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WALTER REED ARMY INSTITUTE OF RESEARCH

Walter Reed Army Medical Center

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ANNUAL PROGRESS REPORT

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ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 809, MILITARY VETERINARY RESEARCH (Development and maintenance of conventional animal colonies)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Laboratory Animals
Department of Veterinary Microbiology
Division of Veterinary Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Major John H. Morris, II, VC

Assistants: John C. Curry, B.S.
Vernon L. Cowell, M.S.
Arthur A. King, B.S.
Captain Alan D. Stevens, VC
Captain Carol M. Lang, VC
1st Lt Norman B. Guilloud, VC
Arthur E. Dorsey, B.S.

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

ABSTRACT

Project No. 3A 0 12501 A 809 Title: MILITARY VETERINARY RESEARCH (Development and maintenance of conventional animal colonies)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Major John H. Morris, II, VC
John C. Curry, B.S.
Vernon L. Cowell, M.S.
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Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

1. Nuclei for 2 closed (barrier sustained) colonies were established with genetically selected mice from 2 different strains. Animals of the new colonies had greater productivity and breeding efficiency when compared with mice in other colonies maintained under similar conditions.

2. Studies were initiated on a highly prevalent and fatal respiratory disease occurring in newly procured dogs. "PPLO" organisms were consistently isolated from the respiratory tract and/or lungs of diseased or dead animals, but were rarely found in the respiratory tract of normal dogs. The etiologic significance of "PPLO" in the disease of dogs is being studied further. The mortality rate in dogs was reduced through use of enriched diets and the application of broad spectra antibiotics.

3. Routine chlorination of drinking water in mouse holding rooms has resulted in a marked reduction in the incidence of fatal Pseudomonas aeruginosa infection in mice that were stressed by irradiation. Installation of a chlorination system for production colonies is now in progress. Efforts are being made also to develop an automatic watering device for rodent colonies to reduce costs and labor and to provide closer hygienic control of water. Similar devices have been utilized advantageously in the large animal colonies.

4. Efforts were made to replace the hermetically sealed metal can as a package for rat and mouse diet with a less expensive disposable package. A polyethylene lined multi-wall kraft paper bag has been found to be satisfactory in preliminary studies.

5. The superiority of a new anthelminthic Teniazine (Phenothiazine & Teniatol) for treatment of nematode infestations in animals of WRAIR sheep flock was demonstrated. Unlike previously employed drugs, Teniazine produces no pronounced and prolonged toxic effects on erythrocytes.

BODY OF REPORT

Project 3A 0 12501 A 809 Title: MILITARY VETERINARY RESEARCH (Development and maintenance of conventional animal colonies)

Description:

1. With the increased demand for mice reared under closed (barrier sustained) colony conditions, it was deemed essential that additional nucleus colonies be derived and developed. Criteria for establishment of these colonies were: increased productivity and breeding efficiency; genetic uniformity; absence of diseases and endoparasites pathogenic to mice.

2. Studies were conducted on causes and method of control of respiratory disease in newly procured dogs. This disease appears during the quarantine period and is characterized by high morbidity and mortality.

3. Outbreaks of lethal Pseudomonas infections in mice stressed by irradiation interfered with evaluation studies on anti-irradiation drugs. Attempts were made, therefore, to control or eliminate Pseudomonas aeruginosa in mice colonies.

4. The high costs of packaging materials for dietary foods used for closed colony rat and mouse colonies and problems posed in their disposal, prompted a search for more suitable containers.

5. The epizootic occurrence of nematode infestations in the WRAIR sheep flock seriously affects the health of animals. The prolonged toxic effects of phenothiazine, the previous drug of choice, limited its usefulness in the control of helminth infestations in the sheep flock. Evaluation studies were conducted, therefore, on a newly available anthelminthic.

Progress:

1. To enhance the production of mice under closed (barrier sustained) colony conditions, two new colonies were established and studied. One, the WRC strain, was established with genetically selected mice from the conventional (open) WRAIR Cinnamon colony. The second was established with one hundred (100) pairs of mice of the ICR strain obtained from the Institute of Cancer Research, Philadelphia, Pennsylvania.

Young were obtained by aseptic caesarean section of pregnant females and were reared by gnotobiotic foster mothers. These young were allowed to mature and subsequently served as nucleus breeding stock for production colonies. Data obtained (Table 1) on these nucleus colonies indicates significant increases in conception rate and number of viable young reared to weaning per female when compared to the Walter Reed Bagg Closed Colony and the Walter Reed Cinnamon conventional colony.

TABLE 1

Strain	No. Pregnant Females	Conception Rate	Viable Young Weaned Per Female
ICR (nucleus)	1296	82.75	11.20
WRC (nucleus)	1344	81.10	6.83
WR Bagg (closed)	97040	71.29	7.20
WRC (conventional)	7800	68.40	3.52

Differences in production as shown in Table 1 are of special interest and reveal definite advantages in the rearing of mice in a barrier sustained environment.

2. Attempts were made to determine the causes of and to reduce the high morbidity and mortality rate of a respiratory disease in procured dogs, occurring during the period of their quarantine. Initial attention was directed on the effect of diet on mortality and morbidity rates. A group of 50 newly procured dogs were selected at random; 25 were given a standard cereal foundation diet supplemented with meat (FDM); the remainder were fed with a commercially available complete diet in pellet form (ST). Animals were observed over a 16-day period. Seven and eight animals in the FDM and ST groups, respectively, became sick, with three deaths in the former and five deaths in the latter group. An average of two pounds weight loss per animal was seen in the ST group, whereas the FDM dogs gained an average of four pounds. No significant advantages of FDM over the ST diet were noted over extended periods.

Upper respiratory signs were usually prominent in sick and moribund dogs. In view of recent reports of "PPLO" organisms in the etiology of respiratory infections of dogs maintained under similar conditions, a collaborative study was initiated by Major Erby L. Massey, USAF, VC, AFIP, to determine the etiologic significance of "PPLO" in outbreaks of disease. "PPLO" was consistently isolated from respiratory tract or lungs of 120 sick or dead dogs, but was rarely isolated from 20 normal dogs employed as controls. The isolated "PPLO" was resistant to sulfaquinoxaline, penicillin, polymyxin B, dihydrostreptomycin, and erythromycin, but was susceptible (in vitro) to chloromycetin, tetracycline, tetramycin, and aureomycin. In a preliminary test, the parental introduction of a "PPLO" isolate in a normal dog produced a respiratory

disease. Antigen and antisera have been prepared from selected strains of "PPLO" for subsequent studies on serologic relationships among the "PPLO" isolates. Employing a complement fixation technique, demonstrable rises in antibody titer were seen in paired sera from ten sick dogs and in the experimentally infected animal. Additional comprehensive bacteriological and virological studies are now in progress to resolve the etiologic significance of "PPLO" and other infectious agents in the respiratory disease of dogs.

During the past year, the mortality rate of dogs was reduced considerably by utilization of enriched diets and by antibiotic therapy of sick dogs. A comparison of mortality rates over a two-year period is shown in Table 2. Current animal husbandry practices are being reviewed from the viewpoint of more effective control of infections in dogs.

TABLE 2

MORTALITY RATES IN PROCURED DOGS

Month	1961-1962 Dogs Rec'd	1961-1962 Mortality	1962-1963 Dogs Rec'd	1962-1963 Mortality
Jul	144	36%	200	21%
Aug	214	38%	200	16%
Sep	313	31%	250	11%
Oct	136	43%	214	18%
Nov	310	38%	185	17%
Dec	250	35%	194	19%
Jan	294	41%	216	21%
Feb	257	26%	227	16%
Mar	224	27%	220	18%

3. Efforts to control the carrier rate of Pseudomonas aeruginosa in the mouse colony population were continued. In previous studies, it was shown that Pseudomonas aeruginosa is primarily spread through drinking water, and that chlorination of water effectively destroyed these organisms in this milieu. On the basis of these findings, chlorination of drinking water was initiated in mouse holding room H, Bldg 83, as in the holding room area on 4th floor, Bldg 40. Since chlorination of water was initiated, there have been no episodes of Pseudomonas aeruginosa infections in mice stressed by irradiation for

evaluation studies of anti-irradiation drugs. A decrease in the prevalence of Pseudomonas aeruginosa in colony mice is indicated on the basis of routine bacteriological examination of animals. Plans have been made to install a chlorination system in the water lines of the rat and mouse production colonies. Automatic watering devices that have been installed in the primate and canine sections are now being considered for use in the rodent colonies. These devices are labor saving and provide more effective control of the sanitary quality of drinking water.

4. Packaging of rat and mouse diets in hermetically sealed metal cans has been expensive and has created a disposal problem. Investigations to find a suitable, less expensive container were instituted. The container chosen must fulfill the following requirements:

a. Heat and moisture resistant when subjected to 212° F. for 15 minutes.

b. Container must have no holes or tears.

c. Must be resistant to wild rodents.

A variety of commercially available packaging materials were investigated, including polyethylene bags; multi-wall kraft paper bags; and a multi-wall bag having an inner layer of polyethylene on aluminum foil, with three layers of multi-wall kraft paper. The latter container fulfilled the requirements listed above. This package has been incorporated in the current diet contract, resulting in a saving of $40 per ton and eliminating disposal problems.

5. The efficiency of a newly available anthelminthic, Teniazine (Phenothiazine & Teniatol) to control endoparasites in the WRAIR sheep flock was studied. It was found to be very effective against Haemonchus, Trichostrongylus, Nematodirus, and Trichuris species. Unlike phenothiazine, it produced no toxic effects on treated animals and had no effect on the fragility of red blood cells. The toxic properties of previously employed phenothiazine compounds limited the usefulness of treated animals as a source of blood for serological tests for three to four months following treatment. The toxic factor also imposed limitations on the application of effective control measures. On the basis of preliminary findings, a more effective endoparasite control program was initiated employing the new anthelminthic.

Summary and Conclusions:

1. Two strains (WRC and ICR) have been established and maintained in a barrier sustained environment. Both strains excel in productivity when compared to the Bagg strain and to the WRC strain maintained under conventional conditions. These strains will be used as nucleus stock in the establishment of future production rooms.

2. Studies were conducted to reduce the incidence of a severe respiratory disease occurring in newly procured dogs. The course of the disease was favorably influenced if animals were given a meat supplemented diet. Preliminary bacteriological studies indicated "PPLO" to be a possible etiologic agent. More comprehensive bacteriological and virological studies are in progress to establish the role of "PPLO" and/or other organisms in respiratory diseases of dogs. Mortality rates have been moderately reduced by use of antibiotics and supplemented diets.

3. Routine chlorination of drinking water in mouse holding rooms has resulted in a marked reduction in the incidence of fatal Pseudomonas aeruginosa infections in mice that are stressed by irradiation. Installation of a chlorination system for production colonies is now in progress. Efforts are being made also to develop an automatic watering device for rodent colonies to reduce costs and labor, and to provide closer hygienic control of water. Similar devices have been utilized advantageously in the large animal colonies.

4. Efforts were made to replace the hermetically sealed metal can as a package for rat and mouse diet with a less expensive disposable package. A polyethylene lined multi-wall kraft paper bag has been found to be satisfactory in preliminary studies.

5. The superiority of new anthelminthic Teniazine (Phenothiazine & Teniatol) for treatment of nematode infestations in animals of WRAIR sheep flock was demonstrated. Unlike previously employed drugs, Teniazine produces no pronounced and prolonged toxic effects on erythrocytes.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 809 MILITARY VETERINARY RESEARCH
(Development and maintenance of germfree animal colonies)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Germfree Research
Division of Basic Surgical Research

Period Covered by Report: 1 July 62 - 30 June 63

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Assistants: Butler, V., Brown, N., B.S., Collins, H., Floyd, B., M.S., Helms, J.B., 1st Lt., VC, Kimler, A., Ph.D., Laundy, R., Capt., VC***, Pearson, W., Capt., MC, Watkins, K., Capt., MC****, and Wren, R.E., A.B.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project 3A 0 12501 A 809 Title: MILITARY VETERINARY RESEARCH (Development and maintenance of germfree animal colonies)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 62 - 30 June 63

Author: Albert Einheber, Ph.D.

Reports Control Symbol: MEDDH-288

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Study phases, described herein, are in preparation, in progress, or are completed with the following objectives: to advance the animal, nutrition and technology of germfree research; to study and compare the constitution (anatomy and biochemistry) and function (behavior, physiology and metabolism) of germfree, control-contaminated germfree, and conventional(ized) animals, when "normal" or after their challenge with physical, chemical or viable noxae; and to learn, regarding the latter, both the possible role of the environmental microorganisms (and their products) and the effect of our modification and/or control of these microorganisms.

BODY OF REPORT

Project No. 3A 0 12501 A 809 Title: MILITARY VETERINARY RESEARCH (Development and maintenance of germfree animal colonies)

DESCRIPTION:

The Department of Germfree Research, continually undergoing revision as to personnel, research interests, and needs by virtue of its organization and nature, makes a primary effort to direct its goals toward the current and/or anticipated needs of military medicine as these may become evident or as they are designated by request.

Study phases are continuing in preparation, in progress, or are completed on:

1) The germfree animal, its technology, production, rearing, maintenance, and nutrition; to ever increase the germfree animal's value to research and our potential for learning its nature.

2) Comparative analyses of the constitution (anatomy and biochemistry) and function (behavior, physiology, metabolism) of germfree, control-contaminated germfree, and conventional(ized) animals.

3) Comparative analyses of the responses (constitution and/or function) of germfree, control-contaminated germfree, and conventional(ized) animals after challenge with physical, chemical or viable noxae, or combinations thereof; to learn the possible role of the microorganisms (and their products) of the internal and external environment, and the effect of their modification and/or control.

Subjects Listed:

Animal Production and Utilization.
Interaction in vivo of S. aureus and E. coli.
New "Animal-Ejector-Sleeve" Technique for Serial Removal of Animals from Isolators.
Technique for Caesarean Section of Mice: Obtaining Germfree Inbred "Hairless" Mice (hr).
Evaluation of New Pellet-form Diets.
Development of a Synthetic Diet.
Ascorbic Acid and Tyrosine Metabolism.
Rat Tissue Serotonin, Histamine and Ascorbic Acid Levels.
Adrenal Physiology: Adrenalectomy.
Inflammation: "Granuloma-Pouch" Technique.
Experimental Burns: Testing of a Suitable Burn Apparatus.
Anesthesia: Development of a Suitable Volatile Anesthesia Machine.
Carbohydrate Metabolism: Diabetes.
Actions of Tetracyclines.
Actions of Penicillin in Guinea Pigs.
Radiation and Radioprotectants.
Tissue Transplantation: Skin Graft.

Subjects Listed (continued):

The Lymphatic System: Morphologic and Humoral Response to Bacterial Antigen and Radiation.
Mucosal Enzymes and Autonomic Nervous System of the Rodent Cecum.
Mucosal Morphology and Cell Renewal of the Rodent Ileum.
Response of the Guinea Pig Ileum In Vitro to Pharmacologic Agents.
Development of Clinical Isolators.

PROGRESS:

Animal Production and Utilization - During the year 1 July 1962 to 30 June 1963, 1500 animals were used for investigative purposes. The source and species of animals are as follows:

Born and reared germfree or delivered by Caesarean section in the department:

rats	117
mice	252
guinea pigs	140

Obtained from Charles River Breeding Laboratories:

rats	149
mice	596

Obtained from Notre Dame (Lobund):

mice	242

Interaction in vivo of S. aureus and E. coli - Studies were done on germfree rats and mice to determine the in vivo effect of E. coli on S. aureus and of S. aureus on E. coli. Groups of germfree animals were monocontaminated with one organism first by adding 40 ml of a 10^9 per ml suspension to their drinking water and sprinkling 10 ml on their diet and bedding. Cecal and stool cultures after 24 hours and every other day for 1 week showed immediate establishment. Using a loop holding 0.01 ml, each clinical specimen was streaked on blood agar and MacConkey and after 24 hours incubation, colony counts done.

After 1 week, the other organism was introduced and cecal and stool cultures were performed using the same technique as above. Cultures were done after 24 hours, every other day for 1 week and weekly up to 1 month.

When E. coli was established first, the introduction of S. aureus resulted in minimal numbers of the latter within 24 hours. After 1 month, no S. aureus could be found. When animals were first monocontaminated with S. aureus, the subsequent addition of E. coli resulted in an immediate and marked reduction of S. aureus. After 1 month, the latter could not be found.

New "Animal-Ejector-Sleeve" Technique for Serial Removal of Animals from Isolators - Heretofore, it has not been possible to remove isolator-maintained animals, germfree or otherwise, repeatedly during the day, as needed, without repeated preliminary sterilization of the isolator locks by peracetic acid fumigation. Apart from the time-delays and labor involved in sterilization and the possible deleterious effects of peracetic acid on the animal being removed each time, such chemical sterilization done often enough over a relatively short period may likewise cause untoward effects in the unused animals remaining in the isolator. In order to overcome this problem, four

plastic "ejector-sleeve" apparatuses (fashioned after the principle of the germicidal trap that has been used for introduction of materials into steel isolators) have been designed, fabricated, installed and used daily for a period of 2 weeks without contamination; by this means, 4-5 rats were individually removed during the day as needed (histamine and serotonin study found elsewhere in this report). The rat remains undisturbed in its normal isolator environment, and only when all is in readiness for its use is it placed in a suitably large water-tight jar which is quickly transferred from the isolator to the external environment via the once-sterilized flexible plastic sleeve, the free jar-exit end of which is continuously and deeply submerged in a germicidal solution contained in a vat sitting outside the isolator. The "ejector-sleeve" and the technique of its use are being improved and simplified by the dictates of continued experience. These "sleeves" will enable removal of animals immediately upon death or sacrifice, permitting their dissection and fixation in formalin outside rather than inside the isolator. This should release isolator space previously occupied by empty specimen bottles and containers of sterilized formalin which when opened release irritating fumes. Thus, the unwieldy, tedious task of preserving the animals or their tissues within the isolator will be obviated. The "sleeve" will also permit serial, short-interval samplings, as for example, are needed for determination of glucose tolerance curves, and for blood appearance or disappearance curves of injected radioprotectants, isotope-labelled materials, antibiotics, etc., and when materials being analyzed are labile. We further intend to attempt to modify the "sleeve" to make it a "dead-end" tube and suitable container into which an animal that has received an isotope, e.g., I^{131}, may be placed and subjected to total-body counting (small-animal scintillation counter and scaler provided by the Div. of Nuclear Medicine now being installed) without its removal from the isolator. Such a procedure will greatly expedite and facilitate this type of investigation and, most importantly, remove the hazard of contamination that accompanies entries into and removals from isolators, and again, obviate the attendant need for repeated sterilization of the isolator locks.

Technique for Caesarean Section of Mice: Obtaining Germfree Inbred "Hairless" Mice (hr) - The surgical and other technological procedures for performing laparotomy and hysterectomy of full-term pregnant conventional guinea pigs are well established and are used routinely for obtaining germfree guinea pigs in our laboratory; this is the only way of presently obtaining germfree guinea pigs because for reasons still unknown, germfree guinea pigs fail to bear young. Until now, Caesarean section of pregnant conventional mice had not been attempted in our laboratory. Our interest in the homograft problem has caused us to anticipate our possible future needs for germfree mouse strains of known genetic constitution. Since the mice in our present germfree colony, ND II and ICR mice, are hybrids and the only mice now available commercially, we have modified our standard guinea pig equipment and have achieved successful germfree delivery of infant mice by Caesarean section of full-term pregnant conventional mice. Unlike newborn guinea pigs which are relatively mature at birth, easily hand-fed, and which promptly fare for themselves, newborn germfree mice are hand-fed only with great difficulty and are therefore best reared (suckled) by germfree foster-mothers.

An 8.5 inch square stainless steel plate attached to and elevated 2 inches above the standard guinea pig operating plate by "feet" interposed at the 4 corners of the plates, has enabled us to perform Caesarean sections on full-term pregnant mice. Multiple holes drilled in the plate and 2 notches made at each corner have enabled us to secure the mouse properly during surgery.

The first strain of inbred mice we are attempting to obtain germfree, are the so-called "hairless" mice (hr) or Hypotrichosis cystica. We are attempting to establish a germfree colony of these mice for several reasons, primary among which are the following: 1) an inbred variety is available in the Walter Reed animal colony; 2) laparotomies, adrenalectomies and other surgical procedures; studies of healing of skin after incision, irradiation, burns, infection; skin bioassay procedures; skin transplantation and parabiosis; induced skin inflammation, etc. usually require preliminary depilation of animals. This is disturbing to the animal because anesthesia is usually required as is the undue handling of the animal in the process of applying and removing the depilatory, not to mention the known irritating and other possibly covert, undesirable effects on the skin of the depilatory itself. Obviously, this time-consuming technicality, greatly magnified in the germfree isolator, will not be required with "hairless" mice. Furthermore, in the healing of skin incisions after depilation, it is known that nutrients are delivered to the wound for restoration of the hair as well as for repair of the incision; ostensibly, such metabolic studies with "hairless" mice should not be confounded by this problem; 3) direct observation of the skin in situ after its experimental manipulation should be facilitated and for indefinite periods because the limitation usually imposed by regrowth of hair is obviated; and finally, 4) the fact that these mice are inbred should prove of special value in comparative studies of the time-course of homograft and heterograft rejection with hybrid mice. Moreover, skin graft exchanges between "hairless" and the usual albino mice are automatically provided with a conspicuous skin graft "marker" equal to that provided by the oft-used exchange of skin between mice of differing hair color.

Three pregnant hr mice were subjected to Caesarean section. One litter was discarded because it was too immature. However, 16 full-term mice were successfully delivered. These 2 litters were given to ND II foster-mothers that had delivered young 2 days previously. Unfortunately, the foster-mothers cannibalized the litters during the next 2 days. The operating tank did remain sterile, however, and was again used 5 days after initial surgery on one hr mouse; again the litter placed with an ND II mother was cannibalized. The operating tank remained sterile post-operatively.

The ND II mothers are notorious for their cannibalistic tendency; their unfortunate use was dictated by their timely availability. In our next attempts, which are now in progress, we plan to use ICR strain foster-mothers

which are less inclined to cannibalize their young. We feel a germfree colony of "hairless" mice is virtually assured once the foster-mother problem is solved. The capability of performing Caesarean section on mice will enable us to obtain specific strains of germfree mice as we need them.

Evaluation of New Pellet-form Diets - The specially fortified rat and mouse diet L-356 presently used in this laboratory is a powder and requires time-consuming handling and preparing. A pre-pelleted form of L-356, presumably of the same composition and nutritional adequacy as the powdered L-356, has become commercially available recently, as has, also, a less costly similarly pelleted diet, L-462. The availability and use of a pre-pelleted diet offers certain advantages: it would facilitate and expedite diet handling; presumably be in a form more palatable for rodents and reduce waste of diet; lessen the hazard of incomplete sterilization; and permit special packaging to insure greater standardization of the autoclaved diet offered to isolator-maintained conventional animals. For these reasons, we have modified our standard annual contractual purchase of the powdered L-356 diet to a quarterly arrangement, pending the results of current tests on samples of the pelleted L-356 and L-462 diets provided us for this purpose by the manufacturer.

The parameters being measured to test the nutritional adequacy of the pelleted diets are body weight gain, reproduction (mating and breeding efficiency), lactation (infant survival and growth).

Conventional, specific pathogen-free male and female Fisher rats, weighing 45-55 grams, maintained in individual cages, and subjected to an alternating schedule of 12 hours light and dark, were offered one of 5 diet preparations and water ad libitum. The rats were weighed once weekly for a total of 5 weighings, during a 28-day observation period. Representative males and females on each of these dietary regimens were mated and continued on the corresponding diets.

Males and females were offered one of the following forms and types of diet: 1) autoclaved, pelleted L-462; 2) non-autoclaved, pelleted L-462; 3) autoclaved, pelleted L-356; 4) non-autoclaved, pelleted L-356; and 5) autoclaved, cake-form L-356 (diet now used).

Using the average daily gain in body weight of Group 5 as the standard for comparison, all groups showed weight gain except Group 1 which showed a slight but definite loss. Comparison of the differences between the average daily weight gains of Groups 1 and 2 and of Groups 3 and 4, suggests that autoclaving had a deleterious effect on the body-weight-promoting properties of pelleted diet L-462, but not that of pelleted diet L-356. The results, therefore, suggest that the autoclaved, pelleted L-356 is an adequate diet as regards "growth rate". An effort is now being made to duplicate the above results using the same procedure as outlined. This study is now in its 3rd week and the results obtained, thus far, closely parallel the above data. To date, two females, one each from Groups 3 and 5, that were mated to males of corresponding groups, have delivered young;

further observations along these lines are in progress. Once we have established the efficacy of the pelleted L-356 diet in the open laboratory, similar studies will be performed with germfree rodents. The results on the pelleted L-356 diet are definitely encouraging.

Development of a Synthetic Diet - The fact that germfree animals are free from viable bacteria does not preclude their immunologic perturbation by antigenic materials, such as dead bacteria, proteins and other polymeric substances contained in the autoclaved semi-synthetic diet they are fed. The development and use of water-soluble chemically-defined "hypoantigenic" diets should increase the value of the germfree animal as an investigative tool. Synthetic water-soluble diets have been devised and successfully fed to conventional rats (Greenstein, J.P., et al. Arch. Biochem. Biophys. 72:396, 1957).

Several experiments have been conducted to determine the ability of synthetic diets #116 and #26 to support life and produce optimal growth. All animals given diets #116 and #26 and water ad libitum had growth rates that were 50% less than control animals eating our standard L-356 diet. It was observed that the animals on diets #116 and #26 (liquid) increased their water intake. Since satiation of thirst by water might have a negative effect on intake of liquid diet, open-colony conventional male rats were fed diet #116 to which 25% more water was added, but the drinking water removed. Weight gains achieved were comparable to that of the control group receiving L-356 diet and drinking water. The average daily weight gain of the animals on the more dilute diet minus drinking water was 4.5 grams as compared with 2.5-3.0 grams obtained with the previous dietary regimen. However, similar experiments with germfree and tank-bred conventionalized Fisher rats that were fed either diet #116 or #26 with 25% more water and no drinking water resulted in average daily weight gains of only 2.5 to 3.0 grams. The reason for the discrepancy in weight gain between the open-colony and the isolator-maintained conventional and germfree rats is not known.

Further studies along these lines are being held in abeyance until personnel availability permits full-time attention to this work, trained personnel and established methods are available for routinely evaluating the "antigenicity" of so-called chemically-defined, sterilized diets prepared in our laboratory or purchased commercially, and acceptable standards of "hypoantigenicity" of such diets are decided upon. Thus far, the synthetic diets prepared and used in this laboratory have not been tested for their antigenicity.

Ascorbic Acid and Tyrosine Metabolism - It has been well established in this laboratory that the germfree guinea pig survives more than twice as long as its conventionalized litter-mate when deprived of ascorbic acid. The mechanisms responsible for this prolonged survival remain to be elucidated. Since ascorbic acid is required for normal metabolism of a tyrosine load, the response of germfree and conventionalized animals to successive tyrosine loads on a scorbutigenic regime can yield information about the time-course of metabolic scurvy in the two groups.

Germfree and conventionalized litter-mates at 2 months of age were placed on a regimen of fresh daily ad lib. diet and water intake. The drinking water contained a quantity of ascorbic acid such that each pig had an intake several times his minimum daily requirement. Each pig was then given by pharyngeal injection a tyrosine load of 0.75 mg l-tyrosine/gram body weight (in a slurry in saline) and urine and feces were collected and analyzed for the following 48 hours. Neither group demonstrated a metabolic abnormality. Animals were then given tyrosine loads on two successive days and urine and feces again analyzed. Conventionalized animals excreted, principally in urine, significant quantities of phenoxy-carboxylic acids indicative of partial metabolism of the load and a relative deficiency of ascorbic acid. Germfree animals manifested no abnormality.

For the second phase of the study, both groups were deprived of ascorbic acid and tyrosine loads given at successive intervals. Urine and feces were collected and analyzed for 48 hours after each load. The conventionalized animals had an abnormal metabolic response within 96 hours, were maximally abnormal by the 11th day, and were dead, with marked gross manifestation of scurvy, by the 28th day. Germfree animals did not show any abnormality until the 11th day (when the conventionalized were already maximally abnormal), became maximally abnormal after the 28th day, and survived to 65-108 days. At death, as noted by others, germfree animals exhibited less marked gross signs of scurvy than conventionalized animals.

These data show that (1) on an intake of excessive ascorbic acid the conventionalized animal has a lower tolerance for a tyrosine load than a germfree animal and (2) when deprived of ascorbic acid, the conventionalized animal manifests the metabolic abnormality sooner and to a greater degree than a germfree animal. The time-course of the metabolic abnormality parallels in a general way the time-course of survival of the two groups. Since the metabolic response to a tyrosine load has been shown to correlate well with tissue ascorbic acid levels, one would suggest that the conventionalized and germfree animals have a different tissue half-life of ascorbic acid when placed on a scorbutigenic diet.

Rat Tissue Serotonin, Histamine and Ascorbic Acid Levels - Ever-increasing important new roles are being postulated for the potent biogenic amines, serotonin and histamine in many areas of normal and abnormal physiology. Schayer has found that the histamine-forming capacity of tissues (histidine decarboxylase activity) increases concomitantly with 'nascent' histamine production during a variety of stressful conditions such as endotoxic or tourniquet shock or cold exposure. He and others have rejuvenated the possibility of histamine's importance in shock states. Serotonin too has been linked in a variety of ways to neuro-, circulatory and smooth-muscle physiology and pathology; its production and presence in high concentration in the enterochromaffin cells also makes it of interest to gastro-intestinal physiologists and pathologists. The latter, obviously puts the problem, so-to-speak, directly in the lap of germfree research. It has been reported that certain antibiotics increase the serotonin content of the intestine.

Since we are interested in separating the antibacterial from the non-antibacterial (pharmacologic) activities of antibiotics by the use of germfree animals, the question arises whether increases in intestinal serotonin would result in germfree animals given these antibiotics; the fundamental problem may relate to differences in tryptophan metabolism in the presence or absence of bacteria. Of further interest to us, are the reports that germfree chickens, rats and mice have greater serotonin, but lower histamine levels in their intestines than corresponding conventional animals; whether these amines are in any way related to the "physiologic" distention of the cecum of germfree rodents is not clear. The latter studies are subject to one of several criticisms: 1) The values for serotonin and/or histamine levels are presented either per unit fresh weight of tissue, or it is not clearly specified that the serotonin concentration is based on dry or wet weight of tissue. Since it has been found that the intestinal wall is less hydrated in the germfree than in the conventional animal, it would seem that the concentration of serotonin should also be calculable on a dry tissue weight basis for proper comparisons to be made, i.e., differences in tissue water content should be taken into account. This has apparently not been done by previous investigators. In our work, we have additionally taken into account that the amine content of the intestine expressed on the basis of the amount of dry matter per unit length of gut, which is a usable substitute for the surface area (Gordon, H.A., et al. Am. J. Physiol. 201:175, 1961), may have even greater functional significance. 2) In some of these experiments, it is not clear whether the conventional animals were run in parallel with the germfree animals, or the comparisons are based on averages of data pooled from animals seemingly examined at random intervals, as available, and compiled in this fashion over a period of years. Moreover, in one of these studies, the conventional animals were not maintained within isolators, but were from the "open" animal colony; we do not know whether this environmental factor is of importance in influencing serotonin and histamine levels, but it is certain, and also our standing policy, that this question is best avoided by providing the same physical conditions for germfree and conventional animals that are being compared.

In addition to the areas already mentioned, our interest in the metabolism, physiology and pharmacology of histamine and serotonin (and their precursors, antagonists, and "releasers") also stems from our projected and in-progress investigations of inflammation, wound healing, irradiation, endotoxins, superior mesenteric artery occlusion (bowel ischemia), burns, and other forms of injury and shock. Of special pertinence is the fact that serotonin has marked radioprotective activity if administered in advance of x-irradiation, and on a weight basis, is one of the most potent radioprotective agents known. Since germfree animals are more resistant to lethal and supralethal doses of irradiation than conventional animals, the question may well be asked whether differences in tissue serotonin content, metabolism, release, etc. between germfree and conventional animals are involved in this. Also, the recent demonstration

in this laboratory (see elsewhere in report) that radioprotectants are effective in x-irradiated germfree mice, raises the question as to whether these agents are operating via liberation, etc. of these amines. However, it has not yet been determined that these radioprotectants are more beneficial for germfree than for conventional animals. Of further relevance to our research program is the fact that while serotonin and histamine are considered 'mediators' of hypersensitivity reactions, their precise mechanism of action is still speculative. In this regard, it is pertinent that the immunologic reaction (rejection) resulting from skin homografts is accompanied by changes (decreases) in serum serotonin concentration that are similar in magnitude to those which occur after administration of endotoxin or during anaphylactic shock (Rosenberg, J.C., Transplant. Bull. 30:531, 1962).

In order to establish baseline values under our laboratory conditions and diet, we have just completed collection of a variety of tissues from 20 germfree and, concurrently, from 19 conventional isolator bred and maintained adult Fisher rats, of the same age, and receiving the same autoclaved L-356 diet, and are determining, by biochemical assay, the concentrations of serotonin, histamine and vitamin C in various tissues. In addition, various organ weights have been obtained and the percentage of water in the tissues is being determined. Since conventional guinea pigs develop scurvy about twice as fast as do germfree guinea pigs when vitamin C is removed from the diet, and the data collected thus far suggest that the normal germfree guinea pig 'from the start' has more vitamin C in certain of its tissues than normal conventional guinea pigs, we thought it of interest to examine the vitamin C content of certain tissues of germfree and conventional rats, which unlike guinea pigs, do not require and therefore do not receive a dietary source of vitamin C because they synthesize their own supply.

The following table is a preliminary comparison of the weight of the brain, spleen and adrenal glands of adult germfree and conventional rats. The organ weights represent fresh weight obtained immediately after killing the rat by decapitation, expressed in g/100 g of "body weight", the "body weight" here refers to total body weight minus the weight of the cecum and its contents. The latter correction is made because of the often enormous disparity in the weight of the cecal contents between germfree and conventional animals, and which if not made may obscure differences. Obviously, the cecal contents represent neither functional nor actively metabolizing tissue. Unfortunately, this factor of difference between germfree and conventional animals has not always been taken into account in previous studies.

Organ Weights (g/100 g "B.W.")

	Brain	Spleen	Adrenals
Germfree	0.986	0.231	0.030
Conventional	0.841	0.208	0.022

The composition of the intestinal flora may bear on the particular results one obtains for the serotonin and histamine levels of the intestinal wall of conventional animals. To characterize this state of affairs as best we can, bacteriograms of the fresh cecal contents (water content determined) obtained by sterile sampling of the conventional rats immediately post-mortem, are being determined. The following types of organisms are being searched for and counted: E. coli, Aerobacter, Pseudomonas, Proteus, Staphylococcus, Micrococcus, Lactobacillus, enterococcus (fecal streptococci), diphtheroids, fungi and anaerobes.

Adrenal Physiology: Adrenalectomy - Extirpation of the adrenal glands renders animals generally more susceptible to stress. Thus, adrenalectomized mice are highly susceptible to experimentally induced infections and to administered endotoxins; the influence of steroids on the reticulo-endothelial system has been studied but is incompletely understood. As regards the therapeutic efficacy of adrenocortical steroids, both gluco- and mineralocorticoids, in clinical and experimental infections and "endotoxinemias", the literature is confusing and conflicting. Due to the well known hypersecretion of steroids following injury and infection and other noxious stimuli, it has been suggested that to discern the "non-specific" effects of the stressors, which relate in large part to the steroids, it is instructive to study the responses, to stressor agents, of adrenalectomized as well as intact animals.

To our knowledge, germfree animals have not been adrenalectomized and their ensuing metabolic and histopathologic states contrasted with that of adrenalectomized conventional animals. In view of the aforementioned, and our continuing interest in traumatic shock and infection, would healing, inflammation, endotoxins, etc., it seems important to us to determine initially the clinical course and survival of adrenalectomized germfree and conventional animals. A fundamental question is whether or not the adrenal glands of the conventional animal are physiologically burdened or benefitted in the net by the ever-present microorganisms and their products. Since it has been demonstrated that following the repeated administration of adrenocortical steroids, conventional animals may die of infection, presumably due to rekindling or exacerbation of so-called latent or "silent" infections, i.e., from microorganisms presumably of endogenous origin, the germfree animal may also serve to help us distinguish the local and systemic "direct" effects of administered steroids from those that are "indirect", i.e., resulting from a "bacterial factor", which may operate covertly in the conventional animal.

To approach these and other problems relating to the adrenals and their secretions, we desired first to establish a suitable adrenalectomy procedure for conventional rats and mice of the strains that will be studied within the germfree isolators. A complication to be anticipated is the variable presence in different strains, especially of mice, of accessory cortical nodules in and about the anatomic locus of the adrenals; thus, depending on the particular mouse strain, the incidence of "rests" may increase or

decrease with age, or they may vary as to anatomic location, over-all number, and with the sex. The alternatives are to perform detailed anatomic studies for such statistics in order to select the animal status that will likely lead to minimal post-adrenalectomy interference by accessory nodules, if such occurs, or to adrenalectomize animals, study their survival, and then do post-mortem examinations. Initially, we chose to do the latter functional study. Since it is known that provision of saline in lieu of drinking water enables adrenalectomized animals to survive (if not subjected inadvertently or otherwise to undue stress such as extremes of temperature, etc.), and that without such provision of sodium, death results, our initial efforts were directed towards the establishment of this pattern with our conventional mice and rats.

Conventional ICR male and female mice 4-6 weeks of age were subjected to bilateral adrenalectomy or to a sham operation. Each of these two groups was divided, one being offered 0.9% saline in lieu of drinking water, the other demineralized water. All animals were offered ad libitum D&G mouse pellets. None of the animals died after three weeks of observation and were killed. Gross examination, post-mortem, of a sample of these mice revealed the presence of nodules which were felt to represent accessory cortical tissue. Thus, the accessory cortical tissue may have accounted for survival after adrenalectomy; the dietary sodium may have also contributed to this.

Since females generally are reputed to have fewer accessory adrenal cortical nodules than males, the above studies were repeated using conventional female Fisher rats weighing 140-160 grams. All rats were given the standard autoclaved L-356 diet ad libitum. After 18 days, there were no deaths. All the animals gained weight. The groups arranged in decreasing order of magnitude of weight gained per day are: Adrenalectomy-saline (0.4 g); sham-adrenalectomy-saline (0.3 g); sham-adrenalectomy-demineralized water (0.2 g); and adrenalectomy-demineralized water (0.1 g). On the 18th day, post-adrenalectomy we, therefore, decided to deny the rats food, but to allow them their respective fluid regimens. The idea was to see whether the sequence of death after superimposition of fasting would reveal which rats were adrenalectomized, and also to detect whether the L-356 diet may have been providing the margin for survival by its ample sodium content. The groups arranged in decreasing order of average number of days survived post-starvation are: sham-adrenalectomy-demineralized water (10 days), sham-adrenalectomy-saline (8 days), adrenalectomy-saline (4.5 days), and adrenalectomy-demineralized water (2.5 days). Since the order of survival is just as one might anticipate, it would appear that "effective" adrenalectomies had been performed, that saline was efficacious in promoting survival of starving adrenalectomized rats, and finally, that the sodium content of the L-356 diet was preventing death of fed, adrenalectomized rats receiving demineralized drinking water. We are presently attempting to obtain a low sodium L-356 diet from the manufacturer. In view of the clear-cut survival pattern pursuant to starvation, we are presently planning similar experiments with germfree and conventional animals.

Inflammation: "Granuloma-Pouch" Technique - Whether inflammation is "harmful" or "useful", and the circumstances under which either or both aspects may prevail have been debated for many years. While it is certain that inflammation is a hallmark of tissue injury, tissue injury may be present in the virtual absence of overt inflammation under conditions of severe systemic debilitation arising from a variety of causes. Our interest in the local response to injury and its components, inflammation, necrosis and their 'mediators', originates from the fact that traumatic and infectious shock states are conditions that are initiated by local stress which usually continues after the shock is cured. Whether or not invasion of microorganisms will occur and clinically important infection become established may be decided by the early ("critical period") local tissue responses that are dependent, in large part, on an adequate nutrient blood flow and delivery of anti-microbial cellular and non-cellular components. Thus, the interactions between local and systemic stress after injury are of importance in determining whether or not shock may subsequently redevelop, the type and efficacy of attempted therapy (including antimicrobial agents), and the ultimate course of healing and convalescence; the superimposition of local and/or whole-body irradiation on other trauma modifies and increases the complexity of these interactions. In past studies of experimentally induced chemical inflammation, germfree animals have not been used, and, frequently neither mention made, nor assurance given, of the sterility of the agents (irritants) or of the techniques by which these agents were introduced into the animal. For these reasons, it is questionable whether studies of so-called "sterile" inflammation induced in this way, or the biochemical and histopathologic sequelae described therefrom have actually always excluded the overt or covert participation of microbial factors. If aseptic procedure has been achieved in some of these studies and microbial factors are in no way involved, it is presently impossible to single out these studies with certainty (vide infra). We are, therefore, comparing the evolutionary and involutionary mechanisms and microscopic morphology of inflammation induced by sterile non-microbial (chemical and physical) as well as microbial (viable and non-viable) agents in germfree and conventional animals.

From among the various experimental preparations available for the study of inflammation, we have, for our initial use, adapted and modified the Selye "granuloma pouch" technique (Selye, H., Proc. Soc. Exp. Biol. Med. 82:328, 1953). The procedures, salient features and principle applications of this technique have been numerously published. Our previous experience with the air-pouch technique has proven helpful (Einheber, A., Am. J. Physiol. 192:258, 1958).

We have used over 150 conventional, pathogen-free, young adult male and female Fisher rats to explore the various procedural aspects of the "granuloma pouch" technique, to modify these to our needs, and have made preliminary observations on the development of the pouch. These studies

are essential before engaging in full-scale experimentation within the germfree isolator. We have made a variety of measurements and observations which are being evaluated. To gain the necessary preliminary experience of performing this technique within the isolators, and of introducing the necessary equipment and materials, 5 germfree and 5 conventional isolator-maintained rats were also subjected to the "granuloma pouch" procedure.

Croton oil -- The irritant, croton oil, has most frequently and successfully been used. It results in considerable exudate formation and a well-defined inflammatory tissue barrier. We considered two problems initially: how to sterilize the croton oil and the corn oil (used as vehicle for the croton oil and as a control injection) to permit their sterile introduction into the isolators and their sterile administration to germfree and conventional animals without loss of phlogogenic potency? What volume to inject and what concentration of croton oil in corn oil to use? We have employed two procedures for sterilization, viz. autoclaving and filtration through millipore filter into a pre-autoclaved ampouling apparatus. Two concentrations, 0.5 and 1.0% of croton oil in corn oil (v/v) were subjected to the above sterilization procedures. The four types of irritant preparation were administered to rats in a volume of 0.5 or 1.0 ml. On opening each ampoule before injection, a sample of the contents was cultured for sterility. Rupture of the millipore filter during aspiration-filtration of the oils into the ampoules, caused some initial difficulty but this has been overcome. Following filtration-sterilization, the product was as clear as before filtration and remained this way on storage in the cold; only filtered oils are now used. By contrast, the croton oil became cloudy on autoclaving but was active in producing inflammation. The contents of all successfully prepared ampoules proved sterile.

Subcutaneous air and croton oil injection -- Since we desire to create, with the highest degree of assurance, "sterile" inflammation in conventional as well as in germfree animals, i.e., to exclude exogenous contamination incidental to production of the pouch, we sterilized, by autoclave, all syringes used for injection of air or croton oil and used sterile hypodermic needles. The depilated intrascapular injection site was prepared with propanol before injection was made. Being concerned that loading the sterile syringe with ambient room air for injection might result in contamination of the air-pouch, we searched the literature for information on the need for any such precautions but were surprisingly unable to find any mention of this seeming problem. Obviously, this is as much a problem in an isolator containing conventional animals as it is in the open laboratory. Our first approximation to a solution of this problem was the following: A vaccine bottle was rubber-capped (new cap), and a hypodermic needle with its hub packed with FG 50 (filter material), jabbed once through the cap into the bottle. The bottle is then autoclaved. On removal to the contaminated environment, the inside of the bottle remains sterile. At the start of a series of air-injections, another sterile needle is jabbed, once and for all, into the cap beside the "filter-needle",

no subsequent punctures being made into the cap. To fill the sterile syringe with air, the plunger, having been driven to its full extent into the barrel prior to autoclaving, is maintained in this position (allowing no room air to enter) and the needle-adapter-end of the syringe inserted snugly into the last-placed needle and the plunger withdrawn. The ambient air, on being aspirated into the syringe, therefore first goes through the "filter-needle", then into the sterile bottle and finally into the syringe. After filling the syringe with the desired volume of "filter-air" (25 ml), it is removed from the needle with care to avoid movement of the plunger that might contaminate the filtered-air contents of the syringe with room air. A sterile hypodermic needle is then placed on the syringe and the air injected subcutaneously through the prepared skin site.

We are presently working on a procedure that is simpler and which should prove more fool-proof, viz., pre-filling the syringe with the required air volume and then autoclaving. The only problem is to find a suitable means for immobilizing the plunger at the desired volume level in the barrel before autoclaving, and for its subsequent easy mobilization at injection.

We injected the diluted croton oil or corn oil in one of two ways: The first method was to leave the needle used for air injection in situ and use it again for croton oil injection. The advantage here is that the air pocket need not be perforated twice; the disadvantage is owing to the viscosity of the oil which precludes the use of a small gauge needle for air injection. Thus, some of the injected air may escape through the needle prior to oil injection. The second method was to use one needle for air, pinching the skin around the needle on its withdrawal and simultaneously pushing the pouch in the direction opposite to or away from the direction of the syringe with the fingers, and then after checking the quality of the air pocket, injecting the oil through a new injection site. We have found that directing the needle for air injection from a cephalad to caudad direction, with the rat's head facing the investigator, for some reason produces more uniform air-sacs. To avoid creating a tract of dermal inflammation and an open wound by the deposition of croton oil on withdrawal of the needle, the empty syringe is always first removed from the needle and the needle allowed to remain in situ to allow the croton oil remaining in its lumen to drain into the pouch before being removed.

Animals -- The rats are anesthetized with nembutal and the dorsum depilated with Nair. The depilatory is then carefully washed off with surgical detergent and water and the animal gently dried. As mentioned, the injection site is prepared with propanol prior to subcutaneous injection. The animals are allowed L-356 diet and water ad libitum before and after preparation.

Observations -- Pouches have been made and studied with air alone, corn oil or croton oil in corn oil at 0.5% and 1.0% concentrations, the oils being pre-sterilized either by autoclaving or by filtering through millipore. Rats have been sacrificed from 15 minutes to 3 weeks after

pouch formation. Some of the parameters observed include: exudate volumes, pouch weights, hydroxyproline and dry weight determinations on pouch tissue and changes in body weight. Intact pouches have been collected at various times (also brachial or axillary lymph nodes) and fixed in cold buffered 10% formalin for following the morphologic evolution and involution of the inflammatory structure, with observations on the specific effect of croton oil, corn oil and air. Exudates have been collected and frozen for chemical analyses. The effects of exudates, fresh and frozen, on E. coli and S. aureus have been examined.

Values for "Granuloma Pouch": Conventional Rats

Treatment	Duration of Inflammation	Sex	Exudate (ml)	Pouch Tissue Wet. Wt. (g)	Pouch Tissue Hydroxyproline (mg/g, Dry Wt.)
0.5 ml of 1% croton oil (filtered)	7 days	Female	9.0 (10)	4.0 (10)	-
		Male	6.0 (7)	3.0 (7)	1.7 (3)
	14 days	Female	15.0 (8)	3.0 (9)	1.7 (9)
		Male	13.0 (8)	3.0 (8)	2.2 (8)
1.0 ml of 1% croton oil (filtered)	7 days	Female	11.0 (5)	5.0 (5)	-
		Male	8.0 (5)	4.0 (5)	-

These preliminary observations suggest that females produce more exudate than males and possibly more inflammatory tissue. The data also suggest that the hydroxyproline content of the inflammatory tissue (males) increases between 7 and 14 days post-inflammation, reflecting an increase in collagen formation. All exudates collected thus far, have proven sterile on culture. Similar observations should prove interesting in germfree animals.

Partition of Total Body Weight Gain Between Inflamed and Non-Inflamed Tissue*

Treatment**	No. and Sex	Percent of Pre-Inflammation Body Weight: Total Body-Weight Gain	Total Pouch Weight Gain ("parasite" tissue)	Final Body Weight Gain Minus Pouch ("host" tissue)
0.5 ml croton oil	5 F	+ 10.0	+ 9.2	+ 1.0
	4 M	+ 10.2	+ 5.4	+ 4.7
1.0 ml croton oil	4 F	+ 11.0	+ 8.9	+ 2.0
	4 M	+ 13.2	+ 7.0	+ 6.1
25 ml of air only	5 F	+ 7.3	-	-
	5 M	+ 11.0	-	-

* Conventional rats, 7 days after 1% croton oil.
** Injected subcutaneously.

To the extent that the granuloma pouch grows at the expense of the normal tissues of the host, it may, so-to-speak, be considered a "parasite". The "parasite" and "host" appeared to share about equally in the total body weight gain of the males; by contrast, the "parasite" accounted for almost all of the total body weight gain of the females. Comparing the total body weight gains of the controls (air only) and rats with the granuloma pouches, the males were unaffected by the inflammation, but the females bearing pouches, on the average, grew more than their controls. Thus, whereas the air-injected males grew more than the corresponding females, this difference is diminished between granuloma pouch-bearing males and females, their over-all growth being comparable. More observations are required to confirm these trends, and may prove of value in our search for differences in similar comparisons between germfree and conventional animals.

Effect of inflammatory exudates on E. coli and S. aureus, in vitro -- Fresh rat pouch exudates were collected 7 days after injection of 0.5 or 1.0 ml sterile 1% croton oil in corn oil. Two 1 ml aliquots of each of the 12 sterile exudates were pipetted into sterile vials, E. coli was added to 1 vial and S. aureus to another. The concentration of organisms was such that 0.01 ml of the final pouch fluid suspension contained 150 organisms. Using a platinum loop calibrated to deliver 0.01 ml, each mixture was subcultured at 0, 2, 4, 6, 20 40 and 88 hours, incubated for 24 hours at 37°C. and a colony count done. The following results were noted: 1) Immediate and marked progressive reduction in the numbers of E. coli, effective up to 6 hours. After 20 hours of exposure, the E. coli counts increased. These results are the antipode of that which obtains when E. coli are placed in a nutrient medium under the same conditions; 2) No inhibition of S. aureus. Aliquots of the fresh exudates were stored at -70°C. for 1 month, and the above study repeated. The only difference noted was about a 25% increase in the E. coli count, at the subculture intervals indicated. Apparently there is some loss of "inhibitory" effect of the exudates on E. coli with storage. In all the above respects, then, the exudates behave qualitatively as do blood sera obtained from rats; possible differences between blood sera and exudates obtained from individual animals will be searched for in germfree and conventional animals.

Histopathology of pouches -- The pouch begins as a partially fluid-filled air-space between the panniculus carnosus and the deep muscles of the back, and along and within its wall the vascular, cellular and exudative events of an acute inflammatory response proceed in classic fashion, with or without irritant, such as the injected croton oil, in both conventional and germfree animals. Five days following production of the pouch an inner proteinaceous membrane surrounds the fluid-filled cavity; exterior to this is a band of collagen and fibroblasts 2 to 4 mm. thick. This cellular but fibrous wall has at its periphery varying areas of acute and subacute inflammatory responses if a diffusible chemical irritant, such as croton oil, has been used in the production of the pouch. At this time, the fluid content is relatively acellular, proteinaceous and may be repeatedly sampled for biochemical studies.

At about 2-3 weeks after production of the injury, contrary to what has been previously described, the wall of the pouch undergoes partial lysis and fragmentation. This latter phase is currently under study.

The granuloma pouch, with its predictable evolution, rather constant morphology and fluid-filled lumen appears to be a most suitable model for the biochemical and morphologic evaluation of inflammatory responses and mechanisms of host-resistance in the germfree animal. The appropriate base-line studies to facilitate this are currently concluding and more extensive experiments with germfree and isolator-maintained rats now are in progress.

Experimental Burns: Testing of a Suitable Burn Apparatus - One of our primary objectives is to study the extent to which bacteria and their products may influence early mortality (shock phase), delayed mortality, and convalescence after burns alone or when combined with ordinarily low-lethal x-irradiation (Levenson, S.M., Einheber, A., and Crowley, L.V., Research in Burns, Public No. 9, AIBS, 1962, p. 143) with the use of germfree and purposely contaminated germfree animals. Towards this goal, we have performed a considerable number of preliminary experiments with conventional mice (Einheber, A., et al., Ann. Progr. Rept. Project No. 6X59-01-001, Task No. 01, 1 Jul 61 - 30 Jun 62) in attempts to standardize a suitable burn (scald) procedure. We have found the scald-type injury, either back-burn (30-35% BSA) or circumferential lower trunk (mid-axillary-distad) burn (ca. 66% BSA), to be a highly reproducible and controllable method for inducing shock (death within 48 hours). However, burn studies with germfree animals have been delayed because of the lack of a suitable burn apparatus and volatile, non-inflammable, anesthetic agent. Among the attributes a burn apparatus should have for use within germfree isolators are: ability to maintain a pre-set water temperature (within a desired range of temperatures) with minimal or no fluctuation over a considerable length of time; circulate the water in such a way as to insure even heat distribution throughout the water container without causing undue water turbulence that would preclude infliction of a reproducible burn wound, viz., its area and severity; be compact, sturdy and mobile; and most importantly, be amenable to chemical or heat sterilization.

An instrument that may serve our purpose is the Bronwill Constant Temperature Circulator (Will Corp.). It is currently being subjected to extensive test.

Anesthesia: Development of a Suitable Volatile Anesthesia Machine - In view of our previous findings (Einheber, A., et al. Ann. Progr. Rept., ibid. 1 Jul 61 - 30 Jun 62) that mice anesthetized with fluothane just before scalding suffer a 24-hour post-burn mortality (44%) intermediate between that of similarly scalded mice anesthetized with ether (22%) or chloroform (62%), we are currently developing and testing a fluothane-vaporizer-anesthesia apparatus that can be sterilized for use in the germfree isolator. The relatively low toxicity of fluothane and the fact

that it has been used successfully for anesthetization of newborn animals (Morley, E. et al. Brit. J. Anesth. 34:776, 1962) is encouraging. A reproducible method for accomplishing uniform levels of anesthesia (volatile) still unattained under germfree conditions, will be an important asset to many phases of our germfree research program. (See neonatal thymectomy elsewhere in report.)

Carbohydrate Metabolism: Diabetes - Consideration of the role of carbohydrate in bioenergetics, of insulin in facilitating the transfer of glucose across cell membranes, hence, in glucose utilization, and of the blood glucose in satisfying, indispensably, the special requirement for it by the central nervous system, amply reveals the specific and universal biological importance of carbohydrate metabolism. It is, therefore, not surprising that derangements of body carbohydrate are found in traumatic shock states, and that carbohydrate metabolism is intimately and importantly concerned in the local and systemic bacterial defense mechanisms, in inflammation and in wound healing. In view of our research interest in these aspects, we are developing suitable procedures for inflicting specific defects to the various biochemical links in the chain of carbohydrate metabolism.

Our first efforts are directed towards the production of insulin deficiency and a diabetic state in both germfree and conventional rodents by the administration of alloxan or by the surgical removal of about 99.5% of the rat's pancreas (Scow, R.O. Endocrinology 60:359, 1957).

Precisely why diabetics as compared with non-diabetics are more prone to develop infections, to suffer a more complex and difficult course during infection, and to require more prolonged and vigorous treatment to be cured of infection is still a mystery. The "diabetic-host"-parasite enigma persists despite the availability and use of insulin and an impressive array of antimicrobial drugs, and despite attempts at its understanding both clinically and experimentally. The results of investigations of diabetes and infection are in many respects conflicting.

Difficulties in resolving the "diabetic-host"-parasite problem are to be expected in view of: the varying complex of metabolic, physiologic and anatomic disturbances that accompany the diabetic state; the still incomplete definition of the biochemical role and metabolism of insulin, and of its interrelationships with the other hormones; the increasing clinical revelations of the existence of a variety of diabetic syndromes, including the recent discovery that "...diabetes mellitus may result from extrapancreatic malfunction of the mechanism regulating insulin activity in blood, and not from a lack of endogenous insulin" (Antoniades, H.N. et al. N. Engl. J. Med. 267:218, 1962).

Unlike "good" actors, most "good" diabetics are "made" during the course of life, rather than born as such. This has led to the suggestion that "...toxic or metabolic factors may appear in man and contribute to the progressive degeneration which is taking place in the beta cells of the human pancreas" (Lazarow, A. Physiol. Rev. 29:48, 1949).

In preparing for our attempts to induce alloxan diabetes in isolator-maintained germfree and conventional mice, we have been establishing a suitable method for sterilization of the alloxan in a dry state without loss of its diabetogenic potency. The instability of alloxan in solution has motivated us to devise means for delivering pre-sterilized alloxan into the isolator and to put it into solution just prior to use. Since the diabetogenic dose of alloxan may vary with the strain of mouse studied (Martinez, C. et al. Proc. Soc. Exp. Biol. Med. 87:236, 1954), we are determining a suitable dosage for conventional ICR mice. The effect of pre-fasting, sex and route of administration (s.c. or i.p.) are being evaluated for their effect on mouse sensitivity to alloxan. Alloxan, apart from its action on the beta cells of the pancreas, is known to damage kidney and liver. For this reason, the dosage we seek to use is one that avoids or minimizes such damage. The clinical course, survival time, and persistence of the hyperglycemia after alloxan are being followed. An ultra-micro method for determination of plasma glucose has been standardized (glucose-oxidase method). Suitable glucose tolerance tests are being devised. Sampling of blood via the mouse's infra-orbital ophthalmic venous plexus with special sterilizable, pre-heparinized glass collecting tubes has proven a safe, reliable, and simple procedure with practice; there is little blood loss as hemostasis is easily achieved by gentle compression of the eye. A method for determining the plasma ketoacids is being set up. Total liver lipid is being measured and histopathologic studies are being conducted on the pancreas, liver and kidneys. The newly devised "animal-ejector-sleeves" (see elsewhere in report) should prove invaluable for prompt removal of serial blood samples for glucose tolerance tests performed within isolators.

