duration than in P. vivax infections, regardless of whether the volunteers were infected by sporozoites or parasitized erythrocytes. This difference

was more evident with the IHA than with the SAFA tests. In most cases the antibody titers decreased after the parasites disappeared. In general, although the parasite density was affected by intermittent anti-malarial chemotherapy, relatively high antibody titers persisted for several months after infection. The sera of some individuals remained positive in both the SAFA and IHA tests throughout the period of observation (up to 315 days) as shown in Table 8.

Table 7

The Course of Falciparum or Vivax Malaria in Volunteers

Infection	Patient initials	Pre-patent period (days)	Day	Peak Parasitemia No. per mm <sup>3</sup> (X 10 <sup>3</sup> )	Last day of parasitemia	Length of observation (days)
Falciparum malaria (sporozoite induced)	K.W.	10	44	4.2	47	315
	J.J.	13	53	12.0	56	146
	J.E.	11	16	74.0	80	163
	R.F.	11	13	<1.0	15	71
Falciparum malaria (blood induced)	L.H.	14	18	19.2	62	202
	J.C.	5	8	17.5	11	128
	S.L.	8	12	115.3	86	93
	J.W.	11	14	77.4	15	37
	D.R.	9	14	75.5	118	129
Vivax malaria (sporozoite induced)	R.P.	17	93	4.5	95	140
	D.D.	15	24	14.2	44	170
	J.D.	13	20	7.4	21	137
	J.C.	16	21	<1.0	22	84
	R.B.	16	24	5.6	31	71
Vivax malaria (blood induced)	R.S.	8	42	2.2	66	115
	R.D.	14	18	5.6	20	94
	W.H.	13	13	<1.0	15	89
	W.P.	11	17	17.6	19	150
	D.R.	4	13	16.4	>43	43

Soluble antigen fluorescent antibody and indirect hemagglutination tests with *P. falciparum* parasitized erythrocyte lysates gave satisfactory and consistent results throughout these studies. These results confirm earlier reports which indicated that these procedures possessed a high degree of reliability. All the infected individuals showed a serologic response with both tests using this antigen. Low level antibodies were detected with the SAFA test in one volunteer (S.L.) and with the IHA test in two individuals (S.L. and J.W.) who had been inoculated with parasitized blood before being included in this project. The time-course development of antibodies followed almost parallel lines with both tests. Antibodies were detected at approximately the same time

Table 8

The Course of Antibody Development in Volunteers Infected with *Falciparum* or *Vivax* Malaria

Infection	Patient	Ab first detected		Maximum Ab level		Last determination Day	Titer		Days** to negative serology	
		Day (Titer)	IHA	Day (Titer)	IHA		SAFA	IHA	SAFA	IHA
<i>Falciparum</i> malaria (sporozoite induced)	K.V.	24*(320)	24*(40)	24(320)	150(320)	315	160	320	>268	>268
	J.J.	27*(80)	27*(20)	60(320)	60(320)	147	20	40	> 90	> 90
	J.E.	34*(320)	34*(160)	34(320)	34(160)	163	40	20	> 83	> 83
	A.F.	14 (160)	21 (1280)	21(320)	21(1280)	56	20	80	> 56	> 56
<i>Falciparum</i> malaria (blood induced)	L.H.	22*(320)	22 (50)	22(320)	98(320)	202	20	160	>140	>140
	J.C.	14 (40)	7 (80)	31(160)	14(1280)	200	<10	40	124	117
	S.L.	0 (10)	0 (20)	18(320)	18(2560)	70	320	640	> 17	> 17
	J.W.	14 (20)	0 (20)	23(320)	23(2560)	37	<10	1280	15	> 17
	P.R.	7 (40)	7 (20)	21(320)	70(320)	100	160	320	Still patent	Still patent
<i>Vivax</i> malaria (sporozoite induced)	R.P.	10 (20)	36 (80)	36(80)	115(320)	140	<10	80	45	> 45
	D.D.	21 (20)	28 (20)	25(320)	42(40)	168	10	<20	>126	126
	J.D.	14 (10)	5 (20)	22(320)	21(40)	150	<10	<20	90	28
	J.C.	14 (10)	7 (20)	26(320)	21(320)	84	20	20	> 62	> 62
	R.S.	25*(320)	25*(40)	25(320)	25(40)	71	<40	<20	> 40	23
<i>Vivax</i> malaria (blood induced)	R.S.	14 (80)	21 (40)	21(320)	21(40)	115	<10	20	29	> 74
	P.D.	21 (320)	21 (40)	21(320)	21(40)	150	<10	<20	112	112
	M.H.	14 (20)	14 (20)	23(80)	23(80)	89	10	40	> 74	> 74
	M.P.	15 (20)	22 (80)	22(320)	22(80)	150	<10	<20	74	20
	D.R.	10 (320)	10 (160)	10(320)	38(1280)	38	320	1280	Still patent	Still patent

\*First determination.

\*\*After patency.

after the onset of parasitemia regardless of whether the volunteers were infected with P. falciparum or P. vivax or whether the infections were sporozoite or blood induced. Therefore, the two tests may be measuring the same antibodies and the differences observed may be quantitative rather than qualitative.

The initial detection of malaria antibodies correlated closely with the first appearance of malaria parasites in the peripheral blood. Neither of the tests indicated that there was any decrease in antibody titer during relapses. This observation is at variance with the report by Lunne et al., who observed with the fluorescent antibody test that relapses in two vivax infections and recrudescences in two falciparum infections resulted in a significant decrease of antibody titers. Compared with P. vivax, the antibody titers in P. falciparum infections were usually higher and persisted for a longer time after the parasites were last seen in the peripheral blood. Whether this was due to the use of an antigen obtained from P. falciparum or whether falciparum malaria elicits a greater antibody response cannot be assessed at this time, since the volunteers constitute a heterogeneous group. However, these results are in general accord with the findings of previous investigations with other serologic methods and suggest an incomplete cross-reactivity between the two parasites resulting in higher antibody levels in the homologous antigen-antibody system.

Since the appearance of antibodies and the attainment of maximum antibody titers did not correspond with a spontaneous decrease in parasitemia, one may surmise that these antibodies were not necessarily protective in nature. However, one must also consider the possibility that antigenic variants may have developed in these volunteers and that this enabled the parasites to multiply in the presence of high antibody levels.

The present investigations indicate again that these tests provide a specific and sensitive method for following the course of antibody development after infection with either P. vivax or P. falciparum. The value of a serologic procedure for malaria is based not only on test specificity, sensitivity and reproducibility, but also on the relative ease with which specimens can be processed. The use of P. falciparum lysate antigen obtained from experimentally infected chimpanzees made it possible to conduct a large number of tests with great economy of time and parasite material. The large number of tests performed in the present studies could be conducted with the amount of antigen normally collected from a single chimpanzee. The results suggest that SAFA and IHA tests using P. falciparum lysate antigen may provide relatively simple and reliable procedures for the serodiagnosis of human malaria. These tests could be used even in small laboratories if the antigens were available from commercial sources.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 106, Antigenic fractionation, serology of malaria

3. Publications

Sadun, E. H. and Gore R. W. Mass diagnostic test using Plasmodium falciparum and chimpanzee erythrocyte lysate. Exper. Parasitol. 23:277-285, 1968.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACRONYM	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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69 01 31	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
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C. TYPE				70		2	
D. KIND OF AWARD				E. CUM. AMT.		50	
21. RESPONSIBLE ORGANIZATION				22. PERFORMING ORGANIZATION			
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RESPONSIBLE INDIVIDUAL				PERSONNEL INVESTIGATOR (Provide SELF if U.S. include last name)			
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TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3544			
23. GENERAL USE				SECURITY AGENCY NUMBER			
Foreign Intelligence Not Considered				DA			
23. (U) Malaria; (U) Fluorescent Antibody; (U) Complement; (U) Complement Fixation Tests; (U) Antigens; (U) Serodiagnosis; (U) Serology; (U) Biochemistry; (U) Hemolysis							
24. (U) Isolation and purification of malaria antigens and investigation of immune response. Development of methods for evaluating immunopathologic response to infection. Antigens evaluated for diagnostic ability and for immunogenic properties. Isolation, characterization and in vivo response to lytic factor obtained from malaria parasites. Immunopathologic response applied to studies on host parasite relationships.							
25. (U) CF and FA technics are used in conjunction with antigen purification methods. Osmotic fragility of erythrocytes, hematocrits, febrile reactions and C-prime levels are used as criteria for appraising immunopathologic response in host. Technical problems include limited availability of parasite material and separation of parasites from blood components.							
26. (U) 69 01 - 69 06. Methods previously developed for separating P. knowlesi and P. falciparum from host red cells and for preparing CF antigens were employed in obtaining specific antigens from P. malariae. Now have capability for differential serodiagnosis of vivax, falciparum and malariae malaria. The three antigens showed strong reactions with homologous antisera but little or no cross reactivity with heterologous sera. In vivo studies on the plasmodial lytic factor (LF) showed that erythrocytes of hamsters injected with LF exhibited a temporary increase of resistance to hypotonic lysis for 4 hrs after injection, but showed abnormal fragility after 5 hrs. Multiple injections of LF significantly reduced hematocrit values and gave rise to febrile reactions. Maximum temperatures increased with each injection with LF. In vivo studies involving longer observation periods are in progress. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.							

PII Redacted

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 107, Malaria antigens

**Investigators.**

Principal: Earl H. Fife, Jr., M.S.

Associate: Albert E. vonDoenhoff, Jr., B.S.

**Description.**

This work unit is concerned with the isolation and characterization of plasmodial antigens, studies on the antigenic structure of various plasmodia, investigations of serologic patterns developed during the course of infection, and elucidation of immune mechanisms associated with this disease. In vitro as well as in vivo methods are employed. In vitro methods are used in (1) development of procedures for separating malaria parasites from host blood components; (2) isolation, purification and identification of plasmodial antigens by physicochemical and serologic methods; and (3) development, improvement and evaluation of serologic procedures for detection of antibodies and for following antibody patterns in infected hosts. In vivo studies include (1) the role of antigen and antibody in certain immunopathologic conditions associated with malaria infection; (2) production of specific antibodies to characterize experimental antigen fractions and to investigate the antigenic relationships of various species of Plasmodium; and (3) investigations on the immunogenicity of the purified antigen fractions with particular emphasis on their potential value as vaccines.

**Progress.**

1. Isolation and fractionation of serologically active malaria antigens. An effective method for separating malaria parasites from host erythrocyte components by selective fragmentation of the parasitized red cells in a French pressure cell has been described in previous reports on this Work Unit (WRAIR Research & Development Reports, 1966 et seq.). During this reporting period, these techniques have been used to acquire a supply of Plasmodium knowlesi, P. falciparum and P. malariae parasite harvests for preparing complement fixing antigens. A notable accomplishment during this period was the successful infection of a splenectomized chimpanzee with P. malariae. Although the parasitemia in this animal did not exceed 5%, repeated bleedings ultimately provided a quantity of parasites sufficient for preparing a supply of antigen adequate for currently programmed studies. The fractionation procedures previously used for the isolation of the P. knowlesi and P. falciparum antigens were employed in the preparation of the P. malariae antigen. The antigen proved to be highly specific for quartan malaria and showed very little cross reactivity with sera from individuals with vivax or falciparum malaria. With the acquisition of the P. malariae antigen, we now have the capability for differential serodiagnosis of vivax, falciparum and malariae malaria.

2. Serodiagnostic tests for malaria. The potential value of purified P. knowlesi and P. falciparum antigens for the serodiagnosis of vivax and falciparum malaria respectively has been indicated in previous reports on this Work Unit (WRAIR Research & Development Reports, 1967, 1968). These antigens were further evaluated during the present reporting period and preliminary studies on the efficacy of the malariae antigen were initiated. The suitability of the Microtiter System for complement fixation tests for malaria was demonstrated in the previous report (op. cit.) and this micro-technic was used exclusively in the studies reported herein. All three malaria antigens showed excellent sensitivity and specificity in parallel tests on representative homologous and heterologous sera (Table 1). Each gave a strong reaction with its homologous antiserum but showed little or no cross reactivity with the heterologous sera. Moreover, in ancillary studies, none of the antigens reacted in tests with sera from healthy individuals or patients with diseases other than malaria. In view of these findings, it was apparent that use of the three antigens in diagnostic complement fixation tests would provide a capability for: 1) Detecting individuals with current or recent malaria; 2) Identifying the species of plasmodium involved; and 3) Identifying individuals with mixed infections.

Collaborative studies with investigators in Vietnam and Uganda provided an opportunity to critically evaluate the tests for malaria with sera from individuals residing in highly endemic areas. Members of the U. S. Army Medical Research Team (WRAIR) Vietnam, observed a high incidence of splenomegaly in Montagnards residing in a village located in the Central Highlands of Vietnam. This disease had a marked resemblance to the "big spleen" syndrome described by investigators in East Africa. Since the latter reportedly was a sequela of P. malariae infection, studies were conducted to determine whether P. malariae could be the etiologic agent of the disease in Vietnam. Accordingly, 21 individuals with Class II or greater splenomegaly (Hackett's classification) were selected for the study. Kala azar was excluded on the basis of negative bone marrow examinations and formol gel tests. A single thick blood film from each patient was examined for malaria parasites and a serum was collected for evaluation in the complement fixation tests for malaria. The findings are summarized in Table 2. Results of the complement fixation tests on these patients indicated an exceptionally high incidence of malaria infection; only 1 failed to react with one or more of the malaria antigens. Infection with P. falciparum was almost universal and there was serologic evidence of a considerable number of individuals with intercurrent infections with P. vivax and/or P. malariae. The incidence of reactions with the P. malariae antigen, however, did not appear to support the hypothesis that the "big spleen" disease was due to quartan malaria.

The results of the thick film examinations contrasted markedly to those obtained with the complement fixation tests. Malaria parasites were demonstrated in only 7 of the group. It was especially curious to note that many of the thick film-negative patients exhibited high malaria antibody titers, particularly in tests with the P. falciparum antigen. These findings illustrate the inadequacy of using single thick film

Table 1  
 Reactivity of P. knowlesi, P. falciparum  
 and P. malariae antigens in complement  
 fixation tests with representative  
 homologous and heterologous sera

Serum Category	Reactions* obtained with indicated plasmodial antigen:		
	<u>P. knowlesi</u>	<u>P. falciparum</u>	<u>P. malariae</u>
<u>P. vivax</u>	R (32)	wr	-
<u>P. falciparum</u>	wr	R (128)	-
<u>P. malariae**</u>	wr	-	R (8)

\*Classified as R(reactive), wr (weakly reactive) or - (nonreactive).  
 The figures in parentheses indicate serum titers.

\*\*Serum from chimpanzee experimentally infected with P. malariae.

Table 2  
 Results of Thick Film Examinations and Complement  
 Fixation Tests for Malaria on 21 Montagnards with  
 Splenomegaly

Plasmodia identified in thick film*	CF results** with indicated antigen		
	<u>P. falciparum</u>	<u>P. knowlesi</u>	<u>P. malariae</u>
Pf, Pm	R(1)	wr	-
Pf, Pv	-	wr	wr
Pv	R(64)	wr	-
Pf	R(>128)	R(4)	-
Pf	R(>128)	R(64)	R(1)
Pf	R(16)	wr	R(1)
Plasm. sp.?	R(4)	R(1)	QNS
0	R(>128)	R(4)	wr
0	R(>128)	-	-
0	R(>128)	-	R(4)
0	R(>128)	R(4)	-
0	R(>128)	R(4)	-
0	R(>128)	R(2)	-
0	R(>128)	R(4)	R(1)
0	R(>128)	-	-
0	R(>128)	-	R(1)
0	R(4)	-	-
0	R(1)	-	wr
0	R(AC)	R(AC)	-
0	R(AC)	R(AC)	R(AC)
0	-	-	-

\*Pf = P. falciparum; Pv = P. vivax; Pm = P. malariae

\*\*R = reactive; wr = weakly reactive; (-) = nonreactive. The number in parenthesis indicate the titer. R(AC) = Reactive but anticomplementary. AC = Anticomplementary.

examinations for epidemiological surveys of malaria in hyperendemic areas, and it is suggested that the complement fixation tests with purified plasmodial antigens provide a much more accurate appraisal of the prevalence of Plasmodium infections in semi-immune populations.

Collaborative studies with investigators at the Lymphoma Treatment Center, Entebbe, Uganda provided further opportunity to evaluate the complement fixation tests for malaria with sera from individuals residing in a hyperendemic area. Although the results of thick blood film examinations on individuals of the group studied were not provided with the serum specimens, investigators at the Center stated that patent parasitemias usually were very low and examination of multiple thick films often was necessary to demonstrate the parasites. This was similar to the experience with individuals living in hyperendemic areas in Vietnam.

In part, these studies were conducted to determine whether malaria, particularly quartan malaria, possibly played a role in the etiology of Burkett's tumor disease. Accordingly, sera were collected from 12 children with Burkett's tumor, and 12 "healthy" controls of the same age group. All were examined in complement fixation tests for serologic evidence of vivax, falciparum and malariae malaria. The results are summarized in Table 3. Results obtained with the tumor patients did not differ significantly from those obtained with the controls. Reactivity with the P. falciparum antigen was a universal finding. It is noteworthy that 5 of the 7 tumor patients and all 5 of the controls that reacted with the P. malariae antigen also gave strong reactions with the falciparum antigen, indicating dual infections with both parasites. Two of the tumor patients showed strong reactions with all three antigens, suggesting intercurrent vivax, falciparum and malariae infections. These findings do not support the hypothesis that malaria plays a role in the etiology of Burkett's tumor. Nevertheless, the results do provide further evidence of the efficacy of complement fixation tests employing purified plasmodial antigens for the differential diagnosis of malaria.

A further potential use of the CF tests for malaria would be the screening of troops returning from duty in endemic areas. It is believed that non-immune individuals on chemoprophylaxis could exhibit a parasitological picture similar to that shown by semi-immunes residing in endemic areas. For example, a soldier could acquire malaria during a period in which he temporarily failed to take the drug. However, recontinuance of the drug at prophylactic (sub-curative) levels could suppress clinical disease and keep the patent parasitemia at very low or nondetectable levels. Such individuals invariably would be overlooked in screening procedures consisting solely of examination of a single thick blood film. It is suggested that concomitant use of CF tests for malaria would minimize this risk. Earlier studies on human volunteers experimentally infected with P. vivax or P. falciparum have shown that CF antibodies appear 4-5 days after appearance of patent parasitemia, rapidly rise to significant titers, and persist for a considerable period of time (45 to 100 days or more) even though curative therapy was initiated immediately after the appearance of parasites in the peripheral blood. On this basis, it is likely infected soldiers receiving suppressive regimens of drug,

Table 3

Comparison of Malaria CF Test Results on Patients  
with Burkett's Tumor with Results on Healthy Controls  
Residing in Same Area of Uganda

Health Status	Number Tested	Number reacting* in CF tests with indicated antigen		
		<u>P. knowlesi</u>	<u>P. falciparum</u>	<u>P. malariae</u>
Burkett's Tumor	12	2 (8 - >128)	12 (8 - >128)	7 (4 - 16)
Healthy Control	12	0	12 (1 - >128)	5 (4 - >128)

\*Figures in parentheses indicate the serum titer range.

would show detectable antibodies but no parasites in the circulating blood. However, these subjects would be expected to relapse and develop frank, clinical malaria when the drug is withdrawn.

Although valuable information has been obtained from human volunteers experimentally infected with selected strains of P. falciparum, P. vivax and P. malariae, it is recognized that certain immunological and serological features of the disease may differ in individuals with naturally acquired infections with "local" strains of the parasites. Arrangements therefore are being made to conduct comprehensive studies on a select group of American soldiers hospitalized for malaria acquired in the field. These investigations are being designed to provide information concerning: 1) the length of the prepatent period; 2) the time of initial appearance of detectable complement fixing antibodies; 3) the antibody patterns exhibited throughout the course of the disease, and their relationship, if any, to the clinical status of the patient; and 4) the persistence of antibodies following radical cure. The latter information is essential for interpreting the results of screening tests for malaria. The results of these studies will be given in a subsequent report on this Work Unit.

### 3. Preservation and storage of malaria parasites and antigens.

Previous reports on this Work Unit (WRAIR Research & Development Report, 1967, 1968) noted that incorporation of 2% polyvinyl pyrrolidone (PVP) with malaria parasite harvests or purified antigens effectively stabilized these products and minimized deterioration during storage. Observations on the stability of these products were continued during the present reporting period. To date, parasite harvests preserved with 2% PVP and held at  $-60^{\circ}\text{C}$ , showed no evidence of deterioration after storage for more than 2 years; complement fixing antigens prepared from these stored parasites were comparable to those obtained from freshly harvested organisms. Similarly, complement fixing antigens that were stabilized with 2% PVP and lyophilized, showed no change in serologic properties after storage at  $3^{\circ}\text{C}$  for more than 2 years. The excellent stability and long storage life of these products assure the ready availability of free parasites for antigen production, and make it feasible to prepare large volumes of antigen that would be required for mass-testing. Observations on the stability of the parasite harvests and antigens will be continued for at least one more year.

### 4. Isolation and characterization of a lytic component of P. knowlesi.

During investigations on the fractionation of malaria antigens on Sephadex gel columns, it was observed that certain low molecular effluents from the column possessed lytic properties. Preliminary observations on the physical properties, kinetics of functional activity, and chemical nature of this lytic factor (LF) have been presented in previous reports on this Work Unit (WRAIR Research & Development Reports, 1966, et seq.). Detailed investigations on the kinetics of LF-induced hemolysis have revealed certain noteworthy features. Little or no hemolysis occurred during the first 4 hours of incubation. This was observed regardless of the concentration of LF or the temperature of incubation. However,

hemolysis progressed in a linear fashion after the initial lag phase, but spontaneously terminated after incubation for 21 hours, even though some intact red cells remained in the reaction mixture.

The spontaneous termination of lysis after incubation for 21 hours was shown not to be due to depletion of the lytic factor; treatment of the remaining cells with fresh LF failed to induce further lysis. However, kinetic studies on a mixture of old cells labeled with Fe<sup>55</sup> and young cells tagged with Fe<sup>59</sup> revealed that the majority of unlysed cells remaining after incubation for 21 hours with LF bore the Fe<sup>59</sup> label. Thus it appeared that the younger cells were more resistant to LF-induced lysis and that hemolysis spontaneously terminated when the population of older cells was depleted.

Physicochemical analyses of the LF have revealed the following characteristics. The factor is a relatively small molecule and appears to have a molecular weight of less than 5000. Quantitative chemical analysis for protein (Lowry test) indicates the presence of a small amount of protein. However, in view of the low molecular weight of the LF, it is believed that these reactions were due to amino acids and/or small peptides rather than protein per se. Trace amounts of carbohydrate also were detected. However, the principal component of the LF appeared to be lipid in nature, with a high cholesterol content. Thin layer chromatography revealed the presence of a variety of lipids belonging to the following classes: phospholipids, free fatty acids, cholesterol, cholesterol esters, and tryglycerides. Resignation of the biochemist responsible for this phase of these investigations necessitated temporary discontinuance of further studies on the chemical characterization of the LF. However, these studies will be continued when the position is filled.

In view of the possible role of the LF in the host-parasite relationships and pathology of malaria, in vivo studies were initiated to determine whether certain clinical features of the disease could be induced by injecting the LF into experimental animals. Fogel, et al (Am. J. Trop. Med. & Hyg., 15 : 269, 1966), working with Rhesus monkeys infected with P. knowlesi, reported that the nonparasitized as well as parasitized erythrocytes showed increased susceptibility to hypotonic lysis. Moreover, the osmotic fragility progressively increased with each cycling of the parasite during the acute stages of the infection. During the course of recent in vitro studies on the kinetics of LF-induced hemolysis, it was observed that LF-treated cells exhibited a similar increase of osmotic fragility during the pre-lytic lag phase. It was of interest, therefore, to determine whether this phenomenon could be produced in vivo by injecting LF into experimental animals. Hamsters weighing 105-125 gm were used in these studies. The hamsters were divided into 8 groups containing 4 animals each. All animals of the first six groups received an IV injection of 2.0 ml of the LF. Animals in group seven received three IV injections of LF administered at 24 hour intervals. Group eight served as controls and received 2.0 ml of 0.9% NaCl solution administered IV. Hamsters of the first six groups were exsanguinated at 1/2, 1, 2, 3, 4, and 5 hours respectively

after injection with the LF. Animals of group seven who received the multiple injections of LF were exsanguinated 4 hours after the last injection. The controls were sacrificed 4 hours after receiving the saline. Immediately after collection, the erythrocytes from each animal were washed to remove the plasma and the osmotic fragilities determined according to the method developed by Allen, et al (Am. J. Clin. Path., 45 : 112, 1966).

Results of these experiments are summarized in Figure 1. Contrary to expectations, the osmotic fragility of the erythrocytes decreased rather than increased for a period following injection of the LF. The cells collected one-half hour after injection were considerably more resistant to hypotonic lysis than the controls. Resistance progressively increased through hour 2 and remained at a relatively high level through the fourth hour. However, there was a significant decrease in resistance after the fourth hour, and cells collected 5 hours after injection with LF were more fragile than the controls. The latter observation showed a striking similarity to the 4-hour lag phase noted in the earlier in vitro studies on the kinetics of LF-induced hemolysis. However, the initial transitory increase of osmotic resistance was an unexpected finding. Erythrocytes from the hamsters receiving the three injections of LF over a 48 hour period and then exsanguinated 4 hours after the last injection, showed osmotic fragility values comparable to those of the animals receiving a single dose of LF and exsanguinated 5 hours later. However, the hematocrit values of the animals receiving multiple injections of LF were considerably lower than those of the other groups. These observations suggested that considerable in vivo hemolysis had occurred and indicated that the anemia associated with acute malaria could be simulated by multiple injections of LF.

During the course of the in vivo studies on the effects of LF on hamster erythrocytes, it was observed that the animals appeared to develop a fever after each injection. Since recurrent fever is a characteristic of malaria in a nonimmune host, the pyrogenic properties of the LF were investigated. The group of hamsters receiving three injections of LF at 24 hour intervals in the previous studies were used for these experiments. Rectal temperatures of each animal were taken immediately after injection of LF and then 1/2, 1 1/2, 2 1/2, and 4 hours later. The results are summarized in Table 4. Following each injection, the temperature progressively rose during the succeeding 4 hours. However, the temperatures had returned to normal before the second and third injections were given 24 and 48 hours later. The degree of febrile response appeared to increase with each injection. Four hours following the first injection with LF, the average temperature increased from 33°C to 35.5°C. After the second injection, the increase was from 33°C to 36.5°C, and an increase from 33 to 37.5°C was observed after the third injection. These studies are being continued to investigate the febrile response to repeated injections of LF over a longer period of time.

Results of these in vivo experiments suggest that the lytic factor (LF) isolated from malaria parasites may in part contribute to certain clinical features of malaria. The ability of the LF to increase the

Figure 1

Osmotic Fragility of Hamster Erythrocytes in Blood  
Collected at Various Periods After  
Injection with Lytic Factor (LF).

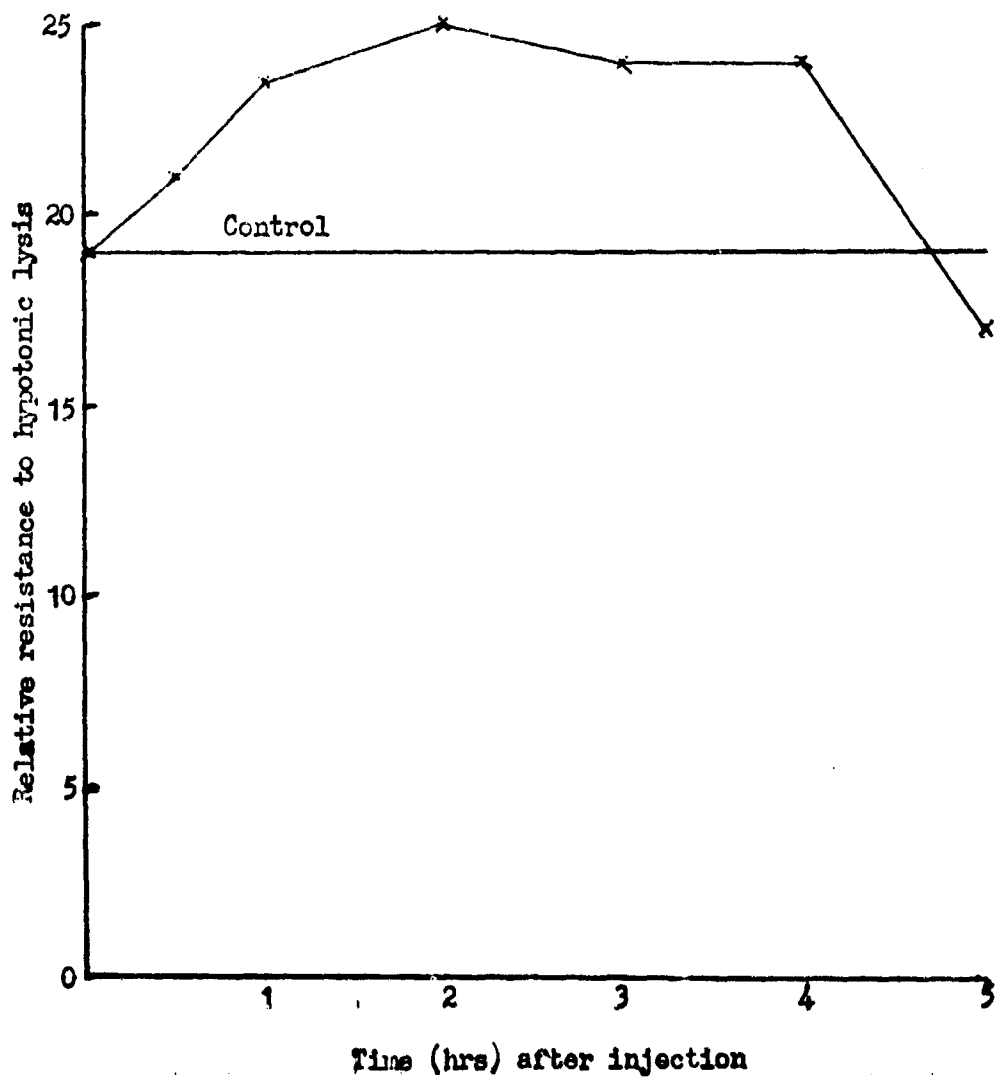


Table 4

Rectal Temperatures of Hamsters After Receiving  
1, 2 and 3 Intravenous Injections of Plasmodial  
Lytic Factor Administered at 24-hour Intervals.\*

Injection No.	Average temperature (°C) at indicated time after injection			
	0 hr.**	1 1/2 hr.	2 1/2 hr.	4 hr.
I (initial)	33	32.5	34	35.5
II (24-hour)	33	32.5	35	36.5
III (48-hour)	33	33	35	37.5
Saline Control	32.5	31	31	32

\*Average temperature for 4 animals

\*\*Temperature immediately after injection



osmotic fragility and ultimately lyse red cells indicates that LF may play a role in the anemia associated with acute malaria infections and possibly contribute to the black water fever syndrome developed in certain individuals. The findings also suggest that the LF could be responsible for the febrile reaction associated with the cycling of the parasites. These studies are being continued to further characterize the LF, investigate its action in vivo, and explore possible methods for inhibiting its biological activity.

#### Summary and Conclusions.

1. The recently described method for separating malaria parasites from host erythrocytes by preferential fragmentation of the red cell membranes under controlled pressure has been successfully used for the isolation of a variety of species of Plasmodium. The procedures have been used for the isolation of P. knowlesi from Rhesus monkeys, P. falciparum from splenectomized chimpanzees, and most recently, P. malariae from a splenectomized chimpanzee. Complement fixing antigens from these parasites now provide a capability for differential serodiagnosis of vivax, falciparum and malariae malaria.

2. The efficacy of purified P. knowlesi, P. falciparum and P. malariae antigens for the serodiagnosis of vivax, falciparum and malariae malaria was evaluated with sera from Vietnamese and Ugandan natives residing in hyperendemic areas. The findings indicated that the complement fixation tests with purified plasmodial antigens were far superior to thick blood film examinations for detection of malaria in semi-immune individuals, and would provide a much more accurate appraisal of the malaria experience in epidemiological surveys of populations residing in hyperendemic areas. The results further suggest that the complement fixation tests may be of value for screening troops returning from duty in malaria endemic areas.

3. Studies on the stability of stored parasite harvests and antigens were continued. Parasite harvests containing 2% FVP and maintained at  $-60^{\circ}\text{C}$ , showed no evidence of deterioration after storage for more than 2 years. Likewise, the purified plasmodial antigen fractions that were stabilized with 2% FVP and lyophilized, showed no change in serologic properties after storage at  $3^{\circ}\text{C}$  for more than two years.

4. Investigations on the functional activity, kinetics of hemolysis, and chemical nature of a plasmodial lytic factor (LF) were continued. Earlier preliminary findings were confirmed, and in vivo studies were initiated. In kinetic studies, an initial lag phase during which no hemolysis occurred always was observed. This was followed by a linear progression of hemolysis which spontaneously terminated after incubation for 21 hours. The spontaneous cessation of lysis was shown to be due to depletion of the older, more susceptible cells of the suspension used in the system. In in vivo studies, erythrocytes of hamsters receiving IV injections of LF showed a temporary progressive increase of resistance to hypotonic lysis during the 4-hour period following injection. However, after this period, the cells became more susceptible to lysis, and the

osmotic fragility 5 hours after injection was considerable higher than that of the controls. It was also shown that injection of LF induced a febrile response in the hamsters. Moreover, the maximum temperature increased with each injection of the factor. The possible relation of the LF to certain clinical features of malaria was discussed.

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 107, Malaria antigens

Publications.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				11. AGENCY ACCESSION#	12. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA 0A6506	69 07 01	DD-R&E (AI) 638	
3. DATE PREVIOUS SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SECURITY	6. WORK SECURITY	7. REGRADING*	8. DDDM INSTN#	9B. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
69 01 31	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	63713A	3A663713D329	00	108			
B. CONTRIBUTING							
C. COORDINATING	1412A(2)						
11. TITLE (Precede with Security Classification Code)							
(U) Study of Malaria and Antimalarial Therapy (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
002 600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
07 64		NA		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
NA				FISCAL YEAR		FUND\$ (in thousands)	
A. DATES/EFFECTIVE:				B. NUMBER*		C. TYPE:	
EXPIRATION:				69		5	
D. NUMBER*				70		3	
E. TYPE:				70		70	
F. KIND OF AWARD:				3		70	
G. CUM. AMT.				70		70	
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22. KEY WORDS (Precede each with Security Classification Code)							
(U) Plasmodium falciparum; (U) Plasmodium; (U) Enzymes; (U) Carbonic Anhydrase; (U) Antimalarials; (U) Chloroquine; (U) Drug Effects; (U) Primaquine.							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Summarize technical paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Basic biochemical activities of malarial parasites will be studied to provide further data for evaluation of drug-resistance of plasmodium falciparum. Analogues of antimalarial drugs will be synthesized where not available for biochemical studies.							
24. (U) Plasmodium berghei will be used as a test organism to evaluate intermediary metabolism of the malarial parasite and responses to antimalarial agents. Special synthesis of additional antimalarial compounds will be done.							
25. (U) 69 01 - 69 06 Using the <u>P.falciparum</u> , Aotus monkey model of malaria, preliminary studies of chloroquine accumulation indicate that the ability to concentrate chloroquine varies inversely with the degree of chloroquine resistance of the parasite; this is in agreement with previous observations of <u>P.berghei</u> . Characterization of CO2 Fixation in chloroquine-sensitive <u>P.berghei</u> is in progress. The enzyme is inactivated by urea probably by dissociation of the enzyme into smaller subunits. The conditions for dissociation of the enzyme into subunits are being investigated. The control of this key enzyme may be dependent on its state of disunity caused by different agents such as chloroquine and quinine sulfate. Chloroquine-resistant <u>P.berghei</u> also demonstrated the ability to fix CO2 with PEP to form oxaloacetate. The similarity or dissimilarity of this key enzyme from chloroquine-sensitive and chloroquine-resistant <u>P.berghei</u> will be investigated. The yield of 10-chloro, 3-methylbenzo(h)quinoline, 4-carboxylic acid was obtained in large enough quantities to proceed with the synthesis to the final compound, 2-(dibutylaminoethyl)-10-chloro-3-methylbenzo(h)quinoline-4-methanol. This compound has been submitted for testing. Work is continuing on the synthesis of 10-hydroxy-and 10-methoxy-3-methylbenzo(h)quinoline, 4-carboxylic acid. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

PII Redacted

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 108, Study of malaria and antimalarial therapy

Investigators.

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Jean E. Matusik, GS-07  
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Description:

This study was designed to study certain biochemical processes in the malaria parasite, to obtain new information relevant to malaria prophylaxis, and to synthesize and test theoretically more ideal antimalarial agents.

Progress:

a. Study of carbon dioxide fixation by Plasmodium berghei. The enzyme phosphoenolpyruvate carboxylase, which is important for carbon dioxide fixation and for survival of P. berghei, has been purified by high-speed centrifugation, ammonium sulfate fractionation, DEAE-cellulose and cellulose phosphate column chromatography. The enzyme is normally very unstable, but it can be stabilized in the presence of 0.4 M phosphate buffer solution during storage. Urea causes inactivation of the enzyme, probably by altering subunit structure. The effect of altered subunit structure on enzymic activity is under investigation.

This enzyme has now been purified over 400 fold with a recovery of 10% and a specific activity of 1.4  $\mu\text{moles}/\text{min}/\text{mgm}$  of protein. It has a  $K_m$  of  $2.6 \times 10^{-3}$  molar for phosphoenolpyruvate and  $1.3 \times 10^{-3}$  molar for magnesium. By sucrose density gradient in low ionic strength buffer, it has a  $S_w$  value of 18 when compared to the standards, catalase and hemoglobin. This corresponds to a calculated molecular weight of 500,000. However, sucrose density gradient in a high ionic strength buffer gives an activity peak with a calculated molecular weight of 250,000. This variation in molecular size dependent upon the ionic strength of the buffer is also seen on agarose columns which separate protein, depending upon their size.

Polyacrylamide gel disc electrophoresis of the purified protein shows a major band and another band at the beginning of the 7% acrylamide gel. These are thought to represent the 250,000 M.W. and 50,000 M.W. forms of

this enzyme. Two very minor bands are also present and thought to be contaminants.

Corresponding with the change in physical characteristics of the enzyme, there is an alteration of the kinetic parameters as the ionic milieu is changed. In one form it is stimulated by Adenosine diphosphate and in another form it is slightly inhibited by ADP. The Michaelis-Menten plot of initial reaction velocity versus magnesium concentration is either hyperbolic or sigmoidal, depending on the form of the enzyme present.

b. Biochemical activity of antimalarial agents. To follow up the earlier observation of antimalarial activity of 1-methyl-3-nitro-1-nitrosoguanidine which was made in their laboratory, the mechanism of action of the compound is being investigated with electron microscopic techniques. Preliminary studies of feasibility have been completed and indicate that the compound causes definite subcellular changes.

c. Chloroquine binding in malaria parasites. Chloroquine-3-<sup>14</sup>C (ring-labeled) was used to study the processes which concentrate chloroquine in mouse red blood cells, normal or infected either with chloroquine-sensitive (CS) or with chloroquine-resistant (CR) Plasmodium berghei. The initial rates of uptake and release of chloroquine-<sup>14</sup>C were both too fast to measure in these studies, yet large gradients could be maintained by the cells. For example, when red blood cells were exposed to  $10^{-8}$  M chloroquine, pH between 7.4 and 7.2, and 22°, steady-state gradients of chloroquine-<sup>14</sup>C were approximately 600:1 (cells:medium) for cells infected with CS parasites, 100:1 for cells infected with CR parasites and 14:1 for normal cells. The processes responsible for these gradients were saturable, in agreement with the proposal of chloroquine binding to cellular constituents. No degradation of chloroquine was detected.

In red blood cells infected with CS parasites, there were at least three classes of binding sites, the apparent intrinsic association constants of which were approximately  $10^8$ ,  $10^5$ , and  $10^3$  M<sup>-1</sup>. Only the class of binding site with low affinity was found in normal red blood cells. Two of the classes of binding sites, those with low and intermediate affinities, were present in red blood cells infected with CR parasites, but high-affinity chloroquine binding was grossly deficient. This deficiency explains the reduced ability of CR parasites to concentrate chloroquine from dilute solutions of chloroquine, and it suggests that chloroquine resistance is due to a decrease in the number, affinity, or accessibility of chloroquine receptor sites on a constituent of the malaria parasite. This receptor site possibly is the site of action of chloroquine.

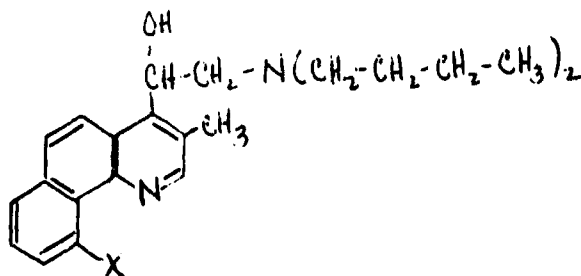
d. Antimalarial drug levels. To study the excretion of chloroquine after prophylactic treatment and to follow the clearance of this drug in the urine, over 5000 analyses for antimalarial drugs have been performed.

The subjects of this study were in large part, troops about to embark for Viet Nam. A sustained residual of chloroquine in the urine was found throughout the seven-day interval between prophylactic doses of chloroquine. All subjects in the study maintained a chloroquine level in the urine after the second dose (second week), but plasma chloroquine levels dropped sporadically to an undetectable concentration in some of these subjects. This divergence of plasma and urine levels suggested other modes of drug clearance in addition to normal metabolism and urinary excretion, and subsequent preliminary studies on chloroquine concentrations in perspiration have provided evidence that the drug may be discharged in substantial quantity through sweat. This study will be continued until the effect on plasma levels of chloroquine loss through heavy sweating can be evaluated.

Studies on chloroquine metabolites continue in an effort to determine the ratio of unchanged drug to that of the major metabolite if, in fact, the principal metabolite is the same in every case. Indications of metabolic differences have been noted periodically in the analyses of body fluids for chloroquine, but too often, the amount of specimen is insufficient to pursue the investigation any further. As specimens for chloroquine analysis are received from Viet Nam and Malaysia, they will continue to be scrutinized for discrepancies in the normal drug metabolic patterns.

e. Chemical study of antimalarial agents. A series of new compounds, which are considered to be potential antimalarials, are being synthesized.

Eight new compounds of the 10-substituted-benzo-[h]-quinoline-4-methanol type (I) will be submitted for the rodent antimalarial screen and for phototoxicity evaluation.

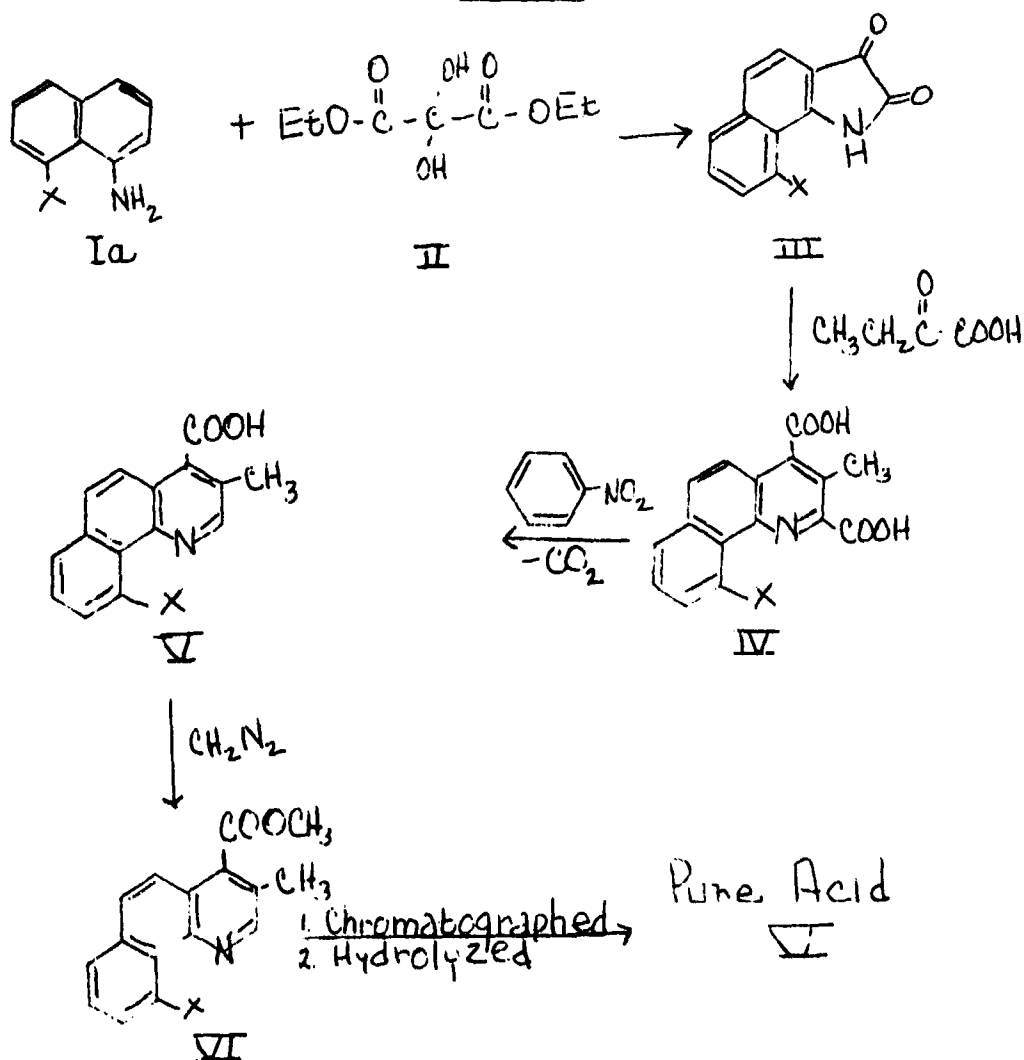


I

Substituent groups in the 3- and 4-positions are held constant, while the group in the 10-position varies. Substituents proposed for this position are -Cl, -Br, -OCH<sub>3</sub>, -OH, -SO<sub>2</sub>NH<sub>2</sub>, -SO<sub>2</sub>CONH, SO<sub>2</sub>Cl, and CF<sub>3</sub>. The 10-chloro-compound has been synthesized and submitted for testing.

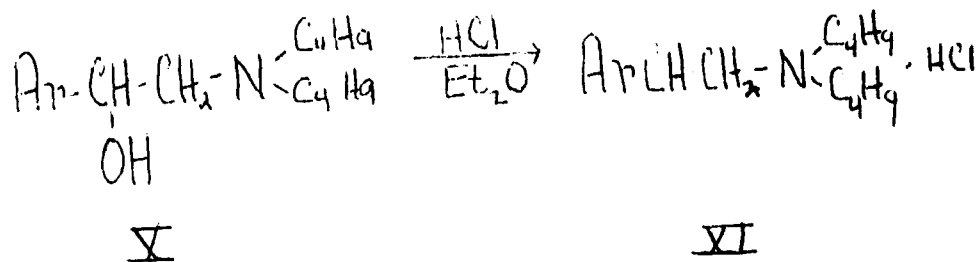
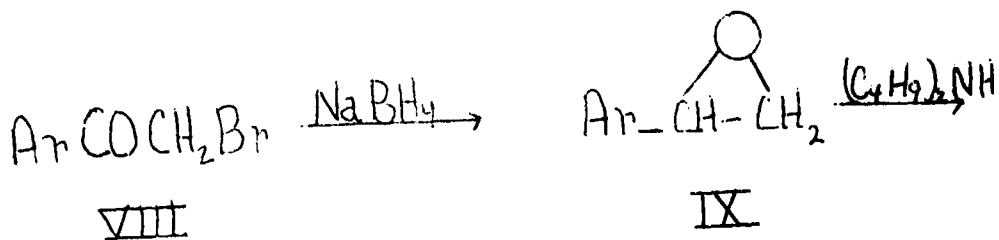
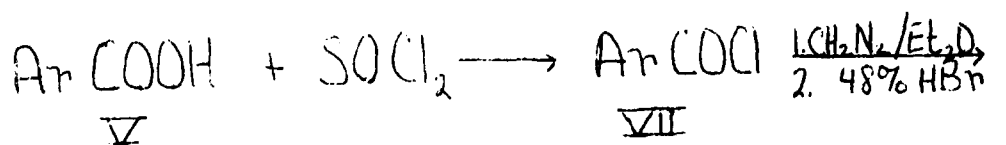
The compounds are made by way of the carboxylic acid (IV) derived from the appropriate benzisatin (III) and  $\alpha$ -ketobutyric acid in a base-catalyzed condensation followed by selective decarboxylation to produce acid V. The crude carboxylic acids so obtained are difficult to purify. They are, therefore, converted to the methyl ester (VI) with ethereal diazomethane, chromatographed over alumina, and hydrolyzed back to the carboxylic acid. The benzisatins are made from the appropriate 8-substituted-1-naphthylamines (Ia) and diethylketomalonnate hydrate (II). The preparations are illustrated in Scheme I.