Over 100 conventional ICR mice have been used in the aforementioned preparatory studies. Of the various dosages tested thus far, 200 mg/Kg B.W. of alloxan monohydrate (non-sterilized) in 0.9% sterile saline (2.5% solution) given s.c. to 24-hour pre-fasted mice was most successful; higher doses of 250-350 mg/Kg B.W., s.c., killed all mice in 72 hours. Of 10 conventional male ICR mice, 4-5 weeks old, given s.c. 200 mg/Kg B.W., 3 died within the first week, the remaining 7 survived for 6 weeks after injection but died shortly thereafter; the saline controls all survived. Lower doses of alloxan are now being tried.

Actions of Tetracyclines - The reversible accumulation of fat in the liver during high dosage or prolonged administration of the tetracyclines is "an interesting but unexplained metabolic effect" (Shils, M.E., Clin. Pharmacol. & Therap. 3:321, 1962). It has been observed in man, dog, rat and mouse. In view of the question of bacterial involvement in certain forms of experimental dietary cirrhosis and its reported delay or prevention by antibiotics as well as the seemingly contradictory observations of exacerbated symptoms of dietary cirrhosis in germfree rats, we are studying the various tetracyclines given in doses we establish to be ordinarily effective in inducing fatty changes in the livers of conventional rodents and intend to contrast these and other effects observed in the latter with those obtained in germfree and control-contaminated

germfree animals. We hope to discern, thereby, whether the fatty infiltration of the liver and other responses to antibiotics are due to their non-antibacterial (pharmacologic) or antibacterial (including coincidental involvement of bacterial products) activities or to both of these activities combined.

In these studies, we are determining and comparing: the nature (composition) of the fat accumulated by the "post-tetracycline" liver, which has, to our knowledge, not been examined; the alteration of food and water intake; the gross and histologic changes of liver, intestine and other tissues; and the body distribution and appearance and disappearance of the tetracyclines by their fluorescent property (liver, skeleton, brain, kidneys, spleen, intestinal lumen and lymph nodes). In view of our interest in the shock of burns and other forms of injury and in irradiation, we also intend to examine some of the other metabolic effects of the tetracyclines, such as their "anti-anabolic" (catabolic?) actions (increased nitrogen and riboflavin loss) which attend their increased toxicity when renal function is impaired, as may happen following injury and shock.

Preliminary to study of tetracycline activity in germfree and control-contaminated ICR mice, we have been standardizing dosages, establishing baseline values and making other incidental observations in conventional ICR mice. We have also initiated preliminary study of the possible salutary effects of cold exposure on tetracycline-induced fatty liver. This idea stems from the fact that: 1) liver extract, crude vitamin B complex, all the purified B vitamins tried singly, DL-methionine with or without choline, BAL, penicillin, heparin and magnesium sulfate have failed to alter a chlortetracycline-induced increase in liver fat in rats (Seto, J.T., et al. Antibiotics & Chemother. 4:666, 1954); and 2) because fatty livers induced in rats by adjustment of the dietary levels of protein and neutral fat are prevented (Treadwell, C.R. et al. J. Nutrition, 63:611, 1957) and even cured (Treadwell, C.R. et al. Proc. Soc. Exp. Biol. Med. 97:434, 1958) by exposure to a cold environment. To our knowledge, the effect of environmental cold on tetracycline-induced fatty liver has not been evaluated. Our previous extensive experience with cold exposure of mice is valuable in doing these experiments (Einheber, A. and Wren, R.E., Ann. Prog. Rept. Project 6X99-26-001, Task 02, 1 Jul 61 - 30 Jun 62). It has been suggested that "tetracycline may be the drug of choice..." in the event of infection in accidental hypothermia (Jones, J.H. et al. J. Path. Bact. 84:433, 1962). This suggestion stems from the results of experiments on penicillin therapy of experimental staphylococcal septicemia in mice exposed to cold. The investigators found ordinarily effective doses of penicillin to be ineffective in such mice and consider the lessened effectiveness to be possibly due to the reduced temperatures of the cold-exposed infected mice. They note that this is true in vitro, as shown by the lessening of effectiveness of penicillin against staphylococci at low incubation temperatures while, on the contrary,

under these conditions, tetracycline and Oxytetracycline are effective against S. aureus and S. viridans, respectively (Jones, J.H. et al. ibid. 1962). Consequently, we are also interested in noting any untoward effects of tetracycline treatment in mice exposed to cold, even though untreated, mice allowed food and water ad libitum under our conditions maintain colonic temperature in the normothermic range when kept at the low ambient temperature (0-4°C.) we employ.

Thus far, only Oxytetracycline and Tetracycline have been used. Chlortetracycline, the most potent fatty-liver-inducing tetracycline, has been unavailable and is to be used in future experiments. Preliminary trials of Tetracycline failed to induce gross evidence of fatty liver in mice with the dosage regimens thus far employed. However, Oxytetracycline, as will be described, has for the most part been found effective in this regard.

The following pertains to each of three experiments to be described in which a total of over 200 conventional male and female ICR mice, 6-7 weeks of age, were used. Injections were given intraperitoneally. Oxytetracycline (OTC) (Pfizer) containing ascorbic acid was dissolved in 0.9% sterile saline, solution pH 1.9, and was administered at a dose of 100 mg/Kg (including contained ascorbic acid 400 mg/Kg). Ascorbic acid (AA) was similarly dissolved, solution pH 2.0, and given at a dose of 400 mg/Kg. Fresh solutions were made daily. With repeated injections, dosage was calculated on the basis of daily body weight. The volume of injection was 10 ml/Kg. Body weights were obtained and food and water intake measured daily. Animals were killed by exposure to carbon dioxide on the day following the last injection. The liver and gastrointestinal tract were examined grossly. In some experiments, a standard portion of liver was saved for total fat analysis (gravimetric method) and for water content (fresh weight minus dry weight) and the remainder fixed in 10% buffered formalin and stained with Sudan III for fat. Other livers were fixed, in toto in 10% buffered formalin as were entire remaining mouse carcasses. Mice were maintained on an alternating 12-hour light-dark cycle. The data assembled to date are presented below.

Experiment No. 1 -- Male mice were divided into 2 groups and treated as follows: Oxytetracycline (OTC) or ascorbic acid (AA) given daily for 1 to 8 days and maintained at room temperature throughout the experiment. The OTC group ate and drank significantly less than the AA group. The OTC and AA lost weight during treatment but the OTC consistently lost more weight than the AA. None of the AA, irrespective of number of injections, showed either liver or GI abnormality; only after 2 injections did OTC show an incidence of pale (fatty) livers, the incidence increasing with the number of injections. The same pattern held true for the incidence and severity of GI distention and bile staining.

Thus far, histologic examination has been done on liver (fat infiltration graded 0 to 4+) from AA and OTC receiving 5 or 8 injections and total liver fat and water content done only after 8 injections. The data in the following table suggest that OTC have more fat and water in their livers than AA.

Group	No. of Inject.	No. Mice	Histol. Grading of Liver Fat*	% Wet Weight Total Liver Fat	% Wet Weight Liver Water Content
AA	5	3	1.5+ (1+ to 2+)	-	-
OTC	5**	3	3.0+ (2+ to 4+)	-	-
AA	8	3	1.1+ (0.5+ to 2+)	4.8 (2.9 to 6.2)	68.2 (66.8 to 69.4)
OTC	8**	3	2.3+ (0.5+ to 4+)	7.7 (7.4 to 8.0)	71.6 (71.6 to 71.6)

* Graded on the basis of comparison of fat found in cytoplasm of liver cells.

** Only one mouse from each of these groups showed true fatty metamorphosis.

Experiment No. 2 -- Male mice were divided into 3 groups and given daily injections for 5 days as follows: Oxytetracycline and kept at room temperature (OTC-RT) or in the cold (OTC-C), or ascorbic acid and kept in the cold (AA-C). The AA-C had consistently higher food and water intakes than the OTC-RT and OTC-C. The OTC-RT and OTC-C groups showed grossly fatty livers, increased total liver fat, increased water content and gastrointestinal distention. The AA group did not manifest liver or gastrointestinal abnormalities after exposure to cold.

Group	No. Pos. Find./No. Obs. Liver*	G-I*	% Wet Weight Total Liver Fat	% Wet Weight Liver Water Content
OTC-RT	3/5	5/5	5.5	69.2
OTC-C	2/3	3/3	5.1	69.9
AA-C	0/5	0/5	4.4	68.0

* Gross positive findings: pale, yellowish livers assumed to be fatty; G-I distention and bile staining.

Experiment No. 3 -- Male and female mice were each initially divided into 3 groups as follows: Oxytetracycline (OTC) or ascorbic acid (AA) given daily for 5 days; untreated (non-injected) mice (U). All mice were observed at room temperature and observed for food and water intake and body weight changes before treatment was begun, and daily during the post-treatment period. At the beginning of treatment, the groups were again divided, one-half continued at room temperature and the other half subjected to cold for the duration of the experiment.

Food and water intake -- At room temperature, the only deviation from pre-treatment levels was found in the OTC males which decreased their food and water intakes. All other mice maintained their normal food and water intakes.

In the cold, males and females, in each group, showed the same corresponding trends: viz., OTC manifested unchanged food intakes and reduced water intakes; AA showed increased food and decreased water intakes; and U showed increased food and unchanged water intakes. The increment of food intake by the male and female U was greater than that of the AA.

The expected effect of cold-exposure, viz., increased food intake (AA and U males and females) and the expected effect of Oxytetracycline, viz., decreased food intake (OTC males, room temp.) were mutually antagonistic in the OTC cold-exposed males. This is to say, the opposing effects on food intake of cold and Oxytetracycline were both operant in the cold-exposed OTC males, the net result being that their food intake was unchanged from the pre-treatment room temperature level. The paucity of data do not permit judgement as to whether or not either opposing effect may, in fact, have dominated to any extent.

In the cold, AA showed a decrease in water intake and U no change. The decrease in water intake by the cold-exposed OTC males and females was, however, much greater than that of the cold-exposed AA and, therefore, not fully accounted for by the ascorbic acid, pH or the "injection procedure" factors per se, i.e., is seemingly due in large part, or in addition, to the Oxytetracycline; this conclusion is further supported by the reduced water intake of OTC males at room temperature but not that of AA males or females. Why the room temperature OTC females did not manifest a decreased food and water intake as did the corresponding OTC males is not known, nor is it clear why the AA males and females reduced their water intake in the cold but not at room temperature. We are attempting to corroborate these trends.

Body weight -- OTC males and females demonstrated a modest weight loss in the cold and at room temperature; in the cold, OTC males showed a greater weight loss than OTC females. Whereas OTC males showed comparable weight losses at room temperature and in the cold, OTC females showed a greater weight loss at room temperature. The U males in the cold and at room temperature manifested comparable increases in body weight which were somewhat larger than that of the U females at room

temperature; the U females showed no change in the cold. Similar increases in body weight were exhibited by AA males and females at both temperatures.

Gross liver and GI findings -- Combining the data for the males and females, OTC demonstrated fewer pale, presumably fatty, livers during cold exposure (3/9) than at room temperature (6/9). The incidence of gastrointestinal abnormality was the same (8/9) for OTC in the cold and at room temperature. AA and U at room temperature displayed no gross liver or GI abnormalities; in the cold, this was true as regards the liver but here the AA (5/10) and the U (1/10) displayed some gross gastrointestinal abnormality.

Group	No. of Pos. Findings*/No. of Observations					
	Males		Females		Male & Female	
	Liver	GI	Liver	GI	Liver	GI
			ROOM TEMPERATURE			
OTC	2/5	5/5	4/4	3/4	6/9	8/9
AA	0/5	0/5	0/5	0/5	0/10	0/10
U	0/5	0/5	0/5	0/5	0/10	0/10
			COLD			
OTC	1/4	4/4	2/5	4/5	3/9	8/9
AA	0/5	3/5	0/5	2/5	0/10	5/10
U	0/5	1/5	0/5	0/5	0/10	1/10

* Positive findings: Pale, yellowish livers assumed to be fatty; GI distention and bile staining.

What the altered gross gastrointestinal picture signifies can only be speculated upon at present. Whether or not the OTC-induced GI alterations at room temperature are identical with or result from the same mechanisms as those in the cold is questioned because ascorbic acid per se (i.p. injection, pH 2.0) caused no GI abnormality at room temperature but apparently did so in the cold.

Summation of the results for the OTC males and females, irrespective of subjection to room temperature or cold during treatment, suggests that the females suffered an over-all greater incidence of gross liver abnormality (6/9) than the males (3/9).

We are currently exploring the possibility of establishing temperature and humidity control for the germfree isolators which would permit studies of extremes of ambient temperatures.

Actions of Penicillin in Guinea Pigs - Conventional guinea pigs die after the oral or parenteral administration of penicillin. Two possible explanations for this phenomenon have been presented: 1) acute toxicity of penicillin and 2) the alteration of the intestinal flora by penicillin which causes an enterocolitis in the animals resulting in death. Therefore, the effect of penicillin in germfree and conventional guinea pigs was studied. We found that germfree guinea pigs are resistant to penicillin which suggests that acute toxicity is not the answer. However, when we contaminated germfree animals with an organism which might be suspected to have caused enterocolitis in a penicillin-treated conventional animal, we did not bring about a colitis. Further study of this phenomenom is in progress. For details, see Annual Progress Report, Project 3A 0 12501 B 813, Task 02, Microbiology (Pathogenesis and immunity in enteric diseases).

Radiation and Radioprotectants - It has been demonstrated that germfree mice and chickens as compared with their corresponding conventional counterparts are significantly more resistant to lethal and supralethal whole body x-irradiation. Continuing studies with germfree and purposely contaminated germfree mice are helping to elucidate the overt and covert roles of microorganisms in post-irradiation morbidity and mortality. A variety of studies have been performed this past year involving 256 mice, including: germfree, E. coli monocontaminated, and conventional ND II mice, and germfree ICR mice. Two previously incompleted studies were concluded: 1) Thirty-day survival of ND II germfree mice subjected to x-ray and 2) survival studies of such animals subjected to supralethal radiation (25,000 r). The greater survival of germfree ICR mice after radiation bears out the findings with germfree ND II mice. A new series of studies has begun on the ability of radioprotective drugs to enhance the tolerance of mice to radiation. Thus far, two drugs have been studied in germfree ICR mice. These drugs were found to produce complete protection against x-ray doses which are 100% lethal (9-12 days) in both conventional and germfree mice. Thyroid function of the germfree and conventional animals measured by I^{131} uptake and retention are in progress. For details, see Annual Progress Report, RD 40-61 Biomedical (NWER) (DASA), WEB No. 03.074, Biological effects at cellular, organ, or total organism level.

Tissue Transplantation: Skin Graft - Despite the continued and extensive study of homograft failure, the discovery of its precise cause(s) and means for its permanent prevention still seem goals for the distant future. A complicating feature in the study of the "homoplasty enigma" is infection of the graft, either in a previously "healthy" site or in the more complex burn wound. A phenomenon of interest to us is the still unexplained longer survival of homografts when transplanted to debilitated patients suffering from uremia or extensive burns. The non-specific effects of stress, chiefly elevated corticosteroid levels, and the general anti-anabolic and/or catabolic status of the patients, have been proposed as tentative explanations for this phenomenon. Noteworthy, from our standpoint, is the fact that while such debilitated patients exhibit longer survival of homografts, they are also more prone to infection. The latter serves to demonstrate the

palpably intimate relationship between the homograft rejection and the bacterial "rejection" (defense) mechanisms of the host.

For these reasons and because the germfree animal has a relatively immature, unstimulated lymphatic and immune system, we are attempting to perform skin transplants within germfree isolators which, when perfected, will allow us to make comparative analyses of homograft rejection, initially, in hybrid germfree and conventional mice (ICR strain). Therefore, we are testing the practicability of a non-suture skin grafting technique in conventional ICR mice. Such a technique should greatly facilitate and expedite this operation within isolators. The method we are exploring entails the use of a skin graft punch of the Waldemar type (Weck) with 1.37 cm cutter, and the preliminary circumferential taping of the depilated trunk of the mouse. Thus, on raising the loose dorsal skin with superficially adherent tape (autoclavable), a fold of skin results between the fingers. The skin punch is worked on this fold and 2 identical-sized discs, backed with tape, are formed. These can be lifted intact and precisely transplanted into corresponding discoid beds of identical dimension that are framed in tape. The latter beds are bared on removal of the skin discs. The other procedural details and advantages of this method for conventional laboratory use have been described (Gottfried, B. Transpl. Bull. 6:427, 1959).

In conjunction with the latter studies, we have been attempting to develop a suitable neonatal thymectomy procedure for germfree mice. Neonatal thymectomy of conventional mice has been found to severely interfere with the development of the capacity to reject skin or tumor homografts. What interests us most, at present, is the finding that thymectomy of the conventional newborn mouse leads to a "runting" syndrome (failure to grow, wasting, diarrhea) and death during the second month of life, and that "these animals often died apparently of intercurrent infections" (Dalmasso, A.P. et al. Proc. Soc. Exp. Biol. Med. 110:205, 1962). In view of this observation, the question we would like to pose experimentally is whether neonatally thymectomized germfree mice would die after the fashion of their conventional counterparts. If they do not die, then apparently a bacterial factor rather than an unknown auto-immune-like reaction is involved. Furthermore, the neonatally thymectomized adult germfree animal would be a most unusual animal to study, and unattainable under conventional circumstances.

The Lymphatic System: Morphologic and Humoral Response to Bacterial Antigen and Radiation - The microscopic, histochemical and immunocytochemical structure of the reticuloendothelial system of germfree, E. coli-monocontaminated, and conventional mice were studied. Particular emphasis was given to the alterations in morphology and function resulting from sublethal whole body x-irradiation and bacterial antigen.

Despite a deficiency in immunologically competent cells, in antibody, and in gamma globulin, the germfree mouse when stimulated adequately, shows morphologic and humoral responses which are qualitatively the same as those of the conventional mouse.

Uptake of bacterial antigen and the ensuing antibody response are essentially the same for germfree and conventional mice. After uptake of locally-injected antigen by the lymph nodes, the antigen may possibly be more slowly disintegrated by the germfree macrophages.

Subletnal x-irradiation of germfree and conventional mice results in an identical lymphatic tissue response as reflected by the same plasma cell reaction and rise in serum gamma globulin. For details, see The Mt. Sinai Hosp. Annual Report, 1 May 62 - 30 Apr 63, Grant No. DA-MD-49-193-61-G24.

Mucosal Enzymes and Autonomic Nervous System of the Rodent Cecum - As is well known, the cecum of the germfree rodent, relative to that of its conventional counterpart, is abnormally enlarged. The microscopic morphology of the cecal mucosa of the germfree guinea pig is likewise different. However, there is still no explanation for the larger cecum of the germfree rodent. In mammals, the cecum is the most poorly innervated area in the gastrointestinal tract. The germfree animal with its enlarged cecum might, therefore, prove an excellent model for studying the growth of the vegetative nervous system, be it cause or consequence of the specific organ enlargement.

The topographic distribution of the following enzymes was compared histochemically in the cecal mucosa of conventional and germfree mice: Alkaline and acid phosphatases, DPNH diaphorase and monamine oxidase. The germfree mouse cecum has more alkaline phosphatase activity than that of the conventional mouse; however, the latter animal's cecum has more acid phosphatase. DPNH diaphorase and monamine oxidase activity of the cecum is apparently no different for the two groups. Alkaline phosphatase may be concerned with the absorption of nutrients from the intestine, and the acid phosphatase may have a defensive function. In the small intestine, the difference in enzyme activity between the two groups are less; only the acid phosphatase appears weaker in the germfree.

Five conventional and 5 germfree isolator-reared and maintained adult rats have been studied with the methylene blue technic of Schabadash to examine the cecal innervation. Normal baseline values with this technique were previously established in open-colony rats and guinea pigs. The results, thus far, suggest that there has been no enlargement of the vegetative network concomitant with that of the organ itself. For details, see Ann. Prog. Rept., Project 3A 0 12501 B 813, Task 09, Physiology, (Gastrointestinal physiology).

Mucosal Morphology and Cell Renewal of the Rodent Ileum - Germfree and conventional mice were given tritiated thymidine i.p. and sacrificed 1, 10, 24, and 48 hours after injection. The terminal ileum was studied histologically and radioautographically.

The conventional mouse as compared with the germfree mouse has: a bulkier and more cellular lamina propria, a greater mitotic rate in the intestinal crypts, and twice the turnover of ileal epithelium.

The lamina propria and intestinal lymphatic tissue of the germfree mice, respectively, contained virtually no reticuloendothelial cells, and virtually no plasma cells or their precursors. For details, see Ann. Prog. Rept., Project 3A 0 12501 B 813, Task 09, Physiology (Gastrointestinal physiology); see The Mt. Sinai Hosp. Annual Report, 1 May 62 - 30 Apr 63, Grant No. DA-MD-49-193-61-G24.

Response of the Guinea Pig Ileum In Vitro to Pharmacologic Agents - The intestinal tract of the germfree and conventional animal is different as regards microscopic morphology, histochemistry and content of the smooth-muscle active amines, histamine and serotonin (see elsewhere in report). We, therefore, deemed it of interest to compare the responses of germfree and conventional guinea pig ileum when challenged in vitro with selected pharmacologic agents, viz., histamine, acetylcholine and atropine. The lack of microbial stimulation of the germfree animal's intestine might affect physiologic differences which might in turn be revealed by the response of the isolated ileum to challange with these agents.

Seven germfree and seven conventional guinea pigs of the same age, and eating the same diet, were paired for sex and weight, time of day and germfree or conventional state. Sixty minutes after removal from the isolators, the animals were killed by a single blow to the back of the neck. Cecal contents were collected under aseptic conditions; cultures of specimens from germfree guinea pigs were uniformly sterile. The isolated ileum was studied under standardized conditions using an established method of assay (Code and McIntire Methods of Biochemical Analysis, vol. III, ed. D., Glick, N.Y., Interscience Publishers, Inc., 1956, pp. 49-98).

No differences were found in the guinea pig ileum responses to histamine, acetylcholine or atropine that could be related to the germfree state. An unusual degree of baseline stability was noted for the germfree gut in one series of experiments that may be significant, but it could not be quantitated by the methods used in these studies. A significant difference was observed in the response to the higher dose of acetylcholine considering contraction above the primary baseline; however, this difference disappeared when adjusted for dynamic relaxation tone.

Development of Clinical Isolators - During the past year, the development of plastic isolators for clinical application of germfree techniques has been continued conjointly (Patient Isolator) with the Dept. of Nursing, WRAIR.

The surgical isolator project was recently transferred to the Albert Einstein College of Medicine, New York.

Surgical isolator -- Clinical trials have demonstrated that it is functionally possible to perform human surgery in the surgical isolator; however, further investigation is necessary before definitive conclusions can be made regarding efficacy and practicability of this system. Areas requiring further investigation, modification, or both, include: 1) finding suitable materials which will eliminate explosive hazards and not limit the choice of anesthesia to non-explosive agents; 2) finding suitable materials which can be sterilized by heat rather than by gaseous or chemical agents; 3) procedures for aseptic glove change; 4) bacteriological testing; and 5) equipment re-design to provide: clear, wrinkle-proof, vision panels for personnel outside the enclosure; relatively noiseless air supply motors; larger incision panels; more space for orderly arrangement of surgical equipment within the isolator; unobstructed light into the operative field; form fitting cuffs; and rapid, aseptic, initial and subsequent entry of sterile surgical supplies.

Patient isolator -- Clinical trials were conducted after numerous modifications were made in the equipment and procedures for care. These trials were initiated under the new subtask to test feasibility of providing nursing care to patients in a closed environment.

The investigators found that the practice of nursing care became feasible after modifications in the procedures and equipment were made. Further investigation is necessary to meet the other objectives of the project proposal.

Because of the ever-increasing clinical orientation of the patient isolator development program, it is being transferred to the Dept. of Nursing, WRAIR. For details of this report, see Ann. Prog. Rept. Project No. 3A 0 12501 A 803, Task 01, Internal Medicine (Nursing care of patients confined within isolator systems).

SUMMARY AND CONCLUSIONS:

Study phases, described herein, are in preparation, in progress, or are completed with the following objectives: to advance the animal, nutrition and technology of germfree research; to study and compare the constitution (anatomy and biochemistry) and function (behavior, physiology and metabolism) of germfree, control-contaminated germfree, and conventional(ized) animals, when "normal" or after their challenge with physical, chemical or viable noxae; and to learn, regarding the latter, both the possible role of the environmental microorganisms (and their products) and the effect of our modification and/or control of these microorganisms.

LIST OF PUBLICATIONS:

Abrams, G.D., Bauer, H. and Sprinz, H.: Influence of the Normal Flora on Mucosal Morphology and Cellular Renewal in the Ileum. A Comparison of Germfree and Conventional Mice. Lab. Invest. 12:355, 1963.

Bauer, H., Horowitz, R.E., and Levenson, S.M.: Quantitative Morphology of the Lymphatic System in Germfree Animals. Fed. Proc. 21:280, 1962.

Bauer, H., Horowitz, R.E., Levenson, S.M., and Popper, H.: The Response of the Lymphatic Tissue to the Microbial Flora. Studies in Germfree Mice. Am. J. Pathol. 42:471, 1963.

Bauer, H., Horowitz, R.E. and Popper, H.: Response of the Germfree Mouse to Bacterial Antigen. Fed. Proc. 22:374, 1963. (Presented at 1963 Annual Meeting, Federation of American Societies for Experimental Biology, 17 April 1963, Atlantic City, N.J.).

Bauer, H., Horowitz, R.E., Paronetto, F., Abrams, G.D., Watkins, K.C., Levenson, S.M. and Popper, H.: Immunologic Competence and Phagocytosis in Germfree Animals with and without Stress. (To be presented at the 1963 Annual Meeting of the American Medical Association, 20 June 1963, Atlantic City, N.J.)

Formal, S., Abrams, G.D., Schneider, H.S., and Laundy, R.: Penicillin in Germfree Guinea Pigs. Nature (in press).

Horowitz, R.E., and Bauer, H.: Gamma Globulin in the Lymphatic Tissue of the Germfree Mouse. Lab. Invest. 11:693, 1962.

Horowitz, R.E., Bauer, H., Paronetto, F., Watkins, K.C., and Popper, H.: The Effect of the Microflora on Lymphatic Tissue Reaction Following Whole Body Irradiation. Studies in Germfree Mice. (Presented at the 1963 Annual Meeting of the American Society of Pathologists and Bacteriologists, 25 April 1963, Cincinnati, Ohio)

Levenson, S.M., Trexler, P.C., Malm, O.J., LaConte, M.L., Horowitz, R.E. and Moncrief, W.H. Jr.: A Plastic Isolator for Operating in a Sterile Environment. Am. J. Surg. 104:891, 1962.

Levenson, S.M., Einheber, A. and Malm, O.J.: Metabolic Aspects of Shock. J. Am. Med. Ass. 181:874, 1962.

Levenson, S.M., Tennant, B., Geever, E., Laundy, R. and Daft, F.: Influence of Microorganisms on Scurvy. Arch. Int. Med. 110:693, 1962.

Levenson, S.M. and Tennant, B.: Some Metabolic and Nutritional Studies with Germfree Animals. Fed. Proc. 22:109, 1963.

ANNUAL PROGRESS REPORT

Project No. 3A-O-12501-A-811, MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

(Arthropod-borne infections)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Division of Communicable Disease & Immunology

Special Activity: U.S. Army - SEATO Medical Research Laboratory
APO 146, San Francisco, California

Department of Entomology

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A-O-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA (Arthropod-borne infections)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Large collections of mosquitoes were made in Bangkok and in southern Thailand in connection with investigations of haemorrhagic fever. Dengue strains and chikungunya have been isolated from Aedes aegypti and chikungunya from Culex pipiens quinquefasciatus. Colonies of the aforementioned mosquitoes were established and maintained. A field program to examine the incidence of rickettsioses in Thailand was initiated. Studies on malaria, with particular reference to Anopheles balabacensis, were undertaken.

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BODY OF REPORT

Project No. 3A-0-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

(Arthropod-borne infections)

Description:

These studies are designed to elucidate the relationship of man, the arthropod vectors and the arthropod-borne disease organisms, as they exist in Southeast Asia. Principal attention has been directed to those diseases which are of potential importance to military forces operating under various conditions of terrain and climate in Thailand. The organisms of main interest have been the arthropod-borne viruses, the rickettsiae and the plasmodia. Most of the studies comprising the investigations have been conducted in the field, to explore the ecological relationships. These field studies have been supported by laboratory investigations of some aspects of arthropod biology. The field studies have, in turn, supplied the material from which isolations of disease organisms have been attempted.

Progress:

1. Arthropod-borne viruses in the Bangkok area (SEATO MEDIC Study #40)

a. From July 1962 to January 1963 a year long cooperative study with the Virus Department was continued in five study areas in the city of Bangkok. These studies were concerned chiefly with the role of mosquitoes in the transmission of hemmorhagic fever. Over sixty species of mosquitoes were collected during this period, a reflection of the diversity of the mosquito fauna of tropical regions and the hightly variable nature of the Bangkok-Dhonburi city complex. By the end of the study year in January 227,863 male and 207,099 female mosquitoes had been taken in the following types of collections: human biting (day and night), animal biting, net traps, resting collections in homes, and light traps. Larval collections were made in some cases to confirm the identification of species. Over 41,000 females from these collections, representing seven of the most important species, were frozen for virus isolation (Table I).

TABLE I

Bangkok mosquitoes preserved for virus isolation July 1962-June 1963

Species	Females preserved
Culex pipiens quinquefasciatus	37,151
Aedes aegypti	4,369
Mansonia uniformis	114

TABLE I (cont.)

Species	Females preserved
Culex tritaeniorhynchus	84
Culex gelidus	32
Mansonia annulifera	8
Anopheles subpictus malayensis	5
Total	41,763

This total does not include specimens collected in a few special studies of mosquitoes attacking horses and cattle. In these, Culex gelidus and C. tritaeniorhynchus were by far the predominant species preserved for virus isolation. The figures given above refer only to the mosquitoes collected in the special virus area study.

b. The most important species, in terms of their attack on man and their house frequenting habits were Culex pipiens quinquefasciatus and Aedes aegypti. Both of these species are present throughout the year in the Bangkok area, the numbers at any time of the year depending chiefly on the available water sources. Culex pipiens quinquefasciatus is by far the most abundant species in the city, being found in all areas from the most urban center of the city to the most rural fringes. It has been found breeding in almost every conceivable type of natural and artificial water container in the city, but it is particularly abundant in the foul water which collects in the street drains and under houses. Aedes aegypti, on the other hand is found in relatively clean water only, especially in the water stored in large jugs around homes in the city which do not have adequate supplies of piped water. Aedes aegypti attack man in Bangkok chiefly during the day and early evening and indoors; while Culex quinquefasciatus is chiefly a nighttime biter, with peaks of biting activity not long after dark, and again before dawn. Almost all complaints of human biting in Bangkok are referable to the latter species. Other Culex species, C. tritaeniorhynchus and C. gelidus form a much smaller part of the mosquito population feeding on man. The anopheline population of Bangkok is relatively low, only Anopheles vagus being present in significant numbers in the city proper. It is capable of breeding in rather foul water. Other species of possible medical importance in Bangkok because of their human biting habits include: Armigeres subalbatus, Mansonia annulifera, M. indiana and M. uniformis. A few specimens of the Anopheles "hyrcanus' and A. 'barbirostris' groups have been taken in biting collections on the fringes of the city, but never in significant numbers. Members of some genera, such as Uranotaenia and Filcalbia do not feed on man, and thus are of no known medical significance. A list of all species collected from July 1962 through the end of the study in January 1963 is presented in Table II. It is worth reiterating that the population and biting data indicate that over 80%, and at some times of the year over 90%, of the mosquitoes likely to bite man in Bangkok are Culex pipiens quinquefasciatus and Aedes aegypti. Exposure to the former is likely to occur during the

hours of darkness, outdoors as well as indoors. The biting activity of Aedes aegypti is largely restricted to indoors, and most activity occurs during daylight hours.

TABLE II

Mosquito species collected in Bangkok July 1962 - June 1963

Aedeomyia catasticta
Aedes aegypti
Aedes albopictus
Aedes dux
Aedes lineatopennis
Aedes mediolineatus
Aedes taeniorhynchoides
Aedes vexans
Aedes w-albus
Anopheles aconitus
Anopheles annularis
Anopheles argyropus
Anopheles barbirostris
Anopheles campestris
Anopheles kochi
Anopheles lesteri
Anopheles nigerrimus
Anopheles peditaeniatus
Anopheles philippinensis
Anopheles sinensis
Anopheles subpictus malayensis
Anopheles subpictus subpictus
Anopheles tessellatus
Anopheles vagus
Anopheles sp. D
Armigeres subalbatus
Culex bitaeniorhynchus
Culex brevipalpis
Culex fuscitarsis
Culex fuscocephalus
Culex fuscanus
Culex gelidus
Culex halifaxii
Culex malayi
Culex mimulus
Culex nigropunctatus
Culex pipiens quinquefasciatus
Culex pseudovishnui
Culex rubithoracis
Culex sinensis
Culex sitiens
Culex tritaeniorhynchus summorosus
Culex whitmorei
Culex sp. 1
Ficalbia chamberlaini
Ficalbia hybrida
Ficalbia luzonensis
Ficalbia minima
Ficalbia sp. 1
Mansonia annulifera
Mansonia crassipes
Mansonia indiana
Mansonia uniformis
Malaya genurostris
Malaya jacobsoni
Toxorhynchites splendens
Uranotaenia atra
Uranotaenia campestris
Uranotaenia edwardsi
Uranotaenia orientalis
Uranotaenia recondita

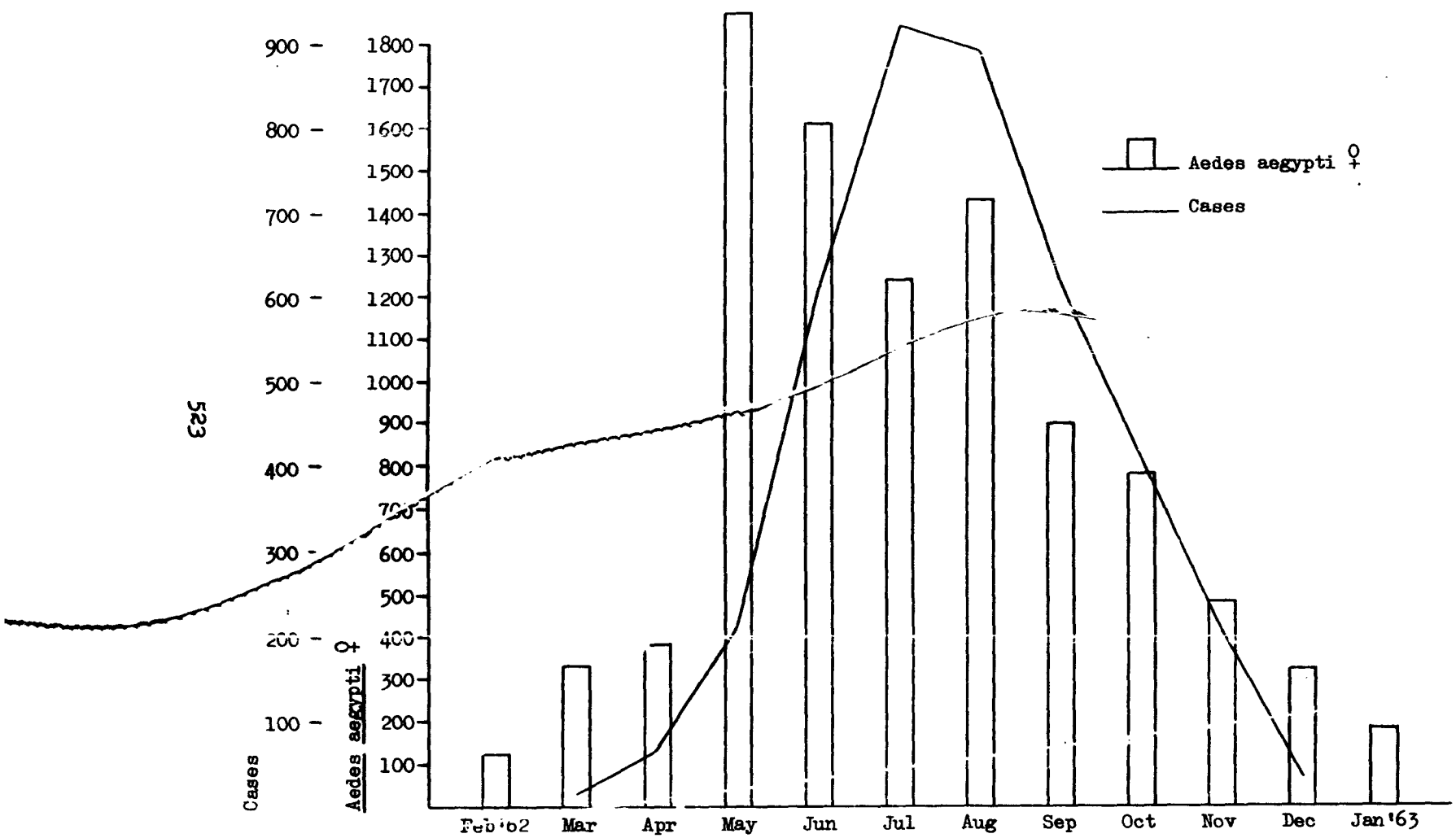
c. A full account of the results of virus isolation attempts utilizing the mosquitoes discussed above will be found in the report of the Virus Department. It should be noted, however, that all isolations confirmed thus far have been from Aedes aegypti and Culex pipiens quinquefasciatus. This may be reflection of the very small numbers of other species examined thus far, but the collections were designed to give as close an approximation as possible of the species which actually attacked man in the city, and it may be anticipated that either of both of these species are the vectors of the viruses which are the etiological agents of hemmorhagic fever, No other species attack man at a rate in Bangkok which could account for the large number of cases in the city. Members of the dengue complex and chikungunya virus were isolated from Aedes aegypti, while chikungunya virus was isolated from Culex quinquefasciatus. Examination of the data by area of collection and made of collection and made of collection is still in progress.

d. On completion of the large area study in January two of the study sites were selected for further examination by human and animal biting and resting collection. One of these is in an area of relatively high income, with a high percentage of foreign residents and adequate piped water supplies. This area was expected to show relatively little virus activity in the main study, but preliminary evidence indicates that virus appeared early in this suburban area, and persisted despite relatively few human cases. The contrasting study area chosen for follow-up study is an area of low income housing in the denser part of the city.

TABLE III

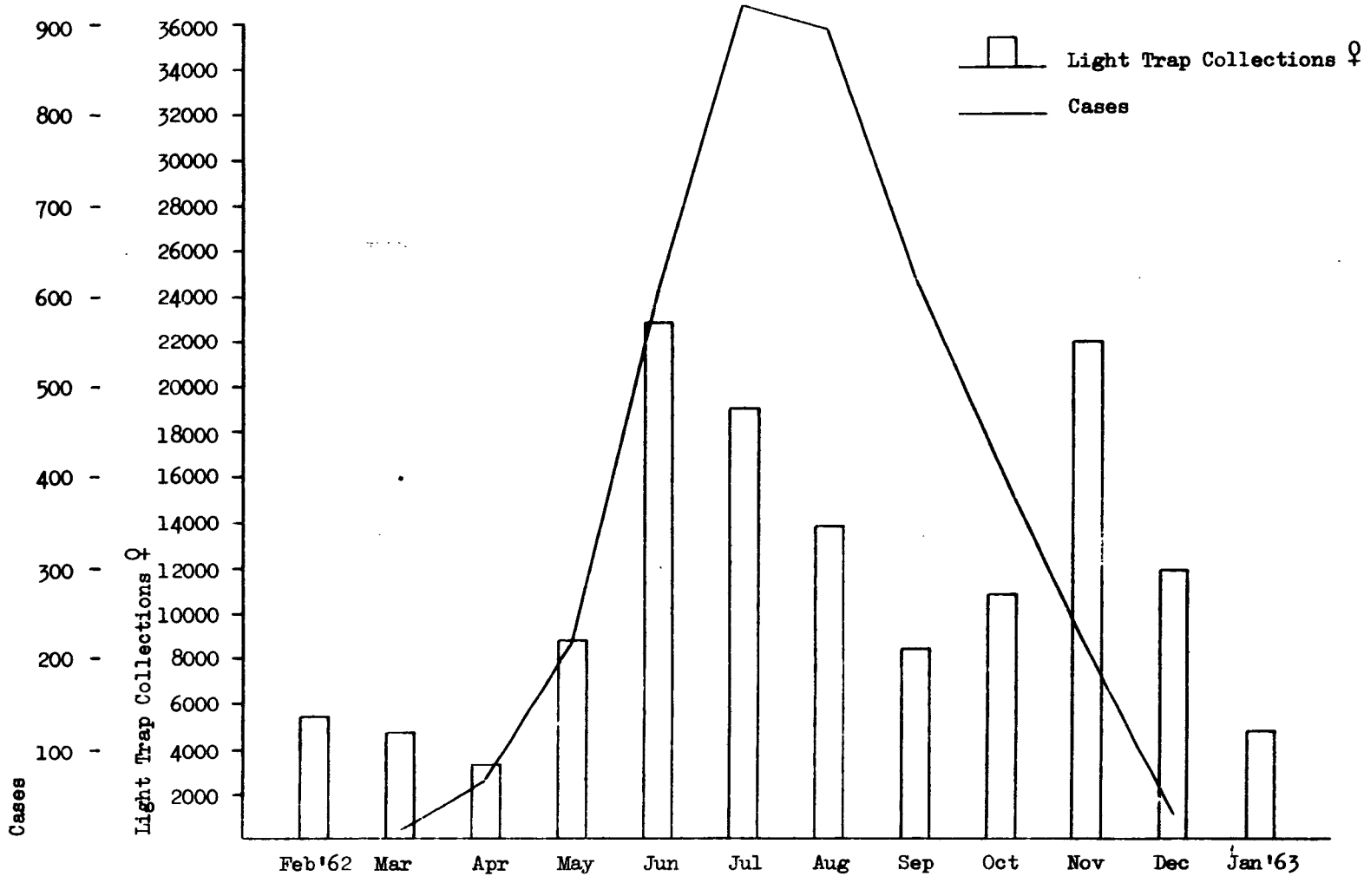
Number of mosquitoes identified per month in Bangkok virus study

Month		Female	Male
July	1962	32,165	31,066
August	1962	36,843	35,857
September	1962	27,626	30,181
October	1962	27,157	29,658
November	1962	38,610	49,157
December	1962	27,071	33,343
January	1963 (end of study)	17,627	18,601
	Totals	207,099	227,863



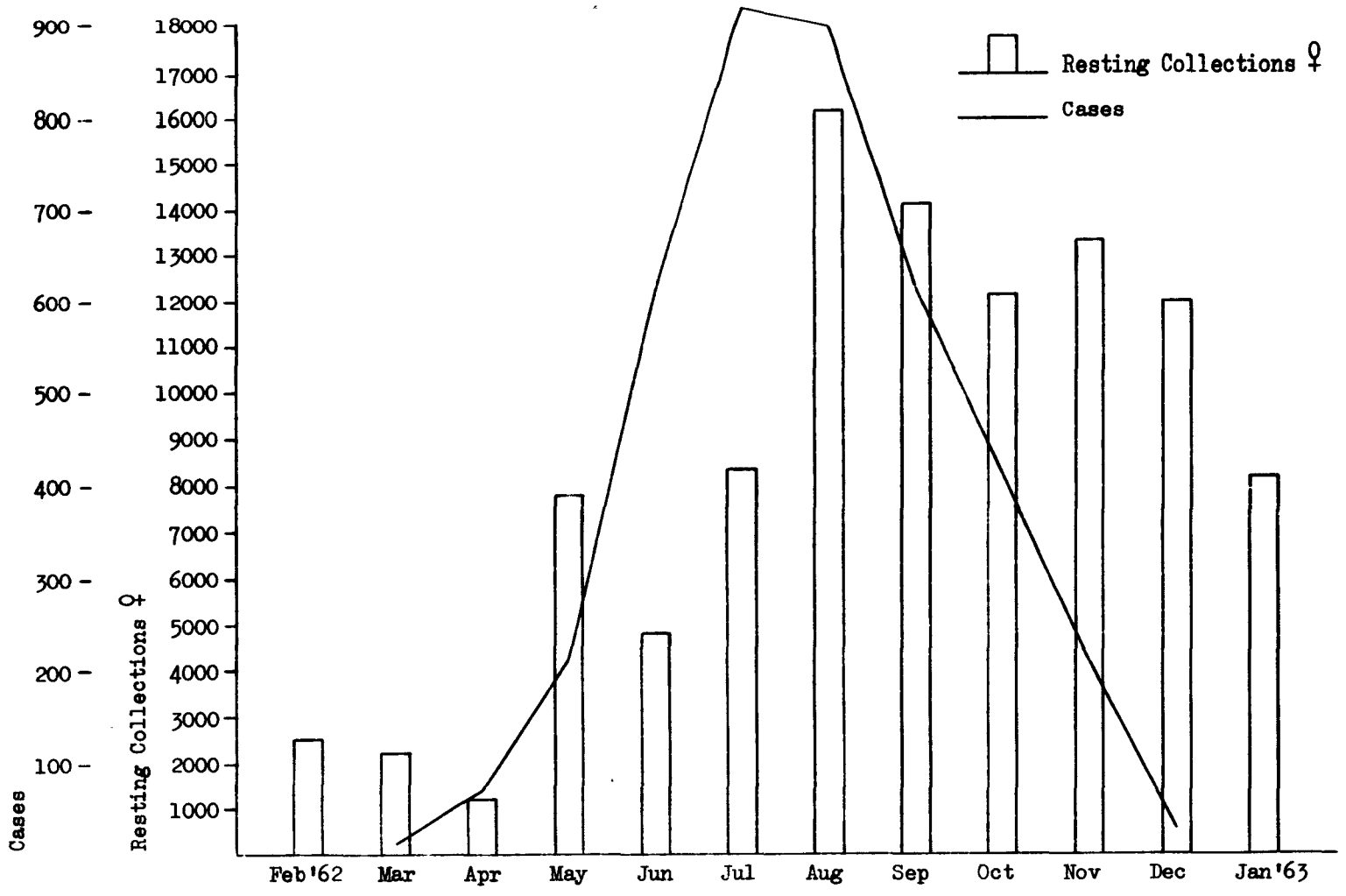
Monthly Collections of Aedes aegypti ♀ and cases of Hemorrhagic Fever

Figure 1



Monthly Light Trap Collections ♀ and Cases of Hemorrhagic Fever

Figure 2



Monthly Resting Collections ♀ and Cases of Hemorrhagic Fever

Figure 3

2. Japanese encephalitis at Bang Phra, Cholburi (SEATO MEDIC Study #41)

a. Mosquitoes were collected several times a week throughout the year at Bang Phra in an attempt to determine the vectors of encephalitis virus among horses on the farm. This facility is maintained by the Thai Red Cross Society to serve as a holding facility for horses used to obtain snake antivenins. In past years horses have died at the farm with symptoms diagnosed as arthropod-borne encephalitis. In addition, evidence of serological conversion to japanese encephalitis or a closely related agent has been obtained. From July 1962 to the end of this reporting period light trap and horse-baited trap collections have been made at weekly intervals. A few larval and human biting collections were also made to determine what species of mosquitoes might be in the area which were not attracted to horses or light. The general trend of the mosquito population as measured by the two types of traps is given in figure 1. The large mosquito population in September-November is related to the greater availability of breeding sites at that time. During the height of the rainy season streams and pools in this sandy coastal area are swept clean of larval concentrations at fairly frequent intervals. During the cool and dry months of January through May there is progressively less and less breeding area available. At the end of the rainy season, however, the water surface situation is fairly stable, and the temperatures at night are high enough to permit considerable mosquito activity.

b. The most abundant species in the bait and light traps were Culex tritaeniorhynchus, C. gelidus and C. fuscocephalus. The percentage composition of the catches of C. tritaeniorhynchus and C. gelidus by month is given in table IV. The markedly lowered percentage for these two species in August and September is due to an increase of several Anopheles species in the collections during those months. The number of some of the Aedes species, such as A. mediolineatus and A. taeniorhynchoides showed several irregular peaks during the year. The viruses isolated at Bang Phra were all isolated during the period of the peak of the mosquito population (October - November). Three were isolated from C. tritaeniorhynchus, one from C. gelidus. A more detailed discussion of these isolations will be found in the report of the Virus Department. Collections through several additional years will be needed to determine the significance of the time of isolation and the peak of the mosquito population.

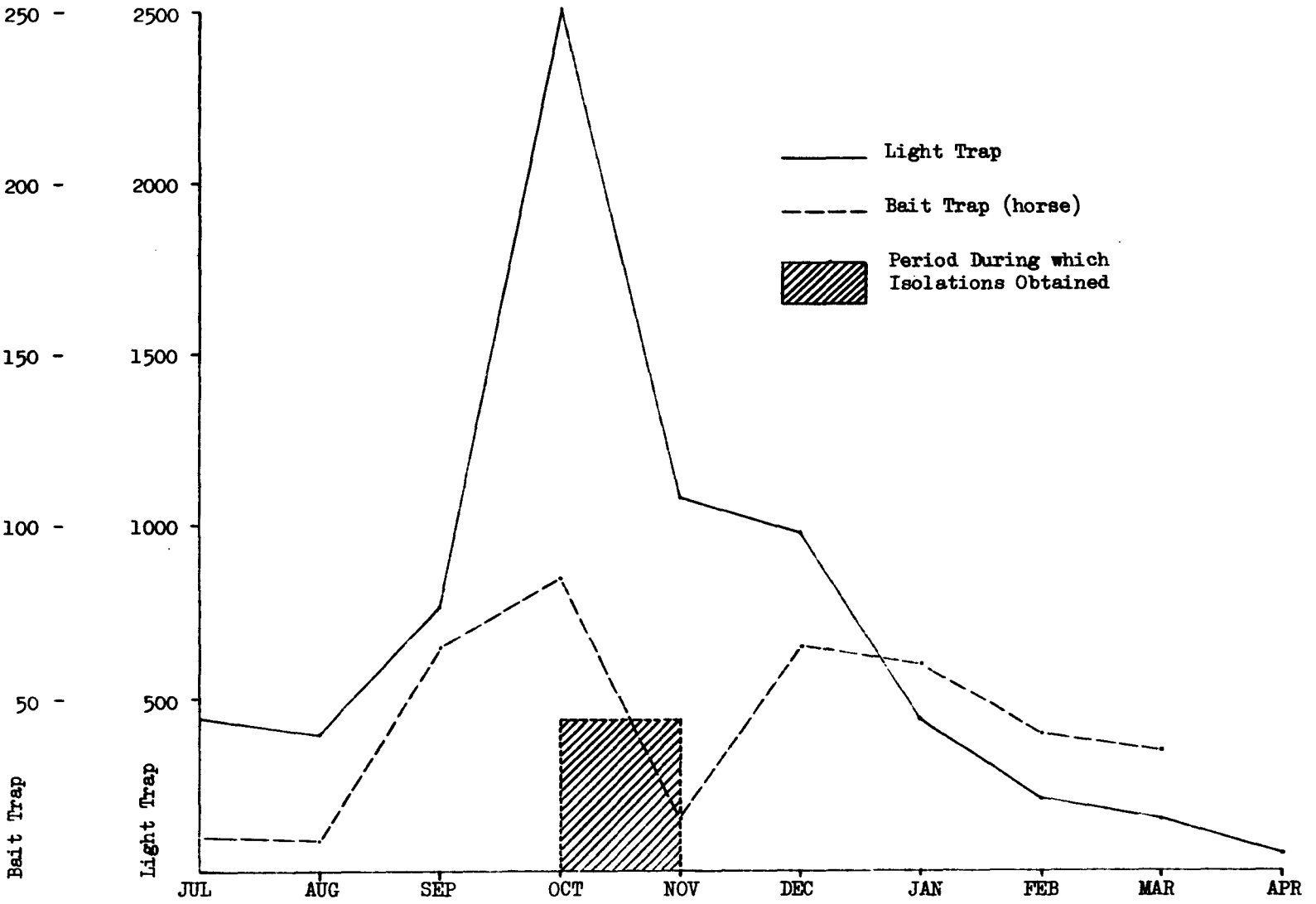


Figure 4. Mosquito Collections at Bang Phra, Cholburi, July 1962 to May 1963

(Females per Trap Night - Light and Bait Trap)

TABLE IV

Percentage composition of female mosquito captures at Bang Phra
July 1962 to April 1963

MONTH	LIGHT TRAP			BAIT TRAP		
	C.gelidus	C.tritaen.	Combined	C.gelidus	C.tritaen.	Combined
Jul. 62	40.0	50.5	90.5	78.9	9.6	88.5
Aug. 62	30.4	54.4	84.8	30.3	6.9	37.2
Sept.62	16.8	56.0	72.8	19.6	4.7	24.3
Oct. 62	35.4	45.3	80.7	38.4	21.3	59.7
Nov. 62	51.4	39.9	91.3	78.3	2.4	80.7
Dec. 62	80.8	12.5	93.3	98.0	0.8	98.8
Jan. 63	74.0	16.0	90.0	91.5	3.0	94.5
Feb. 63	45.5	12.0	67.5	64.1	20.1	84.2
Mar. 63	71.0	18.3	89.3	80.4	6.0	86.4
Apr. 63	66.0	16.4	82.4	not completed		

c. Several human biting collections and larval collections were made at Bang Phra in April, at the height of the dry season. The biting collections were made during the early night hours near the light trap. Over 73% of the females were Culex pipiens quinquefasciatus, despite the fact that this species had been present in only insignificant numbers at Bang Phra in the horse baited trap, and only very small numbers in the light trap. One of the most striking differences between the mosquito captures at Bang Phra and Bangkok, has been the scarcity of Culex pipiens quinquefasciatus at Bang Phra. The sample taken in human biting collections is small (115 females for 11 man - hours of collection), but the results re-emphasize the danger of basing an assessment of the mosquito population on only one or two methods of collection. Both C. gelidus and C. tritaeniorhynchus larvae were collected in large numbers from pools along the course of a highly polluted stream which runs through the horse farm. The stream carries wastes from a tapioca mill, and in the dry season the water is extremely foul. Culex larvae were restricted to slightly less polluted pools along the sandy margin. On the beach along the Gulf of Thailand Anopheles subpictus malayensis larvae were found breeding in large numbers in an open sunlit brackish pool. Identification of additional larvae is still in progress. Much more extensive larval surveys will be made as the 1963 rainy season progresses.

3. Mosquito - borne viruses in other areas of Thailand (SEATO MEDIC Study # 42)

a. As time and supplies of dry ice permitted, mosquitoes were collected and frozen for virus isolation in several areas of Thailand. Some of these collections were made coincident to other studies, some were made at the request of the Ministry of Health, and some were made as part of a newly developed program of sampling of several areas of Thailand on a regular basis. Whenever trained personnel were available the identifications of at least the most common species were made in the field and the mosquitoes were tubed and frozen by species and time of collection. When this was not possible the lots were frozen by time of collection and the specimens were later identified on ice in the laboratory just prior to inoculation. A backlog of frozen material still exists at the time of this report.

b. In Chiengmai collections were made from animal and human bait, in the city outskirts and in the surrounding forests, several times during the year. Over ten thousand specimens were collected for isolation from the city, and a small number of Aedes (Stegomyia) and (Finlaya) species were collected in the forests. Routine collections have been made from a cow baited trap in the suburban area since January 1963, but collections from this source have been at a low level during the dry season.

c. Several collections, not yet processed, were made in Southern Thailand along the main railroad line, centered on Klong Chandi in connection with an outbreak of hemmorhagic fever. These consisted chiefly of Aedes aegypti and Culex pipiens quinquefasciatus, a situation not unlike that found in Bangkok and other concentrations of humans in Thailand. Collections on a larger scale were made in the town area of Klong Chandi and in the forested area approximately twenty kilometers southeast of the town in September. In the town members of the Culex (Culex) group predominated, while in the forested hills Aedes (Finlaya) and (Stegomyia) species, Armigeres species and Heizmannia species predominated. Small samples of the mosquitoes were preserved for isolation, but results are not available as yet.

d. Two very successful collections were made in the coastal region centered around Rayong in Southeastern Thailand in July. The principal species collected resting in houses and in human biting collections were Aedes aegypti, Culex pipiens quinquefasciatus, C. sitiens and Anopheles vagus. Several virus strains were isolated from the first three of these species. I appeared that the Aedes aegypti population was higher on a per house or unit of collection basis in Rayong than in Bangkok at the same time. Culex sitiens was found breeding in brackish pools along the coast, some highly polluted. This species appears in rather small numbers in Bangkok. Thirteen strains of virus were isolated from the twenty-six pools of mosquitoes collected at Rayong, and a discussion of these will be found in the report of the Virus Department.

e. In February a system of surveying a number of sites around Bangkok on a regular year round basis was initiated. Initial collections were made in Rangsit, a Klong-side village North of Bangkok. Collections here were very poor, largely due to the open linear arrangement of the village which permitted a free sweep of the wind through the night. The collections were transferred to Bang Pa In, a more compact town along the Menam Chao Phaya River, approximately fifty kilometers North of Bangkok. Data from the Bang Pa In collections are not available at this time. At Rangsit only eighty female mosquitoes were collected in condition satisfactory for virus isolation. These were chiefly Aedes aegypti, Culex pipiens quinquefasciatus and C. tritaeniorhynchus. This is an extremely poor yield for three man-days of collection. In contrast, four man days of collection in the village of Pakchong on the Korat plateau yielded 918 female A. aegypti and 1240 female C. quinquefasciatus for virus isolation. The same groups of collectors were responsible for these collections. The system of bi-weekly collections in these two areas (Bang Pa In and Pakchong) will be continued through 1963. Several additional areas near Bangkok will be added to the system as time and personnel permit. Processing of the material collected thus far is still in progress.