SCHEME I



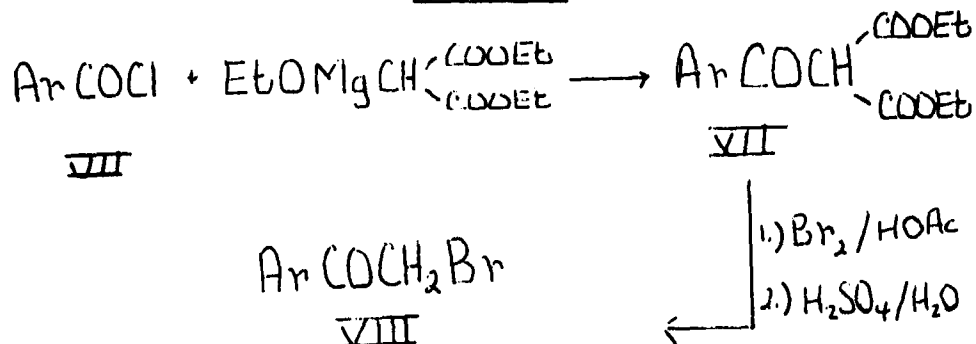
The carboxylic acid V is converted to  $\alpha$ -(dibutylaminomethyl)-benzo-  
[h]-quinoline-4-methanol  $\frac{X}{IX}$  by either of two routes (Scheme II or III).  
In Scheme II, the carboxylic acid is converted to the acid chloride (VII)  
with thionyl chloride, then treated with diazomethane and HBr to produce  
an  $\alpha$ -bromoketone (VIII). An epoxide (IX) results from treatment of the  
bromoketone with sodium borohydride. Refluxing with di-N-butyl amine,  
opens the epoxide ring to produce the final compound. The compounds are  
submitted for testing as the hydrochloride salt (XI).

SCHEME II



Scheme III differs from Scheme II in the preparation of  $\alpha$ -bromo methyl ketone (VIII). The acid chloride (VII) in this procedure is reacted with diethyl ethoxymagnesium malonate, followed by dilute aqueous acid to give crude diethyl acylmalonate (XII). When this compound is treated with bromine,  $\alpha$ -bromomethyl ketone (VIII) is produced.

SCHEME III



Summary and Conclusion:

Phosphoenolpyruvate carboxylase, an enzyme of importance for the survival of Plasmodium berghei, has been purified and partially characterized. Its physical and kinetic characteristics are dependent upon the ionic environment. The characteristics of this enzyme are sufficiently different from those of the enzyme as isolated from other organisms to suggest a thorough understanding of this basic enzyme will help to understand the metabolism of the malarial parasite.

A deficiency of high-affinity chloroquine binding has been discovered in chloroquine-resistant P. berghei. This deficiency explains the reduced ability of chloroquine-resistant parasites to concentrate chloroquine from dilute solutions of chloroquine, and it suggests that chloroquine resistance is due to a decrease in the number, affinity, or accessibility of chloroquine receptor sites on a constituent of the malaria parasite. This receptor site possibly is the site of action of chloroquine.

Studies of chloroquine clearance in the urine after prophylactic treatment revealed discrepancies between plasma and urine chloroquine concentrations in certain subjects. Preliminary searches for the source of this discrepancy provided evidence that the drug may be discharged in substantial quantity in perspiration.

Eight new compounds of the 10-substituted-benzo-[h]-quinoline-4-methanol type are being synthesized. One of these compounds, the 10-chloro-compound, has been synthesized and submitted for testing in the rodent antimalarial screen and in the screens for phototoxicity.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 108, Study of malaria and antimalarial therapy

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**Principal:** Edward C. Knoblock, COL, MSC  
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Huey G. McDaniel, MAJ, MC  
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**Publications:**

1. Siu, P. M. L., Antimalarial activity of 1-methyl-3-nitro 1-nitroso-guanidine. Proc. Soc. Exp. Biol. Med. 129, 753 (1968).
2. Fitch, C. D., Chloroquine resistance in malaria: A deficiency of chloroquine binding. Clin. Res. 17, 367 (1969).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY	3. REPORT CLASSIFICATION
				DA 0A6521	69 06 30	DD
4. DATE PREVIOUS SUMMARY	5. KIND OF SUMMARY	6. SUMMARY SCY	7. WORK SECURITY	8. RECORDING	9. DISSEM. CATEG.	10. SPECIFIC DATA CONTRACTOR ACC.
69 01 31	H. Termination	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
11. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NO.		
A. PRIMARY	69713A	3A663713D829	00	109		
B. CONTRIBUTING						
C. CONTRACTING	242A(2)					
11. TITLE (Prefix with Security Classification Code)						
(U) Pathophysiology of Malaria and Antimalarial Therapy (09)						
12. SCIENTIFIC AND TECHNOLOGICAL AREA						
002600 Biology						
13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE		
65 07	69 06	DA		C. In-f		
17. CONTRACT/GRANT NA				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS
A. DATE/EFFECTIVE:				FISCAL YEAR		70
B. NUMBER:				CURRENCY		70
C. TYPE:				70		2
D. KIND OF AWARD:				70		2
E. AMOUNT:				70		2
F. CUM. AMT.				70		2
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION		
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21. GENERAL USE				ASSOCIATE INVESTIGATOR		
Foreign intelligence considered				NAME: Herman Polet, M.D.		
				NAME:		
12. REVISIONS (Prefix each with Security Classification Code)						
(U) Malaria; (U) Blood; (U) Coagulation; (U) Tissue Culture						
23. (U) Basic studies of the relationship between the parasite and erythrocytes in malaria.						
24. (U) Utilization of a method to culture malarial parasites in vitro per studies of the metabolic requirements of malarial parasites during their intraerythrocytic life cycle, studies of the growth of parasites within red blood cells, and the effect of both drugs and changes in the erythrocytic milieu upon parasitic growth. Studies suspended because of loss of senior personnel trained in tissue culture techniques without replacement.						
25. (U) 69 01 - 69 06 None. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.						

PII Redacted

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 109, Pathophysiology of malaria and antimalarial therapy

**Investigators**

Principal: COL Marcel E. Conrad, MC

Associate: Herman Polet, M.D. and Charles F. Barr

Description

Studies of host parasite relationships, metabolic requirements and growth of the parasite and the effects of antimalarial drugs upon the host and the parasite.

Progress and Results

A method was developed in this laboratory which permitted the "in vitro" cultivation of malaria parasites throughout their intra-erythrocytic life cycle. This system has been shown to be useful for the "in vitro" cultivation of Plasmodium knowlesi, berghei and falciparum. P. knowlesi has been used for most studies because of the availability of biological materials and the synchrony of the parasitemia. The methodology has been provided to other laboratories for drug screening. Basic investigations of the metabolic requirements of the malaria parasite and the effect of antimalarial drugs upon DNA, RNA and protein synthesis were continued in this laboratory.

Erythrocytic forms of Plasmodium knowlesi were incubated in media depleted of various amino acids. The rate of uptake of eighteen isotopically labeled amino acids into plasmodial protein from the media was compared. These studies showed that parasites were capable of degrading hemoglobin to supply their amino acid requirements. Amino acids either absent or in short supply within hemoglobin must be obtained from extra erythrocytic sources for parasitic growth and development. L-isoleucine is not a constituent of hemoglobin and is essential for the growth of P. knowlesi "in vitro". L-O-methylthreonine, an antagonist of L-isoleucine, markedly inhibits the growth of P. knowlesi in cultures containing normal amounts of isoleucine. Preliminary studies in animals show that L-O-methylthreonine is a potent antimalarial drug.

DNA, RNA and protein synthesis by P. knowlesi were quantified in cultures of parasites throughout their intraerythrocytic schizogonic life cycle. The addition of either radiolabeled orotic acid or adenine to cultures showed a slow rate of incorporation of these radionuclides into DNA during the ring and trophozoite stage of

parasitic growth with exponential utilization of these radioisotopes during the phase of nuclear development and division. RNA synthesis occurred at a rate that seemed intermediate between linear and exponential. Protein synthesis was measured by incorporation of radiolabeled amino acids into infected cell cultures and occurred at a linear rate. This combination of exponential synthesis of DNA and linear protein synthesis seemed characteristic of plasmodial development.<sup>1</sup>

The influences of chloroquine, dihydroquinone and quinacrine on the biosynthesis of DNA, RNA and protein synthesis during the intra-erythrocytic growth of P. knowlesi were investigated.<sup>2</sup> Chloroquine inhibited DNA and RNA synthesis more than it decreased protein synthesis. Dihydroquinone and quinacrine had a greater effect upon DNA than on either RNA or protein biosynthesis. These findings suggested that inhibition of DNA replication is the primary mechanism of action of these antimalarial drugs. Erythrocytes parasitized with plasmodia concentrated much more of these drugs than normal red blood cells. The specific antimalarial effect of chloroquine was shown to decrease with progressive development of the plasmodium during the growth cycle. However, the accumulation of chloroquine within infected erythrocytes was independent of the amount of plasmodial DNA, RNA, protein or malarial pigment. Studies of chloroquine uptake by infected red blood cells showed an initial rapid uptake followed by a slow increase to a saturation value after three hours of incubation. Lower temperatures diminished glucose in cultures and metabolic inhibitors markedly decrease chloroquine uptake by infected red blood cells. Intracellular and extracellular chloroquine seem to be in a dynamic equilibrium in which dihydroquinone and quinacrine can participate. The characteristics of chloroquine uptake by plasmodia resemble the uptake of albumin by mammalian cells. Pinocytosis with subsequent accumulation in lysosomes may be the mechanism by which chloroquine is accumulated by plasmodia. This hypothesis is supported by experiments which showed increased chloroquine accumulation in mammalian cells stimulated by sucrose administration to increase the size of lysosomes and the activity of lysosomal enzymes. Since chloroquine resistant plasmodia contain less chloroquine than drug sensitive parasites, the resistance of malaria parasites to chloroquine may be explained by changes in lysosomal activity. Conversely, agents which stimulate lysosomal activity might enhance the effects of antimalarial drugs even with drug resistant strains of plasmodia.

"In vitro" cultures of red blood cells containing P. knowlesi were performed in media containing radiolabeled glucose, pyruvate and acetate. The radionuclides were incorporated into aspartic acid, glutamic acid and alanine of the plasmodial protein but not into other amino acids. This indicates that the malaria parasite has a similar capability as leucocytes in the conversion of sugars into amino acids but that the plasmodia must obtain most of its amino acids from either the host hemoglobin or the plasma.

### Conclusions and Recommendations

Methods for the "in vitro" cultivation of mammalian malaria in nonnucleated erythrocytes were developed. These methods provided a way to screen drugs for antimalarial activity; to study DNA, RNA and protein synthesis by parasites; to investigate the effects of antimalarial drugs upon nucleoprotein synthesis and to measure the essential requirements of parasites for growth and development during their intraerythrocytic life cycle. These studies showed that lysosomal activity may be important in the development of drug resistance and that L-0-methylthreonine may be a useful drug for treatment of malaria. Further studies of these findings are needed. In addition, basic investigation of the culture system is required to permit continuous culture of parasites throughout their entire life cycle. The latter would permit studies of the effects of drugs during the exoerythrocytic stage of the parasitic life cycle. Employment of a senior investigator with a background in tissue culture methods and protein synthesis is required for continuation of this project.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 109, Pathophysiology of malaria and antimalarial therapy

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1. Polet, H., and Barr, C. F.: DNA, RNA and protein synthesis in erythrocytic forms of Plasmodium knowlesi. Amer. J. Trop. Med. Hyg. 17:672, 1968.
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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL	
				DA 0A6522	69 06 30	DD-RSE (AR) 636	
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A. PRIMARY	63713A	3A663713D829	00	110			
B. CONTRIBUTING							
C. CONTINUOUS	1412A(2)						
11. TITLE (Precede with Security Classification Code) <sup>9</sup>							
(U) Modes of Action of Antimalarials(09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>10</sup>							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
65 07		NA		DA		C. In-House	
17. CONTRACT/GRANT				19. RESOURCES ESTIMATE		20. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: Not Applicable				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER:				FISCAL YEAR		CURRENT	
C. TYPE:				68		6	
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18. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Considered				NAME: Ciak, Jennie M. S.			
				NAME: Wolfe, A. D. S. M.			
				DA			
22. KEYWORDS (Precede with Security Classification Code) <sup>11</sup>							
(U) Molecular Pharmacology; (U) Antimalarials; (U) Chloroquine; (U) Quinacrine; (U) Quinine; (U) Nitroakridin 3528; (U) Ethidium Bromide; (U) Berberine; (U) Naphthoquinones							
23. (U) Elucidation of the primary modes of action of chloroquine, mepacrine, quinine, and other antimalarials at the molecular level. From this- Explanation of the nature of resistance to antimalarial drugs, recognition of the relationships between chemical structures and biological actions of such drugs, development of theoretical principles for the design of improved antimalarial compounds.							
24. (U) Experimental studies at 3 levels of biological organization- 1. Molecular biophysical studies on the interaction of antimalarials with their primary macromolecular sites of action, especially DNA, 2. In vitro biochemical studies on the enzymological consequences (inhibitions) of such interactions, and 3. Biochemical, physiological, and morphological studies of the in vivo manifestations of such inhibitions leading to cytostasis or lethality of microorganisms exposed to antimalarials.							
25. (U) 69 01 - 69 06 Nitroakridin 3528 and ethidium bromide labilize ribosomes to heat and inhibit amino acid polymerizations in cell-free ribosome systems. -Berberine, a mutagen and antiprotozoal alkaloid, stabilizes DNA, cosediments with DNA and is intercalated as proved by flow dichroism. -Intercalative drugs, in decreasing order of potency, quinacrine, ethidium bromide, acridine orange, chloroquine and miracil D, eliminate the F lac episome from E. coli. Episomal DNA disappears as the result of treatment with the drugs. -DNA from plasmodium berghei served as primer for the polymerization of DNA catalyzed by standard E. coli enzyme. Chloroquine inhibited the plasmodial DNA-dependent polymerase reaction to the same extent as reported 1966 for calf thymus DNA. The immediate objectives of this project have been realized. Further work in this area is to be performed in a new work unit. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.							

PII Redacted

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 110, Modes of action of antimalarials

Investigators.

Principal: Fred E. Hahn, Ph.D.

Associate: Jennie Ciak, M.S.; MAJ Richard Estensen, MC; Anne Krey, M.S.; Bruce Mann, B.S.; John Olenick, M.S.; CPT John Sutherland, MSC; Alan David Wolfe, S.M.; Betsy Sutherland, Ph.D., USPHS Post Doctoral Fellow

Description.

Scientific experimental studies in depth on the molecular biology, molecular pharmacology, biochemistry, biophysics, microbial physiology and genetics of the actions of antimicrobial drugs, especially anti-malarials, with a view to elucidating modes and mechanisms of drug action at the primary level, explaining phenomena of drug resistance and offering conceptual guidance to both improved methods of chemotherapy with existing drugs as well as rational development of novel chemotherapeutic substances.

Progress and Results.

1. Mode of Action of Chloroquine. A cell-free DNA-polymerase system was obtained which employs DNA from Plasmodium berghei as a primer and standard DNA-polymerase enzyme from E. coli. The polymerization of building blocks on the plasmodial DNA template was inhibited by chloroquine to the same extent as reported earlier (O'Brien, Olenick & Hahn, PNAS 55, 1511, 1966) for this type reaction with calf thymus DNA as primer. Fluorescent or phosphorescent light emission by DNA-bound chloroquine is produced by energy transfer from DNA to the intercalated drug.<sup>1</sup> This also results in a decreased formation of UV-induced pyrimidine dimers in DNA.

2. Mode of Action of Quinine. Biophysical studies on the binding of quinine to DNA have been completed. From spectrophotometric titrations, an absorption isotherm of quinine to calf-thymus DNA has been obtained which shows: (1) Quinine binds to more than one class (probably two) of sites of DNA; (2) The strong binding by intercalation is characterized by an unusually high value of association constant ( $3.6 \times 10^6 \text{ M}^{-1}$ ) and a small number of binding sites (one molecule of quinine per forty base pairs of DNA); (3) The weaker process, probably representing attraction of the quinine cation to phosphates of DNA, involves binding of one drug molecule per nine DNA base pairs. About twice as much quinine is found to be bound to DNA in equilibrium dialysis experiments.

3. Complex of Berberine with DNA. Berberine, an alkaloid with anti-protozoal and mutagenic activity, has a planar molecule of an area of approximately  $50 \text{ \AA}^2$ ; its chemical structure and biological activity suggests that berberine can form a complex with DNA by intercalation. This was confirmed by the following results: (1) Berberine cosediments with DNA in the ultracentrifuge. (2) DNA alters the absorption spectrum of berberine. (3) Berberine stabilizes DNA to thermal strand separation. (4) Flow dichroism of the berberine DNA complex indicates that the planes of DNA's constituent bases and the plane of the berberine molecule lie parallel in the complex.

4. Studies on Quinacrine and Other Heterocyclic Amines. Quinacrine stabilized DNA as well as transfer RNA to thermal strand separation; this is, in part, a function of the aliphatic diamine side chain of the drug.<sup>2</sup> The absorption spectrum of quinacrine, known to be altered by double-helical DNA, is also altered by single-stranded DNA, by transfer RNA, ribosomes, polyadenylic acid and polyguanylic acid.<sup>2</sup> The double helix of polyadenylic and polyuridylic acids is more active in this respect than can be accounted for by the arithmetic summation of the effects of the two components measured singly.<sup>2</sup> Transfer RNA and single-stranded DNA induce a Cotton effect in the optical rotatory dispersion spectrum of quinacrine.<sup>2</sup> The electronic transition which is induced to become optically active is different from the one responsible for the Cotton effect in quinacrine's ORD spectrum when the drug is bound to double-helical DNA. Quinacrine, Nitroakridin 3528, ethidium bromide and chloroquine bind to ribosomes and labilize ribosomes to thermal degradation in vitro.<sup>3</sup> The extent of this labilization is a function of the dimension of the drugs' ring systems. Inhibition by quinacrine of phenylalanine polymerization in a ribosome-poly U cell-free system was partly reversed by increasing concentrations of ribosomes.<sup>3</sup> The acridines inhibited charging of transfer RNA with phenylalanine; ethidium bromide was especially active in this respect. Chloroquine was only weakly inhibitory. The inhibitions observed appear to be functions of the dimensions of the ring systems of drugs in a similar manner as is the labilization of ribosomes reported above. Transfer of energy from DNA to ligands<sup>4,5</sup> such as proflavine, acridine orange, methyl green and ethidium bromide inhibits the formation of UV-induced pyrimidine dimers in DNA. Both the singlet and the triplet energetic states of DNA are precursors of the pyrimidine dimers.

5. Elimination of Episomes by Drugs Intercalating into DNA.<sup>6</sup> Intercalative drugs (ethidium bromide, actinomycin D) are known to produce drastic configurational changes in circular DNA. We speculated that the known "curing" of bacteria of R-factors and the elimination of mitochondria from yeast cells by certain drugs were based on intercalations into circular episomal or mitochondrial DNA. This was tested by studying the elimination of the F lac episome from E. coli by a series of DNA-complexing drugs. In decreasing order of potency, the following intercalative drugs eliminated the episome: quinacrine, ethidium bromide, acridine orange, chloroquine and miracil D.<sup>6</sup> Quinine showed a trend to eliminate F lac; methylene blue and p-rosaniline (the latter

a non-intercalating compound) were non-active. Elimination of the F lac episome was accompanied by a drastic reduction in the amount of episomal DNA quantitated in equilibrium centrifugations of bacterial extracts. Bacterial growth was not affected at drug concentrations which eliminated episomes.<sup>6</sup>

6. Mode of Action of One Antimalarial Naphthoquinone.<sup>7</sup> Studies on the action of 2-hydroxy-3-cyclohexylpropyl-1,4-naphthoquinone have continued. Bactericidal concentrations of the drug produced only a partial decrease in oxygen consumption of B. megaterium. This effect does not account for growth inhibition and killing of the organism by the drug. Naphthoquinone caused immediate and complete inhibition of RNA and protein biosynthesis in B. megaterium but permitted continued incorporation of <sup>14</sup>C-thymine into DNA for at least 20 min after addition of the drug to exponentially growing cultures. Addition of deoxyadenosine to the experimental medium (known to promote the utilization of thymidine by bacteria) resulted, surprisingly, in a complete inhibition of thymidine incorporation by naphthoquinone. The underlying reason is under study.

7. Studies on Ribosomes<sup>8</sup> and on Chloramphenicol Inhibition of Protein Biosynthesis.<sup>9</sup> Immunological studies concerned with structure and function of ribosomes have been completed.<sup>8</sup> An antiserum reacting with a protein component of 50s E. coli ribosome subunits reacted also with the larger of two incomplete subunits which are formed in bacteria under conditions in which protein synthesis does not occur owing to inhibition by chloramphenicol or amino acid starvation. It is assumed that the known degradation of pre-existing ribosomes during chloramphenicol's action makes ribosome proteins available for redistribution and that newly synthesized 23s RNA has a specific binding site for the antigenic component protein. Cultures of Lactobacillus were supplied with radioactive amino acids and their protein biosynthesis was inhibited by 16 or 480 µg/ml of chloramphenicol. Molecular sieve elution analysis of cell-free extracts of these bacteria showed decreasing quantities of a protein fraction with increasing concentrations of chloramphenicol and the appearance of a second radioactive fraction, possibly dipeptides, accumulating during chloramphenicol inhibition.

#### Conclusions.

Chloroquine inhibits in vitro the plasmodial DNA-dependent DNA polymerase reaction. Quinine binds strongly (by intercalation) to DNA, on the average, to one in 40 base pairs; weak binding is about 10 times more frequent. Berberine complexes with DNA by intercalation. Quinacrine and other heterocyclic amines interfere with protein synthesis in vitro by at least two mechanisms: (1) direct interaction with ribosomes, and (2) inhibition of the formation of amino acyl transfer RNA. Intercalative drugs, foremost quinacrine, eliminate episomes from bacteria without inhibiting bacterial growth. This effect is probably caused by configurational changes produced by intercalation into episomal, circular, DNA. Binding of chloroquine, acridines, ethidium bromide and methyl green to DNA interferes with energy transfer along the double helix and, hence,

inhibits the UV-induced formation of pyrimidine dimers. Studies on ribosomes and on chloramphenicol continue with the long range objective of elucidating the mechanism of action of this antibiotic on protein biosynthesis.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 110, Modes of action of antimalarials

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Work Unit 110, Modes of action of antimalarials

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3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ACTY	6. WORK SECURITY	7. RECLASSIFICATION	8. REPORT NUMBER	9. SPECIFIC DATA - CONTRACTOR ACORN		LEVEL OF DISSEMINATION
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11. CONTRIBUTING								
12. CIRCUMSTANCES	1412A(2)							
13. TITLE (Provide CAS Security Classification Only)								
(U) Fine Structure - Malaria Parasites (09)								
14. SCIENTIFIC AND TECHNOLOGICAL AREA								
002600 Biology								
15. START DATE		16. INTERMEDIATE COMPLETION DATE		17. PERIOD AGENCY		18. PERIOD OF REPORT		
09 64		69 06 30		DA		C. In-Hour		
19. CONTRACT/GRANT								
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not applicable						68		30
E. KIND OF AWARD:				F. CURR. AMT.		69		30
20. RESPONSIBLE DISSEMINATOR				21. PERFORMING ORGANIZATION				
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research				
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RESPONSIBLE INDIVIDUAL: Meroney, Col. W.H.				PRINCIPAL INVESTIGATOR (Provide GRAF # U.S. Armed Forces):				
NAME: Meroney, Col. W.H.				NAME: Sprinz, Col. H.				
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2677				
22. GENERAL USE				23. FEDERAL INVESTIGATOR				
Foreign intelligence not considered				NAME: DA				
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. FINDINGS (Provide individual paragraphs identified by number. Provide list of work unit security classification only)								
(U) Malaria; (U) Plasmodium; (U) Electron Microscopy; (U) Freeze Etching; (U) Chloroquine; (U) Drug Effect; (U) Mode of Action								
23 (U) To define the fine structure of various stages of several malaria parasites during metabolism and as modified by Drug action. This is a continuation of work initiated under DA OA 6492, Code Number 61130011 3A013001A91C 01110.								
24 (U) High resolution electron microscopy, freeze-etching and biophysical-biochemical methods to isolate and identify subcellular components of malarial parasites are employed.								
25 (U) 69 01 - 69 06 The report on the relationship of pigment formation to chloroquine resistance has appeared in Proc. Soc. The paper on the mechanism of merozoite penetration has been published in J. Parasitol. The study of the comparative morphology of malaria parasites was presented before the International Work Shop on Malaria and will be published in the Proceedings of the Work Shop. Results obtained to date by the application of the Freeze-Etching Technique have been reported before the Society of Electron Microscopy and the aforementioned workshop. The immediate objectives for this work unit have been achieved and in view of a shift in the research program of the department, this work unit is to be considered as completed. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.								

PII Redacted

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 - Malaria Investigations

Work Unit 112, Fine Structure - malaria parasites

Investigators:

Principal: Colonel Helmuth Sprinz, MC

Associate: Captain Roger Ladda, MC, Masamichi Aikawa, M.D., Major  
Robert T. Cook, MC

Description

The fine structure of different stages of various plasmodia was studied with the aid of the electron microscope. Natural as well as experimental infections served as source of the parasites.

Progress

1. It had previously been asserted that chloroquine resistance in rodent malaria is coupled with depressed pigment formation. Our study established that pigment formation and chloroquine resistance are independent variables (see bibliography).

2. The mechanism of merozoite entry into a host red cell was elucidated. We noted that the red cell membrane on contact with the conoid of the parasite alters its physical characteristics. In the small zone of contact it becomes exceedingly pliable and bulges inward before the advancing merozoite. The cavity thus formed is sealed secondarily. Our study demonstrates unequivocally that the outer parasite membrane is of red cell origin. The origin of this outer membrane has been a disputed and moot point before. A manuscript entitled: "Penetration of erythrocytes by merozoites of mammalian and avian malarial parasites" by R. Ladda, M. Aikawa and H. Sprinz is in press. (J. Parasit. 55: 12 pps 1969). The study has an added significance in that it clearly establishes the indispensable role of the conoid in the entry of a merozoite into a new host cell. If we succeed in our attempt to isolate and identify the active material from the conoid it could result in an entirely new approach to malaria prophylaxis: a vaccine which might prevent merozoite entry.

3. The Freeze-Etching technique has been applied for the first time to the analysis of the fine structure of malarial parasites. This technique permits a three-dimensional reconstruction of the structural organization of the parasite and of its relation to the host red cell. The results so far obtained were presented before the International Work-

shop on Malaria, held at WRAIR and will be shown at the forthcoming national meeting of the Electronmicroscope Society of America (R. Ladda and R. Steere: Freeze-etching of malarial parasites, Proc. 27th Annual Meeting of EMSA, Aug. 2 pps 1969).

4. The major effort of our group of investigators to provide an inventory of the fine structure of different stages of various malarial parasites, derived from natural and experimental infections, is nearing completion. The papers on the reptilian parasite and the comparative study of gametocytes have been published (see bibliography). The paper on rodent malaria was presented before the International Workshop and will appear in *Milit. Med.* 134 (1969) Sympos. Suppl. A paper entitled: "Fine structure of Erythrocytic Stages of Plasmodium Knowlesi", by M. Aikawa, R.T. Cook and H. Sprinz, has been accepted for publication in *Zeitschrift. Zellforsch. u. mikrosk. Anatomie*. Manuscripts on a) the fine structure of P. vivax infection in the owl monkey, with emphasis on the analysis of Schuffner's dots; and b) the formation of microtubules in the cytoplasm of malarial parasites, are in preparation. The work on P. falciparum infection in the chimpanzee has remained incomplete. Dr. Aikawa in his new position at Case Western Reserve University will complete the work he started here on the fine structure of plasmodium - related parasites.

5. The growing knowledge of ultrastructural details permitted a reorientation of emphasis on the study of drug effects on fine structure and parasite development. This aspect of our program will be increasingly performed by three of our graduates in their new civilian locations (Tezakakis [New York]; Aikawa and Cook [Cleveland]) who will report on it independently. While still with us, Aikawa completed a study on chloroquine effect on the erythrocytic stages of P. gallinaceum, 2 papers in collaboration with R. Beaudoin (see bibliography) and prepared the report entitled "Morphologic effects of 8-Aminoquinolines of the Exorythrocytic Stages of Plasmodium Fallax" which he presented at the International Workshop on Malaria.

6. In our work we place increasing emphasis on the correlation of biochemical with biophysical events. In preparation is a paper by Ladda and Sprinz on the morphological effects of chloroquine on human fibroblasts in tissue culture with a study of DNA, RNA and protein synthesis. In collaboration with Capt. Charles Meszoely, M.S.C., the morphologic effects of methylene blue and chloroquine analogues are being studied in rodent malaria by light and electron microscopy. This is an extension of work by Barnes (The Influence of Methylene Blue on the Pentose Phosphate Pathway in Erythrocytes of Monkeys Infected with Plasmodium Knowlesi, *J. of Lab & Clin. Med.*, in press).

7. In continuation of our long standing interest in parasite development in the mosquito a cooperative project with the Department of Entomology has been started on the histochemistry of the mosquito gut (Anopheles stephensi) during digestion and sporogony. This study involves initially the developing of techniques to section fresh frozen mosquito gut.

#### Summary and Conclusion

During the period reported on we were able to maintain work leadership in the field of malarial fine structure. Dr. Aikawa and his former associates at WRAIR were honored by his appointment as Chairman of the session on blood parasites at the International Congress of Protozoology in Leningrad. Due to losses of personnel and shift of research interest the work unit "Fine Structure - Malaria Parasites" is being discontinued. Work in Progress will be taken up under the work unit "Experimental Pathology and Metabolism of Plasmodia."

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(1 April 1968 through 30 June 1969)

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 - Malaria Investigations

Work Unit 112, Fine Structure - malaria parasites

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OA 6536	69 07 01	DD-138 (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>b</sup>	6. WORK SECURITY <sup>b</sup>	7. REGRADING <sup>c</sup>	8. ORG'S INSTN <sup>d</sup>	9. SPECIFIC DATA CONTRACTOR ACCESS	
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12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>g</sup>							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
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20. RESPONSIBLE DDO ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: <sup>h</sup> Walter Reed Army Institute of Research				NAME: <sup>h</sup> Walter Reed Army Institute of Research			
ADDRESS: <sup>h</sup> Washington, DC 20012				ADDRESS: <sup>h</sup> USA Med Comp, SEATO Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic institution)			
NAME: Meroney, COL W. H.				NAME: <sup>i</sup> Gould, D. J.			
TELEPHONE: 202-576-3551				TELEPHONE: NA			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Malaria; (U) Mosquitoes; (U) Primates; (U) Chloroquine							
23. TECHNICAL OBJECTIVE, <sup>j</sup> 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) To conduct field and laboratory studies on malaria with primary interest in chloroquine refractory Plasmodium falciparum.							
24 (U) A balanced laboratory staff is maintained in Bangkok augmented, as necessary, by TDY personnel.							
24 (U) 68 10 - 69 04 Detailed information on the research performed under this work unit is being assembled for publication in the Annual Progress Report, USA Med Comp SEATO, Bangkok, Thailand, which is not yet available.							

<sup>a</sup> Available to contractors upon originator's approval.

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RESEARCH AND TRAINING BY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
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12. SCIENTIFIC AND TECHNOLOGICAL AREAS							
012100 Organic Chemistry							
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19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research ADDRESS: Washington, D. C. 20012				NAME: Walter Reed Army Institute of Research ADDRESS: Division of Medicinal Chemistry Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Meroney, COL W. H. TELEPHONE: 202/576-3551				NAME: Jacobus, Dr. D. P. TELEPHONE: 202/576-2280 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Rothe, COL W. E. NAME: DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U)Malaria; (U)Drugs; (U)Biology; (U)Chemistry							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To manage, to integrate, and provide quality control for the Drug Research Program on Malaria, both in-house and by contract.							
24. (U) To define areas requiring investigation, to develop suitable contract proposals, to follow progress by correspondence or site visits, to guide direction of investigation, to provide for exchange of information, and to continually check findings for verification through independent agencies (both in-house and contract). Two outside advisory groups are utilized.							
25. (U) 69 02 - 69 06 Two IND basic documents and seven supplements were submitted for review. Preclinical workups were instigated on two new IND basic documents with seven supplements in preparation. Four two-day conferences of Test System and Synthesis contractors were organized and conducted. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

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Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 114, Malaria program supervision

Investigators

Principal: David P. Jacobus, M.D.

Associate: Thomas R. Sweeney, Ph.D.

1. The Chemical Synthesis Program

As of the end of FY-69 there were 48 active synthesis contracts, 5 preparations laboratories contracts, 1 contract for the radioactive labeling of compounds and 1 for chemical analytical work. During the year 9 new contracts were let and 29 contracts terminated. Of the 48 contracts presently active, 27 are with academic institutions, 11 with research houses and 10 with industry.

During FY-69 there was a total of 2109 compounds submitted from the synthesis programs and about two-thirds of these were target compounds. As of the end of FY-69 the overall cost per target compound was \$2352. Chemical publications and submitted manuscripts numbered 68.

Synthesis emphasis during the year has coalesced into three main areas: the phenanthrene methanols, the antifols, and the polyhalogen type compounds. One new area opened up in the benzothiapyrans with the finding of activity in this system. The large 4-quinoline methanol area has been de-emphasized. Most other areas not included in the three main ones mentioned above have been or are being phased out.

Some highlights in the synthesis program include

a. The successful application of the Krohnke pyridine synthesis to the preparation of 2,6-diphenylpyridine methanols.

b. The application of photochemical methods to the synthesis of some azaphenanthrenes not easily obtainable via other approaches.

c. New routes to the introduction of the aminoalcohol sidechain in the diphenylpyridine, quinoline and phenanthrene systems via the carboxaldehyde which is formed from the reaction of the aryl lithium with DMF and the conversion of the aldehyde to the epoxide using dimethylsulfonium methylide.

## 2. Preparations Laboratories

There were a total of 42 compounds requested from the preparations laboratories during FY-69. Of this number, 15 were requested in large (1 kilogram or greater) quantities, 17 in intermediate (100-500 g.) quantities and 10 in small (10 g.) quantities. During the year 45 compounds were received in the following quantities - 21 small, 17 medium and 7 large. Some of these compounds were in the pipeline from the previous year's request.

The synthesis of the 9-phenanthrene methanols by present Pschor approaches involves on the order of a dozen synthetic steps with a very low overall yield. With the best compounds, which contain trifluoromethyl substituents, the starting materials are rather ~~are~~ chemicals which are quite expensive even when synthesized in small quantities by relatively convenient and well known laboratory procedures. Thus, to obtain large quantities of the target compounds by present methods would be exceedingly expensive and other synthetic routes are being investigated. One shortcut to the ring system would involve a photo ring closure. This has been investigated to some extent but not extensively. It has the drawback that the reaction as carried out is a high dilution one and unless volumes could be reduced, very difficult engineering problems would be anticipated. A totally new approach involving a readily available starting product is now under study. This necessitates one step with sulfur tetrafluoride which involves formidable engineering and chemical problems which would have to be worked out but which seem soluble. A feasibility study is about to be made on the sulfur tetrafluoride reaction. Good progress on the other steps in this route is being made. Finally, there is the possibility of a pyrolytic approach to the substituted phenanthrene system which will be looked into. Thus, it appears that there is a good chance of eventually avoiding the cumbersome Pschor synthesis. Development of one of these new approaches would be of outstanding significance in pilot plant or commercial production of this type of compound.

## 3. Acquisition of Chemicals

In addition to the synthesized compounds, new chemicals continue to be received as gifts or purchased, and from the No Dollar contractors. Out of a total of about 40,000 compounds received, approximately 23,000 were submitted under the No Dollar Agreement. The rate of acquisition of new agreements fell off noticeably during the year. This decrease reflects the loss of a full time high level professional to promote these contracts. The bottling team collected about 17,000 compounds during the year.

Special chemical structure files have been created for USDA, PCRB Beltsville; CCNSC, NIH; Fort Detrick and Edgewood Arsenal in order to facilitate the trading of compounds for screening. This activity has been very useful for all parties concerned.

#### 4. Communications

Three technical conferences were held at WRAIR for the contractors working in the aminoalcohol and antifolic acid areas, as well as test systems contractors. The purpose of this was to afford the contractors first hand knowledge of each other's work and to exchange technical information through discussion. Equally important, the conferences provided a forum for the exposition of new ideas with respect to types of compounds that might be useful in enhancing anti-malarial activity or decreasing undesirable side effects.

#### 5. Pharmacology and Clinical Program

During the past year the Pharmacology Department supervised the contractors responsible for their pre-clinical studies of the anti-malarial drugs scheduled for clinical trial, the contracts responsible for the metabolism of these agents, and provided the first line of contact with the three clinical centers involved in Phase I and Phase II drug trials. There are 16 primary contracts concerned with the preparation of safety information; 14 primarily concerned with the metabolism and toxicology of the candidate compounds, one with the detailed formulation and one responsible for an independent check on the purity and components of both the raw drug and the formulated final product. One additional contract for the conduct of Phase I trials was added.

During the past year the Pharmacology Department prepared six Investigational New Drug Applications as well as 15 Supplements. Details on each of these drugs are reported in the respective INDs.

In the last 12 months the methodology for analysis of the various drugs in early clinical trials has been expanded to include radio-metabolism studies on absorption, distribution, excretion and metabolic patterns on these drugs in man. The program has been modified so that chemical determinations are expected to be available in addition to the radioisotope techniques. The test system for the evaluation of drugs for phototoxicity was improved. As a result of this increased sensitivity, the phototoxic action of quinine in both mice and swine was unequivocally demonstrated if the drug was given on a repetitive basis. In view of the close

relationship of quinine to the other methanols which have been synthesized in the program, this phototoxic response was expected. Tetracycline in these same systems is also phototoxic. A number of methanol compounds have been developed which retain the high activity of the 2-phenylquinolinemethanols but which lack the phototoxicity shown by other members of this latter group. The best compounds so far were the 9-phenanthrenemethanols. One of these compounds is already in the clinic, and another one is scheduled. The 2,6-diphenylpyridine also lacks phototoxicity and possesses considerable promise. Emphasis was given to the phenanthrenes because this group is the more effective. During the forthcoming year emphasis will be placed upon blocking the metabolic attack on these materials in order to provide an even longer action than that shown by the 3,6-trifluormethyl-9-alpha-piperidyl phenanthrenemethanol.

New test systems have also developed which use human Plasmodia on an almost routine basis. At the University of Chicago a very simple scheme, involving the maintenance of trophozoites in vitro for 24 hours by the simple addition of glucose, has permitted the evaluation of candidate drugs prior to conducting a pre-clinical workup. In this system chloroquine and quinine show markedly different responses in the chloroquine- and quinine-resistant drug strains of falciparum. Likewise, folic acid antagonists show a similar cross resistance if normal and sensitive strains are used for comparison. A parallel system has been developed in which the facilities at Insect Control and Research Company, which are close to the University of Maryland, are used for evaluation of drugs in the sporogenous cycle using both human normal and resistant falciparum strains. Fortunately, the folic acid machinery remains intact throughout the sporogenous cycle so that cross resistance is easily discovered, and agents may be selected for clinical development which have a reasonable chance of success. Lastly, the Actus system has also developed the capability of screening compounds using human normal and resistant falciparum strains. This system is essentially blood induced and so far correlates with clinical results.

Detailed reports from the three clinical centers will provide the specific information on the past year's activity. The major contribution from the University of Chicago during the past year was a continued demonstration of the gametocidal and sporontocidal effect of primaquine administered on a weekly basis. The drug-resistant strains respond as well as the old drug-sensitive strains. The in vitro test was used to survey

an area around Cuiaba in Brazil. In this survey only chloroquine was assayed. Cuiaba is on a dividing line between tributaries of the river Paraguay and the Amazon. All patients from both valleys having circulating falciparum trophozoites had chloroquine resistance either equal to or greater than that manifest by the Camp strain. The in vitro procedure has the advantage of assaying resistance to more than one drug at one time and furthermore determining drug resistance without the complicating factor of the patient immunity which is manifest in the WHO test. The naphthoquinone in the current formulation had local irritant effect upon the gastrointestinal tract which precluded its administration at doses expected to be effective. Those doses which were administered were poorly absorbed. The major side effect expected from the agent, namely prolongation of clotting time, was not observed. The drug had no action on trophozoites, but did have a sporontocidal action. The chemical therefore remains of interest because, when administered systemically, it is now known to affect all phases of the Plasmodia life cycle.

The University of Missouri has continued to study the combination of Kelfizina and Trimethoprim. Chesson vivax remains appreciably more resistant to this combination than the Camp falciparum strain; however, a dose schedule effective in controlling this disease was worked out. The responsiveness of the Camp strain in man was confirmed at the University of California National Primate Center contract using Camp in the Aotus. Nitroguanyl was also taken through Phase I and Phase II studies primarily for investigation of cross resistance. It was sufficiently non-effective that it was not useful even for this limited purpose. Phase I and Phase II studies are underway at the present time on WR-4809, a 7-chloro-4-aminoquinoline which has shown activity in the chloroquine resistant preclinical studies. A commercially discreet compound was also evaluated through Phase I at the University of Missouri. Phase II studies are continuing. The compound appears to be active against both normal Uganda I and chloroquine-resistant Camp strains. At the present time the compound appears to be too rapidly excreted to constitute a single agent in its own right. It does, however, constitute a lead.

The University of Maryland has been concerned with tolerance studies on the diformyldiaminodiphenylsulfone alone, in combination with chloroquine, and in combination with chloroquine-primaquine. At the present time the drug shows a marked potential to produce methemoglobin when co-administered with primaquine. However, it is markedly more effective when given in combination with chloroquine-primaquine than chloroquine-primaquine alone. During the

last year a number of new isolates were also made; the most studied isolates have been the Taylor and Pooley strains from the Kota Tinggi area near Singapore. These strains have shown much more chloroquine resistance than previous isolates and tend to confirm the expanding spread geographically and the increasing magnitude of resistance. During the last year Phase I studies on WR-5677 were conducted by Dr. John Colmore at the University of Oklahoma. The drug is now ready for clinical evaluation. Dr. Thomas Maren has been examining the metabolism of diformyldiaminodiphenylsulfone; specifically, the formyl groups seem to be rapidly excreted through respiration. At the present time there appear to be a number of metabolites which are thought to be comparable to those from DDS.

The clinical treatment of malaria in the field continues to be quinine, pyrimethamine and DDS, the quinine being continued for a period of 10 days, pyrimethamine 50 mgs daily for three days, and DDS throughout the entire hospital stay. Last year it was reported that there were 13,447 cases of malaria with 13 fatalities which were assigned to malaria.

#### 6. Summary and Conclusions

The Malarial Program has in a sense progressed into its third phase. The first phase involved the scale-up in synthesis of known anti-malarial compounds and their evaluation against the new drug-resistant strains which were also in the process of being characterized. The second phase involved the derivatization of neglected leads from World War II. The third phase is the development in the supporting research and screening components of the program and involves the use of human Plasmodia for the selection of agents to be developed either by chemical derivatization or through preclinical study.

We consider the first phase to have been successful by virtue of the combination of DDS with our standard chloroquine-primaquine prophylaxis. The second phase has primarily involved the synthetic development of methanol derivatives and the development of a number of folic acid antagonists, the most important of which are commercially discreet. The antimalarial power of certain of these agents suggests that the second phase will also be successful and hopefully will provide us with new drugs. Both major classes of compounds are, however, related to the old leads; the methanols in that they have a side chain, and the folic acid antagonists to known agents. While these compounds and a number of others, both open and commercially discreet, are effective

against the resistant strains in which they have been tested, they are the same general classes to which resistance has developed. There are from the screening program structurally new classes of materials which are proceeding toward clinical evaluation. The initiation of the third phase, however, will permit the selection for appropriate chemical development of new structure leads which do not show cross resistance against the human strains of interest - a goal long sought in malarial chemotherapy.

Drug resistant malaria continues to be found in new areas, in old areas with greater density, and often with an enhancement in resistance. We continue to believe that a variety of antimalarial drugs will have to be developed to handle the disease which is now widespread in South America and Southeast Asia.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 114, Malaria program supervision

7. Publications - None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL	
				DA 0A6512	69 06 30	DD-R&E (AR) 634	
3. DATE PREP SUBMIT <sup>3</sup>	4. KIND OF SUMMARY	5. SUMMARY CTRY <sup>4</sup>	6. WORK SECURITY <sup>5</sup>	7. ABSTRACT <sup>6</sup>	8A. EMPH DISTN <sup>7</sup>	8B. SPECIFIC DATA - CONTRACTOR ACCESS <sup>8</sup>	9. LEVEL OF SUN A. WORK UNIT
60 01 31	K. COMPL.	U	U	NA	NL	<input type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES <sup>9</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	63713A	3A663713D829		00	122		
b. CONTINUING							
c. CONTINUING	CDOG 1412A(2)						
11. TITLE (Provide with Security Classification Code)							
(U) Field Studies on Control of Mosquitoes of Southeast Asia (TH)							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>10</sup>							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
65 07		CONT		DA		C. In-House	
17. CONTRACT/ORDER				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATE/EFFECTIVE NA				PREFERRED			
b. NUMBER <sup>11</sup>				FISCAL YEAR		b. FUNDS (in thousands)	
c. TYPE				68		2	
d. KIND OF AWARD				69		70	
e. CUM. AMT.						70	
20. RESPONSIBLE ORG ORIGINATOR				21. PERFORMING ORGANIZATION			
NAME <sup>12</sup> Walter Reed Army Institute of Research Washington, D.C. 20012				NAME <sup>13</sup> Walter Reed Army Institute of Research Div of CD and I ADDRESS Washington, D.C. 20012			
RESPONSIBLE PERSONAL Meroney, COL W. H. NAME: 202 - 576-3551 TELEPHONE:				PRINCIPAL INVESTIGATOR (Provide NAME if U.S. Army only) NAME <sup>14</sup> Scanlon, LTC J.E. TELEPHONE: 202-576-3719 SOCIAL SECURITY ACCOUNT NUMBER:			
22. GENERAL USE				ASSOCIATE INVESTIGATOR NAME: Sullivan, CPT M.F. NAME:			
Mosquito Control				U Aedes			
23. TECHNICAL SUBJECTS (List the terms, including U.S. Army symbols, used in the title)							
(U) Anopheles, (U) Deet, (U) Insecticides, (U) Insect repellents (U) Mosquito Control, /							
24. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Provide individual paragraphs identified by number. Provide text of each with Security Classification Code.)							
23. (U) To determine the effectiveness of various standard and newly developed insecticides and repellents against mosquitoes and other arthropods in nature, under field conditions in Southeast Asia, with particular emphasis on Anopheles and other vector species.							
24. (U) Materials to be tested are selected by the United States Department of Agriculture Laboratory in Gainesville, Florida, assigned by law to develop military insecticides and repellents. Materials selected for field trial are those which show promise in screening tests, or standard materials being tested against important vector species. Tests are conducted in conjunction with military units in Southeast Asia. Insecticides are applied as fogs and dusts, and in various formulations against larvae in the laboratory and field. Repellents are tested by skin and clothing application using local volunteers.							
25. (U) 69 01 - 69 06. Treatment of larval breeding sites with Abate insecticide effectively controlled Aedes aegypti on Koh Samui Island, Island for a period of at least 12 weeks. Dengue transmission was virtually stopped and no apparent new transmissions were produced by Aedes albopictus mosquitoes which were not affected by the treatment. This project has been terminated as the technical objectives have been fulfilled							

DD FORM 1498-1 (FOR ARMY USE)

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 122, Field Studies on control of mosquitoes of Southeast Asia

Investigators

Principal: LTC J. E. Scanlon, MSC\*

Associate: Dr. G. A. Mount\*\* and Dr. D. J. Gould\*\*\*

Description

Materials for field study are selected jointly with the Laboratory of Insects Affecting Man and Animals, United States Department of Agriculture, Gainesville, Florida. The testing is accomplished chiefly in Thailand, in cooperation with the US Army Medical Component-SEATO, and Thai military and civilian health authorities. Insecticides are applied as larvicides, and adulticides and observations are made on the effectiveness of repellents on skin, clothing and other materials.

Progress and Conclusions and Recommendations

Refer to 1968 SEATO Medical Research Laboratory Annual Progress Report.

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\* Until 1 March 1969

\*\* Insects Affecting Man and Animals Laboratory, U.S. Department of Agriculture, Gainesville, Florida

\*\*\* US Component, SEATO Medico Laboratory

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 122 Field studies on control of mosquitoes of Southeast Asia

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	3. POINT CONTROL SYMBOL	
				DA OA 6513	69 07 01	DD-R&E (AR) 636	
4. DATE PREV. SUMRY	5. KIND OF SUMMARY	6. SUMMARY SCTY <sup>b</sup>	7. WORK SECURITY <sup>b</sup>	8. REGRADING <sup>c</sup>	9. DIS'N INSTR <sup>d</sup>	10. SPECIFIC DATA CONTRACTOR ACCESS	
69 01 31	D. Change	U	U	NA	NL	<input type="checkbox"/> YE: <input type="checkbox"/> JO	
11. NO./CODES <sup>e</sup>		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY		63713A	3A663713D82c	00	123		
b. CONTRIBUTING							
c. COVER NUMBER		1412A(2)					
11. TITLE (Precede with Security Classification Code) <sup>f</sup>							
(U) Colonization of Anopheline Vectors (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>g</sup>							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
07 65		NA		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCE ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATE/EFFECTIVE: Not Applicable				PRECEDING			
b. NUMBER <sup>h</sup>				FISCAL YEAR		b. FUNDS (in \$thous <sup>h</sup> )	
c. TYPE:				69		1 35	
d. KIND OF AWARD:				70		1 35	
e. AMOUNT:							
f. CUM. AMT.							
20. RESPONSIBLE DOC ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME <sup>i</sup> Walter Reed Army Institute of Research Washington, D.C. 20012				NAME <sup>i</sup> Walter Reed Army Institute of Research Div of CD and I ADDRESS <sup>j</sup> Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Ac. & do not include)			
NAME: Meroney, COL, W. H.				NAME <sup>k</sup> Ward, Dr. R. A.			
TELEPHONE: 202 - 576-3551				TELEPHONE: 202 - 576-3719			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
22. GENERAL USE				ASSOCIATE INVESTIGATORS Peach, CPT M. J.			
Foreign Intelligence Considered				NAME: NAME: DA			
23. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Anopheles; (N) Balabacensis; (U) Colonize; (U) Mosquitoes; (U) Vectors							
23. TECHNICAL OBJECTIVE, <sup>l</sup> 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Establishment of laboratory colonies of anopheline malaria vectors. These colonies will be used for studies of comparative susceptibility to primate and other malaras, genetic and physiologic studies, and as a source of malarial for other in-house and contract research programs.							
24. (U) Mosquitoes are acquired through correspondence with independent investigators or through field collections by Walter Reed Army Institute of Research or SEATO Medical Research Laboratory Personnel. Colonization trials are conducted at Washington, D. C., and optimal rearing procedures are developed for each species colonized. Biologic experiments are conducted.							
25. (U) 69 01 - 69 06. New anopheline colonies were started to replace those destroyed due to a microsporidian infection. Current weekly mosquito production averages 65,000 adults of Anopheles stephensi (3 strains), A. quadrimaculatus, A. balabacensis, Culex gelidus and C. tritaeniorhynchus. Isolations of bacteria from mosquito midguts indicates presence of bacterial succession following digestion of blood meal, with serratia mercescens dominating. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

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Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 123, Colonization of anopheline vectors

Investigators

Principal: R. A. Ward, Ph.D.

Associate: CPT M. J. Peach, MSC

Description

Mosquito colonies are established and maintained for malaria and arbovirus transmission studies, biological investigations on mosquitoes, chemosterilant tests, development of new rearing techniques and as a source of material for other in-house and contract research programs.

Progress

During November 1968 it was observed that excessive larval and adult mortality was present in all the anopheline colonies. Investigations indicated that a microsporidian pathogen, Nosema stegomyiae, was the responsible agent. This protozoan parasite develops within the fat body of larval and adult mosquitoes and is transmitted through contamination of larval habitats by spores. Subsequent review of colony introductions revealed that the parasite was inadvertently introduced with new mosquito strains acquired the previous January or April.

Due to the high level of infection and inability to destroy the pathogen through surface sterilization of contaminated anopheline eggs it was decided to destroy all Anopheles colonies and acquire new strains. All rearing equipment was either discarded or sterilized. Humidity controls were turned off and the temperature raised to 35°C in the insectaries for one week to kill microsporidian spores. As a further precaution both rearing rooms were disinfected with a 25 per cent sodium hypochloride solution and repainted.

At present the following species and geographic isolates of mosquitoes are being reared:

Anopheles stephensi (India, Iraq and Iran)

Anopheles quadrimaculatus (U.S.)

Anopheles balabacensis (Thailand)

Culex gelidus (Malaya)

Culex tritaeniorhynchus (Japan)

Approximately 65,000 adult mosquitoes are produced weekly.

In order to reduce the possibility of the transfer of mosquito pathogens through contaminated mosquito cages several modified mosquito

cages have been constructed with a plastic framework instead of the previously used plywood. These appear satisfactory in terms of fabrication and are presently being evaluated.

The bacterial flora of adult female Anopheles stephensi (India) was identified with the assistance of Miss Sylvia Carey (Dept. of Bacterial Diseases) in order to begin a study on the role of these organisms in mosquito nutrition and host susceptibility. During each of seven time periods list in Table 1, the midguts of six female mosquitoes were removed by sterile dissection and transferred to small vials containing one ml of sterile heart infusion broth. The guts were crushed and inoculated onto the following media: Casman's blood agar, Chocolate brain heart infusion (BBL) agar and thioglycollate broth. Before the mosquitoes were given their initial blood meal, 10 ml of heart blood was obtained from the rabbit blood source of mosquito feeds and subcultured as a control. All controls were negative. The results of the isolations are summarized in Table 1.

#### Conclusions and Recommendations

A microsporidian pathogen, Nosema stegomyiae, was eliminated from the anopheline colonies through sterilization of all rearing equipment and discarding all infected colonies. The possibility of reinfection from new colonies is still strong as the department does not have isolation facilities available for the observation of newly colonized material. Current mosquito production consists of 65,000 adults weekly of three species of Anopheles and two species of Culex. Colonies of additional anophelines and Aedes aegypti will be started in the near future.

In newly emerged adults, bacteria were isolated from only 2 of 6 midguts, suggesting that the isolations found in two guts were contaminants. Bacteria isolated from midguts four hours following a sugar meal or shortly after a blood meal were identical. 24 hours after a blood meal, the bacterial flora began to change with Serratia marcescens appearing and remaining the predominant species. 96 hours later, all bacteria previously present had almost exclusively been replaced by Serratia. These preliminary observations indicate that there may be an ecological succession of bacteria within the midgut of female mosquitoes.

TABLE 1

Bacteria Isolated from Midguts of Anopheles stephensi females  
at various times before and after feeding\*

	No growth after 7 days	Corynebacterium	Klebsiella pneumoniae	Enterobacter liquefaciens	Pseudomonas aeruginosa	Serratia marcescens	Enterobacter cloacae	Enterobacter aerogenes	Pseudomonas sp.
Newly emerged (Unfed)	4	1	1	0	0	0	0	0	0
4 Hours After Sugar Meal	0	0	0	5	2	0	0	0	0
$\frac{1}{2}$ Hour After Blood Meal	0	0	0	5	2	0	0	0	0
24 Hours After Blood Meal	0	0	0	0	0	5	4	0	0
48 Hours After Blood Meal	0	0	0	0	0	5	2	1	2
72 Hours After Blood Meal	0	0	0	1	0	6	1	1	0
96 Hours After Blood Meal	0	0	0	0	0	5	0	0	1

\*Number of females infected with given pathogen out of sample of 6.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 123 Colonization of anopheline vectors

Publications

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTYCL SYMBOL	
				DA OA6514	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCYV <sup>3</sup>	6. WORK SECURITY <sup>4</sup>	7. REGARDING <sup>5</sup>	8. DISB <sup>6</sup> INSTR <sup>7</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS	
69 01 31	D. Change	U	U	NA	NL	<input type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES <sup>8</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	63713A	3A663713D829	00	124			
b. CONTRIBUTING							
c. <del>SECRETARY</del>	1412A(2)						
11. TITLE (Provide with Security Classification Code) <sup>9</sup>							
(U) Genetic Aspects of Sporogony in Mosquitoes (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>10</sup>							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
07 65		NA		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
Not Applicable				PREP/CONS		FUND\$ (in thousands)	
a. DATE EFFECTIVE:		EXPIRATION:		FISCAL YEAR		CURRENT	
				69		35	
b. NUMBER <sup>11</sup>		c. AMOUNT:		70		35	
d. TYPE:		f. CUM. AMT.		1			
e. KIND OF AWARD:							
20. RESPONSIBLE FOR ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME <sup>12</sup> Walter Reed Army Institute of Research Washington, D.C. 20012				NAME <sup>13</sup> Walter Reed Army Institute of Research			
ADDRESS <sup>14</sup>				ADDRESS <sup>15</sup> Div of CD and I Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide RCAN H.U.S. A-codes if available)			
NAME <sup>16</sup> Meroney, COL W. H.				NAME <sup>17</sup> Ward, Dr. R. A.			
TELEPHONE <sup>18</sup> 202 - 576-3551				TELEPHONE <sup>19</sup> 202 - 576-3719			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Considered				ASSOCIATE INVESTIGATORS			
				NAME <sup>20</sup> Rutledge, CPT L.C. DA			
				NAME:			
23. (U) Basic studies are conducted on the infectivity of human and simian malarial parasites to various mosquito vectors of malaria. Special emphasis is placed on the role of genetic and environmental factors in mosquito susceptibility. Mosquito transmission of falciparum and vivax malaria to lower primates is attempted. The results of these studies are applied to the development of test systems for the evaluation of antimalarial drugs.							
24. (U) Matched feedings of anophelines on gametocytic hosts are conducted, followed by dissection of samples of the mosquitoes at intervals thereafter to determine the level and progress of the infection. Mass selection techniques are being employed to identify a species/strain of Anopheles suitable for further study of the genetic basis of the susceptibility of mosquitoes to malaria.							
25. (U) 69 01 - 69 06. Aotus monkeys have been successfully infected with the Chesson strain of vivax malaria by both inoculation of infected blood from man and monkeys and by the bite of infected anopheline mosquitoes. The complete sporogonous cycle of parasite development has occurred in mosquitoes and the second mosquito to primate to mosquito cycle has been observed. The susceptibility of anopheline mosquitoes to simian malaria is partially controlled by genetic factors and this susceptibility in a laboratory population can be reduced by genetic selection procedures. It is therefore possible in principle to produce a genetically resistant pure line. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

PII Redacted

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 124, Genetic aspects of sporogony in mosquitoes

Investigators

Principal: R. A. Ward, Ph.D.

Associate: U. C. Rutledge, MSC and Mr. D. E. Hayes

Description

Basic studies are conducted on the infectivity of human and simian malarial parasites to various mosquito vectors of malaria. Special emphasis is placed on the role of genetic and environmental factors in mosquito susceptibility. Mosquito transmission of falciparum and vivax malaria to lower primates is attempted. The results of these studies are applied to the development of test systems for the evaluation of antimalarial drugs.

Progress

1. Transmission of human malaria to monkeys

Previous attempts to infect mosquitoes with blood induced infections of Plasmodium falciparum (Camp strain) in night monkeys, Actus trivirgatus, have all been negative. This failure to infect mosquitoes has been attributed to the loss of gametocyte infectivity of the Camp strain through numerous blood passages in chimpanzees and night monkeys. To eliminate this failure, future experiments were designed to utilize strains of parasites which had a recent history of mosquito passage in human volunteers or direct sporozoite infections from mosquitoes.

In January 1969 six splenectomized Actus were inoculated with fresh isolates of the Taylor and Poole strains of P. falciparum from Malaya. Infected blood was collected from volunteers at the Maryland House of Correction and each monkey was inoculated with  $3 \times 10^6$  -  $3.2 \times 10^7$  parasites intravenously and intraperitoneally. Less than three hours elapsed between withdrawal of the blood and animal inoculation. Three monkeys were inoculated with each strain. At the end of one month, 3 monkeys in each group had died from non-malarial causes. The two survivors showed no evidence of malarial parasitemia up to four months after inoculation. Two additional night monkeys were bitten by 25 heavily infected Anopheles stephensi mosquitoes (average sporozoite count  $10^8$  sporozoites/mosquito) during May 1969 which had been previously infected with the Poole strain. Both monkeys died before completion of the exo-erythrocytic cycle.

Experiments have been started with the Chesson strain of P. vivax to establish a mosquito - monkey - mosquito cycle of this parasite. This parasite is especially interesting because it is the standard strain of vivax malaria which is used to assess the efficacy of candidate antimalarial

compounds in human volunteers in this country. This strain has been mosquito passed in man for over 25 years and a large volume of literature is available on the biology and response of this strain to chemotherapeutic treatment. If this vivax strain can consistently be cyclically transmitted in the Aotus system it should be an invaluable aid for the analysis of sporonticidal drugs.

Initially three splenectomized monkeys, #388, 395 and 396, were inoculated with parasitized blood of Chesson vivax malaria. The infection became patent between 2 - 15 days after inoculation (Table 1). Mosquitoes (Anopheles stephensi and A. quadrimaculatus) were fed on monkeys when gametocytes were observed. All pools of Anopheles which were fed three weeks after the monkeys were infected showed oocyst development. Although the oocyst counts were low, usually ranging between 1 - 5 per infected mosquito, they were normal in size and resembled P. vivax oocysts from human feeds. Two weeks after infection sporozoites were present in the salivary glands of the mosquitoes. Infected anophelines (ex Aotus #388) were fed on four splenectomized night monkeys and one chimpanzee (#7) to determine the susceptibility of both host species to sporozoites after passage through a heterologous host system. The chimpanzee was used in lieu of a human volunteer due to possible transfer of uncharacterized agents of Aotus monkeys which might be transmitted through anopheline mosquitoes. With the exception of two monkeys which died before completion of a possible exo-erythrocytic cycle, all exposed animals became patent after a 12 - 24 day period. An additional transmission cycle was produced by feeding infected mosquitoes from chimpanzee #7 upon Aotus #67 which became patent 21 days after infection. Abundant gametocytes appeared on day 23 and mosquito infection trials are in progress.

During the initial blood passage from the human volunteer to night monkeys, 11/46 or 24% of mosquito lots applied to the monkeys became infected. On a second monkey to monkey blood passage 12/21 or 57% of the mosquito lots were infected. When mosquitoes were fed on monkeys or the chimpanzee infected by mosquito bite from an infected monkey, 20/21 or 95% of the lots contained infected mosquitoes.

The present observations indicate that a mosquito passaged cycle of Chesson vivax malaria can probably be established without difficulty in the Aotus system.

## 2. Transmission of simian malaria

This investigation utilizes Plasmodium cynomolgi in rhesus monkeys as a model close to Plasmodium vivax in man. The experimental work embraces two inter-related approaches to the problem. The normal variability among and within available species, strains and populations of anopheline vectors of human malaria is studied with respect to susceptibility to infection with Plasmodium cynomolgi. This information is used as a gauge of the heritability of the trait in question and as a guide to the choice of vector species and strains for genetic selection experiments. Genetic selection experiments are conducted with the expectation of isolating genetic factors

for resistance and susceptibility to Plasmodium cynomolgi in pure strains of mosquitoes. The ultimate goals are to determine mode of inheritance and to determine whether such susceptibility and resistance extends to species which are vectors of human malaria.

A paper comparing Anopheles quadrimaculatus, Anopheles balabacensis and four geographic strains of Anopheles stephensi with respect to susceptibility to infection with Plasmodium cynomolgi is in press (Rutledge, Hayes & Ward, 1969). Susceptibility within species and strains was found to fluctuate in time in a manner consonant with genetic theory of population gene frequencies. Heritability of the trait was established by demonstrating significant average differences in susceptibility among vector species and strains. Individuals of Anopheles quadrimaculatus showed the greatest variability, and this species showed greater variability in time than other species and strains. The average susceptibility of Anopheles quadrimaculatus was significantly less than that of other species and strains.

A number of genetic selection experiments with Anopheles quadrimaculatus have been carried out. The eggs of mosquitoes developing relatively low and relatively high numbers of oocysts after feeding on rhesus monkeys infected with Plasmodium cynomolgi are hatched and reared. The adult females of this generation are compared with those of the stock strain with respect to the average number of oocysts developing after the same exposure to infection with Plasmodium cynomolgi. To date the progeny of mosquitoes selected for resistance to malaria have developed an average of 47% of the number of oocysts developed by the stock strain, and the progeny of mosquitoes selected for high susceptibility to malaria have developed an average of 67%. This pattern of response to selection has also been observed in preliminary selection experiments with Anopheles stephensi. The reduced number of oocysts developed by progeny of mosquitoes selected for high susceptibility to malaria is apparently due to environmental effects. Mosquitoes used in selection experiments are necessarily subjected to different conditions of rearing, handling and confinement than the mosquitoes of the stock strain.

To date it has not been possible to routinely impose genetic selection procedures on successive generations of Anopheles quadrimaculatus. Only about 20% of the parent population can be selected for propagation if selection intensity is to be adequate. Since dissection is necessary to determine the number of oocysts present in individuals of the parent population, reproduction of those selected for propagation is limited to a single gonotrophic cycle. Mating, feeding, oviposition, hatching and survival are all adversely affected by the conditions of close confinement, repeated handling and isolation necessary in the selected procedures. In addition, inbreeding itself is known to reduce mosquito fecundity and survival in the first few generations.

Three related investigations supplementary to this project are in the final stages prior to publication: (1) A time series analysis of the infectivity of individual rhesus monkeys infected with Plasmodium cynomolgi

to Anopheles stephensi is nearly completed. Cycles of gametocytemia in the monkey were found to have a shorter period than cycles of infectivity to mosquitoes. Consequently, periods of high gametocyte levels in the monkey do not necessarily coincide with periods of high infectivity to mosquitoes and periods of low gametocyte levels do not necessarily coincide with periods of low infectivity. (2) Experimental work in a study of the numbers of oocysts produced in similar mosquitoes exposed to equal risk of infection has been completed. Zero-constant, bimodal, skewed and normal distributions are observed. The normality of the distribution is more nearly related to the proportion of mosquitoes infected than to the average number of oocysts developed. (3) A study of sporozoite production and survival in the period from the 8th to the 14th day after a mosquito is infected is partially completed. The number of oocysts developed in experimental mosquitoes is not a completely reliable guide to the number of sporozoites that will be produced, since the sporozoite potential of the developing oocysts is sometimes not realized.

#### Conclusions and recommendations

1. Aotus monkeys have been successfully infected with the Chesson strain of vivax malaria by both inoculation of infected blood from man and monkeys and by the bite of infected anopheline mosquitoes. The complete sporogonous cycle of parasite development has occurred in mosquitoes and the second mosquito to primate to mosquito cycle has been observed. Progress has been delayed by a shortage of night monkeys and non-specific mortality of experimental animals. It is recommended that more rigid standards be established for the procurement of these animals and that an exception be made to purchasing animals from the lowest bidder on a contract.

2. The susceptibility of anopheline mosquitoes to simian malaria is partially controlled by genetic factors and this susceptibility in a laboratory population can be reduced by genetic selection procedures. It is therefore possible in principle to produce a genetically resistant pure line. Further progress will depend on allocation of sufficient personnel for mass-rearing and extensive genetic selection of mosquitoes and on improvement of techniques for obtaining maximum propagation of the selected mosquitoes.

TABLE 1

Transmission of *Plasmodium vivax* (Clesson strain) to lower primates

Monkey	Source of infection	Prepatent period (days)	Day of peak parasitemia	Peak parasite count/mm <sup>3</sup>	Day of 1st mosquito infection	Duration of infection (days)	Total days observed
388	Blood ex man	15	30	13,700	22	67	107
395	Blood ex man	9	28	10,100	21	105	107
396	Blood ex man	2	50	13,400	22	107	107
402	Blood ex 396	7	22	14,100	19	30	44 (died)
407	Blood ex #396	12	34	3,400	27	97	97
425	Blood ex #388	9	9	1,200	-	23	26 (died)
394	Sporozoites ex #388	-	-	-	-	-	10 (died)
428	Sporozoites ex #388	12	12	300	-	14	14 (died)
405	Sporozoites ex #388	24	44	14,500	37	54	54 (died)
408	Sporozoites ex #388	-	-	-	-	-	19 (died)
7*	Sporozoites ex #388	13	22	30,200	21	33	68
427	Sporozoites ex #402	42	-	-	-	-	44 (died)
67	Sporozoites ex #7	21	28	10,700	-	28	28

\* Splenectomized chimpanzees used one year previously for *P. falciparum* experiment

Project 3A663713D829, MALARIA PROPHYLAXIS  
Task 00, Malaria Investigations  
Work Unit 124, Genetic aspects of sporogony in mosquitoes

No Publications

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUBMITTAL	REPORT CONTROL SYMBOL	
				DA 0A6515	69 07 01	ED-7000 (12) COS	
3. DATE PREV. SUPPLY	4. KIND OF SUMMARY	5. SUMMARY SECY	6. WORK SECURITY	7. RESEARCHING	8. DDD FORM LISTING	9. SPECIFIC DATA CONTRACTOR ACCESS	10. LEVEL OF SUPP. WORK UNIT
69 01 31	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	63713A	34663713D829	00	125			
b. CONTRIBUTING							
c. COMPONENTS	1412A(2)						
11. TITLE (precede with Security Classification Code)							
(U) Taxonomy and Ecology of Mosquitoes of Southeast Asia (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
07 65		NA		DA		C. In-House	
17. CONTRACT/GUANT Not Applicable				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
4. DATES/EFFECTIVE:				FISCAL YEAR		20. FUNDS (in thousands)	
5. NUMBER:				69		1	
6. TYPE:				70		35	
7. KIND OF AWARD:				8. AMOUNT:		9. CUM. AMT.	
1. CUM. AMT.							
21. RESPONSIBLE DOD ORGANIZATION				22. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research Washington, D. C. 20012				NAME: Walter Reed Army Institute of Research Div of CD and I Washington, D. C. 20012			
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23. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Considered				ASSOCIATE INVESTIGATORS			
				NAME: Tyson, 1LT W. H.			
				DA			

PII Redacted

(U) Anopheles; (U) Ecology; (U) Mosquitoes; (U) Taxonomy; (U) Vectors

23. (U) Taxonomic revision and ecological investigation of the mosquitoes of Southeast Asia. To compile data on the distribution, abundance, habits, disease transmission potential and other aspects of mosquito biology, and to produce monographs, keys and other aids for units in the field mosquito.

24. (U) Mosquitoes are collected by cooperating military and civilian organizations in Southeast Asia and forwarded to a combined Walter Reed Army Institute of Research-Smithsonian Institution Team at the United States National Museum. Definitive identifications are made there and collection data dealing with biology and distribution tabulated for later machine processing. Keys and other identification aids are produced for field units, and for later publication. Specimens are also identified from older collections at various museums, and ecological and disease data abstracted from published literature.

25. (U) 69 01 - 69 06. Completed manuscript on anophelines of Thailand to be published in CY 70. Taxonomic review of subgenus Muscidus of genus Aedes of S.E. Asia completed. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 125, Taxonomy and Ecology of Mosquitoes of SE Asia

Investigators

Principal: LTC J. E. Scanlon, MSC\*  
LTC B. F. Eldridge, MSC, MAJ J. F. Reinert, MSC\*\*  
1LT W. H. Tyson, MSC, MSG E. L. Peyton, AMEDD\*\*\*

Description

Mosquitoes are collected in Southeast Asia by cooperating military organizations and other groups. Other supplementary materials are obtained from existing collections in museums and other institutions. After study taxonomic revisions and descriptions are prepared for all of the mosquitoes of Southeast Asia, with emphasis on the species of medical importance. Sections of the work are published as completed, and keys of value to military entomologists are prepared as required. The eventual aim of the project is the publication of a series of monographs completely describing the mosquitoes of the area. In addition collection and ecological data are recorded later for collation with published data on the ecology of the various species. Studies under this work unit are performed in conjunction with the Smithsonian Institution under U.S. Army Medical Research and Development Command Contract MD-2672. This report covers the in-house portion of the work only.

Progress

1. Anopheles of Thailand

The study of material from Thailand has been completed and a final draft of a book-length work: Anopheles mosquitoes of Thailand, has been completed. Publication of this work should take place in calendar year 1970. Before completion of the manuscript, considerable additional work was accomplished during the report period. One Anopheles species was added to the Thailand fauna, A. kyondawensis, originally described from Burma. An intensive study of the minimus group was completed, with the elimination of three species from the Thailand list, now shown to be morphological variants of other species.

2. Aedes of Southeast Asia

a. Subgenus Diceromyia. A manuscript covering a revision of this subgenus in Southeast Asia has been completed and should be published this year.

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\* Until 1 March 1969  
\*\* Until 1 September 1968  
\*\*\* Until 1 September 1968

b. Subgenus Mucidus. A review of this subgenus has been completed and a manuscript is in rough draft. Illustrations of the species are being made by artists at the 406th Medical Laboratory. A supplementary study involving six species in this group has been undertaken in order to clear up an area of taxonomic confusion.

### 3. Uranotaenia of Southeast Asia

Approximately 2000 specimens of this group were examined from Thailand and the Philippine Islands. Twenty-two species are recorded from the latter area, three of which are new and undescribed. A study of the male terminalia of the genus has been completed, involving the examination of approximately 150 dissected and mounted terminalia specimens.

#### Conclusions and recommendations

Publication of the Anopheles of Thailand should materially assist the identification of malaria vectors of SE Asia. Completion of taxonomic revisions of other mosquito genera of this area will facilitate the identification of closely related vectors of dengue and other arbovirus diseases. Further collections of reared material must be made from other Asian countries before completion of systematic studies can be achieved.

Project 3A663713D829  
Task 00 Malaria Investigations  
Work Unit 125, Taxonomy and Ecology of Mosquitoes of SE Asia

Publications

- Payton, E. L. and R. H. Hochman. 1968. A revised interpretation of the proctiger of male Uranotaenia with a related note on Hodgesia. Proc. Entomol. Soc. Washington 70(4): 376-382.
- Scanlon, J. E., E. L. Peyton and D. J. Gould. 1968. An annotated checklist of the Anopheles of Thailand. Thai National Sci. Papers, Fauna Series No. 2, Bangkok, pp 1-35
- Scanlon, J. E., J. A. Reid and W. H. Cheong. 1968. Ecology of Anopheles vectors of malaria in the Oriental Region. Cahiers O.R.S.T.O.M. Ser. Ent. Med., 6(3): 43-52.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
				DA OA6516	69 07 01	DD-R&E (AR) 696	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. SOURCE SECURITY	7. RESEARCHING	8A. DISC'D INSTN	8B. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF R&E
69 01 31	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES:		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		637134		3A663713D829		00	
b. CONTRIBUTING						126	
c. SPONSORING		1412A(2)					
11. TITLE (Precede with Security Classification Code)							
(U) Cultivation of Mosquito Tissues and Malarial Parasites In vitro (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREA							
002600 Biology							
13. START DATE		14. ANTICIPATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
07 65		NA		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
Not Applicable				FISCAL YEAR		a. FUNDS (in thousands)	
a. DATE/EFFECTIVE:				69		2	
b. NUMBER:				70		1	
c. TYPE:				CURRENT		35	
d. KIND OF AWARD:				I. CUM. AMT.			
20. RESPONSIBLE ORG:				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research Washington, D.C. 20012				NAME: Walter Reed Army Institute of Research Div of CD and I ADDRESS: Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide NAME if U.S. and home preference)			
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TELEPHONE: 202 - 576-3551				TELEPHONE: 202 - 576-3049			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Considered				ASSOCIATE INVESTIGATOR			
				NAME:			
				DA			
				NAME:			
22. REVISIONS (Precede with Security Classification Code)							
(U) Aedes; (U) Anopheles; (U) Mosquitoes; (U) Plasmodium; (U) Tissue Culture							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) To study by means of an In vitro system, the metabolic activities of the mosquito phase of malaria parasites, Plasmodium gallinaceum and P. cynomolgi, the physiology and biochemistry of the interaction between the invertebrate host /Aedes aegypti and Anopheles stephensi respectively/ and parasite, and the factors responsible for host specificity of the parasites. Maintenance and development of the various parasitic stages In vitro would provide a system for collecting parasitic antigens. The possibility exists that such antigens could be utilized for the production of antimalarial vaccines.</p> <p>24. (U) Development of a culture medium which will permit the differentiation and growth In vitro of the invertebrate stages of the above-mentioned parasites. Evaluation of the competence of various organs, tissues and cells from the appropriate mosquito species to provide the parasites with a cellular milieu In vitro comparable to that found In vivo. Biochemical requirements of the parasites will be determined primarily by the employment of chromatographic techniques.</p> <p>25. (U) 69 01 - 69 06. P. cynomolgi oocysts were placed in culture with primary and established cell lines of A. stephensi and compared with the development of control oocysts placed in medium alone. Results were inconclusive as older oocysts in both instances developed to the point of liberating sporozoites while efforts to obtain very young oocysts were unsuccessful due to technical difficulties. Emphasis has been shifted to finding a satisfactory method for obtaining much younger stages for culture. In particular, attempts are being made to isolate gametocytes from the other blood forms on density gradients. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.</p>							

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Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 126, Cultivation of mosquito tissues and malaria parasites  
in vitro

Investigators

Principal: Imogene Schneider, Ph.D.

Associate: CPT David H. Chen, MSC

Description

This investigation is designed to determine the feasibility of employing an in vitro system for the collection of large numbers of malaria sporozoites free or virtually free of host tissue. Such parasites could serve as a source of malarial antigens for immunological studies. In addition, attempts are being made to devise a culture system capable of supplying the necessary nutritional, hormonal and/or other factors for the growth and differentiation of the insect cycle of these parasites. If this were possible studies involving the biochemistry, physiology and host specificity of the invertebrate phases could be readily undertaken. Prior work on culturing the mosquito phases of malaria parasites has been largely confined to studies involving Plasmodium relictum by Ball and Chao (Am. J. Trop. Med. Hyg. 13:181, 1964 and earlier publications). In such studies the younger stages underwent little if any development in vitro whereas the older stages were capable of producing viable sporozoites. In this study two other host-parasite combinations were employed, namely: Aedes aegypti-Plasmodium gallinaceum and Anopheles stephensi-Plasmodium cynomolgi. The parasites were cultured in various media alone and with cells from both primary and established insect cell lines.

As an alternative to the in vitro system for the mass isolation of malaria sporozoites the technique of density gradient centrifugation is being explored. This technique is usually employed to separate subcellular particles but in recent studies has been extended to the isolation of whole cells and organs. The success of these latter studies indicated that the technique might also be applicable to the isolation of the invertebrate stages of plasmodia.

The infected mosquitoes are homogenized in buffered saline and the resulting brei is subjected to a series of filtration and centrifugation steps prior to being placed on a linear gradient. After high speed centrifugation the sporozoites band at the appropriate density in the gradient and are collected by bottom puncture of the centrifuge tube.

Progress

1. Cultivation of malaria parasites and mosquito tissue

In general, the results obtained when the parasites were cultured alone in the various media closely paralleled those of Ball and Chao.

Details are given in the papers cited at the end of this report. Since the culture system employed was a relatively static one it seemed plausible that more substantial results might be obtained by the addition of actively growing mosquito cells to the cultures. A number of mosquito cell lines have been established in other laboratories and were available for this study. In addition, three cell lines derived from first stage larvae of Anopheles stephensi were established in this laboratory. Results obtained by placing the parasites in any of these cell lines was inconclusive. The older oocysts continued their development in cultures both with and without cells. Dissections of the younger oocysts were difficult because of their small size and the number obtained was not sufficient to make valid comparisons. Hence, emphasis has been switched to finding a means of isolating the earliest stages. The most promising approach seems to be that of using density gradients. Kowley et al., (J. Lab. Clin. Med. 70:933, 1967) have shown that it is possible to separate the various blood forms of Plasmodium berghei in different bands of the gradient. The parasitized cells from such a gradient retain their infectivity which suggests that the procedure does not subject them to undue stress. Four gradient runs using blood infected with P. cynomolgi have been made thus far. The gradients are prepared from bovine serum albumin with a density range of 1.02 to approximately 1.15 g/cm<sup>3</sup> at 4°C. Ring forms predominate in the denser portion of the gradient whereas the schizonts, segmenters and gametocytes are considerably lighter. Adequate separation of the forms has not yet been achieved but this can probably be resolved by adjusting the steepness of the gradient.

## 2. Isolation of malaria sporozoites on density gradients

Seventy-nine centrifugation runs have been made employing Aedes aegypti mosquitoes infected with Plasmodium gallinaceum. In addition, approximately 30 runs have been made with Anopheles stephensi infected with P. cynomolgi. Except where noted, the results given below pertain to the former host-parasite system.

Earlier work with this technique employed a linear sucrose gradient with an 11 point range of 31 to 42 percent sucrose. Values for the mean sporozoite density in the peak fraction ranged from 1.156 to 1.158 g/cm<sup>3</sup>. Using a 1000 mosquito sample, up to 10<sup>7</sup> sporozoites could be recovered from the peak fraction and the number of sporozoites recovered per mosquito was estimated at 45,000. (For more details see Chen, D. H. and I. Schneider, 1969).

Further work has centered around two drawbacks encountered with the above technique, namely: (1) the recovered sporozoites, although intact and motile, are no longer infectious and (2) although the numbers of sporozoites isolated far surpass those obtained with the culture technique the preparations are not as clean. Loss of infectivity was attributed to the use of relatively high concentrations of sucrose needed to obtain the appropriate densities. Hence, gradients employing substances other than sucrose were tested. Among these were potassium tartrate, bovine serum albumin (Fraction V), Ficoll and Renografin.

(methylglucamine diatrizoate with additives). The most successful gradients were those utilizing both bovine serum albumin (BSA) and Reno-grafin (R) with the most effective concentrations so far tested being 20% BSA/20% R (density = 1.066 g/cm<sup>3</sup>) and 30% BSA/60% R (density = 1.161 g/cm<sup>3</sup>). In such gradients the peak band of P. gallinaceum sporozoites has an average density of 1.120 g/cm<sup>3</sup>. Sporozoites recovered from these gradients are infectious to chicks.

BSA/R gradients have also been used to isolate P. cynomolgi sporozoites from Anopheles stephensi. Although extensive data are not available the peak bands tend to be situated slightly lower in the gradient than is the case with P. gallinaceum and the density averages 1.124 g/cm<sup>3</sup>. Infectivity of these sporozoites has not as yet been tested.

Less progress has been made in overcoming the second drawback of concomitant isolation of particles having densities very closely approaching or equal to that of the sporozoites. Larger particles have been eliminated by means of two additional centrifugation steps prior to running the gradient. Elimination of the smaller particles has not yet been achieved.

#### Conclusions and recommendations

1. A culture system which will adequately support the growth and differentiation of the invertebrate stages of the malaria parasites has not yet been devised. Various media are capable of maintaining the older stages for a period long enough for them to complete their development. Culturing the youngest and presumably the most responsive stages of the insect cycle would probably yield much more information regarding the nutritional needs of the parasites. Further efforts should be made to obtain these stages in sufficient quantity and purity for such studies.

2. At present, the technique of density gradient centrifugation can be used very effectively to isolate massive numbers of malaria sporozoites from infected mosquitoes. Since the technique avoids the handicap of manual dissection the mass of sporozoites isolated is, to a very large extent, limited only by the numbers of infected mosquitoes available. It, therefore, should prove of considerable value as a preliminary step for both immunological and biochemical studies which in the past have been hampered by the lack of sufficient material. The use of this technique might also be extended to estimating the percentage of infected mosquitoes in a given field sample, assuming further refinements which will allow a tighter banding of sporozoites in the gradient. Efforts should also be made to further purify the final sporozoite preparation.

Project 3A663713D829  
Task 00 Malaria Investigations  
Work Unit 126 Cultivation of Mosquito tissues and malaria parasites  
in vitro

Publications

- Chen, D. H., and I. Schneider. 1969. Mass Isolation of Malaria Sporozoites from Mosquitoes by Density Gradient Centrifugation. Proc. Soc. Exp. Biol. Med. 130:1318-1321.
- Schneider, I. 1968. Cultivation in vitro of Plasmodium gallinaceum Oocysts. Exptl. Parasitol. 22:178-186.
- Schneider, I. 1968. Cultivation of Plasmodium gallinaceum Oocysts in Grace's Cell Strain of Aedes aegypti (L.). II Internat. Colloq. Invertebrate Tissue Culture. Instituti Lombardo. Milan. pp. 247-253.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA 0A6518	69 07 01	DD-R&E (AR) 638	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ECTY <sup>b</sup>	6. WORK SECURITY <sup>b</sup>	7. REGRADING <sup>c</sup>	8A. DISSEM INSTN <sup>c</sup>	8B. SPECIFIC DATA - CONTRACTOR ACCESS <sup>c</sup>	9. LEVEL OF SUM <sup>c</sup>
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2. CONTRIBUTING							
3. SPONSOR/CRK	1412A(2)						
11. TITLE (Provide with Security Classification Code) <sup>e</sup>							
(U) Test Systems for Plasmodium falciparum(09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>f</sup>							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
07 65		NA		DA		D. In-House	
17. CONTRACT/GRAANT				19. RESOURCES ESTIMATE		20. PROFESSIONAL RAN YRS	
NA				UNRECOVERED		B. FUNDS (In thousands)	
18. DATES/EFFECTIVE:				FISCAL YEAR		CURRENT	
a. NUMBER:				69		3	
b. TYPE:				70		70	
c. KIND OF AWARD:				70		70	
19. RESPON. & DO ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D.C. 20012				ADDRESS: Washington, D.C. 20012			
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TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3308			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: MOON, A. P.			
				NAME: DA			
22. KEYWORDS (Provide with Security Classification Code)							
(U) Malaria; (U) Chimpanzee; (U) Immunity; (U) Chloroquine; (U) Gamma Globulin; (U) Isotope; (U) Susceptibility; (U) Owl Monkey							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23(U) Study susceptibility of chimpanzees and other primates to infections of human malaria. Study the characteristics of drug resistant strains, provide high density of parasites for morphological and biochemical studies. Conduct physiological and pathological studies of malaria and provide test animals for chemotherapeutic and immunological investigations.							
24(U) Infect splenectomized, drug treated chimpanzees and other primates with plasmodia of human origin. Observe the extent and duration of parasitemias, study the response of different strains to chemotherapy, study susceptibility to reinfection with homologous and heterologous strains.							
25(U) 69 01 - 69 06 The 14th passage of chloroquine refractory Plasmodium falciparum was repeated a second time in a splenectomized chimpanzee. A mixed plasmodia infection resulted which was successfully treated. Blood frozen for 12 months from the 9th passage failed to infect a splenectomized chimpanzee. Two splenectomized chimpanzees were successfully infected with P. falciparum parasitized blood obtained from Aotus monkeys and maximum parasitemias were 3.6 and 5.4 percent. Chimpanzee infected with P. malariae remained patent for more than 190 days. Using 100 million parasites 43 passages with California isolate of P. falciparum (Camp) were done in Aotus monkeys. Maximum parasitemias were consistently greater than 30 percent with deaths occurring in 7 to 12 days. The chloroquine resistant monterey strain of P. falciparum was obtained and passed 4 times in 8 intact Aotus monkeys. The course of infection was less predictable but parasitemias greater than 20 percent were common. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							
26. AVAILABLE TO CONTRACTORS UPON ORIGINATOR'S APPROVAL							

PII Redacted

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 127, Test systems for Plasmodium falciparum

Investigators.

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1. The use of subhuman primates for experimental studies of human malaria.

A serious obstacle to an adequate understanding of human malaria has been the lack of suitable animal models in which plasmodia could be studied under controlled conditions. Limited success was reported in infecting monkeys with Plasmodium falciparum more than 30 years ago, but only in the past few years have investigators been able to maintain and transmit human plasmodia in subhuman primates. Experimental infections of human species of malaria have been reported in the chimpanzee (Pan satyrus), the gibbon (Hylobates lar) and the owl monkey (Aotus trivirgatus). Therefore, new hosts are now available for providing the basis of a better understanding of human malaria and for producing relatively large quantities of parasites for biochemical and immunologic studies. Severe limitations still exist, however, since these primates must be obtained as feral animals, are available only in limited numbers and adapt poorly to laboratory conditions.

This report describes infections of chimpanzees and owl monkeys with 3 species of plasmodia (P. falciparum, P. vivax and P. malariae) and the various pathologic, immunologic, physiologic and biochemical investigations for which these host-parasite systems were used. Some of the advantages and disadvantages associated with the use of these animals is also discussed.

A. Host Animals

1. Chimpanzees. A total of 77 chimpanzees were purchased for malaria studies, of which 58 were eventually used. Details of their procurement and treatment during quarantine were reported previously.

On release from quarantine, the chimpanzees were caged individually and provided a diet of monkey chow, fresh fruit, vegetables and water. The animals underwent a conditioning period, followed by splenectomy, pre-infection chemotherapy and experimental inoculation with malaria. Each was trained to submit to rectal temperature and bleeding procedures in order to avoid the use of sedation or undue restraint and to minimize the risk of injury to the chimpanzee or the handlers. Splenectomies were performed essentially as described by Elovitz et al. Seven days after splenectomy, or earlier if a spontaneous malarial infection was observed, treatment with chloroquine (7.3 mg. base/kg. i.m. for 3 days) and primaquine (.75 mg. base/kg. i.m. for 14 days) was initiated. Post-treatment examinations for natural malarial infections were performed weekly or

more frequently for a minimum of 30 days before the animals were exposed to infection. During this period, blood for pre-infection hematologic and serologic tests was also collected.

2. Owl monkeys. A total of 136 of 203 owl monkeys procured from Colombia was used in these studies. The monkeys ranged in weight from 450 to 900 grams and both sexes were represented although nearly 5 times as many females as males were used.

Upon arrival, monkeys were quarantined for a minimum of 3 weeks during which time they were tattooed, tuberculin tested and individually treated for any disease which occurred. Following release from quarantine, they were transferred in lots of 20 to 40 to an empty, previously sensitized room where they were housed initially in pairs. Room temperature was maintained between 79 and 85 F with a high relative humidity. The monkeys were provided a diet of monkey chow ad libitum, bananas, apples, and vitamin supplemented water in wide-mouth containers. Live neonatal mice were placed in the cages 3 times a week. After experimental inoculation, each animal was placed in an individual cage.

Fifty-eight owl monkeys were splenectomized to meet the requirements of a particular infection or experimental protocol. Blood for hematologic examination and serum collection was drawn and several blood films from each animal were examined for parasites before experimental inoculation. Pre-inoculation treatment with antimalarial drugs was not given.

## B. Infections

1. Inocula. Several strains and isolates of human Plasmodium spp. were used (Table 1). All fresh inocula were quantified for parasitized red blood cell per cmm. of blood and total parasitized cells injected. No parasite counts were made for inocula from frozen blood but thin smears were examined for morphology of the parasites and host cells.

P. falciparum (Camp.) is a chloroquine resistant strain which was originally obtained from a patient in Malaya, tested in volunteers and forwarded to this laboratory by Dr. Robin Powell. In a previous report this strain was designated as a South East Asian (SEA) strain of P. falciparum. The same report describes the West African (WA) strain of P. falciparum obtained from a patient in Nigeria.

The P. falciparum (Camp./W) strain was isolated from a chimpanzee infected with P. falciparum (Camp.) during the eighth passage. In addition to the passages indicated in Table 1, periodic subinoculations were made 8 times from splenectomized to non-splenectomized owl monkeys and 3 passages were made in non-splenectomized animals. The origin of P. falciparum (Camp./C) and infections in owl monkeys were first described by Geiman, et al. The strain was passed many times in owl monkeys in two California laboratories before its use at this Institute.

Table 1

Inoculations of Chimpanzees (Pan Satyrus) and Owl Monkeys (Aotus trivirgatus) with Human Plasmodia

Parasite		Source of Infected Blood	Host	No. of Animals*	No. of Passages**	Standard Inoculum (parasitized RBC's)	Route of Inoculation
	Camp. (also SEA)	Man	Chimpanzee	49(S)	14	Variable	i.v.
	WA	Man	Chimpanzee	8(S)	2	4 x 10 <sup>7</sup>	i.v.
	Camp./w	Chimpanzee	Owl Monkey	26(S)***	17	1 x 10 <sup>8</sup>	i.v.
<u>Plasmodium falciparum</u>	Camp./C	Chimpanzee	Owl Monkey	45	30****	1 x 10 <sup>8</sup>	i.v.
	Nigera	Man	Owl Monkey	2(S)	--	2 x 10 <sup>7</sup>	i.v./i.p.
	Uganda	Owl	Owl Monkey	3(S)	--	5 x 10 <sup>7</sup>	i.v./i.p.
<u>Plasmodium vivax</u>	Varnes	Man	Owl Monkey	15(S)	10	1 x 10 <sup>7</sup>	i.v.
	Chesson	Man	Owl Monkey	3(S)	0	1 x 10 <sup>7</sup>	i.v./i.p.
<u>Plasmodium malariae</u>	--	Man	Chimpanzee	1(S)	0	3 x 10 <sup>7</sup>	i.v.
	--	Man	Owl Monkey	1(S)	--	6 x 10 <sup>6</sup>	i.v./i.p.
	--	Chimpanzee	Owl Monkey	2(S)	0	1 x 10 <sup>8</sup>	i.v.

\* (S) Splenectomized.

\*\* -- Infection not established; 0 Infection established but not passaged.

\*\*\* Subinoculation: in nonsplenectomized animals not included.

\*\*\*\* Earlier passages not included.

Both the P. falciparum (Uganda) and P. falciparum (Nigeria) isolates were obtained from patients who contracted malaria while in Africa. P. vivax (Varnes) was obtained from a patient recently returned from Korea. The Chesson strain of P. vivax was provided by Dr. David Clyde after its isolation from an infected volunteer. Dr. Peter Contacos provided the isolate of P. malariae, also from a volunteer.

2. Clinical observations and treatment. All animals were observed daily and any abnormalities in behavior or general appearance were recorded. Immediately upon detection of nonexperimental disease, symptomatic treatment was initiated and then followed by more specific therapy when results of laboratory tests became available. After inoculation for experimental malaria infections, blood was collected routinely for parasitologic, hematologic and serologic examination. P. falciparum (Camp.) infected chimpanzees were treated with antimalarial drugs to test the drug sensitivity of the parasites and, later, to suppress high parasitemias. Treatment consisted of intramuscularly administered chloroquine HCl in 3 to 5 doses of 7.3 gm. base/kg. at 24 hour intervals or orally administered quinine in 7 doses of 22 or 73 mg. base/kg. at 24 hour intervals. Transfusion with non-infected, compatible chimpanzee blood and other supportive care was given when required. Experimentally infected owl monkeys were never treated with antimalarial drugs and only rarely treated for concurrent spontaneous disease.

3. Parasitologic determinations. Malaria infections were identified in Geimsa-stained thick and thin blood smears. Quantitative determinations of the parasitemias were made by counting the number of parasitized RBC's per 100 WBC's or 10,000 RBC's. Results were recorded as percent parasitized RBC's per cmm. of blood. Developmental distribution of the parasites and abnormal morphology were also noted.

#### C. Passive Immunization

The general methodology and results of passive immunization studies using chimpanzees infected with P. falciparum was reported previously. West African gamma globulin (WA) was extracted from plasma obtained in Nigeria. It was administered to splenectomized chimpanzees in a manner designed to determine if an immunoprophylactic and immunosuppressive effect could be demonstrated against the Camp. (SEA) and WA strains of P. falciparum. Moreover, plasma was collected from several military patients having a history of infection with P. falciparum contracted while in South East Asia. Results of indirect fluorescent antibody (IFA) tests indicated that the titer of the pooled plasma exceeded 1:500. Gamma globulin (SEA-USA) was extracted by the American Red Cross using a technique described earlier.