4. Colonization of vector mosquito species (SEATO MEDIC Study #43)

a. Colonies of Aedes aegypti and Culex pipiens quinquefasciatus were established on a permanent basis during the year, and have been carried through a number of generations without the addition of further wild caught material. These colonies are intended for use in virus transmission experiments and for a reference standard for insecticide testing. One attempt has been made to transmit Chikungunya virus by the bite of C. quinquefasciatus, with inconclusive results. The insecticide tolerance level of both species was determined. No other demands have been placed on the colonies to date. Eggs from the Aedes aegypti colony were forwarded to the Entomology Department, WRAIR, for establishment of a sub-strain.

b. A small number of female Anopheles minimus were obtained at the close of the rainy season, from an area where United States troops had been exposed to malaria. Attempts to colonize the species from this material were hampered by the distance over which the females had to be shipped, and the small number involved. None of the larvae survived to pupation. This species has not been colonized elsewhere, and additional attempts are planned with the onset of the rainy season. It is doubtless the most important malaria vector in Thailand and additional attempts will be made when adults are available. A large number of larval Anopheles balabacensis, another important vector suspect, were collected in Chantaburi Province in April and May, and these are being reared for stock with which to establish a colony. A satisfactory method has been worked out to transport larvae to the insectary with relatively little loss. Work on the colonization of this species is still underway at the time of this report.

5. Precipitin tests of mosquito blood meals (SEATO MEDIC Study #44)

Approximately two thousand engorged female Aedes aegypti and Culex pipiens quinquefasciatus mosquitoes were removed from the routine Bangkok mosquito collections during the year. Each was smeared on filter paper with accompanying collection data, and the papers were stored in a dessicator under refrigeration. Antisera were prepared to human, cow, horse, chicken, pig, dog and monkey. The latter antiserum is intended chiefly for planned studies on the feeding habits of Anopheles mosquitoes in the forest, but some of the Bangkok mosquitoes are also tested against it. Many of the smears, when leached out in saline in the normal manner have shown no reaction against the antisera used. It is believed that some of the negative results have been due to extended storage of the smears. Both Culex quinquefasciatus and Aedes aegypti have shown a great preponderance of human positive, if the non-reactors are excluded. This was the result expected, since both species are highly anthropophilic. Complete data on the test series with all antisera is not available at the time of this report.

6. Association of Diptera with incidence of trachoma (SEATO MEDIC Study #45)

Large fly traps made to United States Public Health Service specifications were baited with cattle liver, entrails and other attractants were placed in Korat city in Northeastern Thailand, and in five neighboring villages. These traps were placed and removed by World Health Organization personnel engaged in a pilot study of trachoma control in Thai children, and the flies forwarded to Bangkok for separation. In addition, eye-gnat traps of a standard design were placed at each study site, baited with cattle liver. Records were kept of the population of flies at each site, and of the incidence of trachoma by month. All of the flies collected were forwarded to the United States National Museum for identification, after either the number of flies, or a volumetric approximation of number was noted. The percentage of cases of trachoma and the number of flies per trap night are portrayed graphically in figure 5. The relationship by species and by village of collection are still under analysis. The specific identifications of the flies have not as yet been received from the United States National Museum, and the final analysis of the data will await the receipt of this information. The apparent correlation of number of cases of trachoma and number of flies may merely represent the action of a third factor, such as temperature or rainfall, and any conclusions from the study must await specific identification of the various flies which go to make up the curve.

7. Collection of chiggers and ticks for rickettsial isolation (SEATO MEDIC Study #46)

a. This activity was undertaken in its present form in November 1962 as a joint study of the Thai and U.S. Components, SEATO Medical Research Laboratory, WRAIR and the University of Maryland School of Medicine. Prior to that time several lots of chiggers had been frozen for later isolation work, and large numbers of chiggers and ticks had been preserved for taxonomic purposes. Since November 1962 the program has expanded to encompass a number of facets, all with the principal aim of determining the extent of distribution

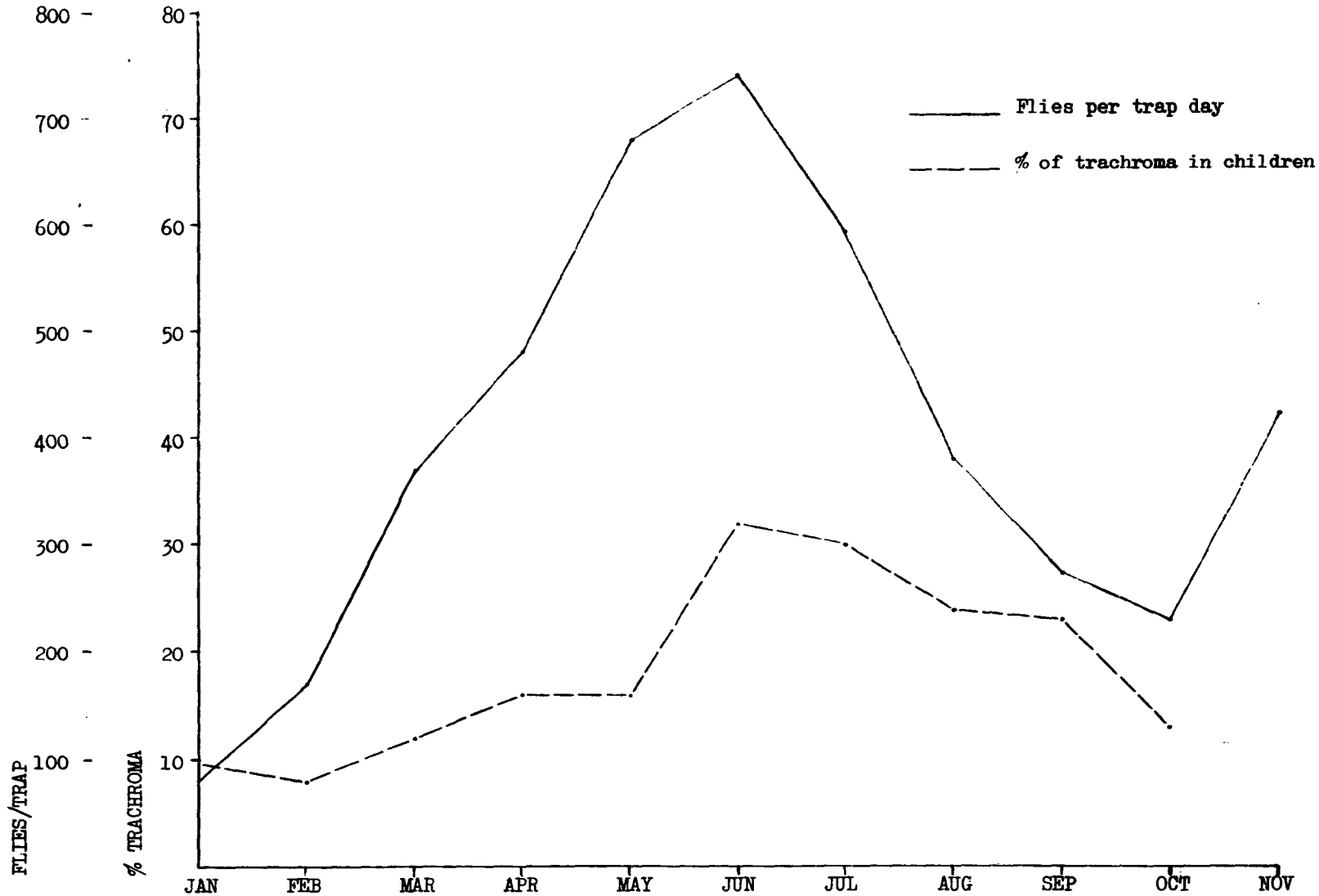


Figure 5. Relation of fly population to trachroma in children

of the rickettsial diseases in natural foci in Thailand. The U.S. Component of the SMRL has primary responsibility for the determination of the trombiculid mites, ticks, and vertebrate hosts, as well as observations on the ecology of the rickettsial diseases. The Thai Component of the SMRL is primarily responsible for the isolation of rickettsiae from vertebrates and arthropods, and for the clinical phases of the investigation. Both components receive support from the cooperating elements of WRAIR and the University of Maryland.

Since November 1962, large scale collections have been made in the vicinity of Chiengmai and Khon Kaen, and at various sites in the vicinity of Bangkok. The latter sites include areas of strategic importance or suspected rickettsial activity within 200 kilometers of the city. There have been sporadic reports of presumed scrub typhus in Thailand since the initial report in 1952, with little documentation. The present studies include the collection of vertebrates for ectoparasites, tissues and blood for serological examination. Humans living in the selected study areas are also bled for serological evidence of exposure to rickettsiae. Chiggers are identified as far as possible in the field and pools inoculated in mice as soon as possible. Minced and ground tissues are also inoculated in the field and aliquots frozen for later inoculation in Bangkok if warrented. Two hundred and sixty three (263) mammals have been so examined this year, mostly various species of rodents (Rattus sp., Menetes sp., Callosciurus sp.) and tree shrews (Tupaia sp.). Blood was obtained from almost all of these animals. In addition, a few rarer animals such as the pangolin (Manis), and civets (family Viverridae) have been examined.

b. Seven strains of rickettsiae have thus far been isolated from these animals. Serological evidence has been obtained indicating a widespred exposure of man and forest and field mammals to scrub typhus and other groups of rickettsiae. Rickettsial isolations have been made from several localities in the Chiengmai area of Northern Thailand, Chantaburi district of Southeastern Thailand and the Ubol region of Northeastern Thailand, along the Laos border. The latter site is particularly important, as the Thai Army experienced an outbreak of a disease while on maneuvers in that area which was in retrospect diagnosed as scrub typhus. This outbreak occurred in 1956, and extended field studies are presently underway to determine the extent of the focus. At the time of this report five additional suspicious lines in mice are also under investigation. These originated from Lopburi and Samutraprakarn in Central Thailand, and Cholburi in Southeastern Thailand. Strains of rickettsiae isolated thus far have included forms virulent for mice, and a few avirulent forms. Those definitely identified as scrub typhus have resisted challenge with the Karp strain of rickettsiae. Aliquots of several of the lines have been sent to WRAIR for further identification and antigenic analysis.

c. Seasonal trends have been noted in the abundance of chiggers and ticks, and in the movements of the mammalian hosts. Chiggers and seed ticks were found abundantly on the hosts at the end of the rainy season (November) when this study was initiated, and have become less abundant as the dry season progressed. Almost no chiggers have been found on the ground during the dry period, contrary to experience further south in Malaya,

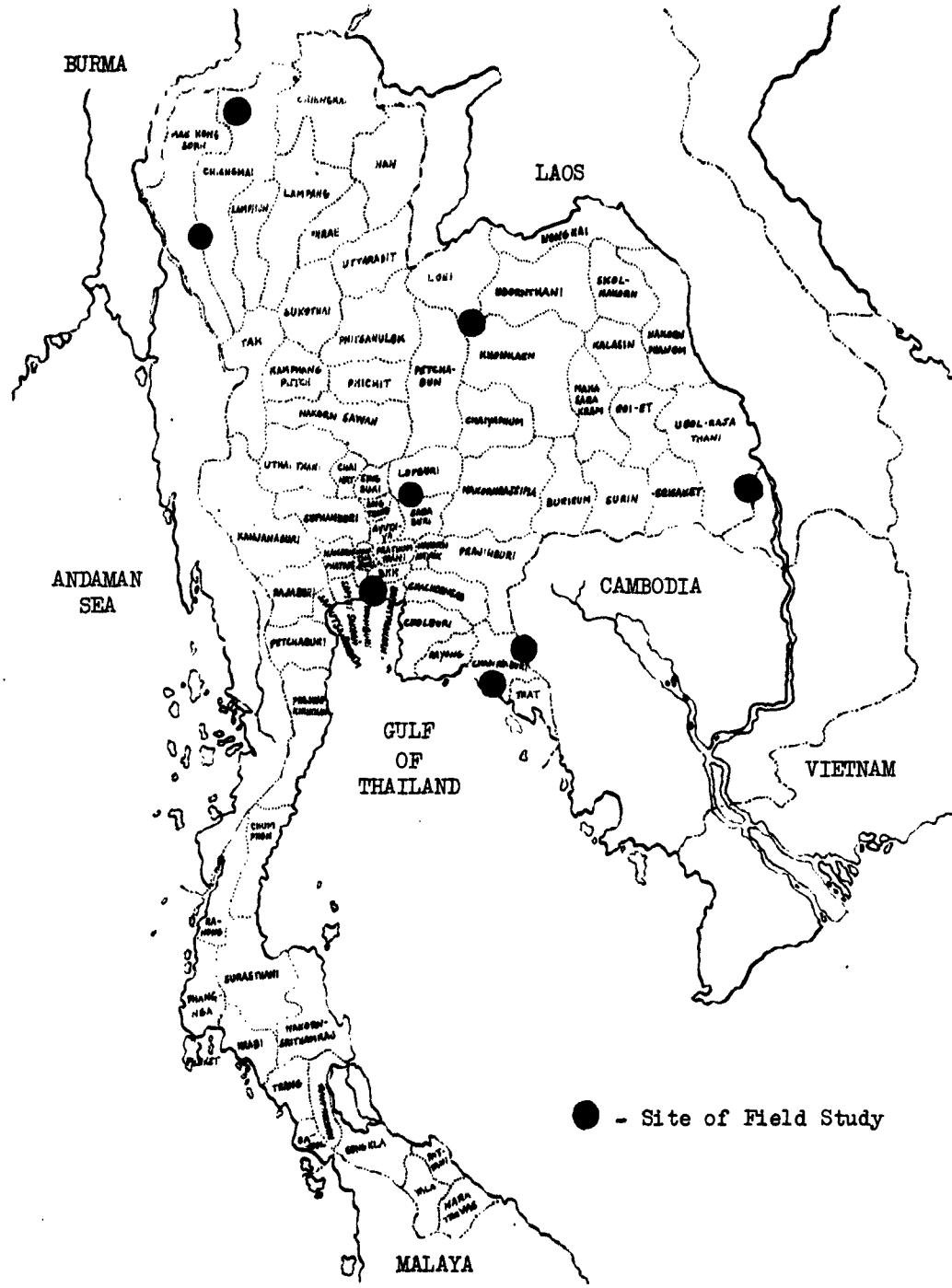


Figure 6. Locations of rickettsial disease surveys

which does not experience the prolonged dry period of Thailand's monsoon climate. During the dry months the small mammals move from the open areas of the forests and concentrate along the few remaining water courses. The association of these trends with scrub typhus and other rickettsioses will be followed through the coming year. Areas where collections have been made thus far are in-dicated in map 1.

8. Anopheles in relation to Malaria. (SEATO MEDIC Study #51)

Weekly collections of Anopheles are being made in two different biotypes in Thailand known to be highly malarious. These are associated with continuous surveys of the state of malaria in the local populations. Female mosquitoes collected in the proper physiological state are dissected for detection of malaria infection and for isolation of plasmodial organisms in clean animals. Collections have been made thus far at several sites in Southeastern Thailand. Small numbers of Anopheles, including A. minimus and A. balabacensis have been collected, but dissections have been negative thus far. This project is closely tied to development of significant numbers of mosquitoes, and the onset of the malaria transmission season in the rainy season.

9. Epidemiology of malaria (SEATO MEDIC Study #52)

This study was initiated in May 1963, with the cooperation of several Provincial hospitals of the Ministry of Health, and several military hospitals under the direction of the Surgeons General of the Thai Armed Services. Patients who appear to resist normal drug therapy are studied under controlled conditions. When a history of large numbers of cases of malaria from a particular district or village is found the area is studied in detail. The study began in late April 1963 and a concentration of cases has been found at the Cholburi Provincial hospital, most of them among forest workers in the jungle foothills of the Province. Collections are presently in progress, and no information is available at the time of this report.

10. Drug resistant malaria (SEATO MEDIC Study #53)

Chemical studies are being carried out of levels of chloroquin and other drugs in the plasma and urine of individuals who appear on clinical and parasitological grounds to be resistant to normal drug thereapy. Plasma sample have been received from U.S. troops on normal prophylactic drugs to serve as a reference standard, and several samples have been received from suspected resistant cases. Tests will be initiated as soon as instruments received from WRAIR in May have been installed and calibrated. A trial of U.S. and Thai volunteers on two dosage levels of chloroquin has been planned to aid in the calibration of the equipment and to serve as a standard for future tests.

Summary and Conclusions:

1. A longitudinal study of the mosquitoes and mosquito-borne virus in five areas in Bangkok was completed in January 1963. During the fiscal year over 400,000 mosquitoes were identified in connection with the study, and over 41,000 were preserved for virus isolation. Viruses were isolated only from Aedes aegypti and Culex pipiens quinquefasciatus, which are by far the most numerous species attacking man in Bangkok. Most isolations, of chikungunya and dengue viruses were from Aedes aegypti, while only chikungunya virus was isolated from Culex. These species thus appear to be the only ones of significance in the transmission of hemmorhagic fever in the city. Work is continuing in some of the areas used in the year long study, in order to follow the progress of the viruses in the mosquito populations this year.

2. Culex gelidus and C. tritaeniorhynchus were found to be the predominant species of mosquitoes at a horse farm in Southeastern Thailand where encephalitis had occurred in equines. Viruses which appear to be japanese encephalitis were isolated from both species at the time at which they were most abundant. These species appear to be the only ones of importance in the transmission of encephalitis in the area, but studies of mosquito populations and infection are continuing.

3. Mosquitoes were collected from several areas of Northern, Southern and Southeastern Thailand for virus isolation. Some of these were taken in connection with specific disease studies, others from routine mosquito collections. A large percentage of the mosquitoes collected in coastal areas of Southeastern Thailand yielded isolations of viruses associated with hemmorhagic fever. Many of the collections remain to be processed.

4. Colonies of Aedes aegypti and Culex pipiens quinquefasciatus have been established and maintained for insecticide testing and virus transmission studies. Initial efforts have been made to establish colonies of Anopheles minimus, A. balabacensis, and other suspected malaria vectors.

5. Antisera have been produced to permit testing of mosquito blood meals. Included in the series thus far are: man, pig, cow, horse, dog, chicken and monkey. Tests performed thus far have been restricted to Bangkok mosquitoes, which show a high proportion of reactions to human antiserum.

6. Eye gnats and other Deptera were collected from Korat city and several surrounding villages, in connection with studies on the incidence of trachoma in children. The gross population levels of the flies appears to show some correlation with the seasonal incidence of trachoma, but final analysis will depend on the specific or generic identifications of the flies, which are not available at this time.

7. Chiggers and ticks and their arthropod hosts were collected from several areas of Thailand. Mammalian tissues and chigger pools were inoculated in mice for the isolation of rickettsiae, and a number of lines of virulent and avirulent rickettsiae were isolated. Some of these appear to be scrub typhus, and specific identification of all of the agents isolated

is still in progress. Serological evidence was obtained for widespread dissemination of several species of rickettsiae in man and wild mammals in several regions of the country. Initial observations indicated that chigger and tick populations fluctuate widely on mammalian hosts by season.

8. Anopheles are being collected in highly malarious villages and forested areas of Thailand, for determination of the species which transmit malaria under differing conditions. Work is too newly undertaken in this project to permit a full report at this time.

9. A surveillance system has been established to permit study of areas of high incidence of malaria in civilian and military communities. Initial bleedings have indicated the presence of significant amounts of malaria in some areas. Work is still in the initial stages, and a complete report is not possible at this time.

10. Chemical determinations of the levels of chloroquin and other anti-malaria drugs in the plasma and urine has been initiated. The tests are to be applied to individuals who appear to have malaria infections which are refractory to the usual drug treatment.

ANNUAL PROGRESS REPORT

Project No. 3A-0-12501-A-811, MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

(Arthropod borne Infections)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D.C.

Division of Communicable Disease & Immunology

Special Activity: U.S. Army - SEATO Medical Research Laboratory
APO 146, San Francisco, California

Department of Virology

Period Covered by Report: 1 July 1962 - 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A-0-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA (Arthropod-borne Infections)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

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Detailed clinical and laboratory studies were completed on hemorrhagic fever cases. During 1962 this disease caused nearly 6000 hospital admissions and 300 deaths. There was evidence of infection of Americans in Bangkok who developed classical dengue fever but in no instance were the manifestations of hemorrhagic fever observed in Caucasians.

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BODY OF REPORT

Project No. 3A-0-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

(Arthropod borne infections)

Description:

The purpose of this task is to study the health significance, epidemiology, pathogenesis, etiology and distribution of arthropod borne virus infections in Thailand. During the report period Thai hemorrhagic fever (THF) was a major public health problem and the following departmental studies were mainly concentrated in this area:

1. Epidemiology of Thai hemorrhagic fever.
2. Clinical and virologic definition of THF syndrome.
3. Clinical course and pathogenesis of Thai hemorrhagic fever.
4. Serologic response to THF infection.
5. Survey for prevalence of arboviruses antibodies among residents of Thailand.
6. Overt and inapparent infections with arboviruses in Americans in Thailand.
7. Virus isolation from wild caught mosquitoes.
8. Inapparent infection rates to THF and other arboviruses among residents of Bangkok.
9. Avian and mammalian resevoirs of arboviruses in Thailand.

Progress:

1. Epidemiology of Thai hemorrhagic fever (SEATO MEDIC Study #1)

a. This study was initiated in April 1962 and has been maintained through the cooperation of the 18 major hospitals in Bangkok and Thonburi, the Bangkok Municipal Health Department, the Ministry of Public Health and the directors of provincial and private hospitals throughout Thailand.

b. In 1962, epidemic Thai hemorrhagic fever occurred widely throughout Thailand as far as 400 kilometers north and 700 kilometers south of Bangkok. Cases outside Bangkok occurred in urban centers in the known areas of distribution of Aedes aegypti. Among residents of the metropolitan

area of Bangkok and Thonburi (2 million population), there were 3,550 hospitalized cases with 154 deaths. Fifty provinces outside Bangkok reported a total of 2316 cases with 153 deaths; the vast majority of these cases occurring in nine provinces located on the Central Plain and along the southwestern coasts of the Gulf of Siam. Nearly all hospital admissions and all deaths outside Bangkok occurred in the same age groups as were reported in Bangkok. In addition, these cases grouped themselves seasonally with the outbreak in Bangkok (April - December). Viruses similar to those of the Bangkok outbreak have been isolated from three of the major provincial outbreaks and cases confirmed serologically from the other six.

c. Age specific attack rates were highest in infants under 1 year (the reliability of diagnosis at this age is questionable) declined in 1 and 2 year-olds and rose again in the 3 to 5 year group finally decreasing nearly to zero after the age of 16. Infections studied clinically and virologically in individuals over the age of 13 or 14 have been very mild and did not conform to the description of severe hemorrhagic fever. Attack rates are almost identical in males and females. The over all case fatality rate in females was slightly higher than in males; mortality rates were highest in girls up to the age of 3 and in 6 - 7 year old boys, tapering off to zero at age 13 in both sexes. Based upon a house-to-house survey of 19 areas of Bangkok with a population of 44,000 persons it was found that hospital admissions reflect only a portion of the total morbidity due to the hemorrhagic fever viruses. It was calculated that approximately 4000 children in addition to those hospitalized were diagnosed as having hemorrhagic fever by physicians in Bangkok and were treated in private clinics or at home. Minor illness associated with the epidemic occurred at an enormous rate. From the study of febrile patients seen in the out-patient department of Children's Hospital it was estimated that nearly 17,000 out-patients had mild illnesses caused by chikungunya or dengue viruses. All other Bangkok hospitals combined see approximately 5 times as many patients as Children's Hospital. Thus, there was an estimated total of 78,000 children seen in all Bangkok hospitals with febrile illness due to HF viruses. In the area survey it was observed that for each child seen in the OPD 1.5 go to private physicians; thus, the total mild illnesses in children seen by OPD and private physicians in Bangkok might be as high as 200,000-this number out of a total of 870,000 children 14 years and under living in the community.

d. Sporadic hemorrhagic fever admissions continued in Bangkok-Thonburi hospitals during the period 1 January - 31 March 1963. Several of these cases were documented serologically. These observations support the widely held suspicion that the viruses of hemorrhagic fever are maintained during interepidemic periods by endemic man-mosquito-man transmission and that disease rates simply reflect relative abundance of susceptible host and vector mosquito.

2. Clinical and virologic definition of THF syndrome. (SEATO MEDIC Study # 2)

a. To assess clinical diagnostic accuracy and to establish the virus etiology of THF in 1962, randomly selected hemorrhagic fever patients admitted to Children's Hospital were studied within 24 hours of hospital admission during the entire THF outbreak. Serum was inoculated without freezing into one-day old mice and blind passed to the third mouse passage. Using exactly the same techniques and on the same days other groups of patients were also studied: a) randomly selected out-patients with miscellaneous febrile disease, b) in-patients with febrile disease but not hemorrhagic fever and c) surgical patients. These latter groups of patients were studied to discover how much minor illness was caused by hemorrhagic fever viruses, to discover the variety of hospitalized febrile syndromes attributable to hemorrhagic fever viruses and finally as a control study of the rate of silent conversion in hospitalized non-medical patients. Paired sera were obtained in over 95% of patients surviving hospitalization.

b. Preliminary results to date may be summarized as follows: (1) 86% of hospitalized hemorrhagic fever was identified as being caused by chikungunya or dengue viruses by virus isolation or HI serology. Dengue viruses accounted for 74% of infection and chikungunya virus for 12%. With the admitted limitations of laboratory procedures for testing for dengue virus infection these percentages are probably low. At any rate, it appears that the clinical diagnosis of hemorrhagic fever under epidemic conditions was highly accurate. Virus was isolated from the blood of 70% of patients with chikungunya disease and approximately 20% of patients with dengue virus infection. (2) Of 93 out-patients studied, 37% had either chikungunya or dengue virus infection. The most common diagnostic categories which were HF virus-positive were upper respiratory infection (pharyngitis, tonsillitis, bronchitis, influenza etc.) fever of unknown origin and suspected hemorrhagic fever. Rates of recovery of virus from blood were nearly 100% for chikungunya infection and 50% for dengue infections. These higher rates of virus recovery reflect the fact that out-patients were studied earlier in the course of disease than were in-patients. (3) Of 102 patients hospitalized with febrile illness other than hemorrhagic fever, 22% had dengue or chikungunya infections. HF virus-positive patients in this category had a great variety of diagnoses e.g. encephalitis, rheumatic fever, pneumonia, but only one diagnosis was seen several times, fever of unknown origin. As the hemorrhagic fever epidemic proceeded, admissions of "misdiagnosed" HF decreased markedly. (4) Of 93 surgical patients studied, 7% developed a diagnostic antibody rise in acute and convalescent sera to either chikungunya or dengue virus. No viruses were isolated. This conversion of non-medical patients suggests several possibilities a) transmission of hemorrhagic fever on hospital premises or, b) infection acquired shortly before or shortly after hospitalization. Since most surgical patients were in hospital for both acute and convalescent bleedings the former hypothesis is more likely. If true, the percentage of virus confirmations attributed to hospitalized febrile disease should be reduced since some of these conversions may be attributed to virus infection acquired after hospitalization.

3. Clinical course and pathogenesis of Thai hemorrhagic fever (SEATO MEDIC Study # 3)

a. A total of 57 THF patients, 16 febrile out-patients, 11 febrile in-patients and 12 surgical patients were subjected to similar historical and physical examination by a single observer. Each of these patients was followed daily for changes in symptomatology and physical signs; other studies include plasmas for liver chemistries, electrolytes, enzyme and steroid levels, CBC, direct platelet count (phase contrast method), hemoglobin, hematocrit, urinalysis and stool benzidine. Twenty-seven THF patients were further studied for blood clotting abnormalities.

b. Preliminary evaluation of results has established that hepatomegaly and liver damage are extremely common in HF; that prothrombin time abnormalities in addition to prolonged bleeding time, thrombocytopenia and poor clot retraction are seen in the severely ill patient; that right-sided pleural effusions are relatively common; that neurological abnormalities (pathologic reflexes, personality change, irritability) are frequent and that lymphadenopathy, while common in HF patients is seen just as frequently in controls.

c. This study has served to emphasize the importance of shock and damage to vital organs in the pathogenesis of the hemorrhagic fever and as a cause of death. Hypovolemia, liver damage and myocardial failure may be more directly related to death than is hemorrhage. Hemorrhage alone rarely, if ever, is the cause of death. The term "hemorrhagic fever" while easy to remember, therefore, does not adequately describe the clinical condition associated with dengue and chikungunya virus infection in Asian children.

4. Serologic response to THF infection (SEATO MEDIC Study #4)

a. From April through December 1962, sera from 604 patients with the clinical diagnosis of hemorrhagic fever were studied. In 86% of cases in which sera were paired at an interval of 14 days it was possible to confirm infection with a group B or a group A virus using the HI technique. High, fixed titers of 1:1280 or greater were highly significantly correlated with clinical HF patients as compared with other febrile and non-febrile controls studied at the same time during the epidemic. It appears HI antibody response following overt HF is considerably greater in magnitude than that following clinically milder infection. Furthermore, high HI titers are short lived and their presence correlated with an illness is highly suggestive of a causal relationship.

b. Late convalescent sera have been collected from 51 patients with virus isolation and 75 patients with serologic conversion to dengue or chikungunya viruses. Material for this study will be collected for a period of one year following the end of the 1962 HF outbreak.

c. Plans for the serologic study of HF include examination of the "simultaneous" group A-group B response noted in 5 - 10% of HF patients, comparative efficiency of diagnosis of CF and HI tests, the specificity of response of serologically "virgin" Caucasians to dengue virus infection and the rate of development and decay of CF and HI antibodies following overt, mild and inapparent infections.

d. By bleeding a large number of school children it was possible to pre-bleed 13 individuals before onset of hemorrhagic fever. It is hoped that analysis of these sera will help to answer the question of whether or not HF occurs as the result of the first experience with any of the dengue viruses.

e. These studies are in progress.

5. Survey for Prevalence of Arbovirus Antibodies among Presidents of Thailand. (SEATO MEDIC Study #5)

a. Hemagglutination inhibition tests for chikungunya and dengue 1 antibodies have been completed on 1498 sera collected throughout North, Central and Northeast Thailand. Criterion for positivety was a titer of 1:20 or greater in the overnight incubation test ($4^{\circ}C$). The degree to which antibodies against other group A viruses are represented in these results is not known. Tests of Americans exposed to JE virus in NE Thailand (Study 6) showed that using dengue virus as an antigen a few sera with JE antibody were missed, but most sera were positive to both antigens.

b. Since most of the individuals bled were young males just inducted into the Army, the population sampled probably was mainly rural; most individuals coming to large urban centers for the first time in their lives. If this surmise is correct the group B antibody measured may be largely due to JE or related viruses.

c. This survey showed that group B antibodies occurred in 90% of persons resident in the Northeast and in 95% of residents of the Central Plain. As the sample moved into North Thailand the percent of persons with group B antibody experience diminished to 36%. Group A antibodies occur at lower levels that group B in all areas tested with the same general trend of lowest incidence in the North. Tests of 2 tribal groups living at altitudes of 3000 feet or higher in North Thailand (the Nan area and east of Maehongsorn) revealed almost no experience with group A or group B viruses as expressed in the HI test.

6. Overt and inapparent infections with Arboviruses in Americans in Thailand (SEATO MEDIC Study #6)

a. This study was conducted with the cooperation of the JUSMAG Medical Unit, the Bangkok Sanitarium and Hospital and with many private physicians in Bangkok. Altogether 76 illnesses in caucasians with adequate specimens have been studied by HI & CF for dengue or chikungunya infections.

Of these, 23 were dengue and 10 were chikungunya infections. Five dengue viruses and 7 chikungunya viruses have been isolated. In general, illness in American adults was more severe than in children. Symptomatically, all of the chikungunya or dengue virus infections which have been studied by this department were either dengue-like or PUO's. Shock, hepatomagaly or life threatening illness did not occur. This supports the hypothesis that Thailand dengue and chikungunya virus infections result in hemorrhagic fever in Oriental children but in Caucasians of any age a completely different disease occurs, dengue fever.

b. In May, 787 American children and adults living in Bangkok were bled; 402 were rebled in November 1962 at the conclusion of the HF outbreak. Twelve per-cent developed HI anitbody to dengue or chikungunya viruses. There was a physician documented morbidity rate of 25% associated with this antibody conversion in Americans and a ratio of overt (or reported) disease to inapparent (or non-reported) disease of 3:1.

c. Two large military units arriving in Thailand have been surveyed similarly: (1) The 809th Engineers quartered in semi-permanent barracks on the Southeastern edge of the Central Plain 60 miles from Bangkok were sampled in February (200) and again in July (100) and September (100), 1962 Approximately 13% of these men converted to group B viruses, probably dengue. (2) The 1st Battle Group, 27th Infantry, on maneuvers through Northeast Thailand from May - August; 100 men were bled on arrival and 87 rebled before departure. Twenty-two percent developed HI antibody probably against Japanese encephalitis virus.

d. In addition to the dengue and chikungunya illnesses which were reasonably common in Bangkok among American Military personnel and their dependents, a few overt diseases among military personnel stationed in other areas of Thailand and Southeast Asia were brought to the attention of this department: (1) A probable case of Japanese encephalitis in a 20 year old Army Corporal was admitted 21 July to the 31st Field Hospital in Korat. He was attached to the 1st Battle Group, 27th Infantry and had been on maneuvers in Northeast Thailand. A single specimen obtained on 6 August had high titer of HI, CF and neutralizing antibody to JE virus. He was transferred to Clark Air Force Hospital on 7 August with residual neurological deficit. Although the discharge diagnosis at Clark Air Force Base Hospital was cerebral malaria it is possible that this represented a case of Japanese encephalitis. (2) Several cases of apparent dengue virus infection occurred in Korat, Northeast Thailand and Chachoengsao, Southeast Thailand. (3) Febrile dengue-like infection among Americans in Saigon and Nha Trang were classified serologically as presumptively dengue, type unknown.

7. Virus isolation from wild caught mosquitoes. (SEATO MEDIC Study #7)

a. During the study period 91,000 mosquitoes of 12 species in 962 pools were processed in suckling mice through 3 blind passages. Nearly all of these mosquitoes were captured in 5 study sites in Bangkok by the

Entomology department. These same sites were studied for evidence of human infection. Over 95% of mosquitoes were of two species, C. quinquefasciatus and A. aegypti. To date 41 viruses have been isolated, 28 of these from mosquitoes captured in Bangkok. Thirteen viruses were recovered from a June outbreak of HF in Rayong on the Southeast coast of the Gulf of Siam, 150 miles from Bangkok.

b. Isolations can be categorized as 1st passage with a short incubation period or 2nd and 3rd passage with a long incubation period. Of the 10 first passage isolates from mosquitoes, 7 from Aedes aegypti are chikungunya; identification of 1 virus is not yet complete; 1 virus from Culex quinquefasciatus is chikungunya and a C. sitiens agent is not chikungunya or Japanese encephalitis virus. Of the 4 agents recovered in 2nd or 3rd passage from C. quinquefasciatus 2 are short incubation period agents which produce CPE in hamster kidney cells but which are not chikungunya. The other two agents are dengue-like. Identification of the other 2nd and 3rd passage agents is still incomplete although all behave like dengue viruses.

c. Although Aedes aegypti represented less than 10% of the total mosquitoes collected from Bangkok, pools tested from this species accounted for 24 out of 28 virus isolations and the over-all rate of recovery from pools was 17%. From 684 pools of Culex quinquefasciatus only 4 viruses (0.6%) were recovered; one of these is chikungunya, 2 are unidentified and 1 doubtful.

d. These data firmly establish Aedes aegypti as the important vector of both chikungunya and dengue viruses to humans in the THF outbreak of 1962. It is speculative whether Culex quinquefasciatus can transmit chikungunya virus at all. Its very abundance makes it likely that it frequently takes an infected blood meal and this may account for the sporadic isolations made. Transmission studies are planned to investigate this point.

e. Dr. Sakol Rohitayodhin of the Pasteur Institute Horse Farm has successfully recovered 5 mosquito agents from 1 pool of Culex tritaeniorhynchus and 4 pools of Culex gelidus captured in light and bait traps at Bangphra in southeast Thailand 80 miles from Bangkok. These traps and other equipment are a part of a collaborative program with the SEATO Medical Research Laboratory. All of these agents killed suckling mice in 3 - 4 days and all produced CPE to high titer in hamster kidney cells. Three of the five viruses have been tentatively identified by neutralization test as Japanese encephalitis virus. These viruses were isolated in October and November from 2 pools of Culex gelidus and 1 pool of Culex tritaeniorhynchus captured in light or bait traps. The fourth virus from Culex gelidus which also produces high titered CPE in hamster kidney cells was not neutralized by either chikungunya or Japanese encephalitis antiserum. The fifth virus, isolated from C. gelidus captured in November is apparently chikungunya virus.

8. Inapparent infection rates to THF and other arboviruses among resident of Bangkok. (SEATO MEDIC Study #8)

a. To study the dynamics of epidemic hemorrhagic fever, 19 study sites were established for case observation. Areas were randomly selected, each containing nearly 400 households (approximately 2000 persons), and representing most of the variations in urban habitat found in the city of Bangkok. Each house was numbered, a census of inhabitants taken and visits made every 4 - 6 weeks to obtain history of hemorrhagic fever among the inhabitants. Random selections of between 10 - 30% of area inhabitants were bled before the HF outbreak and 35% of these persons were rebled at the end of the outbreak (over 2000 persons). Whenever possible a single convalescent blood specimen was obtained from patients with recent hemorrhagic fever. Hemorrhagic fever cases were traced to the source of diagnosis for confirmation, symptoms and physical findings.

b. This study has been a difficult undertaking. Because of the large number of different viral agents involved in the hemorrhagic fever syndrome, it is uncertain whether virus specific inapparent or overt infection rates can be calculated. On the other hand, it will be possible to analyze the combined virus attack rates on the basis of racial or ethnic group, economic status, type of housing area, and previous immunity status and so forth.

c. An ethnic and economic profile of Bangkok has been achieved for the first time. Of 44,923 persons representing 3.38% of the municipal population of Bangkok, 60% were of the Thai racial and ethnic complex, 38% were culturally and racially Chinese; Indians, Filipinos, Malays, Caucasians and others comprised less than 1% of the sample population. Using accepted government civil service wage categories the study population was divided into four economic groups based upon family income: affluent, monthly income of 500 dollars or higher; upper, 75 - 500 dollars per month; middle, 35 - 75 dollars a month; and lower, under 35 dollars per month. Using this classification the sample population distributed itself as follows: affluent - 1.7%; upper - 17.8%; middle - 49.7% and lower 30.8% Of the total number of hemorrhagic fever cases occurring in the areas (147) there was a proportionate distribution of cases among the various ethnic and economic groups. Counting only hospitalized hemorrhagic fever (56) it became apparent that Chinese people sought or gained admission to hospitals more frequently than Thai and that children from the lower economic class were admitted to hospitals for hemorrhagic fever proportionality more frequently than upper class. Whether these admission rates reflect greater severity of disease in lower class and Chinese, greater use of private physicians with home care among the upper income groups and Thais or differences in attack rate in these groups is unknown, but under investigation.

9. Avian and Mammalian Resevoirs of Arboviruses in Thailand (SEATO MEDIC Study 9)

a. Sera from 1032 mammals living in or near Bangkok in 1962 were tested for HI antibody to chikungunya, and Japanese encephalitis. Seventy five percent of the large domestic animals, water buffalo, cattle, horses and pigs had detectable antibody for Japanese encephalitis virus and 25% had antibody against chikungunya virus while bats, common urban rodents, dogs and cats were infrequently positive. Because of limitations in personnel and time it was not possible to rebleed all of these species at fixed intervals during the year so that the time and rate of infection in 1962 could not be established. Fortunately, systematic collections of birds were conducted throughout the year and it is possible to answer this question for avians. Of 1906 birds of 75 species tested, 3 species were collected in large numbers, Blanford's bulbul, magpie robin, and three sparrow. Interestingly, these 3 resident species were most frequently infected with Japanese encephalitis virus. Almost all birds collected in April were negative. However, in June, July and August 50 - 70% of members of these 3 species possessed HI antibody. As collections continued through the end of the rainy season and into the cool dry season (December and January 1963) fewer and fewer birds possessed HI antibody until finally all birds tested were negative again.

b. If the virus being measured in the human, mammalian and avian studies is Japanese encephalitis and not some other group B agent it is now possible to summarize experience with JEV in Thailand as follows: (1) Humans in Northeast Thailand converted to JEV in May - August, 1962. (2) Birds in Bangkok converted to JEV in April - July, 1962. (3) Twenty percent of Australian horses quartered in Bangkok in May, 1960 converted to JEV. (4) Culex tritaeniorhynchus populations in Bangkok build up after the onset of rains (April and May) and peak in July. (5) Three strains of JEV were recovered from Bangphra on elevated, well drained land, 80 miles southeast of Bangkok in October and November, 1962. (6) Peak C. tritaeniorhynchus and C. gelidus populations in Bangphra occurred in October, 1962. Reasons for the variation in population curves in Bangkok and Bangphra are probably related to ground water accumulation and not absolute rainfall (see Entomology report).

c. It is concluded that JE virus is actively transmitted in many areas of Thailand during the period of high vector mosquito populations. The amount of human infection which accompanies this sylvan transmission is not yet known, nor is it understood why so little clinical encephalitis is evident among residents of Bangkok.

Summary and Conclusions:

1. Thai Hemorrhagic Fever (THF) in 1962 caused nearly 6000 hospital admissions and 300 deaths. Over one-half of the cases occurred among residents of the urban Bangkok-Thonburi area (2,000,000 population) other cases occurred throughout the Central Plain and Peninsular Thailand. Various epidemiologic aspects of this outbreak, the largest in the history of Thailand, are presented in the body of the report.

2. Approximately 150 hemorrhagic fever patients hospitalized in Children's Hospital, Bangkok, were studied for virus etiology. To investigate the clinical dimensions of epidemic hemorrhagic fever nearly 100 out-patients with febrile disease, 100 in-patients with febrile disease (not hemorrhagic fever) and 100 surgical patients were studied weekly throughout the outbreaks, using the same virologic techniques. Dengue viruses of several types and chikungunya virus were responsible for nearly all hemorrhagic fever admissions and also a substantial proportion of in- and out-patients with miscellaneous febrile illnesses .

3. The clinical features and pathogenesis of hemorrhagic fever were studied in virologically proven cases. Ninety-six virologically confirmed hemorrhagic fever patients and controls were examined daily by a single observer; serum samples were obtained for biochemical determinations and quantitative blood clotting studies. Final tabulation of results is awaiting identification of virus isolates from cases.

4. To study antibody development and decay in HF infection, 13 sera were collected from patients before they developed hemorrhagic fever; approximately 50 patients were studied with daily bleedings following hospital admission and 70 patients with virus isolation were bled in the late convalescent stage. Serologic studies are in progress.

5. Nearly 1500 residents of North, North Central and Northeast Thailand have been studied for HI antibody to Japanese encephalitis, dengue 1 and chikungunya virus. The percent of HI antibody for group A and group B agents decreases with increasing latitude and altitude.

6. Serial bleedings of Americans in Thailand in 1962 have shown that Japanese encephalitis virus was transmitted in May - August in Northeast Thailand, and that dengue and chikungunya viruses infected 12% of Americans in Bangkok during the THF epidemic. Americans infected in this latter area developed dengue fever not hemorrhagic fever and disease contracted by Caucasian children was remarkably mild.

7. Over 90,000 mosquitoes collected during the THF outbreak from 5 representative study sites in Bangkok were tested for virus. Most of the 41 isolates came from Aedes aegypti suggesting this species is the important vector of both chikungunya and dengue viruses. Three strains of Japanese encephalitis virus have been isolated from Southeast Thailand in October and November 1962.

8. Pre- and post-season bleedings have been obtained from 2000 residents of 19 randomly selected study sites in Bangkok. In these areas 147 children had an illness diagnosed as hemorrhagic fever, and 9 died. Studies are in progress to compare the inapparent infection rate with overt attack rate for the various viruses causing THF.

9. No evidence of dengue virus infection of birds and mammals was obtained in Bangkok during the report period. Antibodies thought to be caused by chikungunya and by Japanese encephalitis virus infection were prevalent in large domestic animals; Japanese encephalitis antibodies were found in several avian species with evidence of virus transmission in April, May and June, 1962.

ANNUAL PROGRESS REPORT

Project No. 3A-0-12501-A-811, MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

Ecology and Control of Diseases Vectors and Reservoirs

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Division of Communicable Disease & Immunology

Special Activity: U.S. Army - SEATO Medical Research Laboratory
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A10112501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA (Ecology and Control of Disease Vectors and Reservoirs)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Large collections of mosquitoes were made in many localities in Thailand in order to determine the number of species, their distribution, abundance, and habits. Some 1100 birds and 1000 mammals were examined for ectoparasites during the year. Seventy-three species of chiggers and 14 species of ticks were found, many of these being new records for Thailand or new to science.

* United States National Museum
** University of Maryland
*** United States Public Health Service
**** Local hire personnel

BODY OF REPORT

Project No. 3A-0-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTH EAST ASIA

Ecology and Control of Disease Vectors and Reservoirs (Arthropods fo Medical Importance in Overseas Areas)

Description:

Arthropods of many types are collected in Thailand, either in special studies relating to the arthropods themselves, or in search of material for the isolation of disease organisms. All of the medically important species, as well as the hosts of ectoparasitic forms, are catalogued, and information is accumulated whenever possible on their habits, distribution and seasonal abundance. All of this information will be prepared for presentation in the form of monographs and distributional lists.

Progress:

1. The mosquito Fauna of Thailand (SEATO MEDIC Study #47)

a. Since July 1962 over 3200 pinned preparations and over 3800 slides of mosquito larvae, pupae and genitalia have been made. A large number of these consist of asociated reared stages of various species. Collections have been made at many localities in Thailand, with most emphasis on the Bangkok and Chiengmai areas. The Central region to the north of Bangkok and the Northeastern region are more poorly represented than the balance of the country, but arrangements have been made to cover these areas more adequately. The genera and included number of species of mosquitoes of Thailand are presented in Table I. Included in the same table are the numbers of each genus now represented in the collections of this organization, and the number of apparently new species in the collection.

TABLE I

Genus	Species: Reported	SMRL Collection	New
Anopheles	57	34	1
Adedes	66	47	15
Aedeomyia	1	1	-
Armigeres	21	17	1
Ayurikitia	1	1	-
Culex	41	28	16*

TABLE I (cont.)

Genus	Species: Reported	SMRL Collection	New
Ficalbia	8	4	3
Heizmannia	10	8	1
Hodgesia	2	-	-
Malaya	2	2	-
Mansonia	9	6	-
Orthopodomyia	5	4	1
Topomyia	8	4	6
Toxorhynchites	8	5	5
Tripteroides	8	6	6
Udaya	1	1	-
Uranotaenia	20	12	4

* Includes a number of Lophoceraomyia which may be known species, analysis of these is still incomplete.

In the listings in Table I the column listing species reported is an edited compilation of published mosquito records for Thailand. As noted in the table, some of the species listed as new may represent inadequately described species. Samples have been sent to specialists with access to the types, and corrections will be made as the identifications are received. Specimens from the present collections and additional reference specimens from other institutions have been sent to the 406th Medical General Laboratory for illustration by artists there. These will be combined with text prepared in this laboratory in the form of a monograph on the Culicidae of Thailand.

b. A special survey of the mosquitoes of the Don Muang International Airport was performed in January and February, at the request of the Royal Thai Air Force. A number of important species were found during the survey, and the results were compiled for use by the RTAF in planning for control purposes.

c. Over two thousand larval, light and bait trap and human biting collections were made in the forested mountains and foothills in the Chiengmai area. These mountains are among the best studied areas from the viewpoint of mosquito taxonomy in Thailand, since a well developed road allows access to several forest types at altitudes up to 5000 feet. However, little was known of the biting habits of the mosquitoes in the forests or of other aspects of their life histories. Light traps were operated simultaneously at various heights on the mountain, and in the town. Simultaneous biting collections were made from human bait on the mountain and in Chiengmai. During the dry season two platforms were

installed for study of the canopy mosquitoes after the onset of the rainy season in June. From November to June when almost no mosquito larvae could be found in natural breeding sites (streams, tree holes, bamboo, etc.) large numbers of Tripteroides, Armigeres and Aedes larvae were collected from artificial breeding sites. These consisted of bamboo sections set at ground level and at various heights in trees, and clay pots set on the ground. Analysis of this large series of larvae is still underway. It appears that the aestivating form of many species of forest mosquitoes is the inseminated female; and that these will lay eggs whenever sufficient water is available. Twenty-nine species of Aedes have been collected biting man in the jungle, of which Finlaya and Stegomyia are the predominant subgenera. Many of these are day biting species. Also included in the biting collections were: nineteen species of Anopheles, ten species of Armigeres, fourteen Culex, three Mansonia and six Heizmannia.

2. The Culicoides of Thailand (SEATO MEDIC Study #48)

During routine study of light trap collections for removal of mosquitoes large number of Culicoides, the biting midges, are encountered. These gnats are serious pests of man and domestic animals and are suspected vectors of viruses and other organisms in some parts of the world. Aliquots of the midges were removed from light trap collections in Bangkok, Chiengmai, Udorn, Don Muang, and Bang Phra. These were sent to the United States National Museum for identification. Six hundred and forty four (644) vials of Culicoides have now been separated and forwarded for identification. All of these are from light trap collections. Only twelve collections of biting specimens have been made, despite extensive day and night biting collections for mosquitoes in many areas of Thailand. Several investigations have been made of complaints of biting under circumstances where Culicoides seemed to be involved, but these have been negative. It appears that the biting midges are at most of local or limited significance as human biters in Thailand. Species previously reported from Thailand or identified thus far from the present collections include: Culicoides anophelis, andrewsi, clavipalpis, oorti, daleki, denmeadi, elbeli, flaviscutatus, flavescens, gewertzi, guttifer, housei, hegneri, hewitti, huffi, humeralis, macfei, exystoma, orientalis, perigrinus palpifer, raripalpis, shermani, shorti, similis and sumatrae. Preliminary examination of the specimens from the present collection indicate that this list of twenty-six species is likely to be tripled.

3. Ectoparasites of the vertabrates of Thailand (SEATO MEDIC Study #49)

a. Relatively little work had been done on the ectoparasites of the mammals and birds of Thailand before the initiation of this study. Thus, work on a number of zoonoses transmissible to man by arthropods was hampered by a lack of knowledge of the systematics of the vertebrates, as well as the ectoparasite. During the year considerable progress was made on check lists and host and locality distribution lists.

b. During the year beginning 1 July 1962, one thousand one hundred and sixty-one (1161) birds were examined for ectoparasites. The majority of the hosts were preserved as study skins, and found to belong to two hundred and fourteen (214) species. Six hundred and seventeen of the hosts were parasite infested, with the following groups represented: Mallophaga-474; chiggers-62 ticks-38; other Acarina - 178; fleas - none; Pupipera - 64; other arthropods-9. Collection of birds is continuing at a low level, with primary emphasis being placed on ground dwelling species which often serve as hosts of chiggers and seed ticks.

c. During the same period eight hundred and thirty-four (834) mammals were examined for ectoparasites. As with the birds, study skins were prepared in the majority of cases. This collection has been examined by a cooperating scientist (Dr. J. Harrison, University of Singapore), and a check list of the mammals of Thailand is in preparation, based on the present collection and published records. A list of the fifty-seven species of mammals examined, and the ectoparasite types found on them is presented in Table II. The mammals listed in Table II were collected from a number of sites in Thailand, chiefly in the Northern and Northeastern regions. In addition, a number of mammals, chiefly rats, were collected within two hundred kilometers of Bangkok, during April and May, 1963 in connection with scrub typhus investigations. A very large percentage of these (Table III) were found infested with chiggers.

d. Chiggers have been by far the most important group of ectoparasites examined thus far, because of the demands of the rickettsial disease project (SEATO MEDIC Study # 46). Identifications have been made in cooperation with Dr. R. Traub, University of Maryland, and Mr. M. Nadchatram, Institute for Medical Research, Malaya. Three thousand nine hundred and thirty-five (3935) permanent slides have been made this year, in addition to temporary mounts prepared in the field. Seventy three (73) species have been identified thus far, from thirty species of vertebrate hosts. All of the material given definite study thus far is from Northern and Northeastern Thailand. It is apparent from this sampling that the number of species of chiggers in the country will be substantially higher than the seventy-three species thus far segregated. Specimens of eight new species of Leptotrombidium have been sent to artists at the 406th Medical General Laboratory for illustration for early publication. The genus Leptotrombidium in Thailand is extremely complex, and will require much additional study. In addition to L. akamushi and L. deliensis sensu strictu, the classical vectors of scrub typhus in Southeast Asia, there are four members of the "deliensis complex" and twelve thus far unnamed species in the collection. Other genera identified thus far include: Microtrombicula, Blankaartia, Siseca, Chiroptella, Reidlinea, Helenicula, Neoschongastia, Laurentella, Schoutedenichia, Walchia, Gahrlepia, Schongastiella, Walchiella, Traubacarus and Acomatacarus.

e. Tick collections are being identified in cooperation with Dr. C. M. Clifford, U.S. Public Health Service. Many of the specimens collected thus far have been larvae and nymphs, for which only generic identifications

are possible at present. Associated reared stages and sibling series will be necessary for definite identification. Adults identified thus far include the following: Amblyomma javanense, Aponoma lucsi, A. pattoni, Argus vespertillionis, Dermacentor auratus, Haemaphysalis dentipalpis, H. doenitzi, H. leachii, H. leachii indica, H. megalaimae, H. traguli, H. wellingtoni, H. hyptricis, H. species s, H. species t, Ixodes granulatus, I. radfordi, I. spinicoxalis, Rhipicephalus haemaphysaloides and R. sanguineus.

f. Other Acarina have been submitted to the Bishop Museum, Honolulu, and distributed by that institution to various specialists. Reports have been requested only for the mites of possible medical importance, chiefly the mesostigmatic mites. The following genera have been found thus far: Haemolaelaps, Laelaps, Echinolaelaps, Pellonyssus, Ornithonyssus, Hypolaspis, Ancystopus, Paraperiglyschus and Hirstionyssus. The collections of these various species are still too scant to permit evaluation of the seasonal and host abundance in Thailand. Also, their medical importance in this area cannot be evaluated as yet.

g. Thirteen species of Anoplura and eleven species of parasitic flies (Pupipara) have been found on birds and mamals in these collections.

TABLE II

THAILAND MAMMAL COLLECTIONS JULY 1962 - JUNE 1963

	No. Examined	No. Parasite infested	Lice	Chiggers	Ticks	Mites	Fleas	Pupipara	Others
Suncus murinus	6	6	1	3	-	4	1	-	-
Crocidura horsfieldi	2	2	-	2	-	-	-	-	-
Tupaia glis	63	59	8	53	30	9	11	1	-
Hylomys suillus	3	3	-	3	-	1	-	-	-
Cynocephalus variegatus	2	0	-	-	-	-	-	-	-
Pteropus vampyrus	7	7	-	-	-	4	-	7	-
Rousettus amplexicaudatus	3	3	-	-	-	2	-	2	-
Cynopterus brachyotis	85	15	-	-	2	3	-	10	-
Rhinolophus luctus	1	1	-	1	-	1	-	-	-
Rhinolophus sp.	4	1	-	1	-	1	-	-	-
Hipposideros sp.	11	11	-	10	1	6	-	6	-

TABLE II (cont.)

THAILAND MAMMAL COLLECTIONS JULY 1962 - JUNE 1963

	No. Examined	No. Parasite infested	Lice	Chiggers	Ticks	Mites	Fleas	Pupipara	Others
Scotophilus temmincki	4	4	-	-	-	2	-	2	-
Bat	88	73	-	8	1	4	-	71	-
Nycticebus coucang	4	2	-	1	1	1	1	-	-
Macaca sp.	1	1	1	-	1	-	-	-	-
Homo sapiens	6	6	-	1	5	-	-	-	-
Manis javanica	1	1	-	1	-	-	-	-	-
Felis bengalensis	3	3	1	2	-	-	-	-	1
Viverricula indica	2	1	1	1	1	-	-	-	1
Paradoxurus hermaphroditus	4	3	-	2	1	-	-	-	-
Arctogalidia trivirgata	6	5	-	5	3	2	-	-	-
Paguma larvata	1	1	-	1	-	-	-	-	-
Arctictis binturong	2	2	-	1	2	-	-	-	-
Melogale personata	2	2	-	1	2	-	-	-	-
Lepus siamensis	8	6	1	-	3	-	4	-	-
Featurista petaurista	7	2	-	-	-	2	1	-	-
Petaurista elegans	1	0	-	-	-	-	-	-	-
Hylopetes phayrei	15	11	8	4	1	3	3	-	-
Hylopetes spadiceus	1	0	-	-	-	-	-	-	-
Collosciurus erythraeus	16	14	-	10	1	5	9	-	-
Callosciurus caniceps	16	14	2	10	1	8	-	1	-
Callosciurus finlaysoni	5	5	2	3	1	3	1	-	1
Callosciurus notatus	2	0	-	-	-	-	-	-	-
Callosciurus lowei	1	1	-	-	-	1	-	-	-
Tamiops macclellandi	36	24	8	9	3	7	14	-	-
Ratufa bicolor	5	4	-	2	1	4	-	-	-
Ratufa affinis	1	1	-	-	1	1	-	-	-
Dremomys rufigenys	1	1	1	-	1	1	1	-	-
Menetes berdmorei	13	13	4	10	6	5	4	-	-

TABLE II (cont.)

THAILAND MAMMAL COLLECTIONS JULY 1962 - JUNE 1963

	No. Examined	No. Parasite infested	Lice	Chiggers	Tick	Mites	Fleas	Pupipara	Others
Rattus norvegicus	33	33	1	-	-	33	1	-	-
Rattus sp.	32	18	2	7	3	17	7	-	1
Rattus rattus	104	89	26	56	11	58	8	-	3
Rattus exulans concolor	81	26	4	2	1	14	17	-	-
Rattus sabanus	6	6	3	6	2	3	1	-	1
Rattus rajah	26	26	2	20	12	26	9	-	3
Rattus niviventer	54	54	10	43	7	54	9	1	3
Rattus berdmorei	6	3	-	3	3	2	-	-	-
Rattus mulleri	1	0	-	-	-	-	-	-	-
Mus famulus	1	1	-	-	-	1	-	-	-
Mus Musculus	4	4	1	-	-	4	-	-	-
Mus pahari	6	5	-	5	1	4	-	-	-
Bandicoto indica	26	22	7	9	11	10	4	-	-
Connomys badius	8	7	4	1	-	6	-	6	6
Rhizomys sumatrensis	2	2	1	2	-	1	-	-	-
Tragulus javanicus	3	2	-	1	1	-	1	1	-
Muntiacus muntjak	1	0	-	-	-	-	-	-	-
Domestic Ox	1	1	-	-	1	-	-	-	-
Total 57 species	834	607	98	301	122	313	107	108	19

TABLE III

MAMMAL COLLECTIONS APRIL 1963 - May 1963

	No. Examined	No. Parasite infested	Lice	Chiggers	Tick	Mites	Fleas	Fly	Others
Tupaia glis	22	18	2	15	6	13	-	-	-
Herpestes javanica	5	5	2	5	-	-	-	-	-
Melogale personata	2	0	-	-	-	-	-	-	-
Menetes berdmorei	33	20	13	20	3	10	2	-	-
Bandicota indica	37	37	5	37	12	10	-	-	-
Rattus berdmorei	7	7	-	5	-	6	-	-	-
Rattus rattus	72	70	12	70	4	35	6	-	-
Rattus exulans	4	3	-	3	1	1	1	-	-
Total 8 species	182	160	34	155	26	75	9	-	-

Fleas have generally been quite scarce, and very few species have been collected. In April and May fairly large numbers of Xenopsylla cheopis were taken from Rattus sp. and the areas in question are being revisited at intervals to determine the size of the population.

4. Insecticide tolerance of mosquitoes in Thailand (SEATO MEDIC Study #50)

a. The insecticide susceptibility of laboratory and wild strains of Culex pipiens quinquefasciatus was tested by means of the adult and larval test kits supplied by the World Health Organization. Aedes aegypti from the laboratory colony, and wild females from Bangkok showed approximately equal susceptibility to DDT, yielding an LC-50 of approximately 1.0% and an LC-90 of approximately 3.0%. These are consistent with results obtained elsewhere. Another chlorinated hydrocarbon, dieldrin, gave an LC-50 of 0.1% for A. aegypti females. The larvae of A. aegypti from the laboratory colony yielded an LC-50 of 0.1% with DDT. Wild larvae have not been tested as yet.

b. Culex pipiens quinquefasciatus adults from the laboratory colony and wild caught females showed approximately the same end points when exposed to DDT; and LC-50 of 0.5% and an LC-90 of 2.0%. The only noticeable indication of enhanced tolerance in the limited series of tests thus far was an LC-50 of 1.6% for dieldrin with Culex pipiens quinquefasciatus females. Dieldrin has been widely sold in Bangkok under a well known propreitary name. This

possible tolerance will be explored further. Culex larvae were tested against DDT and benzene hexachloride. The DDT gave an LC-50 of 0.1% and the BHC gave an LC-50 fo 0.03% and LC-90 of 0.5%.

Summary and Conclusions

1. Over seven thousand specimens of mosquitoes of permanent scientific interest were added to the Department collections this year. Many of these will be used as the basis for illustrations of the approximately three hundred species of Thai mosquitoes in a projected monograph. The Department collections now contain some two hundred species of mosquitoes, including fifty-nine species which may be new to science. A large number of species were found attacking man in the forested areas in day and night biting collections, and large numbers of larvae were attracted to artificial breeding sites in the forest, providing a simple tool for surveying at least part of the forest mosquito population.

2. Over six hundred collections of Culicoides from light traps, and small number from human biting collections, were processed and sent to the United States National Museum for identification. Twenty-six species are now known to occur in Thailand, but preliminary examination of the collections indicated the presence of many more species. Human biting by Culicoides has not thus far been found to be a serious problem in the forests of Thailand.

3. Ectoparasites were collected from approximately eleven hundred birds and one thousand mammals during the year. In the majority of cases, skins of the birds and skins and skulls of the mammals were preserved for further study. Seventy three species of chiggers, many of them new, and fourteen species of ticks have been found. These two groups are the most interesting ectoparasites found from the viewpoint of medical problems. Fleas were generally very scarce.

4. The insecticide tolerance of Aedes aegypti larvae and adults of Bangkok origin is well within normal limits for DDT and dieldrin, as tested by the standard World Health Organization methods. Culex pipiens quinquefasciatus in the Bangkok area appeared to be normally susceptible to DDT in the adult and larval stages, and to benzene hexachloride in the larval stage. Some evidence of tolerance to dieldrin was detected in the adult stage of C. quinquefasciatus in that the LC-50 was found to be 1.6%, the highest concentration offered by the WHO kit.

ANNUAL PROGRESS REPORT

Project No. 3A-0-12501-A-811, MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

(Acute Gastroenteritis)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Division of Communicable Disease & Immunology

Special Activity: U.S. Army - SEATO Medical Research Laboratory
APO 146, San Francisco, California

Department of Bacteriology & Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Sidney Gaines, Lt. Colonel, MSC

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A-0-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

(Acute Gastroenteritis)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Author: Sidney Gaines, Lt. Colonel, MSC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. Salmonellosis was found to be the primary cause of acute diarrhea in Bangkok, Shigellosis playing a relatively minor role. Twenty four different species of Salmonella and 11 strains of Shigella were isolated.

2. Of 686 Escherichia coli strains examined, 124 (18.1%) were shown to be enteropathogenic types. The large majority of these were isolated from children under 2 years of age, the most common types being 025:B19:B23, 0125:B15, and 0119:B14.

3. In a study of normal Thai individuals, it was shown that a significant portion of the population harbors enteric pathogens, particularly the Salmonellae. Of particular interest is the wide variety of Salmonella species isolated.

4. A specially-prepared holding medium for the collection of stool specimens or rectal swabs has been found, in the tests conducted to date, to maintain the viability of pathogenic as well as normal enteric bacterial flora for at least a week following inoculation of the medium. An advantage of this medium is that refrigeration prior to use, and incubation at 37°C following inoculation are not required.

5. A study of the development of diarrheas in a group of U.S. soldiers newly-arrived in Thailand demonstrated that even in an area where diarrheas are endemic, it is possible to keep troops relatively free of enteric infections if proper mess discipline is maintained.

6. Salmonella and Shigella were shown to be responsible for several outbreaks of diarrhea in U.S. and Thai personnel in up-country areas in Thailand during the year.

7. A variety of activities other than research were carried out. Included among these were examination of canal water in Bangkok for vibrios, identification of cultures submitted by various organizations, and bacteriologic examination of a variety of clinical specimens, water samples, foods and other materials.

BODY OF REPORT

Project No. 3A-O-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

(Acute Gastroenteritis)

Description:

Enteric diseases in Thailand are being studied in order to determine the types, distribution, and importance of enteric pathogens involved in diarrheas in Thai nationals and in U.S. personnel.

Progress:

1. Bacteriologic Survey of Stools from Patients with Acute Diarrhea (SEATO MEDIC Study #60)

a. During the period covered by this report, specimens from acute diarrhea patients from 9 Bangkok hospitals cooperating with this laboratory were obtained. A total of 2621 rectal swabs from 1869 patients (414 adults, 1455 children) were submitted to the laboratory. The results obtained show that from 406 (15.5%) of the swabs, 24 different Salmonella species were isolated. The 406 positive specimens were obtained from 305 different patients, indicating that 16.3% of all the patients were positive for Salmonella in one or more specimens submitted. Forty one of the 406 Salmonella were isolated from 40 adults, while the remaining 365 were cultured from 265 children. Thus, 9.7% of the adult patients and 18.2% of the children were positive for these organisms. In a number of instances, 2 different Salmonella species or a Salmonella and a Shigella species were cultured from the same specimen.

b. Of the 24 different Salmonella species isolated, 6 were Group B, 7 Group C, 5 Group D, and 6 Group E. Salmonella montevideo, Group C_1, was by far the most frequent isolate, this species accounting for 231 of the 365 Salmonella isolated from children. In no instance was this organism cultured from adults.

c. Shigella species were isolated from 56 patients (10 adults, 46 children), and included 11 different strains. Ten isolates were obtained from adults and 50 from children. Thus, 2.9% of all the patients examined (2.4% of the adults, 3.2% of the children) were positive for these organisms. Flexner bacilli were the most frequently encountered, accounting for 61.7% of all the Shigella isolated.

d. Numerous paracolon bacilli were cultured from both children and adults, 989 of the 1869 patients examined (52.9%) being positive for one or more of these organisms. Approximately 58% of the adults and 51% of the children yielded paracolons from their stools. As many as 3 different species

were obtained from a number of individuals, sometimes from the same sample. A total of 309 paracolons were cultured from the 241 positive adults, and 1087 from the 748 positive children. Paracolobactrum aerogenoides was the most frequently isolated paracolon species, although significant numbers of P. coliforme and P. intermedium, including Bethesda - Ballerup bacilli, also were found. Providencia strains, on the other hand, were cultured infrequently.

e. In addition to the above, many isolates of Proteus and Pseudomonas species were obtained from the specimens examined. Occasional isolates of Arizona bacilli also were made.

2. Occurrence of Enteropthogenic Escherichia coli in Thailand (SEATO MEDIC Study #61)

In order to determine the kinds and distribution of enteropathogenic Escherichia coli associated with diarrheas in Thailand, a study of these organisms, initiated during the previous year, continued during the time covered by this report. During this period, 686 isolates of E. coli were examined. Employing 12 typing sera provided by The Walter Reed Army Institute of Research (WRAIR), 124 (18.1%) of these organisms were shown to be enteropathogenic. These cultures were isolated from individuals of various ages and from both sexes, the large majority, however, being found in children under 2 years of age. The most common types encountered have been serotypes 025:B19:B23, 0125:B15, and 0119:B14, representing together about 70% of all the types isolated.

3. Bacteriologic Survey of Stool from Normal Individuals (SEATO MEDIC Study #63)

In collaboration with the Department of Public Health Administration and the Department of Epidemiology of the Thai Ministry of Health, and with the Department of Microbiology, School of Public Health, a bacteriologic survey of stools from normal, apparently healthy Thai nationals was initiated. Three communities within a radius of 8 miles from Bangkok were selected for the study. One is rural, another is urban, and the third is a mixture of the 2 types. Approximately 500 persons of all ages and of both sexes from each area have been examined. To date, of 1466 individuals, Salmonella have been isolated from 185 (12.6%), and Shigella from 9 (0.6%). Thirty different Salmonella species have been found, the most frequently isolated ones belonging to Groups B and E. Eight different Shigella species have been isolated. Many paracolon bacilli, including members of the Bethesda-Ballerup and Providence groups, also were recovered from the stools examined. In addition, Proteus and Pseudomonas were cultured frequently, and Arizona bacilli were isolated on occasion. Among the individuals harboring Salmonella, there were a few cases from whom 2 different species were cultured, and in one instance, a Salmonella and a Shigella were isolated from a single stool specimen.

4. Testing & Evaluation of a Holding Medium for Enteric Bacteria (SEATO MEDIC Study #64)

a. Inasmuch as the handling of clinical specimens before they reach the laboratory often decides the fate of the microorganisms present, it is important that the media employed in the collection of these specimens are effective in maintaining the viability of the organisms, particularly if a considerable period of time elapses between collection and examination of the specimens. One of the major problems with collecting media has been the necessity of getting them to the laboratory as soon as possible to avoid loss of sensitive bacteria and overgrowth by interfering organisms. For this reason a joint project on the evaluation of a holding medium for preserving enteric bacteria in clinical material was undertaken with the Department of Bacteriology, WRAIR. This medium has the advantage that minimal equipment is needed in storage and in collection of specimens. Refrigeration for storing the medium prior to use is neither required nor even desirable, and incubation at 37°C following inoculation likewise is not desirable.

b. The medium and specially prepared swabs for obtaining stools or rectal contents have been provided by the Department of Bacteriology, WRAIR, while the Department of Bacteriology & Immunology of this laboratory (SMRL) has been responsible for the collection and processing of the specimens. Duplicate and in some instances triplicate swabs were taken from each stool and placed in either 2 or 3 vials of holding medium. On vial was sent to the Department of Bacteriology, WRAIR, for examination, the second vial was cultured in this laboratory 24 hours after the specimen was obtained, and if triplicate swabs had been taken, the third vial was cultured in this laboratory 1 week later. All vials were kept at room temperature at all times, including during shipment to WRAIR.

c. The results obtained to date on approximately 200 fecal specimens collected in the holding medium are quite promising, inasmuch as the findings are as good or better than the results obtained by the routine procedure employed in this laboratory for the collection and culture of stool specimens or rectal swabs. Eighteen Salmonella, 10 Shigella, 18 enteropathogenic E. coli, 20 Providence bacilli, and many other enteric bacteria have been isolated from the specimens examined. Of interest is the observation that the viability of the pathogenic, as well as normal enteric flora appears to be well maintained for at least one week following inoculation of the holding medium.

5. Diarrhea in U.S. Troops Newly Arrived in Thailand (SEATO MEDIC Study #62)

a. In an attempt to determine the incidence of diarrheas developing in U.S. soldiers entering into an area where diarrheas are endemic, a group of newly-arrived troops were followed during their stay in Thailand near the city of Korat. Baseline information on enteric bacteria in these soldiers was obtained by taking rectal swabs from the men immediately following debarkation from the airplanes which ferried them into the Korat area.

Two or three plane-loads of men arrived each day for a period of 10 days, and a total of 1487 rectal swabs were secured during this time. Two of the men were found to harbor Group E Salmonella, the remainder showing only normal intestinal flora. During the several months the men were in Thailand, they were observed for the development of diarrhea. Rectal swabs, taken from those individuals coming down with severe or mild diarrhea, were sent to this laboratory for culture through the cooperation of the 31st Field Hospital and the Joint Task Force 116 Dispensary in Korat. A surprisingly small number of cases of clinical diarrhea occurred in the troops (according to information from the Medical Officers of the hospital and dispensary servicing the troops), and this laboratory received a total of only 48 specimens for culture. From these specimens 1 Salmonella, 2 Shigella, and 2 Providence strains were isolated. Two enteropathogenic E. coli cultures were found. The remainder were Proteus, Pseudomonas, paracolon bacilli, and a variety of coliforms. Several non-agglutinating vibrios belonging to Heiberg groups I, II, and V also were cultured.

b. Prior to the departure of the troops from Thailand, a random sampling of the men was made to determine whether any had developed asymptomatic bowel populations that included species of Salmonella or Shigella. Swabs obtained from approximately 300 of the men were cultured. In only one instance a Salmonella species was isolated, and in another, an enteropathogenic E. coli strain was found. Paracolon bacilli, coliforms, and Proteus species made up the bulk of the remaining isolations.

c. The small number of diarrhea cases occurring in these troops was probably due to the fact that most meals were taken in the troop messes, with only relatively few meals being eaten in local food establishments. Food preparation and handling in the troop messes was done only by U.S. personnel, while locally hired mess employees were utilized for cleaning and other activities not involving the preparation of food. The results of this study speak well for the sanitary and hygienic disciplines employed in the troop messes, and also for the food discipline shown by the men themselves.

6. Diarrhea in U.S. Personnel in Thailand (SEATO MEDIC Study #65)

a. A number of diarrhea outbreaks in Thai and U.S. personnel in various parts of Thailand were investigated during the period covered by this report. In one explosive outbreak in a U.S. military unit located in the vicinity of Korat, Shigella sonnei 1 was the organism responsible. Specimens from 15 individuals were obtained and of these, 12 were positive for this organism. All of the personnel had eaten all their meals in the company mess, but examination of samples of all the food served in the mess hall during the 2 days prior to the outbreak, along with a water sample from the unit mess failed to show the presence of any pathogenic enteric organisms. Antibiotic therapy consisted of either chloramphenicol or tetracycline, and all patients responded well within 24-48 hours.

b. In 3 other mild outbreaks in both Thai and U.S. personnel, Salmonella species were primarily involved.

7. General Information

a. In addition to research, this department performed a variety of other activities, Included among these were periodic examinations of klong (canal) waters from 10 different klongs in the Bangkok area for the presence of cholera vibrios, inasmuch as cholera has occurred in the past in Thailand, and in view of the extensive cholera outbreaks in Southeast Asia during the past two years. To date, neither true cholera nor EL Tor vibrios have been isolated, although non-agglutinating vibrios have been cultured from some of the samples of klong water.

b. Bacteriologic support has been and is being provided to a number of Thai and U.S. agencies, both civilian and military. Clinical specimens, water samples, food samples, cultures for identification, and other materials have been submitted for bacteriologic examination. In addition, this department has acted in a consultant capacity on bacteriologic matters to several Thai and U.S. organizations.

Summary and Conclusions

1. Salmonellosis was found to be the primary cause of acute diarrhea in Bangkok, Shigellosis playing a relatively minor role. Twenty four different species of Salmonella and 11 strains of Shigella were isolated.

2. Of 686 E. coli strains examined, 124 (18.1%) were shown to be enteropathogenic types. The large majority of these were isolated from children under 2 years of age, the most common types being 025:B19:B23, 0125:B15, and 0119:B14.

3. In a study of normal Thai individuals, it was shown that a significant portion of the population harbors enteric pathogens, particularly the Salmonellae. Of particular interest is the wide variety of Salmonella species isolated.

4. A specially-prepared holding medium for the collection of stool specimens or rectal swabs has been found, in the tests conducted to date, to maintain the viability of pathogenic as well as normal enteric bacterial flora for at least a week following inoculation of the medium. An advantage of this medium is that no special equipment, such as a refrigerator or an incubator, is required for collecting specimens.

5. A study of the development of diarrheas in a group of U.S. soldiers newly-arrived in Thailand demonstrated that even in an area where diarrheas are endemic, it is possible to keep troops relatively free of enteric infections if proper mess discipline is maintained.

6. Salmonella and Shigella were shown to be responsible for several outbreaks of diarrhea in U.S. and Thai personnel in up-country areas in Thailand during the year.

7. A variety of activities other than research were carried out. Included among these were examination of canal water in Bangkok for vibrios, identification of cultures submitted by various organizations, and bacteriologic examination of a variety of clinical specimens, water samples, foods, and other materials.

ANNUAL PROGRESS REPORT

Project No. 3A-0-12501-A-811, MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

Parasitic Diseases

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D.C.

Division of Communicable Disease & Immunology

Special Activity: U.S. Army - SEATO Medical Research Laboratory
APO 146, San Francisco, California

Department of Medical Zoology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: D. E. Wykoff, Major, B.S., M.S., Ph.D.
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Assistants: S. Vajrasthira, M.D., MPH-TM*
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S. Soavakontha, M.D.*
M. M. Winn, MSgt, AMEDS

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

* Bangkok School of Tropical Medicine

ABSTRACT

Project No. 3A-0-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

(Parasitic Diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: D. E. Wykoff, Major, Ph.D,
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. A total of 5003 residents of remote northeast Thai villages were examined for the presence of hepatic and intestinal parasites. Based on a single stool specimen concentrated by the formalin-ether technic 3906 (78%) were found to harbor Opisthorchis viverrini. Excluding children under the age of 11, the average prevalence was 86%. It is now conservatively estimated that 3.5 million persons in Thailand are infected.

2. The snail intermediate host of O. viverrini in Thailand has been found to be Bithynia goniomphalos (Morelet 1866) of which over 150,000 have been collected and examined. The cercaria has been identified. The life cycle of this parasite has been observed for the first time.

3. Eighteen hundred and ninety-six fresh water fish have been obtained, identified and examined for metacercariae of O. viverrini. Pla measadaeng (species being determined) and Mystacoleucus artridorsalis harbored the largest average number of metacercariae per fish (over 100). The metacercariae were most prevalent in Hampula dispar (93% of 155 specimens) and M. artridorsalis (95% of 18 specimens). None of the fish collected in Bangkok were infected, all positive fish coming from the North East.

4. In order to determine whether non-domesticated animals and birds are natural reservoir hosts of O. viverrini in northeast Thailand, a total of 448 such animals have been trapped. None was found to harbor this parasite. Approximately 30% of 100 dogs were infected as were some 60% of 60 cats.

5. Studies have been made to determine the usefulness of hamsters as experimental definitive hosts. Data on prepatent period, percentage of metacercariae developing to adulthood, eggs per gram feces and eggs per worm per day have been gathered.

6. While it is believed that all human hepatic trematodes in northeast Thailand are O. viverrini, a study has been undertaken to study the morphological variations within this species.

7. Various clinical symptoms are believed caused by O. viverrini. However, only few data have been gathered on their specific relationship. A series of liver function tests, coupled with stool and physical examinations, have been undertaken on patients in the Udorn hospital. The data from the first 490 patients have not yet been fully studied and conclusions are not yet possible.

* Bangkok School of Tropical Medicine

BODY OF REPORT

Project No. 3A-0-12501-A-811 Title: PREVENTIVE MEDICINE

(Parasitic Diseases)

Description:

The primary purpose of these investigations was to study the various biological and clinical aspects of the human liver fluke Opisthorchis viverrini in Thailand.

Progress:

1. The prevalence of O. viverrini in remote northeastern Thai villages. (SEATO MEDIC Study #20)

a. A study designed to determine the prevalence and distribution in areas near the Thai-Lao border, based on single stool specimens concentrated by the formalin-ether technic.

b. Data in Table I indicate that of 5003 examinees, 4285 (86%) harbored parasites; 80% of all examinees harbored helminths and 21% harbored protozoa. A total of 3906 persons (78%) were found to be infected with O. viverrini. Data in Table II (age - sex distribution of O. viverrini) indicate that an average of 74% of this population of 4683 harbored the liver fluke. Excluding age group 0-5 the average prevalence was 84%, while the prevalence in persons over age 10 was 86%. The populations represented in Tables I and II are the same, the differences in figures reflect data from two villages not yet analyzed for age-sex incidence.

c. The prevalence of O. viverrini is uniformly high throughout the areas near the Thai-Lao border. It can now be estimated conservatively that 3.5 millions of persons are infected in Thailand and likely several millions more in the neighboring countries.

TABLE I

	Number	%
No Persons Examined	5003	
No. with Parasites	4285	85.6
No. with helminths	4005	80.0
No. with protozoa	1025	20.5
Opisthorchis	3906	78.1
Hookworm	960	19.2
Strongyloides	474	9.5
Taenia	151	3.1
Whipworm	24	0.5
Ascaris	23	0.5
E. histolytica	43	0.9
E. coli	441	8.8
E. nana	338	6.8
Giardia	368	7.4

TABLE II

Age-Sex Distribution of Opisthorchis Infections in Ten Remote North East Thai Villages

	MALES			FEMALES			TOTAL		
Age	No.	+	%	No.	+	%	No.	+	%
0-5	421	151	35.9	412	68	16.5	833	219	26.3
6-10	427	290	67.9	352	311	88.4	779	601	77.2
11-15	320	293	91.6	293	272	92.8	613	565	92.2
16-20	197	176	89.3	255	229	89.8	452	405	89.6
21-25	159	144	90.6	180	155	86.1	339	299	88.2
26-30	170	150	88.2	332	177	53.3	502	327	65.1
31-35	122	109	89.3	109	99	90.8	231	208	90.0
36-40	123	107	87.0	127	116	91.3	250	223	89.2
41-45	90	83	92.2	80	71	88.8	170	154	90.6
46-50	92	80	87.0	85	79	92.9	177	159	89.8
51-55	47	43	91.5	73	67	91.8	120	110	91.7
56-60	52	48	92.3	45	40	88.9	97	88	90.7
61-65	29	27	93.1	15	14	93.3	44	41	93.2
66-70	21	19	90.5	14	11	78.6	35	30	85.7
70	22	21	95.5	19	16	84.2	41	37	90.2
Tot/Ave	2292	1741	76.0	2391	1725	72.1	4683	3466	74.0

Prevalence Excluding 0-5 Year Group = 84.3%

Prevalence Excluding 0-10 Year Group = 86.2%

2. Snail intermediate hosts (SEATO MEDIC Study #21)

a. A study designed to determine which snails act as first intermediate hosts of O. viverrini in Thailand, the proof of this to be the elucidation of the entire life cycle.

b. During the past year, two members of the Family Bithynidae, one considerably more common than the other, were found in the endemic area. The more common species was collected and placed in small water-filled dishes for a period of from 2 to 12 hours. It was found that 10 types of cercariae were shed by this more common snail species, but none were recognized from the literature. Of these 10, two cercariae generally resembled those of O. filineus and Clonorchis sinensis, i.e. lophocercous with two pigmented eyespots. Since each snail shed only a single type of cercaria, those harboring these two types were placed in separate aquaria. Several species of cyprinoid fish (Cyprinus carpio and Carassius aulatus) were purchased in Bangkok where there was little chance of natural infection. These were sent to Udorn where 10% were selected at random and carefully examined for metacercariae. In all cases these fish were found to be free of natural infection, and were placed in the aquaria with the selected snails. Within a few minutes these cercariae attached themselves to the scales and gills of the fish, shed their tails, and within four hours were starting to be encapsulated. Development of the metacercariae was permitted to continue in the fish for a period of from 2 to 4 months, after which the fish were sacrificed and examined. One of the two types of lophocercous-eyespot cercariae produced metacercariae which were morphologically indistinguishable from those of O. viverrini. These metacercariae were then fed to hamsters and cats which were first proved free of natural infection by repeated stool examinations. A period of from 2 to 3 months was permitted to elapse and these animals were then sacrificed. Trematodes recovered from the livers of these animals were identified as Opisthorchis viverrini. The snails have been identified as Bithynia (Bulimus) (Digoniostoma) goniomphalos (Morelet 1866). The cercaria, under study, is lophocercous with two pigmented eyespots.

c. The life cycle of Opisthorchis viverrini has been observed for the first time. The findings do not rule out the possibility that snails other than Bithynia goniompholos may also act as first intermediate hosts. A study of the inter-molluscan phases of the cycle is being initiated, as is a study of the cercarial morphology.

3. Fish intermediate hosts of Opisthorchis viverrini in Thailand (SEATO MEDIC Study #22)

a. This study was designed to determine which fish harbor the metacercariae of O. viverrini and to determine the relative importance of these positive species in the transmission of the disease.

b. A total of 1751 fresh water fish have been carefully examined for the presence and numbers of O. viverrini metacercariae. Fish were obtained both from the endemic area and from Bangkok. None of those from

the latter area were found to be infected. The data in Table III indicate that of 133 Pla maesadaeng (genus and species being determined) 50% harbored metacercariae with an average of 16 per fish. Of 155 Hampala dispar (Pla Suud), 93% were positive with an average of 20 metacercariae per fish. The largest average number of metacercariae per fish (105) was found in Pla Pog, the genus and species of which are under study.

c. The findings indicate the relative importance of several species of fresh water fish. Inasmuch as the results fluctuate with the seasons, further studies will be made periodically throughout the year.

TABLE III

EXAMINATION OF FRESH-WATER FISH FROM THAILAND FOR METACERCARIAE OF OPISTHORCHIS VIVERRINI

1 Oct - 31 Dec 1962

Scientific name:	Common name:	Number Examined	Obtained From:	With No.	O.v. %	Ave.no.meta. per fish:
1. Being determined	Pla Namong	36	SN	1	2.8	12
2. Puntius gonionotus	Pla Dra Pien Kau	19	SN	1	5.3	48
3. Hampala dispar	Pla Suud	6	SN	5	82.5	8
4. Puntioplites proctogysron	Pla Gramang	6	SN	0	0	-
5. Osteochilus hasseltii	Pla Soi Nok Kau	13	SN	0	0	-
6. Puntius orphoides	Pla Gam Cham	8	SN	0	0	-
7. Puntius spilopterus	Pla Howakaeng	6	SN	0	0	-
8. Labiobarbus	Pla Sua Sai	5	SN	0	0	-
9. Being determined	Pla Drapien Sai	26	SN	9	34.5	12
10. Mystacoleucus artridorsalis (Fowler)	Pla Khiyok Nhom Lhang	18	Udorn	17	94.5	103
1 Jan - 31 Mar 1963						
11. Being determined	Pla Na Mong	46	SN	2	4.34	0.28
12. Being determined	Pla Pog	235	SN	189	80.42	105.17
13. Being determined	Pla Maesadang	133	SN	67	50.37	16.15

TABLE III (cont.)

EXAMINATION OF FRESH-WATER FISH FROM THAILAND FOR METACERCARIAE OF OPISTHORCHIS VIVERRINI

1 Jan - 31 Mar 1963

Scientific name:	Common name:	Number Examined	Obtained From:	With No.	O.v. %	Ave.no.meta. per fish:
14. Being determined	Pla Sew	164	SN	17	10.36	0.61
15. Puntius goniono-tus	Pla Dra Pien Kau	34	SN	1	2.941	2.52
16. Hampula dispar	Pla Suud	155	SN	125	92.59	20.41
17. Puntioplites proc-togyaron	Pla Gramong	112	SN	9	8.04	0.14
18. Osteochinlus has-seltii	Pla Soi Nok Kau	100	SN	-	4.34	0.28
19. Puntius orphoi-des	Pla Gam Cham	99	SN	27	27.27	1.25
20. Puntius spilop-terus	Pla Kowakaeng	93	SN	-	-	-
21. Labiobarbus Lineatus	Pla Sau Sai	28	SN	1	3.57	0.21
22. Being determined	Pla Drapien Sai	104	SN	37	35.57	1.96
23. Being determined	Pla Soi Kriept Daeng	77	SN	-	-	-
	Pla Soi Kriept Kau	18	SN	-	-	-
24. Being determined	Pla Sua	28	SN	-	-	-
25. Being determined	Pla Chalad	5	SN	-	-	-
26. Pungius viehoe ver	Pla Drapien	100	SN	7	7	1.85
27. Hampula dispar	Pla Suud	2	BKK**	-	-	-
28. Osteochilus hasseltii	Pla Soi Nok Kau	32	"	-	-	-
29. Puntius veiheoveri	Pla Drapien	73	"	-	-	-
30. Being determined	Pla Measadang	17	"	-	-	-
31. Being determined	Pla Mau Thai	17	"	-	-	-
32. Being determined	Pla Ka Yeng	4	"	-	-	-

TABLE III (cont.)

EXAMINATION OF FRESH-WATER FISH FROM THAILAND FOR METACERCARIAE OF OPISTHORCHIS VIVERRINI

1 Jan - 31 Mar 1963

Scientific name:	Common name:	Number Examined	Obtained From:	With O.v. No.	%	Ave.no.meta. per fish:
33. Being determined	Pla Nua On	5	BKK**	-	-	-
34. Being determined	Pla Kra Dee	5	"	-	-	-
35. Being determined	Pla Chalad	16	"	-	-	-
36. Puntioplites proctogysron	Pla Gamang	13	"	-	-	-
37. Labiobarbus	Pla Sau Sai	26	"	-	-	-

* Lake at Sakol Nakorn
** Bangkok

4. Animal Reservoir Hosts (SEATO MEDIC Study #23)

a. This study was designed to provide information on (1) which animals are natural reservoir hosts of O. viverrini, (2) ectoparasites for the Department of Entomology (3) blood specimens for the Department of Virology, (4) information on the species and distribution of animals in northeast Thailand, (5) kidney and liver specimens for Leptospira work, (6) infected animals for the study on the pathogenesis of opisthorchiasis (see report by the Department of Pathology) and (7) to provide preserved mounted skins for the U.S. National Museum.

b. The following types of animals have been captured in the scrub area of northeast Thailand. Bat (insect eating) 2, Bat, (fruit) 119, rats (both Rattus exulans and R. rattus) 140, bandicoots 75, vultures 8, mongoose 2, porcupine 1, birds 4, pangolin 1, palm civit 1, monkey 1, flying squirrel 1. Larger animals in heavy brush nearer the Thai-Lao border have been avoided as much as possible because of inadequate weapons. Tigers and bears have been seen but with one of the two 12 ga. shotguns loaded with # 9 shot for birds, it seemed unrealistic to attempt capture of the larger animals with the other 00-buckshot-loaded shotgun. All animals were handled so as to provide the information required in (a) above. Livers were examined for Opisthorchis but none were found.

c. While semi-domesticated dogs and cats harbor O. viverrini it is interesting to note that none have been found in the wild animals collected. Fish are eaten more commonly by wild animals in this area of the world than might be expected, because rice and fish constitute the bulk foodstuffs available for consumption both by man and animals. Limited experimental infections of wild animals will be undertaken during the coming year.

5. Experimental Laboratory Hosts (SEATO MEDIC Study #24)

a. A study designed to provide basic biological information on the use of laboratory animals as experimental definitive hosts.

b. A total of 1450 Opisthorchis eggs were measured. The mean length was 28.0 (21.9 to 32.2); the mean width was 16.4 (11.5 to 21.9). Cubic values (length times square of width) ranged from 3516 to 14902 with a mean of 7650 cubic units. The prepatent period in hamsters averaged 24 days. The percentage of administered metacercariae which were subsequently recovered as adult worms in the liver ranged from 40 to 94% (mean 70%). Animals were given graded numbers of metacercariae (50, 100 and 200) and the percentage development to adult by group was 51.80 and 69%, respectively. For these same group, the average number of eggs per worm per day were 89.18 and 4. As shown in Table IV the number of animals used was small, but it appears that the larger inocula resulted in the production of fewer eggs per worm. However, even in animal #18 which had an average of only 1 egg per worm per day being passed in the stool viable adult worms were recovered at autopsy. These findings may stem from the small size of the hamster liver and parallel studies are to be undertaken in rabbits.

c. The findings begin to provide a general background for experimental infection of hamsters. The small number of eggs per worm per day in hamsters is subject to further study.

TABLE IV

EXPERIMENTAL INFECTION OF HAMSTERS WITH METACERCARIAE OF OPISTHORCHIS VIVERRINI

Animal No.	No. of Metacercariae	Prepatent period (da)	Days after infection when sacrificed	No. Adult worms recovered	% Metacercariae developing to adults	Average E/GF/D*	Average GF/D**	Average total daily egg output per gram	Average No. E/W/D***
1.	50	14	30	20	40	N/C	N/C	N/C	N/C
2.	50	23	30	24	48	N/C	N/C	N/C	N/C
3.	50	22	45	23	46	2667	0.6696	1768	78
4.	50	25	E	-	-	-	-	-	-
5.	50	25	60	34	68	3276	1.0248	3369	99
6.	50	25	E	-	-	-	-	-	-
7.	100	25	30	94	94	N/C	N/C	N/C	N/C
8.	100	25	30	52	52	N/C	N/C	N/C	N/C
9.	100	25	45	64	64	1658	1.0632	1763	28
10.	100	25	45	83	83	2085	0.7790	1624	20
11.	100	25	60	97	97	804	1.0542	848	9
12.	100	25	60	87	87	1452	0.8792	1276	15
13.	200	24	30	117	59	N/C	N/C	N/C	N/C
14.	200	24	30	139	69	N/C	N/C	N/C	N/C
15.	200	22	45	155	78	1238	0.8066	998	6
16.	200	24	45	135	68	685	0.8445	578	6
17.	200	24	60	130	65	701	0.8230	577	4
18.	200	24	60	149	75	203	0.8592	174	1

E Animal escaped

* Eggs per gram feces per day. Average of 15 counts, made 3 times daily for the five days preceeding date of sacrifice

** Grams feces per day. Average total daily fecal output measured daily for the five days preceeding date of sacrifice.

*** Average number of eggs per worm per day.

N/C Not counted: duration of infection too short.

6. Morphology of Opisthorchis viverrini (SEATO MEDIC Study #25)

a. Differentiation of O. viverrini and O. felineus is often difficult for major specific points of differentiation are lacking. Various investigators have stated what they believe to be the most important differences, but there is a lack of agreement among these workers. The comparative characteristics of O. viverrini include more lobulated testes and ovaries, a different type and distribution of the vitellaria, greater proximity of the testes and ovary, extention of the intestinal ceca into the extreme posterior end, location of the posterior testes near the tip of the intestinal ceca, and the relatively more elongated esophogus. This study is designed to more carefully compare these differences.

b. Adult O. viverrini have been collected from human autopsies, naturally infected cats and dogs, and from experimentally infected hamsters. Camera lucida drawings have been made to demonstrate the variation of each characteristic mentioned above. Comparative drawings of O. felineus have likewise been prepared. None of the single morphological differences appears to constitute and absolute guide for differentiation.

7. Clinical manifestations of opisthorchiasis (SEATO MEDIC Study #26)

a. Over 90% of the adult population in the Udorn area harbor hepatic trematodes and it would not be surprising to find certain clinical symptoms and syndromes associated with this infection. Ascites, edema, enlargement of the collateral circulation and of the abdomen, gastro-intestinal disturbances, anorexia,malaise and cachexia are believed to be caused by this parasite. However, only few data have been gathered on the specific rolationship of the symptoms to the presence of the parasite. This study is being undertaken to determine whether Opisthorchis causes a specific disease, and if so to define its nature.

b. At the Provincial Hospital, Udorn Thani (Dr Kasem, Director), all patients are examined by one of our staff (Dr Sastri) and the following information is recorded on a card prepared for this purpose - name, age, sex, address, ethnic background (Thai, Thai-Lao, Thai-Vietnam, etc), occupation, symptoms, results of physical, stool, blood count and blood chemistry (liver function) examinations. These data will subsequently be transferred to IBM cards. At the present some 60 patients are being studied weekly. The results are not yet ready for this report.

Summary and Conclusions

1. A total of 5003 residents of remote northeast Thai villages were examined for the presence of hepatic and intestinal parasites. Based on a single stool specimen concentrated by the formalin-ether technic 3906 (78%) were found to harbor Opisthorchis viverrini. Excluding children under the age of 11, the average prevalence was 86%. It is now conservatively estimated that 3.5 million persons in Thailand are infected.

2. The snail intermediate host of O. viverrini in Thailand has been found to be Bithynia goniomphalos (Morelet 1866) of which over 150,000 have been collected and examined. The cercaria has been identified. The life cycle of this parasite has been observed for the first time.

3. Eighteen hundred and ninety-six fresh water fish have been obtained, identified and examined for metacercariae of O. viverrini. Pla measadaeng (species being determined) and Mystacoleucus artridorsalis harbored the largest average number of metacercariae per fish (over 100). The metacercariae were most prevalent in Hampula dispar (93% of 155 specimens) and M. artridorsalis (95% of 18 specimens). None of the fish collected in Bangkok were infected, all positive fish coming from the North East.

4. In order to determine whether non-domesticated animals and birds are natural reservoir hosts of O. viverrini in northeast Thailand, a total of 448 such animals have been trapped. None was found to harbor this parasite. Approximately 30% of 100 dogs were infected as were some 60% of 60 cats.

5. Studies have been made to determine the usefulness of hamsters as experimental definitive hosts. Data on prepatent period, percentage of metacercariae developing to adulthood, eggs per gram feces and eggs per worm per day have been gathered.

6. While it is believed that all human hepatic trematodes in northeast Thailand are O. viverrini, a study has been undertaken to study the morphological variations within this species.

7. Various clinical symptoms are believed caused by O. viverrini. However, only few data have been gathered on their specific relationship. A series of liver function tests, coupled with stool and physical examinations, have been undertaken on patients in the Udorn hospital. The data from the first 490 patients have not yet been fully studied and conclusions are not yet possible.

ANNUAL PROGRESS REPORT

Project No. 3A-0-12501-A-811, MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

Parasitic Disease

The Pathogenesis of *Opisthorchis viverrini* Infections

I Morbid Anatomic Changes in Naturally Infected Cats and Dogs in Udorn

II The Pathogenesis in Laboratory Infected Cats

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Division of Communicable Disease

Special Activity: U.S. Army - SEATO Medical Research Laboratory
APO 146, San Francisco, California

Department of Pathology
Department of Medical Zoology

Period Covered by Report: 29 January 1963 through 30 June 1963

Principal Investigators: Sylvanus W. Nye, Captain, USAF, MC
D. E. Wykoff, Major, Ph.D.

Assistants: B. Laixuthai, M.C.*
C. Harrinsuta, M.D., Ph.D.**
P. Juttijudata, M.D.**

Report Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

* Royal Thai Army Hospital
** Bangkok School of Tropical Medicine

ABSTRACT

Project No. 3A-O-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA (Parasitic Diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 29 January 1963 through 30 June 1963

Authors: S. W. Nye, Captain, USAF, MC
D. E. Wykoff, Major, Ph.D.
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P. Juttijudata, M.D.**

Report Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

The SEATO Medical Research Laboratory is studying the life cycle of Opisthorchis viverrini and the infection in hamsters and rabbits. Study of the pathogenesis in the natural and experimental definitive hosts has not been undertaken. In the first part of this study the morbid anatomic changes in cats with natural infections will be described and correlated with the intensity of the infection and the extent of disease. Second portion of the study has not been completely designed and will study the reaction of the host tissues to known numbers of metacercaria and a known duration of infection. Eleven cats have been collected in Udorn and autopsied. Before the animals were autopsied similar cats were studied to determine the intensity the infection in each animal and to estimate the severity of the infection in the autopsied animals. The autopsies have been completed but histopathological studies have not started.

* Royal Thai Army Hospital
** Bangkok School of Tropical Medicine

BODY OF REPORT

Project No. 3A-0-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA (Parasitic Diseases)

Description:

The first part of this study will describe the morbid anatomic changes in natural infections with Opisthorchis viverrini and an attempt will be made to correlate the intensity of the infections with the extent of disease.

Cats and a few dogs will be obtained by the Department of Medical Zoology in villages highly endemic for Opisthorchis viverrini. The animals will be housed separately and the stool of each animal examined daily for four days. Animals not infected will be withdrawn from the study. Five cats will be separated and the average number of eggs/gram of feces/worm will be determined by stool counts and by counting all of the worms in the liver of these animals. Animals to be autopsied will be studied for three days with stool counts so that the number of worms/animal may be estimated. The animals will be sacrificed and a complete autopsy will be performed. Frozen and formalin fixed specimens will be preserved from all organs. Thick and thin blood smears will be made and the lungs will be cultured for bacteria as well as for fungus. A complete bacteriological survey of each animal will be done. The lungs will be perfused with formalin for further study of parasitic infections. Fixed and frozen tissues will be returned to Bangkok of hostopathological study.

Progress:

Equipment for autopsy and bacteriological study was shipped to Udorn 12 May and 11 infected cats were autopsied. 3 infected cats have had stool counts and will be sacrificed shortly so that accurate counts of the worms in the livers can be made. Unaffected cats are being held for the second part of the study.

Summary and Conclusions:

None

ANNUAL PROGRESS REPORT

Project No. 3A-O-12501-A-811, MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

Microbiology and Pathology

Pathological and Etiological Survey of Naturally Occuring Infection and Infestations

Study Pathological Survey of Vertebrate Fauna Trapped in Udorn and Chiengmai Regions

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Division of Communicable Disease

Special Activity: U.S. Army - SEATO Medical Research Laboratory
APO 146, San Francisco, California

Department of Pathology

Period Covered by Report: 29 January 1963 through 30 June 1963

Principal Investigator: Sylvanus W. Nye, Captain, USAF, MC

Report Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A-0-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

Microbiology and Pathology

Pathological and Etiological Survey of Naturally Occuring Infection and Infestations

Study Pathological Survey of Vertebrate Fauna Trapped in Udorn and Chiengmai Regions

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 29 January 1963 through 30 June 1963

Author: Sylvanus W. Nye, Captain, USAF, MC

Report Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

The SEATO Medical Research Laboratory is at present collecting large numbers of wild animals in the Chiengmai, Bangkok and Udorn areas for study of ectoparasites and endoparasites. This study will combine the information obtained by other SEATO projects with the histopathologic lesions found in the viscera of the trapped animals. At the time these animals are checked for ectoparasites, intestinal parasites, blood parasites, leptospira and Rickettsia, the tissues are put into 10% buffered formalin. At present only 15 to 20 animals have been received in Bangkok and the histopathologic preparations have not been completed.

BODY OF REPORT

Project No. 3A-0-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

Microbiology and Pathology

Pathological and Etiological Survey of Naturally Occuring Infection and Infestations

Study Pathological Survey of Vertebrate Fauna Trapped in Udorn and Chiengmai Regions

Description:

This project hopes to correlate the information being obtained by projects in the Entomology and Medical Zoology departments with the histopathological lesions in the viscera of the animals they collect. A list of the pathologically identifiable diseases of the animals trapped will be prepared. Animals are being trapped by the Entomology Department in Udorn, Chiengmai and Bangkok. These animals are checked for ecto-parasites, intestinal parasites, blood parasites, leptospira and Rickettsia. At the time of that examination heart, lungs, liver, spleen, kidneys, bladder, rectum and gonads are put into buffered formalin. Specimens are returned to Bangkok and histologic preparations will be made in the Royal Thai Army pathology laboratory. The slides prepared will be examined by the principal investigator. Correlations will be made with the Entomology and Medical Zoology departments. It is hoped that the information obtained by histopathologic examination will help to establish whether parasitised animals are diseased and therefore indicate the significance of the parasitism to the host animals. The nature of the disease in the reservoir host may help to explain the incidence of transmission of the infecting agent to man and domestic animals.

Progress:

15 - 20 specimens have been received.

Summary and Conclusions:

No results are available at this time.

BODY OF REPORT

Project No. 3A-0-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

Microbiology and Pathology

Pathological and Etiological Survey of Naturally Occuring Infection and Infestations

Study Pathological Survey of Vertebrate Fauna Trapped in Udorn and Chiengmai Regions

Description:

This project hopes to correlate the information being obtained by projects in the Entomology and Medical Zoology departments with the histopathological lesions in the viscera of the animals they collect. A list of the pathologically identifiable diseases of the animals trapped will be prepared. Animals are being trapped by the Entomology Department in Udorn, Chiengmai and Bangkok. These animals are checked for ecto-parasites, intestinal parasites, blood parasites, leptospira and Rickettsia. At the time of that examination heart, lungs, liver, spleen, kidneys, bladder, rectum and gonads are put into buffered formalin. Specimens are returned to Bangkok and histologic preparations will be made in the Royal Thai Army pathology laboratory. The slides prepared will be examined by the principal investigator. Correlations will be made with the Entomology and Medical Zoology departments. It is hoped that the information obtained by histopathologic examination will help to establish whether parasitised animals are diseased and therefore indicate the significance of the parasitism to the host animals. The nature of the disease in the reservoir host may help to explain the incidence of transmission of the infecting agent to man and domestic animals.

Progress:

15 - 20 specimens have been received.

Summary and Conclusions:

No results are available at this time.

ANNUAL PROGRESS REPORT

Project No. 3A-0-12501-A-811, MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

(Tissue Culture Techniques)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Division of Communicable Disease & Immunology

Special Activity: U. S. Army - SEATO Medical Research Laboratory
APO 146, San Francisco, California

Department of Virology

Period Covered by Report: 1 July 1962 - 30 June 1963

Principal Investigators: Major Scott B. Halstead, MC
Dr. Pairatana Sukhavachana*
Dr. Ananda Nisalak**

Assistants: Dr. Rapin Snitbhan*
SSG Merlyn J. Funkenbusch

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

* Department of Microbiology, School of Public Health, Bangkok, Thailand.

** Local hire personnel

ABSTRACT

Project No. 3A-0-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA (Tissue Culture Techniques)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Major Scott B. Halstead, MC
Dr. Pairatana Sukhavachana*
Dr. Ananda Nisalak**
Dr. Rapin Snitbhan*
SSG Merlyn J. Funkenbusch

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Primary tissue explants (hamster kidney cells and macaca irus kidney cells) support the growth of high mouse-passage strains of dengue viruses types 1 - 6. Growth of virus is detected by development of resistance of cells to secondary challenge with a CPE producing virus. In addition, several stable cell lines (grivet monkey kidney, MK-2 rhesus kidneys and a stable pig kidney line) show sentitively superior to primary cell explants for growth of dengue viruses. It is hoped that these techniques will provide a tissue culture system suitable for primary isolation of dengue viruses.

* Department of Microbiology, School of Public Health, Bangkok Thailand

** Local hire personnel

Summary & Conclusions:

Primary tissue explants (hamster kidney cells and macaca iris kidney cells) support the growth of high mouse-passage strains of dengue viruses types 1 - 6. Growth of virus is detected by development of resistance of cells to secondary challenge with a CPE producing virus. In addition, several stable cell lines (grivet monkey kidney, MK-2 rhesus kidneys and a stable pig kidney line) show sentitively superior to primary cell explants for growth of dengue viruses. It is hoped that these techniques will provide a tissue culture system suitable for primary isolation of dengue viruses.

List of Publications:

Halstead, S.B. and Yamarat, C. Hemorrhagic Fevers of South-East Asia. Proc., 7th Int. Cong. Trop. Med. Mal., Rio de Janeiro. In publication.

ANNUAL PROGRESS REPORT

Project No. 3A-O-12501-A-811, MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

Zoonoses of Military Importance

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D.C.

Division of Communicable Disease & Immunology

Special Activity: U.S. Army - SEATO Medical Research Laboratory
APO 146, San Francisco, California

Department of Veterinary Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Thomas J. Keefe, Captain, VC
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Tubtim Vichitnetinaya, B.S.*
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

* Research & Education Division, Department of Livestock Development

** Local hire personnel

ABSTRACT

Project No. 3A-0-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

Zoonoses of Military Importance

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D.C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Thomas J. Keefe, Captain, VC
Achit Chotisen, D.V.M., Ph.D.*

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. a. A total of 341 wild rodents trapped in different parts of Thailand were culturally examined for leptospirosis. Twelve isolates have been obtained, and five of these have been identified as being in the grippotyphosa or javanica serogroups.

b. Isolation attempts were recently started to recover leptospires from kidneys of cattle and water buffalo collected at the Bangkok abattoir. Serological data from 121 cattle and swine disclosed 50% and 20% leptospiral agglutinins respectively. Duplicate tubes of 129 cattle cultures and 111 water buffalo cultures were examined for presence of leptospires. During this study 45 cattle kidneys and 29 water buffalo kidneys were passed into hamsters with no deaths attributable to leptospires. One isolate was recovered from a water buffalo kidney.

c. Surface water samples have been collected from two sites of effluent drainage (part of a canal system) from a large swine farm at Bangkae, Thailand. From 20 November 1962 to 20 March 1963, 69 water samples have been inoculated into 345 hamsters in an attempt to recover leptospires. All kidney culturing attempts to recover leptospires from these inoculated hamsters have been negative. This study was conducted during the dry season when no rain had fallen in the area since the advent of the study.

2. A serological survey for leptospirosis among the livestock of Thailand has disclosed a high prevalence of leptospiral agglutinins in cattle and swine. 50% of 156 normal cattle and 20% of 143 normal swine tested had leptospiral agglutinins. Agglutinins in cattle were demonstrated for 17 of 18 serogroups tested, the predominant ones being andaman, butembo,

pomona, hyos, and wolffi. Agglutinins in swine were demonstrated for 8 out of 18 serogroups tested; the predominant ones being bataviae and pomona at 31%, and alexi at 10% of the reactors.

* Research & Education Division, Department of Livestock Development

BODY OF REPORT

Project No. 3A-0-12501-A-811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

Zoonoses of Military Importance

Description:

The isolation of leptospires from various ecotypes in Thailand. SEATO MEDIC Study No. 80.

Progress:

1. The isolation of leptospires from various ecotypes in Thailand. (SEATO MEDIC Study #80)

a. (1) A study designed to develop a leptospiral program and diagnostic capability. Rodent kidney culturing was begun primarily to standardize laboratory technique.

(2) A total of 341 wild rodents were cultured for leptospirosis. Ninety-five of these (designated RCF) were trapped in the Chiengmai, Khon-Kaen, Loei areas. Two hundred and forty-six of these (designated RCA) were trapped at Bangkae, Thailand, surrounding a large swine farm. A WRAIR survey team collected the RCF cultures, inoculating kidney slices usually into each of six tubes of Fletcher's medium. The first 100 cultures of the RCA series were kidney slices inoculated into duplicate tubes of Fletcher's medium. There were no isolates from this latter group of cultures. In the continuing RCA series (101-136), 1:10 and 1:100 suspensions of each kidney grinding were prepared. One drop of each suspension was inoculated into each of three tubes of Fletcher's medium. The percentage of isolates was still unsatisfactory. In the continuing RCA series (136-246), one drop of a 1:10 suspension of kidney tissue was inoculated into each of three tubes of Fletcher's medium. Also, kidney plugs, using Pasteur pipetes, were inoculated into each of three tubes of Fletcher's medium. Serological tests, cultural examinations, and serogrouping were conducted according to previously described methods. Isolates were obtained from the kidneys of 12 rodents. Seven of these have not yet been serogrouped. Four isolates have been serogrouped, javanica; one isolate has been serougrouped, grippotyphosa. Isolates are sent back to WRAIR for serological confirmation and serotyping.

b. (1) A study designed to obtain qualitative and quantitative information on leptospiral shedding by domestic livestock. In view of animal husbandry practices causing close human-domestic animal relationships, this data will be an important epidemiological supplement to rodent transmission studies.

(2) Water buffalo and cattle kidneys had been picked up at the Bangkok abattoir for primary culturing and hamster inoculation. Specimens were procured from baskets of kidneys placed in a cold storage room of the abattoir. Kidney capsules were stripped in a sterile manner, and kidney plugs removed with a sterile Pasteur pipete. These plugs were: (a) Inoculated into Fletcher's medium - one tube, (b) Ground with Tenbroek grinder and inoculated into Fletcher's medium - one tube, (c) Tenbroek grinding inoculated into weanling hamsters. Kidney plugs and suspensions from 129 cattle kidneys were inoculated into duplicate tubes of Fletcher's medium. No leptospires were isolated from these cultures. Contamination was about 10%. Specimens from 45 cattle kidneys were inoculated into weanling hamsters. No hamsters died between 6-21 days. Kidney specimens from 30 of these hamsters were passed a second time. Again, no deaths occured between 6-21 days. Kidney plugs and suspensions from 111 water buffalo kidneys were inoculated into duplicate tubes of Fletcher's medium. One isolate (BCA 10) was obtained from these cultures. Contamination was about 15%. Specimens from 29 water buffalo kidneys were inoculated into weanling hamsters. No hamster died between 6-21 days. Kidney specimens from 21 of these hamsters were passed a second time. Again, no deaths occured between 6-21 days. The one isolate obtained in this series confirms suspicions that domestic livestock are a contributing source of pathogenic leptospires. After completion of preliminary work, attempts to recover leptospires from cattle, swine, and water buffalo kidneys have temporarily ceased. A revised protocol for the handling of abattoir specimens, and a smoothly functioning, rennovated kitchen will enable this series to recommence.

c. (1) A study designed to determine whether canal water coursing through a swine farm will support viable leptospires, and whether any relationship exists between recovery rate and rainy periods.

(2) Surface water samples were collected from two sites of effluent drainage (part of a canal system) from a large swine farm at Bangkae, Thailand. These were concentrated to 5 cc. aliquotes by filtration, and inoculated 1 cc. into each of five weanling hamsters. On a series of 69 water samples the following data was obtained:

# Samples	1st Pass. Hamsters	Die 1-5 days	Die 6-21 days	Cultures Matured	Cultures +	Cultures -
69	345	11	12	12	0	12

	2nd Pass. Hamsters	Die 1-5 days	Die 6-21 days	Cultures +	Cultures -
	2	1	1	0	1

This study was conducted during the dry seasor when no rain had fallen in the area since the advent of the study. This study will recommence in the rainy season when rain-washed land would be apt to contribute leptospires into the canals.