The SEA-USA gamma globulin was administered intramuscularly daily for 4 days to 3 chimpanzees in doses of 50 mg./kg. of body weight and 2 chimpanzees were given the same amount of non-immune gamma globulin. On the second day of treatment (day 0) the 5 chimpanzees were inoculated

with SEA P. falciparum (Camp.) infected chimpanzee blood containing  $4 \times 10^7$  parasitized RBC's. Daily parasitemias were compared to determine if an immunoprophylactic effect resulted from the use of SEA-USA gamma globulin.

#### D. Active Immunization Study

Protection of chimpanzees against P. falciparum infections was reported earlier. After treatment and apparent elimination of a primary blood induced SEA P. falciparum (Camp.) infection, 4 chimpanzees were challenged with the same strain and 3 were challenged with the WA strain of P. falciparum.

Active immunization of owl monkeys against P. falciparum (Camp./C) was attempted by inoculation of parasitized blood irradiated by exposure to a dose of 25,000 r. Fourteen monkeys were given 4 intravenous inoculations of 2-3 ml. of irradiated RBC's containing  $1-5 \times 10^9$  parasitized RBC's at weekly intervals. Equivalent amounts of non-infected identically irradiated RBC's were administered to 11 other monkeys. A third group of 11 animals was not treated. During the immunization period several monkeys from each group died apparently due to nonexperimental causes. The survivors were challenged 9 days after the last immunization by intravenous inoculation of P. falciparum (Camp./C) infected blood containing  $10^8$  parasitized RBC's.

#### E. Other Studies

Additional methods used in specific experiments presented in this paper will be included, when pertinent, in the presentation of results.

##### 1. Chimpanzees

a. Pre-infection findings. A period of several weeks was required to prepare new chimpanzees for experimental infections. During this time 19 animals either died or were found to be unsuitable for use. The primary cause of death in 5 cases was diplococcal pneumonia, whereas other agents and accidents were implicated in 10. Four additional chimpanzees were rejected because they were not amenable to frequent handling.

Natural malaria infections (6 P. schwetzi, 1 P. reichenowi, 1 P. malariae and 3 unidentified) were detected after splenectomy in 11 of the chimpanzees used. These were apparently eradicated by antimalarial treatment. Several animals had microfilariae and one had trypanosomes.

Although exposure to chimpanzees has been implicated as the source of human cases of infectious hepatitis, only one diagnosis of infectious hepatitis was made in persons having had contact with this colony. That occurred in an individual whose exposure to other possible sources of infection was considerably greater than his single contact with one chimpanzee. Seven experimental chimpanzees housed in an isolated animal

room were euthanized after tuberculosis was diagnosed. Tuberculin skin tests were consistently negative in the remainder of the chimpanzees. All chimpanzees were infected with a variety of intestinal helminths and protozoa among which Strongyloides stercoralis, Endamoeba histolytica and Balantidium coli were frequently identified.

b. P. falciparum. Splenectomized chimpanzees were shown to be susceptible to infection with both WA P. falciparum and SEA P. falciparum (Camp.). Fourteen passages of the latter strain were made in 41 animals. The infections in 24 animals were allowed to run their natural course or were not treated until a high level of parasitemia was reached (Table 2). Despite the variable size of inocula, the prepatent periods and the days of maximum parasitemia were within a relatively short, predictable range. The maximum parasitemias were consistently high after the fifth passage, and appeared to be unrelated to the size of the inocula. During the fifth passage, 4 of 5 untreated animals developed elevated temperatures. Of these, all but one demonstrated evidence of chills at 48 hour intervals during the periods of maximum parasitemia. One chimpanzee died as a result of the infection. During the next 9 passages, fever and apparent chills were observed in 7 of 11 animals. One of these animals was not treated and died, while 2 others were given chloroquine and transfused. A marked anemia invariably developed concurrently with the logarithmic increase of parasitemia. During the period of maximum parasitemias in untreated animals, erythrocyte counts less than 1.5 million per cmm. of blood, hematocrits less than 12 percent and hemoglobin values less than 3.0 gm per 100 ml. of blood were common. A characteristic course of parasitemia and accompanying anemia is graphically shown in Fig. 1.

In all infections, only early trophozoites were observed in the peripheral blood during the first few days of infection. Later, as parasitemias increased, mature trophozoites and schizonts were seen. Gametocytes were seen only occasionally near the peak of parasitemia and were always immature in appearance. Recrudescences frequently occurred. They were characterized by the reappearance of parasites in the peripheral blood, usually at a low level, and by a moderate decrease in the number of erythrocytes. Parasites were found in one chimpanzee 6 months after inoculation. Although chloroquine in therapeutic doses failed to produce radical cure of the 6 chimpanzees treated, a marked suppressive effect accompanied by typical alterations in parasite morphology was noted.

The effect of parasitemia on the osmotic fragility of chimpanzee erythrocytes was determined and reported previously. As in other species of mammalian malaria, the osmotic fragility increased significantly as the parasitemia increased. Blood clotting defects were studied in only a few infections but no evidence of the defects demonstrated in human infections was found.

Table 2

Infections of Splenectomized Chimpanzees  
with Plasmodium falciparum (Camp.)

Passage Number	Number of Animals	Day of Maximum Parasitemia	Maximum Parasitemia (Parasitized RBC's per cmm/10 <sup>5</sup> )	Remarks
1	3	11-19	0.1 - 4.0	No Rx
2	1	18	3.7	No Rx
3	3	10-23	5.2 - 9.1	No Rx
4	1	14	3.4	No Rx
5	5	6-10	0.1 - 16.0	No Rx, 1 Died
6	1	16	3.6	B,T
7	2	12,12	5.1, 10.0	No Rx, 1 Died
8	2	13,13	7.1, 8.2	B,T
9	1	11	5.6	No Rx
10	1	11	14.0	B,T
11	1	12	5.6	No Rx
12	1	14	9.1	B,T
13	1	17	8.4	B,T,C
14	1	9	8.6	B,T,C

No Rx - No treatment

B - Bled on days of maximum parasitemia

T - Transfused

C - Treated with chloroquine

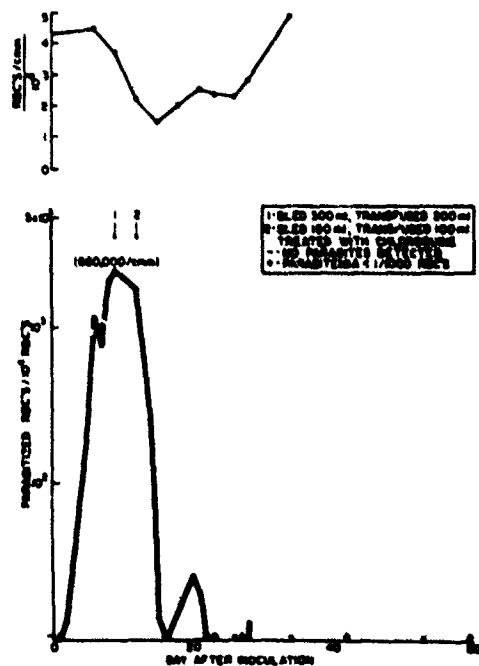


Fig. 1. Parasite and RBC counts from a splenectomized chimpanzee infected with P. falciparum (Camp.) during the fourteenth passage.

The serum biochemical changes which occurred during the course of infection were also studied. A significant increase in transaminases (SGP-T and SGO-T) and a decrease in the SGP-T/SGO-T ratio was related to the increase in number of parasites in the peripheral blood. The highest values were observed during maximum parasitemia. The results of electrophoretic analyses indicated a decrease in albumin, alpha 1 and beta globulin and a significant increase in alpha 2 and gamma globulins with a consequent decrease in A/G ratio.

Despite the immaturity of the few gametocytes observed in the peripheral blood, *Anopheles stephensi* were allowed to feed several times on days when arietocytes were detected. No oocysts were found on subsequent mosquito dissections.

In an effort to determine if passage in chimpanzees had altered the host specificity, two rhesus monkeys (*Macacca mulatta*) were splenectomized and inoculated with  $4 \times 10^7$  parasitized RBC's from a *P. falciparum* (Camp.) infected chimpanzee. Blood films examined 3 times per week during a 12 week period failed to demonstrate evidence of infection. Infected blood from a chimpanzee was used to establish one of the first *P. falciparum* infections in the owl monkey.

Attempts to reinfect splenectomized chimpanzees indicated that a primary infection of *P. falciparum* induced protection against challenge with the homologous strain but not with the heterologous strain of this parasite. Previously reported passive immunization studies indicated treatment with WA gamma globulin apparently had no effect against the SEA *P. falciparum* (Camp.) infections whereas it did produce an immunoprophylactic and immunosuppressive effect against the WA strain of *P. falciparum*. Three of 5 chimpanzees infected with SEA *P. falciparum* (Camp.) were treated prophylactically with SEA-USA gamma globulin (Fig. 2a). There was a decrease in the slope of parasitemia line and an indication that the maximum parasitemia attained was lower in the SEA-USA gamma globulin treated animals. The immunoprophylactic effect appeared to be greater than that shown for the heterologous system (Fig. 2b) and different from that shown for the other homologous system (Fig. 2c). The prolonged prepatent periods and delayed increase in parasitemia reported in the latter case were not observed.

Quantities of parasitized blood were collected from infected chimpanzees for the purpose of harvesting the parasite or parasite by-products. Blood with relatively low numbers of parasites was provided for the presentation of slides for diagnostic training and for a few trials of *in vitro* cultivation.

Highly parasitized blood was obtained primarily for the preparation of antigens to be used in established immunodiagnostic malaria tests and for the development of new tests.

Thin films of parasitized blood were used in the indirect fluorescent antibody (IFA) test to establish the titer and its persistence in

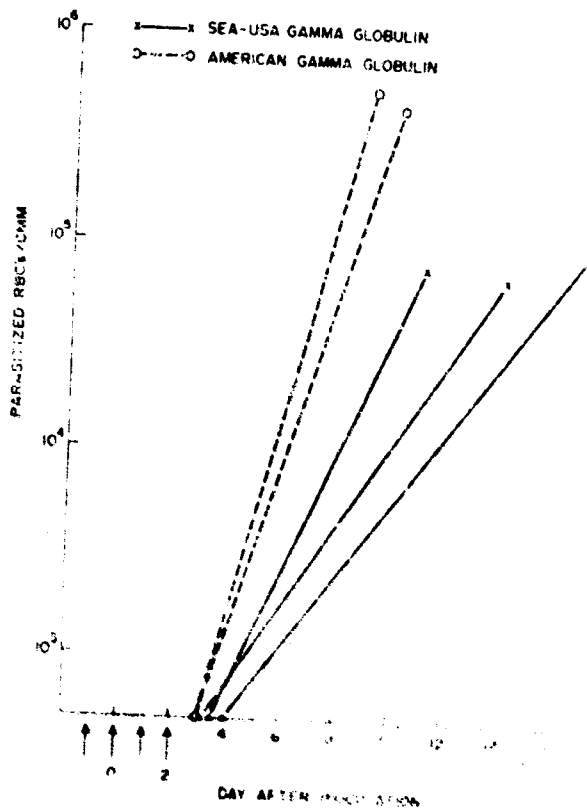


Fig. 2a. Parasitization of red blood cells in mice with SEA-USA gamma globulin in relation to the amount of gamma globulin administered with a SEA strain of *P. berghei*.

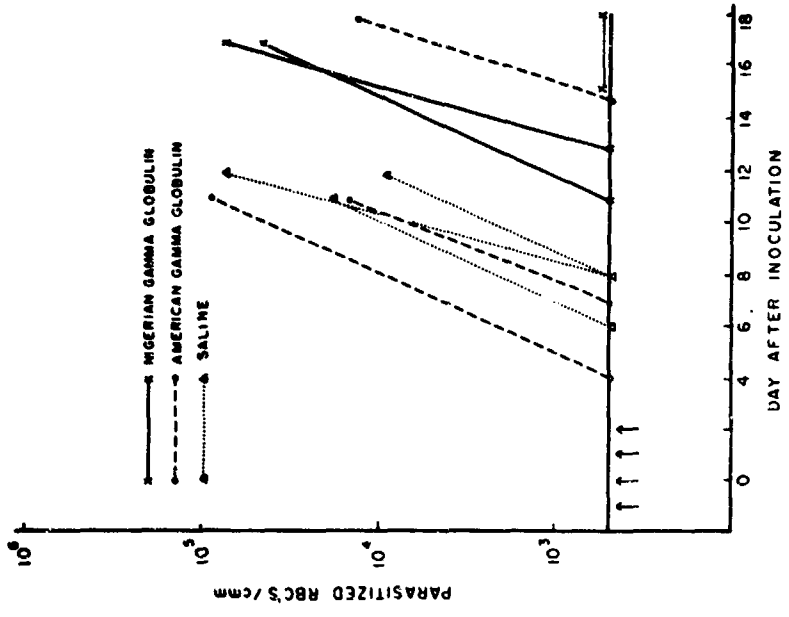


Fig. 2c. Immunoprophylactic effect of WA human gamma globulin in splenectomized chimpanzees infected with a SEA strain of P. falciparum.

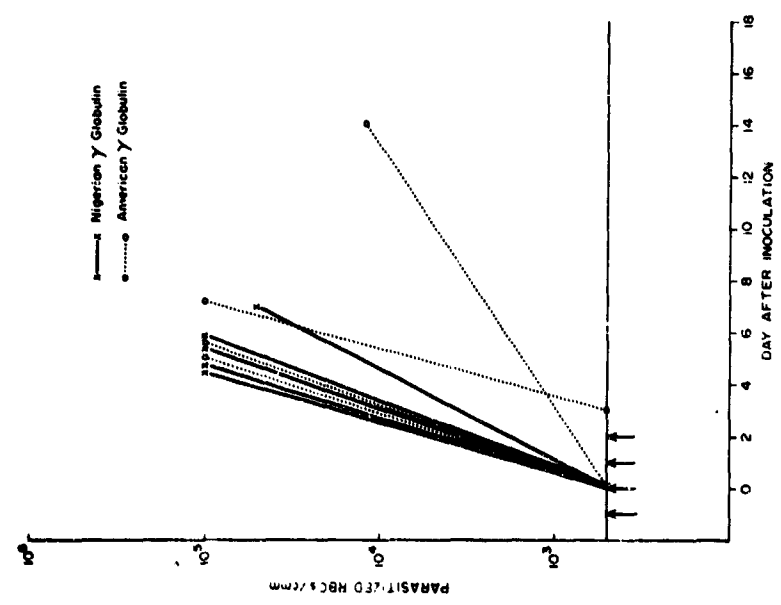


Fig. 2b. Immunoprophylactic effect of WA human gamma globulin in splenectomized chimpanzees infected with a WA strain of P. falciparum.

P. falciparum and P. vivax infected servicemen who had recently returned from Vietnam. The results indicate that human malarial parasites from chimpanzees provide satisfactory results when used as sources of antigen for this test.

Antigen for the complement fixation test was prepared from SEA P. falciparum (Camp.) infected chimpanzee blood using a method described by D' Antonio et al. The specificity and sensitivity of this test were evaluated and the pattern of antibody production in P. falciparum infected human patients was determined. The appearance and development of antibody was in close agreement with data obtained using the IFA test. Disrupted freed SEA P. falciparum (Camp.) parasites were fractionated and a soluble fraction was used successfully in a hemagglutination test to detect antibody in human sera.

A soluble antigen fluorescent antibody (SAFA) test was developed for the serologic diagnosis of human malaria using lysates of parasitized erythrocytes from P. falciparum infected chimpanzees as antigen. These lysates were separated by ion exchange column chromatograph and one fraction (No. 4) was found to confer to this test a high degree of sensitivity, specificity and reproducibility. Since a very small amount of antigen was required, approximately 50,000 SAFA tests could be performed with the parasite material normally collected from a single infected chimpanzee.

A micro-method indirect hemagglutination (IHA) test was recently developed using lysates of parasitized erythrocytes from SEA P. falciparum (Camp.) infected chimpanzees after separation by column chromatography as described for the SAFA test. The most active antigen for this test was found to be in fraction No. 3 of the preparation. Since fraction No. 4 was selected for the SAFA test, antigen for both tests could be supplied from the same volume of infected blood. Results of comparative tests using sera from human volunteers suggest that the SAFA and IHA tests follow similar time course development antibody curves. The micro-hemagglutination test, like the SAFA test, required a very small amount of antigen.

c. P. malariae. A single splenectomized chimpanzee without a history of natural malaria was inoculated with P. malariae. An infection was established which persisted for more than 120 days (Fig. 3). After a prepatent period of 8 days, the parasitemia increased rapidly and on day 23 reached a maximum of 96,000 parasitized RBC's per cmm. Another peak of 41,000 parasitized RBC's per cmm. was detected on day 64. There was no clinical indication of paroxysms although schizogony, as demonstrated by changes in relative parasite maturity, was synchronous enough to enable the detection of a 72 hour cycle. In the earlier phase of infection, parasite morphology was typical of that described for P. malariae. For approximately 2 weeks, however, beginning at the peak of parasitemia, an increased number of atypical asexual forms was observed. The trophozoites were more ameboid and the host cells were slightly enlarged.

Fewer of the parasites were seen as the parasitemia decreased. Mature microgametocytes were identifiable on the fifth day of patency. On day 29 there were 10% per unit of blood, the maximum concentration observed. Anopheles stephensi were allowed to feed on the chimpanzee on six different occasions without successfully establishing a mosquito infection.

A moderate anemia associated with the acute phase of the infection was influenced by repeated blood collections. Infected blood was used for the preparation of slides for teaching purposes and parasites were harvested and processed for antigen to be used in the complement fixation (CF) test. Evaluation of this antigen in the CF test is still incomplete. Blood was also drawn for injection into 2 owl monkeys in an attempt to evaluate this animal as an experimental host.

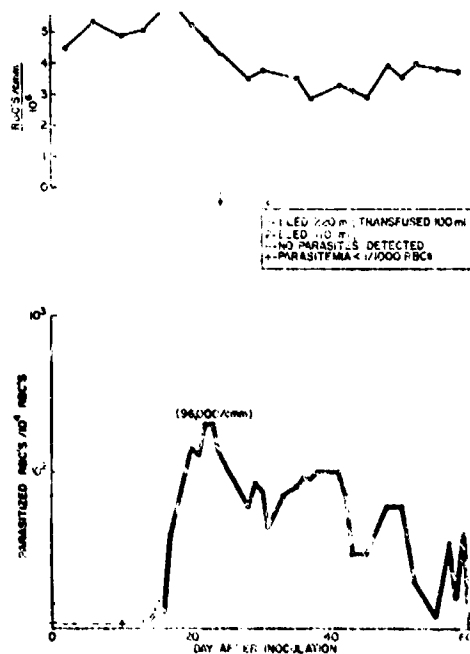


Fig. 3. Parasite and RBC counts from a splenectomized chimpanzee infected with P. malariae.

## 2. Owl Monkeys

a. Pre-infection findings. Of the 203 owl monkeys procured for the purpose of studying malaria infections of human origin, 67 or 33 percent died before being experimentally inoculated. Twelve died as a result of traumatic injury, post surgical complications or laboratory accidents. Of the remainder, pathologic and bacteriologic examinations established that pneumonia was most often the cause of death, with Klebsiella pneumoniae the predominant organism isolated. The usual gross pathologic observations were acute or partially resolved pneumonic lesions, ulcerations of the gastric mucosa, larvae of Pentostoma spp. and adult filarial worms. Short black thorns were frequently noted in the subcutaneous tissues

and occasionally longer thorns, up to 2 cm. in length, were found penetrating the body wall. Lesions, including intranuclear inclusion bodies, characteristic of Herpesvirus hominis or Herpesvirus T. were occasionally found histologically. Uncertainty concerning the incidence of viral infections and their possible role as a primary cause of death prevented us from establishing a definitive diagnosis in a number of instances. It was apparent, however, that non-experimental morbidity and mortality rates in these monkeys were high. In addition, an indeterminate number of owl monkeys infected with plasmodia sustained non-experimental infections which prevented us from achieving a proper interpretation of the experimental data.

Intradermal palpebral tuberculin tests in these animals were consistently negative. No evidence of pre- or post-splenectomy malarial infections was detected before experimental inoculation. Microfilariae were detected in six monkeys and trypanosome infections in two others. Intestinal helminthic and protozoan infections were rare and did not include species of significant zoonotic importance.

b. P. falciparum. Two African isolates of P. falciparum, Uganda and Nigeria, were not infective when inoculated into splenectomized owl monkeys. No parasites were detected during the observation period of 120 days.

Owl monkeys were susceptible to two isolates of P. falciparum, Camp./W and Camp./C, from chimpanzees. On initial isolation, P. falciparum (Camp./W) inocula produced infections in 2 of 3 splenectomized owl monkeys. The prepatent periods were 48 and 59 days and maximum parasitemias were 1.5 and 11.5 percent respectively. No parasitemias were detected within 90 days in one splenectomized and in two non-splenectomized monkeys. In 5 subsequent passages in 10 splenectomized monkeys (Table 3), using inocula of  $10^8$  parasitized cells, the prepatent periods ranged between 1 and 4 days. Maximum parasitemias ranged from 1 percent or less in 4 animals to 25 percent in one animal. Eleven further passages in 13 splenectomized animals were made using inocula of the same size (Table 3). Prepatent periods were 0 to 3 days. Only two animals failed to develop a high parasitemia and one of these died with a 7.0 percent parasitemia 7 days after inoculation. Nine monkeys that died had terminal parasitemias of 22.5 to 86 percent. The mean parasitemia was 41.2 percent and the mean survival time was 16.4 days. The characteristic course of parasitemia in these animals is shown graphically in Fig. 4. Two monkeys survived maximum parasitemias of 18.5 and 20 percent and both infections remained patent for more than 90 days.

Periodic subinoculation from heavily parasitized splenectomized monkeys to 14 non-splenectomized monkeys resulted in short term infections, but parasitemias never exceeded 1.0 percent. By using inocula of  $10^9$  parasitized RBC's, infections were passaged 3 times in non-splenectomized animals. The heaviest infection detected was 13 percent.

Table 3

Infections of Splenectomized Aotus trivirgatus  
with Plasmodium falciparum (Camp./W)

Passage Number	Number of Animals	Maximum Parasitemia			
		<1	>1 to 5	>5 to 20	>20
1-6	13	4	3	5	1
7-17	13	1	0	2	10

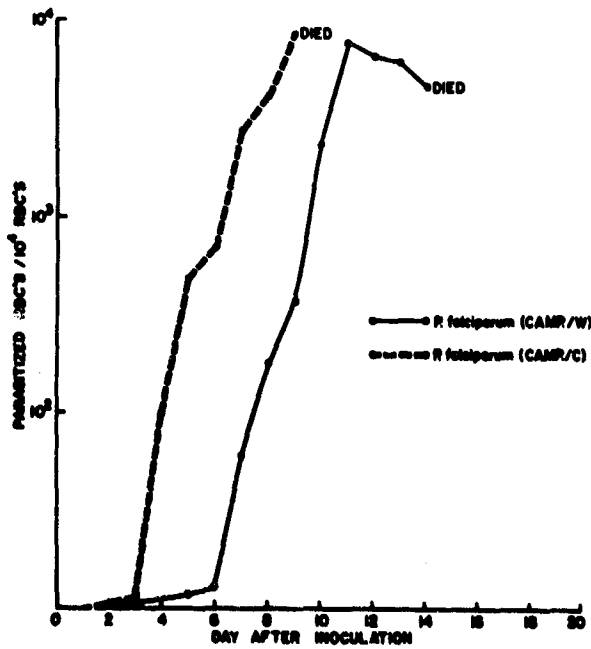


Fig. 4. Parasite counts from a splenectomized owl monkey infected with P. falciparum (Camp./W) and a non-splenectomized owl monkey infected with P. falciparum (Camp./C).

The P. falciparum (Camp./C) isolate was passaged 30 times in 45 non-splenectomized monkeys in this laboratory. Although the isolate had already been passaged many times in non-splenectomized animals before it was received, the parasitemias during the first seven passages were unpredictable (Table 4). Conversely, subsequent passages produced very predictable parasitemias with prepatent periods of 0 to 2 days, followed by a rapid increase in parasite counts to a terminal peak parasitemia of more than 30 percent parasitized RBC's. All the animals succumbed to the infection after 6-13 days (mean = 9.3 days). A typical course of parasitemia is shown in Fig. 4, and data from four passages in 6 animals are presented in Fig. 5.

Table 4

Infections of Non-Splenectomized Aotus trivirgatus  
with Plasmodium falciparum (Camp./C)

Passage Number	Number of Animals	Maximum Parasitemia			
		<1	>1 to 5	>5 to 20	>20
1-7	12	3	2	3	4
8-30	33	3	0	0	30

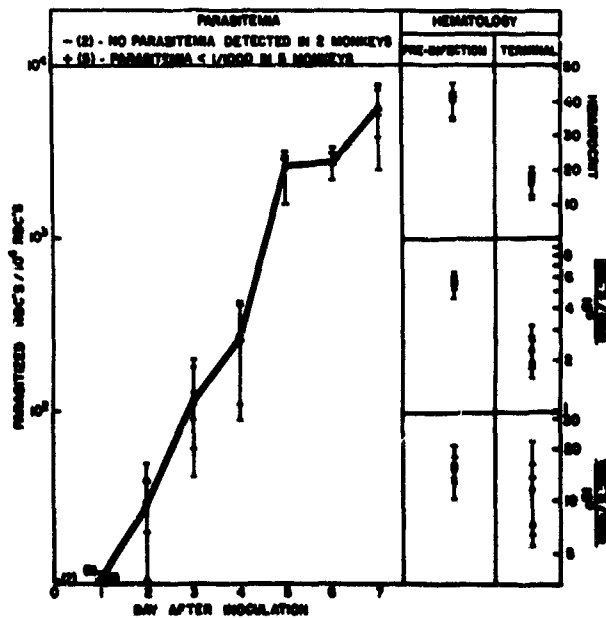


Fig. 5. Parasitologic and hematologic data from seven non-splenectomized owl monkeys infected with P. falciparum (Camp./C).

A comparison of the Camp./W and Camp./C infections (Fig. 4) shows significant differences as well as striking similarities. Prepatent periods were approximately the same, but, despite the fact that Camp./W infections were passaged in splenectomized owl monkeys, the period during which the number of parasites increased logarithmically was delayed approximately 5 days, probably accounting for the increased longevity of the host. Once the increase began, the rate was the same for both strains.

The morphological features of parasites from both isolates were similar and conformed to the original description of P. falciparum in the owl monkey. The infections were rarely synchronous although a 48 hour cycle was usually apparent. At infection levels of less than 5 percent, the population of parasites consisted predominately of small, typical ring forms. Above 10 percent, all asexual forms were represented. Only immature gametocytes were seen occasionally in those animals which survived at least 11 days.

Despite the paucity of gametocytes (morphologically immature), mosquitoes were fed on infected monkeys having gametocytes in an effort to establish the exogenous portion of the cycle. The results of 18 feedings by either A. stephensi or A. balbacensis were consistently negative.

Severe anemias which accompanied the increases in parasitemias (Fig. 5) were evidenced clinically by pale mucous membranes, cold extremities and a tendency for the monkey to tire quickly and to develop marked respiratory distress. Despite these signs, the animals remained alert and active with good appetites until 24-48 hours before death. At that time they became severely depressed and weak, assuming a hunched, head down attitude. Signs of classical paroxysms were not observed. Body temperatures were so highly variable that significant changes could not be detected.

Gross and histopathologic findings were remarkably consistent. Splenomegally and widespread distribution of pigment were seen. The spleen, when present, and liver were blue-black in color, the lungs were gray, and the lymph nodes were grey to black. The presence of malaria pigment in the cells of the reticuloendothelial system was confirmed histologically. A varying degree of hepatic fatty metamorphosis was common and centrilobular necrosis was occasionally observed. No intra or peri-vascular lesions were found although the cardiac endothelium was noted to be heavily pigmented. The central nervous system was normal in appearance except for the presence of malaria pigment. Death appeared to be a result of the anemic state of the monkey.

Preliminary studies using irradiated parasitized cells from P. falciparum infected owl monkeys to actively immunize normal monkeys were conducted (Table 5). Three days following challenge, 3 animals died of a generalized bacterial infection and others were obviously ill. Each of the affected monkeys had a swollen discolored leg where the challenge injection had been made. Bacterial cultures indicated that the challenge

inocula were contaminated with Pasteurella multocida. Two more monkeys died on the fourth day despite initiation of antibiotic therapy. Deaths on day 7 and later were attributed to the experimental infection. Despite the complications encountered, the parasitologic results showed that 4 intravenous immunizations at weekly intervals using  $10^9$  parasitized cells exposed to 25,000 r provided a significant degree of protection to challenge with a viable inoculum of  $10^8$  parasitized RBC's.

Table 5

Challenge of Aotus trivirgatus with Plasmodium falciparum (Camp./C)  
Following Four Immunizations with Irradiated Parasitized  
and Non-Parasitized Erythrocytes

Immunization	Monkey Number	Day of Death	Maximum Parasitemia	
			Day after Challenge	Percent Parasitized RBC's
Irradiated Parasitized RBC's	1	3	--	0
	2	4	--	0
	3	8	5	4.5
	4	19	18	3.4
	5	27	21	1.0
	6	35	15	9.6
	7	--	21	1.6
	8	--	--	0
	9	--	--	0
Irradiated Non-Parasitized RBC's	10	3	3	+
	11	9	8	49
	12	9	8	35
	13	10	9	18
	14	31	29	33
None	15	3	3	++
	16	4	4	++
	17	7	6	48
	18	8	7	62
	19	8	7	73
	20	8	7	76
	21	8	7	78

\*Positive on thick

Several other physiologic, immunologic, morphologic, pharmacologic and biochemical studies of P. falciparum infections in the owl monkey are in progress.

c. P. vivax. Studies with P. vivax (Varnes) were limited to maintenance of the infection by serial passage in splenectomized owl

monkeys and description of the parasitemia and concurrent clinical signs which developed. Nine passages were made in 13 animals. The first infection was established in one of the 2 splenectomized monkeys inoculated with infected human blood. The prepatent period was 35 days and parasites were still present in the peripheral blood 127 days later. The highest level was detected on day 63 when the parasitemia reached 28,000 parasitized RBC's per cmm. blood. The prepatent period in subsequent infections, using inocula containing 10<sup>7</sup> parasitized RBC's, was much shorter, ranging from 0 to 7 days. Maximum parasitemias of 40,000 to 90,000 parasitized RBC's per cmm. of blood were detected between 18 and 36 days after inoculation. Most of the infections remained patent throughout the observation periods of up to 180 days. After the acute phase of the infection parasitemias rarely exceeded 20,000 parasitized RBC's per cmm. of blood and usually were less than 5,000 per cmm.

Parasites in all stages of development were morphologically characteristic of P. vivax in man. Although a few mature gametocytes were usually observed a few days after the monkeys became patent, no attempts were made to infect mosquitoes.

There were no overt clinical signs observed during the course of these infections. Minimal hematologic changes were noted, but these were not always consistent and were difficult to evaluate in view of the requirement for frequent bleeding. The only gross pathologic abnormality associated with the infections was widespread tissue pigmentation which histologically appeared to be confined to the cells of the reticuloendothelial system.

Parasitized blood from infected monkeys was used successfully as antigen for IFA tests conducted with sera from humans and owl monkeys infected with P. vivax.

Recently, splenectomized owl monkeys were shown to be susceptible to blood induced P. vivax (Chesson) infections. The initial prepatent periods were shorter and acute parasitemias somewhat higher than observed in P. vivax (Varnes) infections.

d. P. malariae. A single splenectomized owl monkey which was inoculated with P. malariae infected human blood did not become patent prior to death (22 days later) from causes apparently unrelated to the malarial injection. P. malariae infected chimpanzee blood was injected into 2 splenectomized owl monkeys and parasites identified as P. malariae were detected 100 days later.

The reported susceptibility of the splenectomized chimpanzee to blood induced P. falciparum (Camp.) was confirmed. The predictable prepatency, the high parasitemias and the relatively large size of the chimpanzee made this animal an ideal host from which to collect large quantities of P. falciparum parasites when needed for experimental studies. Thus, it was possible to conduct extensive biochemical, ultramicroscopic

and serologic studies including the fractionation and isolation of diagnostic antigens and the development of new serologic tests.

From these studies at least two promising tests, the SAFA and the micro-method IHA tests, were developed. The SAFA test lends itself to semi-automatic processing and requires minute quantities of antigen which can be supplied readily from a limited number of infected chimpanzees. Therefore, it has great potential for mass screening of sera in blood banks, debarkation points for soldiers returning from malarious areas, endemic areas where malaria eradication programs are in progress and boundary zones between malaria-free and endemic areas.

Our investigators have shown that large numbers of P. falciparum parasites required for antigenic analyses and for biochemical studies can be readily obtained from infected chimpanzees. Parasites for in vitro studies can also be obtained from these animals. However, our experience indicates that since a constant supply of parasite material is essential for continuity of effort in in vitro cultivation this requirement could not always be fulfilled.

The predictable prepatency and subsequent parasitemias of P. falciparum (Camp.) infections in splenectomized chimpanzees made it possible to conduct experiments with both active and passive immunization. Although the number of chimpanzees studied was too few to draw definitive conclusions on statistical grounds, the non-sterilizing immunity demonstrated appeared to be similar to that observed in man living in hyperendemic areas. Also, the apparent lack of protection induced by heterologous strains of P. falciparum was similar to what was observed in semi-immune individuals who were experimentally inoculated with heterologous strains of P. falciparum.

West African human gamma globulin had a prophylactic effect against WA P. falciparum infections in chimpanzees but not against SEA strain infections. However, SEA-USA gamma globulin produced less protection in a homologous system than was demonstrated with the homologous West African system. The SEA-USA gamma globulin may have had a lesser inherent protective quality since it was obtained from patients having a history of a single infection with one or two recrudescences rather than from hyper-immune individuals. Our results indicated immunogenic differences between the WA and SEA strains and proved the potential value of the chimpanzee as an experimental host for falciparum malaria.

Results of chloroquine administration to chimpanzees infected with a chloroquine resistant strain suggest that the chloroquine refractory characteristics of the parasite remain unaltered. After 14 passages, P. falciparum (Camp.) infections in splenectomized chimpanzees are reproducible to such an extent as to suggest considering this host-parasite system as a model for critical testing of new antimalarial drugs, prior to clinical tests.

Results of pathophysiologic studies in chimpanzees infected with P. falciparum (Camp.) indicate that the observed serum biochemical changes

are similar to those occurring in man infected with P. falciparum. Also, P. falciparum (Camp.) infections in the chimpanzee increased the osmotic fragility of the uninfected erythrocytes. Although fever, chills and death were observed in malarious chimpanzees, none of the more dramatic human clinical signs of renal failure, CNS involvement and black water fever were observed. Similarly, although blood clotting defects are demonstrated in human infections, they were not found in infected chimpanzees. To these considerations, one must add the failure to infect mosquitoes from P. falciparum infected chimpanzees.

The chimpanzee is considered a natural host of P. malariae and has been the subject of several experimental infections by this parasite in the past. The single study reported here indicates that infections in splenectomized chimpanzees are similar in many respects to human infections and that the failure to obtain mosquito infections was likely to be the result of a low concentration of gametocytes. As was to be anticipated, the parasitemias observed in the splenectomized chimpanzee were much lower than those demonstrated in P. falciparum infections. Despite this, there were enough parasites on the days of maximum parasitemia for studies requiring large quantities of material. The results were encouraging and the establishment of further infections of P. malariae is being contemplated.

The susceptibility of the owl monkey to blood induced P. falciparum (Camp.) infections was confirmed. The failure to establish infections with two African isolates of P. falciparum are in agreement with the difficulties reported by other laboratories in establishing P. falciparum infections in these monkeys even when the inocula contained large numbers of parasitized RBC's and precautions were taken to avoid deleterious effects on viability and infectivity of the parasites inoculated. On the other hand, a number of strains and isolates from many parts of the world have been established with relative ease. Variations in subspecies of monkeys and differences in physical condition, environment and diet may account for the differences in owl monkey susceptibility to isolates of P. falciparum if, in fact, disparities exist.

P. falciparum (Camp.) infections in owl monkeys after the first six or seven passages were consistent and reproducible, but the gametocytes detected were morphologically immature and failed to infect mosquitoes. This contrasts with the work of Collins, et al. in which A. freeborni mosquitoes were fed on 2 splenectomized P. falciparum-infected owl monkeys. The mosquitoes became infected and subsequently the infections were transmitted to two volunteers. Explanations for these differing results may reside in the different species of mosquitoes used, in the lower concentration of gametocytes and in the fact that the P. falciparum (Camp.) isolate has a history of consecutive blood induced passages first in volunteers, then chimpanzees and finally in owl monkeys. There is considerable evidence that blood passages in abnormal hosts may result in lowered gametocyte production and reduced mosquito infectivity.

Because of its smaller size the owl monkey was not as satisfactory an experimental host for producing large volumes of P. falciparum parasitized blood as was the chimpanzee. However, in some laboratories it may be practical to obtain parasites by pooling blood from several infected owl monkeys. The small size and ready availability of the owl monkey made it possible to design experiments requiring relatively large numbers of concurrently infected animals for quantitative studies, or experiments requiring many individually infected monkeys on a regular schedule.

An example of the former type of study was the attempt to actively immunize owl monkeys with irradiated parasitized RBC's from P. falciparum infected monkeys. However, the difficulty experienced because of concurrent bacterial and/or viral infections demonstrates some of the major problems encountered in dealing with the owl monkey in the laboratory. When healthy, well conditioned animals become available, active and passive immunization experiments, in vivo drug studies and other similar investigations will become more practical.

Infections of P. falciparum (Camp./C) were regularly induced in owl monkeys for a series of studies, all of which are still in progress. These include pathophysiological and immunological studies of infections in the owl monkey and biochemical, pharmacological and morphological studies of parasites free of the host cells or of the parasitized RBC's in cultures. Results to date have been encouraging.

Blood induced P. vivax (Varnes) and P. vivax (Chesson) infections in splenectomized owl monkeys were similar to vivax malaria infections reported earlier in this host. The success reported in cyclically transmitting P. vivax from owl monkey to owl monkey and to man suggests that this is a very satisfactory host for P. vivax. Limited pathophysiological and immunological studies of the infections were initiated and are still in progress. Because of the monkey's size and the relatively low parasitemias observed, no experiments requiring large numbers of parasites are contemplated at this time.

The reported susceptibility of the owl monkey to P. malariae infections was recently confirmed. Comparative data are limited to the long prepatent period of approximately 100 days and the morphology of the parasites which was typical of P. malariae in all cases.

In comparing the relative usefulness of chimpanzees and owl monkeys for studies with human plasmodia, the results of our extensive studies, in general show that the chimpanzees can be used to great advantage as a source of parasites and for critical investigations requiring small numbers of animals. On the other hand, the owl monkey is ideally suited for those investigations requiring several animals or a constant supply of limited numbers of parasites.

Of the four species of great apes, chimpanzees are the most readily obtainable for research purposes in the United States. However, they

are relatively rare and their indiscriminate use for any purpose is not justified. They are expensive to purchase and even more costly to maintain since they require special housing and care. Because of their size and tremendous strength, untrained chimpanzees are hazardous to handle. They also represent a zoonotic problem since they may harbour many organisms infectious to man including the virus of infectious hepatitis. The requirement for splenectomy and anti-malarial treatment prior to initiation of an experimental malaria infection subjects the animals to risky procedures and increases the time which they must be maintained in the colony. However, once adjusted to the laboratory environment, chimpanzees are extremely hardy animals which heal from surgical procedures quickly and respond well to treatment. Moreover, the trained chimpanzee is amenable to the required bleeding and examination procedures with only minimal restraint and encouragement.

In contrast to chimpanzees, owl monkeys are small, readily available and relatively inexpensive animals. They require little in the way of special housing, are easy to handle, although subject to injury, and require no training. However, these monkeys adapt slowly to the laboratory environment provided and are highly susceptible to stress and to infectious diseases. Their proven susceptibility to fatal infections of Herpesvirus hominis requires that precautionary measures be taken to protect owl monkeys from undue human exposure. In turn, although tuberculosis and protozoan and helminthic infections are apparently not a zoonotic problem, some owl monkeys are collected in yellow fever endemic areas and apparently all are subject to infection by Herpesvirus T. There is no evidence to date that these monkeys carry yellow fever and the significance of the latter infection as far as man is concerned is not known.

There have been no reports in the literature of spontaneous malarial infections in the owl monkeys nor were any detected in this laboratory before or after splenectomy, thus eliminating the need for pre-infection anti-malarial treatment. One isolate of P. falciparum (Camp./C) produced fulminating infections in non-splenectomized animals thus reducing the requirement for this surgical procedure.

Once the means are found to establish and maintain healthy laboratory conditioned owl monkeys, the seemingly unlimited potential of this animal as an experimental host of human malaria can be more fully exploited.

( ) Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

( ) Work Unit 127, Test systems for Plasmodium falciparum

2. Publications.

No publications.

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23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Precede individual paragraphs identified by number, precede text of each with security Classification Code.) 23(U) To evaluate the role of humoral and cellular factors in determining susceptibility of hosts to rodent malaria, for the maintenance of the complete life cycle of malaria in the laboratory, to find a laboratory animal suitable and for the production of large amounts of infected blood for immunological and biochemical studies.  24(U) Test a variety of rodent species for natural susceptibility to P. berghei. Attempt to increase susceptibility by splenectomy and chemical treatment. Standardize the course of infections quantitatively. Evaluate the mechanism of antibody action on host and parasite, and characterize antibodies responsible for these activities. Study the effects of antibody on the parasite and on the host.  25(U) 69 01 - 69 06 Neonatally thymectomized rats infected with P. berghei develop higher percent parasitemias and have a higher percent mortality than sham operated animals. The degree of lymphocyte suppression in a thymectomized group can be used as an index of impairment of the host response to infection. The increased morbidity and mortality in thymectomized rats could not be explained on the basis of decreased phagocytic activity or antibody production. These studies suggest that thymic dependent cellular immunity is important in the development of acquired resistance to P. berghei infection. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

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Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 128, Natural and acquired immunity in rodent malaria

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1. Plasmodium berghei infection in thymectomized rats.

Rats that survive a Plasmodium berghei infection demonstrate a strong resistance to reinfection when rechallenged with parasites of the same strain. It is assumed that acquired immunity plays some role in controlling the initial and in preventing subsequent infections. This is supported by studies which demonstrate the presence of protective antibody in the serum of previously infected animals. Enhanced phagocytic activity of the reticuloendothelial system may also be involved in this resistance to reinfection.

Neonatal thymectomy of the rat is known to alter the animals immune responses. One of the major effect, that on cellular immunity, is manifested by delayed skin homograft rejection and impaired delayed type hypersensitivity reactions. Thymectomized rats also have decreased antibody production following immunization with certain antigens.

A preliminary report indicates that thymectomized rats have a higher mortality from P. berghei than non-thymectomized controls. No information is available concerning the mechanism by which neonatal thymectomy might alter the immune response to this parasitic infection. The following study confirms the higher mortality from P. berghei infection in thymectomized rats and evaluates the antibody response and phagocytic activity in animals. The susceptibility of thymectomized and non-thymectomized rats to reinfection was also studied.

Animals. WRCF rats derived from the Wistar strain were used throughout the study. Litters were weaned at age 20-28 days. All animals were fed a standard diet. The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

Surgical Procedure. Thymectomy was performed within 36 hours of birth. Hypothermic anesthesia was utilized as previously described. Sixty to 70 percent of the animals in each litter were randomly chosen for thymectomy and the remainder were sham-operated. Animals with

stitch abscesses or those cachetic in appearance were excluded from the study. All animals that were alive at the end of the experiment were necropsied to verify the results of surgery.

White Blood Cell Counts. In some experiments repeat total WBC and differential counts were obtained from the tail blood of the rats between 20 and 30 days of age and the mean values for each determined. The median lymphocyte count of thymectomized animals in any particular experiment was determined and the rats were then subdivided into high and low lymphocyte groups.

Infection. The N.Y.U. 2 strain of Plasmodium berghei was maintained in the laboratory with bi-weekly passage in young rats. Groups of experimental animals were infected at 28 to 45 days of age by intraperitoneal injection of parasitized erythrocytes. Progress of the infection was determined by counting parasitized cells per 500 erythrocytes on Giemsa stained smears obtained every 2 to 3 days from tail bleedings.

Antibody determinations. Serum was obtained by orbital sinus bleedings 11 to 13 days after infection. Indirect fluorescent antibody titers were determined by methods previously described. Indirect hemagglutinating activity of the sera was studied by using tanned sheep red blood cells sensitized with a soluble P. berghei antigen.

Carbon Clearance. The non-specific phagocytic capacity of the reticuloendothelial system was measured by determining the rate of carbon clearance from blood. Rats were injected intravenously with 10 mg of colloidal carbon per 100 mg body weight; blood specimens were obtained at 5 minute intervals and the carbon level and rate of disappearance calculated.

Post-Operative Mortality. Within 72 hours after surgery 29 percent of the operated animals had died. This figure represented death due to cannibalism, blood loss and the surgical procedure. By 14 days an additional 6 percent had succumbed due to undetermined causes and thereafter the mortality rate from causes other than malaria was negligible. The mortality rates of the thymectomized or sham-operated animals were not significantly different.

Effect of Thymectomy on Carbon Clearance. Forty-four day old sham-operated and thymectomized animals were studied for rates of carbon clearance. The average clearance rate in the thymectomized group was slightly faster than that observed in the sham-operated rats.

Effect of Thymectomy on the Lymphocyte Count. Previous workers have shown that thymectomized animals have a decreased population of lymphocytes. As noted in Table 1, considerable variation in the lymphocyte counts was observed in all groups. Only when the thymectomized

animals were divided was there a group identifiable by a significant suppression of the number of circulating lymphocytes. At autopsy up to 40 percent of the animals in some of the high lymphocyte thymectomized groups contained a small remnant of thymic tissue. Thymic remnants were found in less than 5 percent of the animals in the low lymphocyte groups.

Table 1

White Blood Cell and Lymphocyte Counts  
in Normal, Sham-Operated and Thymectomized Rats

Group	WBC mm <sup>3</sup>	Lymphocytes mm <sup>3</sup>
Normal (31)*	9135 ± 1508**	6897 ± 1133
Sham-operated (15)	9783 ± 1933	6945 ± 1401
Thymectomized (10) (high lymphocyte)	9411 ± 2595	5912 ± 1753
Thymectomized (11) (low lymphocyte)	6390 ± 865	3784 ± 1389

\* Number of animals per group.

\*\* Mean ± one standard deviation.

Effect of Thymectomy on *P. berghei* Infection. In Experiment No. 1, thymectomized and sham-operated animals at 40 days of age were injected intraperitoneally with  $2 \times 10^7$  parasitized erythrocytes. The course of the infection is shown in Figure 2. The level of parasitemia in the high lymphocyte thymectomized rats was the same as that noted in the sham-operated controls. The low lymphocyte thymectomized group had a higher parasitemia for a longer duration and two of the 11 animals died. At age 82 days the groups were reinjected intraperitoneally with  $3 \times 10^8$  parasitized cells. No parasites were seen in the peripheral smears from any of the animals. In repeat experiments using the same conditions, the percent parasitemias of the high lymphocyte thymectomized group was intermediate between the sham-operated and low lymphocyte thymectomized groups. The percent mortality from infection in thymectomized and sham-operated animals is shown in Table 2. The mortality in unselected thymectomized rats was, in all experiments, higher than the mortality in sham-operated controls. When the thymectomized animals were divided into a high and low lymphocyte group, the low lymphocyte group had the higher percent mortality.

Normal animals, comparable to those in Experiment No. 1 in both age and weight, were injected with the same number of parasites and the course of infection was similar to that observed in the sham-operated and high lymphocyte thymectomized groups. Five of the normal animals with the highest mean lymphocyte count were compared with the five normal animals having the lowest mean lymphocyte count. The percentages of parasitized RBC's in these two groups of normal animals were essentially the same throughout the infection.

Table 2

Percent Mortality in Thymectomized and Sham-Operated Rats Infected with P. berghei

Experiment	Infected* at Age	Percent Mortality
Number 1	40 Days	
Sham-operated		0 (15)
Thymectomized		
High lymphocyte		0 (10)
Low lymphocyte		18.2 (11)
Number 2	28 Days	
Sham-operated		33 (15)
Thymectomized		
High lymphocyte		50 (10)
Low lymphocyte		100 (10)
Number 3	45 Days	
Sham-operated		0 (15)
Thymectomized**		41 (27)

\*  $2 \times 10^7$  parasitized erythrocytes.

( ) Number of animals per group.

\*\* No separation according to lymphocyte count was done.