2. Serological Survey of leptospirosis (SEATO MEDIC #81)

a. This study was designed as a part of a continuing study to determine the extent, distribution, and potential significance of leptospirosis in an area which is important militarily.

b. A serological survey for leptospirosis among the livestock of Thailand has disclosed a high prevalence of leptospiral agglutinins in cattle and swine. Data obtained up to the present is listed in the following charts:

Serum tested:

Species	Number tested	Number Positive	Percent positive
Cattle	156	79	50.6
Swine	143	29	20.3
Rodents	25	6	24

Four-fold dilution started at 1:25
Source: Rajaburi, Cholburi, Nakorn-Pathom
Species: Cattle

	Dilution						
	25	100	400	1600	6400	25600	%Reactors
1. L. andaman	10	0	0	0	0	0	12.6
2. L. butembo	8	1	0	0	0	0	11.3
3. L. celledoni	1	0	0	0	0	0	1.3
4. L. bataviae	2	3	1	0	0	0	7.6
5. L. pomona	6	1	1	0	0	0	10.1
6. L. djasiman	1	1	0	0	0	0	2.5
7. L. hyos	5	3	1	0	0	0	11.3
8. L. autumnalis	4	0	0	0	0	0	5.1
9. L. ballum	1	0	0	0	0	0	1.3
10. L. caniccola	2	2	1	0	0	0	6.3
11. L. icterohemorrhagiae	1	0	0	0	0	0	1.3
12. L. pyrogenes	2	0	0	0	0	1	3.7
13. L. alexi	0	1	0	1	0	0	2.5
14. L. grippotyphosa	0	0	0	0	0	0	0
15. L. borincana	1	1	0	0	0	0	2.5
16. L. wolffi	3	5	1	0	0	0	11.3
17. L. javanica	4	0	0	0	0	0	5.1
18. L. australis	0	0	1	1	0	1	3.7

Four-fold dilution started at 1:100
Source: Rajaburi, Cholburi, Nakorn-Pathom
Species: Swine

	Dilution						
	100	400	1600	6400	25600	102400	%Reactors
1. L. andaman	0	0	0	0	0	0	0
2. L. butembo	2	0	0	0	0	0	6.8
3. L. celledoni	0	0	0	0	0	0	0
4. L. bataviae	5	3	1	0	0	0	31
5. L. pomona	7	2	0	0	0	0	31
6. L. djasiman	0	0	0	0	0	0	0
7. L. hyos	0	0	0	0	0	0	0
8. L. autumnalis	0	1	0	0	0	0	3.4
9. L. ballum	1	1	0	0	0	0	6.8
10. L. caniccola	1	1	0	0	0	0	6.8
11. L. icterohemorrhagiae	0	0	0	0	0	0	0
12. L. pyrogenes	0	0	0	0	0	0	0
13. L. alexi	2	0	0	0	0	0	10.3
14. L. grippotyphosa	0	0	0	0	0	0	0
15. L. borincana	1	0	0	0	0	0	3.4
16. L. wolffi	0	0	0	0	0	0	0
17. L. javanica	0	0	0	0	0	0	0
18. L. australis	0	0	0	0	0	0	0

Summary and Conclusions:

1. a. Twelve leptospiral isolates have been obtained from a series of 341 rodent cultures. Serogrouping is conducted at this installation, with serotyping and confirmation of results by the Veterinary Division at WRAIR. This study provided the basis for training technicians in the proper handling and culturing of leptospiral specimens during the developing of a leptospiral diagnostic capability with the Research and Education Division of the Department of Livestock Development.

b. One leptospiral isolate was recovered from a series of 111 water buffalo kidney specimens. No leptospiral isolates were recovered from a series of 121 cattle kidney specimens. Errors in sampling technique, an insufficient number of replicate cultures per specimen, and un-controlled handling of glassware all contributed tc low isolate yields in the study. A revised culturing protocol, and a smoothly functioning rennovated kitchen, should increase isolates in the work when the study re-commences.

c. Sixty-nine (69) surface water samples from two sites of effluent drainage from a canal system coursing through a large swine farm were inoculated into 345 weanling hamsters with no leptospiral isolates resulting. This study was conducted during a 4 month period during which time no rain had fallen on the study area. This study will re-commence during the rainy season when rain-washed land would be apt to contribute leptospires into the canal.

2. A serological survey for leptospirosis among the livestock of Thailand has disclosed that 50% of 156 normal cattle tested and 20% of 143 normal swine had leptospiral agglutinins. Water buffalo sera have not yet been tested. All of these sera were collected from areas about 70 kilometers south of Bangkok. This study will continue on domestic livestock sera collected from northern and north-eastern provinces to determine the qualitative and quantitative presence of leptospiral infection in these areas.

ANNUAL PROGRESS REPORT

Project No. 3A-0-12501-A-811, MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

Veterinary Medicine and Health of Animals

Development and Maintenance of Conventional Animal Colonies

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D.C.

Division of Communicable Disease & Immunology

Special Activity: U.S. Army - SEATO Medical Research Laboratory
APO 146, San Francisco, California

Department of Veterinary Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Thomas J. Keefe, Captain, VC
Lenly D. Wetherald, S/Sgt.(E-6), Vet.Spec.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A-0-12501-A-811

Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

Veterinary Medicine and Health of Animals

Development and Maintenance of Conventional Animal Colonies

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Thomas J. Keefe, Captain, VC
Lenly D. Wetherald, S/Sgt.(E-6),Vet.Spec.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. a. Continually increasing the production of laboratory mice and Hamsters has constituted the main effort with these animals. Rooms have been re-modeled and re-screened. Racks, cages, food hoppers, water bottles, food and bedding storage facilities have been added as needed. Facilities to boil water were added in the cleaning line to clean water bottles.

b. On 10 April, mites of the genus Ornithonyssus sp. were noticed in all of the mouse and hamster colonies. Cleaning of rooms and equipment, residiual spraying with 5% DDT, and dusting the animals with Dri-Die was about 50% effective. Since the use of 1% Malathion solution in the bedding, no mites have been noticed.

2. Initial attempts at keeping monkeys were generally unsatisfactory. Diarrhea, particularly within a group of monkeys new to the colony, would usually develop within a month after entrance into the colony. Shigella dysenteriae and Shigella flexneri, were each isolated from 2 monkeys which had died. Improved management practices and a controlled use of anti-diarrheal drugs have eliminated this problem.

BODY OF REPORT

Project No. 3A-0-12501-A-811

Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA

Veterinary Medicine and Health of Animals

Development and Maintenance of Conventional Animal Colonies

Description:

The primary purpose of this work is to provide healthy experimental animals to all departments of the SEATO Medical Research Laboratory. Conventional animal colonies are developed, maintained and expanded; or animals are purchased and maintained in keeping with the needs of the various departments.

Progress:

1. The development and maintenance of conventional animal colonies (SEATO MEDIC Study No. 85)

a. (1) This work is designed to determine and implement satisfactory methods for raising and maintaining laboratory animals.

(2) In keeping with the expansion of the animal colonies, approximately the following number of cages were designed and constructed locally:

(a) 790 hamster cages
(b) 800 mouse cages
(c) 6 guinea pig cages
(d) 100 rabbit cages
(e) 40 hamster shipping cages

Forty (40) monkey cages were re-constructed to improve durability. One-half of the mouse colony was installed in a re-modeled, air-conditioned room. The small hamster colony was moved into a re-modeled, air-conditioned room and expanded. The animal colony office was re-modeled and air-conditioned to also include a drug cabinet and stainless-steel treatment/operating table. The following graphs indicate the yearly production figures for mice and hamsters:

Graph # 1.

A - Mouse production, litters/day

Points 1 - 2: Indicates production based upon average set-ups of 560 females/week.

Points 2 - 3: Indicates decreased production due to decreased set-ups while aging female breeders for future breeders.

Points 3 - 4: Indicates production based upon average set-ups of 790 females/week.

Points 5 - 6: Indicates decreased production due to mite infestation, and possibly rise in ambient temperature.

B - Hamster production, litters/week

Points 1 - 2: Indicates rise in production of hamsters from a maintenance colony to one in which 25 females are set up every week.

Points 2 - 3: Indicates normal production based upon 25 - 30 females set-up every week.

Points 3 - 4: Indicates increased production from 30 set-ups each week to 120 set-ups.

Graph # 2.

A - Mice animals/litter:

Indicates constant litter size arrived at by selective breeding with mainly P2 and P3 generations, with a large number of animals.

B - Hamsters, animals/litter

Points 1 - 2: Indicates rather constant litter size arrived at by rather selective breeding with mainly P2 and P3 generations, with a moderate number of animals.

Points 2 - 3: Indicates decreased litter size arrived at by less selective breeding with P1, P2 and P3 generations. Pressure to increase colony size required the utilization of P1 animals for breeders.

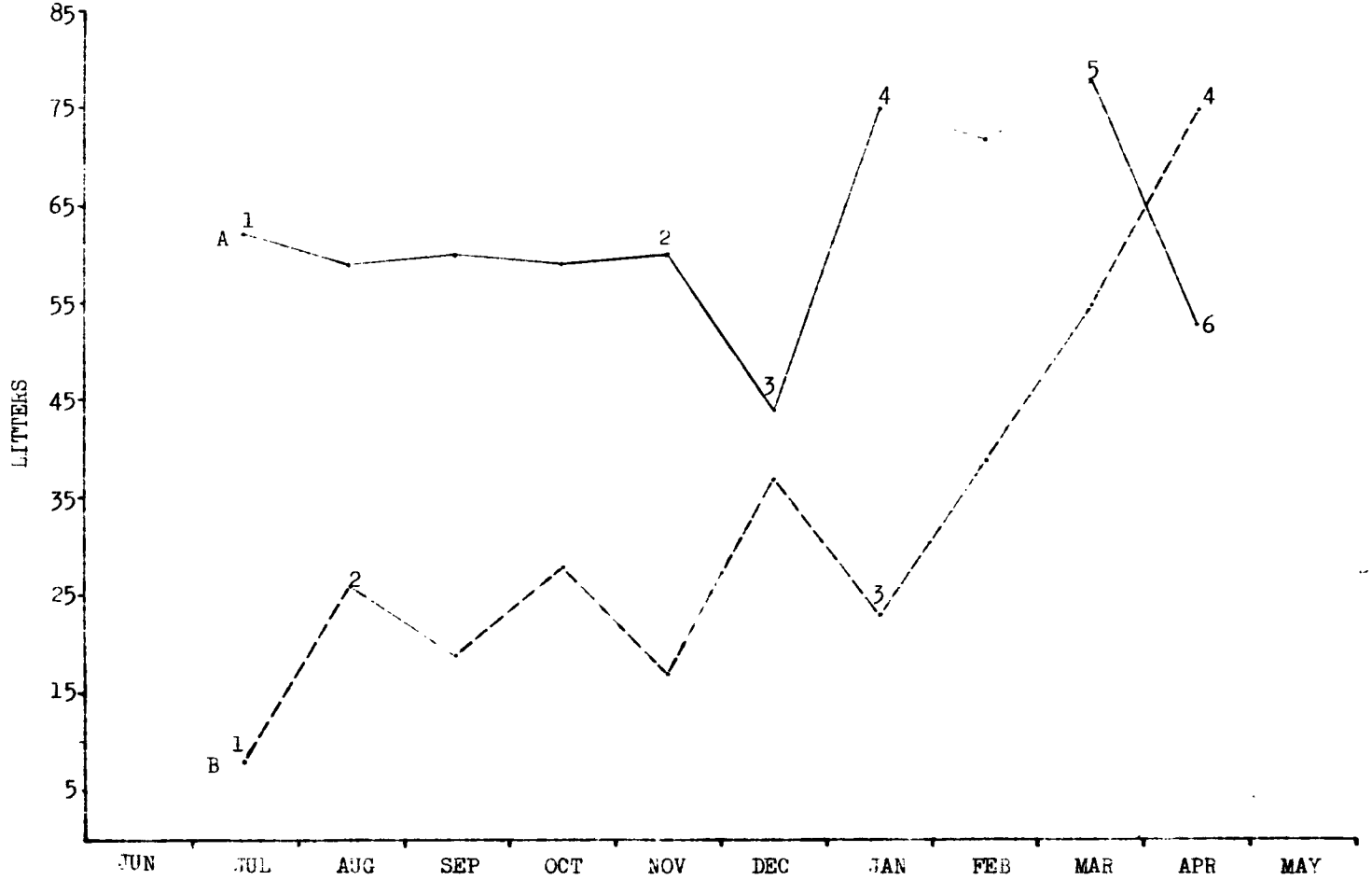
Points 3 - 4: Indicates an increasing litter size arrived at by using predominantly P2 and P3 generations for breeders.

GRAPH # I

LEGEND

(A)———— Mice, listed as average litters/day

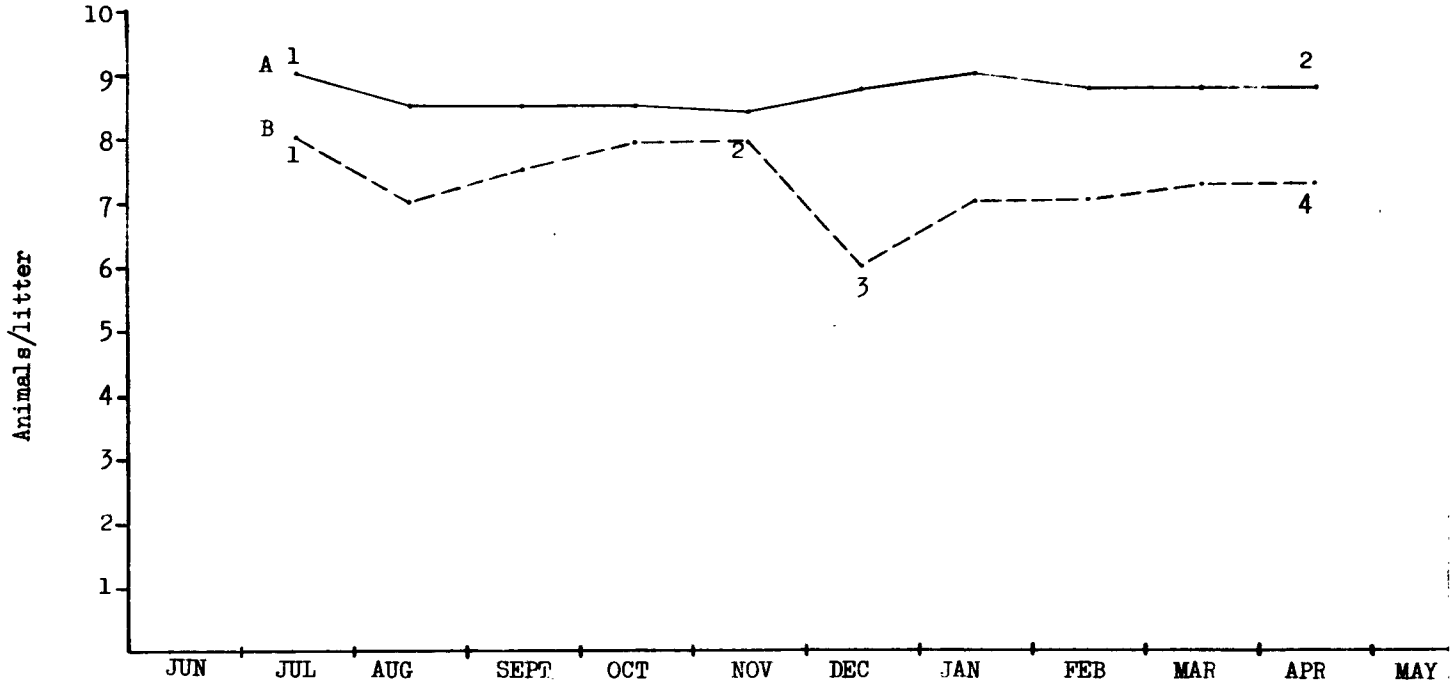
(B)— — — — Hamsters, listed as average litters/week



GRAPH # 2

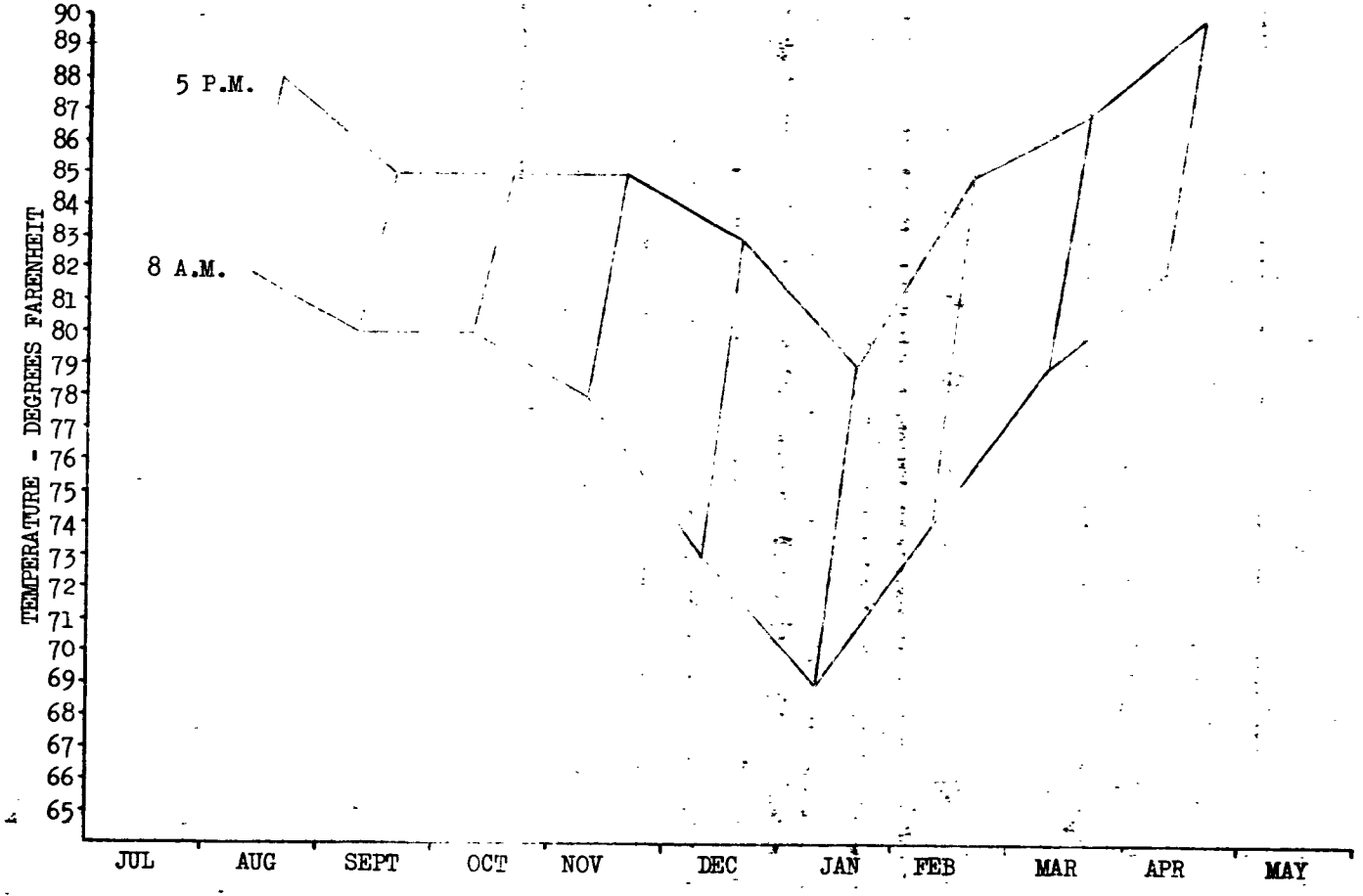
A _____ Mice, listed as animals/litter

B _ _ _ _ _ Hamsters, listed as animals/litter



AVERAGE A.M. & P.M. MONTHLY TEMPERATURES

OUTDOOR MOUSE COLONY



Graph # 3.

Indicates AM and PM average monthly temperatures in outdoor mouse colony. Because of manipulations of the breeding colonies (increasing set-ups between November and February, and the mite infestation) attempts to correlate increases or decreases in animal production with decreases or increases in ambient temperature are not possible.

Litters of suckling mice issued between 1 July 1962 and 30 April 1963 equal 10,608. Weanling mice issued between 1 July 1962 and 30 April 1963 equal 17,759. Hamsters issued between 1 July 1962 and 30 April 1963 equal 2,413.

b. On April 10, 1963, mites of the genus Ornithonyssus sp. were noticed in all of the mouse and hamster colonies. The mites were identified generically by Major John E. Scanlon, MSC, Chief, Department of Entomology, SEATO Medical Research Laboratory. Samples of the mite were sent to the Bishop Museum for positive identification. In retrospect, mouse production was noticed to have dropped off from a high of 79 litters/day in March to 53 litters/day by April 10. Concomitantly, seasonally warm days of 90°F had started. These high ambient temperatures may have added to the decrease in production.

Immediately, all the affected colonies were treated. Entire half-corridors were treated as units. All cages and racks from a room were removed and cleaned. Simultaneously the room was scrubbed and sprayed with 5% residual DDT. All animals were dusted with Dri-Die, For the entire operation (6 days) about 8,000 animals were individually dusted, and 8,000 cages individually cleaned. Two days after the operation ceased, mites were again noticed. Within one week, they were about 50% as prevalent as originally. Henceforth, as cages were routinely removed from rooms and cleaned, the new bedding was sprayed with 5 ml. of 1% Malathion solution.

After the first treatment, no live mites were noticed. This treatment was repeated, and then stooped. No mice or hamsters were lost due to any of the treatments or manipulations. During the course of cleaning the animal colony, a wild rat was flushed from a storage rack. Drains are now rat-proof, and local food is kept in rat-proof bins.

c. Prior to October 1962, poor results with maintaining monkeys were thought to be enhanced by a diet consisting wholly of bread, sweet potato and bananas. Oranges, cucumbers, peanuts, and crabs were added to this diet. Within one month after this change in diet, the monkeys had filled out and were noticeably more lively. Vitamins, (ABDEC drops) were spread on bread twice a week. All in-coming monkeys were given low level doses of terramycin in the drinking water for 10 days after arrival. All monkeys that developed soft stools were marked. The diet was decreased and kaopectate was administered, t.i.d. orally. One day later, if the diarrhea still

continued, paregoric was added to the kaopectate, along with Na and K salts, and stool specimens collected for bacteriological culturing. If the diarrhea still persisted, therapeutic levels of antibiotics were started until the diarrhea stopped.

Since October 1962, only 5 monkeys have developed a diarrhea lasting longer than 24 hours. These have all responded within 48 hours to treatment orally with broad-spectrum antibiotics. Since October 1962 no monkeys have died within the animal colony as a result of diarrhea.

Summary and Conclusions:

a. The continued expansion of all departments within the SEATO Medical Research Laboratory will necessitate further expansion of laboratory animal production. When mouse production again reaches the February '63 level of production, then production will have reached its maximum within present facilities. The completion of the new wing to this laboratory will provide additional, though not adequate, room for projected expansion.

Inquiries are being made for the procurement of still additional space for laboratory animal production.

b. A mite infestation was encountered within the mice and hamster colonies during the month of April 1963. Since the use of 1% Malathion solution in the bedding, no mites have been seen. It is still too early to foresee the long-range effectiveness of the control measures.

c. The maintaining of healthy monkeys within the colony of the SEATO Medical Research Laboratory has been made considerably easier by increasing the dietary protein and instructing the animal caretakers to recognize and report all animals off food or developing diarrhea. A controlled regimen of therapeutics for the handling of diarrhea cases has eliminated losses due to diarrhea with the monkeys.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 811 Military Medical Research Program in Southeast Asia (Rickettsial Diseases in Thailand).

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Rickettsial Diseases
Division of Communicable Diseases and Immunology

Period Covered by Report: 22 October 1962-30 June 1963

Principal Investigators: Bennett L. Elisberg, M.D.
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F. Marilyn Bozeman, M.S.
Major John E. Scanlon, MSC +
Captain Vichai Sangkasuvana, MC (RTA) ++
Major Samnieng Buspavanich, MC (RTA) ++
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 811

Title: Military Medical Research Program in Southeast Asia (Rickettsial Diseases in Thailand)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 22 October 1962-30 June 1963

Authors: Bennett L. Elisberg, M.D.
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Collaborative studies by WRAIR, University of Maryland School of Medicine, and the SEATO Medical Research Laboratory carried out in Thailand, initiated a continuing program to evaluate the existing and potential military importance of rickettsial diseases in that country. Preliminary results indicate widespread endemicity of Q fever, scrub typhus and an unidentified member of the spotted fever group of rickettsioses.

+ U.S. Component, SEATO Medical Research Laboratory, Bangkok, Thailand.
++ Royal Thai Army Medical Service, Thai Component, SEATO Medical Research Laboratory, Bangkok, Thailand.
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BODY OF REPORT

Project No. 3A 0 12501 A 811 Title: Military Medical Research Program in Southeast Asia (Rickettsial Diseases in Thailand).

Description: Rickettsial Disease Investigations in Thailand.

During the period November, 1962 to mid January, 1963, members of the Department of Rickettsial Diseases, WRAIR, in collaboration with the U.S. and Thai Components of the SEATO Medical Research Laboratory, Bangkok, and the Department of Microbiology, University of Maryland School of Medicine, initiated a project to evaluate the existing and potential military importance of rickettsial diseases in Thailand.

Progress:

a. Although much has been learned in the past about the ecology of Rickettsia tsutsugamushi in Burma to the north and west, and in Malaya to the south, virtually nothing is known about the endemicity of scrub typhus throughout most of Thailand. Information about the occurrence of clinical scrub typhus gathered from reports in the literature and from conversations with Thai military and civilian physicians may be summarized as follows: Between 1943 and 1959, a total of six cases and three outbreaks of scrub typhus have been reported from localities within four of the five distinctive physiographic regions of Thailand. Single unconfirmed cases have been reported from Suras Thani (Medical Journal, (Thailand), 8:(6)630-636, November 1959), and Klong Chandi (Major S. Halstead, MC, USA, personal communication), two towns in the eastern portion of the Peninsula region in south Thailand. Other cases have been diagnosed clinically in Sarapi (Dr. Guyar, McCormick Hospital, Chieng Mai, personnel communication) and in San Patong, two towns located south of Chieng Mai in the Northern Hill region of the Continental Highland in northwest Thailand. A single case of clinical scrub typhus was recognized in Utai Thani in the central valley (Medical Journal (Thailand) 8:(52)482-488, 1957). The Japanese reported outbreaks of scrub typhus in their troops stationed on Phuket Island off the west coast of peninsular Thailand, and in the Kwai Noi valley in the Western Mountain section of the Continental Highland region (cited by Audy, J.R., 1949, Institute for Medical Research, Federation of Malaya, Bulletin 1:34). On the Khorat Plateau region in northeast Thailand, an outbreak, confirmed by positive Weil-Felix reactions, occurred among Thai Army personnel on training operations at Chong Mek near the Laotian border (Journal of the Medical Association (Thailand), 41:103-109, No. 2, March 1958). Another serologically confirmed case came from the Ban Pong District of Nakorn Pathom, west of Bangkok in the central valley (Journal of the Medical Association (Thailand) 35:(6)9-25, December 1952). It was from the same general area of the last mentioned illness that Traub and his associates (Am. J. Trop. Med. & Hyg., 3:(2)356-359, 1954) reported the infestation of rodents with the known vector, Leptotrombidium deliensis. These workers recovered two strains of Rickettsia tsutsugamushi, one from a pool of tissues from Rattus rattus thai, and another from a pool prepared from Bandicota. No cases of scrub typhus have been reported from the Southeast coastal region. Epidemic and murine typhus have been reported in Thailand, but the frequency of occurrence and distribution are unknown.

Nothing was known of the presence of Q fever or tick-borne rickettsioses.

b. During the three month stay of the team from WRAIR, it was possible to (1) establish a laboratory to carry out rickettsial investigations in the existing facilities of the SEATO Medical Research Laboratory in Bangkok, (2) train Thai military personnel in the techniques of isolation and identification of scrub typhus, and (3) undertake a preliminary survey for rickettsial diseases in several strategically important areas of Northern Thailand. Field investigations were carried out at Huai Mae Sanam and Doi Chieng Dao in the Northern Hill section of the Continental Highland in northwest Thailand, and at the Khon Kaen-Loi border and Chong Mek on the Khorat Plateau in Northeast Thailand. In each of these locations, animals were trapped or shot, and their ectoparasites collected and sorted. The larval trombiculid mites and the ticks removed from the animals were shipped from the field, in either the living or frozen state, to Bangkok where attempts were made to recover rickettsiae. Suspensions of chiggers were inoculated intraperitoneally into adult white mice for the isolation of R. tsutsugamushi and tick suspensions inoculated into guinea pigs for the recovery of Q fever and spotted fever group rickettsiae. Aliquots of the pools of chiggers and ticks were preserved in alcohol for definitive identification. White mice used to recover R. tsutsugamushi were kept for at least 28 days, after which they were challenged with a known lethal strain of scrub typhus to detect inapparent infections with non-pathogenic strains. Animals captured alive were exsanguinated and the sera collected for serologic tests. Their liver and spleens were removed, quick frozen and forwarded to the Bangkok laboratory where they were processed for isolation of R. tsutsugamushi. Pieces of kidney were inoculated into Fletcher's medium for cultivation of leptospira. Specimens of blood were obtained from local residents and border police wherever possible and the sera tested for evidence of prior rickettsial infections. The ecological circumstances encountered in each of the habitats sampled were documented by photographs and data collected from reports of the Thai Forestry, Mining and Agriculture and Soil Chemistry Departments.

c. Only the data specifically relevant to the rickettsial disease investigations will be reported here. It should be noted that opportunities provided by the field investigations were well exploited. Much valuable information on the mammalian and avian fauna were collected, as well as on the mosquitoes, Anoplura, other parasitic Diptera, and mesostigmatic mites of Thailand to further the studies being undertaken by the U.S. Component of the SEATO Medical Research Laboratory on the "Ecology and Control of Disease Vectors and Reservoirs."

d. The tabulation of specimens presented in Table 1 does not reflect the true extent of the mammalian, avian and entomological collections made. Included among the 160 animals indicated in Table 1 are representatives of at least 16 different species of mammals. The animals most frequently trapped were Tupaia glis, Rattus rajah and species of Rattus rattus. These mammals were the most heavily parasitized by Leptotrombidium chiggers. Arrangements have been made by the U.S. Component with Dr. J.L. Harrison, University of Singapore and with Mr. Lim Boo Liat, Institute for Medical Research, Kuala Lumpur, for identification of the mammalian collections from study skins and skulls prepared for this purpose. The ticks are currently being studied by Dr. C. Clifford, Rocky Mountain Laboratory, Hamilton, Montana.

Table 1

SUMMARY OF SPECIMENS COLLECTED FOR RICKETTSIAL DISEASE STUDIES, THAILAND, NOV-DEC, 1962

STUDY REGION	Number Animals Trapped or Shot	Isolations Attempted on Animal Tissues		Isolations Attempted on Chiggers #		Isolation Attempts on Ticks			HUMAN SERA COLLECTED
		Number of Animals	Number of Pools Prepared	Number of Animals Infested	Number of Pools Prepared	Number of Animals Infested	Number of Pools Prepared		
							Larvae	Nymphs Adults	
Huai Mae Sanam	23	16	4	15	21	5	3	1	93
Chieng Dao	27	22	7	18	23	5	5	1	50
Khon Kaen-Loei	87	79	23	25	28	26	11	11	49
Chong Mek	23	23	7	1	1	0	0	0	8
TOTALS	160	140	41	59	73	36	19	13	200

Includes 2 pools of Laelaptid mites.

e. Identification of aliquots of the pools of chiggers inoculated into white mice which were preserved for taxonomic study have been completed on the collections made from Huai Mai Sanam and Chieng Dao. In the first mentioned area, the pools which varied in composition from 150 to 300 chiggers contained approximately 75 to 100% Leptotrombidium deliensis. Only one or more of four other species of Leptotrombidium were found infesting the animals. Greater diversity was encountered in the infestation of the small mammals in the Chieng Dao region. Here the L. deliensis content of the pools varied from 25 to 99% and at least eight other species of Leptotrombidium were found in the pools. The taxonomic studies of the chiggers from the Khon Kaen-Loei border area have not been finished. A tabulation relating types and degrees of infestation to mammalian species is awaiting the completion of the identification of the ectoparasites by the entomologists and the skins and skulls of the animals by the mammalogists.

f. Reports received to date from the SEATO Medical Research Laboratory of the results of their continuing efforts to recover R. tsutsugamushi from the chiggers and animal tissues indicate the failure to demonstrate scrub typhus in any of the 73 pools of chiggers processed. Tentative isolations of strains of scrub typhus from one pool of animal tissues from the Huai Mae Sanam and the Chong Mek areas have been recorded, but these data await confirmation.

g. It was not possible to carry out the program to recover rickettsiae from ticks due to the occurrence of an epizootic of unknown disease which virtually wiped out the colony of guinea pigs in Bangkok. The frozen pools of ticks and tick suspensions were returned to WRAIR where further studies are currently in progress.

h. Much difficulty was encountered during the leptospiral isolation attempts on wild mammal kidneys. The majority of the Fletcher's cultures inoculated in the field became contaminated with extraneous bacteria. However, three strains of leptospira were recovered from Rattus rattus sp. The strain obtained from a rat trapped in Doi Chieng Dao belongs to the L. grippotyphosa serogroup and the other two strains recovered from rats trapped in the Khon Kaen-Loei region are members of the L. javanica serogroup. Definitive identifications of these strains are being made by the Division of Veterinary Medicine, WRAIR.

i. The results of serological testing of the animal sera with spotted fever and typhus group, and Q fever antigens are presented in Table 2. Evidence of prior rickettsial infection indicated by the finding of complement-fixing antibody titers of 1:5 or greater was found in the sera of only two of the 16 species of animals available for testing; i.e., Tupaia glis and Rattus rajah. The only other rodent that was trapped in comparable numbers was Rattus rattus sp. and all tests on these sera were negative, as were tests on sera from 33 other mammals comprising 13 different species. Spotted fever group antibodies were found in sera from both Tupaia and Rattus rajah trapped in three of the four areas studied (Chieng Dao, Khon Kaen-Loei and Chong Mek). The typhus group and Q fever antibodies were found only in Tupaia sera. Typhus antibodies, probably of the flea-borne type were found in the sera of animals from Huai Mae Sanam, Khon Kaen-Loei and Chong Mek. Q fever antibodies were demonstrated only in animal sera from the Khon Kaen-Loei location.

Table 2

RESULTS OF RICKETTSIAL SEROLOGICAL SURVEY ON WILD ANIMAL SERA
THAILAND, NOV-DEC, 1962

Animals* Species	Number Tested	Number with Comp-Fix Antibodies** S.F. Group	Typhus Group	Q Fever
Tupaia sp.	35	9	4	4
R. rajah	41	8	0	0
R. rattus sp.	29	0	0	0

* Sera from 33 other mammals including representatives of 13 different species were negative.
** Complement fixing antibody titers of 1:5 or greater.

j. Similar complement fixation tests were performed on the Thai human sera collected during the field investigations (see Table 3). Evidence of prior infection with some member of the spotted fever group of rickettsioses was found in only eight sera, but positive reactors were among the collections made in each of the four regions indicated. Q fever antibodies were found in human sera obtained in the Huai Mae Sanam area and Khon Kaen Forest Reserve. Typhus group antibodies were demonstrated in only four of the blood specimens drawn from border police in northwest Thailand. Although it has long been suspected that Q fever and spotted fever group rickettsiae should be in Thailand, these serological results provide the first real evidence of their existence in that country.

k. Microscopic agglutination tests on the Thai human sera are to be carried out for leptospirosis? by the Division of Veterinary Medicine.

l. The human sera were also examined for scrub typhus antibodies using the indirect immunofluorescent test developed last year in this laboratory (Annual Report WRAIR, 1 July 61-30 June 62). In 43 of the 194 sera tested, a 1:10 dilution of serum resulted in significant fluorescence with either the Karp or Gilliam antigen, or both. Titers of 1:40 or greater with one or the other, or both antigens, were obtained in 25 of the specimens. The finding of positive reactors among the sera collected in all four locations suggests a widespread endemicity of scrub typhus in Thailand.

Summary and Conclusions:

During the third quarter of 1962, a program for the investigation of rickettsial diseases in Thailand was initiated in the SEATO Medical Research Laboratory and members of the Thai Component trained to continue the laboratory aspects of the study. The demonstration of Q fever and spotted fever group antibodies in the sera of resident human populations and wild small mammals and scrub typhus immunofluorescent antibodies in the human sera indicate a widespread endemicity of these rickettsioses in Thailand.

Table 3

RESULTS OF RICKETTSIAL SEROLOGICAL SURVEY ON HUMAN SERA
THAILAND, Nov-Dec, 1962

Location	Complement Fixation Tests				R. tsutsugamushi I.F. Test		
	Number tested	Number positive*			Number tested	Number positive	
		S.F.	Typhus	Q fever		1:10	≥1:40
Huai Mae Sanam	89	4		3	93	15	8
Border Police N.W. Thailand	50	1	4		49	15	6
Khon Kaen Forest Reserve	40	2		1	44	13	9
Border Police Chong Mek	8	1			8		2
TOTALS	187	8	4	4	194	43	25

* Complement-fixing antibody titers of 1:5 or greater.

ANNUAL PROGRESS REPORT

Project 3A O 12501 A 811, Military Medical Research Program in Southeast Asia. (Anopheline Vectors for Laboratory Transmission of Drug-resistant Falciparum Malaria)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Entomology
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 - 30 June 1963

Principal Investigators: Alf S. Alving, M.D.*
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Captain R. D. Powell, MC**
Ronald A. Ward, Ph.D.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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**Army Medical Research Project, The University of Chicago, Stateville Penitentiary, Joliet, Illinois

ABSTRACT

Project No. 3A 0.12501 A 811 Title: Military Medical Research Program in Southeast Asia. (Anopheline Vectors of Drug-resistant Falciparum Malaria)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 - 30 June 1963

Authors: Alf S. Alving, M.D.*
Captain G. J. Brewer, MC**
Captain R. D. Powell, MC**
Ronald A. Ward, Ph.D.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

A suitable experimental vector, Anopheles stephensi, has been found for the mosquito transmission of drug-resistant Falciparum malaria strains. This mosquito is easily reared in the laboratory and consistantly shows extremely high sporozoite infections.

*Department of Medicine, The University of Chicago, Chicago, Illinois
**Army Medical Research Project, The University of Chicago, Stateville Penitentiary, Joliet, Illinois

BODY OF REPORT

Project No. 3A 0 12501 A 811 Title: Military Medical Research Program in Southeast Asia. (Anopheline Vectors of Drug-resistant Falciparum Malaria)

Description: This subtask was initiated to find an easily reared mosquito species that could be readily infected with chloroquine-resistant strains of Falciparum malaria and would transmit these strains for chemotherapy studies.

Progress:

1. Anopheline vectors for laboratory transmission of drug-resistant Falciparum malaria.

a. During preliminary attempts in August 1962 to find suitable experimental vectors for drug-resistant strains of Falciparum malaria, it was found that Anopheles quadrimaculatus and A. freeborni were partially or wholly refractory to infection with Plasmodium falciparum. A. albimanus, a Central and South American vector of falciparum was then established at WRAIR and in November a subcolony was established at the Stateville Penitentiary for experimental transmissions. Light midgut and low sporozoite densities (1-2+) were achieved in this species after feeding on infected volunteers. In general, mosquito infections in this species were not appreciably higher than A. quadrimaculatus.

b. Earlier studies on avian malaria had indicated that drug-resistance in a malaria strain did not alter its mosquito transmissibility, therefore it was thought that resistance would not be a factor in this situation. It is known that falciparum strains exhibit varying degrees of infectiousness towards different anopheline species and that they will usually develop only in vectors from contiguous geographic areas in contrast to vivax strains which show no such specificity. A survey of colonized strains of anophelines being maintained in laboratories throughout the world (Ward and Kitzmiller, in press) indicated that two Oriental vectors, A. stephensi and A. sundaicus were available from several overseas institutions. Since stephensi is more readily reared in the laboratory than sundaicus and has been used for experimental malarial infections in India a colony of A. stephensi was established at WRAIR in January 1963. Experiments carried out at Stateville in March with a subculture of this strain demonstrated that very high infections (4+) could be established in stephensi fed upon a carrier infected with the Viet Nam chloroquine-resistant falciparum strain. Five volunteers exposed to the bite of these mosquitoes nine days after mosquito infection all developed malaria. Similar transmissions have been obtained with stephensi infected with drug-resistant parasites strains from Thailand and Malaya. Since A. stephensi is so easily reared in the laboratory and can be infected with ease it appears to be an ideal experimental vector for these drug-resistant strains of P. falciparum.

Summary and Conclusions:

An Asian malaria vector, Anopheles stephensi, has been brought to WRAIR for biological studies and the Army Malaria Unit at Stateville Penitentiary for the mosquito transmission of drug-resistant Falciparum malaria. It is an easily reared species and provides an excellent vector for chemotherapy studies.

Publication:

Ward, R. A. and Kitzmiller, J. B. A list of laboratory colonies of Anopheline mosquitoes. Mosquito News (in press), 1963.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 811, MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA (ZOONOSES)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Veterinary Microbiology
Division of Veterinary Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

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ABSTRACT

Project No. 3A 0 12501 A 811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA (ZOONOSES)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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1. Preliminary culture typing tests were completed on 484 of 804 leptospiral isolates obtained in a study of natural foci of leptospiral infections in Malaya. Sixty different serotypes were disclosed. Limitations were disclosed in the use of hamster inoculation technics to determine the infectiousness of natural waters and soils. Studies were initiated to develop a multivalent leptospiral bacterin for human use in Southeast Asia. An experimental bacterin is now being evaluated in laboratory animals.

2. Studies on leptospirosis in Thailand were initiated. A laboratory for this purpose was established in Bangkok and efforts of the Thai Department of Livestock Development, the School of Tropical Medicine and School of Medicine in Chieng Mai have been coordinated with those of the WRAIR component in the SEATO laboratory. A considerable number of serums from domestic animals have been collected. Tests to date have disclosed presence of agglutinins in approximately 40 per cent of the samples. Numerous isolates from trapped rodents have been obtained. More than 100 human cases have been seen in Chieng Mai. In preliminary culture typing on 23 isolates from rodents, 3 different serotypes were disclosed, representing members of the autumnalis, javanica, and grippotyphosa groups.

3. In a survey of melioidosis antibodies in normal Thai and S. Vietnam personnel, significant reactions were found in 6 to 23% of the samples--providing evidence of the widespread occurrence of melioidosis infections in these areas. Procedures for handling, storage, and bacteriological and serological examination of specimens for melioidosis were developed for projected epidemiological studies.

BODY OF REPORT

Project No. 3A 0 12501 A 811 Title: MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA (ZOONOSES)

Description:

1. Studies on leptospirosis were conducted in Malaya and Thailand to elucidate epidemiologic and ecologic factors that govern formation of endemic or epidemic loci of infections, to determine the prevalence of disease and the potential infection hazards in areas not heretofore studied and to develop effective prophylactic measures.

2. Studies on melioidosis were initiated to evaluate its public health significance in Southeast Asia, which comprises epizootic and enzootic areas of this disease.

Progress:

1. During the period of this report, approximately 600 strains of leptospira isolated in Malaya were submitted to WRAIR for culture typing. To date, a total of 804 strains from Malaya have been submitted. They comprise primarily isolates from various surface waters and soils, but include isolates from wildlife and from human cases of leptospirosis. To date, preliminary culture typing has been completed on 458 isolates. This procedure involves initial screening of isolates against a battery of type anti-leptospira serums. On the basis of observed cross-reactions, strains are then tested with additional antiserums to disclose further antigenic relationship. During the past year, it was possible to halve the number of test antiserums employed in culture typing and still get adequate tests on cultures. This revision, which has resulted in a considerable reduction in work, was made possible through an analysis of previous culture typing results on Malayan strains. A summary of culture typing findings is given in Table 1.

There were sixty characteristic patterns of cross-reactivity presumptively reflecting the presence of the same number of serotypes. The relationships of isolated types to recognized serotypes will be determined after all isolates are screened by cross-agglutination tests. No doubt, a few of these types will be found to be identical. Many, if not most, will be related to recognized types; however, it is evident from findings, e.g. the disclosed reaction patterns, that a large number of new leptospiral serotypes are represented in this series.

Eleven of the isolates were obtained from the same number of human patients, that were seen primarily in a British Military Hospital. In this group, only two of the patients were infected with the same serotype. Ten different serotypes were represented amongst the 11

TABLE 1

Preliminary Classification of 458 Leptospiral Isolates for Malaya

Serogroup	Type	No. of Isolates
Icterohaemorrhagiae	54i	73
	75-373	21
(105 strains)	M-33	7
	M-94	2
	M-108	1
	368	1
canicola	M-53	15
	M-23	9
(46 strains)	M-302	7
	M-9	5
	257	4
	M-172	2
	M-200	2
	M-18	1
	M-357	1
pyrogenes	M-50	9
	M-84	9
(51 strains)	55-271	9
	29	6
	111-561	5
	198-998	2
	M-36	2
	M-48	2
	M-97	2
	247	1
	M-31	1
	M-211	1
	M-88	1
	193-988	1
javanica	33SB	1
	267-1348	1
(3 strains)	M-107	1
autumnalis	M-63	13
	M-275	7
(41 strains)	M-15	6
	13KKB	3
	M-359	3
	Blythe	2
	M-83	1
	M-89	1
	M-7	1
	M-12	1
	M-239	1
	M-391	1
	M-360	1
australis	M-85	32
	267-1349	9
(46 strains)	GP-6	3
	GP-62	1
	392	1
grippotyphosa	M-56	21
	GP-58	7
(35 strains)	14	7
bataviae	20-96	47
	267	23
(78 strains)	264-1333	8
hebdomadis	61 Ham	47
	256	4
(53 strains)	31a	1
	193-972	1

isolates. This finding, coupled with the disclosed variety of types in natural foci of infection, extends the previous findings on the multiplicity of types found in Malaya. The correlation of serological findings with ecological and epidemiological data will be made when field studies are completed.

Hamster inoculation technics were used to obtain the leptospiral isolations from water to establish the relative infectiousness of the same or different loci of leptospirosis under different environmental conditions. The determination of leptospirosis in inoculated hamsters is based on the occurrence of death or severe illness between the 8th and 14th days post-inoculation with the subsequent demonstration of organisms in tissues. It was presupposed that infections did not occur in experimental animals that gave no signs of disease. It was deemed desirable to check this hypothesis and serums from surviving hamsters were forwarded to WRAIR. Microscopic agglutination tests were performed on 95 selected hamsters with the greatest risk of exposure. Selection was based on a history of recovery of cultures from companion hamsters inoculated with the same or comparable samples of water. Significant agglutinin titers were detected in two animals; 17 others had partial or questionable agglutinins. It was apparent that not all infections were detected with the present technic. To obtain an index of the percentage of missed infections, further serological study is required.

The disclosure of remarkably large numbers of different serotypes in Malaya in nature and also in patients seemingly ruled out the applicability of vaccine prophylaxis in this area. This concept was based primarily on considerable empirical information that cross-protection occurs only amongst serotypes that are related antigenically. However, on the basis of other experimental data in this laboratory, it was believed that a multivalent vaccine could be developed for use in Malaya and other areas of multiple leptospirosis and would afford protection against infections with 95 to 90 per cent of the serotypes. For this purpose, 5 strains, encompassing cross-agglutination reactions with all of the types found in Malaya, were carefully selected for the preparation of a bacterin. The strains selected were from the following serogroups: autumnalis, grippotyphosa, pyrogenes, hebdomadis and bataviae. The experimental vaccine consisted of a pool of washed cells from each type suspended in 0.1% formalized saline, containing a total of 350 X 10^6 cells per ml, or 70 X 10^6 organisms of each serotype per ml. One half of the preparation was freeze-dried, the remainder was stored in the fluid state. Tests in experimental animals to evaluate the vaccine are now in progress.

2. Studies on leptospirosis in Thailand were initiated by the U. S. Army Component of the SEATO laboratory in Bangkok in cooperation with the Division of Veterinary Medicine, WRAIR. Liaison with Thai veterinary government officials was established and resulted in the establishment of a leptospira laboratory by the Department of Livestock Development of Thailand. This laboratory is staffed in part by U. S. Army personnel of the Veterinary Corps assigned to SEATO. In January 1963, technical assistance was provided to establish cultural and serological methods and technics in the new leptospira laboratory.

The initial efforts of this laboratory were directed to the collection of large numbers of serum samples from the livestock population. Serological studies disclosed the prevalence of leptospiral antibodies in approximately 40 per cent of the animals. Subsequently, cultural and serological studies were initiated on trapped rodents and other wildlife and the activities of the leptospira laboratory were coordinated with those of Dr. Chamlong of the School of Tropical Medicine in Bangkok and Dr. Bundham of the School of Medicine in Chieng Mai, who were currently doing studies on human leptospirosis. Dr. Bundham, during approximately an 18 month period, uncovered over 100 human cases from whom numerous isolates were obtained. A comprehensive report of these animal and human leptospirosis studies will be included in the annual report from the U. S. Army Component in SEATO. Arrangements have been made to forward leptospiral isolates to WRAIR for typing. To date, preliminary culture typing tests were completed on the first group of 23 isolates obtained from rodents. The source and tentative identification by serogroup of these isolates are as follows:

Area	Host	No. Exam.	(Serogroup) Identification
Rajaburi	Rat (Not identified)	19	13 javanica 6 autumnalis
Bangkok	Rat (Not identified)	1	1 javanica
Chieng Mai	Black Rat	3	1 grippotyphosa 2 javanica

Members of the autumnalis and bataviae serogroups had been reported previously in Thailand. This was the first evidence of the presence of javanica and grippotyphosa serotypes in this area.

3. Efforts to develop cultural and serological technics for use in epidemiological investigations of melioidosis in Southeast Asia were continued. In the previous annual report, it was noted that a sonic-vibrated antigen for use in complement-fixation tests (disclosed by Nigg, et al, J. Bact. 82:159, 1961) was found to be highly sensitive and specific for detection of melioidosis antibodies. The antigen, however, was not stable when stored in the frozen state. New lots of antigen were prepared that had comparable sensitivity and specificity. After a three month storage period at 5°C, the stability and sensitivity of the antigen has not declined. This antigen was employed for initial tests to determine the presence of melioidosis antibodies in natives of Southeast Asia. Tests were conducted on serums from Thai army recruits (collected by Major E. Blair, WRAIR), normal Thai located in the northern sector of the country (collected by Dr. B. Elisberg and Captain G. Rapmund, WRAIR) and S. Vietnam troops (collected by Dr. H. Noyes, WRAIR). A summary of test results is shown in Table 2.

TABLE 2

Survey of CF Antibodies for Melioidosis in Human Beings in Thailand and S. Vietnam

Source of Serums	No. Exam.	Positive No.	Positive %	Questionable Reactions
Thai soldiers (normals)	52	4	7.7	1
Thai civilians	186	44	23.6	14
S. Vietnam soldiers	103	6	5.8	9
Control (WRAIR personnel)	100	1	1.0	0

The percentage of positive findings in different groups samples in Thailand and S. Vietnam ranged from 5.8 to 23.6%. In addition, questionable reactions were elicited in a large proportion of the serums from these areas. In contrast, only 1 of 100 serums obtained from normal WRAIR personnel was positive. These findings provide strong presumptive evidence of the widespread occurrence of asymptomatic cases of melioidosis in Thailand and Vietnam. It is noted that the CF antibodies elicited by melioidosis are believed to be detectible for only a few months after infection, according to Nigg. If such is the case, the positive titers reflect relatively recent infections. Arrangements have been made to collect more representative serum samples in these areas as well as in Malaya from indigenes, as well as from American troops for a more comprehensive serological survey.

On the basis of available clinical information on melioidosis, the most probable location of clinical cases is in patients with undiagnosed pneumonic disease, particularly those with tuberculosis-like signs. Diagnosis in these cases could be affirmed or denied by bacteriological examination of sputum. To facilitate the detection of cases, it was deemed advisable to develop technics for handling, shipping and storage of clinical material for bacteriological examination and to develop more discriminate bacteriological technics for the isolation and differentiation of the organism. Specific attention was directed to handling and culturing of sputum samples.

Initial studies were conducted to determine the optimum conditions necessary for maintaining the viability of melioidosis (and also glanders) organisms in sputum samples. Three different Pseudomonas pseudomallei (melioidosis) and two different Actinobacillus mallei (glanders) strains were used in this study. Sputum obtained from WRAH was homogenized with glass beads of pancreatin and divided into two parts, one of which was untreated; to the second, antibiotics were added. Aliquots containing no or various concentrations of antibiotics were seeded with suspensions of organisms to provide final concentrations

ranging from 10^1 to 10^4 cells per ml. One part of each test sample was stored at 5°C, a second at 30°C for 1 week, after which time, measured volumes were planted on agar medium for recovery of organisms. It was found that both glanders and melioidosis organisms remained viable in untreated and antibiotic-treated sputum, whether samples were stored at 0° or 30°C, even when organisms were present in relatively low concentration (10 per ml). Furthermore, treatment of contaminated sputum with penicillin G or crystalline polymyxin B singly in final concentrations of 1000 u/ml did not affect the viability of organisms. In combination, the two antibiotics could be used in final concentrations of 400 u each, without affecting glanders or melioidosis organisms. Treatment of sputum with antibiotics resulted in the suppression of other microorganisms from sputum and allowed almost a pure cultural recovery of glanders and melioidosis organisms. Pancreatin did not affect the viability of the organisms studied. It was also found that a holding medium (Stuart's), seeded with contaminated sputum could be effectively used to maintain viability of melioidosis and glanders organisms.

Selective and differential media were also studied for the rapid isolation and identification of organisms of melioidosis and glanders. The most effective mediums for this purpose were a crystal violet agar (described by Millar, et al, J. Bact. 55:115, 1948) and Hendrickson's blue agar medium (J. Bact. 28:597, 1934) and a penicillin-polymyxin nutrient broth medium.

A standard operating procedure for the handling, shipment, and bacteriological examination of sputum (as well as specimens) for melioidosis and glanders has been mimeographed and copies thereof were forwarded to WRAIR laboratories in Thailand and Malaya. Basic groundwork for the proposed melioidosis studies in Southeast Asia are now completed.

Summary and Conclusions:

1. During the course of studies on the ecological and epidemiological factors that govern the infectiousness of natural foci of leptospiral infections, 804 isolates were obtained primarily from surface waters and soils and forwarded to WRAIR for typing. Preliminary culture typing tests were completed on 458 isolates and the presence of approximately 60 different serotypes were revealed. These findings extend previous knowledge of the multiplicity of serotypes found in Malaya. Hamster inoculation technics were employed to determine the presence of leptospira in water or soil samples and recoveries were obtained only when hamsters became moribund 8 - 14 days post-inoculation. It was apparent from serological tests conducted on inoculated hamsters that developed no manifest disease, that some of the animals were infected. Determination of the percentage of missed cases in test hamsters requires further study. Studies were initiated to develop a multivalent

leptospiral bacterin for use in man in Southeast Asia. Five strains, encompassing cross-agglutination reaction of all types found in Malaya were selected for this purpose. A formalized washed-cell bacterin has been prepared and is now being evaluated in experimental animals.

2. Studies on leptospirosis in Thailand were initiated. A laboratory for this purpose was established in Bangkok and efforts of the Thai Department of Livestock Development, the School of Tropical Medicine and School of Medicine in Chieng Mai have been coordinated with those of the WRAIR component in the SEATO laboratory. A considerable number of serums from domestic animals have been collected. Tests to date have disclosed presence of agglutinins in approximately 40 per cent of the samples. Numerous isolates have been obtained from trapped rodents. More than 100 human cases have been seen in Chieng Mai. In preliminary culture typing on 23 isolates from rodents, five different serotypes were disclosed, representing members of the autumnalis, javanica, and grippotyphosa groups. Strains from the latter two groups had not been found previously in this area.

3. Preliminary serological tests, employing a new complement fixation technic for melioidosis, were conducted on samples of serums obtained from groups of normal Thais and S. Vietnam personnel. The prevalence ratio of positive reactions in these groups ranged from 5.8 to 23.6%. This finding provided presumptive evidence of the widespread occurrence of melioidosis infections in this area. Procedures for the handling, storage, and bacteriological examination of specimens for melioidosis were developed. Development of the cultural and serological technics for the epidemiological investigations of melioidosis in Southeast Asia has now been completed.

List of Publications:

Rubin, H. L., Alexander, A. D., and Yager, R. H. Melioidosis - A Military Medical Problem? Military Medicine, In Press.

ANNUAL PROGRESS REPORT

Project No. 3A O 12501 A 811, Military Medical Research Program in Southeast Asia
(Viability Studies on Enteric Bacteria in a Semi-solid Transport Medium)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Bacteriology
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Sylvia G. Cary, M.S.
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Assistants: Charles Harkins, SFC
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12601 A 811 Title: Military Medical Research Program in Southeast Asia (Viability Studies on Enteric Bacteria in a Semi-solid Transport Medium)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Security Classification: UNCLASSIFIED

Preliminary results from a comparison study, conducted with the SEATO Medical Laboratory, on the recovery of pathogenic enteric bacteria from rectal swabs and fecal specimens held for varying periods in a modified Stuart Transport Medium is discussed.

BODY OF REPORT

Project No. 3A 0 12501 A 811 Title: Military Medical Research Program in Southeast Asia (Viability Studies on Enteric Bacteria in a Semi-solid Transport Medium)

Description: The purpose of this task is to discover, develop and evaluate laboratory procedures for the accurate and rapid etiologic diagnosis of acute infectious diseases, with particular reference to those of real or potential value for use in field or other specialized military laboratories.

Progress: Results of preliminary experiments (Annual Report 1962) on the use of a modified Stuart Transport Medium for the collection and transportation of rectal swabs and fecal specimens have warranted further testing of the medium under conditions of actual usage. Such a study was initiated in 1962 with the SEATO Medical Research Laboratory (US Component), Bangkok, Thailand. Duplicate enteric specimens were collected in the modified transport medium and one of each was mailed to WRAIR where it was examined bacteriologically for the presence of pathogenic enteric bacteria. Similar studies were performed on the other specimen in Bangkok within 24 hours of collection. To date 155 specimens from Thailand have been processed. The average time in transit from Bangkok to Washington, D. C. was six days. Actual times between collection and processing at WRAIR varied between 6 and 59 days, although the majority of specimens were processed within 20 days.

WRAIR isolations of salmonellae and shigellae compared favorably with SEATO results on specimens held for 10-12 days, however, too few positive specimens were found by either laboratory to assess the significance of these findings. Isolations from specimens held in transport media for longer periods fell off precipitously, as compared with SEATO results, although Shigella boydii 4 and Shigella flexneri 4 were recovered from specimens held for 20 and 59 days, respectively. Few non-agglutinable vibrios (NAG) survived storage in the medium, which may be due, in part, to their inhibition by Pseudomonas aeruginosa appearing in 26 per cent of the specimens reported by SEATO to contain NAG vibrios. However, in view of favorable reports obtained from independent comparison studies made by Lt. Col. Sidney Gaines, SEATO Laboratory, testing will be continued until a sufficient number of positive specimens are obtained to determine the optimal period of storage permitted by this medium.

Summary and Conclusions: Preliminary results from a comparison study, conducted with the SEATO Medical Laboratory, on the recovery of pathogenic enteric bacteria from rectal swabs and fecal specimens held for varying periods in a modified Stuart Transport Medium is discussed.

ANNUAL PROGRESS REPORT

Project No. 3A O 12501 A 811, Military Medical Research Program in Southeast Asia
(Bacteriologic Survey of Battle Wounds)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Bacteriology
Division of Communicable Diseases and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Howard E. Noyes, Ph.D.

Assistant: Richard B. Ransford, PFC

Reports Control Symbol: MEDDH-288

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ABSTRACT

Project No. 3A 0 12501 A 811 Title: Military Medical Research Program in Southeast Asia (Bacteriologic Survey of Battle Wounds)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Howard E. Noyes, Ph.D.
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The authors spent the period from 3 March 1963 through 20 April 1963 in Viet Nam to participate in the performance of a survey among Viet Namese battle casualties. During this period approximately 200 wounds were characterized in terms of type of wound, type of missile, prior therapy, time lapse between wounding and sampling and quantitative and qualitative bacteriologic flora. In addition approximately 125 presumably normal human sera were obtained for assay of clostridial antitoxins.

BODY OF REPORT

Project No. 3A 0 12501 A 811 Title: Military Medical Research Program in Southeast Asia (Bacteriologic Survey of Battle Wounds)

Description: Bacteriologic and related studies were carried out on Viet Namese battle casualties at the Cong Hoa Military Hospital in Saigon and at the Phong Dinh Province Hospital at Cantho. Samples were obtained in most instances on admission to the hospital.

Progress: Quantitative and qualitative bacteriologic studies were carried out to determine the microflora of these wounds in relation to anatomic location, geographic area, type of wound, type of wounding device and the distance from it at the time of wounding, effect of prior therapy, and the effect of the time lag between wounding and sampling. Additional studies were carried out to determine the microflora of infections acquired in the hospitals, antibiotic sensitivities of "house strains" of bacteria and assay of normal sera from Viet Nam soldiers for the presence of clostridial antitoxins.

Summary and Conclusions: Portions of these studies are still in progress and a final report will be submitted in the near future.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 811, Military Medical Research Program in Southeast Asia
(Clinical Observations on Thai Hemorrhagic Fever)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Bacteriology
Division of Communicable Disease and Immunology

Department of Metabolism
Division of Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Mark R. Margiotta, Major, MC

Assistants: K. G. Barry, Lt Col, MC
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 811

Title: Military Medical Research Program in Southeast Asia (Clinical Observations on Thai Hemorrhagic Fever)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

A prospective clinical evaluation of 62 patients with proven Thai Hemorrhagic Fever was accomplished during the Bangkok epidemic of 1962. Multiple system involvement was present and particularly prominent were signs of circulatory, gastrointestinal and hematologic disturbances.

During epidemic periods, the clinical diagnosis of this disease syndrome should not be difficult; during interepidemic periods a febrile illness of sudden onset with multisystem involvement including hemorrhagic phenomena, hepatomegaly without splenomegaly, and circulatory instability should be strongly suggestive of Thai Hemorrhagic Fever.

BODY OF REPORT

Project No. 3A 0 12501 A 811 Title: Military Medical Research Program in Southeast Asia (Clinical Observations on Thai Hemorrhagic Fever)

Description: To accomplish careful clinical evaluation of a small number of randomly selected patients with Thai Hemorrhagic Fever, all of whom would be studied extensively from an etiologic standpoint. During August, September and October of 1962 the clinical manifestations of Thai Hemorrhagic Fever were studied in 62 patients in whom concurrent extensive virologic studies established the existence of a current dengue or chikungunya infection.

Progress: Clinical Observations on Thai Hemorrhagic Fever.

The first recognition of the disease syndrome known as Thai Hemorrhagic Fever occurred with a small outbreak among Bangkok children in 1954. Since then the disease has occurred in epidemic proportions during the rainy seasons of 1958, 1960 and 1962 and has occurred sporadically during interepidemic periods. For as yet undetermined reasons Thai Hemorrhagic Fever appears to occur only in indigenous, oriental children under 15 years of age. Extensive clinical and laboratory investigations during the 1958 epidemic (Hammon, et al. Trans. Assoc. Amer. Physicians 73: 140-155, 1960.) yielded considerable evidence, including virus isolation and serologic studies, implicating dengue or dengue-related viruses and a group A virus similar to the chikungunya virus as etiologic agents. Recent and current entomological investigations have indicated an association between Thai Hemorrhagic Fever and Aedes aegypti mosquitos. During epidemic periods, dengue virus isolations have been made from A. aegypti pools and not from other species. The chikungunya virus has been isolated from Culex fatigans but not from A. aegypti. It is generally believed that A. aegypti represents the principal disease vector.

Although extensive clinical studies have previously been reported the lack of a sufficient number of cases with adequate etiologic diagnoses has detracted from their value. Despite general agreement among most observers as to certain clinical aspects including fever, hemorrhagic phenomena, gastrointestinal tract involvement, and the tendency to peripheral vascular collapse, there has been much difference of opinion concerning the relative frequency and importance of the various clinical features.

During the rainy season epidemic of 1962, randomly selected patients at the Bangkok Children's Hospital were studied extensively from clinical, clinical laboratory, and virologic standpoints. Patients were admitted to the study and control groups every other day according to a prearranged schedule. (See Table 1).

TABLE 1

Category	No. of Patients	Description
1. Surgery	1	Admission to surgical service during previous 24 hrs.
2. Non-hemorrhagic Fever	1	Admission to medical service during previous 24 hrs.; admitting diagnosis other than Thai Hemorrhagic Fever.
3. Fever of unknown cause	1	Outpatient clinic patient with fever of undetermined cause.
4. Hemorrhagic Fever	2 or more	Admission to medical service during previous 24 hrs.; admitting diagnosis of Thai Hemorrhagic Fever.

A total of 94 patients were admitted to the study, 62 of whom had unequivocal evidence of a current dengue, chikungunya or combined infection. The case distribution within each category and the summarized virologic results are shown in Table 2.

The prominent clinical features can most conveniently be considered under separate headings. Fever was invariably present, usually was moderate, and there was no "typical" fever curve. Malaise and lethargy were almost universally present. The course of the disease encompassed a spectrum from mild to severe. There was one death.

(1) Circulatory: Acute circulatory failure (or shock) was the most feared and life threatening manifestation and occurred in 14.5 per cent of patients. It is likely that the acute circulatory failure is due to a marked sudden decrease in blood volume secondary to capillary abnormalities and fluid shift from the intravascular space to the interstitium.

(2) Gastrointestinal: No patient failed to manifest some symptom or sign referable to the enteric canal or accessory organs. Abdominal pain (70.7 per cent), vomiting (82.3 per cent), constipation, anorexia (79 per cent) and hepatomegaly (77.4 per cent) were among the common features. Melena occurred in seven patients, all of whom had marked thrombocytopenia.

(3) Hematological: Thrombocytopenia, often to a severe degree, was present, at least transiently, in 79.2 per cent of patients. Petichiae and purpura were common features and the standard Rumpel-Leeds test was positive in three-fourths of the patients.

(4) Neurological: Nonspecific neurological abnormalities such as headache, lethargy, and restlessness were common but more definitive indicators of neurologic involvement were not usually observed. Coma occurred in ten patients, usually in association with acute circulatory collapse (seven of ten).

(5) Respiratory: In general, our data did not suggest prominence of respiratory tract involvement but a definite pleural effusion of unexplained cause was noted in six of fifteen patients examined roentgenologically.

Clinical laboratory studies indicated that a polymorphonuclear leukocytosis was present in the majority of patients and an early lymphocytic response was not uncommon. Elevations of SGOT levels were noted in 88.7 per cent of patients and values over 200 units were associated with particularly severe cases.

Summary and Conclusions: A prospective clinical evaluation of 62 patients with proven Thai Hemorrhagic Fever was accomplished during the Bangkok epidemic of 1962. Multiple system involvement was present and particularly prominent were signs of circulatory, gastrointestinal, and hematologic disturbances.

TABLE 2

Category	Patients	THF	Virus Isolates	Diagnostic Titer Rise	High Fixed Antibody Titer
Surgery	12	1	-	1	-
Non-Hemorrhagic Fever	11	1	-	1	-
Fever of Unknown Cause	16	11	5	10	1
Hemorrhagic Fever	55	49	10	36	13

During epidemic periods, the clinical diagnosis of this disease syndrome should not be difficult; during interepidemic periods, a febrile illness of sudden onset with multisystem involvement including hemorrhagic phenomena, hepatomegaly without splenomegaly, and circulatory instability, should be strongly suggestive of Thai Hemorrhagic Fever.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 811, Military Medical Research Program in Southeast Asia
(*Pasteurella pestis* Antibody in Human and Rodent Sera from Viet Nam)

Reporting Installation: Walter Reed Army Institute of Research
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Washington 12, D. C.

Department of Bacteriology
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: James H. Rust, Jr., Ph.D.

Assistants: Tom Sanders, SFC
John W. Zibreg, SP4

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 A 811 Title: Military Medical Research Program in Southeast Asia (*Pasteurella pestis* Antibody in Human and Rodent Sera from Viet Nam)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: James H. Rust, Jr., Ph.D.
Tom Sanders, SFC
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Human sera (131) and rodent sera (63) from Viet Nam were screened for antibodies to *Pasteurella pestis* by the micro-complement fixation and micro-hemagglutination tests.

BODY OF REPORT

Project No. 3A 0 12501 A 811 Title: Military Medical Research Program in Southeast Asia (*Pasteurella pestis* Antibody in Human and Rodent Sera from Viet Nam)

Description: This task involves the study of the antigens and properties of *Pasteurella pestis* and the associations of this organism with the rodent reservoir, the insect vector, and the human host in order to elucidate the environmental and biological mechanisms of infection and resistance.

Progress: A number of sera from various areas of Viet Nam were examined by the micro-hemagglutination (HA) and micro-complement fixation (CF) tests described in the 1962 Annual Report.

Serological data from these sera for antibodies to *Pasteurella pestis* are presented in the following table:

VIET NAM (PLAGUE) SERA*

No. of Specimens	Species	Area	HA	CF
105	Human	Saigon	2 pos	Neg
25	Human	Phu Bon	Neg	Neg
1	Convalescent plague	?	Neg	Pos
10	Rodent	Phu Loc	2 pos	Neg
8	Rodent	Minh Mang	Neg	1 pos
21	Rodent	Ly-Thai To	Neg	Neg
14	Rodent	Nguyen Thien Thuat	Neg	Neg
10	Rodent	Bui Vien	Neg	Neg

* Sera submitted by Major Eugene Feeley, Medical Laboratory Hq., USASGV, APO 143, San Francisco, California.

All of these sera were screened by the micro-serological CF and HA test, using chemically purified *P. pestis* capsular material (FI) as the antigen.

Repeated serological observations on animals infected with, or immunized against, P. pestis suggest that complement fixing antibody appears early, e.g., within four or five weeks, and then rapidly declines. On the other hand, hemagglutinating antibody appears somewhat later and remains elevated for some time. Therefore, it is not surprising that the single human convalescent serum has only a relatively low complement fixing titer and no hemagglutination titer. Also, serological results from the rodent sera indicate that the Minh Mang area was recently involved in an epizootic while the Phu Loc (A) area experienced an epizootic probably some months previous. It should be noted that although about 50 per cent of the 105 Viet Namese soldiers had been vaccinated within the last three years, only two showed evidence of plague antibody.

Dates of sera collection and other pertinent information concerning this material is not available, therefore, no absolute conclusions can be drawn from this data. Further studies on these sera are contemplated pending receipt of additional information.

Summary and Conclusions: Sera specimens from 131 humans and 63 rodents were examined for plague antibodies by the newly developed micro-hemagglutination and complement fixation tests. Possible significance of the positive sera specimens is mentioned.

ANNUAL PROGRESS REPORT

Project: 3A 0 12501 A 811

MILITARY MEDICAL RESEARCH PROGRAM
IN SOUTHEAST ASIA
(Social-psychiatric-medical research)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Psychiatry
Division of Neuropsychiatry

Period Covered by Report: 1 July 1962 - 30 June 1963

Principal Investigator: Lt Col Kenneth L. Artiss, MC

Assistant: Lt Col Jerrold L. Wheaton, USAF, MC*

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

* 5th Epidemiological Flight, USAF, Tachikawa AF Base, Tokyo, Japan.

ABSTRACT

Project No. 3A 0 12501 A 811

MILITARY MEDICAL RESEARCH PROGRAM IN SOUTHEAST ASIA
(Social-psychiatric-medical research)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 - 30 June 1963

Author: Lt Col Kenneth L. Artiss, MC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

The report includes investigative efforts in Bangkok, Thailand and Northwest Thailand from Chieng Mai, north to the Burma border; northeast Thailand from Pitsanuloke, north through Uttradit Prae, Nan, Pua. It includes interviews with 260 Special Forces soldiers coming out of action in Laos and a comparison of the medical problems seen in the aforementioned areas with those seen in South Viet Nam to include Saigon and Dalat, with a brief stop at Danang.

Problems investigated regarding the feasibility of further social-psychiatric-medical research, included widespread anemia in some cases complicating mental illness, malnutrition correlated with illiteracy, social attitudes which prevent sanitary measures, problems of education and communication, medical laboratory facilities and communication difficulties between United States personnel and indigenous peoples.

The use of American Special Forces troops in indoctrinating roles with hill tribes was investigated both from the viewpoint of hill tribes in situ and of 260 Special Forces soldiers interviewed, in groups, at the point of evacuation from Laos. Certain aspects of this material are classified and have been reported elsewhere.

BODY OF REPORT

Project No. 3A 0 12501 A 811

MILITARY MEDICAL RESEARCH PROGRAM
IN SOUTHEAST ASIA
(Social-psychiatric-medical research)

Description:

a. The responsibility for socio-psychiatric studies as part of a total field team feasibility study, was given to the above-named investigators. With the question of possibilities for further research constantly in mind, the investigators travelled widely in Thailand, using all varieties of transportation, preferring to obtain firsthand information whenever possible. The itinerary included far North Thailand to the Burma border at Maesai, to the far Northeast Thailand border area at Pua and a series of two dozen locations in the interior.

b. Findings were compared with similar areas in South Viet Nam.

Progress:

The central medical problem of this entire area appeared to be that of anemia. Clinically we found widespread anemia both in and out of hospitals and seriously complicating many problems of mental illness. Anemia appeared to stem from a variety of sources, malnutrition, parasitic infestation, malaria, and inadequacy of both education and communication. Other investigators have observed that the average hemoglobin in Thailand is approximately 60% of normal and it is our impression that this degree of anemia will be found prevalent throughout the entire Southeast Asia complex, Burma, Thailand, Laos, Viet Nam, Malay.

The problems of nutrition have been exhaustively dealt with in a recent Nutrition Survey by the Interdepartmental Committee on Nutrition for National Defense in cooperation with the National Institutes of Health. However, it would be a serious oversight to consider this problem as one of nutrition alone. As an example, we inquired of a mother of a child near death from malnutrition as to what she thought was her child's problem. Her answer was "She vomits." Further inquiry revealed that both mother and father were lacking in sufficient education to possess the conceptual framework for even such an idea as malnutrition, to include their own social responsibility. In short, they simply did not conceive of themselves and the diet they provided as being in any way related to the problems of their dying child.

This situation is repeated in variation with a large percentage of the medical problems in the area. For example, the vast majority of persons resist efforts to introduce sanitation into their living. Apparently they resist such efforts because once again they are unable to conceptualize the relationship between sanitation and disease. They behave as if sanitary measures were rules of behavior invoked by a higher authority which should be avoided whenever the authority is not looking.

Again, it is naive to look at these problems simply as a matter of education because there are more teachers available than are being used in outlying communities. The teachers themselves refuse to live in these outlying communities because they are so isolated by the totally inadequate roads, transportation, news and other communications media that they cannot long remain comfortable without access to persons of their own intellectual level. For example, a teacher who can visit with another equally well educated companion over a weekend will be likely to stay on the job in an isolated community. If, however, this teacher is unable to visit because of totally inadequate roads, unable to obtain newspapers, mail, or other periodicals for the same reasons, etc., then the teacher eventually transfers his interests to an area where his living is more comfortable. In pursuing these issues with local health authorities and officials, it appears that the relationship of sanitation, education, and communication is an interlocking one. Apparently the medical problems of this area are in fact sociological problems and will be successfully approached only if sociological methods of attack are included. E.g., the furnishing of tons and tons of vitamin tablets is no solution.

Adequate indigenous medical efforts are seriously handicapped by inadequate medical training and an almost complete absence of meaningful laboratory facilities. Such absence leads to an empirical practice of medicine in which the only test for a diagnosis is response to medication. As is well known, such practices lead to increasingly "sloppy" diagnosis. The armed forces in these areas suffer from similar privations and are considered to be inadequate to cope with the medical problems which would result from any serious combat endeavor.

Interviews with 260 United States Special Forces soldiers being evacuated from Laos revealed several items of distinct medical importance.

First, the Special Forces medic is unquestionably the most valuable person on the team for making friends and introducing operations in a new territory.

Second, adequate professional (M.D.) supervision of these medics is almost completely lacking. The only medical officer available to them for direct consultation was in Okinawa and there were no arrangements made for systematic supervisory and consultative tours on the part of professional medical officers.

Third, due to certain factors of a classified nature, Special Forces are not being used in the manner for which they have been trained and this has seriously damaged morale, especially of the officer group.

Fourth, without exception, every Special Forces group interviewed maintained that their effectiveness would have been increased an average of 200-300 percent had they received appropriate training in language and customs prior to entering upon their assignment.

Visits were made to two separate hill tribes in Thailand and two separate tribes in South Viet Nam. In addition, consultations were held with Harold M. Young and Gordon S. Young, considered the leading anthropological authorities in the area. Furthermore, all Special Forces interviewed were queried concerning their experience with hill tribes. In general it can be said that certain hill tribe people apparently can be trained as self defense forces to defend territory which is well known to them, if, and only if, strong and constant leadership is provided. The hill tribe people face such common problems and have such a common history that they are relatively indistinguishable whether found in Thailand, Laos, or Viet Nam. Generally, American soldiers have more confidence in the fighting performance of hill tribe people than they do of indigenous so-called valley people, such as Thai, Lao and Vietnamese. The hypothesis that serious and extensive research efforts of a social-anthropological-psychiatric nature in this area are vitally needed was amply supported by the evidence of this trip.

The vital necessity for increased research efforts in an attempt to understand the relevant factors affecting the behavior of indigenous personnel is brought to sharp focus daily in South Viet Nam. It is clearly evident that cultural factors can help explain the fact that so-called friendly indigenous people will not reveal the local presence of Viet Cong ambush troops to Vietnamese and American military units, whereas "terror" explanations alone do not suffice.

Summary and Conclusions:

Problems requiring research in Southeast Asia do not appear to be particularly exotic. They include widespread anemia, correlated with malnutrition, parasitic infestation, malaria, inadequacy of education and communication, and absence of such a conceptual framework as would support the abstraction known to us as "sanitation." Medical problems appear to be inextricably locked-in with sociologic-psychiatric ones, suggesting that appropriate solutions require a multidisciplinary approach.

Special Forces medics are useful, but minimally supervised. Hill-tribe people, while not nationalized, do not appear in great contrast with many indigenous peoples who, also illiterate, lack "nationalistic" ideals. Special Forces soldiers were not overly optimistic regarding the self-defense potential of hill tribes over that of indigenous "nationals."

The hypothesis that increased medical research efforts were needed was amply supported.

Publications:

Artiss, K. L. Brief Report on Socio-psychiatric-medical Problems in Southeast Asia, circulated to AMEDS personnel on a need-to-know basis, 1962.

ANNUAL PROGRESS REPORT

Project: 3A 0 12501 B 813, Army Medical Basic Research in Life Sciences

Task 02, Microbiology (Metabolic Patterns of Pathogenic Bacteria)

Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
	Department of Bacteriology Division of Communicable Disease and Immunology
Period Covered by Report:	1 July 1962 through 30 June 1963
Principal Investigator:	James H. Rust, Jr., Ph.D.
Assistants:	Tom Sanders, SFC John W. Zibreg, SP4
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No.: 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task No. 02 Title: Microbiology (Metabolic Patterns of Pathogenic Bacteria)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: James H. Rust, Jr., Ph.D.
Tom Sanders, SFC
John Zibreg, SP4

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. Purified murine toxin from Pasteurella pestis inhibits the respiration of liver mitochondria isolated from both the rat and the rabbit. Toxin has little or no effect on the respiration of rabbit sarcosomes. However, when rabbit sarcosomes are disrupted with either sodium desoxycholate or sonic vibration, their respiration is inhibited by toxin.

2. Toxin causes the heart mitochondria of the rat to swell but has little or no effect on those of the rabbit. This suggests that one of the effects of murine toxin may be at the mitochondrial membrane level.

BODY OF REPORT

Project No.: 3A 0 12501 B 812 Title: Army Medical Basic Research in Life Sciences

Task No. 02 Title: Microbiology (Metabolic Patterns of Pathogenic Bacteria)

Description: Metabolic studies on the plague murine toxin involves the examination of effects of toxin on host metabolism in attempts to delineate the mechanism of action of this toxin and its role in the pathogenesis of plague.

Progress:

1. Effect of P. pestis Toxin on Disrupted Heart Mitochondria from Toxin Resistant Animals.

The obvious question that now arises concerns the site of action of toxin inhibition of mitochondrial respiration. If a permeability factor were involved, then disruption of heart mitochondria from the toxin resistant rabbit should result in the inhibition of the respiration of such mitochondria upon the addition of murine toxin. The results of experiments involving the effect of toxin on the respiration of rabbit heart mitochondria disrupted with desoxycholate demonstrated that disrupted mitochondria are inhibited by toxin. A summary and a comparison of the results of experiments using sonically disrupted mitochondria as well as mitochondria disrupted by sodium desoxycholate are given in Table I. The addition of toxin alone had essentially no effect on the respiration of unaltered rabbit heart mitochondria whereas the addition of toxin to rabbit heart mitochondria subjected to sodium desoxycholate inhibited respiration by approximately 50 per cent when compared with respiration in the presence of desoxycholate alone. Addition of toxin to sonically disrupted mitochondria gave similar results, although the per cent inhibition appears to be somewhat lower than that with desoxycholate disrupted preparations. It should be noted that desoxycholate alone causes some inhibition when succinate is used as substrate.

2. Effect of Toxin on Mitochondrial Swelling.

Measurement of mitochondrial swelling caused by P. pestis toxin was made using the Beckman DU spectrophotometer. Swelling was indicated by changes in optical density at 520 mu. The diluting fluid consisted of 0.32 M sucrose containing 0.025 M tris (Hydroxy-methyl) aminomethane (tris) buffer, pH 7.4. All reagents were placed in the cuvette and the experiment was initiated by adding 0,1 ml of mitochondrial suspension. The reaction mixture was incubated at room temperature.

An attempt has been made to correlate the effect of toxin on mitochondrial respiration with its effect on mitochondrial swelling. The effect of active toxin and inactivated toxin (heated to 100° C, for 30 minutes) on the swelling of heart mitochondria from the rat is shown in Figure 1. The decrease in optical density demonstrates that toxin

TABLE I

EFFECT OF TOXIN ON THE RESPIRATION OF DISRUPTED RABBIT HEART MITOCHONDRIA

Method of Disruption	Toxin	O_2 consumed (μM/L/sec/mg protein)			Inhibition	
		Endogenous	Alpha-ketoglutarate	Succinate	Alpha-ketoglutarate %	Succinate
None	absent	0.058	-	1.03	-	-
None	2.0 mg	0.075	-	0.942	-	8.6*
$5x10^{-5}$ desoxycholate	absent	0.041	-	0.442	-	57.1*
$5x10^{-5}$ desoxycholate	2.0 mg	0.063	-	0.175	-	59.8**
None	absent	0.051	0.233	—	-	-
None	2.0 mg	0.040	0.240	—	0.0*	-
$5x10^{-5}$ desoxycholate	absent	0.094	0.247	—	0.0*	-
$5x10^{-5}$ desoxycholate	2.0 mg toxin	0.068	0.144	—	42.6**	-
None	absent	0.090	0.350	—	-	-
None	2.0 mg toxin	0.054	0.370	—	0.0+	-
Sonicated-3 minutes	absent	0.062	0.353	—	0.0+	-
Sonicated-3 minutes	2.0 mg toxin	0.055	0.232	—	34.2++	-

Experimental conditions as described in Table I.

* Compared to control without desoxycholate.

** Compared to control with desoxycholate.

\+ Compared to non-sonicated control.

++ Compared to sonicated control.

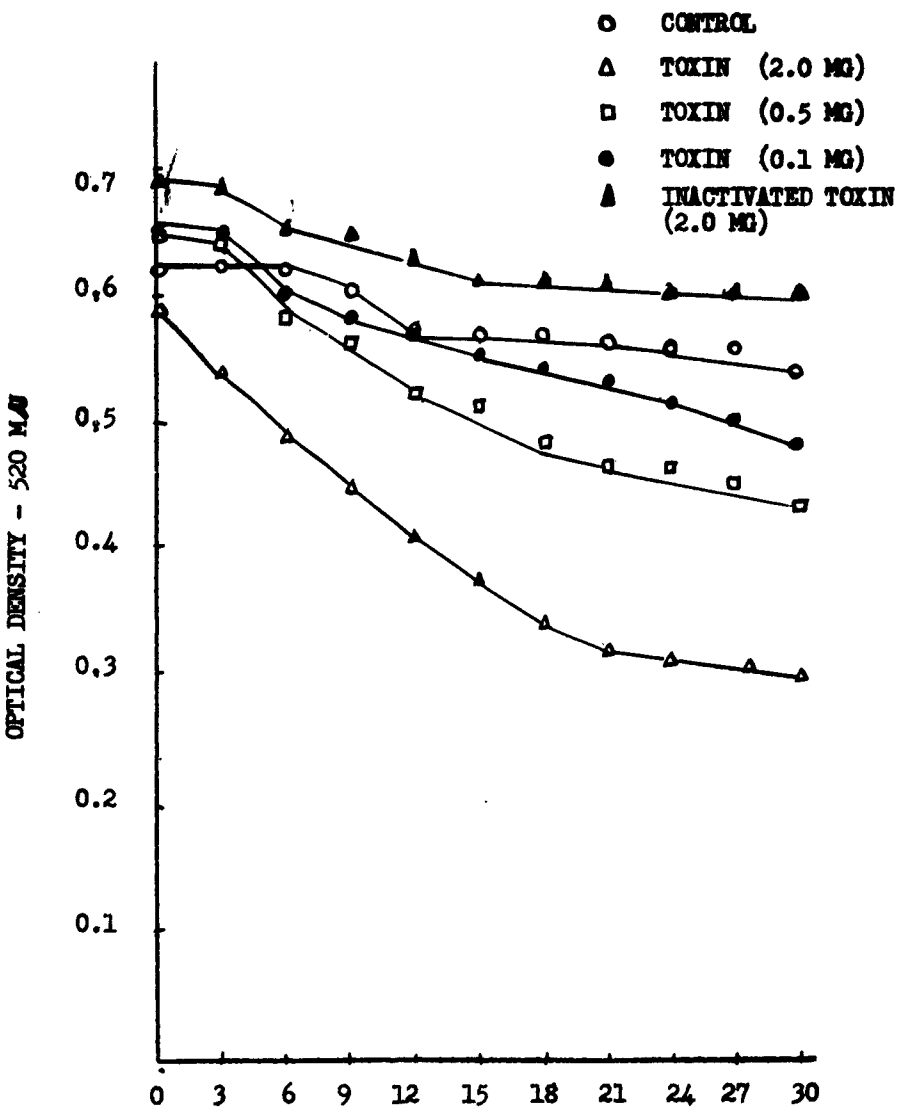


FIGURE 1

EFFECT OF PLAGUE MURINE TOXIN ON RAT HEART MITOCHONDRIA

causes rat heart mitochondria to swell considerably, whereas there is no effect on rabbit heart mitochondria. As expected, inactivated toxin has no effect on the mitochondria from either the rat or the rabbit.

Summary and Conclusions:

1. Preliminary observations on respiration of toxin resistant rabbit heart mitochondria and toxin susceptible rat heart mitochondria indicated that the species specific action of plague toxin may reside at the membrane level of heart mitochondria. Further evidence for this hypothesis was obtained by demonstrating that the resistant mitochondria, when disrupted by either desoxycholate or sonication, were rendered susceptable to the action of toxin.

2. Observed differences of the effects of murine plague toxin on swelling of rabbit and rat heart mitochondria suggest a species specific site of action at the level of the mitochondrial membranes. The precise mechanism of these effects is yet to be elucidated.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 B 813, ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task No. 02, Microbiology (Characterization of leptospiras)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Veterinary Microbiology
Division of Veterinary Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: A. D. Alexander, Ph.D.

Assistants: G. E. Wood, M.S.
R. J. Byrne, D.V.M.*
Francis Yancey, M.S.*
John Rigg, D.V.M.

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

* Department of Veterinary Science, University of Maryland, College Park, Maryland

ABSTRACT

Project No. 3A 0 12501 B 813 Title: ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task No. 02 Title: Microbiology (Characterization of leptospiras)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: A. D. Alexander, Ph.D.
G. E. Wood, M.S.
R. J. Byrne, D.V.M.
Francis Yancey, M.S.
John Rigg, D.V.M.

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

1. Studies were initiated to purify and chemically characterize a hemolytic toxin produced by specific leptospiral serotypes. It was deemed advisable to use allantoic fluid from infected chick embryo eggs as the starting substance for the chemical separation of hemolysin. Methods for producing optimum amounts of hemolysin in infected eggs were determined and several lots of hemolysin with high activity have been produced for projected fractionation studies.

2. In vivo experiments in sheep on cross protection between leptospiral hemotoxins derived from antigenically diverse strains have been completed. Additional data were obtained to demonstrate that animals infected with a hemolysin-producing strain were refractory to hemolytic effects produced by the parenteral administration of partially purified leptospiral hemolysin from an homologous or antigenically-different strain. Furthermore, it was shown that infection with a non-hemolytic strain--serotype hardjo--does not afford protection against the effects of hemolysin. The toxic effects of culture supernatant fluid of serotype hardjo in sheep were again noted in a significant proportion of animals previously exposed to infection with a serologically heterologous type. These effects were seen rarely in animals previously infected with the homologous strain or in controls inoculated with culture medium. These untoward effects are presumably attributed to anaphylaxis. In vitro studies to test this hypothesis are now in progress.

BODY OF REPORT

Project No. 3A 0 12501 B 813 Title: ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task No. 02 Title: Microbiology (Characterization of leptospiras)

Description:

1. The disclosure of a hemolytic exotoxin in cultures of specific leptospiral serotypes was reported previously from this laboratory. Earlier studies provided evidence that the toxin was a protein with enzymatic and antigenic properties. Furthermore, it was demonstrated that leptospiral hemolysin can provoke characteristic disease signs of anemia and hemoglobinuria in sheep. To obtain more information on the nature, mode of action and properties of hemolysin, studies were initiated to purify and chemically characterize this subject.

2. Cross neutralization between hemotoxins from antigenically distinct leptospiras were previously demonstrated in vitro. Initial in vivo studies conducted in sheep provided presumptive evidence of cross immunity amongst antigenically different hemolysin-producing strains with regard to hemolytic manifestations. To elucidate findings in previous studies, additional experiments were conducted.

Progress:

1. In view of problems posed in the separation of leptospiral hemotoxin from proteins of serum ingredients (10%) of leptospiral mediums, it was deemed advisable to utilize allantoic fluid from experimentally infected embryonated chick eggs. The allantoic fluid contains very little proteinacious substance. The presence of hemolysin in this milieu from infected eggs had been demonstrated previously.

The method for obtaining optimum yields of hemolysin in eggs was determined. In the present procedure, 0.2 ml of a culture of serotype pomona is inoculated into the allantoic fluid of 8-10 day old eggs via the air sac. After five days incubation of eggs at 37°C, the allantoic fluid is removed, and centrifuged to separate leptospiral cells. The supernatant fluid, containing the toxin is removed, dialyzed successively against several changes of cold water and finally versus physiological salt solution. It is then placed in stoppered glass containers and stored at -60°C. Several lots of hemolysin with potent activity have been prepared in this manner. Purification studies will be initiated on this starting material employing various protein separation methods.

2. It was previously noted that sheep recovered from experimentally induced infections with hemolysin-producing serotypes canicola or pomona were generally protected against the action of hemotoxins from serologically homologous or heterologous types. On the other hand, infections with serotype hardjo, a non-hemolytic strain, afforded no protection against leptospiral hemolysin. During these studies, it was noted also that an extract of culture supernatant fluid from serotype hardjo provoked severe toxic manifestations in animals exposed previously to canicola or pomona, but not in controls or in animals that had been infected with the homologous strains. The toxic manifestation of culture supernatant fluid of serotype hardjo was unexpected. To resolve questions posed in earlier experiments, additional cross-protection studies were conducted in 8-12 month old sheep with serotypes hardjo and pomona.

Sheep ascertained to be serologically negative for leptospirosis were divided into 3 groups. Eight animals in each of 2 groups were infected with serotypes pomona and hardjo respectively, by inoculation of 5 ml dose, S.C. of a 10-14 day old culture. A third group of control animals, comprising 14 individuals, were similarly inoculated with culture medium. Following infection, only 1 of 8 animals infected with serotype pomona had signs of anemia and hemoglobinuria. All animals in this group had a febrile response. No signs of disease were seen in animals infected with serotype hardjo. Twenty-eight days after infection, 4 animals from each of two groups were given an intravenous injection of 40 ml of a partially purified pomona hemolysin. The hemolysin preparation was obtained by fractionation of culture supernatant fluid with ammonium sulfate. An analogous (non-hemolytic) preparation from cultures of hardjo was given similarly to the remaining 4 animals in each of the groups. Of the control animals, 4 were given pomona hemolysin, 8 the hardjo preparation and 2 were given a similar preparation obtained from culture medium.

Animals that were infected with serotype pomona were immune to action of hemolysin. Pomona hemolysin elicited typical signs of anemia and hemoglobinuria in control animals and in animals that had been exposed to serotype hardjo. Inoculation of the hardjo preparation into sheep exposed to pomona resulted in the death of 3 or 4 animals. Death occurred approximately 18 hours after inoculation. In contrast, this preparation produced death in only 1 of 4 sheep exposed to hardjo and in 1 of 8 sheep previously inoculated with medium.

The above and previous findings are given in Table 1 and summarized in Table 2. Previous exposure to a hemolytic strain conferred immunity against effects of the hemotoxin in 7 of 8 animals challenged with a homologous hemolysin and in all 4 animals challenged with a hemolysin from a heterologous strain. In contrast, only 1 of 8 animals exposed to a non-hemolytic strain were refractory to effects of hemolysin. The toxic effects of the preparation from hardjo were significantly higher in animals previously infected with serologically heterologous strains (5 out of 6) than in animals infected with the homologous strain (1 out of 6). There was no evidence of persistent toxic reactions following inoculations of analogous preparations of media.

Table 1

Results of Cross-Protection Studies in Sheep on Leptospiral Hemolysins

Exposure of Sheep	Challenge 26 Days Post Exposure	Signs No. Negative / No. Challenged	Signs* in Non-Protected Animals
Infected with pomona (hemolytic strain)	pomona hemolysin	5/6	H., A., Death (4 day)
	canicola hemolysin	2/2	
	hardjo concentrated culture fluid	1/5	H., A., Death 3/4 (18 hr)
	concentrated media**	3/3	
Infected with canicola (hemolytic strain)	pomona hemolysin	2/2	
	canicola hemolysin	2/2	
	hardjo concentrated culture fluid	0/1	Death (24 hr)
Infected with hardjo (non-hemolytic strain)	pomona hemolysin	1/6	H., A.
	canicola hemolysin	0/2	H., A., Death (24 hr)
	hardjo concentrated culture fluid	5/6	Death (24 hr)
Media control	pomona hemolysin	0/5	H., A.
	canicola hemolysin	0/1	H., A.
	hardjo concentrated fluid	8/9	Death (24 hr)
	concentrated media**	3/3	

*Abbreviations: H. = hemoglobinuria, A. = anemia
**Includes heat inactivated pomona hemolysin given to 2 and 1 sheep in top and bottom groups respectively.

Table 2

Summary of Cross-Protection Studies on Leptospiral Hemolysin

Exposure of Sheep	Challenge (26 Days Post Exposure)	No. Protected / No. Challenged
hemolytic strains (*pomona* or *canicola*)	homologous hemolysin	7/8
	heterologous hemolysin	4/4
	non-hemolytic prep. (hardjo)	1/6
	medium (control)	3/3
non-hemolytic strain (*hardjo*)	hemolysin (*pomona* or *canicola*)	1/8
	non-hemolytic preparation (hardjo)	5/6
	medium (control)	3/3
medium (control)	hemolysin (*pomona* or *canicola*)	0/6
	non-hemolytic preparation (hardjo)	8/9
	medium (control)	3/3

Summary and Conclusions:

1. Studies are now in progress to purify and chemically characterize the hemolytic toxin produced by leptospiras. The starting material for fractionation of hemolysin is allantoic fluid from infected chick embryo eggs.

2. Cross-protection studies were conducted in sheep that were experimentally infected with 2 hemolytic, but antigenically different, leptospiras (serotypes pomona and canicola) and a third antigenically distinct non-hemolytic strain (serotype hardjo). Infection with a hemolytic strain conferred immunity against the hemolytic effects of partially purified hemotoxins from homologous and heterologous strains. Animals infected with the non-hemolytic strain were susceptible to action of hemolysin. A preparation from a non-hemolytic strain (serotype hardjo) elicited toxic effects in a significantly higher proportion of those sheep previously infected with pomona or canicola than in animals infected with the homologous types. These effects are believed to be due primarily to anaphylaxis.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 B 813 ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task 02, Microbiology (Role of bacteria and endotoxins in shock)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Germfree Research
Division of Basic Surgical Research

Period Covered by Report: 1 July 62 - 30 June 63

Principal Investigators: Albert Einheber, Ph. D.

Assistants: Robert E. Wren, B.A.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 B 813 Title: ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task 02 Title: Microbiology
(Role of bacteria and endotoxins in shock)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 62 - 30 June 63

Authors: Albert Einheber, Ph.D.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Mice in shock following hind limb tourniquets survive after saline therapy, but not after any amount or type of therapy yet tried if the mice are subjected to tourniquets on all 4 limbs. The investigators have taken this to suggest that factors beyond fluid, electrolyte and protein disturbances are involved in this "irreversibility". Since other researchers have implicated a bacterial factor in tourniquet shock, this contention should be amenable to test with germfree mice once the therapeutic refractoriness of conventional mice subjected to 4 limb tourniquets is substantiated. To initially standardize tourniquet injury in conventional ICR mice (a strain not used for this previously but now available germfree), tourniquets were applied to the fore limbs, hind limbs, or to all 4 limbs for 2 hours with resultant 24 hour mortalities of 0%, 30% and 100%, respectively. Thus, application of both fore and hind limb tourniquets markedly potentiates the lethal tendency of the separate injuries. Hind limb tourniquets applied for 2.0, 2.5 or 3.0 hours, resulted in 24 hour mortalities of 25%, 60% and 100%, respectively. Therefore, three hour hind limb tourniquets produced the same 24 hour mortality (100%) as did the 2 hour quadrilateral tourniquets. Therapeutic studies are in progress.

BODY OF REPORT

Project No. 3A 0 12501 B 813 Title: ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task 02 Title: Microbiology
(Role of bacteria and endotoxins in shock)

DESCRIPTION:

Adequate amounts of isotonic sodium salts have been found to be highly effective in promoting survival of mice after they have received "one lethal dose" of burn or tourniquet injury or hemorrhage. However, it has been reported (Rosenthal, S.M. The Biochemical Response to Injury, CC Thomas, 1960, p. 397) that few mice survive more than one lethal dose of trauma (tourniquet to four legs) "in spite of therapy with up to 30% body weight saline even when supplemented with plasma or whole blood, and antibiotics." The difficulty of explaining "why doubling the trauma is not to some extent counteracted with double the amount of therapy", has led Rosenthal to consider this as evidence that factors in addition to fluid, electrolyte and protein disturbances are acting in shock. If we can substantiate these findings with four leg tourniquets, then it would be of interest to repeat these experiments with germfree mice to determine whether or not a bacterial factor is involved, i.e., whether these mice are more tolerant of this injury than conventional mice. Our previous experience with bilateral-hind limb tourniquet injury in monocontaminated and germfree mice (Levenson, S.M., Einheber, A., and Malm, O., J. Am. Med. Ass. 181:874, 1962) has provided us with the necessary procedures for readily doing these experiments in the germfree isolator once the preliminary conventional mouse experiments with four leg tourniquets, with which we have had no previous experience, bear out the above findings. Since the strain of mouse used may be a factor, and we have no experience with tourniquet injury of ICR mice, which will be used in the germfree experiments, we have been studying tourniquet injury of conventional ICR mice, with the intent of attempting therapy once the injury, in terms of mortality, is standardized.

PROGRESS:

Conventional male ICR mice, 5-6 weeks old, were used. To inflict tourniquet shock, we used our adaptation of the Rosenthal mouse tourniquet technic in unanesthetized mice. All tourniquets were applied with a No. 5 cork-borer over which a No. 30 Eberhard Faber rubber band, pre-lubricated with glycerine, was wrapped 6 turns. Food and water were allowed ad libitum prior to injury, but none thereafter. Animals that lived beyond 48 hours after tourniquet release were considered survivors.

Experiment No. 1: ICR mice were divided into 3 groups. Group I received 2 hours of bilateral hind limb ischemia, Group II received 2 hours of quadrilateral-limb ischemia and Group III received 2 hours of bilateral fore limb ischemia.

Hours Post-Tourniquet Release	Mortality, % Group I (20 mice)	Group II (12 mice)	Group III (5 mice)
0-9	0	50	0
21	15	100	0
24	30	100	0
48	75	100	0

The results indicate that ischemia to all 4 limbs is a highly lethal procedure. The mortality following 4 limb tourniquets far exceeds the sum of the individual mortalities resulting from bilateral hind limb and bilateral fore limb tourniquets.

Experiment No. 2: All mice received bilateral hind limb ischemia. The duration of tourniquet application was varied: Group I (2.0 hrs), Group II (2.5 hrs), and Group III (3.0 hrs). Animals surviving 48 hours after tourniquet release were considered survivors.

Hours Post-Tourniquet Release	Mortality, % Group I (8 mice)	Group II (10 mice)	Group III (8 mice)
0-9	0	0	25
21	13	40	88
24	25	60	100
48	38	100	100

Increases in mortality proportional to the duration of tourniquet application are discernible at 24 hours. Also at 24 hours, 3 hour bilateral hind limb ischemia in this experiment proved as highly lethal (100%) as did the 2 hour quadrilateral-limb ischemia in Experiment No. 1. Therapeutic studies are in progress.

SUMMARY AND CONCLUSIONS

Mice in shock following hind limb tourniquets survive after saline therapy, but not after any amount or type of therapy yet tried if the mice are subjected to tourniquets on all 4 limbs. The investigators have taken this to suggest that factors beyond fluid, electrolyte and protein disturbances are involved in this "irreversibility". Since other researchers have implicated a bacterial factor in tourniquet shock, this contention should be amenable to test with germfree mice once the therapeutic refractoriness of conventional mice subjected to 4 limb tourniquets is substantiated. To initially standardize tourniquet injury in conventional ICR mice (a strain not used for this previously but now available germfree) tourniquets were applied to the fore limbs, hind limbs or to all 4 limbs for 2 hours with resultant 24 hour mortalities of 0%, 30% and 100%, respectively. Thus, application of both fore and hind limb tourniquets markedly potentiates the lethal tendency of the separate injuries. Hind limb tourniquets applied

for 2.0, 2.5, or 3.0 hours, resulted in 24 hour mortalities of 25%, 60% and 100%, respectively. Therefore, three hour hind limb tourniquets produced the same 24 hour mortality (100%) as did 2 hour quadrilateral tourniquets. Therapeutic studies are in progress.

LIST OF PUBLICATIONS:

Levenson, S.M., Einheber, A. and Crowley, L.V. Some Effects of Whole Body X-Irradiation and Thermal Injury. Research in Burns, Publication No. 9, AIBS, 1962, p.143.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 B 813, Army Medical Basic Research in Life Sciences

Task 02, Microbiology (Genetic mechanisms in the enteric bacteria)

Reporting Installations: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Bacterial Immunology
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 - 30 June 1963

Principal Investigators: L. S. Baron, Ph.D.
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No: 3A 0 12501 B 813 — Title: Army Medical Basic Research in Life Sciences

Task 02 — Title: Microbiology (Genetic mechanisms in the enteric bacteria)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 - 30 June 1963

Authors: L. S. Baron, Ph.D.
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. Reciprocal recombination tests involving Escherichia coli Hfr donors and Salmonella typhosa hybrids produced by previous E. coli matings showed that recipient ability of these hybrids is increased when their integrated E. coli chromosome segment matches the lead region of the back-crossing Hfr chromsome.

2. Episomic elements derived from E. coli may be transferred to species of Proteus resulting in a marked molecular heterogeneity in the Proteus DNA. The proper structure of the B-galactosidase enzyme of Escherichia is correctly interpreted by episomally-infected Proteus, although the regulatory functions of the transferred Escherichia lactose genes are impaired.

3. The ability to utilize lactose found in Proteus strains isolated from natural sources has been found to be due to an episomic element.

4. Donor strains of Salmonella, either F^+ or Hfr types, have been prepared using the classical fertility factor, F, of Escherichia coli, as a promotor of genetic recombination between Salmonella. The results of these studies describe characteristic features of the Salmonella mating system which are applicable to the investigation of the genetic basis of virulence in the enteric bacteria.

BODY OF REPORT

Project 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task 02 Title: Microbiology (Genetic mechanisms in the enteric bacteria)

Description: The purpose of these studies is to investigate the genetic characteristics of the metabolic and antigenic changes occurring in the enteric bacteria as a consequence of genetic recombination, episomic transfer, and transduction in order to examine the genetic basis of virulence.

Progress:

1. Recipient Ability of Salmonella typhosa in Crosses with Escherichia coli.

a. Salmonella typhimurium hybrids from matings with Hfr strains of Escherichia coli K-12 exhibit increased fertility on backcross with the E. coli parent. This has been shown to represent selection of pre-existing high frequency recipients from an otherwise sterile population by the first round of mating. Examination of this phenomenon in strains of S. typhosa, however, has indicated that, in this species, selection is not involved, the increased backcross frequency being due to the presence of E. coli genetic material in the hybrids. The first evidence of this fact came from the examination of lactose positive (lac+) S. typhosa hetergenotes formed from E. coli x S. typhosa matings. Such heterogenotes, on EMB lactose agar, segregate lactose negative (lac-) clones which have lost the E. coli genetic material. On backcross with the E. coli parent, none of the lac- segregants tested showed any increase in fertility.

b. Employing the E. coli Hfr W1895, which is able to utilize both lactose and arabinose (ara^+), and transfers these genetic markers in the order o... lac^+ ... ara^+, we obtained S. typhosa hybrids which had stably integrated the lead marker lac^+, but not the more distal marker ara^+. These lac^+ hybrids, on backcross with W1895 for the distal marker ara^+, showed a 200-fold increase in recombination frequency over the control crosses, in which previously unmated S. typhosa 643WSr was used as the recipient.

c. Another class of S. typhosa hybrids, possessing ara^+ but not lac^+, was obtained from a mating with W1895. On backcross with W1895 for lac^+, these ara^+ hybrids failed to show any increase in recombination frequency. This again indicated that high frequency recipients were not being selected on the initial cross.

d. The failure of ara^+ hybrids to show fertilize increase with W1895, as had the lac^+ hybrids, was resolved by crossing them with E. coli Hfr Hayes, which transfers in the order o... ara^+... lac^+. In this cross, again with selection for lac^+, the ara^+ hybrids now showed a 265-fold

increase in fertility. Thus it appeared that the increased fertility of the hybrids occurred when the E. coli chromosome segment which they had integrated matched the proximal region of the backcrossing Hfr chromosome. As would be predicted by this concept, the lac^+ hybrids, which had shown increased fertility on backcross with W1895, showed no increase on backcross for ara^+ with Hayes. This same series of reciprocal crosses was repeated with lac^+ and ara^+ S. typhosa hybrids derived from initial mating with Hfr Hayes, and their behavior was essentially similar to that of the hybrids produced by W1895.

e. Data obtained by printing individual S. typhosa clones on selection plates spread with E. coli Hfr donors indicated a relative homogeneity of the S. typhosa population with respect to the ability to mate initially at low frequency with an E. coli Hfr. This frequency is about 2000-fold less than that obtained in an E. coli Hfr x E. coli F^- cross, and the nature of this difference was investigated.

f. The ability of S. typhosa to conjugate with E. coli was examined by employing the sexducing E. coli strain 2586 F_0-lac, which transfers the lac^+ gene as an episomic element. Comparing the recombination frequency of a 2586 F_0-lac x S. typhosa cross with that of a 2586 F_0-lac x E. coli F^- cross, only a 4-fold increase was observed in the latter. Since conjugation is a prerequisite for episome transfer, it was inferred that E. coli conjugates with S. typhosa almost as efficiently as it does intraspecifically.

g. Since the small difference in conjugal ability could not account for the 2000-fold difference in recombination frequency observed when the Hfr donor was employed, the process of transfer was investigated. This was accomplished by assaying the mating mixtures for production of B-galactosidase by the bacterial merozygotes. Since the z-gene of the lac^+ region is expressed almost immediately upon entering the recipient cell, without the requirement of chromosomal integration, enzyme production could be used as an indication of chromosome transfer. Enzyme was readily detectable in E. coli F^- merozygotes and S. typhosa merozyotes in the matings involving the episome donor. Where the Hfr donor W1895 was employed, however, enzyme was not detectable (within the sensitivity limit of the assay) from the S. typhosa mating; only the E. coli F^- merozygotes produced enzyme in this instance. These experiments indicated that transfer of the lac^+ gene from W1895 to S. typhosa was accomplished with low efficiency or was abortive.

2. Episomic Elements in Bacteria.

a. Studies from this laboratory have previously demonstrated that episomic elements may be transferred from E. coli to Serratia marcescens despite the marked divergence in the guanine and cytosine (GC) content of the deoxyribonucleic acid (DNA) of these organisms. Members of the Proteus group exhibit DNA base compositions which differ markedly from all other members of the Enterobacteriaceae and during the past year we have transferred several episomic elements to Proteus species

and characterized the DNA and B-galactosidase found in episomally infected Proteus cells.

b. The episomic elements transferred to Proteus species were F-lac, F_0-lac, F^1-13 and R-factor, all initially described in Escherichia Salmonella. The F-lac and F_0-lac elements consist of an infectious factor, F, which has incorporated the genes governing lactose utilization (lac^+). F^1-13 carries, in addition to F, the genes governing lac^+ as well as the genes governing the synthesis of alkaline phosphatase and adenine. The R-factor consists of an infectious element, RTF, and the drug resistant loci for streptomycin (SM), chloramphenicol (CM), tetracycline (TC) and sulfonamide (SU). Initially all of the episomic elements are markedly unstable in Proteus but by applying rigorously selective conditions, stable clones of each may be isolated.

c. DNA was extracted from the Escherichia and Salmonella strains used as the donors of the episomic elements to Proteus. The DNA of each was examined by the technique of CsCl density-gradient ultracentrifugation and the buoyant densities of the banded DNA determined. The DNA of all of the Escherichia and Salmonella donors exhibited buoyant densities of 1.710±.001 gm/cm (equivalent to 50% GC) with essentially gaussian, unimodal molecular distributions. DNA extracted from Proteus species prior to episomal infection display buoyant densities of 1.698 gm/cm^3 (39% GC) with a small base compositional heterogeniety and a unimodal molecular distribution. DNA extracted from episomally infected Proteus cells, however, show a marked molecular heterogeniety in that in addition to the 1.698 gm/cm^3 (39% GC) band, they all have additional or satellite bands of DNA. In Proteus F-lac, F_0-lac and F^1-13 DNA preparations, this satellite band has a bouyant density of 1.710 $gm.cm^3$, 50% GC - equivalent to the base composition of the strains from which the episomic elements were derived. Proteus R-factor DNA exhibits two satellite bands of buoyant density 1.711 gm/cm^3 and 1.718 gm/cm^3 equivalent to 51% and 58% GC respectively. The satellite bands of Proteus RFT and F^1-13 comprise about 8% of the total DNA while Proteus F-lac and F_0-lac satellite bands represent about 3-4% of the total DNA extracted.

d. The Proteus F-lac, F_0-lac and F^1-13 strains all exhibit the lac^+ phenotype but despite daily restreaking and selection for lac^+ they continually segregate out lac^- cells at a frequency of about 0.3%. They exhibit, therefore, the properties of segregating diploid heterozygotes in which all episomic elements are being maintained autonomously rather than in the integrated state. All of the lac^- segreants examined to date appear to have lost completely their episomic elements. Concomitant with the loss of the episomic element there is a loss of the satellite DNA band. The lac^- cells were observed to accept episomic elements in subsequent matings about as readily as did the original Proteus recipient strains. Upon reinfection with the episomic element, the satellite band(s) are again apparent. It seems likely, therefore, that the satellite DNA bands associated with episomal infection represent the transferred genetic element.

e. B-galactosidase synthesis by Proteus strains harboring lac$^+$ episomic elements was examined in an effort to determine whether enzyme synthesis was affected by a diverse cellular environment and if Proteus can decode information contained in DNA from a cell with different base composition. Table 1 shows enzyme synthesis of Escherichia donor strains episomally infected Proteus strains in a synthetic medium with and without the inducer isopropyl-thiomethyl-B-d-galactoside (IPTG). It may be profitable to recall that four genes, i,o,y, and z, have been described in the lac locus. The genes z and y provide the genetic information for the structures of B-galactosidase and galactoside permease respectively. Normally the i gene produces a repressor substance which by its action on the o gene controls the functioning of the z and y genes. The presence of inducer such as IPTG antagonizes the repressor and permits enzyme synthesis. Enzyme synthesis in the absence of inducer occurs in strains unable to make repressor and in o mutants, termed o^c, which have become insensitive to repressor. In Table 1, a typical inducible E. coli diploid strain is taken as a standard for comparative purposes. E. coli F- lac is inducible and produces roughly 3 X as much enzyme as the diploid strain. This increased synthesis may be interpreted as indicating the presence of more than one episomic element/cell. The E. coli F$_o$-lac strain carries on o^c mutation and exhibits some constitutive synthesis and a high level in the presence of inducer. Proteus species are normally lac$^-$ and do not produce any B-galactosidase with or without IPTG in the growth medium. Proteus F-lac cells despite the presence of the repressor gene display a constituitive synthesis of enzyme and are relatively insensitive to inducer. Moreover, the amount of enzyme produced is only about 1/4 of that produced by an E. coli diploid cell. Proteus F$_o$- lac cells show a somewhat higher level of enzyme synthesis but are also insensitive to inducer. On the other hand, the enzyme produced by lac$^+$ Proteus cells is essentially identical to that produced by E. coli K-12 in regard to K_m, thermal inactivation and immunological specificity. Thus the genetic information for determining the correct structure of Escherichia B-galactosidase resides in and is apparently correctly interpreted by Proteus although the regulatory function of Escherichia lac$^+$ genes in Proteus may be impaired.

3. A lac$^+$ Episomic Element in Naturally Occurring Strains of Proteus.

a. At the same time that we were conducting experiments on laboratory transfer of lac$^+$ episomes to Proteus, we were attracted to a report on lac$^+$ Proteus species isolated from hospitalized patients. We obtained a number of these strains for examination. All of them harbor a genetic element, termed P-lac, which is readily transmissible to lac$^-$ species of Escherichia as well as to Salmonella, Shigella, Serratia and even Vibrio comma. The P-lac element carries the lactose genes as the only known genetic determinants. The infectious component of P-lac is related to F and confers low frequency chromosomal fertility on recipient strains of E. coli but not Salmonella. Unlike F-lac and other episomic elements in Proteus, P-lac is remarkably stable but may be "cured" by treatment with acridine orange. In CsCl density gradients, DNA extracted from Proteus P-lac shows a satellite band comprising about 10% of the total DNA with a bouyant density of 1.710 gm/cm^3 (50% GC). This band is not present in acridine "cured" P-lac cells

but appears in *Proteus* cells concommitant with P. *lac* infection. B-galactosidase production by *Proteus* P-*lac* cells is constitutive and occurs at levels higher than those observed in *Proteus* F-*lac* or F_o-*lac* strains as shown in Table 2. The P-*lac* enzyme is similar to that of *E. coli* K-12 in a number of characteristics but differs in its thermal stability. It seems likely, therefore, that the *Proteus* *lac*$^+$ strains from hospitalized patients acquired their *lac*$^+$ property by infection from an organism of dissimilar DNA base composition, probably of the genus *Escherichia*.

b. It is becoming increasingly clear that episomal infection occurs readily in nature and is not just a laboratory "trick". It should be recalled that the episomic elements F_o-*lac*$^+$ and R factor were initially reported in strains isolated from nature F_o-*lac*$^+$ was found in a *lac*$^+$ typhoid strain isolated from a gastroenteritis case while R factor was found in drug resistant cases of shigellosis. Now we have P-*lac* in *Proteus* strains most of which were encountered in urinary tract infections. Episome transfer is the least demanding of genetic transfer systems in bacteria since the transferred genes do not have to interact with the resident chromosome but may replicate autonomously. This mode of gene transfer, therefore, makes possible the dissemination of genetic information between populations of bacterial cells which normally lack a common mating system and which differ in the composition of their DNA. At the practical level episomic elements such as F_o-*lac* and P-*lac* pose a problem to the diagnostic bacteriologist. Moreover, the case of R-factor is most significant in regard to infectious diseases. Not only are R-factors responsible for the emergence of drug-resistant strains of *Shigella* in Japan but drug resistant strains of *Salmonella typhimurium* due to an R-factor were encountered recently in England during a hospital epidemic. Moreover, preliminary data on the *lac*$^+$ *Proteus* strains from hospitalized patients indicates that some of these also harbor R-factors.