Effect of Thymectomy on Antibody Response. The effect of thymectomy on the antibody response to P. berghei during the infection was studied by using indirect hemagglutinating and fluorescent antibody techniques. Serum was obtained from 11-13 days after infection and the results are shown in Table 3. The hemagglutinating titers were obtained from pools of serum collected 13 days after infection. Serum from the thymectomized group was positive at a greater dilution than the serum collected from the sham-operated animals at a time when the percent

parasitemia was higher in the thymectomized group. In another group of rats fluorescent antibody titers were determined on individual serum samples collected 11 days after infection. The mean titer for each group is shown in Table 3. No significant difference in fluorescent antibody titers was noted.

Table 3

Antibody Response to *P. berghei* Infection  
in Thymectomized and Sham-Operated Rats

Group	Titer	Percent Parasitemia
	Indirect Hemagglutinating	
Thymectomized (11)	320*	27
Sham-operated (8)	80	12
	Fluorescent Antibody	
Thymectomized (11) (low lymphocyte)	41	22
Thymectomized (10) (high lymphocyte)	56	15
Sham-operated (15)	50	18

( ) Number of animals per group.

\* Titer expressed as the reciprocal of the highest positive dilution.

**Discussion.** These studies in neonatally thymectomized rats demonstrate an impaired host-response to *P. berghei* infection and are in agreement with earlier observations. Reinfection of the thymectomized animals failed to demonstrate the continued presence of this impaired host-resistance. The variable response noted in thymectomized rats could be largely eliminated by dividing the animals into high and low lymphocyte groups. Small thymic remnants were detected in a greater number of rats in the high than in the low lymphocyte group. This offers some explanation for the variability noted in unselected thymectomized animals.

The effect of neonatal thymectomy in rodents has been extensively studied. It is accepted that the primary effect is on cellular immunity as manifested by delayed skin homograft rejection and impaired delayed type hypersensitivity reactions. This effect has been documented in the rat. Conflicting evidence is available concerning the effects of neonatal thymectomy on the

antibody response in rats. Immunoglobulin levels are not depressed, however, some evidence indicates that the production of a specific class of immunoglobulin is selectively inhibited, but this has not been confirmed by other workers. In view of these findings the importance of thymic-dependent antibody production is uncertain.

The exact mechanism by which thymectomy alters the host response to P. berghei infection is not known. A non-specific decrease in the phagocytic capacity of the reticuloendothelial system could explain the higher parasitemias noted in the thymectomized animals, but this is unlikely in view of the increased rate of colloidal carbon clearance noted in thymectomized rats. These results are consistent with the earlier report of enhanced clearance of colloidal carbon in thymectomized rats after an initial leading dose. Another possible explanation might be a decreased antibody response to the parasite. In these studies the measurable antibody level in thymectomized rats infected with P. berghei was equal to or greater than the antibody level in sham-operated groups (Table 3). Although it is recognized that the techniques used to measure the antibody response in these studies might be unsatisfactory for the detection of a specific type of immunoglobulin, it seems unlikely that the increased parasitemia and higher percent mortality (Table 2) can be attributed to impaired antibody production. No direct measurement of cellular immunity is available but the effect of neonatal thymectomy on this system is widely accepted. The impairment of cellular immunity could conceivably result in increased susceptibility to the effects of P. berghei infection and in the above studies an alternative explanation is not available. These experiments support the concept that cellular immunity participates in the development of acquired resistance in P. berghei infected rats.

## 2. Cell-mediated immunity in rats infected with Plasmodium berghei.

The role of humoral immunity in rodent malaria is well established. Following the observation of Corradetti that rats after recovery from P. berghei infection were resistant to reinfection, suggesting the development of acquired immunity to this parasite, other investigators obtained evidence that a humoral factor, presumably antibody, was an important participant in acquired immunity. These observations have been extended and recent studies have attempted to identify the type or types of immunoglobulin that function as protective antibody. The following studies were undertaken to investigate the capacity of immune lymphoid cells to transfer protection to recipient animals infected with P. berghei. This communication demonstrates that immune lymphoid cells are capable of conferring resistance to recipient animals, compares the relative protective capacity of lymphoid cells obtained from different sources, and suggests that macrophages are incapable of transferring protection.

Animals. The animals used in the following experiments were inbred male rats of either the Lewis or Fischer strain. In each experiment, the cell donors and cell recipient rats were of the same inbred strain. All animals were fed a standard diet and the principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

Plasmodium berghei infection. The NYU-2 strain of P. berghei was maintained by weekly passage in young rats. Parasites obtained from animals one week after infection were used as the infecting inoculum in all experiments.

Immune rats. Adult rats of either the Lewis or Fischer strain were inoculated with  $3 \times 10^7$  erythrocytes parasitized with P. berghei. Thirteen to 15 days after infection the animals had cleared the parasites from the peripheral circulation and on rechallenge with P. berghei were found to be resistant to reinfection as previously reported. In these studies, rats that had recovered from P. berghei infection were considered to be immune animals and lymphoid cell obtained from them, as described below, are referred to in the text as immune lymphoid cells.

Collection and handling of cells from donor rats. The thoracic duct in rats weighing 300-350 gms was cannulated as described by Wilson. Lymph was collected for 24 hours at  $4^{\circ}\text{C}$  into Ringer's lactate solution containing 25 units of heparin per ml.

Peritoneal macrophages were collected from rats 48 hours after the intraperitoneal injection of beef heart infusion broth fortified with proteose peptone as described by Fishman.

The anterior and posterior cervical, mediastinal, inguinal and mesenteric lymph nodes were excised from rats immediately after light ether anesthesia and exsanguination. The nodes were placed in a chilled nutrient solution, minced and teased into small pieces. The mixture was then passed through a 40-gauge wire screen which retained the fibrous stroma on the screen and allowed the cells to pass through. The spleens were removed from the same animals and a cell suspension was obtained in an identical manner as described for cell extraction from lymph nodes. In some experiments one pool of cells was made from lymph nodes and spleens and is referred to as lymphoid cells. Cells obtained from either lymph nodes or spleens are referred to as lymph node or splenic lymphoid cells.

The cells collected from the thoracic duct, peritoneal cavity, lymph nodes, spleens or lymph nodes and spleens were separated by centrifugation washed twice in Ringer's lactate and then resuspended in this solution. An aliquot of each pool was counted in a hemocytometer, stained with Giemsa stain for differential cell counting and also strained with 0.10% trypan blue. The cells excluding this dye were considered to be viable. Whenever cell viability was less than 98% the total cell count was adjusted so that this value always represents the viable count for each pool.

The cells obtained from the thoracic duct were essentially all small lymphocytes with an occasional polymorphonuclear leukocyte noted. The cells obtained from lymph nodes and spleens were primarily large and small lymphocytes (85-90%) but macrophages (5-10%) and polymorphonuclear (PMN) leukocytes (5-10%) were also present. The cells obtained from the

peritoneal cavity of rats contained 70-75% macrophages, the remainder consisting of PMN leukocytes and mononuclear cells.

Recipient animals. Rats weighing 75-85 gms were injected intravenously with cells (pretreatment) and then injected intraperitoneally with P. berghei. The type and number of cells used for pretreatment and the interval between pretreatment and infection are described in the text for each individual experiment.

Quantitation of parasitemia. Thin blood smears were made from tail blood of individual rats every second and third day throughout the course of infection. The slides were dried, fixed and stained with Giemsa stain. The percent parasitemia was determined from the proportion of 500 counted erythrocytes that contained parasites. For each experimental group a mean value for a given day was calculated.

Protective effect of lymphoid cells. In the initial studies the lymph nodes and spleens were removed from donor animals 35 days after challenge with P. berghei infection. The lymphoid cells were obtained from these organs as described and then recipient animals were pretreated by the intravenous injection of these cells. Pretreatment control animals were injected with cells obtained from normal lymph nodes and spleens. Seven days after pretreatment all animals were challenged with a P. berghei infection and the resultant parasitemia followed (Fig. 1). The two groups receiving either  $24 \times 10^7$  normal lymphoid cells or no pretreatment had a similar course of infection with peak parasitemias on day 13; the group that received normal cells cleared the parasites at a slightly faster rate than the group that received no cell pretreatment. The animals that received  $25 \times 10^7$  immune lymphoid cells had only an occasional parasite on smears obtained from peripheral blood. The rats that received  $2.5 \times 10^7$  immune lymphoid cells had a peak parasitemia on day 4 and this had cleared by day 13.

It was important to determine how soon after an infection the lymphoid cells acquired the capacity to transfer protection to the recipient animals. It was also of interest to determine if a latent period between pretreatment with cells and infection with P. berghei was necessary for the demonstration of protection. Cells were obtained from donor animals 13, 27 and 62 days after challenge with P. berghei. Groups of rats were injected intravenously with equal numbers of cells and injected with P. berghei within 30 minutes. The resultant parasitemias were then studied (Fig. 2). The experimental groups receiving lymphoid cells obtained from donor animals 27 or 62 days after challenge with P. berghei had low grade parasitemias which after day 5 was less than 0.5%. The experimental group that received lymphoid cells from donor animals 13 days after infection had an average parasitemia which reached the peak at 9% on day 9 and then rapidly subsided. In contrast, both control groups had significantly higher parasitemias which reached a level of 50% parasitization of erythrocytes on day 15. The rats pretreated with normal cells were able to overcome the parasitemia, but the 3 control rats that received

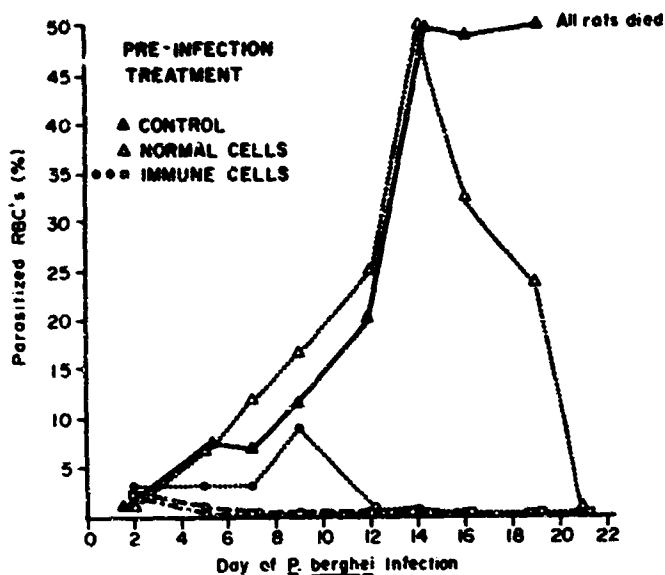


Figure 1. The course of *P. berghei* infection in rats, each pretreated with  $24 \times 10^7$  normal lymphoid cells ( $\Delta$ );  $25 \times 10^7$  ( $\square$ ) or  $2.5 \times 10^7$  ( $\circ$ ) lymphoid cells obtained from rats 35 days after challenge with *P. berghei*. Control rats ( $\blacktriangle$ ) received no pretreatment.

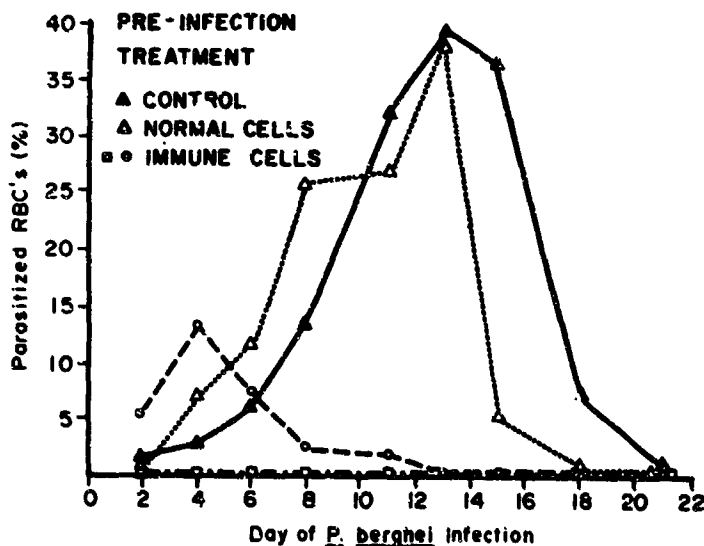


Figure 2. The course of *P. berghei* infection in rats, each pretreated with  $12 \times 10^7$  lymphoid cells obtained from rats 13 days ( $\bullet$ ), 27 days ( $\circ$ ) and 62 days ( $\square$ ) after challenge with *P. berghei*. Control rats ( $\blacktriangle$ ) received no pretreatment or  $12 \times 10^7$  ( $\triangle$ ) lymphoid cells obtained from normal animals.

no cells succumbed to the infection. In similar experiments, the length of time after challenge with P. berghei that lymphoid cells maintain the capacity to transfer protection to recipient animals was extended to 105 days. In these studies the lymphoid cells obtained from animals challenged with P. berghei 105 days previously transferred the same degree of protection as observed with the 27 or 62 day lymphoid cells.

Identification of protective lymphoid cells. An attempt was made to identify the type of cell responsible for passive transfer of protection against P. berghei. Lymphoid cells were obtained from either the lymph nodes of the pleens of animals previously challenged with P. berghei. Comparable numbers of these cells were injected intravenously into recipient animals, then the animals were injected with P. berghei and the course of infection was followed (Fig. 3). The group that received splenic lymphoid cells had a low grade parasitemia with the highest level being 6% of the peripheral erythrocytes parasitized on day 4. This group rapidly cleared the parasites and after day 11 only an occasional ring form was seen in the blood smears. The group that received lymph node cells had a higher average percent parasitemia which reached the peak at 25% on day 11 and then decreased gradually. A similar course of infection was observed in the group receiving normal lymphoid cells or no pretreatment. The control groups had significantly higher parasitemias which reached the highest levels on day 15. The control group receiving no pretreatment cleared the parasitemia at a slightly faster rate than the normal cell recipients and both control groups overcame the infection 3 to 4 days before clearing was noted in the group that received immune lymph node cells. This type of experiment was repeated in groups of 5 rats each. Lymphoid cells ( $9 \times 10^7$ ) were obtained from either the lymph nodes or spleens of immune animals. They were injected into recipient rats and the subsequent P. berghei infection was studied (Fig. 4). The group receiving splenic lymphoid cells was protected against P. berghei infection. The group receiving lymphoid cells obtained from lymph nodes was partially protected as compared to the group receiving no pretreatment.

Lymphoid cells were also harvested by thoracic duct drainage. Thoracic duct cells from the same group of donor animals that supplied lymphoid cells from the nodes and spleens were obtained and compared for their ability to transfer protection to recipient animals (Fig. 5). The group of rats that received the thoracic duct lymphocytes was not protected as indicated by comparable levels of parasitemia in this group and the control rats. The groups of rats that received lymphoid cells from lymph nodes and spleens were protected throughout the course of infection. It is noteworthy that the group pretreated with thoracic duct lymphocytes received 3 times more cells than the group pretreated with the low dose of lymphoid cells. There was no evidence of protection in the former group in contrast to complete protection in the latter.

It was known that in addition to lymphoid cells, a small percentage of macrophages were present in the cell populations obtained from the lymph nodes and spleens. To study the effect of macrophages on the

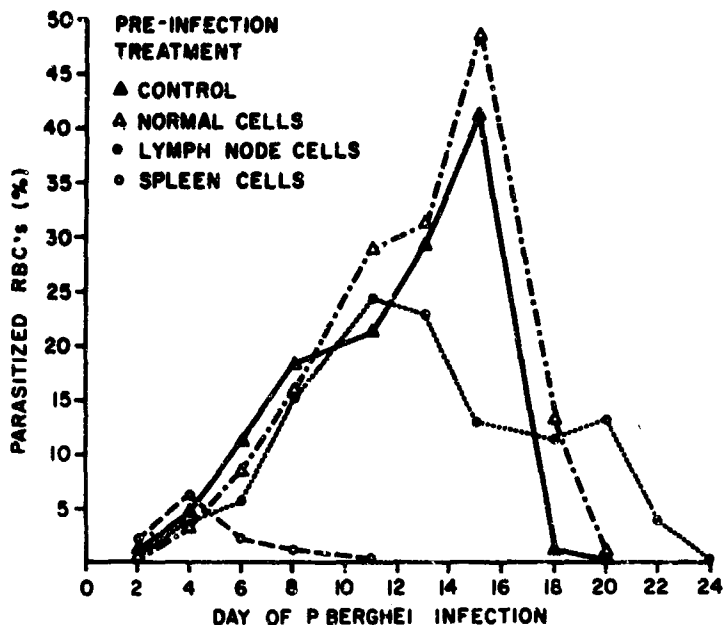


Figure 3. The course of *P. berghei* infection in rats, each pretreated with  $5 \times 10^7$  (○) lymphoid cells obtained from the spleen or  $4 \times 10^7$  (●) lymphoid cells obtained from lymph nodes. The nodes and spleens were removed from donor animals 80 days after challenge with *P. berghei*. Control rats (▲) received no pretreatment or  $5 \times 10^7$  (△) lymphoid cells obtained from normal animals.

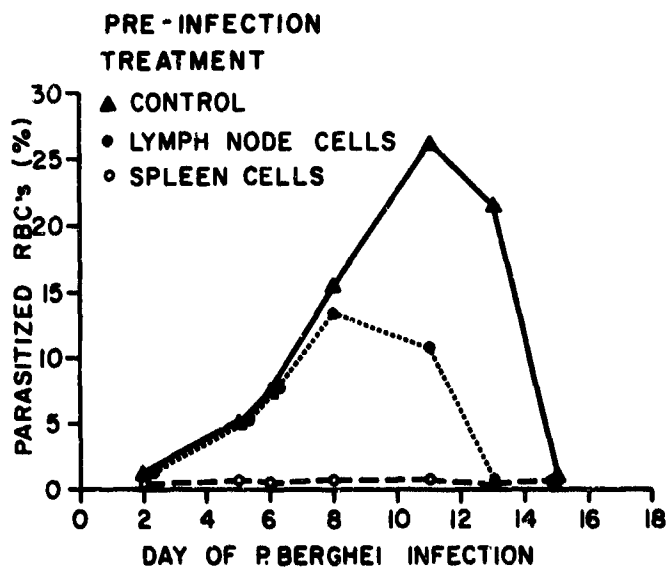


Figure 4. The course of *P. berghei* infection in rats, each pretreated with  $9 \times 10^7$  (●) lymphoid cells obtained from the spleen or  $9 \times 10^7$  (○) lymphoid cells obtained from lymph nodes. The nodes and spleen were removed from donor animals 69 days after challenge with *P. berghei*. Control rats (▲) received no pretreatment.

P. berghei infection these cells were obtained from the stimulated peritoneal cavities of normal and immune rats. Rats were then pretreated with macrophages and infected with P. berghei. No significant difference in parasitemia was noted between any of the groups (Fig. 6).

Under the conditions used in these experiments, lymphoid cells were capable of transferring protection to recipient animals only when the cells were obtained from donor animals previously challenged with P. berghei. In the initial studies (Fig. 1), recipient rats were pretreated with lymphoid cells 7 days prior to infection. With the long interval between pretreatment and infection it was possible that the observed protective effect could be the result of active immunization. Since lymphoid cells were obtained from the lymph nodes and spleens of previously infected animals, the pretreatment inoculum could contain processed P. berghei antigen or small numbers of parasites. Both of these possibilities could significantly alter the course of infection if pretreatment of recipient animals occurred a number of days prior to infection. To reduce this possibility to a minimum, the interval between pretreatment and infection was decreased to 30 minutes or less in later experiments. The protective effect of immune lymphoid cells was the same whether the interval between pretreatment and infection was 7 days or 30 minutes (Figs. 1 and 2). Thus, it seems highly probable that the observed protection against P. berghei in the pretreated animals was due to the effect of passively transferred immune lymphoid cells.

The development of immune lymphoid cells in donor animals occurred as early as 13 days following challenge (Fig. 2) and persisted for at least 105 days. The lymphoid cells obtained 13 days after challenge with P. berghei were not as protective on a per cell basis as those obtained 27 or more days after infection of the donor animal. It is of interest that the level of parasitemia in the donor animals begins to decline rapidly sometime between 10 and 15 days and it is during this time that lymphoid cells with some protective capacity are first demonstrated.

Pools of lymphoid cells were initially obtained from a combination of lymph nodes and spleens. When lymphoid cells were collected from either lymph nodes or spleens excised from a single group of donor animals and then compared for protective effect on a per cell basis (Figs. 3 and 4), it was apparent that the splenic population had a greater protective effect in recipient animals than the population obtained from lymph nodes. In addition, thoracic duct lymphocytes had no demonstrable protective effect under the conditions studied (Fig. 5). These results indicate that the fixed cell populations obtained primarily from the spleen and also the lymph nodes are capable of transferring protective immunity to recipient animals. In contrast, lymphocytes obtained from the thoracic duct, in the numbers used, were unable to transfer protective immunity. This may indicate that the small lymphocyte population obtained from the thoracic duct contributes little, if any, protection in this model system. The cell populations obtained from the spleen and lymph nodes contain many medium and large sized mononuclear cells, presumably lymphocytes, and it may be that these cells transfer the observed protection.

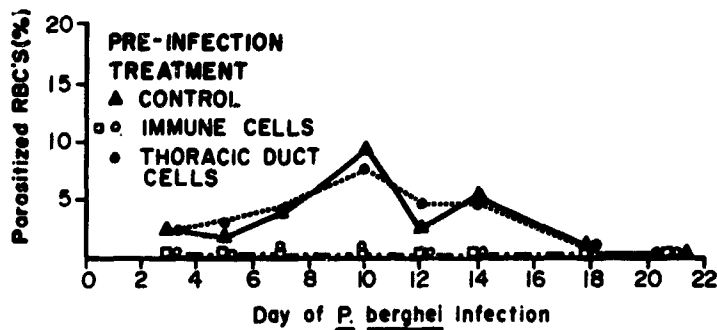


Figure 5. The course of *P. berghei* infection in rats, each pretreated with  $12 \times 10^7$  (◻) or  $1.2 \times 10^7$  (○) lymphoid cells obtained from lymph nodes and spleens; or pretreated with  $3.6 \times 10^7$  (●) lymphoid cells obtained from thoracic duct drainage. The cells were obtained from donor animals 46 days after challenge with *P. berghei*. Control animals (▲) received no pretreatment.

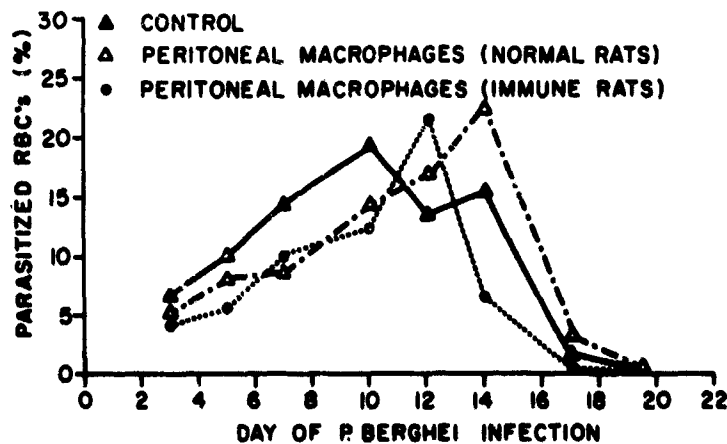


Figure 6. The course of *P. berghei* infection in rats, each pretreated with  $7.2 \times 10^6$  (△) peritoneal macrophages from normal rats,  $7.2 \times 10^6$  (●) peritoneal macrophages obtained from donor animals 21 days after *P. berghei* challenge, or rats (▲) that received no pretreatment.

It is not known whether the greater protective effect observed with splenic cells is due to a quantitative or a qualitative difference between these cells and those obtained from lymph nodes. It may be possible to answer this question when complete cell fractionation is achieved with both lymph node and spleen cell populations.

A certain number of macrophages are present in the cell population obtained from both lymph nodes and spleens. What effect these cells have on the course of infection in pretreated animals is not known. In an attempt to evaluate the effect of macrophages, this type of cell was obtained from the stimulated peritoneal cavities of immune and normal rats and studied for protective effect (Fig. 6). No significant difference in any group was observed but this does not exclude the role of macrophages in passive transfer of immunity. Peritoneal macrophages are not necessarily identical to splenic macrophages and the quantity used may have been insufficient to demonstrate protection. The previously mentioned cell fractionation of the splenic population can be expected to shed light on this question.

Although the ability to passively transfer immunity to P. berghei with a lymphoid cell population has been demonstrated, the effector mechanism of this protection has not been characterized. The ability of transferred cells to produce antibody is well documented and as previously cited, humoral antibody is known to contribute to the acquired resistance to P. berghei infection. Therefore, it can be assumed that some degree of enhanced antibody production occurs in the pretreated animals. At the present time it is impossible to definitively separate the effects of humoral or cellular immunity in the development of acquired resistance to P. berghei infection. However, previous investigators who compared transferred peritoneal exudates with lymph node cells in rabbits and mice, have found a similar antibody response in the recipient animals. The peritoneal exudate cells as studied in this report were unable to transfer protection to the recipient animals. This suggests that a mechanism, presumably cellular immunity, in addition to antibody production, contributes to the development of acquired immunity in P. berghei infected rats.

### 3. Characteristics of protective antibodies in rats infected with Plasmodium berghei.

Plasmodium berghei infections in rats elicit an immunity which controls the course of a primary infection and confers resistance to later homologous challenge. The degree of resistance developed depends in part on the age of the host, older animals being more resistant than younger animals. There is indirect evidence that cellular immunity may be involved in this resistance to P. berghei infection. Other reports implicate humoral factors, presumably antibodies as participants in the expression of this acquired immunity. The role of humoral factors in suppressing mouse and rat infection of P. berghei has been evaluated in two different test systems. Both systems show the greatest anti-parasitic or protective effect in sera from rats receiving repeated immunizing infections. Although several classes of rat antibody have been described

only IGG has been identified as containing antibody protective against malaria infection. In the following study, rat anti-P. berghei serum obtained from animals at different times following infection was fractionated by ammonium sulfate precipitation, gel filtration, and ion exchange chromatography. These fractions were then tested for the ability to suppress the infection in a mouse test system.

Animals. The mice used in the experiments were ICR strain males weighing 20-25 gms. WRCF rats derived from the Wistar strain were used throughout the study for the preparation of antiserum. All animals were fed a standard diet.

Test systems. Globulin fractions of pooled rat antisera were evaluated for protective activity by methods quite similar to those reported previously for evaluating unfractionated antisera. Groups of 5 mice each were injected intravenously with  $2 \times 10^7$  parasitized RBC's; one hour later experimental mice were injected intravenously with 0.5 ml immune globulin. Controls were either left untreated or were injected with normal or control rat globulin. Parasitemias in individual test mice were determined from blood smears prepared on days 3 and 4 after infection, i.e., by determining the percentage of infected RBC's in a sample of 200 counted cells.

The indirect hemagglutination test using antigens extracted from lysates of P. berghei erythrocytes was performed by previously described technic.

Production of anti-P. berghei and control sera. Antiserum was prepared by inoculating 150-200 gram WRCF rats intraperitoneally with the NYU-2 strain of P. berghei which had been maintained in young rats for more than 3 years. Antisera (designated as antiserum No. 1) was obtained from rats 18 days after they had been inoculated with  $1 \times 10^8$  parasitized RBC's. Serum from rats given 4 inoculations of  $1 \times 10^8$  parasitized RBC's per inoculation on days 0, 21, 28 and 35 and bled on day 43 was designated as antiserum No. 2. Control sera was obtained from rats inoculated with diluted normal rat blood following the above schedules. Antiserum No. 3 was collected from rats immunized over a prolonged period (2-5 mos.); these rats were given 5 to 9 immunizing inoculations ranging in size from  $1 \times 10^7$  to  $6 \times 10^7$  parasitized RBC's. Other antisera were obtained from rats 6, 12 and 18 days after inoculation with  $1 \times 10^8$  parasitized RBC's. Control sera (CS6, 12, 18) were obtained from rats on days 6, 12 and 18 after inoculation of diluted normal rat blood. Normal rat serum (NS) was obtained from untreated rats. Serum was collected by cardiac puncture after the rats were anesthetized with ether. The blood was allowed to clot at room temperature; the clot was then ringed and allowed to retract overnight at  $4^\circ\text{C}$ . The following morning the samples were centrifuged and the serum was collected and pooled.

Fractionation of serum. Salt precipitation. The globulins were precipitated from serum at room temperature by adding an equal volume of saturated ammonium sulfate with constant stirring. The precipitate was removed by centrifugation, washed twice with 50% saturated ammonium

sulfate and then suspended in phosphate-buffered saline (PBS), pH 7.2. The ammonium sulfate was removed by passage of the material over a column of Sephadex G-25 or prolonged dialysis. The globulins were routinely suspended in PBS to 3 times the concentration of the original serum. Globulins precipitated from serum taken early in the course of *P. berghei* infections (i.e., 6, 12 and 18 days) were labeled IG 6, IG 12, IG 18A and IG 18B, respectively. Control globulins from rat sera obtained 6, 12 and 18 days after an infection of dilute normal rat blood were designated CG 6, CG 12, and CG 18, respectively. Globulins obtained from hyperimmune rat serum were labelled HIG 1 (4 inoculations) and HIG 2 (5-9 inoculations) while control globulin from rats receiving 4 injections of dilute normal rat blood were labelled CG-H. Normal globulin (NG) was prepared from sera obtained from untreated rats.

Gel filtration. The 3 globulins were separated on the basis of molecular size by gel filtration using 100 x 2.5 cm columns packed with Sephadex G-200. The elution pattern, developed with phosphate buffered saline pH 7.2 was determined by spectrophotometry at 2800 angstroms; the eluates containing the 19S and 7S protein peaks were concentrated by ultrafiltration at 40°C.

Anion-exchange chromatography. The peak obtained by gel filtration and known to contain the 7S immunoglobulins was further fractionated by ion exchange chromatography. Columns (40 x 2.5 cm) were packed with DEAE Sephadex A-25 and the proteins eluted sequentially with the following phosphate buffers: 0.01 M pH 7.5; 0.02 M pH 6.2 and 0.05 M pH 5.5.

Identification of rat immunoglobulins. The terminology used to describe immunoglobulins present in the various fractions is as previously reported. Immunoelectrophoresis was performed according to the method of Scheidegger, with the apparatus described by Wieme. The plates were developed with rabbit antisera that would recognize IgG,\* IgA and IgM rat immunoglobulins.

In the initial studies, salt precipitation of the globulins (HIG 1 and 2) from hyperimmune rat serum (Nos. 2 and 3) was performed to determine if these proteins contained the protective activity. The ability to suppress the parasitemia was readily demonstrated with the resuspended globulins, and in addition salt precipitation provided a convenient means of concentration. The suppression of the parasitemia in the antiserum treated mice was transient and parasitemias rose rapidly in both treated and control mice after day 3 with all mice succumbing to the infection.

Studies with hyperimmune 19S and 7S globulin fractions. HIG 1 and HIG No. 2 (3x globulins) were separated into 19S and 7S fractions by gel filtration on Sephadex G-200 columns. Mice receiving fraction No. 1 (19S globulins) developed parasitemias which were not significantly different from those of mice receiving NG. In mice injected with fraction

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\*IgG is used to describe IgGA and IgGB.

No. 2 (7S globulin), a marked and significant suppression of the parasitemia resulted. Another experiment with 7S and 19S fractions from HIG No. 1 confirmed these results.

Studies with subfractions of hyperimmune 7S globulin. Since the protective activity was found in 7S globulins from hyperimmune sera, further fractionation of these proteins was accomplished. 7S globulins from hyperimmune serum (No. 2) were equilibrated with a starting buffer, applied to a DEAE Sephadex column and eluted with phosphate buffer. The two distinct protein peaks obtained were then evaluated for protective activity. Also tested were HIG 1 (unfractionated globulins), Sephadex G-200 fraction No. 1 (19S) from the same hyperimmune sera (No. 2), and normal 3X globulins (NG).

The 19S fraction obtained from antiserum No. 2 again had no protective effect when compared with normal globulin. The major amount of protective activity was present in subfraction No. 3 of the DEAE fractionation procedure and approximately equalled that of the 3X HIG. Subfraction No. 1 also inhibited the level of parasitemia but to a lesser degree than the HIG or subfraction No. 3. Similar results were obtained in a repeat experiment utilizing the same fractionation technics. Subfraction No. 2 showed only equivocal activity.

Time course development of antibody. A group of rats was injected with  $1 \times 10^6$  parasitized RBC's and one third of these animals were sacrificed and bled at each of the following intervals: 6, 12 and 18 days post-infection. The 3X globulins from rats infected 12 days had a morbidly protective effect in suppressing parasitemias. Parasitemias in mice treated with 6 day globulins were low but not significantly suppressed. In this experiment, globulins from 18 day infections did not show an anti-parasitic effect. In a later experiment, however, globulins obtained at this time (18 days post-infection) had a significant anti-parasite effect. It should be noted that parasitemias in these 2 groups were markedly different. Three groups of control rats were bled 6, 12 and 18 days respectively after injection with diluted normal RBC's. Globulins from these 3 pools showed no suppressive activity.

Infractionation of 12 day globulins. Sephadex G-200 separation of IG No. 12 into 19S and 7S fractions (#1 and #2 respectively) was obtained. When these fractions were studied in the test system, the 19S fraction had a small but insignificant amount of protective effect. The 7S fraction was more effective in inhibiting the parasitemia, but neither fraction was as potent as the unfractionated globulins.

Comparison of immune and hyperimmune globulins. The 19S and 7S fractions obtained from early antiserum (IG-1) and hyperimmune antiserum (HIG #3) were compared for their ability to suppress mouse parasitemias. Unfractionated HIG #3 (from hyperimmune serum) was more potent than the unfractionated IG #1 (early antiserum) and the same relationship was observed with their 7S fractions (Fractions #2). In contrast, the 19S fraction from the early antiserum appears to have some protective activity. This was not noted in the 19S fraction from hyperimmune serum.

Identification of rat immunoglobulins. Sephadex G-200 fractionation of 3X globulin preparations resulted in 2 major peaks. Fraction #1 contained IgM immunoglobulin, X2 macroglobulin and occasionally a trace of IgG. Fraction #2 contained IgG and IgA rat immunoglobulins. Further fractionation of the IgG and IgA mixture was obtained by DEAE Sephadex chromatography. Subfractions #1 and #2 contained only IgG immunoglobulin. Subfraction #3 contained electrophoretically fast IgG and IgA.

Indirect hemagglutinating titers. The IHA titers of the normal 3X globulin and unfractionated and fractionated 3X globulin preparations from immune serum are listed in Table 4. Early IHA activity appeared to be greatest in Sephadex G-200 fraction #1 (19S). On the other hand IHA activity in hyperimmune sera appeared to be concentrated in fraction #2 (7S). A degree of correlation appears to exist between the IHA titer and the protective effect of the 3X globulin or its fractions. An exception is noted with IG 18. Although this globulin preparation had a relatively low IHA titer, protective effect was demonstrated in the test system. In addition, fraction #2 was more protective than fraction #1 and the IHA titers were <20 and 20 respectively. A recent report indicates that hemagglutination titers may fall during or shortly after the crisis in parasitemia.

In the present experiments, ammonium sulfate-precipitated globulins from normal and immune rat sera were tested for the ability to suppress *P. berghei* parasitemia in a mouse test system. Groups of infected mice that received no treatment and those that received normal rat globulins had similar levels of parasitemia, whereas mice receiving immune globulins or fractions thereof showed significant suppression of parasitemias. In addition, the globulins obtained from rats that were injected with non-parasitized erythrocytes were unable to suppress a *P. berghei* parasitemia. This indicated that infection with *P. berghei* elicited humoral factors capable of suppressing the parasite. Antibodies belong to the gamma globulin class of serum proteins, and it is reasonable to assume that the salt-precipitated globulins contained whatever serum antibodies were present in the donor animal. Recent studies with hyperimmune serum have demonstrated that rat anti-*P. berghei* protective antibodies are members of the IgG class of immunoglobulins. The present experiments are in agreement with this observation. However, the most potent fractions contained electrophoretically fast IgG and IgA. This introduces the possibility that IgA antibody may also have antiparasitic activity. Studies utilizing Fc piece specific anti-IgA antisera to remove this immunoglobulin from preparations being tested for protective activity could establish the importance of IgA in antiserum.

No evidence is available in the literature concerning the protective role of IgM antibody in rodent malaria. When antiserum was obtained early (12 to 16 days) after *P. berghei* infection and then separated into 19S and 7S fractions, there was an indication that 19S fraction may contain some protective activity but there was no evidence of activity in 19S fraction from hyperimmune serum. This suggests that IgM antibody may play an important role in suppressing the parasitemia, particularly

since it was demonstrated (12-18 days) at a time when rats are clearing the parasites from their circulation.

A degree of correlation was noted between the IHA titer and protective activity of the various globulin preparations (Table 4). At the present time it is assumed that the IHA titer reflects the presence of many different antibodies, and further work is necessary to establish the exact relationship between this serologic test and the protective effect of antiserum.

Table 4

Indirect Hemagglutinating Titers of Rat Anti-P. berghei Globulins

Specimen	Titer		
	Whole globulin (3X)	Sephadex G-200	
		FR. 1 (19S)	FR. 2 (7S)
Normal globulin (NG)	<20	-	-
Control globulins (CG) 6, 12, 18, CG-H	<20	-	-
Hyperimmune globulins (HIG)			
HIG No. 1	1280	20	1280
HIG No. 2	5280	160	2560
Immune globulins (IG)			
IG-6	320	-	-
IG-12	320	160	<20
IG-18A	80	-	-
IG-18B	40	20	<20
DEAE Chromatography of G-200 FR. 2 (7S)			
	1	2	3
HIG-1	320	160	1280

- Not done

\* Values represent the reciprocal of the greatest dilution giving a positive result. All titers were obtained with 3X globulin preparations.

4. Aspects of immunity in mice inoculated with irradiated Plasmodium berghei.

Attempts to induce protective immunity in experimental hosts by the use of irradiated malarial parasites have been relatively successful. Both sporozoites and blood forms of Plasmodium gallinaceum when irradiated and injected into chickens have conferred a resistance against a challenging infection of parasites of the same strain and stage. Recent

experiments have shown that rats previously treated with irradiated P. berghei infected erythrocytes develop an increased resistance to challenge. There was a 10-30% recovery rate among mice immunized with irradiated blood forms of P. berghei. In addition these mice had extended mean survival times and delayed parasitemias when compared to control animals. Many mice immunized with irradiated sporozoites of P. berghei survived a usually lethal sporozoite challenge, although protection did not extend to blood induced challenges.

The experiments described here are an extension of previous work in which irradiated P. berghei blood forms were used to induce a protective immunity in mice. The dose relationships between immunizing and challenge inocula have been investigated along with experiments designed to determine the nature and duration of protection. Since it has been suggested that antigenic differences between sporozoites and erythrocytic forms may preclude immunization against mosquito induced malaria with blood forms of plasmodia, experiments were also conducted to determine whether or not immunization by irradiated blood forms would protect mice from sporozoite induced challenges.

ICR strain mice weighing 20-25 grams were used. The NYU-2 strain of P. berghei maintained in our laboratory for over 4 years by blood transfers in ICR mice was used in all experiments involving blood induced infections. The preparation of inocula for immunization and challenge with P. berghei (NYU-2) was conducted as previously described. Experiments designed to test the effect of sporozoite challenge on animals immunized with blood forms were done with the 17X strain of P. berghei yoelii. This isolate was received from R. Killick-Kendrick in 1967, and has been maintained by alternate blood transfers in white mice and mosquito transmission by Anopheles stephensi. Mosquitoes were fed on mice during the first 3-4 days of patency, maintained at 27°C and provided with 10% sucrose solution. Samples were dissected 7 days after feeding and salivary gland infections were on day 10.

A sporozoite inoculum for challenge was prepared by triturating pools of 50 female Anopheles stephensi mosquitoes in 5 ml of chilled Medium 199. The ground mosquito-parasite mixture was transferred to a centrifuge tube and lightly spun for a few seconds to remove the coarse mosquito debris. The supernatant fluid with the sporozoites was placed in a vaccine bottle and kept in an ice bath until used. In general, no more than 20 minutes elapsed between the collection of the sporozoites and the inoculation of the mice.

In most experiments, animals were immunized on days 0, 3, 7, 10 and 14 and challenged on day 21. Treated control mice received an equal volume of irradiated normal mouse erythrocytes by the same schedule. All control and immunizing inocula were exposed to 20 kilorads and given intraperitoneally (I.P.) in a 0.5 ml volume. Blood induced challenges were given I.P. in a volume of 0.5 ml while sporozoite challenges were given in 0.1 ml volumes. Parasitemias were determined by counting the number of parasitized RBC's in 200 total RBC's on Geimsa-stained slides.

Reticulocytes were stained with brilliant cresyl blue by mixing 2 drops of stain and 1 drop of blood in a small tube. After 10 minutes staining time, the tubes were agitated and a smear was made from the mixture. These slides were then counterstained with Wright's stain which allowed for distinction between host cell reticulum and parasite material.

To determine the presence of subpatent parasitemias, subinoculations were performed by diluting 2-3 drops of tail blood from each animal with 0.5 ml of saline citrate. Two mice were then inoculated intravenously with this suspension. Protection was usually evaluated by comparative analysis of survival time, percent mortality and course of parasitemia in experimental and control animals.

#### Protection Induced by Irradiated Parasitized Blood

In the first experiment, aliquots of irradiated *P. berghei* parasitized blood ( $1 \times 10^8/0.5$  ml) and irradiated normal blood were centrifuged at 1500 rpm for 20 minutes. The plasma was then decanted from some aliquots of both the normal and infected blood and the cellular portions of each group were then resuspended as shown in Table 5. After 5 injections of the various inocula the mice were challenged along with untreated controls. All the animals became patent. Extended median and mean survival times were observed in each group which had received inocula containing irradiated parasitized cells. These groups also included a significant number of animals which survived the challenging infection. No survivors appeared in the group of mice receiving plasma from infected mice although the range of mortalities was increased slightly in both groups. Irradiated cells and plasma from normal mice alone or in combination did not protect. Survival times for these groups were similar to those of the untreated control group.

#### Dose Relationships

To investigate the dose relationships between immunization and challenge, an experiment was designed in which 4 different levels of immunization were tested against 4 different levels of challenge. A total of 240 mice was divided into 6 groups. The first 4 groups were immunized with 5 injections of  $1 \times 10^8$ ,  $1 \times 10^6$ ,  $1 \times 10^4$  or  $1 \times 10^2$  irradiated *P. berghei* parasitized mouse cells respectively. The animals of the fifth group received irradiated normal mouse RBC's, and those of the sixth group served as untreated controls. Seven days after the last immunization 10 animals from each of the above groups were challenged with  $1 \times 10^8$ ,  $2 \times 10^6$ ,  $2 \times 10^4$ , and  $2 \times 10^2$  viable *P. berghei* parasitized mouse cells respectively. The results (Table 6) show that immunization with a total of  $5 \times 10^8$  parasitized cells protected mice against all 4 levels of challenge. At each challenge dose the mean survival time of these mice was significantly increased over that of controls. The number of animals surviving the challenge ranged from 0% in the animals receiving the largest challenge ( $1 \times 10^8$ ) to 60% in the group given the smallest challenge ( $2 \times 10^2$ ). Animals immunized with  $5 \times 10^6$  irradiated parasitized cells showed no resistance to the heaviest challenge ( $1 \times 10^8$ )

with mortality rates very similar to those of the controls. These animals, however, showed increases in median survival times over those of treated controls by 3.6, 5.5 and 5.3 days when challenged with the 3 smaller doses. The 5.5 and 5.3 day extensions were statistically significant by rank test. Animals immunized with  $5 \times 10^4$  and  $5 \times 10^2$  irradiated parasitized cells showed no protection against any of the 4 levels of challenge. There were no survivors in either of these 2 groups or in the treated or untreated control groups. Mean percent parasitemias have been plotted for the groups that showed a significant increase in survival time. Percent parasitemias of controls and immunized groups whose mortalities did not differ significantly were plotted as a range of means. In animals immunized with 5 injections of  $1 \times 10^8$  irradiated parasitized cells and challenged with  $1 \times 10^8$  parasitized cells, the initial parasitemia appeared to rise in mature erythrocytes and to undergo a minor crisis on day 4 or 5. Soon after this time, increased numbers of basophilic erythrocytes began to populate the blood and the parasitemia appeared to rise and remain in these basophilic cells for the remainder of the infection. A similar pattern was seen when animals immunized with the same number of irradiated parasitized cells were challenged with  $2 \times 10^6$  parasitized cells except that the crisis of parasites was delayed. When challenged with  $2 \times 10^4$  or  $2 \times 10^2$  parasitized cells, animals immunized with  $5 \times 10^8$  and  $5 \times 10^6$  showed lower parasitemias when compared with the other groups. Animals immunized with  $5 \times 10^8$  irradiated cells generally developed lower parasitemias after challenge than did mice immunized with  $5 \times 10^6$  irradiated cells. At the 2 lower challenges 3 immunized mice ( $5 \times 10^6$ ) showed no patent parasitemia at any time. Parasitemias of control groups and immunized groups not showing protection rose rapidly, and parasites were found predominantly in mature erythrocytes at all 4 challenging doses.

Table 5

Protection Induced by Irradiated Parasitized Blood

Treatment	Survival Time (Days)		Mean Day of Death	No. Survivors/ No. Challenged
	Median	Range		
Parasitized Blood	>59	20->59	22.5	7/13
Normal Blood	10	9-12	10.1	0/15
Parasitized Cells and Saline	23	9->59	20.0	4/15
Normal Cells and Saline	10	8-15	10.6	0/15
Parasitized Cells and Normal Plasma	21	20->59	21.3	5/15
Normal Cells and Parasitized Plasma	12	8-19	13.5	0/14
Parasitized Plasma	10	8-18	11.3	0/15
Normal Plasma	10	8-14	10.3	0/15
No Treatment	10	9-14	10.7	0/30

Table 6  
Dose Relationships between Immunization and Challenge

Group No.	Treatment	Survival Time (Days)		Group Median Minus NRBC Median	Mean Day of Death	Survivors/Challenged
		Median	Range			
Challenge Dose - $1 \times 10^8$						
1	$(1 \times 10^8) \times 5$	15.0	10-17	+8.5	14.5	0/10
2	$(1 \times 10^6) \times 5$	7.0	5-10	+0.5	7.2	0/10
3	$(1 \times 10^4) \times 5$	6.5	5-13	0.0	7.9	0/10
4	$(1 \times 10^2) \times 5$	6.0	5-9	+0.5	6.5	0/10
5	(NRBC) $\times 5$	6.5	5-13	--	7.3	0/10
6	No treatment	6.0	5-10	-0.5	6.4	0/10
Challenge Dose - $2 \times 10^6$						
1	$(1 \times 10^8) \times 5$	18.0	8->178	+10.0	17.6	2/10
2	$(1 \times 10^6) \times 5$	11.0	6-19	+3.0	11.9	0/10
3	$(1 \times 10^4) \times 5$	7.5	6-17	-0.5	9.3	0/10
4	$(1 \times 10^2) \times 5$	7.5	6-16	-0.5	8.5	0/10
5	(NRBC) $\times 5$	8.0	6-12	--	8.3	0/10
6	No treatment	9.5	7-16	+1.5	10.5	0/10
Challenge Dose - $2 \times 10^4$						
1	$(1 \times 10^8) \times 5$	19.5	14->178	+10.5	20.0	1/10
2	$(1 \times 10^6) \times 5$	15.0	9-23	+6.0	14.6	0/10
3	$(1 \times 10^4) \times 5$	9.0	8-18	0.0	10.3	0/10
4	$(1 \times 10^2) \times 5$	9.5	9-13	+0.5	9.8	0/10
5	(NRBC) $\times 5$	9.0	8-10	--	9.1	0/10
6	No treatment	10.0	9-15	+1.0	10.9	0/10
Challenge Dose - $2 \times 10^2$						
1	$(1 \times 10^8) \times 5$	>178	19->178	>166.0	25.0	6/10
2	$(1 \times 10^6) \times 5$	19.0	10->178	+7.0	17.4	2/10
3	$(1 \times 10^4) \times 5$	12.0	10-19	0.0	12.9	0/10
4	$(1 \times 10^2) \times 5$	12.0	10-14	0.0	11.8	0/10
5	(NRBC) $\times 5$	12.0	11-14	--	12.1	0/10
6	No treatment	12.5	10-15	+0.5	12.6	0/10

### Duration of Protection

To test the duration of the protection produced by the irradiated parasitized cells, 180 mice were divided into 3 groups and given the following treatments. The first group received 5 injection of  $1 \times 10^8$  irradiated parasitized mouse cells, while those of the second group were given an equal volume of irradiated normal mouse cells on the same days. The animals of the third group were kept as untreated controls. A challenge dose of  $2 \times 10^4$  viable *P. berghei* infected mouse cells was given to 10 animals from each group 7, 28, 56, 84, 119 and 189 days after the last immunization (Table 7). All animals became patent after challenge at each time interval. Protection was evidenced by increased survival times and by the survival of animals after challenge. The ranges of mortalities for immunized and control groups do not overlap for the 7, 28 and 56 day challenges. When mice were challenged 84 days or later after immunization, some immunized animals died within the mortality range of the controls even though the number of survivors appeared to increase between days 56 and 119. Protection could still be demonstrated 189 days after immunization, although only one animal survived the challenging infection at this time. Little variation was found in control groups except for a slight increase in both median and mean survival times over the 6 month period. The survival times of the irradiated normal cell control animals were decreased as compared to the untreated controls.