Table 1

B-galactosidase Production by *Proteus* Strains Harboring Episomic Elements

Strains	Genotype	Enzyme Units -IPTG	Enzyme Units +IPTG
E. coli haploid	$i^+o^+z^+y^+$	1	100
E. coli F-lac$^+$	$i^+o^+z^+y^-/F\text{-}i^+o^+z^+y^+$	1	309
E. coli F_0-lac$^+$	$i^+o^+z^+y^-/F_0\text{-}i^+o^cz^+y^+$	5.4	267
P. mirabilis parent	-/-	1	1
P. mirabilis F-lac$^+$	$-/F\text{-}i^+o^+z^+y^+$	2.8	22.1
P. mirabilis F_0-lac$^+$	$-/F_0\text{-}i^+o^cz^+y^+$	36	41

Table 2

Properties of B-galactosidase from *Proteus* Cells Harboring Episomic Elements

	B-galactosidase		
	E. coli	*Proteus* F-lac$^+$	*Proteus* P-lac$^+$
K_m	1.64×10^{-4}M	1.75×10^{-4}M	1.61×10^{-4}M
K_{590c}	.16 unit/min.$^{-1}$	.16 unit/min^{-1}	.03 unit/min^{-1}
S_{w20}	16.1	15.4	15.9
Immunological Unit	3,700	3,500	?

4. Genetic Recombination Between Species of Salmonella

a. Three donor (male) cultures of Salmonella typhimurium were employed in crosses with the same recipient (female) strain of S. typhimurium. A recipient strain of Salmonella typhosa 643w was used for interspecies hybridization studies. S. typhimurium Hfr 305 has its point of origin close to the marker for isoleucine, followed by the marker for rhamnose, etc, with xylose as one of the last markers to be transferred. Hfr 15-11 and recipient strain S. typhimurium sw1291, were both derived from the same strain, TM-9, a culture previously established as being fertile in crosses with E. coli K-12 donors. Hfr 15-11 which has its origin near proline, was isolated in the following manner, E. coli Hfr P4X-6 and S. typhimurium TM-9 are crossed with selection being made for the distal marker lactose. Since it has been determined that the lactose marker and the sex factor are closely linked in the P4X-6 strain, this mating results almost invariably in the isolation of lactose positive recombinents of the S. typhimurium strain possessing Hfr donor ability. These S. typhimurium males exhibit the identical orientation of chromosomal transfer as does the P4X-6 E. coli Hfr strain. Hfr 19 represents the same donor type in S. typhimurium strain LT-2.

b. In these experiments, crosses between Hfr 15-11 and SW1291, a mating within the same strain, yielded hybids at the highest frequency, generally about ten-fold better than the recombination frequencies observed in the interstrain matings. In addition, transfer of all markers at readily detectable frequencies was obtained in the intrastrain matings regardless of the chromosomal location of the selected marker. Thus complete transfer of the chromosome took place in a proportion of the hybrids which arose in the intrastrain matings. On the other hand, interstrain matings give rise to decreasing numbers of hybrids for the distal markers of the chromosome. This is illustrated by the case of an LT-2 donor crossed with a TM-9 recipient (Hfr 19 mating with SW 1292), where the last marker lactose is not detected, and the prior distal marker galactose is only rarely detectable. Essentially the same sort of results were observed in the interstrain cross, Hfr 305 X SW1291, where transfer of the terminal markers falls off considerably to the point where occurrence of the last marker xylose in the hybrids is an extremely rare event.

Linkage relationships in intrastrain and interstrain matings of S. typhimurium cultures show an extensive transfer of markers along the chromsome. Large relatively stable diploid regions covering mainly the early markers were also observed in the intrastrain crosses. Interspecies matings between S. typhimurium and S. typhosa, on the other hand, present a totally different picture when compared to the results obtained in crosses between S. typhimurium strains. Only rarely does more than the selected marker appear in any of the S. typhosa hybrids. Thus linkage analysis of the recombinants from interspecies hybridizations indicates a lack of the more complete genetic homology which is observed in the intraspecies matings. Further confirmation of genetic inhomologies detectable in conjugation experiments is afforded by the extremely low frequencies of recombination and the

instabilities of the hybrids which are the outcome of crosses between the S. typhimurium Hfr strains and F-strains of either E. coli or Shigella flexneri as recipients.

Summary and Conclusions:

1. Recombination experiments involving Escherichia coli K-12 Hfr strains and Salmonella typhosa hybrids produce by previous mating with E. coli demonstrated that recipient ability is increased in these hybrids when their integrated E. coli chromosomal segment matches the lead region of the Hfr chromosome. Data obtained from replica platings has indicated that the S. typhosa population is probably homogeneous with respect to the ability to mate initially with E. coli at low frequency. The evidence indicates that conjugation between E. coli and S. typhosa is efficient but that in such crosses transfer of genetic material is accomplished with low efficiency or is abortive.

2. The episomic element F-lac and the resistance transfer factor (RTF) originally described in Escherichia and Shigella respectively can be transferred to strains of Proteus mirabilis by conjugation. Usually these genetic elements are markedly unstable in Proteus but repeated plating under rigorously selective conditions can be used to obtain stable clones. DNA extracted from the P. mirabilis parental strain, P. mirabilis F-lac and P. mirabilis RTF have been examined by CsCl density gradient centrifugation. P. mirabilis DNA has a GC content of 38%, while DNA from Proteus F-lac has, in addition, a band of about 3% of the total DNA at 50% GC. DNA extracted from Proteus harboring the RTF episome shows two additional bands, one at 58% GC, the other at 50% GC, in addition to the main band of 38% GC.

3. Several strains of Proteus isolated from patients are unusual in that they possess the ability to ferment lactose (lac^+). It has been found that the lac^+ character behaves as an episomic element that is readily transmissable at high frequency to Escherichia coli, Salmonella, Serratia marcescens, other species of Proteus, and even to Vibrio cholerae. Examination of DNA extracted from these lac^+ strains of Proteus revealed the presence of a large satellite band which represents about 10% of the total DNA and corresponds to a GC content of 50%. These results confirm the fact that genetic transfer occurs readily in nature.

Publications:

1. Wohlhieter, J. A., Brinton, C. C., and Baron, L. S. Utilization of Carbohydrates by Piliated and Non-Piliated Bacteria. J. Bacteriol. 84: 416 (1962).

2. Falkow, S. and Baron, L. S. An Episomic Element in a Strain of Salmonella Typhosa. J. Bacteriol. 84: 581 (1962).

3. Falkow, S., Rownd, R. and Baron, L. S. Genetic Homology Between Escherichia Coli and Salmonella. J. Bacteriol. 84: 1303 (1962).

4. Wohlhieter, J. A., Falkow, S., Citarella, R. and Baron, L. S. Characterization of DNA from Proteus Strains Harboring Episomes. Abst. Biophys. Soc. 7th Meeting (1963).

5. Johnson, E. M., Falkow, S., and Baron, L. S. Recipient Ability of Salmonella Typhosa in Genetic Crosses With Escherichia Coli K-12. Bacteriol. Proc. 1963.

6. Baron, L. S., Ryman, I., Falkow, S., and Krishnapillai, V. Genetic Recombination Between Species of Salmonella. Bacteriol. Proc. 1963.

7. Falkow, S., Wohlhieter, J. A., Citarella, R., and Baron, L. S. Transfer of Episomes to Proteus. Bacteriol. Proc. 1963.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 B 813 Army Medical Basic Research in Life Sciences

Task 02, Microbiology (Etiology of Viral Hepatitis)

Reporting Installation:	**Walter Reed Army Institute of Research** **Walter Reed Army Medical Center** **Washington 12, D.C.**
	Department of Virus Diseases **Division of Communicable Disease & Immunology**
Period Covered by Report:	**1 July 1962 through 30 June 1963**
Principal Investigators:	**Lt Colonel E. L. Buescher, MC** **Captain H. L. Weinberger, MC** **Captain M. S. Artenstein, MC** **Captain A. T. C. Bourke, MC**
Assistants:	**M. L. Wohlhieter, B.S.** **V. M. Edwards, B.S.** **Sfc J. M. McCown** **Pfc D. Bennett** **Pfc W. Burnette**
Reports Control Symbol:	**MEDDH-288**
Security Classification:	**UNCLASSIFIED**

ABSTRACT

Project 3A 0 12501 B 813 — Title: Army Medical Basic Research in Life Sciences

Task 02 — Title: Microbiology (Etiology of Viral Hepatitis)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D.C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Lt Colonel E. L. Buescher, MC
Captain H. L. Weinberger, MC
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. Attempts to recognize transmissible agents from throat washings, stools and sera obtained during the acute phase of infectious hepatitis in Korea in 1962 were made. Efforts to show interference for Polio Virus II, rubella, herpes simplex and ECHO Type II viruses were uniformly negative. No evidence for recent infection with leptospires was shown for the donor patients.

2. Attempts to isolate serum hepatitis virus by demonstrating interference with the cytopathogenic effects of polio type 2, herpes simplex and Eastern equine encephalomyelitis viruses were made. Attempts were also made to recognize an agent by double interference technique using combinations of rubella and polio type 2 viruses, and dengue type I and polio type 2 viruses. One experiment was conducted in fluid cultures of human embryonic kidney cells and the remainder in fluid cultures of African green monkey (Cercopithicus aethiops) kidney cells. Acute phase serum taken from a patient in the Walter Reed General Hospital served as the potential source of homologous serum jaundice virus. The results to date have been negative.

3. The interference technique was used in attempts to recover an agent from serum and throat wash of two patients with infectious mononucleosis. Four cell culture systems as well as suckling mice were utilized. No agent was recovered.

BODY OF REPORT

Project 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task 02 Title: Microbiology (Etiology of Viral Hepatitis)

Description: Purpose - to define the etiology and the factors influencing the occurrence, of infectious hepatitis, serum hepatitis, and other acute and chronic infections which localize in the liver of man and animals.

Progress:

1. Etiology of Infectious Hepatitis: Studied specimens were derived from a series of 25 cases of laboratory and biopsy proved overt infectious hepatitis patients in U.S. Army troops in Korea 1962. These were obtained by Major M. Conrad MC, Division of Medicine of this Institute. Acute throat washings and stools on 10 individuals were received frozen in dry ice for isolation attempts. Stool specimens alone were received on an additional 4 men as well as serial serum specimens on all 25 men.

a. Initial attempts at viral isolation were made using throat washings and stools from each of 5 patients. Each specimen was inoculated (after treatment with antibiotics) into four cell culture systems: African green monkey kidney, human embryonic kidney, WI-26 (continous diploid lung) and diploid skin. Cultures were maintained with medium consisting of equal parts M199 and EME to which 2% chick serum had been added. After 18-38 days incubation cultures were tested for hemadsorption and/or challenged with known viruses. The scheme for these experiments, and the uniformly negative results are summarized in Table I.

Table I

Scheme for Attempts at Isolation of Infectious Hepatitis Virus from Throat Washings and Stool

Cell Culture, Passage	Incubation period	Results CPE:	Other Tests
GMK 1	19 days	neg:	Challenge Polio II, 8 days, neg.
2	28 days	neg:	Had. GP rbc, 11 days, neg.
			Challenge E-11, 11 days, neg. ✓
3	18 days	neg:	
4	14 days	neg:	
HEK 1	19 days	neg:	Challenge Polio II, 8 days, neg.
2	28 days	neg:	Had. GP rbc, 11 days, neg.
			Challenge E-11, 11 days, neg.
WI-26 1	19 days	neg:	Challenge Polio II, 8 days, neg.
2	38 days	neg:	Had. GP rbc, 11 days, neg.
			Challenge E-11, 11 days, neg.
			Last 10 days, cultures rolled at 32°C
Skin 1	19 days	neg:	Challenge Polio II, 8 days, neg.
2	28 days	neg:	Had. GP rbc, 11 days, neg.
			Challenge E-11, 11 days, neg.

✓ Duplicate cultures also challenged at 11 days with rubella virus, and rechallenged at 18 days with E-11 virus, neg.

b. Acute serum from the same 5 patients were inoculated at dilutions of 1:2 and 1:10 into the same cell culture systems as above, and followed as indicated in Table II. No transmissible agents were recovered.

c. Another group of 5 acute stool specimens were inoculated into GMK, primary rabbit kidney, and WI-26 cell cultures after treatment with Genitron. Each specimen made up to a 20% suspension was treated in the following manner: one volume of stool suspension and one-half volume of Genitron were mixed thoroughly on a Vortex mixer for 10 minutes, and centrifuged for 10 minutes in the cold at 1500 RPM. The results of this test series are shown in Table III.

Table II

Attempts at Recovery of Infectious Hepatitis Virus from Acute Phase Serum, (Diluted 1:2 and 1:10).

Cell Culture, passage	Incubation period	Results CPE:	Other Tests
GMK 1	20 days	neg:	Challenge E-11, 11 days, neg.
2	20 days	neg:	Challenge E-11, 11 days, neg.
3	14 days	neg:	Challenge E-11, 11 days, neg.
HEK 1	6 days	?	
2	14 days	?✓	
WI-26 1	20 days	neg:	

✓Control cultures passaged simultaneously: similar CPE seen in these control cultures.

Table III

Attempts at Recovery of Infectious Hepatitis Virus from Genitron Treated and untreated stool specimens.

Cell Culture, Passage	Incubation Period	Results CPE:	Other Tests
GMK 1	15 days	neg:	Challenge E-11, 11 days, neg.
2	19 days	neg:	
RK 1	14 days	neg:	Challenge herpes s., 11 days, neg.
2	19 days	neg:	
WI-26 1	11 days	?	Toxicity (Genitron)
2	14 days	neg:	
3	17 days	neg:	

d. Convalescent serum specimens from the 25 cases were tested in the Division of Veterinary Medicine at WRAIR. Microagglutination tests for leptospirosis employing 18 screening antigens were nonreactive.

2. Etiology of Serum Hepatitis: Studies to isolate the virus of homologous serum jaundice (SH) from an acute phase serum taken from a patient at the Walter Reed General Hospital were also made using the principle of interference with the cytopathogenic effects (CPE) of a number of viruses.

a. Acute phase serum at 1:1 and 1:10 dilutions was inoculated into fluid cultures of African green monkey (Cercopithicus aethiops) kidney (GMK) cells. After incubation periods of 13, 31, and 34 days at 37°C.100 TCD_{50} of polio type 2 virus were inoculated into each culture tube. No interference withe the CPE of the challenge virus was observed. In one experiment, what appeared to be a synergistic effect was noted in GMK cultures similarly challenged with poliovirus after incubation periods of 63 and 77 days. Tubes previously inoculated with acute phase SH serum developed CPE characteristic of poliovirus 24 to 48 hours prior to that observed in control cultures. This observation has yet be verified. A similar series of experiments were conducted to demonstrate SH virus by its interference with the CPE of herpes simplex and Eastern equine encephalomyelitis viruses following incubation periods ranging from 8 to 13 days at 37°C. They were also unsuccessful.

b. One unsuccessful attempt was made to demonstrate either interference or synergism by SH virus with polio type 2 virus in fluid

cultures of human embryonic kidney cells following an incubation period of 31 days at 37°C.

c. Two experiments were conducted to demonstrate SH virus by its interference with the growth of rubella virus in GMK fluid cultures. Following the inoculation of the acute phase SH serum, culture tubes were incubated for 7 and 14 days respectively prior to challenge with 10, 100 and 1000 interfering doses (IID_{50}) of rubella virus. After a further 5-day incubation period, they were again challenged with 100 TCD_{50} of polio type 2 virus. The second challenge served to detect the growth of rubella virus. The acute phase serum failed to interfere with the growth of rubella virus.

d. In one experiment, efforts were made to demonstrate SH virus by its interference with the growth of dengue type I virus in GMK fluid cultures. Cultures inoculated with acute phase serum were incubated for 11 days at 37°C and then challenged with 10, 100 and 1000 IID_{50} of dengue virus. After a second incubation period of 10 days, they were each challenged with 100 TCD_{50} of polio type 2 virus. The second challenge served to detect the growth of dengue virus. The acute phase serum failed to interfer with the growth of dengue type I virus.

3. Etiology of Infectious mononucleosis: Attempts were also made to recover the etiologic agent of infectious mononucleosis utilizing viral interference. Throat washings and serum taken during the acute phase of illness from two patients with classical infectious mononucleosis were studied. This material was inoculated into cell cultures as listed in Table IV. Cultures were observed for cytopathic effect and if absent, challenge with a known virus was performed. In addition, the specimens were inoculated into 2 to 3 day old mice which were challenged 9 days later with a lethal virus, Russian Spring-Summer Encephalitis virus.

Table IV

Attempts at Recovery of Infectious Mononucleosis Virus

Cell Culture	Temperature	Challenge Virus
1. Human epithelial carcinoma (Hep2)	36°C	Parainfluenza type 3 Herpes simplex Adenovirus type 7
2. Grivet monkey kidney	36°C	ECHO 10 (Reovirus) Coxsackie A 9 Parainfluenza type 3 Adenovirus type 7 Herpes simplex
3. Human diploid lung (WI 26)	32°C 36°C	ECHO 11 ECHO 11
4. Bovine embryonic kidney	36°C	Influenza A

Results: No cytopathic effects were noted in the cell cultures and no interference with any of the challenge viruses was demonstrated in cell cultures or in mice.

Summary and Conclusions:

1. Attempts to recognize transmissible agents from throat washings, stools and sera obtained during the acute phase of infectious hepatitis in Korea in 1962 were made. Efforts to show interference for Polio Virus II, rubella, herpes simplex and ECHO Type II viruses were uniformaly negative. No evidence for recent infection with leptospires was shown for the donor patients.

2. Attempts to isolate serum hepatitis virus by demonstrating interference with the cytopathogenic effects of polio type 2, herpes simplex and Eastern equine encephalomyelitis viruses were made. Attempts were also made to recognize an agent by double interference technique using combinations of rubella and polio type 2 viruses, and dengue type I and polio type 2 viruses. One experiment was conducted in fluid cultures of human embryonic kidney cells and the remainder in fluid cultures of African green monkey (Cercopithicus aethiops) kidney cells. Acute phase serum taken from a patient in the Walter Reed General Hospital served as the potential source of homologous serum jaundice virus. The results to date have been negative.

3. The interference technique was used in attempts to recover an agent from serum and throat wash of two patients with infectious mononucleosis. Four cell culture systems as well as suckling mice were utilized. No agent was recovered.

Publications: None

ANNUAL PROGRESS REPORT

Project: 3A O 12501 B 813, Army Medical Basic Research in Life Sciences

Task 02, Microbiology (Significance of PPLO in Human Disease)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Bacteriology
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Ruth G. Wittler, Ph.D.

Assistants: Robert C. O'Connell, M.S.*
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task No. 02 Title: Microbiology (Significance of PPLO in Human Disease)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

The mode of growth and reproduction of Mycoplasma laidlawii was followed in detail by phase contrast microscopy. Growth curves, size of filterable particles, viability, mass, and chemical composition was compared for PPLO and L form strains. Whereas growth curves for certain PPLO and L forms showed somewhat different patterns, the size of the smallest viable filterable particles for both groups of organisms was the same.

The source of widespread PPLO contamination in tissue cultures has been investigated. The evidence strongly suggests that in laboratories handling diverse tissue cultures, cross contamination occurs, via aerosols during trypsinization and feeding procedures, from one pPLO infected cell culture to another. An aerosol containing a single PPLO cell is apparently capable of initiating infection of a tissue culture.

The original report on the presence of a viable microbial agent in the blood of primary rheumatic fever patients has been confirmed in collaboration with Drs. E. A. Mortimer and W. R. Mantel, and isolation and identification of the agent is being pursued.

The type culture collection of PPLO and L forms is being enlarged, and a recent check on strains lyophilized three years ago showed that they retained high viability and typical morphology.

BODY OF REPORT

Project No. 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task No. 02 Title: Microbiology (Significance of PPLO in Human Disease)

Description: As in previous years the objectives of the research of this section are to elucidate the nature and behavior of PPLO, L forms and transitional forms of bacteria, to determine their interrelationships, and to clarify their pathogenic significance in chronic and recurrent disease processes in man. During the past year especial emphasis has been placed on basic research on the biological, biophysical and biochemical attributes of PPLO and L forms in an effort to arrive at more rational and systematic methods for their detection in and cultivation from the human host.

Progress:

1. Morphology, growth and reproduction of PPLO. The growth and mode of reproduction of Mycoplasma laidlawii, type B, was observed by phase contrast microscopy continually for 24 hours and photographed at frequent intervals. The organisms used for these experiments were grown under optimal conditions in serum-yeast-infusion broth which allowed rapid multiplication, maximum titer, close to 100 per cent viability, and striking morphologic uniformity. The following features in the mode of reproduction of M. laidlawii were revealed by the special techniques employed.

a. The smallest reproductive units were not less than 180 mμ. There was no evidence of reproduction from units of 100 mμ or less in size as postulated by some investigators.

b. Only one reproductive unit was required to produce a colony.

c. M. laidlawii multiplied on agar in exactly the same manner as in broth and had the same size, shape, and thickness. There was no evidence during the first 24 hours on agar that the organisms formed large, thin, or flat cells in which many tiny reproductive units developed, as postulated by some investigators from fixed and stained specimens.

d. The individual cell divided by putting out a protoplasmic projection into which material from the mother cell flowed. A constriction formed between mother cell and projection, thus forming a daughter cell. Each daughter cell remained attached to the mother cell by a tiny protoplasmic connection so that chains were formed.

e. The so-called "filaments" observed by some investigators in stained specimens consisted either of the chains of multiplying organisms or of non-reproducing protoplasmic threads produced by tearing organisms

from the chains. The chains showed no branching.

f. Organisms anywhere along the chain could divide and multiply.

g. When M. laidlawii was grown under sub-optimal conditions in the absence of serum and yeast extract, small granules of about 160 mμ were occasionally formed in degenerating cells about to lyse. However, only one such granule per cell actually grew and multiplied; the other granules degenerated and disappeared after lysis of the mother cell.

h. The size of the individual cells in a broth culture was consistently dependent on the age of the broth culture. For instance, three hour old cells measured approximately 0.7 μ in diameter, six hour old cells approximately 0.5 μ, nine hour old cells 0.35 μ and twelve hour old cells 0.22 - 0.25 μ.

i. In collaboration with Dr. H. J. Morowitz in the Biophysics Department of Yale University all the foregoing information was repeated and confirmed. Furthermore, filtration analyses using Millipore membrane filters of graded pore size, plate counts for titration of viable organisms, and chemical analyses for protein, RNA, and DNA were carried out at three hour intervals on growing cultures of M. laidlawii. Of particular interest were the results on three hour old cultures where the individual cells had increased about 2.5 times in diameter without yet showing any increase in number. At the three hour period chemical analyses showed that protein had increased approximately 1.9 times, RNA approximately 2.6 times, and DNA approximately 1.7 times. Morphologic observations suggested an alteration of the surface of three hour old cells, since they were noted to stick to everything they touched. This property was not apparent at other periods of the growth curve.

2. Partical size and growth curve characteristics of PPLO and L forms. A study was conducted on the variations in size of the filterable elements of PPLO and L forms after various intervals of growth ranging from 12 to 120 hours. Millipore membrane filters from 0.22 μ to 0.6 μ pore diameter were used with a negative filtration pressure of 100 mm of mercury. Growth curves were plotted for each unfiltered culture or filtrate using standard dilution and plate count techniques. The PPLO species tested were Mycoplasma arthritidis, M. laidlawii, type B, M. hominis, type 1, and M. spumans, in addition to one L form derived from Gaffkya tetragena. Results showed the number of viable organisms in unfiltered cultures reached a peak titer between 12 and 24 hours but that the relative number of smaller viable particles which passed the finer filters increased with the age of the culture, i.e., for M. arthritidis the small particles reached their maximum number after 36 hours of growth. After 12 hours of growth there was more than a 7 log difference in titer between the unfiltered culture and the filtrate that passed the 0.22 μ membrane, but after 36 hours of growth there was only a 3 log difference in titers between unfiltered and filtered material thus indicating that the majority of the cells had become very small indeed. Studies on the Gaffkya L form showed that very large numbers of small viable forms filterable through the 0.22 μ membrane were produced

by this strain and reached peak titer in 24 hours. Moreover, there was only a 1 log difference in titer between unfiltered and filtered cultures. These data challenge the present distinction between PPLO and L forms based on the concept that L forms do not have viable small units capable of passing 0.22 μ filter membranes. Present studies, however, suggest that production of the smallest filterable elements of the PPLO levels off after reaching a peak titer whereas similar filterable elements of the Gaffkya L form may have more than one peak in titer. Experiments are now in progress on the effect of varying the pressures used for filtration between 100 and 400 mm of mercury with M. laidlawii and the L form of Gaffkya as the test organisms. Whereas the results of these tests have been consistent and predictable with the Gaffkya L form, the results with M. laidlawii have been erratic. The morphologic investigations described in paragraph 2 on size and configuration of the cells of M. laidlawii in early growth stages will be pursued in conjuntion with the filtration analyses in an effort to clarify its unconventional growth habits, and its relationship to PPLO on L forms.

3. Studies on biology of PPLO. A series of experiments was conducted with the following three general objectives: (1) to compare simultaneously but by different methods viable counts and microbial crop mass for PPLO cultures of various ages; (2) to compare growth curves of dissimilar strains of PPLO as a possible means of elucidating major biologic differences; (3) to determine optimal nutritional and environmental requirements of PPLO using alterations in the normal growth curve as the key indicator. Of the various methods tested nephelometry with the Coleman Model 14 Universal Spectrophotometer was least cumbersome and gave significant and reproducible estimates of cell mass when used directly on broth cultures of M. laidlawii. Nephelometric and viable count growth curves were strikingly similar during seven day growth periods. Comparison of two human genital strains (M. hominis, type 1) with a saprophytic sewage strain (M. laidlawii) by plate count titrations made at frequent intervals during incubation showed growth curves of markedly different configuration. M. laidlawii remained viable with little drop in titer during 11 days of incubation whereas the human strains frequently dropped to a titer of less than 10 organisms within three days. When a few organisms did survive for as long as 11 days, reversion from PPLO to corynebacteria occasionally took place. Studies on nutritional factors showed that incorporation in standard serum-yeast-infusion media of potassium phosphate buffers (1.8 per cent concentration) killed M. laidlawii very rapidly. However, potassium phosphate buffers in a concentration of 0.45 per cent in the noninfusion casamino acid medium of Abrams with serum and yeast did not exert the marked killing effect although growth was slightly suboptimal. Further work in this area is required.

4. Source of PPLO contamination of tissue cultures. PPLO contamination of tissue cultures has been investigated to determine source of the PPLO and factors which contribute to the widespread nature of the contamination. The hypothesis that PPLO infections of tissue cultures are caused by the production of L forms from bacterial contaminants in the presence of antibiotics was examined experimentally. Using two

approaches, an attempt was made: (1) to induce PPLO contamination of tissue cultures by infecting them with bacteria in the presence of antibiotics, and (2) to determine the source of PPLO contamination in a laboratory that carried antibiotic-free cell cultures. Corynebacterium sp. and Sarcina sp. both isolated from PPLO infected tissue cultures were used to infect cell cultures in the presence of antibiotics. No PPLO were isolated from these cultures over an eight month period. Fifteen strains of PPLO isolated from a variety of antibiotic-free tissue cultures carried in one laboratory were found to be serologically similar by complement fixation. The possibility was examined that the similarity of the isolates resulted from cross contamination from one cell culture to another by a PPLO strain from a single infected tissue culture that entered the laboratory. Using the Andersen Air Sampler it was demonstrated that aerosols containing PPLO could be produced during the trypsinization of PPLO infected tissue cultures. The minimal infective dose of PPLO for tissue cultures was calculated from plate counts and from terminal dilution experiments in two cell lines. It was determined that the same unit of PPLO was responsible for formation of a colony on agar as was responsible for infection of a tissue culture, i.e., one PPLO cell. On the basis of these results it was postulated that cross contamination occurs via aerosols from one PPLO infected cell culture to another and that this probably accounts for the widespread contamination of tissue cultures with PPLO.

5. Studies on etiology of rheumatic fever. Studies on the etiology of rheumatic fever have continued. In collaboration with E. A. Mortimer of Western Reserve University and Metropolitan General Hospital, Cleveland and W. M. Marted of the Streptococcal Reference Laboratory, Colindale, England, the presence in plasma of primary, acute rheumatic fever patients of an agent that forms deep granular colonies has been confirmed. The combined efforts of the three laboratories are now concentrated on obtaining suitable methods for cultivation, isolation and identification of this microbial agent.

6. Type culture collection of PPLO and L forms. In collaboration with W. A. Clark and E. F. Lessel of the American Type Culture Collection, the type collection of PPLO and L form strains has increased in number with the addition of PPLO strains from infected tissue cultures. Strains that have been in the lyophilized state since 1960 and stored at +4°C. have recently been checked for viability and retention of morphologic characteristics. All lyophilized strains have grown out well and retained typical features. New methods for preparing strains for lyophilization and for storage thereafter are being tested.

Summary and Conclusions:

1, 2 and 3. Investigations of the biological, biophysical and biochemical attributes of PPLO and L forms have been carried out using phase contrast microscopy for following details of growth and mode of reproduction; filtration analyses and plate counts for determining particle size and viability in relation to rate and titer of growth; and standard chemical

analyses for determining alterations in protein, RNA and DNA content during the growth cycle. These studies have clarified much that has been problematic in the biological behavior of PPLO and L forms. The information gained from these basic studies is being applied to the problems of detection, cultivation, isolation and identification of PPLO and related bacterial variants, such as, L forms and transitional forms, from tissues and from the infected human host.

4. One of the mechanisms involved in widespread PPLO contamination of tissue cultures has been shown to be cross contamination via aerosols from one infected cell line to another.

5. Work on the isolation and identification of the microbial agent found in the blood of acute rheumatic fever cases is being actively followed.

6. The type collection of PPLO and L forms now deposited at the ATCC is being expanded, and viability checks on strains lyophilized three years ago have been highly satisfactory.

List of Publications:

1. O'Connell, R. C., 1963. A study of factors contributing to widespread contamination of tissue cultures with Pleuropneumonia-like organisms. Ph.D. Dissertation, University of Maryland.

2. O'Connell, R. C., Wittler, R. G. and Faber, J. E., 1963. Aerosols as a source of widespread Mycoplasma contamination of tissue cultures. In preparation.

3. Wittler, R. G. Methods for Primary Isolation of the Mycoplasmataceae. Presented at Amer. Soc. Microbiol. Medical Division Round Table on Diagnostic Bacteriology, Cleveland, 1963.

ANNUAL PROGRESS REPORT

Project 3A O 12501 B 813 Army Medical Basic Research in Life Sciences

Task 02, Microbiology (Pathogenesis and immunity in enteric diseases)

Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
	Department of Applied Immunology Department of Bacterial Immunology Department of Bacteriology Division of Communicable Disease and Immunology
	Department of Experimental Pathology Division of Special Activities
	Department of Germfree Research Division of Basic Surgical Research
	Department of Gastroenterology Division of Medicine
Period Covered by Report:	1 July 1962 through 30 June 1963
Principal Investigators:	Capt G. D. Abrams, MC Louis S. Baron, PhD Maj Marcel Conrad, MC S. Falkow, PhD Capt W. E. Farrar, MC S. B. Formal, PhD Maj E. Jacobson, MC Capt J. Kalas, MC E. H. LaBrec, PhD Capt R. Laundy, VC H. E. Noyes, PhD Col H. Sprinz, MC
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Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project 3A 0 12501 B 813

Task 02

Title: Army Medical Basic Research in Life Sciences

Title: Microbiology(Pathogenesis and immunity in enteric diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. Experiments to infect monkeys with Shigella flexneri indicate that the incidence of clinical symptoms can be increased if the animals are injected with morphine following challenge.

2. Study of an avirulent mutant of a virulent strain of Shigella flexneri 2a has shown this mutant to be similar to the virulent parent in its virulence for mice and rate of growth in both the ligated guinea pig ileum and in broth.

3. An Hfr Shigella flexneri 2a has been isolated from a mating between a donor strain of E. coli and a recipient strain of S. flexneri 2a.

4. A single sublethal dose of CCl_4 renders guinea pigs abnormally susceptible to the effects of bacterial endotoxin.

5. Attempts to elucidate the nature of the factor in normal guinea pig liver capable of inactivating bacterial endotoxin are in progress.

6. Cr^{51}-labeled bacterial endotoxin was not adsorbed from rabbit loops infected with Vibrio comma.

7. Conventional guinea pigs die after receiving penicillin by oral or parenteral routes. Germfree guinea pigs survive such treatment.

8. Studies of the mechanism of shock produced by endotoxin from an enteric pathogen, Shigella flexneri, were conducted employing pharmacological agents and measuring effects with several hemodynamic parameters.

9. The hemodynamic effects of endotoxin injected by various routes were investigated during constant-flow perfusion of the canine small intestine.

10. Animals injected with reserpine and bacterial endotoxin develop a propulsive diarrhea. Only compounds which prevent the discharge of post-ganglionic adrenergic fibers are effective substitutes for reserpine in producing this syndrome.

11. Guinea pigs with diarrhea induced by reserpine and endotoxin have a decreased capacity to absorb Na^{22} from the gastrointestinal tract.

12. Further studies on the nonspecific fluorescence have indicated that the mechanism may involve an electrostatic attraction between the auxochrome groups of the dye and the reactive groups of the highly basic amino acids arginine and tryptophan.

BODY OF REPORT

Project 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task 02 Title: Microbiology (Pathogenesis and immunity in enteric diseases)

Description: The pathogenesis of enteric diseases is studied in order to find better methods of prevention and treatment in this group of diseases.

Progress:

1. Experiments to study dysentery infections in monkeys have commenced. Initial findings indicated that only 25 percent of monkeys receiving 5×10^{10} S. flexneri per os displayed symptoms of shigellosis. This is in agreement with the observations of others. However, as we found with guinea pigs, the subcutaneous administration of morphine could affect the resistance of animals to infection. Thus by injecting morphine after oral challenge with 5×10^{10} S. flexneri, the incidence of clinical symptoms of monkeys was increased to over 90 percent. Using this procedure, the dose necessary to evoke symptoms in 50 percent of challenged monkeys is approximately 5×10^{7} bacteria. Since we were now able to produce clinical symptoms in high proportion of monkeys challenged with S. flexneri an attempt was made to render animals resistant to challenge. The first technique which has been tested involved oral immunization with a living avirulent culture. This culture is a colonial mutant of a virulent strain of S. flexneri 2a and is avirulent for both monkeys and guinea pigs. Some animals were fed daily doses of 5×10^{10} avirulent bacteria for a period of 28 days, while others were given the vaccine for 14 days. The vaccine produced no evidence of illness with the exception of two animals in whom pneumonia resulted due to the introduction of bacteria into the respiratory tract. These animals were not included in the study. Animals were challenged with 5×10^{10} virulent bacteria within 5 days of receiving the final oral immunizing dose. The results of this experiment are summarized in the following table. From this experiment, it is evident that oral vaccination with a living

The resistance of monkeys to shigellosis following the oral administration of a living avirulent vaccine

Animal number	Material fed	Number of feedings	Diarrhea after challenge	Positive cultures for 7 days after challenge
7-3	Vaccine	28	No	4/7
7-6	"	28	No	2/7
7-8	"	28	No	4/7
7-13	"	14	No	4/7
7-14	"	14	No	4/7
7-1	Broth	28	Yes	5/7
7-9	"	28	No	4/7
7-10	"	28	Yes	6/7
7-11	"	28	Yes (died)	2
7-12	"	28	Yes	3/7

strain can protect monkeys against the symptoms of shigellosis but does not significantly affect the shedding of the organism by the host. The mechanism whereby the animal fed living avirulent cells becomes resistant to shigellosis is not known. Circulating antibody may be ruled out, because a high percentage of so-called normal monkeys have detectable levels of antibodies against the specific challenge organism at the time of challenge. The rationale for using the oral route of immunization was to evoke a predominantly copra-antibody response; while this might have been achieved, we were not able to detect antibody in stools using the hemagglutination technique.

2. We have previously shown that an opaque(O) colonial variant of a translucent(T) guinea pig virulent strain of S. flexneri 2a, is avirulent for the starved or CCl_4 treated guinea pigs. This strain, which kills less than 10% of orally challenged guinea pigs, had also been found to be unable to multiply in the small intestine of treated guinea pigs while the parental strain could multiply. Further studies were carried on to learn more of the factors associated with this loss of virulence. Studies of intestinal tissues from animals infected with the avirulent O variant by histological and fluorescent antibody techniques showed that this strain produced only a slight degree of inflammation, while the virulent T parent produced inflammation, interstitial edema and blunting of villi. Fluorescent antibody studies have previously shown that the T strain frequently invades the mucosa and is found in the lamina propia. Mucosal invasion has never been demonstrated in tissue from animals fed the O strain. The inability of the O strain to multiply in the small intestine suggested that this strain grew slower than the T strain or perhaps was sensitive to the environment in the small intestine. In vitro growth studies in both complex and synthetic media revealed no differences in the growth rate of either strain and both strains were recovered in equal numbers from infected ligated guinea pig ileums. The similarities between the growth of both O and T in vitro and in the ligated guinea pig ileum raised the possibility that the virulence of the T strain was due to a greater toxicity than that found in the O strain. Toxicity tests in mice showed that the LD_{50} of AKD preparation of both strains was similar. Virulence tests in mice show a 10-fold greater LD_{50} for saline suspensions of O strain as compared to the T strain, however, in mucin no differences were seen. We have previously suggested that a major defense mechanism against shigellosis was small intestinal motility and that the high numbers of the T strain recovered from the small intestines of orally challenged guinea pigs was related to increased intestinal transit time. To measure transit in guinea pigs challenged either O or T, radioactive chromic^{51} chloride was incorporated in the challenge suspension. Measurement of tag retained in various portions of the bowel showed that after 24 hrs, animals challenged with T retained 11.6% of the tag in the small bowel while those fed O retained only 1.2%. These studies indicate a longer transit time in animals infected with T, permitting multiplication and the production of microulcers.

3. In order to study the effects of the incorporation of Shigella genetic material into E. coli and Hfr S. flexneri 2a was isolated as a result of a mating between an Hfr E. coli and F$^-$ S. flexneri 2a and selecting for the terminal marker. This Hfr S. flexneri was found to recombine at a frequency of 0.4% with multiply deficient strains of E. coli with selection for the most proximal marker. While this frequency is high it is 10-fold less than comparable intraspecific E. coli matings and is approximately the same as interspecific Hfr

E. coli x S. flexneri matings. Hybrid analysis shows less than 10% linked transfer of unselected markers which is also comparable to the reverse interspecific Hfr E. coli x S. flexneri mating. Intraspecific S. flexneri mating show high frequencies of recombination; however, definitive data require the production of multiply deficient Shigella strains for finer analysis.

4. Previous studies indicated that pre-treatment with a sublethal dose of CCl_4 rendered guinea pigs abnormally susceptible to the lethal action of bacterial endotoxin. In the present study the relationship between hepatic morphology and endotoxin susceptibility was investigated, and the ability of homogenates made from normal and damaged livers to inactivate endotoxin was measured, using intravenous injection of the 11 day chick embryo as a bioassay for endotoxin.

a. Maximum liver necrosis was observed 48 hrs after injection of 0.15 ml CCl_4 subcutaneously. Fatty change was pronounced at 72 hrs, and at 72 and 96 hrs intense regenerative activity was evident. At one week the livers appeared essentially normal.

b. Susceptibility to lethal endotoxin shock was appreciably enhanced by 12 hrs after CCl_4 treatment, and was maximal at 48 hrs. By 96 hrs the animals had regained essentially normal resistance to endotoxin.

c. Homogenates made from normal livers were able to detoxify considerable amounts of endotoxin in vitro. Livers from animals given CCl_4 48 hrs previously were virtually inactive in this regard. One hundred and twenty hours after CCl_4 treatment the livers exhibited activity not significantly different from normal controls.

d. These findings indicate that endotoxin susceptibility following CCl_4 administration parallels the degree of morphologic damage. In addition, livers of animals poisoned with CCl_4 show little ability to inactivate endotoxin, but this capability returns as hepatic regeneration takes place.

5. Studies of the nature of the factor in normal guinea pig liver capable of inactivating bacterial endotoxin are in progress. Intravenous injection into 11-day chick embryos is used as an assay system for endotoxin.

a. Cell-free, mitochondria-free homogenates of liver prepared in a Waring blendor show appreciable endotoxin-inactivating activity. When the cells are disrupted by more gentle means it is not possible to localize this activity within a specific nuclear, mitochondrial, microsomal or supernatant fraction; slight activity is found in all fractions. Mitochondrial activity is enhanced by addition of ATP or malate. Since both ATP and malate are involved in the oxidation of fatty acids, it seems likely that activation of the lipid moiety is an essential feature of the enzymatic degradation of endotoxin, and that the lipid is involved in the toxicity of endotoxin. (The only ATP-dependent enzyme which might be involved in degradation of the polysaccharide portion of endotoxin is hexokinase, and this was shown to be devoid of inactivating potency.)

b. A variety of crystalline enzyme preparations, including takadiastase, salivary amylase, lipoxidase, phospholipase C and alkaline phosphatases from calf intestine and E. coli were tested and found to be inactive.

6. An accepted procedure which has been employed to detect the adsorption of bacterial endotoxin from the intestinal tract is to label the toxin with Cr^{51} and then to measure this isotope in the various organs of the test animal. Using this technique, investigators have failed to detect adsorption of endotoxin from the bowels of animals under a variety of experimental conditions. An attempt was made to determine if Cr^{51}-labeled endotoxin would be absorbed from a loop of rabbit intestine during various stages of an inflammatory process. We previously had shown that if a ligated segment of the rabbit ileum was infected with Vibrio comma, an acute inflammatory reaction occurred which proceeded to necrosis. Rabbit intestinal segments were infected with V. comma and at the same time Cr^{51}-labeled endotoxin was injected into the closed loop. Animals were then sacrificed at various times, and radioactivity of isolated loops and viscera determined. Radioactivity of the remaining animal (i.e. the animal minus the loop) was never greater than normal even at times when the loop was necrotic. Furthermore when Cr^{51} was injected together with the Vibrios, it did not leave even the necrotic loop. Thus, it seems possible that Cr^{51} itself may inhibit the absorption of the endotoxin or perhaps is split off from the endotoxin in the bowel and remains in the bowel while the endotoxin is adsorbed.

7. Guinea pigs usually die within 7 days following the oral or parenteral administration of a single dose of penicillin. Some consider that death is due to a direct toxic effect of the drug. Others feel that the animals succumb to an enterocolitis which is associated with alteration of the enteric flora: Following the dose of penicillin, the bacterial flora of the bowel changes from one which is predominantly gram-positive to one that is mostly gram-negative. The effect of sodium penicillin G was tested on Hartley guinea pigs raised in the germfree as well as the conventional state. The results of these experiments are summarized in the following table:

Deaths in conventional and germfree guinea pigs following the subcutaneous injection of penicillin

Dose	Conventional	Germfree
600mg	6/11*	1/4
120	7/9	0/3
24	28/34	0/13
4.8	9/5	
.96	7/15	
NaCl	0/8	

*Deaths
Total

All of the conventionally reared animals which died did so within 7 days after receiving the penicillin, and exhibited gross and microscopic lesions of enterocolitis. Gram-negative organisms were isolated in large numbers from their caecal contents. In contrast, only one germfree animal succumbed after receiving

penicillin. The exact cause of death was not determined. It may not have been related to the administration of penicillin since germfree guinea pigs occasionally die unchallenged by any specific experimental regimen. It was noted previously that germfree guinea pigs survive oral challenge with a strain of Escherichia coli isolated from a healthy human being. The effect of contaminating germfree animals with a culture of E. coli isolated from a conventional animal which became moribund and exhibited enterocolitis 5 days after receiving penicillin was next studied. All of the 6 germfree guinea pigs contaminated with this culture were apparently well for one week. At the end of that time, 3 were injected with a single 600 mg dose of penicillin and the remaining 3 received saline. All survived for 7 days, and at sacrifice, large numbers of E. coli were present in their intestinal tracts. These animals exhibited no gross or microscopic lesions of enterocolitis. Thus these limited data indicate that germfree guinea pigs are more resistant to the effects of penicillin than are their conventionally reared counterparts. In this regard these observations fail to support the thesis that conventional animals succumb because of a direct toxic action of the drug, for one would expect the germfree animal also to be susceptible to such an action. On the basis of the work of others and the present study, it is likely that alteration of the intestinal flora plays a part in bringing about the death of penicillin treated, conventionally-reared guinea pigs. However, the fact that the presence of large numbers of gram-negative organisms in the intestine did not cause death of what were once germfree guinea pigs, indicates that other factors must be investigated.

8. The shock syndrome produced by the endotoxin of enteric pathogens is characterized in dogs by systemic arterial hypotension, portal venous hypertension and an increase in the resistance to blood flow across splanchnic organs. It was found that pre-treatment with phenoxybenzamine abolished the portal hypertension and markedly diminished the elevated splenic vascular resistance induced by the endotoxin of Shigella flexneri. It was also found that dichloroisoproterenol and Nethalide® markedly attenuated the abrupt hypotension induced by endotoxin injection. Pre-treatment with compound 48/80, reserpine, antihistaminics, cyproheptadine and atropine did not consistently alter the hemodynamic response to endotoxin.

9. The hemodynamic affects of endotoxin injected by various routes were investigated during constant-flow perfusion of the canine small intestine. One LD_{50} (0.6 mg/Kg) of S. flexneri endotoxin was injected into either the superior mesenteric artery (6 dogs), a mesenteric vein (6 dogs) or the right common femoral vein (6 dogs). Qualitative changes were the same in each of the three groups: an increase in portal vein pressure (mean for 18 dogs: 120%) within 5 minutes after injection, a 34% increase in both superior mesenteric artery pressure and intestinal vascular resistance, and a decline in mean systemic arterial pressure of 35 mm Hg within 10 minutes after injection. By 30 minutes hemodynamic measurements had returned toward pre-injection values. There were, however, quantitative differences among the three groups dependent upon route of administration. Portal vein pressure increase was greatest following injection of endotoxin into the mesenteric vein. The elevation in mesenteric artery pressure and intestinal vascular resistance was most prolonged in the group in which endotoxin was administered into the superior mesenteric artery. The most profound fall in systemic arterial pressure was observed in those animals in which endotoxin was injected into a

femoral vein. These findings suggest that the hemodynamic effects of endotoxin vary quantitatively with the route of administration and that these effects are greatest in that portion of the circulation exposed to the highest initial concentration of endotoxin.

10. It was previously demonstrated that when guinea pigs were given high doses of reserpine (0.3 mg/kg) followed in 16-24 hrs by an LD_{50} of bacterial endotoxin, an acute propulsive watery diarrhea developed. Neither snake venom nor bacterial exotoxins cause a similar syndrome. The present work demonstrates that only compounds which prevent the discharge of post-ganglionic adrenergic fibers are effective in substituting for reserpine in this procedure as indicated in the following table. The fact that both guanethidine and syrosingopine are effective indicates that the action of endotoxin is peripheral and helps to explain why spinal cord and vagal section failed to prevent diarrhea. Since guanethidine has little if any effect on depleting serotonin, the mechanism of this diarrheal syndrome is further narrowed to one of adrenergic blockade. This is further supported by the inability of serotonin inhibitors to prevent the diarrheal syndrome and by the fact that the injection of catecholamines prevents overt diarrhea in reserpine-endotoxin animals.

Effect of various adrenergic blocking agents and/or an LD_{50} of *E. coli* O-111 endotoxin on the fecal excretion of guinea pigs

Material injected	No. of animals	Average wt. of animals	Fecal weight in 90 minutes (range in grams)
Control	5	350	0.1-0.9
Reserpine	5	350	0.3-1.0
Guanethidine	5	350	0.2-1.2
Syrosingopine	5	350	0.1-0.8
Phenoxybenzamine	5	350	0.4-1.0
DCI*	5	350	0.1-0.9
Endotoxin	5	350	0.3-1.1
Reserpine + endotoxin	5	350	1.6-8.7
Guanethidine + endotoxin	5	350	3.7-6.9
Syrosingopine + endotoxin	5	350	1.7-8.2
Phenoxybenzamine + endotoxin	5	350	0.7-1.4
DCI + endotoxin	5	350	0.9-1.1

*Dichloroisoproterenol

11. The finding that the injection of reserpine and endotoxin induce acute watery diarrhea prompted the study of sodium and water absorption in these animals. The present work is concerned only with the absorption of orally administered sodium from the gastrointestinal tract of guinea pigs. This was done by measuring the amount of Na^{22} in the carcass of animals after the gastrointestinal tract had been removed. Normal guinea pigs and those previously injected with reserpine adsorbed 70-80 percent of Na^{22} within 80 min. On the other hand, guinea pigs receiving reserpine and endotoxin or endotoxin alone had adsorbed only 15-25 percent of the Na^{22} 160 min. after it was fed. In the case of the reserpine-endotoxin animals, Na absorption was inhibited due to a marked decrease in intestinal transit time; the decrease in adsorption by the endotoxin treated animals was due to marked delay in the gastric emptying time. In experiments in which the intestine was ligated at various levels in order to eliminate transit time as factor, all animals (normal, endotoxin, reserpine, reserpine-endotoxin) absorbed Na^{22} to an equal degree.

12. We previously reported (Annual Report, 1962) that the nonspecific fluorescence of polymorphonuclear leucocytes (eosinophils (PMNE) and neutrophils (PMNN)) could be eliminated by the expedient of incubating tissue sections or bone marrow smears, fixed in non-polar solvents, in dilute HCl before staining with the appropriate fluorescent antibody (FA) solution. We also reported that the granules of these leucocytes in solvent-fixed, HCl-treated, sections lost their affinity for Eosin Y. However, in both cases, if sections or smears were fixed in 10% formalin, HCl had no effect, the leucocyte granules retained their affinity for FA or Eosin Y. We concluded that the mechanism of nonspecific fluorescence might be due to an electrostatic attraction of the free auxochrome groups of the fluorescein dye molecule, attached to the antibody protein, for the basic cytoplasmic proteins of tissue. We have continued this work in an attempt to explain the mechanism involved.

a. Several histochemical procedures for demonstrating protein bound amino acids were employed to determine whether HCl treatment affected the demonstration of certain amino acids. The granules of PMNE have been reported to contain lysozyme, which contains high concentrations of arginine and tryptophan, 13% and 12% respectively. On this basis, histochemical procedures to demonstrate these protein bound amino acids were selected to determine what effect HCl treatment had on their demonstration in tissue.

b. The PMNE granules were positive for arginine and indole (tryptophan) in guinea pig ileum sections prepared from frozen specimens, fixed in acetone, alcohol, and 10% formalin. However, when alcohol or acetone-fixed sections were treated with HCl prior to the histochemical test, the reactions for indole and arginine in the granules were negative, while the demonstration of these granules in formalin-fixed sections was not affected by HCl treatment.

c. From this we selected several standard histochemical techniques designed to block the free reactive groups of amino acids in tissue proteins to see whether they would also prevent the reaction between the fluorescein-labeled antibody and the leucocyte granules. The results showed that peracetic acid which blocks the indole reaction also blocked the nonspecific fluorescence reaction. Methylation of free carboxyl groups or nitrosation of

free primary amino groups failed to prevent NSF of PMNE granules. However, the NSF of PMNE granules was prevented by prolonged nitrosation of proteins by oxidation with nitrous acid (HNO_2). Deitch, J. Histochem. and Cytochem., 1961) concluded in her work on demonstration of arginine in tissue proteins that prolonged nitrosation also blocked the guanidinium group of arginine. The results of our experiments indicates that NSF of leucocyte granules may be due to a complexing of the fluorescein dye molecule (conjugated to antibody protein) with the highly basic amino acids arginine and tryptophan.

Summary and Conclusions:

1. Experiments to infect monkeys with Shigella flexneri indicate that the incidence of clinical symptoms can be increased if the animals are injected with morphine following challenge.

2. Further study of an avirulent mutant of a virulent strain of Shigella flexneri 2a has shown this mutant to be similar to the virulent parent in its virulence for mice and rate of growth in both the ligated guinea pig ileum and in broth. Studies using radioactive chromic^{51} chloride show that the transit time is greater in guinea pigs fed the virulent parent than guinea pigs fed the avirulent mutant, and suggests that the virulent parent can slow small intestinal motility during a stage in the infectious process.

3. An Htv Shigella flexneri 2a has been isolated from a mating between a donor strain of E. coli and a recipient strain of S. flexneri 2a. This Htv S. flexneri 2a recombines with F$^-$ strains of E. coli at frequencies comparable to matings between Htv E. coli and F$^-$ S. flexneri, and also recombines with other F$^-$ Shigella at high frequencies.

4. A single sublethal dose of CCl_4 renders guinea pigs abnormally susceptible to the effects of bacterial endotoxin. Endotoxin susceptibility, hepatic morphology and in vitro endotoxin - inactivity ability of liver homogenates were studied at intervals up to one week after administration of CCl_4. A close temporal relationship among these factors was found. At the height of the hepatic necrosis the animals were most sensitive to endotoxin, and liver homogenates were unable to detoxify endotoxin. With regeneration of liver tissue the animals regained normal resistance to endotoxin and homogenates of their livers were active in degradation of endotoxin.

5. Attempts to elucidate the nature of the factor in normal guinea pig liver capable of inactivating bacterial endotoxin are in progress. Preliminary results indicate that the process is enzymatic in nature. The activity has not been localized within a specific subcellular fraction, but appears in all fractions. Mitochondrial activity is enhanced by adding ATP or malate to the reaction mixture, suggesting that oxidation of fatty acids is an essential feature of this phenomenon and that the lipid moiety of endotoxin is intimately concerned with its toxicity.

6. Cr^{51}-labeled bacterial endotoxin was not adsorbed from rabbit loops infected with Vibrio comma.

7. Conventional guinea pigs die after receiving penicillin by oral or parenteral routes. Germfree guinea pigs survive such treatment.

8. Studies of the mechanism of shock produced by endotoxin from an enteric pathogen, Shigella flexneri, were conducted employing pharmacological agents and measuring effects with several hemodynamic parameters. It was found that the alpha and beta adrenergic vascular receptors appear to participate in the development of endotoxin shock.

9. The hemodynamic effects of endotoxin injected by various routes were investigated during constant-flow perfusion of the canine small intestine. These effects vary quantitatively with the route of administration and are greatest in that portion of the circulation exposed to the highest initial concentration of endotoxin.

10. Animals injected with reserpine and bacterial endotoxin develop a propulsive diarrhea. Only compounds which prevent the discharge of postganglionic adrenergic fibers are effective substitutes for reserpine in producing this syndrome.

11. Guinea pigs with diarrhea induced by reserpine and endotoxin have a decreased capacity to absorb Na^{22} from the gastrointestinal tract. This failure to absorb sodium can be explained by the marked decrease in intestinal transit time which is characteristic of these animals.

12. Further studies on the nonspecific fluorescence of polymorphonuclear eosinophil (PMNE) and polymorphonuclear neutrophil (PMNN) resulting from interaction of fluorescein-labeled antibody with the cytoplasmic granules in these cells has indicated that the mechanism of interaction may be an electrostatic attraction between the auxochrome groups of the dye and the reactive groups of the highly basic amino acids arginine and tryptophan found in the granule protein.

List of Publications:

1. Kalas, J. P., and Jacobson, E. D. Effect of certain pharmacological agents on endotoxin shock. Federation Proc. 22:629, 1963.

2. Jacobson, E. D., and Kalas, J. P. Role of the alpha and beta vascular receptors in endotoxin shock. Clin. Research, 11:209, 1963.

3. Jacobson, E. D., Dooley, E. S., Scott, J. B., and Frohlich, E. D. Effects of endotoxin on the hemodynamics of the stomach. J. Clin. Invest. 42:391, 1963.

4. Farrar, W. Edmund, Jr., and Jacobson, E. D. Influence of route of administration on the hemodynamic effects of endotoxin. Clin. Research 11: 208, 1963.

5. Formal, S. B., Abrams, G. D., Schneider, H., and Sprinz, H. Experimental shigella infections. VI. Role of the small intestine in an experimental infection in guinea pigs. J. Bacteriol. 85:119-125, 1963.

6. Schenider, H., and Formal, S. B. Spontaneous loss of guinea pig virulence in a strain of Shigella flexneri 2a. Bact. Proc., 66, 1963.

7. Formal, S. B., and Falkow, S. Charles Arthur Stuart 1893-1962. J. Bacteriol., 85:259-261, 1963.

8. Formal, S. B., Abrams, G. D., Schneider, H., and Laundy, R. Penicillin in germfree guinea pigs. Nature. In press.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 B 813 ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task 02, Microbiology (Mode of action of antimicrobial agents)

Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
	Department of Molecular Biology Division of Communicable Disease and Immunology
Period Covered by Report:	1 July 1962 through 30 June 1963
Principal Investigators:	Jennie Ciak, M.S. Fred E. Hahn, Ph.D. Richard E. Hartman, Ph.D. Roberta S. Hartman, Ph.D. Alan David Wolfe, S.M.
Assistants:	PFC Peter Matthews, B.S. PFC Robert Usry, B.S. Herbert Whitfield
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 B 813 — Title: Army Medical Basic Research in Life Sciences

Task No. 02 — Title: Microbiology (Mode of action of antimicrobial agents)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. The pattern of biosynthesis of ribonucleic acids in bacteria whose growth and protein synthesis are inhibited by streptomycin is severely distorted. Ribosomal RNA synthesis, like protein synthesis, ceases after approximately 1/3 duplication time, and a low-molecular RNA accumulates which analytically resembles transfer-RNA.

2. The distortion in RNA synthesis, as well as the bactericidal effect caused by streptomycin, are prevented by chloramphenicol. Combinations of the two drugs have the same effects as chloramphenicol alone.

3. The DNA-like RNA which accumulates in chloramphenicol-exposed bacteria is heterogenous and does not possess chromatographic or centrifugal properties markedly different from those of conventional categories of bacterial RNA. -- The direct observation of E. coli B/r in a micro-diffusion cell, which permits the observation by oil-immersion phase contrast microscopy of organisms before, during and after exposure to any dialyzable agent, has revealed that a two-hour exposure results in very little death among those cells which did not divide, whereas, only about 25% of those cells resulting from a division during exposure showed any evidence of viability during an observation period of 2-4 hours after removal of the drug.