Table 7  
Duration of Protection Produced in Mice by Immunization with  
Irradiated *P. berghei*

Day of Challenge	Group	Survival Time (Days)		Mean Day of Death	Survivors*/No. Challenged
		Median	Range		
7	Immunized	20	14->269	18.8	3/10
	NRBC's	9	8-11	9.2	0/10
	No Treatment	9	8-10	9.2	0/10
28	Immunized	20	16->248	21.4	1/10
	NRBC's	9	8-15	9.9	0/10
	No Treatment	10	9-16	10.3	0/10
56	Immunized	>220	16->220	17.7	6/10
	NRBC's	9	9-16	10.4	0/10
	No Treatment	9	9-11	9.6	0/10
84	Immunized	29	9->192	21.1	3/10
	NRBC's	10	8-12	9.6	0/10
	No Treatment	11	9-16	11.4	0/10
119	Immunized	>157	11->157	18.3	6/10
	NRBC'S	11	10-17	11.3	0/10
	No Treatment	12	9-16	12.6	0/10
189	Immunized	20	9->87	19.0	1/9
	NRBC's	9	9-15	10.5	0/10
	No Treatment	11	9-14	11.0	0/10

\* Based on last day of change.

### Subinoculation and Rechallenge of Survivors

Blood from 13 immunized mice which had survived a challenge of  $2 \times 10^4$  *P. berghei* infected cells was subinoculated into normal mice 3 times over a period of 4 months beginning 1-3 months after challenge. Three mice had parasitemias on the first subinoculation. However, no parasites were detected in recipient mice following the second or third subinoculation. The surviving mice were then rechallenged with  $2 \times 10^7$  *P. berghei* infected cells 5-7 months following the primary challenge along with 13 untreated controls of the same age. Percent parasitemias rose rapidly in control animals and also in two animals which had not become patent after the primary challenge. These two experimental mice died on days 5 and 6 after challenge while the average day of death for the control animals was 8.6 days. Parasitemias rose less rapidly in 9 of the experimental animals all of which underwent a crisis 6-9 days after challenge. Following this crisis 3 different courses of parasitemia ensued. Three of these mice died during ascending parasitemias after the crisis on days 14, 16, 18 and after challenge. Three other mice survived this ascending parasitemia. Their parasitemia levels were relatively high for some time, then reached a low point at approximately day 48. One of these animals later developed a high percent parasitemia. Most of the parasites in the other 3 experimentals disappeared soon after the initial crisis, but the animals remained patent at low levels throughout the observation period (83 days). The remaining two experimental mice did not undergo a detectable early crisis in parasitemia and remained patent, usually at low levels, for the duration of the experiment. Eight of the 13 experimental animals survived for longer than 83 days after the second challenge.

### Cell Type Parasitized

Geimsa stained blood smears revealed that parasites developing in animals challenged soon after immunization were primarily basophilic erythrocytes presumed to be reticulocytes. On the other hand, parasites in control animals, although rapidly parasitizing available basophilic cells, developed mainly in mature erythrocytes. The course of parasitemia in animals immunized with 5 injections of  $1 \times 10^8$  irradiated parasitized cells and challenged with  $2 \times 10^4$  viable *P. berghei* infected cells one week after the last immunization were plotted. Reticulocytes were stained with brilliant cresyl blue and the smears were counterstained with Wright's stain. Total RBC counts were done to assess the extent of the anemia in immunized and control animals. Control animals became patent on the 4th day after challenge and the parasites rapidly invaded the available reticulocytes and also large numbers of mature cells, the latter accounting for most of the parasitized cells. RBC counts fell rapidly as the parasitemia progressed. Most of the control animals died without showing any elevation in reticulocyte count. Immunized animals, in contrast, were not patent on day 4, but were patent on day 6. Their parasites were found mainly in reticulocytes and only a relatively small percentage of the mature RBC's were parasitized at any time. RBC counts in these animals decreased at a more gradual rate and, as a reticulocytosis

developed, the parasites were restricted for the most part to the reticulocytes. In animals which survived the challenge infection, parasites were cleared from circulation even though there were large numbers of reticulocytes in the blood.

After challenge in one experiment, blood from immunized mice was subinoculated into normal mice. The resulting infections appeared to follow a normal course. The recipient mice rapidly developed a high parasitemia, largely in mature cells, and died as a result of the infection.

When animals were challenged approximately 4 months after immunization greater numbers of parasites were found in mature cells. Immunized animals which died within the control range could not control the parasitemia in mature cells and succumbed to infections predominantly in mature cells. Parasitemias in some immunized mice which initially developed in mature cells underwent a crisis with a corresponding fall in percent parasitemia. As reticulocytes began to increase in numbers in these animals, the parasitemia usually rose again, and parasites were found mainly in reticulocytes. In other animals, however, the parasitemia did not rise to high levels in reticulocytes even though these cells were available. Total RBC counts done on immunized and control animals challenged 4 months after immunization showed that the anemia in immunized animals produced by the challenging infection developed more rapidly than when the challenge was given soon after immunization.

#### Effects of Immunization

In 3 separate experiments hematocrits were done on animals during the first, second and third week of immunization. Similar results were obtained in each experiment. Each experimental and control mean and range represents results obtained with 30 mice, while the normal mean and range was established from values obtained from 90 untreated mice, 30 of which were done each week. A slight decrease in mean hematocrit values was noticed in immunized groups during the second and third week of immunization. Mean values for control mice receiving injections of irradiated normal blood remained about the same as those of the untreated animals for all 3 weeks.

In two of the above experiments the percentage of reticulocytes in the peripheral blood was determined on different animals in each of 3 groups. Each experimental and control mean represents values obtained from 20 mice while the normal mean and range determinations were based on data from 60 untreated mice, 20 of which were done each week. Although animals injected with irradiated normal blood showed no reticulocytes and their mean values remained near those for untreated mice, immunized animals developed a reticulocytosis which became evident during the first week of immunization. The reticulocytes appeared to increase during the second week and was still present at the time of challenge during the third week of immunization.

Table 8  
 Serum Protein Values of Immunized and Control Mice in Grams Percent-Mean (Range)

Group	No. Animals	Total Protein	Albumin	alpha <sup>1</sup>	alpha <sup>2</sup>	beta	gamma
Immunized	51	5.18 (4.60-6.00)	1.84 (1.44-2.37)	.58 (.37-.81)	.53 (.28-.78)	1.55 (1.30-2.26)	.69 (.50-1.01)
Control	48	5.26 (4.30-6.10)	1.95 (1.65-2.35)	.63 (.34-.93)	.53 (.33-.79)	1.72 (1.32-2.12)	.42 (.24-.72)

Total body weights of immunized and control animals were recorded in one experiment. Each mean value is based on data from 50 mice. No differences in weight between immunized and control animals were found at any time during the immunization period.

Immunized and control mice in two separate experiments were sacrificed on day 21 after 5 immunizations. Serum from immunized animals was tested for a passive protective effect and the results were reported elsewhere. Total protein and serum electrophoresis values of sera from immunized and untreated control mice are shown in Table 8. Mean values for total protein, albumin, alpha<sup>1</sup> and beta globulins showed slight decreases when compared to the control values. Alpha<sup>2</sup> globulins were at comparable levels in both groups. However, a 39% increase in the concentration of gamma globulin was found in sera from the immunized group. The wet weights of spleens, livers and kidneys from these animals were determined and are shown in Table 9 as their average percent total body weight. Spleens in immunized mice increased in weight approximately 100% over both treated and untreated control animals during the period of immunization. These spleens were also darker in color than those of the control animals and resembled spleens from mice early in the course of active *P. berghei* infections. Ranges of liver and kidney weights overlapped in all 3 groups although the average liver was slightly heavier in immunized mice.

Table 9

Organ Weights After Immunization

Group	No. Animals	Percent Total Body Weight		
		Spleen	Liver	Kidney
Immunized	75	.89	6.26	1.45
NRBC Control	36	.46	5.58	1.37
No Treatment	72	.44	5.98	1.49

Blood Stage Immunization vs. Sporozoite Challenge

Four separate experiments were conducted using approximately  $1 \times 10^8$  irradiated *P. berghei yoelii* infected RBC's as the immunizing agent and  $1 \times 10^3$  -  $1 \times 10^9$  sporozoites of the same strain as the challenging organism. Each experiment included an immunized group of mice as well as 2 groups of control mice, one injected with irradiated normal blood and the other untreated. Table 10 shows the immunization schedule, day of challenge, and the number of mice in each group which became patent after challenge. It is evident that the immunization with irradiated blood forms had no apparent effect on the number of animals which became patent after challenge since approximately the same percentage of animals

developed parasitemia in both immunized and control groups. The parasites developing in immunized mice, however, persisted for much shorter periods of time and parasitized smaller percentages of RBC's than did those developing in either control group. This same pattern of low patent parasitemias of short duration in animals immunized with irradiated blood forms was evident in all 4 experiments.

Table 10

Patency Rates of Immunized and Control Mice  
Challenged with Sporozoites of P. berghei yoelii

Experiment No.	Days of Immunization	Day of Challenge	No. Patent/No. Inoculated		
			Immunized	NRBC'S	No Treatment
1	0,4,7,11,14	40	9/10	3/4	4/5
2	0,4,7	12	3/5	5/5	4/4
3	0,7,15,22	29	5/9	9/10	3/5
4	0,4,7,11	26	4/5	1/5	3/4
Total	-	-	21/29(72%)	18/24(75%)	14/18(77%)

The injection of irradiated parasitized blood into mice produced a significant immunizing effect. Presumably the irradiated parasitized erythrocytes are responsible for the development of this resistance, although the effects of other irradiated blood elements from infected animals are not known. Passive transfer of sensitized lymphocytes has been shown to confer protection to a challenging infection of P. berghei in rats, but the relatively small number of irradiated leukocytes present in the immunizing inocula, which is obtained from donor mice early in infection, would probably not be sufficient for the development of immunity. The slight protective effect seen in some animals after injection with irradiated plasma from infected mice may be due to parasitized erythrocytes or cell free parasites which were not completely removed from the plasma during centrifugation, since in previous experiments no protective effect was elicited in rats by the intraperitoneal injection of filtered plasma from infected donors.

The immunity produced in mice was found to depend on the size of the inoculum in both immunization and challenge. While immunization with the largest number of irradiated parasitized cells produced resistance in mice to a wide range of challenges, immunity produced by fewer irradiated cells could be overwhelmed by large challenge doses and could be demonstrated only when the challenging inoculum was reduced.

Relatively small numbers of irradiated parasitized cells did not stimulate detectable protection in mice.

Even though ICR mice are extremely susceptible to the NYU-2 strain of P. berghei, immunization with irradiated parasitized blood forms induced a resistance which could be detected for at least 6 months. Mice cured of active P. berghei infections by chemotherapy have been shown to be resistant to challenge for at least 3 months. The immunity appeared to wane in some immunized animals challenged a long time after immunization. These mice developed high parasitemias and died soon after challenge. The number of survivors in some of these groups, however, was greater than when challenges were given soon after immunization. A secondary response initiated by a more severe infection early after challenge may have protected these animals, although the age resistance to P. berghei previously described in mice was observed in control animals and may have influenced the survival rate in immunized groups.

The pattern of parasitemia developing in immunized mice after challenge was basically different from that of control animals. Whereas the latter succumbed to a rapidly ascending parasitemia predominantly in mature RBC's, parasites in immune mice were restricted primarily to reticulocytes and did not greatly increase in numbers until a reticulocytosis developed in the immunized animals. It has been suggested that parasitized reticulocytes predominate in the circulating blood in some P. berghei infections because parasitized mature cells are removed more rapidly. However, since RBC levels fell more rapidly in control than in immunized mice, it is likely that parasites did not invade a large number of mature cells which were subsequently removed. The mechanisms of acquired immunity probably restricted the parasites primarily to reticulocytes where the parasites were sheltered from the host's response. Experiments reported elsewhere indicate that the parasites persisting after challenge in mice immunized with irradiated parasitized cells are antigenically different from those found in control mice. In many animals which survived the challenging infection, parasites were cleared from the blood even though large numbers of reticulocytes were present. A few immunized mice challenged with small inocula did not become patent although all control mice succumbed to the challenge.

Mice immunized with irradiated parasitized cells develop a reticulocytosis, a slight decrease in hematocrit values, enlarged spleens and elevated levels of serum gamma globulin. The increase in spleen weight was probably a direct result of the increased activity involved in the sequestration and destruction of irradiated parasitized cells by this organ. The hypersplenism may have in turn been responsible for both the decrease in hematocrit values and the reticulocytosis since mild hemolytic anemias have been described in rats with hypersplenic conditions. The disparity in blood values between immunized and control mice was apparently not due to subpatent parasitemias which had developed in immunized mice, since repeated subinoculation of blood from these mice produced no infection in recipient animals. Elevated serum gamma globulin levels have been previously shown in mice made resistant to P. berghei

by curative therapy of active infections. The passive protective effect exhibited by serum from mice immunized with irradiated parasitized cells may indicate a specific effect of acquired immunity.

Immunized mice which survived the challenging infection apparently underwent a complete cure and remained strongly resistant for long periods of time, since their blood remained free from parasites as determined both microscopically and by subinoculation. This is in agreement with findings reported elsewhere in which true residual immunity without stimulation by surviving parasites was found in both rats and mice.

Experiments with avian and rodent malarias have shown that protection could be induced against sporozoite challenge by prior treatment with attenuated sporozoites. This protection did not extend to challenges with blood forms of the same strains of parasites. Richards, on the other hand, found that antigen prepared from erythrocytic parasites of *P. gallinaceum* conferred immunity against both erythrocytic and sporozoite challenges. In our experiments with rodent malaria, immunization with irradiated blood forms protected mice against sporozoite as well as blood induced challenges. Our results further suggest that the mechanisms of resistance might be different in animals immunized with sporozoites than in those immunized with blood forms. In the former the survivors of sporozoite challenges did not usually become patent although these animals showed no immunity to blood induced challenges. Conversely, when irradiated blood forms were used for immunization, challenge with sporozoites produced patency but the resulting parasitemias were rapidly controlled. This suggests that immunity in these animals was directed primarily against erythrocytic forms rather than the sporozoites or the pre-erythrocytic forms.

##### 5. Some characteristics of *Plasmodium berghei* "relapsing" in immunized mice.

Many investigators have attempted to immunize against malarial infection by injecting hosts with non-living parasite materials prepared by a variety of technics, injected by several routes and accompanied by selected adjuvants. Zuckerman has reviewed these attempts with mammalian malaria and concluded that in no case did such vaccination procedures provide "complete protection against infection with viable homologous parasites." It has been widely assumed that failure of vaccination procedures is due to the technics used in preparing antigens, i.e., protective antigens were either inadvertently omitted from the vaccine or denatured during processing.

In an attempt to increase immunogenicity of vaccines, other workers have immunized mice by injecting living *P. berghei*. Since infected mice die so rapidly, effective acquired resistance in this host can only be elicited by special methods, e.g., attenuation of the parasite, so that the host can acquire an effective immunity before it is killed by the parasite. After a short uninterrupted course of *P. berghei* infection,

mice have been treated with atebriane or primaquine to eliminate parasitemias; the development of immunity in such mice is evidenced by marked suppression of parasitemias following challenge infections. Jerusalem has shown that primary *P. berghei* parasitemias in mice can be controlled by eliminating or minimizing the host dietary intake of PABA, and that this treatment is immunogenic. Another method of preventing fulminant infections is to irradiate the parasites with x-rays. Although parasites have not shown to be able to multiply after exposure to high doses of x-ray, they do circulate for a period of time after injection and elicit protective immune response in mice and rats. Weiss and Degiusti have shown that rodent parasites can be attenuated by *in vitro* culture in a medium containing hamster serum; these parasites did not produce patent infections but did stimulate an immunity to challenging infections. In the work cited above, resistance to challenge was measured by one or more of the following: prolonged prepatency, delayed increase in parasitemia, lower peak parasitemia, earlier decline in parasitemia and reduced mortality of the host. In none of these experiments, however, was the immunity absolute and lasting. In general, challenging parasites did establish themselves in the immunized host, and frequently these parasites persisted for weeks to months. Immunization with delayed drug treatment of infection, and irradiation of parasites has been obtained by other workers using other host-parasite situations.

It has been postulated that parasites persisting in chronically infected animals are variants resistant to the hosts immunity. Cox has described properties of relapsing *P. berghei* parasites that differ from those of the parent strain; changes in virulence and immunogenicity as well as susceptibility to latency-inducing treatments. Relapse strains of *P. knowlesi* have been shown by *in vitro* agglutination tests to differ from a parent strain, i.e., persistently relapsing populations differ antigenically from each other and from a parent strain. It is assumed that such immunological specificity applies as well to protective antibodies, shown by Coggeshall and Kumm to be present in infected monkey. Similar differences in immunogenicity have been described for different variants of *P. cynomolgi*. Monkeys actively immunized against some variants showed immunity to homologous challenge; in some cases there was apparent cross immunity between related strains.

The present work uses a passive protection system in an attempt to determine if acquired humoral factors (antiserum) can distinguish between stock parasites and (variant) parasites subinoculated from immunized animals with persisting infections.

Mice used in these experiments were ICR strain males generally weighing 20-25 gm.

Immunization of mice: Mice were immunized with x-irradiated parasites or irradiated normal mouse RBC's according to previously methods.

Antiserum testing of stock and derived parasites: Parasites multiplying in control (non-immunized) and experimental (immunized) groups of mice were subinoculated into groups of normal 20-25 gm mice by intravenous injection of  $2 \times 10^7$  parasitized RBC (PRBC) contained in 0.2 ml. One hour after infection, mice were injected with either antiserum or normal serum (0.5 ml per mouse). Blood smears were prepared from test animals on day 3 and the level of parasitemia determined. In Experiment No. 2, parasite subinocula prepared from experimental and control mice contained  $2 \times 10^6$  PRBC/0.2 ml. Some recipients of this inoculum were bled on the fourth day for material to be passaged; inocula for this and subsequent passages (made every 3-4 days) contained  $2 \times 10^7$  PRBC/0.2 ml. Some parasites subinoculated in Experiments 1 and 2 were tested with normal rat serum or rat antiserum. The rat antiserum used in this testing came from a pool of serum from rats receiving a series of 5-9 injections of  $1 \times 10^6$  to  $6 \times 10^6$  PRBC's over a period of 2-5 months.

Protective effect of antiserum (MAS-1) from mice immunized with x-irradiated stock parasites. An experiment was done to determine whether mice injected with x-irradiated stock parasites would produce antibodies detectable in a passive protection test. A group of mice was immunized with the standard five injections of irradiated stock parasites and bled one week after the last injection. Pooled serum from these mice was tested against stock parasites in each of five mice. Control sera (obtained from either normal unimmunized mice or mice repeatedly injected with irradiated normal mouse RBC's) were tested at the same time, each serum injected into five mice infected with stock parasites.

There was some indication that antiserum from immunized mice had a suppressive effect against stock parasites. Thus, on the third day after infection and treatment with antiserum MAS No. 1, experimental mice showed a mean parasitemia of 23%. This figure was significantly less than that (33%) for the 5 control mice injected with normal mouse serum but not significantly different from figures of 30% and 32% respectively for control mice receiving no serum and control mice receiving sera against irradiated normal RBC's. An additional experiment was done increasing the sample size.

Effect of mouse antiserum (MAS-2) on parasites isolated from immunized and non-immunized mice. This experiment was done to confirm a previous report that injecting of irradiated stock parasites elicits an active immunity against a challenging infection of stock parasites, to confirm that such treatment would also elicit protective antibodies against stock parasites, to determine if parasites could persist in immunized mice and, if so, to isolate these parasites and test them for sensitivity and antiserum. A group of 50 mice was immunized by standard procedures. One week after the last immunizing infection, 40 of these mice were exsanguinated and their blood pooled for serum (MAS-2). The remaining 10 mice were each challenged with stock parasites ( $2 \times 10^4$  PRBC's) along with 10 unimmunized mice of the same age.

Table 11  
Course of Infection Following Challenge\* of Mice Immunized with X-irradiated Stock Parasites

Experiment-1 Group (No. of mice)	Mean Parasitemia on Day																
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20-23	24
Unimmunized AC (5)	5	13	42	69													
BC (5)	8	19	54														
CC (5)	6	11	40														
DC (5)	7	17**															
Immunized A (5)	4	7	6	13	17	20	25	35									
B (5)	4	4	8	8	13	13**											
C (5)	6	4	5	10	16	21	29	42									
D (5)	5	5	8	19	19	26											
Number of Deaths on Day																	
Unimmunized	9	10	11	12	13	14	15	16	17	18	19	20-23	24				
Immunized	4	5	6														
				1	0	1	0	5	1	3	1	0	1				

\*Infected with  $2 \times 10^4$  parasites.

\*\*5 mice used for parasites.

Active immunity in immunized mice: Table 11 shows that infection of x-irradiated stock parasites stimulates an immunity effective against a challenge with stock parasites but that this immunity was only transiently protective. Control mice had rapidly progressive parasitemias between days 5 and 7, and all of these mice died before the 12th day (mean survival time of 10 days). In contrast, immunized mice had a delayed and relatively slowly progressive infection which did not reach parasitemias of 20% or more until the 11th day. Moreover, 2 of the experimental mice survived, and those that died had a prolonged mean survival time (15 days).

Protective antiserum in immunized mice: Antiserum from mice immunized with irradiated stock parasites was tested against two types of parasites, those isolated from control mice and those isolated from experimental mice. In the 6th day of infection a group of 5 control mice (mean parasitemias of 17%) was sacrificed and their blood pooled. Standard inocula ( $2 \times 10^7$  PRBC's) prepared from this blood were injected into 30 mice. Ten of these mice were then treated with mouse antiserum (MAS-2), 10 with normal mouse serum and 10 were left untreated. Another group of 30 mice was similarly infected with parasites subinoculated from 5 experimental mice on the 11th day, at which time their parasitemias averaged 13%. Three subgroups of these recipient mice were then treated with MAS-2, normal mouse serum, and left untreated respectively.

As can be seen in Table 12, MAS-2 had different effects on parasites isolated from control and experimental mice. Normal mouse serum had no effect on the course of parasitemia in mice infected with parasites from the control group, but MAS-2 significantly suppressed these parasites on days 2-4. On the other hand, parasites isolated from experimental mice were only slightly affected by this same antiserum; suppression was relatively slight and significant only on day 2. It should be noted that the two isolates increased at comparable rates in control mice.

Table 12  
Effects of Mouse Antiserum (MAS-2) on Parasites Isolated from Immunized and Non-immunized Mice

Parasites Isolated from	Injected with*	Parasitemia on Day		
		2	3	4
Unimmunized mice	uninjected	6	22	51
	normal rat serum	6	23	48
	rat antiserum	4**	12**	29**
Immunized mice	uninjected	5	25	57
	normal rat serum	5	26	58
	rat antiserum	5	21**	53

\* 0.5 ml serum injected i.v. one hour after injection of  $2 \times 10^7$  PRBC's.

\*\* Significantly less than means for two control groups.

Table 13  
 Course of Infection Following Challenge\* of Mice Immunized with X-irradiated Stock Parasites

Experiment-2 Group (No. of mice)	Mean Parasitemia on Day															
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Unimmunized CAX (10)	7	24**														
CCX (10)	5	20	35	52												
AX (10)	3	18	12	27		35**										
CX (10)	2	6	8	11		16	24	41	40							
	Number of Deaths on Day															
					9	10	11	12	13	14	15	16	17	18	19	20
Unimmunized					5	7	1	1	1							
Immunized							1	0	0	0	1	1	4	3	1	2

\*Infected with  $2 \times 10^4$  PRBC's.

\*\*5 mice sacrificed for parasites.

Effect of rat antiserum (RAS-1) on parasites isolated from immunized and non-immunized mice: In this experiment immunized and control mice were challenged with stock parasites as in the previous experiment; parasites were challenged with stock parasites as in the previous experiment; parasites isolated from these two groups were tested for sensitivity to the same antiserum, in this case a rat antiserum (RAS-1) repeatedly shown to be effective against stock parasites. Table 13 again shows the immunogenicity of x-irradiated parasites; as noted before, however, this immunity only delayed the course of the challenging infection and most of these mice died. Parasites were subinoculated from 5 control mice (mean parasitemia of 25%) on day 6 and from experimental mice (mean parasitemia of 21%) on day 10. These isolates were tested in the first passage for sensitivity and RAS-1.

Table 14 shows that the rat antiserum had different effects on parasites isolated from control and experimental mice. Parasites isolated from control mice were markedly and significantly suppressed by RAS-1, a suppression of 6%, 24% and 36% on days 2, 3 and 4 respectively. On the other hand, RAS-1 had a relatively slight effect on parasites isolated from experimental mice, and this suppression noted only on days 2 and 3 (suppressed by 20% and 6% respectively).

Table 14

Effects of Rat Antiserum (RAS-1) on Parasites Isolated from Immunized and Non-immunized Mice

Parasites Isolated from	Injected with*	Parasitemia on Day		
		2	3	4
Unimmunized mice	normal			
	rat serum	8	32	57
	rat antiserum	2**	8**	21**
Immunized mice	normal			
	rat serum	7	33	66
	rat antiserum	5**	27**	61**

\* 0.5 ml serum injected i.v. one hour after injection with  $2 \times 10^7$  PRBC's.

\*\* Significantly less than mean for controls injected with normal rat serum.

Effectiveness of rat antiserum on parasites passaged after isolation from immunized and non-immunized mice: In this experiment, parasites were again isolated from control and experimental mice. These parasites were passaged during the following 18-20 weeks and tested repeatedly during this interval for sensitivity and RAS-1. In this case there were two types of control animals, those immunized with irradiated normal mouse RBC's and those left unimmunized. Parasites were subinoculated from these two groups on days 7 and 8 when their parasitemias averaged 28%

and 32% respectively. Parasites were subinoculated from immunized mice on day 14 at which time the parasitemias averaged 27% in donors.

Table 15 shows results comparable to those noted in the previous experiment, i.e., parasites isolated from control mice were markedly sensitive to RAS-1, but parasites isolated from immunized mice showed no significant sensitivity to this same rat antiserum. Moreover, these characteristics remained stable during passage of the parasites.

Table 15  
Effectiveness of Rat Antiserum (RAS-1) on Parasites Passaged After Isolation from Immunized and Non-immunized Mice

Passage No.	% Suppression* of Parasites Isolated from		
	Unimmunized Controls	Controls Immunized with Normal RBC's	Immunized Mice
1	17	21	0
2	19	18	3
3	21	16	3
4		20	4
6		24	1
8		18	5
18			2
20		21	

\*Differences between mean % parasitemia in mice treated with normal rat serum and mean % parasitemia in mice treated with rat antiserum.

The present work confirms reports of the immunogenicity of x-irradiated stock parasites, which stimulate humoral and probably cellular factors of acquired immunity. This immunity is not, however, completely protective, and although challenging infections are significantly suppressed, parasites persist and eventually increase rapidly.

Why challenging parasites persist in immunized mice is not clear. It is possible that immunological and non-immunological stresses associated with challenging infection make the immunized host more susceptible to the parasite and/or that the parasite may have undergone certain changes facilitating survival in the immune environment. It is of interest, therefore, that the antiserum used in passive protection tests were effective against stock parasites and against parasites multiplying in control mice but not against parasites persisting in immunized mice. On the other hand, Wellde et al. have shown that such relapsing infections are found in reticulocytes.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 128, Natural and acquired immunity in rodent malaria

6. Publications.

Briggs, N. T., Wellde, B. T. and Sadun, E. H. Variants of Plasmodium berghei resistant to passive transfer of immune serum. Exper. Parasitol. 22:338-345, 1968.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACQUISITION	2. DATE OF SUMMARY	3. REPORT CONTROL SYMBOL	
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23. RESPONSIBLE ORG ORGANIZATION				24. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Inst of Res Washington, D.C. 20012				NAME: Walter Reed Army Inst of Res Div of CD and I Washington, D. C. 20012			
RESPONSIBLE PERSONAL				PRINCIPAL INVESTIGATOR (Provide NAME if U.S. Army/Army Reserve)			
NAME: MERONEY, COL W. H.				NAME: SADUN, E. H., Sc.D.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3308			
25. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: MOON, A.P.			
				NAME: DA			
26. REVISIONS (Provide DATE and REASON for each REVISION 6-73)							
(U) Malaria; (U) Gamma Globulin; (U) Biochemistry; (U) Antibody; (U) Fluorescent; (U) Isotope; (U) Metabolism							
27. TECHNICAL OBJECTIVE, 28. APPROACH, 29. PROGRESS (Provide individual paragraphs identified by number, provide rest of each with Security Classification Code.)							
23(U) To determine how energy requirements are met within the parasite.							
24(U) Use ultramicro techniques to determine changes in serum enzymes in germfree and conventional mice and rats. Study the effect of infection on the uptake and distribution of radioisotope-labelled amino acids, study the levels of enzyme activity in tissue extracts and alterations in protein and free amino acid constituents of blood and urine, study the development of relapses, and the pattern of parasitemias and fluorescent antibodies prior to, during, and following therapy. Investigate the use of immune gamma globulins as an adjuvant to chemotherapy in humans infected with drug resistant malaria.							
25(U) 69 01 - 69 06 Plasmodium knowlesi, P. cynomolgi and P. berghei were found to contain cytochrome oxidase activity, but P. knowlesi which had been freed of white blood cells, platelets and host erythrocytes had no appreciable tricarboxylic acid and pentose phosphate pathway functions. Theoretical yields of lactate did not result from glucose utilizations under atmospheres of nitrogen or air, and less lactate resulted aerobically than anaerobically indicating the existence of functional ancillary pathways. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

PII Redacted

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 129, Host responses to malaria

Investigators.

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1. Cytochrome oxidase activity in platelet-free preparations of *Plasmodium knowlesi*.

Although cytochrome oxidase is widely distributed in mammalian tissues, it is not present in many mammalian parasites. For example, no cytochrome oxidase activity could be demonstrated in the filarial worm, *Litomosoides carinii*, which is known to require oxygen for survival. Conversely, the whipworm, *Trichuris vulpis*, contains cytochrome oxidase but survives for longer periods when maintained under anerobic conditions. Other helminths, such as *Ascaris lumbricoides*, *Hymenolepis diminuta* and *Moniezia expansa* contain no demonstrable cytochrome oxidase and obtain energy primarily from an anaerobic metabolism.

Many parasitic protozoa also lack any demonstrable cytochrome oxidase activity. These include blood parasites such as *Trypanosoma congolense*, *T. vivax*, *T. rhodesiense*, *T. gambiense*, *T. evansi*, *T. equinum* and *T. equiperdum*.

Cytochrome oxidase activity has been reported in only two of the plasmodia, *Plasmodium cathemerium* and *P. berghei*. Interpretation of the findings with *P. cathemerium* are complicated by the use of intact red blood cell preparations containing parasites, making it necessary to determine activities by differences. In addition, the older assay procedures employing ascorbate and p-phenylenediamine coupled to oxygen uptake are relatively non-specific in comparison with methods now available. It is of equal importance that, although cytochrome oxidase activity is not demonstrable in mature erythrocytes, other blood components do possess this enzymatic activity. This is particularly true of the blood platelets which contaminate most preparations of malarial parasites, since they are similar in size and density to the plasmodia and cannot be separated from them by means of differential or gradient centrifugation. Unless platelets are excluded, cytochrome oxidase activity cannot be attributed solely to the parasite. Platelets possess an active aerobic metabolism and are rich in the enzyme systems of the tricarboxylic acid cycle and pentose phosphate shunt, which causes further confusion in attempts to evaluate the enzymatic constitution of malarial parasites. The studies reported here describe methods for obtaining *P. knowlesi* preparations essentially free of platelets and white blood cells with minimum loss of free parasites. Preparations obtained by the method described were then assayed enzymatically for cytochrome oxidase activity.

Collection of parasitized cells and separation from platelets. Macaca mulata monkeys were infected by intravenous transfer of fresh P. knowlesi. When at least 25% of the red blood cells were infected, 10 mg of adenosine diphosphate in one ml of saline was injected intravenously. This resulted in a marked reduction of circulating platelets as was previously reported to occur in rabbits, cats and man. After 20 minutes, animals were exsanguinated by cardiac puncture and the blood was collected in a heparinized bottle (0.67 mg heparin/ml blood) immersed in ice. To obtain a platelet free preparation, a technique was devised in which the whole blood was passed through a column of glass beads prior to centrifugation. The use of glass bead columns for quantitation of platelet aggregation in normal blood has been reported by O'Brien and Heywood. The glass beads (0.012 -0.11 mm; Scientific Products) were washed by immersion in concentrated sulfuric acid, rinsed copiously with water until free of acid and dried at 85°C. The beads (1.5 grams per ml blood) were packed with light tamping into a glass column (approximately 19 mm internal diameter) supported by a plug of glass wool. Flow of blood through the column was maintained at approximately 19 drops/minute by positive air pressure.

To remove white blood cells, the eluate from the column of glass beads was immediately passed through a column of dry cellulose powder (CF 11 Whatman Column Chromedia) in a ratio of one gram of cellulose per 10 ml of initial blood. In the reported control experiments, non-parasitized blood was treated in an identical manner. All operations were performed at 4°C. Eluates from cellulose columns were centrifuged at 4,340 x g for 15 minutes. The supernatant fluid was discarded and the pellet which contained both free and intracellular parasites was washed twice by suspending to the original volume in 0.057 M sodium phosphate buffered saline (pH - 7.4) containing 0.2% glucose. The pellet was subjected to a final wash with the high potassium medium for use with free plasmodia as described by Trager. The particulate fraction was made to a 20% (v/v) suspension in this medium.

Parasites were released from red blood cells by selectively disrupting the latter during passage through a French pressure cell at 2,000 p.s.i. Unbroken red blood cells were separated from the free parasites by centrifugation at 125 x g for 10 minutes. Parasites were harvested from the supernatant fluid by centrifugation at 27,000 x g for 15 minutes and separated from soluble red blood cell components by washing with Trager's medium until a clear and colorless fluid was obtained (3 to 4 washes).

Cell-free extracts of P. knowlesi were prepared by suspending the washed parasites in 0.05 M potassium phosphate buffer (20% v/v), and passing through a French pressure cell at 18,000 p.s.i. For determinations of cytochrome oxidase activity, the cell-free extracts were centrifuged at 27,000 x g for 15 minutes and the supernatant fluid and pellet which was resuspended in 0.05 M phosphate buffer (pH = 7.4) were assayed separately according to the procedure of Smith. Cytochrome c (Sigma Chemicals, St. Louis) was reduced by the addition of sodium hydrosulfite. Excess hydrosulfite was removed by shaking and aeration.

The percentage of reticulocytes was determined in a fresh red cell suspension stained supravitaly with 1% (w/v) Brilliant Cresyl Blue in 0.85% (w/v) saline. Platelets were quantitated by means of phase microscopy. Both leucocytes and blood parasites were estimated in methanol-fixed blood films, stained with Giemsa's stain. To determine the parasitemia, at least 200 red blood cells were examined and the percent of these that were infected was calculated. The number of white blood cells per 100 oil immersion fields was also counted to approximate leucocyte levels at various stages in the procedure. Protein was assayed according to the method of Lowry.

Non-parasitized blood was withdrawn from nine monkeys and subjected to several operations in an effort to determine the effects of these procedures on platelet levels and the cytochrome oxidase activity of the remaining blood components (Table 1). When blood was passed through a filter paper column, there was a marked decrease in white blood cell content. Many of the platelets were also removed by this procedure. However, the number of platelets remaining was sufficient to interfere with further biochemical studies. The injection of adenosine diphosphate (ADP) into monkeys before bleeding had no effect on white blood cell levels, but there was a noticeable decrease in circulating platelets. Unfortunately, the resulting decrease could not be quantitated accurately because of platelet aggregation. When blood from these monkeys was obtained after the injection of ADP and further subjected to passage through glass bead and filter paper columns, the platelet levels were found to be extremely low. These preparations allowed a more meaningful determination of the cytochrome oxidase activity in red blood cell debris freed of white blood cells and platelets. With this method, 70 to 100% of the cytochrome oxidase activity was removed from the whole blood preparations. The low levels of enzymatic activity remaining in most of the platelet free preparations may be due to the small number of reticulocytes still present.

Samples of blood infected with *P. knowlesi* were obtained from each of seven monkeys when the parasites were at specific stages of development. Platelets and white blood cells were removed by the techniques described above, and cytochrome oxidase activities were determined (Table 2). There was a large variation in the specific activities of these preparations indicating the need of further study. Regardless of the stage of development, all parasite preparations were found to possess cytochrome oxidase activity which was inhibited completely by cyanide at a concentration of  $1 \times 10^{-3}M$ . These activities were in all instances significantly higher than the corresponding non-parasitized blood samples from which white blood cells and platelets were removed (compare Tables 1 and 2). On the other hand, if platelets and white blood cells were not removed, then, the cytochrome oxidase activity of the parasites could be masked by the corresponding activities of these blood components; thereby, not allowing for dependable enzymatic assays of the plasmodia. In contrast with most cytochrome oxidase preparations obtained from mammalian tissues, the parasite enzyme remains in the supernatant fluid to a large extent even after centrifugation at 27,000 x g for 15 minutes. The significance of this

Table 1

Separation of White Blood Cells, Platelets and Cytochrome Oxidase from Erythrocytes

Treatment of Blood	Component Measured*	Monkey Number								
		1	2	3	4	5	6	7	8	9
	WBC	69	136	107	89	132	70	183	223	228
Untreated	Platelets	-	-	-	312,000	430,000	333,000	521,000	703,000	544,000
	Cytochrome Oxidase							0.584	0.782	1.082
Filter Paper Column	WBC	4	1	4	0	15	3	-	-	-
	Platelets	-	-	-	17,000	15,000	35,000	-	-	-
ADP Injection	WBC	4	3	7	2	1	0	2	6	3
Glass Bead and Filter	Platelets	-	-	-	0	0	0	4,000	1,000	0
Paper Columns	Reticulocytes	-	-	-	1	0.5	0.8	0.8	0.5	0.8
	Cytochrome Oxidase	0.091	0.017	0.052	0.016	0.028	0	0.117	0.182	0.093

finding awaits further study. These results show that cytochrome oxidase is a constituent of the parasite; they also emphasize the importance of removing platelets prior to the assay of enzymatic activities of the parasite.

Table 2

Cytochrome Oxidase Activities in Stages of P. knowlesi

Stage	% Parasitemia	Cytochrome Oxidase Activity		
		$\mu$ Moles Cyto. c Oxidized/Min/Mg Protein		$\mu$ Moles Cyto. c Oxidized/Min/ML Parasitized Blood
		Supernatant	Residue	
Rings (95%)	31	6.47	-	0.287
Rings (75%)	40	6.36	3.49	3.41
Rings (80%)	70	4.12	4.17	2.75
Trophozoites (95%)	65	39.5	0.0	6.83
Schizont (95%)	40	17.8	0.72	3.53
Schizont (95%)	21	1.15	0.69	0.93
Schizont (95%)	13	1.28	0.0	0.57

The metabolic and terminal respiratory pathways in the plasmodia are still not understood. A great deal of confusion exists in the literature in relation to these pathways. Earlier investigations employing primarily manometric measurements indicate the presence of Krebs' cycle activity in P. gallinaceum and P. lophurae. In addition, P. knowlesi, P. lophurae and P. cathemerium were reported to possess a cyanide sensitive respiration. Cyanide sensitive respiration does not necessarily mean that cytochrome oxidase is present because the respiration of L. carinii is very sensitive to cyanide yet this parasite has no cytochrome oxidase activity. McKee et al, found that P. knowlesi was sensitive to carbon monoxide inhibition in the dark, suggesting a heavy metal containing catalyst in terminal respiration. This inhibition however was not reversed consistently by light, as would be expected if cytochrome oxidase were present.

Recent investigations employing more sophisticated isotopic techniques have indicated that tricarboxylic acid cycle activity was absent in P. berghei and in P. vinckei. The additional finding that P. knowlesi accumulates lactate as an end product of glucose dissimilation further indicates a limited oxidative metabolism, since complete aerobes oxidize substrates solely to carbon dioxide and water.

Dixon pointed out the importance of eliminating platelets in blood parasite preparations before conclusions could be reached in regard to the enzymatic activity of the parasite. He reported that the lactate dehydrogenase activity of T. rhodesiense preparations was directly proportional to the number of platelets present. Similar precautions should be applicable to preparations of the malarial parasites. Therefore, methods reported above were devised to allow for the essentially complete removal of both platelets and white blood cells from P. knowlesi. These preparations have been shown to possess cytochrome oxidase activity by means of a specific enzyme assay procedure. This activity can now be attributed directly to the parasite.

Although the presence of cytochrome oxidase in P. knowlesi indicates the ability of this organism to activate molecular oxygen, this does not by itself allow for the conclusion that the organism possesses a functional cytochrome mediated electron transport system. While true mitochondria have not been reported to P. knowlesi, "double membrane" bodies have been shown to occur. These may or may not contain cytochrome oxidase. Further studies employing parasite preparations free of platelets and white blood cells are required to elucidate the functionally significant metabolic pathways which exist in plasmodia.

## 2. Glycolytic and cytochrome oxidase activity in plasmodia.

In early studies of the cultivation of human malaria, Bass and Bass and Johns stated that anaerobic conditions are necessary for survival of the parasites. However, these reports were difficult to evaluate, since survival data were based on observations of parasites in stained blood films. Anfinson et al. found that P. knowlesi in monkey red cells multiplied equally well with 0.37% oxygen as they did with 20% oxygen, and that there were fewer degenerate forms at the lower oxygen concentration. Although the effects of complete anaerobiosis have not been reported, McKee suggested that lowering the oxygen supply below 0.4% diminished growth and multiplication of P. knowlesi, and Trager demonstrated that air had a favorable effect on the survival of P. lophurae in chicken red blood cells.

Most of the evidence in the above reports indicates that plasmodia depend on oxygen for survival, although the possibility still exists that their requirements may be microaerophilic. If plasmodia are aerobic, then the tricarboxylic acid (TCA) cycle may be acting as the major energy yielding terminal respiratory system. Evidence has been presented indicating that this pathway is present in some plasmodia, but recently the existence of the TCA cycle in P. berghei and P. vinckei has been questioned seriously.

In an effort to resolve these conflicting reports, properties of the terminal respiratory pathway of P. knowlesi were examined and parasite preparations were assayed for cytochrome oxidase. Other investigators reported the presence of cytochrome oxidase in P. cathemerium and P. berghei.

The present studies extend the earlier findings of Scheibel and Miller who employed white blood cell and platelet-free preparations, and a more specific assay procedure to demonstrate the existence of cytochrome oxidase in P. knowlesi.

Blood from infected mice and monkeys was processed to obtain parasite preparations which were essentially free of white blood cells and platelets. Twenty minutes before infected monkeys were bled by cardiac puncture they were injected intravenously with 10 mg adenosine diphosphate (ADP) in 1 ml saline to promote clumping of platelets. Blood was collected from the mice in the same manner, except that the adenosine diphosphate was added to the drawn blood. Chilled bottles containing 0.67 mg heparin/ml blood were used to collect blood from the monkeys. For mouse blood 0.2 mg heparin/ml was added to the bottles before collection. White blood cells and platelets were removed by passing the blood through glass beads and powdered filter paper columns according to procedures described previously. Red blood cells were then separated from the eluates by centrifugation at 4340 x g for 15 minutes and the supernatant fluids were discarded. The pellets containing both free and intracellular parasites were resuspended to a concentration of 20% (v/v) in the high potassium medium, and the red blood cells were ruptured in a French pressure cell to release the parasites. P. knowlesi were released at a pressure of 2,000 p.s.i. Intact red blood cells were separated from the free parasites by centrifugation at 125 x g for 10 minutes. The parasites were then harvested from the supernatant fluid by centrifugation at 4,340 x g for 25 minutes and taken to a 10% (v/v) suspension in the appropriate high potassium medium. These cells infected monkeys both before and after incubation. P. knowlesi parasites prepared in this manner were then added to the respirometer for metabolic determinations.

Parasites used for cytochrome oxidase assays were prepared in a similar manner, except that cells obtained from the powdered filter paper column eluates were washed twice with 0.057 M sodium phosphate buffered saline, pH 7.4, containing 0.2% glucose and once with the appropriate high potassium medium. P. cynomolgi were released from the red blood cell at 1,500 p.s.i., and P. berghei at 2,5000 p.s.i. In addition, after passage through the French pressure cell and removal of unbroken red blood cells, the parasites were harvested by centrifugation at 27,000 x g for 15 minutes and washed with the high potassium medium until a clear and colorless supernatant fluid was obtained.

Cell-free extracts of P. knowlesi, P. cynomolgi and P. berghei were prepared by suspending the washed parasites in 0.05 M potassium phosphate buffer (20% v/v), and passing them through a French pressure cell at 18,000 p.s.i. For determinations of cytochrome oxidase activity, the cell-free extracts were centrifuged at 27,000 x g for 15 minutes and the supernatant fluid and residue, which was resuspended in 0.05 M phosphate buffer (pH = 7.4), were assayed separately according to the procedure of Smith. Cytochrome c (Sigma Chemicals, St. Louis) was reduced by the addition of sodium hydrosulfite. Excess hydrosulfite was removed by aeration.

Cells were incubated in conventional Warburg vessels in a Gilson Differential Respirometer for 30 minutes at 37°C in an atmosphere of air or nitrogen as indicated. Respiratory carbon dioxide was trapped in alkali in the center well. Reactions were terminated by the addition of perchloric acid to a concentration of 3%. Before assays were made for radioactivity, the respiratory carbon dioxide trapped in the center wells was redistilled by acidification in Warburg vessels. The liberated carbon dioxide was then trapped in hyamine hydroxide. Radioactivity was determined on aliquots by scintillation counting in Bray's solution.

The incubation medium was neutralized with KOH, and lactate was quantified enzymatically by a modification of the method of Lowry et al. Lactate dehydrogenase (Worthington, Inc.) was diluted with 0.9% NaCl rather than albumin and the buffer used was 2-amino-4-methyl-1-propanol, pH 9.7.

Glucose was assayed spectrophotometrically at 340 m $\mu$  by a variation of the method of Slein. The reaction mixture contained 40  $\mu$ moles glycyl glycine buffer (pH 7.4), 0.8  $\mu$ moles MgCl<sub>2</sub>, 3.75  $\mu$ moles adenosine triphosphate (ATP) and 0.3  $\mu$ moles triphosphopyridine nucleotide (TPN), 0.56 units of hexokinase and 0.23 units of glucose-6-phosphate dehydrogenase (Boehringer and Soehne) in 0.05 M glycyl glycine buffer (pH 7.4). The total volume was 0.8 ml. Glucose 3,4 C<sup>14</sup> was obtained from New England Nuclear Corporation, and glucose 1-C<sup>14</sup> and glucose 6-C<sup>14</sup> from Nuclear Chicago Corporation. Protein was determined by the procedure of Lowry et al.

The principles of laboratory animal care as promulgated by the National Society of Medical Research were observed.

Cytochrome oxidase activity in plasmodia. Regardless of the medium in which the parasites were prepared, cytochrome oxidase activity was found to be present in P. knowlesi, P. cynomolgi and P. berghei (Table 3). This was evidenced by the ability of the parasites to reoxidize reduced cytochrome c. Cyanide (10<sup>-3</sup>M) inhibited the enzymatic activity of all preparations, further establishing the identity of cytochrome oxidase. All activities in the parasitized samples were higher than in the corresponding non-parasitized samples from which white blood cells and platelets were removed. The cell preparations were a mixture of ring, trophozoite and schizont stages of the parasite, and therefore, the possibility that a single stage was responsible for all the oxidase activity cannot be ruled out.

Glucose-C<sup>14</sup> utilization, lactate and CO<sub>2</sub> production. The presence of cytochrome oxidase does not by itself establish the parasites as aerobes. Therefore, attempts were made to determine whether or not a Pasteur effect was demonstrable in these parasites, and whether or not the tricarboxylic acid cycle was operating as an energy yielding pathway

Table 3

## Cytochrome Oxidase Activities in Plasmodia

Species	Media	Percent Parasitemia	Cytochrome Oxidase Activity		
			µ Moles Cytochrome Oxidized/min/mg Protein		µ Moles Cytochrome Oxidized/min/ml Parasitized Blood
			Supernatant Fluid	Residue	
<u>P. knowlesi</u>	Bowman's	72.0	16.7	0.0	5.12
<u>P. cynomolgi</u>	Bowman's	20.5	13.3	0.0	4.34
<u>P. cynomolgi</u>	Trager's	25.0	9.67	2.54	1.60
<u>P. berghei</u>	Bowman's	30.0	1.22	4.13	1.32
<u>P. berghei</u>	Trager's	32.5	1.15	0.0	0.67

of major significance. The Pasteur effect i.e. a more rapid utilization of substrate anaerobically than aerobically which results from a competition between aerobic and anaerobic pathways for common rate limiting cofactors, is characteristic of aerobic tissues. Cells which do not rely upon oxygen for energy metabolism do not exhibit such an effect. Free parasites, suspended in either Bowman's medium or Trager's medium (Table 4), were incubated in the presence of various species of  $C^{14}$ -glucose. Glucose disappearance, lactate formation and the incorporation of radioactivity into respiratory  $CO_2$  were measured. Under the conditions employed no significant difference was found between the aerobic and anaerobic utilization of glucose (Table 4) which indicates the absence of a true Pasteur effect. The addition of coenzyme A (CoA) and reduced glutathione (GSH) was found to have no significant effect on the aerobic versus anaerobic utilization of glucose or lactate accumulation. It is of interest also that in no case did the quantities of lactate formed account for the amounts of glucose disappearing which indicates either the accumulation of glycogen or of other products. Glycogen, however, has not been found in P. berghei, P. knowlesi, P. gallinaceum or P. cynomolgi. In all experiments, lactate accumulation was higher in an anaerobic environment demonstrating that differences do exist between the aerobic and anaerobic metabolisms.

The possibility of a complete oxidation of significant amounts of glucose to carbon dioxide can be ruled out on the basis of the low incorporation of radioactivity from the 1, 6, 3 and 4 carbons of glucose

Table 4

Glucose C<sup>14</sup> Utilization and the Formation of Lactate  
and CO<sub>2</sub> by Plasmodium knowlesi

Expt.	Substrate	Glucose Utilized		Lactate Formed		Glucose C <sup>14</sup> Incorporation into CO <sub>2</sub>	
		Mμ Moles/ml	Mμ Moles/ml/mg	Mμ Moles/ml	Mμ Moles/ml/mg	Mμ Moles/mg Protein	Percent
A	Glucose-1-C <sup>14</sup>	521.0	103.7	130.6	26.0	2.44	0.55
		501.0	99.7	71.6	14.2	3.18	0.75
	Glucose-3,4 C <sup>14</sup>	446.0	88.7	108.6	21.5	4.25	1.19
		437.0	87.0	61.1	10.2	10.45	2.70
	Glucose-3,4 C <sup>14</sup> +CoA + GSH	639.0	137.9	120.6	24.0	2.96	2.00
		639.0	137.9	113.6	22.6	4.63	3.14
B	Glucose 1 C <sup>14</sup>	1200.0	168.0	625.0	87.5	1.69	1.00
		1160.0	162.0	340.0	47.5	3.47	2.14
	Glucose 6-C <sup>14</sup>	1060.0	148.0	605.0	84.7	0.28	0.19
		760.0	106.0	247.0	34.6	0.84	0.79

into respiratory  $\text{CO}_2$ . These findings imply that a tricarboxylic acid cycle could not operate under the conditions of these experiments, since the cyclic mechanism requires the liberation of two molecules of carbon dioxide for each turn of the cycle. Similarly, it would be expected that a  $\text{C}_2$  compound such as acetate would not be accumulating in large quantities, as this also would require the evolution of carbon dioxide or another one-carbon compound.

Effects of glucose concentration on lactate formation. In experiments to determine the effects of glucose concentration on the utilization of this substrate and on the formation of lactate, no effect on the aerobic vs. anaerobic utilization of glucose was demonstrable (Table 5). As before, the presence of air did not alter the rate of disappearance of the substrate regardless of concentration, although there appeared to be greater utilization at higher concentrations. On the other hand, the ratio of glucose utilization to lactate formation was increased considerably at the higher substrate concentrations both in the presence and absence of air. The possibility arises, therefore, that increasing the concentration of glucose may facilitate the use of ancillary pathways not involving lactate.

Although mature erythrocytes do not contain cytochrome oxidase or tricarboxylic acid cycle activities, white blood cells and platelets are rich in both of these enzyme systems. For this reason, it is possible that many early investigators attributed to plasmodia the enzymatic activities of white blood cells and platelets which were present as contaminants. The procedures used in the present investigation effectively removed the platelets and white blood cells and thereby demonstrated that the cytochrome oxidase activities of P. knowlesi, P. cynomolgi and P. berghei are indeed properties of the parasite.

An apparent discrepancy was observed concerning the existence of a Pasteur effect. The rate of glucose utilization was unaffected by the presence of oxygen, indicating the absence of a Pasteur effect was present. The possibility remains that glucose entering the parasite was constant, but that one portion formed lactate and another was diverted independently to some other component. Admission of air could then alter the relative quantities of each component formed, resulting in an apparent Pasteur effect related to lactate formation but not to glucose disappearance. Furthermore, increasing the glucose concentration served to increase the ratio of glucose utilization to lactate formation, which may also result from a differential effect of substrate concentration on each pathway.

Evidence has been obtained, in these studies, indicating that products other than lactate are formed. In homolactate fermentations, two moles of lactate are formed for each mole of glucose utilized, but in none of these experiments did lactate account for the quantity of glucose which disappeared. Although acetate has been reported to be a

Table 5  
Effects of Glucose Concentration on Lactate Formation

Concentration of Glucose	Atmosphere	Medium	$\mu$ Mole Glucose Utilized/mg Protein	$\mu$ Mole Lactate Produced/mg Protein	Glucose: Lactate
Low	N <sub>2</sub> Air	Trager's	.0263	.0406	1:1.5
		Trager's	.0255	.0250	1:1.0
Low	N <sub>2</sub> Air	Trager's	.0176	.0187	1:1.1
		Trager's	.0157	.0151	1:1.0
Low	N <sub>2</sub> Air	Trager's	.0143	.0254	1:1.8
		Trager's	.0214	.0248	1:1.2
High	N <sub>2</sub> Air	Bowman's	.0271	.0140	1:0.52
		Bowman's	.0277	.0207	1:0.75
High	N <sub>2</sub> Air	Bowman's	.0937	.0230	1:0.25
		Bowman's	.0955	.0122	1:0.13
High	N <sub>2</sub>	Trager's	.1580	.0861	1:0.54
		Trager's	.1340	.0410	1:0.31

product of free P. gallinaceum, it appears unlikely that significant quantities of any C<sub>2</sub> compound would have accumulated in these experiments, since so little respiratory carbon dioxide was recovered. Similarly, under the conditions employed, neither the tricarboxylic acid cycle nor the pentose phosphate pathway could operate to any large extent because each of these requires the elaboration of carbon dioxide. The small differences between the incorporation of radioactivity from the 1 and 6 carbons of glucose into respiratory CO<sub>2</sub> (Table 4) may have resulted from the presence of small quantities of red blood cell (RBC) stroma in the parasite preparations. Red blood cell hemolysates are known to catalyze the pentose phosphate pathway.

Although the reported findings are in general agreement with those of previous investigators, they should not be considered applicable to other plasmodia until further experimental work is accomplished. Factors such as the physiological status of the organisms, incubation media, cofactor requirements, etc., cannot be fully evaluated at present. These factors may account for the variation in results of plasmodial metabolism studies as reported in the literature.

### 3. Resistance produced in owl monkeys by inoculation with irradiated Plasmodium falciparum.

Attempts to induce immunity to malaria have been undertaken by numerous investigators with varying degrees of success. The inconclusive results obtained with killed vaccines have been attributed to the loss or denaturation of functional antigens during the preparation of the vaccines or to the rapid elimination of parasites in the host. Sinton postulated that the amount and rate of development of immunity to malaria may depend upon the amount and duration of antigenic stimulation. Whereas limited success was achieved by numerous investigators in their attempts to immunize animals with killed parasites, positive results were reported in immunizing mice with a strain of Plasmodium berghei which had been rendered noninvasive by incubation in a tissue culture medium containing hamster serum.

The irradiation of parasites interferes with their physiologic processes and frequently inhibits their normal development and multiplication. The dose of irradiation needed to suppress multiplication appears to be considerably smaller than the lethal dose or that required to affect the plasmodia's metabolic processes. Therefore, irradiation can be used to take advantage of special immunizing properties of living parasites in the absence of the pathogenic effects of replicating parasites.

Immunization by the use of irradiated plasmodia has been reported by Ceithaml and Evans. They found that irradiated erythrocytes parasitized with P. gallinaceum when injected into normal chickens conferred resistance to a challenge with normal parasites of the same strain. Subsequent studies indicated that resistance developed in rats, and in

mice immunized with irradiated erythrocytic forms of P. berghei. The degree of acquired resistance was influenced by the number of immunizing exposures and detectable levels of fluorescent and protective antibodies were produced. Appreciable protection and high levels of sporozoite agglutination titers could be induced in birds by immunization with ultraviolet irradiated sporozoites of Plasmodium gallinaceum. These observations were confirmed by Richards and extended to P. berghei by Nussenzweig et al. The partial protection obtained in mice with a single immunizing dose of irradiated sporozoites of P. berghei could be increased with repeated immunizing injections. The incubation of sporozoites of P. berghei in sera from immunized animals produced a considerable loss of infectivity of the parasite.

Since host parasite relationships differs strikingly in different hosts infected with different plasmodia, it is impossible to extrapolate the above results obtained with bird and rodent malaras to human infections. However, the recent discovery that owl monkeys (Aotus trivirgatus) are susceptible to infection by P. falciparum of human origin has opened the way for direct experimentation with this parasite of man. Blood induced P. falciparum infections with predictable parasitemias and duration of survival time have been carried out routinely. Because of its smaller size, ready availability and susceptibility to blood induced infections with P. falciparum, owl monkeys are well suited for immunization studies with this parasite. However, some difficulties have been experienced with these animals because of their susceptibility to viral and bacterial infections.

The present series of experiments was undertaken to determine whether protective immunity could be induced in owl monkeys by inoculation of irradiated P. falciparum-infected erythrocytes.

A total of 72 owl monkeys of both sexes, ranging in weight from 450 to 900 gm at the beginning of each experiment, was used in these studies. Of these, 14 were used as sources of parasites for immunization and challenge of the experimental animals. The remaining 58 monkeys were used in 3 experiments. Seventeen of these animals died from causes unrelated to malarial infection between the time they were selected for these studies and the time of challenge, and are not included in the tables. Details about the quarantine, diet, caging and general handling of the animals are given in detail elsewhere. In conducting the research described in this report, the principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

The Camp strain of P. falciparum was used in these experiments. This strain has been maintained by serial transfer of infected blood in nonsplenectomized owl monkeys. Uniformly fatal infections are produced in these animals by intravenous inoculation of approximately  $1 \times 10^8$  parasitized red blood cells. With this dose, the parasites can be detected in the peripheral blood of nonimmunized animals approximately

2 days after inoculation. The parasitemia increases rapidly and the animals die about one week later. Since high parasitemias of 25 to 75 percent usually result from these infections, a delay of even a few days in the development of an infection or a reduction in the percent parasitemias indicates that the inoculum has been substantially destroyed or that the parasite multiplication rate has changed.

Donor monkeys for P. falciparum parasitized erythrocytes were exsanguinated by cardiac puncture. The blood was then transferred to a centrifuge tube in an ice bath. Normal blood was usually collected from the femoral veins of 2-3 healthy monkeys. Both the parasitized and normal blood were centrifuged at 1,500 rpm for 20 minutes and the plasma was decanted. The packed cells were then reconstituted to the desired concentration with cold saline. An inoculum containing between  $5 \times 10^8$  and  $2 \times 10^9$  infected RBC's per ml and an equal volume of nonparasitized cells were placed in each of a number of plastic tubes. The tubes were then placed in the irradiator and exposed to 25,000 rads. A "Gammacell 220" cobalt irradiator described by Rice and Smythe was used as the source of gamma radiation. This 1,300-C source delivered a dose rate calculated by the Fricke ferrous sulfate method of Weiss et al. of approximately 15,000 rads per minute. The procedure for irradiation was essentially the same as that described previously. Each inoculum was kept in an ice bath until the time it was placed in the irradiator and afterwards until the animals were injected. All immunizing and control inocula were given intravenously. In the first experiment, the monkeys were given 4 inoculations ranging from  $2 \times 10^9$  to  $9 \times 10^9$  irradiated parasitized red cells whereas in the other experiments inocula contained between  $5 \times 10^8$  and  $2 \times 10^9$  irradiated parasitized red cells.

One week after the last immunizing dose, all animals were challenged intravenously with  $1 \times 10^8$  parasitized nonirradiated RBC's in a 0.5 - 1 ml volume. The monkeys were usually examined daily for the presence of malarial parasites in thick and thin smears stained with Giemsa stain. Parasite densities were estimated and recorded as percent parasitemias. If parasites were visible on the smears but were not sufficient for making quantitative determinations, they were recorded as less than 0.1 percent. All animals were observed daily for any abnormalities in behavior or general appearance. In the first experiment, symptomatic treatment was initiated immediately upon detection of concurrent infections. In the subsequent experiments no treatment was given to any of the monkeys regardless of their health status. Antibody levels were measured in some animals at various intervals by the indirect hemagglutination test (IHA) employing as antigen a P. falciparum infected erythrocyte lysate, with strict adherence to the published method.

The first experiment was designed to determine whether inoculations with parasitized blood exposed to 25,000 r would stimulate a demonstrable resistance to a challenge infection with nonirradiated parasites. Since clinical observations were subjective, the parameters used in determining resistance were survival time, percent mortality, and parasitemias.

Eighteen monkeys were divided into three groups. During the immunization period one monkey from each group died. There was no evidence that immunization contributed to these deaths or that it had any ill effect on the animals that survived. Four monkeys were inoculated 4 times with irradiated parasitized RBC's, 5 others were given irradiated nonparasitized RBC's and 6 others served as untreated controls. In those animals which received irradiated parasitized cells, parasites were usually detectable 24 hours after inoculation. However, no persisting infections were ever evident on repeated blood smear examinations. All the animals were challenged one week after the last immunizing inoculation with  $1 \times 10^8$  nonirradiated parasitized RBC's. All monkeys of the control groups became patent 1 or 2 days after challenge. In all but one (No. 12) the parasitemia increased rapidly and 10 days after challenge the animals were dead. The parasitemia in monkey No. 12 remained low until day 21 and the animal died 30 days after challenge with a parasitemia of 35.0 percent. None of the immunized monkeys developed a demonstrable parasitemia. Two animals in this group (Nos. 2 and 3) died on days 3 and 4 with acute septicemia, and 2 survived to the end of the experiment, 90 days after challenge. The 5 monkeys that died between 3 and 4 days after challenge without developing high parasitemia (Nos. 2, 3, 6, 8 and 15) were noted to have developed swelling in the leg at the site of inoculation 2 days after challenge. Blood and tissue cultures revealed that these animals had become infected with a gram negative bipolar rod similar to Pasteurella multocida and penicillin-streptomycin treatment was initiated in all animals at that time. Antibiotic therapy had no apparent effect on the course of malaria infection in the animals. The source of the bacterial infection was believed to have been the donor monkey since plasma from this animal was found to harbor the same organism.

Although the results of this first experiment were very suggestive, they were not conclusive because of concurrent bacterial infections. Therefore, a second experiment essentially similar to the previous one was undertaken. A total of 22 owl monkeys was divided into 3 groups. Twelve monkeys died prior to challenge and were omitted from tabulation. Of the remaining 10, 5 animals received 4 weekly doses of irradiated parasitized RBC's, 5 others were not treated. All the animals were challenged one week after the last immunization with  $1 \times 10^8$  nonirradiated parasitized RBC's. The 2 monkeys which had survived the challenge from experiment No. 1 were rechallenged at this time (90 days after the primary challenge). All of the nonimmunized animals became patent 1 or 2 days after challenge, rapidly developed high parasitemias and died within 9 days after challenge. Conversely, the 5 immunized monkeys developed lower parasitemias and 4 had increased survival times. Deaths occurred with relatively low parasitemias and one of the animals in this group was still alive 150 days after challenge. One of the two monkeys which were rechallenged 90 days after the primary challenge never developed a patent parasitemia (No. 1). Only occasional parasites were observed in the other animal between the 12th and 23rd day after challenge (No. 4). Both of these monkeys were still alive 150 days

after rechallenge.

The results of the first two experiments are summarized in Table 6. The animals that died within 5 days, probably from causes other than malaria, are omitted. Among the immunized animals (Group I) two never developed a patent parasitemia. Of the 5 in which parasites were seen in the peripheral blood, 1 survived after a transient infection and the others died with low parasitemia. Conversely, 12 of the 13 animals which were nonimmunized (Groups II and III) developed fulminating infections and died shortly afterwards with a very high parasitemias.

Table 6

Protection Induced in Owl Monkeys by 4 Weekly Inoculations of Irradiated Parasites (Experiments I & II)

Group (Treatment)	No. of Animals	Patent Parasitemia		Survivors		
		No. of Animals	Peak Percent Mean Range	No. of Animals	Percent	Survival (days)
I Irradiated Parasitized RBC's	7	5	2.6 1.0-9.6	3	43.8	35
II Irradiated Normal RBC's	4	4	36.0 25-49	0	0	8.5
III Untreated Controls	9	9	60.3 33-78	0	0	8.0

In view of these results, an additional experiment involving 18 owl monkeys was designed to determine whether a detectable degree of acquired resistance could be induced by fewer immunizing inoculations. Two animals died prior to challenge and were omitted from tabulation. Of the remaining 16, one monkey received a single dose of irradiated parasitized RBC's (Group I, 4 Group II) were given three inoculations of irradiated parasitized cells, 4 Group III were given irradiated cells from uninfected monkeys and 7 Group IV were used as untreated controls. All the animals were challenge one week after the last immunizing inoculation with approximately  $1 \times 10^8$  nonirradiated parasitized RBC's.

All monkeys became patent within 3 days after challenge. The parasitemias in each of the animals of Groups I, III and IV and in 2 monkeys of Group II increased rapidly and all animals died between 6 and 17 days after challenge. Conversely, the parasitemia levels in 2 of the animals which received 3 inoculations with irradiated parasitized cells (Group II) remained relatively low. They died apparently of untreated upper respiratory disease on days 27 and 29.

In order to find out whether malarial antibodies could be detected in the owl monkeys immunized with irradiated infected blood, serum specimens from these animals and from 18 nonimmunized controls were tested by the indirect hemagglutination test employing as antigen lysates from P. falciparum infected erythrocytes. No antibodies were detected in any of the 27 animals before immunization (Table 7). Antibodies were detected in 4 of the 10 immunized animals before challenge. Three of these are animals which survived the challenge infection. After challenge no antibodies were detected in the nonimmunized controls up to the time they died.

Table 7

The Detection of Antibodies in Owl Monkeys at  
Given Times after Immunization with Irradiated P. falciparum

Monkey No.	Results of IHA Titer (reciprocal)							
	Pre immuni- zation	Pre challenge	Time after challenge (weeks)					
			1	4	5	6	10	12
1	<20	80	80	2560	1280	1280	1280	2560
2	<20	<20	died					
3	<20	<20	died					
4	<20	160	320	1280	2560	10240	640	1280
318	<20	160	320	5120	ND	ND	ND	ND
328	<20	<20	<20	died				
314	<20	<20	<20	died				
327	<20	<20	<20	<20	ND	died		
367	<20	80	80	died				
Unimmunized controls (18 animals)	<20	<20	<20	died				

ND = Not done

An analysis of the foregoing experiment indicates that, in general, an acquired resistance to infection with P. falciparum developed in owl monkeys after 4 weekly immunizations with irradiated malarial parasites of the same strain. The resistance was sufficient to inhibit the multiplication of the plasmodia and to permit some animals to survive otherwise lethal challenge with nonirradiated parasites. A less striking degree of acquired immunity was observed following 3 weekly immunizations and no detectable immunity resulted from a single immunizing dose. These results, indicating that protection is directly related to the number of immunizing inoculations, are in agreement with and extend those previously reported for rodent malaria.

In view of the relative ease with which protection can be induced by irradiated parasites, it is somewhat surprising that control of malaria by artificial immunization has proved to be so unrewarding. The literature on the subject reveals that in most parasitic infections attempts at immunization with vaccines prepared from dead parasites or parasite extracts have given rather disappointing results as compared with immunity stimulated by natural or experimental infections. It seems likely that immunization with irradiated parasites may be similar to that produced with living non-attenuated parasites in nonfatal infections. Thus, it has been postulated that antigenic substances associated with living actively metabolizing parasites are important for producing a strong immunity. Therefore, it is possible that vaccination with irradiated parasites may result in an acquired immunity as great as or even greater than that produced by infection with the living non-attenuated parasites.

The present experiments do not permit us to determine whether acquired immunity in malaria is stimulated by the irradiated parasites per se or by other materials present in the parasitized erythrocytes. Cox suggested that substances elaborated by injected erythrocytes or erythrocyte substances modified by infection may become antigenic. These nonspecific "antigens" are present in the serum of animals during the acute stages of Plasmodium and Babesia infections and render the animals resistant to challenging infection with homologous or heterologous parasites.

The injection of serum, plasma or globulin from animals with acute malaria or babesiosis into normal animals of both homologous and heterologous species was found to cause severe anemia. Zuckerman suggested that enzymatic products of the intracellular parasites might be released into the blood stream and damage the cells. No detailed investigations were conducted to determine whether the process of immunization produced an anemia in our owl monkeys. However, limited hematological observations have failed so far to suggest such an eventuality in these animals.

It was of interest for theoretical and diagnostic considerations to determine whether antibodies are produced in the vaccinated animals to be detected by the indirect hemagglutination test. The results indicated that 4 owl monkeys were able to produce a detectable level of antibodies even when the normal development and multiplication of plasmodia was interrupted by irradiation. These results are in agreement with those reported from mice injected with irradiated P. berghei and other animals with irradiated helminths. It is of interest to notice that the 3 immunized animals which survived the challenge were among those which developed detectable levels of IHA antibodies prior to challenge. Serum from mice immunized with irradiated P. berghei had an immunosuppressive effect when passively transferred to susceptible mice. At present, one cannot state whether the protection stimulated by immunization with irradiated P. falciparum is primarily or exclusively humoral in nature. Recent studies by various investigators suggest that multiple mechanisms are involved in malarial immunity. The role of humoral factors in malaria is well established. Moreover, a degree of correlation was noted between the IHA titer employing parasitized erythrocyte lysates and protective activity of various globulin preparations. However, other factors such as stimulation of the reticuloendothelial system and interferon may play an important role in protection against malaria. Recent investigations have strongly suggested that acquired immunity to P. berghei in the rat involves the development of cell-mediated immunity in addition to the production of protective antibody. Neonatal thymectomy, which primarily effects the cell-mediated immunologic response, reduced the resistance of rats to P. berghei. Immunity has been passively transferred with sensitized lymphoid cells from lymph nodes and spleen. Conversely, thoracic duct lymphocytes or macrophages obtained from the stimulated peritoneal cavities of immune rats had no demonstrable protective effect. The relative role of cellular, humoral and nonspecific factors in immunization plasmodia is being investigated.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 129, Host responses to malaria

4. Publications.

No publications.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION NO.	2. DATE OF COMPLETION	3. REPORT NUMBER
				DA 0A6523	69 07 01	DD FORM (ARJ) 655
4. DATE PREV SUMMARY	5. KIND OF SUMMARY	6. SUMMARY ACTY	7. WORK SECURITY	8. REGRADING	9. DISPOSITION	10. SPECIFIC DATA CONTRACTOR ACCESS
68 10 31	D. Change	U	U	NA	NL	<input type="checkbox"/> YES <input checked="" type="checkbox"/> NO
11. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	63713A	3A663713D929	00	130		
b. CONTRIBUTING						
c. CHANGES	1412A(2)					
11. TITLE (Precede with Security Classification Code)						
(U) Literature of Malarial Test Data (09)						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS						
002600 Biology						
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07 65	NA	DA		C. In-House		
17. CONTRACT/GRANT	18. RESOURCES ESTIMATE	19. PROFESSIONAL MAN YRS		20. FUNDS (In Dollars)		
NA	PRECEDING	2		70		
a. DATES/EFFECTIVE:	b. NUMBER	FISCAL YEAR	CURRENCY			
		69				
c. TYPE:	d. AMOUNT:	70				
e. KIND OF AWARD:	f. CUM. AMT.	2		70		
19. RESPONSIBLE DOD ORGANIZATION			20. PERFORMING ORGANIZATION			
NAME			NAME			
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			SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE			22. ASSOCIATE INVESTIGATORS			
Foreign Intelligence Considered			NAME:			
			NAME: DA			
23. REVIEWS (Precede Each with Security Classification Code)						
(U)Malaria; (U)Chemical; (U)Chemistry; (U)Pharmaceutical; (U)Literature						
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Precede individual paragraphs identified by number. Precede rest of each with Security Classification Code.)						
23. (U) To maintain in machineable form all biological information associated with the test programs supported by the malarial project.						
24. (U) Preliminary machining of approximately 50 percent of data is accomplished at the source. The remainder of preliminary machining and final processing of all data are done at Walter Reed. The chemical typewriters are used in association with conventional key-punch machines. Programs have been written to allow processing of both biological and chemical into one output.						
25. (U) 68 10 - 69 06 A total of 28 additional programs were written to handle biological data. This included program for one new test system and major modifications in one existing test system. This also included programming to allow in-house processing of data from one test system in which all data was previously completely processed at source. Microfilming of biology data sheets was accomplished during this period and computer programming for the maintenance of file index was delivered. Currently 40 hours of 1401 computer time and 18 hours of 7090 time are required per week to maintain and process new data. Records for 175,000 compounds and data from 725,000 biological tests are stored on 34 tapes. Files are updated weekly with 1000 new compounds (or combinations) and 4000 biological tests. For technical reports, see Walter Reed Army Institute of Research Annual Report, 1 Jul 68 - 30 Jun 69.						

PII Redacted

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 130, Literature of Malarial Test Data

**Investigators**

Principal: David P. Jacobus, M.D.

Associate: Edgar H. Eckermann, LTC, VC; Gen Jue, CPT, MSC, Robert Pick, CPT, MSC; Alfred P. Feldman; John F. Waters, Daniel F. Boehel, 1LT, MSC

Description

The development of computer programming for storage and retrieval of drug screening data, drug inventory, and shipping history has been an important aid in guiding the antimalarial drug development program of the Division of Medicinal Chemistry. Programming for the control of chemical inventory assists in the distribution of samples and compound information and makes possible central coordination of testing programs and selection of compounds for test. The ability to interface biology and chemistry files permits searching of files by chemical structure as well as by activity, time, submitter, and various combinations of these parameters.

Progress

A. Inventory Control

The computer system for control of chemical inventory has been completed and put into operation. Computer Usage Corporation was contracted to program and maintain this system. The use of an additional identification number (bottle number) for each submission has made it possible to maintain tight control on drug lot. A cross reference to these bottle numbers with Walter Reed numbers makes it possible to identify all lots of a particular chemical structure. The use of bottle numbers for reference to submitters and test systems also functions as an additional safeguard for the handling of "commercially discreet" information. All shipping lists and labels are machine generated, which facilitates production of machine generated management report of samples overdue for shipment, receipt, or test results. All "backlog" samples received before the current inventory control program was operational and their shipping histories have been incorporated into the system. Recent improvements in all three systems have allowed the inventory to interface on a searchable basis with chemistry and biology.

B. Biological Data

Contracts for computer programming were continued with Advanced Computer Techniques and Service Bureau Corporation. Programming was produced for

a new secondary screening system at the University of Georgia operated by Dr. Paul Thompson, an In-Vitro system measuring inhibition of nucleic acid synthesis operated by Dr. Knox Van Dyke at the University of West Virginia and an insect repellent test system maintained by the Department of Agriculture. The data processing system used by the Illinois Institute of Technology for handling secondary test system data underwent major revision to allow all processing to be done at WRAIR and to make it possible to combine this file with other biology files and interface it with chemical structure files. Modifications of the system used to process data from the mosquito - P. gallinaceum system was begun to allow processing data from similar systems using human and sub-human primate strains of malarial parasites. This modification will also make it possible to process data when leucovorin is added to the system in order to determine if drug activity can be reversed. All new programs and all modifications include necessary routines to permit interfacing with chemistry files. A general merge has been completed for all biology systems and a general print program is presently being written. Preliminary studies have been completed for the creation of an executive type routine capable of accepting any edited biology file for processing through the general merge and print programs. This will materially reduce the time involved by Division of Medicinal Chemistry personnel in monitoring production runs.

#### C. Interface of Biology and Chemistry Files

The addition of new test systems and the continued high throughput in the basic screens has resulted in massive biology files that makes the interface with chemistry cumbersome and time consuming, particularly where one or more sorts are involved. A partial solution to this problem was the production of programming that permits the matching of a file of selected records against up to three biological files so only related records are selected. The creation of a combined biology file for search by a chemistry file, however, remains unwieldy and a study is being conducted to provide programming that permits the merging of a short biology record with chemistry with the necessary expansion occurring post-merge.

#### D. Microfilm Storage of Data

All raw data (data recording sheets) have been recorded on microfilm. All new data sheets are submitted for microfilming immediately following keypunching. Due to limited storage space, all data files that are no longer updated are being placed on microfilm. The computer is utilized for indexing all data to permit immediate retrieval from the microfilm.

#### Summary and Conclusions

All test systems have been programmed and can be processed through a general merge. A general print is near completion. Inventory and all biology systems can be interfaced with chemical files, however, due to the rapidly increasing size of the files this is becoming increasingly difficult and is requiring more computer time. The major effort in

reducing this problem is being directed at delaying expansion of records until after the interface of biological and chemical files. All files can be searched by date, submitter, structure, biological parameters and/or chemical structure.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 130, Literature of Malarial Test Data

Publications

None.

DAQA6525

69 07 01

1. DATE OF REPORT	2. KIND OF REPORT	3. SUMMARY STATEMENT SECURITY	4. SECURITY CLASSIFICATION	5. REPORT NUMBER	6. DA FORM NUMBER	7. SOURCE DATA DISTRIBUTION CODES	8. LEVEL OF EFFORT
31 01 69	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> A <input type="checkbox"/> B <input type="checkbox"/> C <input type="checkbox"/> D	1. CODE ONLY
9. PROGRAM ELEMENT		10. PROJECT NUMBER		11. TASK AREA NUMBER		12. WORK UNIT NUMBER	
63713A		3A663713D829		00		132	
13. CONTROLLING NUMBER		14. REPORT NUMBER		15. REPORT NUMBER		16. REPORT NUMBER	
1412A(2)							

(U) CLINICAL STUDIES OF HUMAN MALARIA (09)

17. SUBJECTIVE AND TECHNOLOGICAL AREAS			
00 2600 BIOLOGY			
18. ESTIMATED COMPLETION DATE	19. FUNDING AGENCY	20. PERFORMANCE METHOD	
11 65	DA	C. In-House	
21. CONTRACT/ORDINARY		22. RESEARCH ESTIMATE	23. PROFESSIONAL PAY YRS
a. DATE/EFFECTIVE: NA b. NUMBER: c. TYPE: d. KIND OF AWARD: e. RESPONSIBLE DOD ORGANIZATION:		69 70	2 2
e. AMOUNT: f. COM. AMT.:		70 70	70 70
24. NAME: Walter Reed Army Institute of Research Washington, D.C. 20012		25. NAME: Walter Reed Army Institute of Research Division of Medicine Washington, D.C. 20012	
26. RESPONSIBLE INDIVIDUAL NAME: Meroney, COL W. H. TELEPHONE: 202-576-3551		27. PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic Institution) NAME: Teschan, COL P. E. TELEPHONE: 202-576-3529 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]	
28. GENERAL USE Foreign Intelligence Considered		29. ASSOCIATE INVESTIGATORS NAME: Canfield, LTC C. J. RACE: DA	

PII Redacted

(U) Malaria; (U) Antimalarials; (U) Parasite; (U) Red Blood Cell

23. (U) - Study clinical disease of acute falciparum and vivax malaria, assess various modes of antimalarial therapy with respect to clinical responses and radical cure, study pathophysiology of the disease.

24. (U) - Document clinical features of acute disease, evaluate available therapeutic agents with respect to clinical response and radical cure, evaluate renal, erythropoietic, hormonal and fluid and electrolyte changes in acute disease.

25. (U) 69 01 - 69 06. Further studies on ferrokinetics in acute malaria suggests that the previously observed decrease in red cell incorporation of Fe 59 are corrected by treatment of the disease with quinine and DDS with or without added pyrimethamine. Pyrimethamine appeared to delay reticulocyte response in anemic patients and this could be partially corrected by the concurrent administration of folic acid. A field trial of Trimethoprim 1.5 sulfalene was begun in Vietnam for acute falciparum malaria. There were 4 relapses and 1 failure to respond in a group of 25 patients. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 132, Clinical studies of human malaria

**Investigators.**

Principal: COL Paul E. Teschan, MC

Associates: LTC Craig J. Canfield, MC; LTC William J. Cirksena, MC;  
LTC Howard I. Keller, MC

**Description:** The objective of this work unit was to assess the clinical response of patients to acute falciparum and vivax malaria. Not only was the effectiveness of specific drug regimens being evaluated in terms of suppression or cure, but also various aspects of the pathophysiology of the disease were studied. The particular fields of interest have been: therapeutics, fluid and electrolyte ("space studies"), erythropoietic, and endocrine.

**Progress:** Admissions to Walter Reed General Hospital for acute malaria have continued in small numbers. No clinical studies have been attempted on these patients due to the uncomplicated nature of their infections and the prompt response to accepted treatment regimens.

Previous work on the pathophysiology was summarized and published.<sup>1,2</sup>

Anemia in patients with acute falciparum malaria has continued to be an interesting problem and investigations were performed in the Republic of Vietnam during the reporting period. Ferrokinetic studies showed that a previously documented abnormality of radioiron incorporation was generally corrected by eradication of parasites with appropriate treatment. Pyrimethamine though did appear to delay reticulocyte response in patients who were anemic and this probably was partially corrected with folic acid. The administration of folic acid did not decrease therapeutic efficiency of combination therapy with quinine DDS and pyrimethamine.

A field trial with Trimethoprim and Kelfizina was completed in 25 patients with acute falciparum malaria in Vietnam. There were four relapses and one failure to respond.

**Conclusions:** Continued evaluation of the recovery from the anemia of malaria is necessary. Folic acid does not appear to decrease therapeutic efficacy and may contribute to earlier recovery. Trimethoprim and Kelfizina do not appear to be as satisfactory for treatment of acute falciparum malaria as quinine pyrimethamine and DDS.

BIBLIOGRAPHY

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 132, Clinical studies of human malaria

1. Brooks, M.H., Kiel, F.W., Sheehy, T.W., and Barry, K.G.: Acute pulmonary edema in falciparum malaria: A clinicopathological correlation. *New Eng. J. Med.* 279: 732, 1968.
2. Brooks, M.H., Malloy, J.P., Bartelloni, P.J., Sheehy, T.W., and Barry, K.G.: Quinine, pyrimethamine and sulphorthodimethoxine: Clinical response, plasma levels and urinary excretion during the initial attack of naturally acquired falciparum malaria. *Clin. Pharm. & Therapeutics*, October 1968.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTRACT NUMBER	
				DA 0A6483	69 07 01	ED-700 (22) 603	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. REGARDING <sup>e</sup>	8. DISPH. DISTR <sup>f</sup>	9. SPECIFIC DATA CONTRACTOR ACCESS <sup>g</sup>	10. LEVEL OF SUR <sup>h</sup>
31 01 69	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO. OF DESI <sup>i</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	63713A	3A663713D829	00	133			
b. CONTRIBUTING							
c. CONTINUING	1412A(2)						
11. TITLE (Precede with Security Classification Code) <sup>j</sup>							
(U) ACUTE RENAL INJURY AND FAILURE IN MALARIA (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>k</sup>							
012900 PHYSIOLOGY      012600 PHARMACOLOGY							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
07 63		NA		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: NA				FISCAL YEAR		3	
c. TYPE:				69		100	
d. KIND OF AWARD:				70		3	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D.C. 20012				Division of Medicine			
RESPONSIBLE INDIVIDUAL				ADDRESS: Washington, D.C. 20012			
NAME: Meroney, COL W. H.				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
TELEPHONE: 202-576-3551				NAME: Teschan, COL P. E.			
21. GENERAL USE				TELEPHONE: 202-576-3529			
Foreign Intelligence Considered				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
				ASSOCIATE INVESTIGATORS			
				NAME: Olsson, LTC R. A.			
				DA			
22. REVERSE (Precede with Security Classification Code) <sup>l</sup>							
(U) Anuria; (U) Kidney; (U) Shock; (U) Mannitol;							
(U) Renal Function; (U) Renal Tubule; (U) Malaria Endocrine							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security Classification Code.)							
23. (U) - To establish a rational approach in the prevention and treatment of acute renal failure associated with malaria.							
24. (U) - Animal models with malarial infections are used to study physiologic alterations with special emphasis on the pathophysiology of acute renal failure.							
25. (U) - 69 01 - 69 06. Due to procurement delays urgently needed equipment ordered in August 1968 has not been received. There is therefore no progress in this project. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

PII Redacted

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 133, Acute renal injury and failure in malaria

Investigator: COL Paul E. Teschan, MC

Description: An animal model that develops acute renal failure during malaria is utilized to study the pathophysiology sequence of acute renal injury. Clinical, laboratory, and morphological observations have been correlated in the Rhesus monkey infected with P. knowlesi malaria. The morphological baseline for these studies has been established by studies of normal monkey kidneys by means of light and electron microscopy and by histochemical technics.

Progress: This work unit has been inactive during the reporting period.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL	
				DA OA6531	69 07 01	DD-RS-E (A) 636	
3. DATE OF SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. RESEARCHING <sup>e</sup>	8. DISSEM INSTR <sup>f</sup>	9. SPECIFIC DATA CONTRACTOR ACCESS	
69 01 31	D. Change	U	U	NA	NL	<input type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES <sup>g</sup>		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
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b. CONTRIBUTING							
c. OTHERS		1412A(2)					
11. TITLE (Use code with Security Classification Code) <sup>h</sup>							
(U) Malaria Screening Systems (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>i</sup>							
002300 Biochemistry 008800 Life Support							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
10 66		NA		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
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d. KIND OF AWARD:				CURRENT		5	
e. AMOUNT:				70		150	
f. CUM. AMT.							
20. RESPONSIBLE DO - ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Inst of Res			
ADDRESS: Washington, DC 20012				ADDRESS: Div of Nucl Med			
				Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic institution)			
NAME: Meroney, COL W.H.				NAME: Mahin, LTC, D. T.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2211			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Considered				Johnson, LTC, M. C.			
				NAME:			
				NAME:			
				DA			
23. KEYWORDS (Provide each with Security Classification Code)							
(U) Malaria Plasmod SPP; (U) Mass Screening Techniques; (U) Automation.							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Provide individual paragraphs identified by number, precede text of each with security Classification Code.)							
23. (U) The measurement of antimalarial drug effectiveness against the malaria parasite in the erythrocytic phase in vitro.							
24. (U) Automatic wet chemical analysis subdivided into an incubation phase and an analytical phase is being employed to define the ability of the malaria parasite within the red cell to utilize nutrients or produce metabolites before and after incubation with chemical compounds under test for antimalarial activity.							
25. (U) 69 01-69 06 Screening of compounds for antimalarial activity continues. A study of the extracellular amino acid requirements of non-infected cells (embryonic turkey brain) in tissue cultures and of cells infected with the exoerythrocytic stages of P. fallox is in progress. P. berghei infected mice and hamsters were exposed to oxygen at pressures from 1.8 to 3.0 atm absolute for varying periods of time, to observe effects on parasitemias, hematocrits, survival and relative organ weights. The course of infection with P. berghei in male hamsters has been studied sequentially in terms of parasitemia, hematology, blood chemistry, relative organ weights, tissue enzyme levels and selected urinary constituents. Recent observations would indicate that plasma lactate levels are progressively elevated in the course of infection. In mice exposed to a hypoxic environment in varied time relation to malaria challenge, duration of survival after malaria challenge depended on the time after termination of hypoxia when the mice were infected. This correlated inversely with the Fe-59 erythrocyte uptake by the ex-hypoxic mice at the time of infection. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.							

PII Redacted

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 134, Malaria screening systems

Investigators.

Principal: LTC Dorsey T. Mahin, MC

Associate: LTC C. R. Angel, MSC; A. R. Berman, B.S.; N. D. Brown, B.S.; J. I. Davis, B.S.; MAJ J. E. Del Favero, VC; A. Einheber, Ph.D.; S. Garson, Ph.D.; LTC M. C. Johnson, MC; CPT T. F. McLeod, MSC; R. E. Wren, B.S.; Division of Medicinal Chemistry, D. P. Jacobus, M.D., LTC E. Eckermann, VC; R. Beaudoin, Ph.D. (NMRI); G. C. Huff, Ph.D. (NMRI).

Description.

The objective of this work unit is to provide automated or semi-automated screening systems to study the effects of chemical compounds on the metabolism of Plasmodium species, "in vitro." A broad spectrum of developmental studies provides basic developmental input to the work unit.

Progress.

1. Screening activities.

A previous report (WRAIR Annual Reports 1966-67; 1967-68) briefly described the development of an automated in vitro procedure for screening antimalarial compounds. This project was undertaken jointly with the Division of Medicinal Chemistry, WRAIR, and the Department of Pharmacology, West Virginia University. The latter unit currently participates in the Army Research Program on Malaria under contractual arrangement and is engaged, principally, in the automated mass screening of antimalarials. In-house activities during this period included a correlative evaluation of in vitro and in vivo test results, and a series of studies designed to evaluate and improve the performance of the automated test system. Screening operations have proceeded on a limited basis; a total of 1337 drugs have been tested and the results have been submitted to the Division of Medicinal Chemistry in machine readable format.

Instrumentation employed in the automated screening procedure includes a train (or trains) of interconnected Technicon and laboratory designed modules as follows: sampler, proportioning pump and manifold, incubator, dialyzer, heating bath, colorimeter, recorder and information

processing system (Infotronics). Various modifications of the proportioning pump, incubator, dialysis plates and metering valves have significantly improved the performance of the system.

The screening procedure is comprised essentially of two phases: incubation and analysis. At two minute intervals, small samples of parasitized (*Plasmodium berghei*) mouse red blood cells suspended in glucose-free buffer and solutions of glucose and drug are sampled and mixed in a continuous flow, air-segmented stream, which also incorporates a 90-second saline wash interspersed between test mixtures. Appropriate control mixtures (drug omitted) are included. Each compound is tested in a final concentration of 60 mg percent and each test series includes, for comparative purpose, a standard antimalarial agent, quinine dihydrochloride. The final mixture contains  $15 \times 10^7$  parasitized red cells per .6 ml sample.

Mixtures are then pumped sequentially through an incubator coil ( $37^{\circ}\text{C}$ ) for 1 and 1/2 hours, after which they are twice dialyzed. The dialysate stream is apportioned into separate analytical channels for determination of glucose, lactic acid, and alpha-amino nitrogen. Carbon dioxide determination is performed on an undialyzed blood sample.

In order to obtain an estimate of possible analytical (non-metabolic) interference produced by individual test compounds, each compound is incubated with known quantities of glucose, lactic acid, amino acid and  $\text{CO}_2$  in a cell-free mixture. A ten percent reduction in the measurable quantities of these constituents subsequent to incubation is regarded as unacceptable analytical interference and the individual test result is rejected. Indices calculated from these measurements are used to characterize metabolic inhibition (control vs. test results) of the red cell-parasite complex produced by individual drugs. Indices are calculated by subtracting the experimental utilization or production rate from the appropriate control rate, and expressing this as a percent of the control rate.

Recently, a preliminary attempt was made to determine the extent of correlation between the results of: 1) the *in vitro* systems and the primary mouse screen of Dr. L. Rane (University of Miami), and 2) the two automated *in vitro* antimalarial screens (in-house and West Virginia University). For this purpose, 115 drugs representing a broad spectrum of activity in the in-house test system were selected. Of these, comparative data were available for 42 drugs processed in all three systems. Evaluation of the results show that, of the four *in vitro* indices now used, lactate production was most frequently affected by (sensitive to) drug exposure but overall correlation was least impressive. Eight of the 42 drugs were active in the mouse screen and six of these eight drugs were correspondingly active *in vitro* (45 to 68% inhibition of lactate production). However, of the remaining 34 drugs, all non-active in the mouse screen, 25 inhibited lactate production in the *in vitro* system (inhibitions from 47 to 89%). Agreement between results obtained with other *in vitro* activity ratings and the mouse screen ratings were

as follows: glucose consumption, 83%; CO<sub>2</sub> production, 54%; alpha-amino nitrogen, 65%. Agreements between in vitro results (in-house and West Virginia) were as follows: glucose consumption, 58%; lactate production, 78%; alpha-amino nitrogen, 80% (CO<sub>2</sub> measurements are not performed by West Virginia University).

Statistical methods were used to examine: 1) reproducibility of media glucose and lactate measurements after media had been incubated under the different conditions required during each assay period, 2) the ability of calculated indices to separate and rank drug activities according to results obtained in single assay periods and according to pooled results from different assay periods. To perform these analyses, quinine, primaquine and chloroquine activities, determined during five assay periods over a two month interval, were used.

Reproducibility of media glucose and lactate measurements were best described by the average and range of the coefficients of variation (C.V.). Ten replicate determinations were made during each assay period for each of the following conditions: media and drug alone; normal blood and media alone; normal blood plus media and drug; parasitized blood alone and parasitized blood plus media and drug. Coefficients of variation as averaged for each condition during each assay period were between 0.9% and 2.1% with a range from 0.6% to 4.4%. These low coefficients indicate that good reproducibility has been achieved.

Simple averaging of all lactate inhibition indices during all assay periods produced the following order: quinine (25.4%), primaquine (11.8%), chloroquine (9.8%). Primaquine interfered with glucose determination and therefore only quinine (34.6% inhibition) and chloroquine (19.1% inhibition) were ranked according to glucose inhibition indices.

Analyses of variance techniques showed that overall differences in drug effect could be clearly demonstrated. Inhibition indices from each assay period were considered separately in these analyses. Overall differences between lactate inhibition indices were significant at the 1% level for all five assay periods. Differences between glucose inhibition indices were significant at the 1 to 2.5% levels for these periods. No other analyses of glucose results were made since an analysis of variance on two drugs orders the drugs. Quinine inhibited to a greater extent than chloroquine in all five assay periods. In the case of lactate inhibition indices least significant differences (l.s.d.) were calculated. These varied, for the five periods, between 4 and 8.5% (5% level). It was only for the third assay period, where there was a least significant difference of 4%, that ordering of quinine (34.1% inhibition), primaquine (9.3% inhibition) and chloroquine (5.1% inhibition) was possible. For the other assay periods it was only possible to show that quinine caused more inhibition than the other two drugs. The non-parametric Kruskal and Wallace ranking test was used, and was no better than analysis of variance in separating drug activity.

A second analysis of variance utilizing pooled lactate indices from the five assay periods was done, with each period considered as a block and each set of two (glucose) or three (lactate) drugs as a replicate. Significant differences could not be shown due to a large "interaction" error term.

In summary, the statistical analyses showed that conditions of incubation and autoanalyzer operation are well controlled, as shown by the low coefficients of variation for glucose and lactate data from replicate samples. In a particular assay period, drug activities could be separated if average inhibition indices were 8% apart. Indices pooled from separate assay periods could not be used to separate drug activity with statistical significance. However, inspection of the data suggests pooling of indices from additional assay periods would allow such separation.

The above statistical analyses can be used to determine the minimal number of experimental units needed to show whether manipulation of the infected mouse, the blood, the incubation media, etc., will increase the efficiency of the system in ranking drug effects. However, such efforts are not useful unless increased efficiency is associated with competency to identify new antimalarials and competency to reject ineffective agents. As reported above, comparison of antimalarial activities of 42 drugs in a mouse *P. berghei* system and in the presently described system demonstrated no such competency. Further in vitro, in vivo comparisons are planned this year.

## 2. Effect of hypoxia on the course of Plasmodium berghei infection in mice.

Knowledge of the dynamic interplay between malaria parasite and host erythron is fundamental to an understanding of the innate mechanisms of host immunity and the overall disease process. Moreover, it is significant from the standpoint of new and better approaches to the management of the disease. Accordingly, some investigators have sought to manipulate erythroid tissue homeostasis of rodents in advance of challenge with *P. berghei* and to study the effects of such manipulation on the course of infection. Thus, to induce anemia, animals have been bled repeatedly, irradiated, or injected with phenylhydrazine. To induce polycythemia, animals have been infused with red cells. Experimental enhancement and depression of host erythropoiesis have therefore required the use of two distinctly different procedures. These procedures are technically cumbersome and unphysiological. They may also involve considerable handling of the animals, which alone can influence the course of the infection.

In the present study, the single stimulus, hypoxia, was used to alter erythropoiesis. Mice manifest increased erythropoiesis during, and for a short period after, exposure in a hypoxic environment. However, several days after termination of hypoxia, erythropoiesis is depressed, presumably due to bone marrow readjustment to a normal gaseous environment.

This pattern of stimulated and then depressed erythropoiesis was observed by Lange *et al.* (Proc. Soc. Exp. Biol. Med. 122:761, 1966) whose simplified technique for exposure of mice to prolonged periods of hypoxia has greatly facilitated the present work. The principal device involved in Lange's procedure is a siliconized rubber membrane enclosure within which hypoxia is produced by virtue of the membrane's selective gaseous permeability properties.

In the present study, the Lange enclosure was used to subject 7-8 week old female CD-1 mice, in groups of ten, to either one or three weeks of continuous hypoxia (<10% oxygen concentration, range 7 to 9%, corresponding to an altitude between 24,000 and 28,000 feet). At different intervals following termination of hypoxia, the mice were inoculated with  $1 \times 10^7$  parasitized red blood cells (*P. berghei*, NYU-2 strain). The course of infection in these ex-hypoxic mice (ex-HM) was then compared to that of concurrently infected controls (CM) maintained under normal atmospheric conditions (ground level). Concurrently, other groups of ex-HM (1 week of hypoxia) and CM, instead of infection, were given approximately 0.1  $\mu$ Ci of  $^{59}\text{Fe}$  (ferrous citrate) i.p. for determination 24 hours later of percent  $^{59}\text{Fe}$  uptake by spleen and red blood cells. These values were used as indices of the level of erythropoiesis extant at the time of  $^{59}\text{Fe}$  injection.

The results of this study indicate that Lange's technique for the induction of hypoxia (<10% oxygen concentration) and polycythemia in mice is practical and effective. Either an enhanced or depressed erythropoiesis occurs, depending on the time elapsed subsequent to the termination of hypoxia. The spleen and red cells of ex-HM show depressed uptake of  $^{59}\text{Fe}$  on the fourth day after the end of one week of hypoxia. Ex-HM infected with *P. berghei* on the fourth day after the end of one week of hypoxia showed significantly prolonged survival; parasitemia was suppressed and the development of hypothermia was delayed. Mice exposed to three weeks of hypoxia and also infected on the fourth day after the end of hypoxia also showed a considerably prolonged survival time. In contrast, uptake of  $^{59}\text{Fe}$  by spleen and red cells of ex-HM tended to be increased within one hour or on the first day after the end of one week of hypoxia; when ex-HM were infected with *P. berghei* at these times, survival time was shortened, hypothermia developed more quickly, and parasitemia increased more rapidly. Mice inoculated with *P. berghei* on the seventh day after the end of one week of hypoxia did not show a significantly altered mean survival time even though the percentage uptakes of  $^{59}\text{Fe}$  by spleen and red cells were below normal at the time of inoculation, and despite a delay in the development of hypothermia and evidence of some suppression of parasitemia.

The present findings indicate that hypoxia is a useful tool for exploring the relationship between malaria parasite and status of the host erythron, and further investigations using this model are warranted. Observations on post-infection prolongation or shortening of survival time in relation, respectively, to a decreased or increased state of

erythropoiesis at the time of infection may be interpreted on the basis of the oft-mentioned predilection of *P. berghei* for immature erythrocytic forms and to differences in their numerical availability to the parasite. However, present observations provide no specific information to support or to refute this explanation, nor do the observed correlations necessarily indicate a cause and effect relationship with length of survival subsequent to infection with malaria.

It must be emphasized that while the physiological status of the mouse at the time of infective challenge in the post-hypoxia period clearly appears to influence the subsequent course of malaria infection, the precise mechanisms involved are unknown. Therefore, while it is tempting to explain the results on the basis of differences in the host erythron other physiologic and metabolic alterations consequent to hypoxic exposure may be involved.

The present findings and results from similar studies may bear on the use of "anti-fol" type antimalarial drugs that may suppress host erythrocyte production. The greater definition of humoral regulators (stimulators and inhibitors) of erythropoiesis and the availability of such factors in quantity for reversibly activating or inhibiting erythrogenesis may lead to their use together with antimalarial drugs in the prevention and control of malaria.

### 3. Exoerythrocytic stages of Plasmodium fallax in tissue culture: Alterations in extracellular and intracellular amino acids.

This study, undertaken in collaboration with Dr. C. G. Huff (NMRI) and his associates, was designed to probe the extracellular free amino acid requirements of non-infected cells (embryonic turkey brain) in tissue culture and of cells infected with the exoerythrocytic or tissue stages of avian *Plasmodium fallax*. Subsequent studies may include the use of tracer methodology and relate to other metabolic requirements.

Whereas intra-erythrocytic malarial parasites can be separated from their host cells, suitable separative techniques have not been devised for other intracellular stages. However, it should be noted that free-floating merozoites can be obtained in variable numbers and were employed in parts of the experiments. Knowledge of the overall metabolism and nutritional requirements of exoerythrocytic malarial parasites will facilitate a rational approach to the chemoprophylaxis of malaria. Moreover, such information will permit physiological comparisons of the erythrocytic and exoerythrocytic stages.

A detailed report of the culture methods used in this study was presented by Davis, Huff and Palmer (Exp. Parasitol. 19:1, 1966). Quantitative analysis for free amino acids was made on the following: 1) fetal calf serum, pre- and post-incubation; 2) fresh medium, pre- and post-incubation; 3) supernatant medium after incubation with

infected and non-infected cells; 4) cells, infected and non-infected, after incubation, removal of medium, freezing, thawing and sonication; and 5) merozoites. The results of these analyses seem to indicate that percentage loss of amino acids, with the possible exception of lysine, in the fresh (non-inoculated) media during incubation was quite minimal. A further comparison of the values shown for lysine in the supernatant (inoculated) media, infected and non-infected, indicates a net change which may well be due to release associated with the proteolytic activities of the parasite.

Final amino acid levels in the supernatant media represent the net result of both uptake and release of amino acids from the host parasite complex. However, it is interesting to note that comparative (infected versus non-infected) values for approximately half of the amino acids show minimal differences. For an individual amino acid this may indicate either a minor requirement for extracellular amino acid or that uptake and release occur at nearly similar rates.

A requirement for extracellular methionine in the erythrocytic form of *P. knowlesi* was demonstrated by Fulton and Grant (Biochem. J. 63:282, 1956). They reported the requirement for cystine was apparently met within the red cell, either through methionine or by proteolysis of hemoglobin. Unfortunately, the present data pertaining to exoerythrocytic *P. fallax* do not permit useful speculation concerning methionine and cystine requirements. However, it may be noted from the work of several investigators that a variety of cells in tissue culture cannot convert methionine to cystine, and therefore shows a requirement for both amino acids.

Perhaps the most significant observation in this study relates to the striking reduction in glutamine content of the supernatant after incubation with infected cells and the concomitant elevation in concentrations of glutamic acid. Comparing infected and non-infected supernatant media, metabolic influence of the parasite is evident. It is also conceivable that glutamine may serve as an important limiting factor in the survival of infected cultures. It is well known that glutamine participates in a wide variety of metabolic reactions, many of which are associated with the transfer of its amide nitrogen and consequently lead to the formation of glutamic acid. The role of glutamine as an essential growth factor for animal cells in tissue culture is well documented. It appears that certain cells in culture possess limited glutamine synthetase activity and, in this respect, are similar to several microorganisms. On the other hand, the biosynthesis of glutamic acid is readily accomplished through a number of metabolic routes.

#### 4. Hepatic microsomal drug-metabolizing enzymes and malaria infection.

It was recently reported (Einheber, A., Wren, R. E., Rosen, H. and Martin, L. K., *Nature* 215:1489-1491, 1967) that plasma ornithine carbamoyltransferase activity increases progressively and dramatically during the first 6 days of P. berghei infection. Since this urea cycle enzyme is normally almost exclusively confined to liver and is found in the mitochondria, these findings suggested that hepatocellular damage occurs quite early after malaria infection, perhaps before morphological alterations are demonstrable. To examine whether early functional changes also occur in other subcellular entities of the liver (viz., the microsomes), the durations of hexobarbital-induced narcosis in control and P. berghei-infected mice were compared. In addition, just before or just after P. berghei infection, mice were given a course of phenobarbital treatment that ordinarily stimulates the liver to increase its weight and the activity of its microsomal drug metabolizing enzymes. The objective was to examine whether the effects of such treatment can modify the course of malaria infection, and vice versa.

It was found that hexobarbital sleeping time (HST), 100 mg/Kg i.p., becomes significantly prolonged by day 3 of infection (10 million parasitized RBC i.p. on day 0) and increases markedly thereafter. Mice given 100 mg/Kg of phenobarbital sodium i.m. daily for three days showed a significant increase in fresh liver weight, due to approximately equal increases in liver water and dry mass, and a significant decrease in HST. This course of treatment was begun before (days -3, -2, and -1) or just after (days 0 (1 hr), 1, and 2) infection on day 0 with P. berghei (10 million parasitized RBC). These treatments did not significantly alter the mean survival time, nor the degree of parasitemia on days 3 and 5 when compared with saline-treated P. berghei-infected controls. However, both the pre- and post-malaria phenobarbital regimens "normalized" the HST of malaria-infected mice on day 3 of infection and caused significant increases in liver weight.

In summary, the prolonged HST occurring in mice early after P. berghei infection can be counteracted by pre- or post-infection treatment with phenobarbital. These results suggest that liver metabolism of hexobarbital is depressed by P. berghei infection, and that this is probably related specifically to a depression in the activity of the microsomal drug-metabolizing enzymes because phenobarbital treatment, which ordinarily stimulates an increase of these enzymes, corrects the "defect" in HST. These findings are germane to drug management and prevention of malaria infection, and particularly to possible non-polar (lipid-soluble) antimalarials which may be metabolized by and/or may stimulate the hepato-microsomal drug-metabolizing enzymes.

## 5. Physiological alterations in hamsters infected with Plasmodium berghei.

Infections with P. berghei in mice and in young rats are almost invariably fulminant and rapidly fatal; however, a more prolonged clinical course of infection occurs in hamsters (Yoeli, Trans. Roy. Soc. Trop. Med. Hyg. 59:255, 1965). The present study was undertaken to determine if hamsters infected with P. berghei would provide a suitable and convenient model for the investigation of malarial pathophysiology. A review of the literature indicates that little information is available in this regard.

The course of infection with P. berghei in male hamsters was studied sequentially in terms of parasitemia, hematology, blood chemistry, relative organ weights, tissue enzyme levels and selected urinary constituents.

Separate groups of hamsters were bled from the heart under light ether anesthesia at intervals of 4, 7, 12, 17 and 21 days subsequent to intraperitoneal inoculation with  $2 \times 10^7$  parasitized red blood cells. Parasitemia and hematocrit determinations were made on blood obtained from the orbital venous plexus. Non-infected controls were included at each interval. The spleen, liver and kidneys were quickly removed, chilled in ice, blotted and weighed. Homogenates were prepared from weighed samples of liver and kidney for the measurement of enzyme activities. Glutamic-oxalacetic transaminase was determined by the method of Tonhazy et al., (1950), and glucose-6-phosphatase was measured according to Harper (1965). The procedure of Lowry et al., (1951) was used for protein analyses. Separate samples of the individual organs were weighed after drying for 24 hours at 100°C.

Sera were usually prepared within 3 to 4 hours following the collection and refrigeration of bloods and subsequently frozen (-20°C) until analyzed. Ultramicrotechniques were used in the analysis of the following serum constituents: serum glutamic-oxalacetic transaminase (SGO-T); serum glutamic-pyruvic transaminase (SGP-T); urea nitrogen; creatinine; cholesterol; total bilirubin; uric acid; total protein, and relative protein concentrations. Alpha-amino nitrogen, glucose and lactic acid were determined by automated wet chemical analytical procedures.

Separate groups of infected and non-infected male hamsters were used in a study of excretion rates for various urinary constituents. Hamsters were placed in individual stainless-steel metabolism cages and urine collections were made under toluene. Food, but not water, was withheld during the collection period. Urines were centrifuged clear, and measured; protein and pH were estimated by Hema-Combistix reagent strips. The urines were frozen at -20°C until analyzed for urea nitrogen, total alpha-amino nitrogen, creatinine, and uric acid, by automated wet chemical analytical procedures. Amino acid analyses were carried out according to the method of Rosen et al., (Analyt. Biochem. 4:213, 1959) using an automatic amino acid analyzer.

The first death among infected hamsters occurred on the 14th day of infection. Any survivors were sacrificed on day 21. Hepatosplenomegaly was an invariable finding on necropsy. Parasitemias progressed from a mean of 1.9% on day 4 of the infection to 37.5% on day 21. Reticulocytes rose to a mean level of 36.7%. Hematocrits, during the same interval, decreased from 51.7% to 15.3%. Individual hematocrits as low as 9.0% were observed. Thus, the hamster, like the mouse, develops an intense anemia in the course of infection with *P. berghei*. In contrast, parasitemia in the hamster is relatively mild compared to that in the mouse. It would appear, therefore, that the hamster may be well suited for the study of "excessive blood loss" as it occurs in malaria (i.e., anemia relatively in excess of parasitemia). Of significance in this regard is a syndrome found in about half the infected hamsters and seen between days 12 and 14 of the infection. Urines voided by these animals measured 2 to 3 times the volume (24 hrs.) of the controls and showed a characteristic dark brown coloration which tested positive for hemoglobin. This usually subsided within 1 to 2 days. Infected hamsters also exhibited a significant increase in urinary alpha-amino nitrogen.

Hypoalbuminemia and hyperglobulinemia have been found repeatedly in human and experimental malarias; similar observations were made in the present study. In addition, infected hamsters showed progressive elevations in serum enzymes (SGO-T particularly), urea nitrogen, creatinine and lactic acid. Levels of serum glucose and uric acid were decreased. A small reduction in serum cholesterol was noted on day 12 which was subsequently followed by a significant rise on day 21. Levels of glutamic-oxalacetic transaminase and glucose-6-phosphatase in liver and kidneys of infected hamsters were moderately decreased in the course of the infection.

#### 6. Tissue anoxia in experimental malaria.

Maegraith (Brit. Med. Bull. 8:28, 1951; Riv. Parassit. 20:317, 1959) and his associates have long believed that tissue anoxia leading to degenerative changes, as in centrilobular hepatic necrosis, plays a prominent role in the pathogenesis of malaria. Maegraith believes that the development of tissue anoxia results both from critical changes in local blood circulation and from defective utilization of oxygen by the affected cells. Except under extreme conditions, anemia appears to be of minimal significance. More recently, the same group of investigators has shown that an inhibition of oxidative metabolism occurs in isolated liver mitochondria of mice infected with *Plasmodium berghei* and of monkeys infected with *P. knowlesi*. Somewhat comparable, although less pronounced inhibition was obtained when normal mitochondria were incubated in sera from malaria-infected animals (mice and monkeys). This was tentatively attributed to a toxic factor present in the sera of infected animals either elaborated by the parasite or resulting from an altered host metabolism.

Hall (Proc. Soc. Exp. Biol. Med. 122:1240, 1966) has shown that anoxic liver damage is associated with an increased intracellular acidity. This apparently results from an accumulation of lactic acid in the early stages of anoxia and leads to an inhibition of oxidative enzyme activities in liver mitochondria. An accumulation of acid was demonstrated by a decrease in the titratable buffer capacity and pH of the liver. Buffering by the addition of Tris or THAM to the incubation medium largely prevented the inhibition of enzyme activities associated with anoxia.

In the present study, preliminary observations have been made in further exploring the possible role of tissue anoxia and related phenomena in the pathogenesis of experimental malaria. As noted in a previous report, the intraperitoneal administration of THAM to *P. berghei* infected mice increased their survival time by 1 to 2 days although parasitemias were not significantly altered. Subsequent observations have revealed that the course of *P. berghei* infection in hamsters is characterized by a progressive decrease in the concentration of plasma glucose and by a concomitant increase in levels of lactic acid. This apparently derives from the glycolytic activities of the parasite as well as the inadequately perfused and, therefore, oxygen deficient tissues of the host. An attempt will be made to measure *in vivo* the intracellular pH of tissues from *P. berghei* infected animals; the DMO procedure of Waddell and Butler (J. Clin. Invest. 38:720, 1969) will be used.

The synthesis or resynthesis of glucose from lactic acid (gluconeogenesis) normally proceeds through the Cori (lactic acid) cycle in mammalian liver and kidney, whereas glycogenesis occurs chiefly in liver and muscle. Extensive liver damage resulting in lowered glycogen content has been reported in rats infected with *P. berghei* (Mercade and Von Brand, Exp. Parasitol. 3:259, 1954), although the organ retained its ability to synthesize glycogen when glucose or other carbohydrate sources were provided. Accordingly, the present finding of increased plasma lactate levels in *P. berghei* infected hamsters may reflect a disturbance in mechanisms of gluconeogenesis or in the related control mechanisms postulated by Krebs (Proc. Roy. Soc. Ser. B 159:545, 1963).

Hyperbaric oxygenation or oxygen under high pressure (OHP) has been used in a wide variety of experimental and clinical situations encompassing a broad spectrum of objectives. For example, OHP has received considerable attention in recent medical literature because of its theoretical potential in the treatment of diseases involving anoxia. The rationale involved is that large quantities of oxygen can be forced into physical solution in blood plasma, thus supplementing the oxygen-carrying capacity of the red cells. The dissolved oxygen may then cross from the intravascular to the extravascular and intracellular spaces by diffusion and thereby become available to the anoxic or hypoxic cells. Tissue oxygen tension studies in experimental animals have indicated that an almost immediate rise in tension occurs

when the animals are exposed to OHP; this is followed by a slower fall in tension when the pressure is discontinued (Ackerman et al., Proc. 3rd Intern. Conf. Hyperb. Med. 1966). Weglicki et al., (Proc. 3rd Intern. Conf. Hyperb. Med. 1966) have observed that excess lactate levels associated with exercise under OHP at 3 atmospheres absolute (ata) were significantly lower than those levels in the same animals exercised at 1 ata in air. Finally, the inhibitory effects of OHP on certain enzymes, particularly the glycolytic sulfhydryl enzyme, glyceraldehyde-3 phosphate dehydrogenase, have been well documented.

A study was recently undertaken to determine the effects of OHP on the course of P. berghei infection in mice and in hamsters. Various regimens of exposure to OHP, including repeated short applications, have been employed at different intervals in the course of infection. Exposure to OHP involves the use of a small cylindrical hyperbaric chamber with a diameter of 6 inches, a length of 16 inches, and a total volume of approximately 450 inches<sup>3</sup>.

Therefore only a small number of animals (approximately 16 mice or 8 hamsters) can be accommodated during a single exposure period. The continual circulation of gases is maintained by a constant influx of pure oxygen and by a constant exhaust at a rate of 10 liters per minute. Preliminary observations relate, particularly, to survival time and levels of parasitemia; the results of plasma lactate analyses are presently unavailable. Striking alterations in survival time or in parasitemias have not occurred with the regimens of OHP treatment employed thus far.

#### Summary and Conclusions.

Malarial projects included further development of an in vitro drug screening procedure: experiments involving the effects of hypoxia altered erythrocyte production on Plasmodium berghei infection in mice; exoerythrocytic stages of Plasmodium fallax in tissue culture; hepatic microsomal drug-metabolizing enzymes and malaria; physiological alterations in hamsters infected with Plasmodium berghei; and tissue anoxia in experimental malaria.

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 134, Malaria screening systems

Publications.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL	
				DA 0A6534	69 07 01	DD-DSR (AR) 655	
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b. CONTRIBUTING							
c. CONTINUING		1412A(2)					
11. TITLE (Precede with Security Classification Code) <sup>9</sup>							
(U) Experimental Pathology and Metabolism of Plasmodia (09)							
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00 26 00 Biology							
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18. RESPONSIBLE DOD ORGANIZATION				19. PERFORMING ORGANIZATION			
NAME <sup>12</sup> Walter Reed Army Institute of Research				NAME <sup>13</sup> Walter Reed Army Institute of Research			
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TELEPHONE <sup>18</sup> 202-576-3551				TELEPHONE <sup>19</sup> 202-576-2677			
20. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME:			
				DA			
21. TECHNICAL OBJECTIVE, 22. APPROACH, 23. PROGRESS (Publish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To study metabolic pathways in malarial parasites maintained in vitro. To correlate metabolic and morphologic effects of anti-malarial drugs, and to isolate, purify and identify by chemical, physical and morphologic means the subcellular components of malarial parasites. To study sporogenesis and its modification by drug action.							
24. (U) Biophysical, biochemical and morphologic techniques are employed.							
25. (U) 69 01-69 06 The studies in P. berghei infected hamsters of sequential histologic and enzymatic changes in the liver, of the time-sequence of the appearance, aggregation and disappearance of malaria pigment in liver and lung, and of the renal pathology have been published in the A.M.A. Archives of Pathology. The study of the fate of the malaria pigment bearing macrophages is in press. Studies of drug effects on sporozoite development are continued under contract. Related to this project are studies on the morphology and enzyme histochemistry of the mid gut of control and infected Anopheles stephensi in progress. The study on the pentose-phosphate pathway and its relation to drug action is in press. Six additional papers on malarial fine structure and drug effects were accepted for publication and four appeared in print. Parasite fractionation studies are continuing. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

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Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 - Malaria Investigations

Work Unit 135, Experimental pathology and plasmodial metabolism in malaria

Investigators:

Principal: Colonel Helmuth Sprinz, MC

Associate: Major Robert Cook, MC; Major Robert Rock, MC

Description

A multidisciplinary approach was employed in the study of experimental malarial infection and of the metabolism of plasmodia in vitro and in vivo. We were particularly concerned with the effects of anti-malarial drugs on parasite morphology and metabolism.

Progress

1. The studies in P. berghei infected hamsters of sequential histologic and enzymatic changes in liver, lung and kidneys are completed and continuing to appear in print. In the preceding annual report publication of the paper of Sesta et al on malarial nephropathy was listed. We can now report publication of two additional papers in the A.M.A. Archives of Pathology by Jervis et al on the liver and by MacCallum on the lung (see bibliography). The two manuscripts on macrophage response in this experimental model have been published (see bibliography). Work in this area has been drastically curtailed and only a single study on the pathology of P. falciparum infection in the owl monkey is in progress.

2. Our major effort was directed towards biochemical - biophysical analysis of plasmodia and the correlation of these with morphologic findings.

a) We performed extensive quality control experiments on the isolation of P. knowlesi from its host erythrocytes. It was demonstrated that no methods yet devised have led to demonstrably clear parasite preparations. Subsequently, we studied the fractionation of P. knowlesi by equilibrium density gradient centrifugation. It was shown that smooth microsomal fractions operationally free of host cell contaminants can be obtained. In addition, we have demonstrated the presence of a soluble enzyme in P. knowlesi capable of hydrolyzing denatured monkey hemoglobin. These studies were presented before the Third International Workshop on Malaria (R.T. Cook, M.Aikawa, R.C. Rock, W.Little, and H. Sprinz, The isolation and fractionation of Plasmodium knowlesi. Third International Workshop on Malaria, Mil. Med., Suppl, for Sept. 1969).

At the present time experiments are in progress to develop an in vitro protein synthesizing system of P. knowlesi, suitable for drug inhibition studies. These studies will continue to be pursued by Dr. Cook at Case Western Reserve University.

b) The current principal area of investigation by Major Rock is a systematic study of the lipid composition and metabolism of the malarial parasite, Plasmodium knowlesi. This organism has a high lipid content (chiefly in cell membranes and organelles) which increases markedly during the intraerythrocytic growth of the parasite.

As in other areas of plasmodial metabolism, the parasite probably derives much of its lipid precursors from the extra- and intra-cellular environment of the host erythrocyte, but contamination of parasite lipid fractions by host red cell membrane (which contains 97% of the total red cell lipid) has made investigation of individual parasite lipids difficult.

By using a microsomal fraction of the parasite (which is free of host cell material), the lipid composition of this component of the parasite can be studied by biochemical methods currently under investigation in our laboratory (including determination of microsomal protein, glycoprotein, and carbohydrate as well as lipid). The results derived from parasite microsomes can then be compared to those obtained from the host Rhesus monkey red cell (which has been extensively investigated in our department). Lipid and protein synthesis by parasite microsomes can be investigated using radioactive tracers, (such as  $^{32}\text{P}$  and  $^{14}\text{C}$ -glycerol for phospholipid synthesis and  $^{14}\text{C}$ -labeled amino acids for protein synthesis). In such a system, the effects of antimalarial compounds can also be examined at the subcellular level of organization.

c) Chemical and morphological characterization of DNA isolated from avian, rodent and simian malarial parasites. This aspect of our research is guided by Capt. Ladda and is being performed in collaboration with Major Estensen and Dr. Wohlhieter.

#### Summary and Conclusions

We have entered a very exciting but very difficult area of research in malaria. While progress has been excellent - we are the leader in this area - the road ahead appears difficult. Our problems are compounded by the non-availability of replacements. With the leaving of Dr. Cook this work unit will have a single full-time investigator, Major Rock. It is only thru consolidation with the other work unit that we shall start the next fiscal year with two full-time investigators: Rock and Ladda. This number is less than the number of investigators available when the malaria program was initiated.

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Task 00 - Malaria Investigations

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malaria

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				DA 0A6535	69 07 01	DD-FRM (AF) 538	
4. DATE PREV SUMMARY	5. KIND OF SUMMARY	6. SUMMARY SCTY <sup>4</sup>	7. WORK SECURITY <sup>5</sup>	8. REGRADING <sup>6</sup>	9. DISSEM RESTRI <sup>7</sup>	10. SPECIFIC DATA- CONTRACTOR ACCESS	
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C. CONTINGUING		1412A(2)					
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(U) METABOLIC AND ENZYMATIC STUDIES OF NORMAL AND MALARIA INFECTED RED CELLS (09)							
13. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>10</sup>							
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NAME <sup>12</sup> Walter Reed Army Institute of Research				NAME <sup>13</sup> Walter Reed Army Institute of Research			
ADDRESS <sup>14</sup> Washington, D.C. 20012				ADDRESS <sup>15</sup> Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic personnel)			
NAME <sup>16</sup> Meroney, COL W. H.				NAME <sup>17</sup> Teschan, COL P. E.			
TELEPHONE <sup>18</sup> 202-576-3551				TELEPHONE <sup>19</sup> 202-576-3529			
GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Considered				ASSOCIATE INVESTIGATORS			
				NAME <sup>20</sup> Canfield, LTC C. J.			
				NAME <sup>21</sup>			
				DA			
23. KEYWORDS (Provide SSAN with Security Classification Code) <sup>22</sup>							
(U) Malaria; (U) Antimalarials; (U) Parasite; (U) Red Blood Cell							
23. TECHNICAL OBJECTIVE, <sup>23</sup> 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Provide level of each with Security Classification Code.)							
23. (U) - Document metabolic alterations of human and animal red blood cells when infected with malaria parasites and to assess the effect of antimalarial drugs on these alterations. To characterize the effect of antimalarial drugs on P. Knowlesi schizogony.							
24. (U) - Study the uptake of certain amino acids by infected red blood cells, measure folic acid reductase in parasite suspensions, to measure the effect of antimalarial drugs on morphologic growth, lactate production and 14-C methionine incorporation in in vitro schizogony, and to study red cell flux of sodium.							
25. (U) 69 01 - 69 06 - P. Knowlesi infected monkey red cells show elevated distribution ratios for isoleucine and methionine, but apparently normal values for cystine, leucine, and histidine. Increases were found in incorporation into protein of all these amino acids. Technical difficulties precluded assay of folic acid reductase and this project has been abandoned. Sodium influx is increased in erythrocytes from malarious monkeys and combined with the known sodium efflux defect accounts for the elevated intracellular sodium. Incubation of normal cells with malarial plasma induces a sodium efflux inhibition. A secondary antimalarial drug screening system utilizing the in vitro culture of P. Knowlesi parasitized red cells has been developed which is effective for all classes of drugs tested except sulfones, sulfanilamides and folic acid antagonists. These drugs can be screened though in a modified system employing labelled orotic acid incorporation into DNA and RNA. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

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Project 3A663713D829 MALARIA PROPHYLAXIS

Task (0) Malaria Investigations

Work Unit 136, Metabolic and enzymatic studies of normal and malaria infected red blood cells

Investigators: COL Paul E. Teschan, MC; LTC Craig J. Canfield, MC;  
MAJ Michael J. Dunn, MC; Gerald J. McCormick, Ph.D.;  
Esther P. Jorolan, Ph.D.

Description.

The objective of this work unit is to study the pathophysiologic alteration induced at a cellular level by malaria infection. The specific areas of study at the present include: 1) red cell sodium flux; 2) parasite and red blood cell folic acid reductase; and 3) amino acid uptake and incorporation by infected red blood cells.

Progress.

Sodium influx is increased in erythrocytes from malarious monkeys and combined with the known sodium efflux defect accounts for the elevated intracellular sodium. Incubation of normal cells with malarial plasma induces a sodium efflux inhibition. Therapy of infected monkeys with chloroquine reverses these cation abnormalities over the course of one week.<sup>1,2</sup>

A radioassay for folic acid reductase (FAR) using <sup>14</sup>C-folic acid as substrate was attempted. Technical difficulties has led to abandonment of this project.

The uptake from plasma and incorporation into protein of several labelled amino acids by parasitized (*P. knowlesi*) red blood cells from rhesus monkeys has been compared to that of normal monkey blood. The amino acids used were isoleucine, methionine, leucine, cystine and histidine.

Distribution ratios for isoleucine and methionine were increased.

Incorporation into protein by malaria blood was greatest for isoleucine (150 times normal), followed by methionine (30 times normal), leucine and histidine (16 times normal) and cystine (4 times normal) after two hours of incubation.

A system for study of antimalarial drugs using in vitro cultures of intraerythrocytic forms of *P. knowlesi* has been developed and is actively being used for secondary screening. Young ring forms of the parasite introduced into this system will predictably develop into mature schizonts. During this 18-hour growth period lactic acid is produced and labelled amino acids are incorporated into trichloroacetic-acid-precipitable parasite protein. The effects of drugs on morphologic maturation, lactic

acid production and the incorporation of these amino acids have been observed. Dose response curves have been obtained with known anti-malarials which correlate well with clinical experience. A modified system employing labelled orotic acid incorporated has been developed which permits evaluation of sulfones, sulfonilamides and folic acid antagonists.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 136, Metabolic and enzymatic studies of normal and malaria  
infected red blood cells

1. Dunn, M. J.: Alterations of red blood cell sodium transport during  
malarial infection. Clin. Res. April 1968.

2. Dunn, M. J.: Alteration of red blood cell sodium transport during  
malarial infection. J. Clin. Invest. 48: 674-684, 1969.

PROJECT 3A062110A830  
BIOSENSOR SYSTEMS

Task 00  
Biosensor Systems

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL	
				DA OB6441	69 07 01	DD-RSS (AR) 635	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. RECLASS <sup>e</sup>	8. DEDN INSTN <sup>f</sup>	9. SPECIFIC DATA CONTRACTOR ACCESS <sup>g</sup>	10. LEVEL OF SUM A WORK UNIT
69 01 31	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A WORK UNIT
10. NO./CODES <sup>h</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62110A	3A062110A830	00	055			
b. CONTRIBUTING							
c. CONTRIBUTING	1412A(2)						
11. TITLE (Precede with Security Classification Code) <sup>i</sup>							
(U) Development and Evaluation of Improved Biological Sensor Systems (21)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>j</sup>							
001700 Animal Husbandry 011800 Operations							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
09 67		07 72		DA		C. In-House	
17. CONTRACT/GRANT <sup>k</sup>				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATE/EFFECTIVE: NA EXPIRATION:				FISCAL YEAR		FUNDING (in thousands)	
b. NUMBER:				69		4	
c. TYPE:				70		4	
d. KIND OF AWARD:				e. AMOUNT:		300	
f. CUM. AMT.							
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D. C. 20012				Bio-Sensor Research Team			
				ADDRESS: Edgewood Arsenal			
				Maryland 21010			
				PRINCIPAL INVESTIGATOR (Precede SSAN if U.S. Academic Institution)			
RESPONSIBLE INDIVIDUAL				NAME: Castleberry, COL M.			
NAME: Meroney, COL W. H.				TELEPHONE: 301-895-3350 Ext 27226			
TELEPHONE: 202-576-3551				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Considered				NAME: Morris, LTC J. H.			
				NAME: Dearing, CPT S. J.			
				DA			
24. REVIEWS (Precede EACH with Security Classification Code)							
(U) Breeding; (U) Dogs; (U) Genetics; (U) Selection							
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To develop a more intelligent and sensually acute dog which is physically and temperamentally better suited for military purposes than is now generally available.							
24. (U) Critically evaluated AKC registered breeding stock purchased especially for this purpose are selectively bred to produce superior progeny. These are in turn closely evaluated by recognized tests designed to reveal the superior individual. Line breeding combined with progeny testing of each generation will be utilized to accomplish the objective.							
25. (U) 69 01 - 69 06 A visit was made to Fort Gordon to observe the techniques employed in the training of tunnel and mine detection dogs by the use of food reinforcement. Six enlisted personnel visited Fort Benning to observe the final week of training for both scout dogs and handlers prior to overseas deployment. Two German Short Haired Pointers were purchased and placed in training to detect mines, booby traps, and ambushes using food reinforcement training techniques. A program was initiated providing 24 hour care of bitches immediately prior to, during and for 5 weeks following whelping. Since January, 93 offspring have been produced. Pelvic and elbow radiographs of all German Shepherd progeny over 6 months of age were examined by our consultant. Of these 35 puppies, only 3 were dysplastic in the hips, none in the elbows. Construction of the permanent dog handling facility began 9 May. Completion date is 9 Sep. Two AH/PRT-4 transmitter units used for hidden alert purposes on off leash scout dogs were perfected by mounting a mercury make-brake switch. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.							

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Project 3A062110A830 BIOSENSOR SYSTEMS

Task 00, Biosensor Systems

Work Unit 055 Development and evaluation of improved biological sensor systems

Investigators

Principal: COL Merida W. Castleberry, VC

Associate: LTC John H. Morris, VC; CPT Stuart J. Dearing, MSC

**OBJECTIVE:** To develop a more intelligent and sensually acute dog which is physically and temperamentally better suited for military purposes than is now generally available.

**BACKGROUND:** This study is being made in response to the requirements of the recently approved US Army QMDO, "Detector System, Military Dog". (USACDC Action Control Number 12527). Seven breeds of dogs, including crosses, were recently studied by the University of Maryland for behavioral evaluation and selection for army breeding and training (Army Contract No. DADA 17-68-C-8015). As recommended in the final report of that study, and because of the years of military experience gained with the German Shepherd Dog, this breed has been selected for primary breeding emphasis.

**APPROACH:** Critically evaluated AKC registered breeding stock purchased especially for this purpose are selectively bred to produce superior progeny. These are in turn closely evaluated by recognized tests designed to reveal the superior individual. Line breeding combined with progeny testing of each generation is being used to accomplish the objective. Training evaluation of other breeds is being accomplished. Limited cross breeding for desirable characteristics is programmed.

**PROGRESS**

A. **Facilities:** A host-tenant agreement with Edgewood Arsenal was concluded and approval of the MTDA by OPJ was received in August 1968. The following month this Department became independently operational at Edgewood Arsenal with the arrival of its first six enlisted men. Temporary kennel, whelping, and evaluation areas were made ready and received the initial breeding stock and puppies. Construction of permanent facilities was initiated in May 1969. Estimated completion date is 9 September 1969.

B. **Breeding Program**

1. **Kennel population:** One hundred and forty seven dogs are now on hand. Except for four German Short Haired Pointers, all are German

Shepherd Dogs. The latter includes twenty three breeding bitches and four males. The remaining one hundred and twenty are puppies ranging up to 11 months of age. Transferred to Veterinary Medical Division, WRAIR, but remaining available to the Department are the following:

<u>BREED</u>	<u>NUMBER</u>
Labrador Retriever	43
Standard Poodle	20
Airedale	10
English Pointer	9
Bloodhound	1

2. Genetics: A pedigree analysis was completed on the foundation breeding stock and breeding plans for the first generation were devised. Arrangements were completed for American Kennel Club registration of all stock and progeny.

3. Puppy evaluation: Puppy testing procedures were formulated and the final format reviewed by Drs. J.P. Scott and J.L. Fuller of the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. Evaluation of 1st generation progeny was initiated in October. To date, 84 puppies have completed the evaluation phase. Of this number 15 have been rejected and transferred to Walter Reed Army Institute of Research. Twenty of the more outstanding individuals are being considered as potential breeders. The remaining dogs have been placed in holding phase and will be shipped, at eleven months of age to Fort Benning or elsewhere for military dog training. The first shipment of 5 dogs is scheduled for July 1969.

#### 4. Special projects

a. A pilot study for determining the feasibility of using dogs for locating clandestine marijuana was allocated to the Department by the Provost Marshall General Office (OTPMG). This project was successfully completed and the final report submitted to OTPMG on 14 October 1968. Effectiveness of a dog in detecting marijuana was shown in demonstrations conducted for the Director of Inspection Services, LT GEN Erton, and members of the Drug Abuse Board.

b. Primarily because of their typical "point", the possibility of utilizing bird dogs for off-leash detection purposes is being investigated. Preliminary work was conducted with the English Pointer. In early training these proved to be temperamentally unsatisfactory and two German Short Haired Pointers were substituted and are now in an advanced stage of training. These dogs are being trained to detect mines, booby traps, and ambushes. The dogs are being trained to "sit" within two feet of a mine or booby trap. They indicate the presence of an ambush with the typical foot-in-the-air bird dog point.

c. The possibility of regulating the onset of estrous and ovulation in the anestrus bitch by hormonal administration is being studied. Preliminary work has been directed to the establishment of effective dose levels. Using six month old beagles, clinical signs of estrus have been noted and ovarian follicular stimulation determined by laparotomy. Formation of ova is, as yet, undetermined.

d. Two AH/PRT-4 transmitter units used for hidden alert purposes on off-leash scout dogs were improved for demonstration purposes, by mounting a mercury switch to the shoulder harness. Thus, on the "sit" alert, all transmission stops. Upon returning to all four feet, contact is reestablished and transmission resumes.

#### DISCUSSION

1. First generation matings have proven very successful. The incidence of hip dysplasia has shown a substantial decrease from the expected frequency. Pelvic radiographs of all German Shepherd progeny over six months of age were examined by our consultant, Dr. Wayne Riser. Of these 35 puppies, only three were dysplastic. Further test matings will need to be made in order to cast further light on the exact mode of inheritance. Various crosses between certain genetic lines have produced first generation offspring closely approximating the coloration and physical structure set forth in the QMDO. The conception rate during the last year has been over 80%.

2. Effectiveness of the puppy evaluation procedures must await the training feed-back from Fort Benning and, subsequently, Vietnam. It is pointed out, however, that the evaluation procedures closely parallel those developed primarily by and which are so successful for the Guide Dogs for the Blind organization.

3. Since bird dogs have a very characteristic alert, are easily managed by different handlers, and hunt off leash naturally, it was assumed that these would make superior scout dogs. Their rather unspirited response to the grind of scout dog training has tended to mitigate the early enthusiasm for these dogs. Sufficient promise is being shown, however, to warrant continuation of the project. Consideration is being given to the possible advantages to be gained by crossing the German Shepherd Dog with the German Short Haired Pointer.

**CONCLUSION:** The principles of selectively breeding for desired characteristics have known for many years and have resulted in man's ability to genetically develop anything from a dwarf tree to a seventeen gallon cow. It can be assumed that this project will result in a remarkably improved military dog.

**RECOMMENDATIONS:** None

Project 3A062110A830 BIOSENSOR SYSTEMS

Task 00 Biosensor Systems

Work Unit 055 Development and evaluation of improved biological sensor systems

PUBLICATIONS.

None.

**PROJECT RD 41-51  
NUCLEAR WEAPONS EFFECTS RESEARCH**

1262

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	3. REPORT CLASSIFICATION	
				DA 0A6473	69 06 30	DD-FORM (REV) 683	
4. DATE PREV. SUMMARY	5. KIND OF SUMMARY	6. SUMMARY SCY*	7. WORK SECURITY*	8. REGRADING*	9. DIFFERENTIAL*	10. SPECIFIC DATA CONTRACTOR ACQUIS*	11. LEVEL OF SUMMARY
69 01 31	Completion	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. DOCUMENT
12. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
				NWER-440 DASA 03.008			
13. TITLE (Protect with Security Classification Code)							
(U) Durability of Behavior Following Lethal Radiation Exposure (09)							
14. SCIENTIFIC AND TECHNOLOGICAL AREA*							
G17100 Weapon Effects 013400 Psychology 014100 Radiobiology							
15. START DATE		16. ESTIMATED COMPLETION DATE		17. FUNDING AGENCY		18. PERFORMANCE METHOD	
07 62		06 69		DH		C. In-House	
19. CONTRACT/GRANT				20. RESOURCE ESTIMATE		21. PROFESSIONAL MAN YRS	
Not Applicable				FICIES		22. FUNDING (in thousands)	
23. DATES/EFFECTIVE				FISCAL YEAR		24. FUNDING	
25. NUMBER*				68		1	
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27. KIND OF AWARD				28. DURA. AMT.		29. FUNDING	
						25	
30. RESPONSIBLE SCS ORGANIZATION				31. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Division of Neuropsychiatry Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide GRAF if U.S. Anatomic Pathologist)			
NAME: Meroney, COL W. H.				NAME: McIlwain, MAJ J.T.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-5262			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
32. GENERAL USE				33. ASSOCIATE INVESTIGATORS			
				NAME: Hawkins, T.D., M.S.			
				DA			
34. KEY WORDS (Protect with Security Classification Code) (U) Radiation; (U) Incapacitation; (U) Behavior; (U) Performance; (U) Psychology; (U) Primates							
35. TECHNICAL OBJECTIVE, 36. APPROACH, 37. PROGRESS (Protect with Security Classification Code)							
<p>23. (U) Ultimate aim of this research is the specification and time course of those behavioral patterns which deteriorate following massive and lethal doses of ionizing radiations and how those behaviors are best maintained. These are necessary and critical steps for an analysis of radiation effects; the lack of such information is impeding accurate military and civil defense planning.</p> <p>24. (U) Before considering the effects of the radiations on complex behavior patterns it was necessary for this laboratory to determine the precise time course of general behavioral incapacitation through utilization of a highly-motivated, shock avoidance task. Proceeding from this basic data, current work has focused on development of more complex behavioral tasks calling for a series of closely-spaced, discrete decisions on visual pattern discrimination problems. Measures such as reaction time, accuracy, nature of errors, visual acuity, and duration of effective performance are variables which are being investigated.</p> <p>25. (U) 69 01 - 69 06 Performance of primates under fixed and titrated reaction time schedules has been investigated over extended periods. This project has now been completed and is being phased out. Appropriate administrative steps are in progress for termination. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.</p>							

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Project R.D. 41-51 NUCLEAR WEAPON EFFECTS RESEARCH

Work Unit 03.0081, Durability of behavior following lethal radiation exposure.

Investigators.

Principal: MAJ Joseph C. Sharp, MSC (Jul 68 - Dec 68)  
MAJ James T. McIlwain, MC (Jan 69 - Jun 69)  
Associate: T. Daryl Hawkins, B.S., M.A.  
John Schrot, B.S., M.A.

Description.

The aim of this study has been the analysis of changes in complex behavioral patterns following high doses of ionizing radiation. The overall experimental program was designed to investigate three separate but related behavioral problems: (1) differential effects in specific performance domains including visual pattern discrimination, vigilance tasks, reaction time tasks and attention tasks; (2) effects on acquisition and retention of new behaviors; and (3) effects on behaviors motivated by positive and negative reinforcers. The ionizing radiation exposures were designed to simulate the mixed spectra of a nuclear blast. The main thrust of the experimental activity has been to measure the degradation in behavior (incapacitation) following such a mixed radiation exposure.

Progress.

Detailed reports of earlier experiments may be found in previous WRAIR Annual Progress Reports, WRAIR Technical Reports and open literature publications from this laboratory. These sources document experiments exploring dose and spectral effects on behavioral incapacitation, effectiveness of a radioprotective compound (N-Decylamino-ethanethiosulfuric acid) in preventing radio-incapacitation in primates, performance degradation in complex match-to-sample problems and comparison of radio resistance of behaviors reinforced by food or water as opposed to intra-cranial stimulation.

Pilot studies had suggested that reaction time measures might be sensitive respondents to the incapacitating action of the mixed radiation exposure. Extensive study of reaction time performance in primates was undertaken in FY 69 to provide behavioral baselines for studies of radiation effects. It has been shown that several schedule variables control the reaction time distribution in animals performing in a titration schedule. This is a schedule in which the maximal rewarded latency ("criterion") is made shorter for successful responses and longer for unsuccessful responses. In this program, the animal controls his own schedule. This schedule is particularly sensitive to external perturbation and provides a sensitive general measure of performance decrement.

The most noticeable effect of schedule manipulation is the production of bimodal response time distribution with certain parametric manipulations. In particular, choice of intertrial interval and criterion titration parameters are crucial to the appearance of this phenomenon. Extensive study of these parameters has identified those values which reproducibly correlate with bimodal reaction time distributions.

The development of this behavioral baseline is the last task carried out in this work unit. With its completion, the project has been phased out and appropriate administrative steps taken to terminate support. A technical report of the year's work is in preparation.

#### Summary and Conclusions.

Objective behavioral programs have been developed to study the time course of incapacitation following massive doses of spectrally mixed ionizing radiation, simulating nuclear weapons effects. The experiments have shown that certain behaviors, particularly those associated with avoidance, are very resistant to incapacitation effects. Substitution of non-satiating reinforcement (intra-cranial stimulation) also reduces the incapacitation following large radiation doses. The protective action of a particular chemical agent has been verified. Reaction time schedules have been developed to use in radiological experiments.

R.D. 41-51 03.0081 BIBLIOGRAPHY

Sharp, J. D., Kelly, D. D. and Brady, J. V. "The radio-attenuating effects of n-Decylaminoethanethiosulfuric Acid in the Rhesus Monkey in H. Vagtborg (Ed.) The Use of Subhuman Primates in Drug Evaluation. The University of Texas Press, 1968, pp 338-346.

Unclassified

Security Classification

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<i>(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)</i>		
1. ORIGINATING ACTIVITY (Corporate author) Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington, D. C. 20012		2a. REPORT SECURITY CLASSIFICATION Unclassified
6. REPORT TITLE RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES, INCLUDING BIOCHEMISTRY, COMMUNICABLE DISEASES AND IMMUNOLOGY, INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE		2b. GROUP NA
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11. SUPPLEMENTARY NOTES NA	12. SPONSORING MILITARY ACTIVITY U. S. Army Medical Research and Development Command Washington, D. C. 20315	
13. ABSTRACT The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigations are included on the DD Form 1498-1 introducing each work unit report.		

DD Form 1473

REPLACES DD FORM 1473, 1 JAN 61, WHICH IS OBSOLETE FOR ARMY USE.

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14. KEY WORDS	LINK A		LINK B		LINK C	
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Biological Sciences Medical Sciences Biochemistry Communicable Diseases Immunology Internal Medicine Nuclear Medicine Physiology Psychiatry Surgery Veterinary Medicine						

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