BODY OF REPORT

Project No. 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task No. 02 Title: Microbiology (Mode of action of antimicrobial agents)

Description: A study of the molecular biology, biochemistry and microbiology of bacteria under the incluence of antibiotics in order to elucidate the modes of action of these antimicrobial agents.

Progress:

1. Mode of action of streptomycin.

a. It has been reported in detail from this laboratory (Hahn, et al, Biochim. Biophys. Acta, 61:741, 1962) that streptomycin inhibits the synthesis of protein in susceptible bacteria while the synthesis of RNA and of DNA, in overall analytical terms, continues. These effects would seem to resemble those produced, e.g., by chloramphenicol but the following exceptions are noteworthy. (1) Streptomycin kills bacteria rapidly while loss of viability in chloramphenicol-exposed cultures occurs much more slowly. (2) Nucleic acid synthesis occurs at decreasing rates and after little more than one duplication time becomes minimal. (3) Inhibition of protein synthesis is immediate and strong but requires approximately 1/3 of a duplication time to become complete.

b. During the report period, research efforts were concentrated on the elucidation of the pattern of RNA synthesis in streptomycin-exposed bacteria. When E. coli, strain Gratia, growing in mineral medium, was supplied with streptomycin and 30 sec pulses of Uracil-C^{14} were given at predetermined time intervals to individual cultures in series, the rate of uracil incorporation into RNA was shown to drop rapidly and to re-establish itself at a value approximately 50% of that of the pre-streptomycin rate. Pulse labelling which originally was introduced into molecular biology as a method of detecting messenger-RNA synthesis (Gros, et al, Nature, 190:581, 1961) actually labels also other categories of bacterial RNA (Midgley and McCarthy, Biochim. Biophys. Acta, 61:696, 1962) and the results of the present pulse-labelling studies can, therefore, not be interpreted as specific indications of a decrease in the formation of messenger-RNA.

c. Next, the distribution of RNA in different fractions of pre- and post- streptomycin bacteria was studied. E. coli, Gratia, growing in a liquid synthetic medium was exposed to streptomycin for 60 minutes. Bacteria were then harvested and disrupted by sonic oscillation; the sonicate was freed from debris by low-speed centrifugation, the supernatant liquid was dialyzed and then subjected to differential centrifugation. The ribosomal sediment (100,000 x g/ 3 h) contained 30 per cent more RNA (by ribose analysis) in post-streptomycin bacteria than was present in a pre-streptomycin control. The supernatant of the ribosomal fraction showed an increase in RNA of 100 per cent in the post-streptomycin cells. This supernatant was then subjected to extensive high-speed centrifugation (100,000 x g/ 15 h).

Most of the RNA remained in the supernatant of such centrifugations. It is concluded that much of the "streptomycin-RNA" is of relatively low molecular weight since it does not sediment under the conditions listed above. This finding had been anticipated in preliminary studies by other authors (Eaton and Caffrey, J. Bact., 81:918, 1961; White and Flaks, Fed. Proc., 21:412, 1962).

d. The relatively small increase in RNA of the ribosomal fraction, as well as in the 15 h sediment, suggested that the synthesis of ribosomal RNA (bound to ribosomes or free) was affected by streptomycin. This was investigated by supplying Uracil-C^{14} at and for various time intervals after addition of streptomycin to experimental cultures, isolating the ribosomal fraction and studying the distribution of RNA and of radioactivity either in the undegraded fractions or in phenol extracts of aliquots of the same fractions. It was found that the formation of ribosomal RNA and its assemblage into ribosomes decreased rapidly and became minimal approximately at the 20th minute following streptomycin addition. The time course of this decline was remindful of that of the decline of protein synthesis in streptomycin-treated bacteria. However, an RNA, lighter than 16S ribosomal RNA became associated with the ribosomes, and while the formation of this material also decreased with time, this decrease was distinctly slower than that in the formation of conventional ribosomal RNA.

e. It is concluded that in streptomycin action the normal pattern of bacterial RNA synthesis becomes severely distorted. Formation of ribosomal RNA and assemblage of ribosomal particles cease approximately at the same time as does protein synthesis. A non-sedimentable RNA accumulates in quantity, and a heterogenous non-ribosomal RNA becomes associated with the existing ribosomal particles. These two RNAs might be transfer-RNA or messenger-RNA respectively, and the nature of these two materials is now under investigation.

f. Among the various hypotheses attempting to explain the action of streptomycin we subscribe to that of Spotts and Stanier (Nature, 192:633, 1961) who assume a direct interaction of streptomycin with the ribosomes (i.e., with the protein-synthesizing machinery) of bacteria; the present results are compatible with the assumption of White and Flaks (Fed. Proc., 21:412, 1962) that streptomycin interrupts protein synthesis by an initial involvement in a fraudulent attachment of messenger-RNA to the ribosomes.

2. Effects of chloramphenicol upon streptomycin-exposed bacteria.

a. It is well established that chloramphenicol protects bacteria from loss of viability owing to the action of streptomycin (e.g., Jawetz, et al, Am. J. Med. Sci., 222:404, 1951). When chloramphenicol is supplied at time intervals to cultures of E. coli dying from the effects of streptomycin, the surviving cells are protected (Plotz and Davis, J. Bact., 83:802, 1962). This interference phenomenon involving two drugs that are both inhibitors of protein formation poses a perplexing problem and also raises the question whether the bactericidal effect of streptomycin can be adequately explained by the action of the drug on protein formation.

b. It has been found in this laboratory that the distortion of the pattern of bacterial RNA synthesis caused by streptomycin is prevented by

chloramphenicol. When both antibiotics are supplied to experimental cultures of E. coli simultaneously, the pattern of RNA synthesis resembles that of bacteria that have received chloramphenicol alone. Chloramphenicol, thus, does not only supersede the overall microbiological effect of streptomycin but also some of its more detailed effects at the molecular level.

c. Streptomycin, chloramphenicol, or a combination of both antibiotics were added to exponentially growing cultures of E. coli, Gratia, in synthetic medium. The cultures received, also, Uracil-C^{14} at the time of addition of the antibiotics. After 60 min incubation, the bacteria were harvested and fractionated as described in Section 2, para d. of this Report.

TABLE I

Incorporation of radiouracil into E. coli RNA under the individual or combined influence of streptomycin and chloramphenicol

Additions	cpm/aliquot of ribosomal fraction	cpm/aliquot of 15 h sediment	Total cpm
Streptomycin	10,848	6,491	17,339
Chloramphenicol	22,105	20,587	42,693
Strep+Chloramph.	23,799	16,879	40,678

Table I lists the relative amounts of RNA synthesized during the individual or combined actions of streptomycin and chloramphenicol. The large quantity of RNA in the 15 h sediment of chloramphenicol-treated bacteria represented the nucleid acid of the abnormal "chloramphenicol particles" described by Kurland, et al, (J. Mol. Biol., 4:388, 1962).

d. Examination of Table 1 reveals that chloramphenicol superseded completely the action of streptomycin on ribosomal RNA synthesis and did so largely for the synthesis of RNA found in the 15 h sediment. Finally, the overall amounts of RNA synthesized in chloramphenicol action, or by bacteria that were exposed to a combination of chloramphenicol and streptomycin, were almost identical, while streptomycin-exposed cells produced only 40 per cent of this quantity of RNA.

e. These observations can be related to important and difficult theories concerning the genetic regulation of RNA synthesis (Stent and Brenner, Proc. Nat. Acad. Sci., US, 47:2005, 1961; Kurland and Maaløe, J. Mol. Biol., 4:193, 1962; Alföldi, et al., J. Mol. Biol., 5:348, 1962) but a discussion of this topic exceeds the framework of this Report. Suffice it to say that inhibitions of protein synthesis at different reaction steps could plausibly have different effects upon the mechanisms that regulate the synthesis of different categories of RNA.

f. Finally, it should be noted that the supersession of the actions of streptomycin by chloramphenicol could perhaps be explained by assuming that both drugs act sequentially upon different steps of the pathway of protein synthesis and that chloramphenicol, by acting "earlier" prevents a "later" lethal event involving streptomycin action. This, however, is an abstract idea and will require concrete experimental resolution of the sequence of reactions

3. Mode of action of chloramphenicol.

a. In the Annual Report, 1961-62 and in two publications (Hahn and Wolfe, Fed. Proc., 21:384, 1962, Biochem. Biophys. Res. Comm., 6:464, 1962), it was reported that the labile portion of ribonucleic acid that accumulates in chloramphenicol-exposed Bacillus cereus was dissimilated upon removal of the drug into a mixture of free purine and pyrimidine bases. The composition of this mixture suggested that these RNA-constituents were derived from an RNA whose chemical composition was complementary to that of the DNA of the organism. Analogous results have been reported (Chantrenne, Arch. Intern. Physiol. Biochem., 69:745, 1961) for B. cereus exposed to azaguanine, the action of which resembles that of chloramphenicol. We have proposed the hypothesis that DNA-like RNA accumulating under conditions in which protein synthesis is inhibited, represents unused messenger-RNA.

b. Independent evidence of the accumulation of DNA-like RNA in bacteria under the influence of chloramphenicol has been brought forth since by a number of other investigators (Nomura, et al, J. Mol. Biol., 4:376, 1962; Midgeley and McCarthy, Biochim. Biophys. Acta, 61:696, 1962; Ishihama, et al, J. Mol. Biol., 5:251,1962).

c. Simultaneous and independent efforts in this laboratory have been directed at recognizing and studying distinct molecular species of RNA that accumulate in B. cereus in the presence of chloramphenicol. Advanced and modern techniques were adopted for that purpose. Mass cultures of B. cereus to which chloramphenicol had been added 60 min earlier were fragmented either by sonic oscillation or in a Mickle disintegrator. The nucleic acids were extracted from the disrupted bacteria with phenol and were either centrifuged into a preconstructed sucrose density gradient at high speed or were chromatographed on a column made up of discrete layers of methylated albumine-coated diatomaceous earth (Mandell and Hershey, Anal. Bioch., 1: 66, 1960). Characteristic diagrams are obtained in both procedures which separate the transfer-RNA, the two ribosomal RNAs and (in the chromatographic procedure) also the DNA of the extracted cells.

d. In order to distinguish between pre-chloramphenicol RNA and RNA synthesized in the presence of the drug, Uracil-C^{14} was added together with chloramphenicol to the experimental cultures, and the individual components of the centrifugal and chromatographic separation procedures were quantitated spectrophotometrically and counted for radioactivity.

e. Chloramphenicol-exposed B. cereus continued to synthesize transfer RNA, as well as ribosomal RNA. No singular prominent RNA component was discovered in chloramphenicol-exposed bacteria whose properties differed markedly from those of normal RNA. Simultaneous work in other laboratories with E. coli has furnished evidence, that DNA-like RNA, accumulating in chloramphenicol action is heterogenous and not easily distinguished from the major RNA-constituents of the cell (Midgeley and McCarthy, Biochim. Biophys. Acta, 61:696, 1962). Our own results are in accord with these findings.

f. During the report period, theories concerning the nature of DNA-like RNA have undergone a further complication in that some investigators are inclined to consider this material to be only messenger-RNA, while others believe that much of it is precursor material for other categories of bacterial RNA. In either event, heterogeneity of DNA-like RNA is a plausible finding. It is obvious that the nature of the different RNA-components of the cell, the regulation of their biosynthesis and transitions, and the nature of their involvement in protein biosynthesis represent a major theme in current molecular biology and that the results of much basic work will be required in order to integrate the effects of chloramphenicol into the emerging picture. One such a basic effort is under way in this Department and will be reported as part of the In-House research program of the WRAIR; because of its ultimate relevance to the studies reported here, a preliminary publication (#5) by Hartman and Allison is listed below.

g. It has been shown that E. coli B/r continues to divide in the presence of "bacteriostatic" concentrations (50 µg/ml) of chloramphenicol (Allison, et al, loc. cit.). Analysis of the growth curves obtained showed that this continued growth could not be described as being the result of any simple assumption concerning the characteristics of the population as a whole, for example, the assumption that all those cells past a certain "age" had the ability to divide once. Further, direct observations indicated that

the fate of a cell could not be predicted from its state of cellular division at the time of the introduction of the antibiotic. To clarify this situation it was desirable to obtain information concerning the fate of statistically significant numbers of individual cells observed before, during and after exposure to the drug. For this purpose a micro-diffusion cell which permits observation by oil-immersion microscopy was devised (Hartman and Hartman, J. Bact., 84:595, 1962), and the collection of such data is underway.

h. All investigators concerned with microscopic observation of microbiological growth are faced with the problem of deciding what constitutes the final step of cell division; there is no consensus on this problem. Electron microscopic study of sectioned cells has demonstrated that the gram-negative bacteria divide by a pinching-in of the cell wall and the cell membrane together, thus leaving the two daughter cells in communication (sometimes with the nucleus incompletely divided) until the new cell walls are complete (Glauerb, Brit. Med. Bull., 18:245, 1962). Therefore, for the purpose of correlation with biochemical events, we use for the criterion of division the rounding of both of the new ends. Of course, complete separation must be used when comparing data with plate counting for viable cells or electronic counting of total cells. It is the former criterion which was used to collect the data discussed below, although calculation on the basis of complete separation leads to the same qualitative results.

i. Complete data are available on a total of 26 cells, each of which had either shown substantial growth or was a daughter cell of an observed division in the absence of the drug, and was, therefore, presumed viable. After exposure to a concentration of 50 ug/ml of chloramplenicol in BHI growth medium for two hours at room temperature (two generation periods for a non-aerated mass culture of this strain in BHI at room temperature), 11 of the 26 cells had divided and 15 had not. After removal of the drug, observation was continued for a period of two to four hours. Of the population of 37 cells which were present at the time of the removal of the drug, 19 recovered (by the criterion of division or very substantial growth) and 18 did not. This parent population of 37 cells can be divided into two sub-populations characterized by whether the cell was or was not a result of division in the presence of the drug. In the sub-population of 22 members which are the result of division in the presence of the drug, only 5 recovered while 17 did not. In the sub-population of 15 cells which had not divided in the presence of the drug (although they may have grown substantially and started to divide), 14 of the cells recovered and 1 did not. It can be shown that the probability of drawing these two sub-populations from the parent population of 37 cells by chance is vanishingly small. Therefore, we conclude that division in the presence of 50 ug/ml of chloramphenicol is deleterious to the cells, and that the resulting population is a mixed population of cells in two significantly different physiological states. Data which are not yet statistically significant indicate that this effect is not related to growth and early stages of division, but to the final stage. This suggests that the cells are unable to recover from some mistake in the division of the resources of the mother cell.

Summary and Conclusions:

1. The pattern of biosynthesis of ribonucleic acids in bacteria whose growth and protein synthesis are inhibited by streptomycin is severely distorted. Ribosomal RNA synthesis, like protein synthesis, ceases after approximately 1/3 duplication time, and a low-molecular RNA accumulates which analytically resembles transfer-RNA.

2. The distortion in RNA synthesis, as well as the bactericidal effect caused by streptomycin, are prevented by chloramphenicol. Combinations of the two drugs have the same effects as chloramphenicol alone.

3. The DNA-like RNA which accumulates in chloramphenicol-exposed bacteria is heterogenous and does not possess chromatographic or centrifugal properties markedly different from those of conventional categories of bacterial RNA. -- The direct observation of E. coli B/r in a micro-diffusion cell, which permits the observation by oil-immersion phase contrast microscopy of organisms before, during and after exposure to any dialyzable agent, has revealed that a two-hour exposure results in very little death among those cells which did not divide, whereas, only about 25% of those cells resulting from a division during exposure showed any evidence of viability during an observation period of 2-4 hours after removal of the drug.

List of Publications:

1. F.E. Hahn, Jennie Ciak, A.D. Wolfe, R.E. Hartman, J.L. Allison and Roberta S. Hartman, "Studies on the Mode of Action of Streptomycin. II. Effects of Streptomycin on the Synthesis of Proteins and Nucleic Acids and on Cellular Multiplication in Escherichia coli." Biochim. Biophys. Acta, 61: 741-749, 1962.

2. J. Ciak and F.E. Hahn, "Concurrent Morphological and Biochemical Events in Penicillin-Exposed Staphylococcus aures." Science, 137:982-983, 1962.

3. Roberta S. Hartman and Richard E. Hartman, "Microdiffusion Chambers Permitting the Oil-Immersion Microscopy of Growing Microorganisms." J. Bact., 84:595-596, 1962.

4. F.E. Hahn, "Psychoactive Material in the Serum of Schizophrenic Patients." Serological Fractions in Schizophrenia, pp 57-62, Harper & Row, 1963.

5. Richard E. Hartman and James L. Allison, "Isopycnic Fractionation of sRNA by Fractional Recrystallization in a Density Gradient." Abstract, Biophysical Society, Seventh Annual Meeting, MB11, 1963.

6. Alan David Wolfe and Fred E. Hahn, "Biosynthesis of RNA in Bacteria Exposed to Streptomycin." Fed. Proc., 22:462, 1963.

ANNUAL PROGRESS REPORT

Project No. 3A 12501 B 813, ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task 02	Title: Microbiology (Bio-assay of biologically active substances by tissue culture)
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C. Department of Cellular Physiology Division of Basic Surgical Research
Period Covered by Report:	1 July 1962 through 30 June 1963
Principal Investigators:	Andre D. Glinos, M. D. Robert B. Greer, III, Capt, MC
Assistant:	Robert J. Werrlein, B. S.
Reports Control Symbol:	MEDDH-288
Security Clasification:	UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 B 813, ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task 02	Title: Microbiology (Bio-assay of biologically active substances by tissue culture)
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
Period Covered by Report:	1 July 1962 through 30 June 1963
Authors:	Andre D. Glinos, M. D., Robert B. Greer, III, Capt., MC, and Robert J. Werrlein, B. S.
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

Glycerol and dimethylsulfoxide are being used as protective agents in an attempt to preserve the viability of tissue culture cells after freezing in liquid nitrogen. The purpose of this work is to provide a stable preservation technique for cells of known characteristics. Comparative studies are being undertaken to evaluate biochemical and genetic similarities and differences before and after freezing. Additional work is being done on the possible biochemical effect of protective substances at temperatures as low as -196° C.

BODY OF REPORT

Project No. 3A 0 12501 B 813, ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task 02 Title: Microbiology (Bio-assay of biologically active substances by tissue culture)

Description:

For over twenty years it has been known that glycerol offers a measure of protection to cells and tissues which are subjected to extreme cold. Recently a second compound, dimethylsulfoxide, has been used in the freeze-preservation of living cells. Both of these compounds are toxic in moderate concentrations, but in concentrations of 5% to 15% their ability to preserve cells from damage by freezing outweighs their toxicity and establishes their usefulness in this technique.

In this laboratory a method is being evaluated for preserving viable L-strain mouse fibroblasts in liquid nitrogen at temperatures ranging from -150° C to -196° C. When perfected, such a procedure would eliminate much of the routine daily work of "farming" stock of identical cells which would not be subjected to constant manipulation, with the risks both of introducing infection as well as genetic variability, which may happen with frequent manipulation and constant subtle changes in extracellular environment. In order for this technique to be useful, therefore, the following criteria must be met:

(a) Cells may be kept for indefinite periods at -150° C. without loss of viability.

(b) A reasonable percentage of frozen cells must survive thawing and must grow normally thereafter.

(c) There must be no latent damage to cells which will appear after a number of cell generations.

(d) Cells after thawing must be genetically and biochemically identical to their unfrozen counterparts. In other words, freezing be shown not to select out a "hardy" population with different characteristics than the parent cells.

Progress:

Thus far a number of experiments have been performed utilizing glycerol as the preservative in the freezing process. Briefly, cells have been made up in a concentration of 2-3 million cells per ml. in a medium containing 15% horse serum, 7.5% glycerol, and 77.5% minimal essential medium. This has then been divided into a number of 2 ml. sterile ampules which were heat-sealed. The cells have then been subjected to a 1° C a minute temperature drop from ambient to -80° C, and then placed in liquid nitrogen vapor at -150° C until a convenient time for thawing. Ampules have then been thawed quickly at 37° C and diluted with routine suspension culture medium to a final cell concentration of 400,000 cells per ml. The population kinetics of these cells have been followed in detail.

Referring back to the several criteria mentioned earlier, the following results are apparent even after a relatively few experiments:

(a) Cells have been kept in the frozen state for as long as 25 days, thawed, and grown routinely with no difficulty. The slopes of the growth curves of frozen cells in suspension culture, when graphed logarithmically, are identical with those of unfrozen control.

(b) Between 15% and 50% of the frozen cell population are damaged sufficiently by freezing to prevent their growth. The remainder grow normally. There is every reason to suspect that 15% mortality or less will be the ultimate cell loss as techniques are perfected. Interestingly enough, when cell counts are made immediately before freezing and after thawing and dilution, they are identical, indicating that the above-mentioned damage is not due to physical destruction of cells by ice crystals, or by rupture of cells due to osmotic forces as the glycerol is diluted. This suggests that the "freezing lesions" is a biochemical one. Lovelock has shown (Proc. Royal Soc. (B), 147: 427, 1957) that lipoprotein complexes, which are held together by weak association forces and contain up to 60% water, are denatured by freezing, although glycerol exerts considerable protection from denaturization in most instances studied. A possible explanation for the "freezing lesion" we see may lie in lipoprotein alterations. Since the external cell membrane, as well as the many intracellular membranes, are all made up in large part of lipoprotein, it may be that freezing causes altered permeability of various membranes, leading to loss of essential metabolites, or loss of barrier function to toxic by-products, which then leads to delayed cell destruction. It may also be that other substances, such as dimethylsulfoxide, may offer more protection to biochemical as well as physical damage. These hypotheses will be subjected to considerable investigation in the coming year.

(c) There appears to be no latent damage following freezing, once the surviving cells have begun to grow. Cultures have been carried for 35 days after thawing, through ten cell divisions, with no alteration in their growth curves during this period. The ten cell division test is fairly standard in tissue culture biology for determining "indefinite viability," and we therefore have little doubt that, once thawed and growing, frozen L-strain cells may be cultured indefinitely, as is possible with their unfrozen counterparts.

(d) Whether frozen L-strain cells remain genetically and biochemically identical to unfrozen controls remains to be shown in the coming months. Techniques are at hand in our laboratory for evaluating these parameters, and work in this direction is already in progress. Thus far there is reason to believe, from pilot studies already done, that chromosomal distributions in frozen and unfrozen cells are similar, and within the range of normal variation. Both have the majority of chromosome counts between 50 and 60, with modal peaks between 54 and 56. Interestingly enough, mitotic counts suggest that late mitotic figures (telophase and anaphase) are rarely found following freezing, perhaps indicating that cells in late mitosis are more susceptible to damage by freezing.

Summary and Conclusions:

Various chemical substances, including glycerol and dimethylsulfoxide, are being studied as protective agents in the freeze-preservation of viable tissue culture cells in liquid nitrogen. The purpose of this work is to provide a stable preservation technique for indefinite storage of cells of known characteristics. Results to date indicate that the technique is reasonably efficient, and that cells surviving freezing are a random sample of the initial pre-freezing population. Further work will be done to try to uncover the biochemical nature of the "freezing lesion," as well as to characterize and compare the biochemical activity of unfrozen and frozen cells.

List of Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 B 813 ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task 02, Microbiology (Methods for electron microscopy of entities related to infection)

Reporting Installation:	**Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.**
	Department of Molecular Biology Division of Communicable Disease and Immunology
Period Covered by Report:	**1 July 1962 through 30 June 1963**
Principal Investigators:	**Richard E. Hartman, Ph.D. Roberta S. Hartman, Ph.D.**
Assistant:	**PFC Robert Usry, B.S.**
Reports Control Symbol:	**MEDDH-288**
Security Classification:	**UNCLASSIFIED**

ABSTRACT

Project No. 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task No. 02 Title: Microbiology (Methods for electron microscopy of entities related to infection)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Richard E. Hartman, Ph.D.
Roberta S. Hartman, Ph.D.
PFC Robert Usry, B.S.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. An evaporation source has been developed for shadowcasting specimens for electron microscopy which has an angular aperture of less than 0.3°, thus bettering the approach to the ideal point source by a factor of five to ten over conventional sources.

2. Studies of the structure of monomolecular protein films have shown that resolutions of 5 Å are possible with such specimens. A method for substantially reducing the usual specimen contamination by "cracked" diffusion-pump oil has been important in achieving these results.

3. Further work has confirmed the characterization of the ferritin-containing inclusion bodies found in human intestinal epithelium as previously reported.

BODY OF REPORT

Project No. 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task No. 02 Title: Microbiology (Methods for electron microscopy of entities related to infection)

Description: The demonstrated resolution of currently manufactured electron microscopes approaches atomic dimensions. Studies are reported here which concern the development of methods which will provide useful information about the molecular structure of biological entities.

Progress:

1. Micro Effusion Chamber.

a. "Shadowcasting" of specimens for electron microscopy is a standard procedure in which heavy metal is evaporated in vacuo from a small-diameter source onto a specimen inclined at an angle to the source. Regions where metal is deposited have high electron scattering cross sections relative to regions where no metal is deposited. Since the specimen is inclined at an angle to the metal source, particles which have elevations above the surface of the supporting membrane prevent the deposition of metal behind them, with the result that electron micrographs of such specimens have the appearance of objects photographed on a plane surface and illuminated by bright sunlight with the sun near the horizon. The resultant "shadows" contain information concerning the shape of the particles studied, the amount of such information being determined by the sharpness of the shadows.

b. Ideally, the evaporation source should be a point. However, in practice all metal evaporating sources are somewhat extended, and the resulting shadows are less than ideally sharp. The angle subtended by the source at the specimen is thus a measure of its excellence. The best of conventional sources is one in which metal is evaporated from the tip of a "hairpin" of tungsten wire. Such a source has a dimension of from 2 to 3 mm, and, because only a small amount of metal can be evaporated, the specimen must be placed no more than 5 cm away from it. Such a source, therefore, subtends an angle of from 2^{o} to 3^{o}. Although such a source is quite satisfactory for most purposes, difficulties were encountered, for example, in studies of the structure of Ferritin-globulin conjugates, which indicated a need for sharper shadows.

c. For this reason a new source was developed which consists of a small-diameter carbon rod which has been drilled to provide a small diameter cylindrical chamber. When heavy metal, such as Pd and Pt-Pd alloys, is placed in the chamber and heated by passage of an electric current, metal evaporates from the orifice in its end. Rods having annular openings as small as ½ mm can be used to shadow specimens placed at least 10 cm away. Such a source subtends an angle of about 0.3^{o} and is thus 5 to 10 times better than more conventional sources. Such sources are now used routinely for shadowcasting in this laboratory.

2. The use of protein monolayers as specimen supports.

a. The use of protein films spreading as monomolecular films on aqueous surfaces as vehicles for the uniform dispersion of particulate specimens and the subsequent transfer of such films to supporting membranes for study in the electron microscope was first demonstrated some ten years ago (Hartman, et. al., J. Appl. Phys., 24:90-92, 1953). This procedure has since been widely used. Later, the authors demonstrated that if such films are transferred to a supporting membrane consisting of a carbon film containing numerous small holes (diameters of 1 μ or less) the protein films have sufficient strength to support both themselves and the particulate matter over the open holes (Hartman, R.E. and Hartman, R.S., J. Appl. Phys., 26:1394, 1955). Such films have been shown by optical means to have an average thickness of approximately 7 Å (Langmuir, Schaefer and Wrinch, Science, 85:76, 1937).

b. Although the theoretical importance of a specimen having a thickness of only 7 Å units both for the study of the characteristics of electron images and for the study of structures having dimensions of these orders is obvious; the practical importance was limited by instrumental difficulties. Many of these limitations have been removed by design changes which have been incorporated into the present generation of electron microscopes, of which our Siemens' Elmiskop I is one of the first and still one of the best. A persistent difficulty (which has not been eliminated in these instruments) is the build up of contamination on the specimen as a result of interaction of the electron beam with residual diffusion-pump oil in the column. Such contamination builds up under normal operating conditions on the illuminated portion of the specimen at rates which greatly exceed 10 Å per minute, so that a 7 Å specimen very rapidly becomes more contamination than specimen. We have, however, learned a number of tricks which greatly reduce this contamination rate. The most important of these is the introduction of a very small flow of dry nitrogen introduced into the microscope column just below the specimen stage. This results in a positive flow of gas away from the specimen and toward the pumps and serves to drive the pump oil away from the specimen. This reduces the contamination rate from well over 10 Å per minute to well under 1 Å per minute.

c. The above change in operating procedure, although not an ideal solution to the contamination problem as it requires the operation of the instrument at pressure above the optimum, has made possible the production of electron micrographs showing extremely high resolutions. At present the method is being studied to learn what its potentialities may be. The following preliminary results may be reported:

(1) In a bovine plasma albumin film in which the albumin had been lightly stained with phosphotungstic acid before spreading, not only were the individual phosphotungstate ions well resolved (somewhat less than 10 Å units in diameter) but structures considerably smaller than these ions were also resolved.

(2) Specimens containing DNA spread with bovine plasma albumin on 10^{-5} M uranyl nitrate show DNA fibers well resolved with some indication of the structure of the fibers.

(3) A specimen of bovine plasma albumin spread on 10^{-6} M uranyl nitrate shows resolved structure having dimensions of less than 5 Å. These appear to be the individual uranyl ions. In addition, in the through-focus series of one of these specimens, the out-of-focus images show diffraction rings having diameters of 5 Å and less which are obviously related to the fundamental structure of the electron image. These results indicate that with the development of suitable chemical markers the method can be used for the direct study of macromolecular structure in the electron microscope.

3. Ferritin-containing bodies in human intestinal epithelium.

a. Using more conventional techniques of electron microscopy, the characterization of the ferritin-containing inclusion body (F-body) discovered in the absorption cells of human intestinal epithelium has been completed. There is no change from the preliminary characterization (see Annual Progress Reports, WRAIR, 1 July 1961-30 June 1962). However, a few clues have been collected which suggest that the F-body comes to the apical cytoplasm from the golgi area. Based on frequency of observation and geometrical considerations, it has been concluded that there is one or more per cell (at least in the upper four feet of the gut).

b. The F-body is of considerable interest because ferritin has been assigned a prominent role in various hypotheses concerning the regulation of absorption of iron from the gut. These hypotheses have been under attack because some attempts to isolate ferritin from the epithelium have failed and because the various detailed studies of the intestinal epithelial cells of the mouse and rat which are in the literature do not mention or demonstrate ferritin. We have also demonstrated ferritin in rat jejunum, but it is much less plentiful than in the human cells. We have no evidence in favor of any particular hypothesis of iron absorption, but it is obvious that any hypothesis which has been discarded on the grounds that there is no ferritin in the absorptive cells needs to be re-examined.

Summary and Conclusions:

1. An evaporation source has been developed for shadowcasting specimens for electron microscopy which has an angular aperture of less than 0.3°, thus bettering the approach to the ideal point source by a factor of five to ten over conventional sources. This source is now being routinely used in this laboratory.

2. A method for substantially reducing the rate at which the "cracked" diffusion pump oil builds a contaminating layer on the electron microscope specimen permits the utilization of the high initial magnification of the Siemens' Elmiskop I for the study of the fine structure of protein

monomolecular films stretched over the holes in carbon supporting membranes. Results to date indicate that (1) resolutions better than 5 A can be obtained with such specimens and (2) upon development of suitable chemical markers, these methods can be used for the study of macromolecular structure.

3. The completion of the characterization of the ferritin-containing inclusion bodies found in the absorptive cells of the human intestine confirmed the earlier description. Further, it is concluded from geometrical considerations and the frequencies of observation that there is probably one or more in every cell, at least in the upper four feet of the gut. Their function is unknown, but clearly any hypotheses regarding the regulation of iron absorption from the gut must take into account the presence of ferritin in the absorptive cells.

List of Publications:

1. R.E. Hartman and R.S. Hartman, "A Small-Diameter Evaporation Source for Shadowing with Platinum and Platinum-Alloys." Proceedings of the Vth International Congress for Electron Microscopy, ed. S. Breese, Academic Press, New York: 1962, p FF-4.

2. R.S. Hartman, M.E. Conrad, R.E. Hartman, R.T. Joy and W.H. Crosby, "Ferritin-Containing Bodies in Human Small Intestinal Epithelium." Blood (in press).

ANNUAL PROGRESS REPORT

Project: 3A O 12501 B 813, Army Medical Basic Research In Life Sciences

Task: 03 Biochemistry (Vital Biochemical Activity In Health and Disease)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Biological Chemistry
Division of Biochemistry

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No: 3A O 12501 B 813 Title: Army Medical Basic Research In Life Sciences

Task: 03 Title: Biochemistry (Vital Biochemical Activity In Health & Disease)

Reporting Installation: Walter Reed Army Institute of Research
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Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

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Extension of previous studies has allowed the following observations:

α-tocopherol is active-without the formation of intermediary metabolites - in stimulating oxidation of many substrates by rat liver homogenates. In mitochondria, tocopherol prevents the decline of succinate oxidation by preventing oxaloacetate accumulation, either by preventing its formation or by shunting it through an unknown pathway.

Bacterial endotoxin is inactivated by liver fractions. Enzymatic evidence indicates the lipid moiety is necessary for toxicity.

Countercurrent distribution developments allow purification of alanine and tyrosine RNAs from yeast. Structural differences were demonstrated.

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Species specificity for 14 amino acids showed striking differences, although several homologies appeared.

The tyrosine-α-ketoglutarate "inhibitor" found in rat liver has been partially purified on DEAE cellulose. Kinetic studies have shown combination of the factor with enzyme and substrate.

The erythrocyte membrane of rats was found fifty times more concentrated with trans isomers of unsaturated fatty acids than adipose tissue.

Polarography was successfully used to demonstrate interaction between insulin, chromium, and the mitochondrial membrane. Additional electroanalytical research allowed development of coulometric methods for phosphate, calcium, ammonia, magnesium and ferrous iron. Selenium electrode processes have been described and a synthetic diet for producing selenium deficiency has been studied.

BODY OF REPORT

Project No: 3A O 12501 B 813 Title: Army Medical Basic Research In Life Sciences

Task: 03 Title: Biochemistry (Vital biochemical activity in health and disease.)

Description: The series of studies described are an extension of previous work in basic biochemical research designed to better describe the fundamental processes of biochemical activity relating to intermediary metabolic functions and factors which have a vital role in the regulation of these functions.

Progress:

a. Metabolites of Tocopherol in vitro:

In animals it has been shown that a deficiency of tocopherol results in a variety of symptoms including heart and liver necrosis, muscular dystrophy, kidney and brain damage. Obviously, tocopherol must play a very central role in mammalian metabolism and the elucidation of this role would be of great importance. The introduction of unsaturated fats into diets to inhibit heart and arterial diseases creates an increased requirement for tocopherol, which deserves detailed study.

Incubation of tocopherol with rat liver homogenates can prevent a metabolic lesion characterized by an inability to oxidize a variety of substrates. Water-soluble vitamins have been shown to be converted to an active form before action in metabolism occurs, i. e., thiamine is converted to thiamine pyrophosphate, niacin to NAD, NADP, and riboflavin to FMN and FAD. Such an active form for tocopherol has long been postulated. When C^{14} -tocopherol is incubated under conditions shown to prevent the metabolic lesion, a compound can be isolated from the incubation medium after saponification, which after chromatography on a silicic acid column was shown to be tocopherol itself. This was demonstrated by paper chromatography in three different solvent systems and by infrared and ultraviolet spectrophotometry. It was necessary to perform many of the operations under a stream of nitrogen to prevent the accumulation of artifactual metabolites. It was interesting that extraction with petroleum ether before saponification did not remove any radioactivity-indicating that the tocopherol was bound. Treatment with glutathione released some of the tocopherol. This indicates that tocopherol is bound in part to sulfhydryl groups in the protein. Only tocopherol can be extracted prior to saponification with $CHCl_3$-CH_3OH; therefore, it does not exist in ester or phosphate linkage. It is evident from these results that tocopherol is its own form in this in vitro system. Since no dimer of tocopherol could be detected, it is also obvious that tocopherol is not acting as an antioxidant in this system.

b. Relation of Tocopherol to Succinate Oxidation in Mitochondria:

In the presence of NAD, a cofactor not required for succinate oxidation, oxygen uptake will decline when tocopherol is not present in rat liver mitochondria. This can be prevented by dietary or in vitro supplementation of the vitamin. It can also be prevented in vitro by the addition of many compounds which are known to remove oxaloacetate (OAA), a potent inhibitor of succinate oxidation. This coupled with the fact that NAD will stimulate formatinn of OAA from malate indicates that tocopherol is involved with decreased accumulation of OAA. This was demonstrated upon direct measurement of OAA accumulation. Whether or not tocopherol is present, oxaloacetate can be removed equally well. Furthermore, malate plus pyruvate will form citrate with equal facility in the presence or absence with tocopherol. Such evidence indicates that tocopherol does not affect the formation of citrate from OAA, a possible pathway. The question now is whether tocopherol is affecting an alternative pathway for removal of OAA or preventing its formation. Succinate-1, 4-C^{14} when incubated in the usual way will release twice as much $C^{14}O_2$ in the absence of tocopherol. This indicates that tocopherol is not stimulating decarboxylation of OAA, but does not distinguish between an effect on formation or removal by another pathway. The amino acid accumulation resulting from the C^{14} succinate incubation is unaffected by tocopherol indicating that transamination may not be a factor. Further evidence in this regard is required.

c. Effect of Endotoxin on Enzyme Action:

Bacterial lipopolysaccharides (endotoxins) are of great interest in infectious diseases. These toxins, still effective after the death of the bacteria, produce many symptoms such as fever and diarrhea. A knowledge of the structure and toxic principle of these compounds, would contribute much toward the chemotherapy of bacterial diseases.

It has previously been shown that the liver is able to inactivate endotoxins to some extent. With the collaboration of W. E. Farrar from Applied Immunology, an investigation was made as to the mode of action of the liver in detoxifying the bacterial lipopolysaccharide. It was hoped that a knowledge of the detoxifying factor or enzyme would give an answer to the question as to whether either the lipid or polysaccharide moiety of the endotoxin was primarily toxic. The experiment was performed by incubating the endotoxin (from S. typhimurium) with the guinea pig liver preparation for one hour and then making a proper dilution to give what

should be a $2xLD_{50}$ dose intravenously to eleven day chick embryos. Liver homogenates were quite active in detoxification. Fractionation of the homogenate into mitochondria, microsomes, and supernatant fractions revealed that the activity could not be localized but the individual activity was far less than that of the whole homogenate. It was found that supplementation with ATP and/or malate would activate these fractions. Acetone powders of the whole liver could also be activated by ATP and malate. The properties of this system indicated that a fatty acid oxidation system was involved. An investigation of the literature revealed striking similarities to Kornberg's fatty acid activation system with guinea pig liver i. e. the inability to localize activity in only one fraction of the liver and the inability to obtain substantial purification of the enzyme from the acetone powder.

To show that fatty acid oxidation was taking place, the resulting hydroxamates were measured and showed that 50% of the fatty acid esters in the lipopolysaccharide were activated. These results strongly suggest that the oxidation of fatty acids inactivate the toxicity of the lipopolysaccharide. This, coupled with the inability for takadiastase (a polysaccharide-splitting enzyme) to inactivate endotoxin, indicates the lipid is a necessary ingredient for toxicity.

d. Mechanism of Protein Biosynthesis:

One of the most important functions of the cell is to synthesize proteins since proteins make up the enzymes, several hormones and other vital components of cells. Thus, the study of the mechanism of protein biosynthesis will facilitate better understanding of diseases such as cancer and viral infections.

The role of DNA and RNA in protein biosynthesis is well established. It is believed that the sequence of nucleotides in RNA determines the sequence of amino acids in proteins. It thus becomes important to study the structures of nucleic acids.

Using one solvent system for the initial countercurrent distribution of yeast S-RNA and another for redistribution of various fractions isolated from the first distribution, alanine and tyrosine-RNA have been purified to such an extent that they appear to be homogeneous. Two dimensional mapping procedures for the separation and identificat ion of oligonucleotides obtained after pancreatic RNase digestion of these two RNAs showed that there are major qualitative and quantitative differences, in their oligonucleotide contents. Complete structural studies of these two RNAs and the purification of other RNAs is in progress.

It is shown that amino acid incorporation into S-RNA by activating enzymes is species specific and also depends upon the amino acid in question. The problem of species specificity thus becomes important from the point of view of the degeneracy and universality of the genetic code contained in nucleic acids.

Species specificity of S-RNA and amino acid activating enzymes from rat liver, yeast and E. coli were studied for 14 amino acids. A wide range of specificity was observed; however, several homologies appear to exist. Thus it appears that the genetic code for protein synthesis is degenerate; however, this degeneracy is restricted.

It has been shown that 'RNA Core" contains streptolysin-S enhancement activity. A solvent system has been developed in which the 'RNA Core" is distributed. One fraction which has approximately 70% guanylic acid contains all the streptolysin-S enhancement activity. Further efforts to isolate this factor in purified form and studies of the mechanism of this toxin production are in progress.

A solvent system in which one can separate native DNA from denatured DNA has been developed.

e. Studies of Tyrosine Transaminase Activity:

It was shown previously that tourniquet injury causes a rise in the activity of tyrosine -α ketoglutarate transaminase in rat liver and that this change is due to the inactivation of an "inhibitor" rather than to an actual increase in the quantity of enzyme. An "inhibitor" of the transanimase has been demonstrated in rat and human plasma. In addition some evidence had been obtained that a substance existed in plasma and probably in liver which inactivated the inhibitory factor.

In order to identify these factors and determine their relation to the development of traumatic shock attempts have been made to separate the enzyme and inhibitor and to purify both factors.

A preliminary crude separation of enzyme was accomplished by running a .05 M phosphate buffer (pH 7.35 or 8.0) extract of liver through a DEAE cellulose column and eluting with 0.3 M buffer at the same pH. Approximately 60% of the inert protein is separated from the active enzyme fraction by this procedure. Recoveries of enzyme activity from extracts of liver of normal rats have appeared to range from 88% to 167%. Precipitation of the active fraction with .6 saturated ammonium sulfate neutralyzed to pH 7.4 has resulted in 100% - 130% recovery of activity. Since the recoveries have been over 100%, it is evident that by these two steps some separation of inhibitor and enzyme has been accomplished.

When extracts of liver from rats that had been subjected to tourniquet injury were put through the same purification process, recoveries were below 100%.

Knox and Lin have shown that when tyrosine is injected into rats there is a rise in the activity of tyrosine α-ketoglutarate transaminase. This increase in enzyme activity has been described as an "adaptive" increase in the quantity of enzyme. When extracts of liver from rats treated in this manner are put through a DEAE cellulose column at pH 7.9 only 25% of the activity is recovered. After the bulk of the protein has been washed through the column with .05 M buffer, a second protein fraction is obtained. This "second" peak is not observed with extracts either from normal rats or from rats subjected to tourniquets. The pH of the first portion of eluate containing the bulk of protein was 7.9; the pH of the fraction containing the second peak was 9.2. This would indicate that the injection of tyrosine into rats caused an increase in a basic protein, possibly a histone.

The inhibitor factor has been separated from the proteins of plasma by extraction with 1 to 3 alcohol-ether mixture at a pH of 1.5. Neutral fat is separated by extracting the ether solution with sodium carbonate solution. The inhibitor is then extracted into ether after neutralization of the carbonate. Since as yet there has been no method developed to determine "inhibitor" quantitatively, the percent recovery has not been determined.

When increasing quantities of plasma or albumin are added to an enzyme reaction mixture, the inhibition obtained is not directly proportional to the amount of inhibitor added. The curve resembles a parabola with a maximum dependent on the enzyme extract. The "maximum" obtained with sucrose extracts of rat liver has varied from 11 to 80% inhibition. The average for extracts of normal rat liver is approximately 50% inhibition. The average for extracts of liver from animals that had been subjected to tourniquets is lower approximately 35-40%. The reaction of solutions of partially purified enzyme has been inhibited 84% by 2 ml human plasma. The percent inhibition produced by a given quantity of plasma is independent of the quantity of enzyme within the limits of the method of determination.

A study of the kinetics of the reaction has indicated that the inhibition produced by the plasma factor is a coupling type where there is a combination of enzyme, inhibitor and substrate.

f. Biochemistry of trans Isomers of Unsaturated Fatty Acids:

A literature search showed that neither the source nor possible functional role(s) of trans isomers of unsaturated fatty acid ("trans F. A. 's") have been demonstrated. These compounds are found in rather high concentration in mammals; human liver has been reported to contain up to 14. 4% and human atheroma up to 8. 8 percent of trans F. A. The trans isomer of any given fatty acid has a higher melting point than its cis isomer; the incorporation of trans into body fat will lead to a "harder" fat than will its cis isomer. In a given cell, the highest concentration of trans F. A. appears to be in the structural lipid of the cell membrane. Recently, it has been shown that the transport rate of both isomers across various membranes (e. g. , in the gut or in adipose tissue) is quantitatively the same, but that the turnover time of the trans is considerably slower than that of the cis, indicating possible preferential retention or accumulation of trans F. A. In the chemical laboratory, conversion of cis to trans is accomplished by heating the cis form in the presence of selenium metal. Selenium is known as a trace element essential to the nutrition of several mammalian species.

Work is now in progress to determine the source of trans F. A. 's found in mammalian tissue. The effect of dietary selenium of the concentration of trans F. A. 's are being studied in the rat. Future plans include studies of the effect of these compounds in the erythrocyte membrane and other membranes to determine possible functional roles in the body.

Much of the present work has been occupied with conducting a thorough literature search, adapting techniques for assay of the trans bond, assaying lipid components of various test diets for suitability of use, and running preliminary experiments.

The method of choice for assaying the trans bond is that of infrared spectrophotometry. Trans absorbs strongly at 10. 36 u with relatively little interference by other structural groups. The method is quite sensitive and fairly rapid.

Rats were put on diets of high and low trans F. A. 's, to attempt to modify the trans F. A. content. Many commercially available dietary lipids contained a high proportion of trans F. A. 's; one margarine contained nearly 1 millimole of trans F. A. per gram. Several samples of lard showed less than 0. 01 micromole per gram, which is the lower limit of resolution. Commercial lard was found to be a suitable source of low-trans F. A.

Preliminary diet experiments were disappointing; the amino acid source for the diets, Torula yeast, was found to contain sufficient trans F. A. to statistically overshadow the high and low lipid components of the diet. Work now in progress has fat-free soybean protein as the amino acid source.

g. Evaluation of Dietary Salt Mixes:

A series of studies were undertaken to evaluate the different salt mixtures commercially available since senior investigators had observed inconsistency of the growth rate of experimental rats. In most instances the salt mixes used were questioned. Most investigators use a specific salt mix - generally the result of previous associations - and it is not uncommon for investigators to compare results while overlooking the fact that a different salt mix was used.

The preliminary study compared the effect of three salt mixes on growth rate of weanling rats. A casein diet containing fifteen percent protein was used. Table I shows the results obtained during a 28 day trial.

Table I - Growth Data of Experiment I

Salt Mixture	n	Ave. Initial Wt.	Ave. Final Wt.
Fox-Briggs	10	43.3	203.7
Jones-Foster	10	43.2	188.5
USP XVI	10	43.4	197.2

There appeared to be no statistical difference between the growth rate of the rats receiving the Fox-Briggs and the USPXVI salt mixes. The performance of both was slightly superior to that of the rats receiving the Jones-Foster salt mix.

A second study consisted in evaluating two different salt mixes designed to meet the requirement of a selenium-free diet. Since it appears that selenium is a contaminant when sulfur containing derivatives are used, all sulfate salts were eliminated from the mixture. Composition of the diets is shown in Table II.

Table II - Percent Compositions of the Salt Mixtures

Ingredients	Salt Mixtures		
	WRAIR 1	In common	WRAIR II
$Ca(HPO_4)_2$		60.00	
$CaCO_3$		15.00	
KCl		9.00	
$MgCO_3$		4.00	
NaCl		3.00	
$MnCl_2 \cdot 4H_2O$		.80	
Ferric citrate		.50	
$ZnCO_3$		.10	
KI		.001	
Cupric acetate	.06		.12
Sucrose	7.539		7.479

On WRAIR #I, the initial weights of the rats was 40.2 grams. This increased to 142.5 grams after 28 days. All rats fed WRAIR #II mix died within 3 weeks with symptoms of typical copper toxicity (eye lesions, diarrhea, etc.). Growth rate on WRAIR #I is not favorable in comparison with other mixes.

Seven salt mixes are currently being compared. These include Fox-Briggs (1), Jones-Foster (2), USPXVI (3), HMW (4), Briggs (5), Modified Fox-Briggs and Modified WRAIR I. The modified Fox-Briggs differs from the original in that the calcium phosphate dibasic is of the N.F. Grade. The Modified WRAIR I has twice as much zinc and 33% more sodium chloride than the original.

Casein diets at three levels of protein (8, 12 and 16 percent) are being used to compare the seven salt mixes. Diets based on Torula yeast and Drackett protein are also being used to compare the same salt mixes. The Torula yeast diets contain thirty percent yeast and about fifteen percent protein. The Drackett protein diets contain sixteen percent protein.

Results of this experiment should give a better understanding of what salt mixes to use under a given set of circumstances and may serve as a guide for comparing data between studies from different laboratories which use different salt mixes in rat diets.

h. Electroanalytical Applications to Biochemical Assay:

The determination of sub-microgram quantities of selenium in biological samples is becoming increasingly important as the role of selenium as an essential nutrient in trace quantities is being investigated. A polarographic procedure has been developed which will qualitatively detect as little as 0.01 microgram selenium in a tissue sample. The method shows promise of being a quantitative tool and of being made qualitatively much more sensitive.

Polarography also offers a convenient method to study biologically important interactions of substrates and metal ions. This has been applied in the case of chromium-insulin interaction with mitochondrial membrane groups.

Clinical application of coulometric methods of analyses has not been fully developed. This method offers the advantage of requiring no standard solutions, thereby reducing technician error. It has the added advantage of being sensitive and very precise. Therefore, methods have been investigated for coulometric titration of various clinically important substances including phosphate, ammonia, calcium and magnesium, and ferrous iron.

Polarographic Determination of Selenium in Biological Samples.

The electrode processes of the three pH dependent polarographic waves of Se (IV) have been elucidated by constant-potential coulometry. The first wave in acid medium is due to the process:

$$H_2SeO_3 + Hg + 4H^+ + 4e^- \longrightarrow HgSe + 3H_2O$$

The second wave in acid medium is due to further reduction of this electrolysis product: $HgSe + 2H^+ + 2e^- \longrightarrow H_2Se + Hg$.

The third wave in alkaline medium corresponds to a six electron reduction to selenide ion.

Advantage has been taken of the first two electrode processes to develop a sensitive hanging drop polarographic method for detection of selenium. An acid electrolyte at pH 2.8 is employed. The potential is set on the diffusion current plateau of the first wave and the electrolysis at the hanging drop electrode allowed to proceed for a predetermined time. The potential is then scanned cathodically causing reduction of the HgSe plated on the drop surface and giving rise to a cathodic peak corresponding to the second polarographic wave. This method is very selective for the detection of selenium as there is apparently no other substance giving rise to such a cathodic peak following electrolysis. It is also very sensitive. As little as 0.01 microgram of selenium in a tissue sample can be detected and even less in a non-biological sample. Unfortunately, the method is not yet sufficiently quantitative at very low selenium levels although the recovery of selenium in the digestion procedure appears to be quantitative. There is no apparent linear relationship between peak height or area and concentration at very low levels of selenium; however, the method shows promise of being quantitative at higher concentrations of selenium. This is presently being investigated. Other ramifications of the method may be able to extend the range of quantitation to low values. These will be investigated.

The method of sample preparation is important in eliminating loss of selenium. The presence of nitric acid in the digest eliminates loss. However, sulfuric acid must be absent. Nitric acid-perchloric acid digest mixture is used. It is important to heat very strongly when perchloric acid fumes are reached to oxidize traces of organic matter which give rise to interfering polarographic waves. Even so, enough organic matter remains to give an appreciable residual current, limiting the sensitivity of detection to 0.01 micrograms, a level about 10 times greater than the detection limit of a synthetic sample.

Polarographic Study of Mitochondrial-Chromium-Insulin Interactions.

This investigation has been completed. The mitochondrial polarographic wave was found to be an anodic wave due to the oxidation of the mercury electrode by sulfhydryl groups of the mitochondrial membrane:

$$RSH + Hg \longrightarrow RSHg + H^+ + e^-.$$

The same wave is obtained with several mitochondrial sources including rat liver and brain and dog liver mitochondria. These sulfhydryl groups form a complex with insulin as evidenced by the shift of the mitochondrial polarographic wave in the presence of insulin. The shift of the wave is enhanced by the addition of chromium (III). The proposed mechanism for the role of chromium on the interaction of insulin with membrane sulfhydryl groups involves a complex formation between insulin and chromium (III) which then further complexes with the mitochondrial membrane to increase permeability to the non-polar glucose molecule. Studies reported elsewhere elaborate on this proposal.

Polarographic Determination of Gold in Blood and Serum.

Iron and copper in the blood samples interfere with the polarographic determination of gold. Therefore, the gold must be separated prior to analysis. The samples is first dry ashed at 500° C. and then evaporated nearly to dryness with aqua regia. It is then transferred to a separatory funnel with 3 M hydrochloric acid and extracted with ether. The ether fraction is evaporated just to dryness and then transferred to a 10 ml volumetric flask with 2 M potassium hydroxide. This solution is immediately polarographed. If the solution is allowed to stand for a period of time, slow decomposition of the polarographically reducible auric hydroxide complex results.

Argentometric Titration of Orthophosphate.

The clinical determination of orthophosphate is an important one. A coulometric method would be desirable and an investigation was conducted to attempt to apply coulometric generation of silver ions to the titration of phosphate in serum samples. Synthetic samples of orthophosphate were titrated both volumetrically and coulometrically with silver ion and both amperometric and potentiometric end-point detection methods were investigated. Tenth molar sodium acetate in 80% ethanol was used as electrolyte. Using an amperometric end-point detection, 1.7×10^{-3} M orthophosphate could be titrated. The presence of 0.2% gelatin was necessary to prevent depolarization of the indicating electrode by the silver phosphate precipitate. An indicating potential of 0.00 V. vs S. C. E. was employed. Using a potentiometric end-point detection, a limit of 2×10^{-4} M orthophosphate could be titrated. Unfortunately, these levels of concentration are too high to be applicable to phosphate determination in biological samples. Other methods are being investigated involving precipitation of magnesium ammonium phosphate or ammonium phosphomolybdate and titration of the ammonia in these precipitates using the coulometric method to be described.

Direct Amperometric End-Point Detection for Coulometric Titration of Microgram Quantities of Ammonia.

A coulometric titration of as little as 14 micrograms of ammonia with electrogenerated hypobromite has been described in the literature. However, this laboratory has not been able to reproduce the indirect amperometric end-point detection method employed. Therefore a direct amperometric procedure has been developed which is applicable to as little as 1.4 microgram ammonia. Two platinum foil indicating electrodes are used with 150 mu impressed between them. Hypobromite is generated in a buffer medium of pH 8.5. The indicating current is recorded directly in this alkaline medium and plotted vs time of generation. An abrupt rise in current results at the end-point

and the inflection point is taken as the end-point. The electrode reaction is sufficiently rapid to allow the current to be plotted automatically on a recording polarograph. This method should be applicable to several clinically important nitrogenous sources in biological samples and several are to be investigated. Preliminary work has been done on the determination of urea following incubation with urease. Unfortunately the sample cannot be titrated directly because urease produces a large blank. However, a distillation of the formed ammonia prior to titration appears to give very good results.

Coulometric Titration of Calcium in the Presence of Magnesium.

Calcium content in serum is a frequent clinical determination. Therefore a coulometric method would be desirable. A volumetric titration of calcium in the presence of magnesium with ethylene glycol bis-(β-aminoethylether) tetroacetate (EGTA) has been reported. A potentiometric end-point was employed. A method for coulometrically generating EGTA from Hg-EGTA has been developed in this laboratory. It has been employed for the titration of down to 0.1 mg of calcium. This is sufficiently sensitive for calcium levels in serum. However, any magnesium in this system appears to titrate slightly, causing a decrease in the potentiometric end-point break and a shift of the inflection point away from the equivalence point. The amount of this distortion depends on the amount of magnesium present. However, if a sufficient excess of magnesium is added initially to the solution before preelectrolysis, the shape of the potentiometric curve will not be altered significantly upon the addition of magnesium equivalent to the calcium present in the sample. Thus, a 5 to 10 fold excess of magnesium is added to the solution before titration to "buffer" it. This method, unfortunately, is not applicable to levels of calcium and magnesium found in serum samples. An amperometric end-point detection system is therefore being investigated which shows promise of having no interference from magnesium.

Amperometric End-Point Detection in Coulometric Titration of Ferrous Iron With Ceric Ion.

In the usual coulometric titration of ferrous iron with ceric ion, a potentiometric end-point detection is employed. With small amounts of iron, however, this detection system is extremely sluggish. Therefore, work has been conducted in this laboratory to develop an amperometric detection of the end-point. A successful method has been developed in which the indicating electrode has a potential of +0.90 V. vs S. C. E. The end-point occurs at zero current. A break in the current-time curve occurs as the current changes from anodic to cathodic and therefore, it is neither essential to use a zeroed galvanometer nor to generate titrant close to the end-point. Also the indicating electrode process is sufficiently rapid to allow the current to be recorded automatically on a polarographic recorder. Down to 1 ug of ferrous ion has

been titrated but the limit of titration has not been fully investigated. The possible application of this technique to determination of ferrous iron in serum samples is being investigated.

Summary and Conclusions: The addition of tocopherol to rat liver homogenates enables the latter to maintain the oxidation of many substrates for an hour and a half. It does so without being converted to any active form other than being bound to the sulfhydryl groups of the homogenate.

In mitochondria, tocopherol prevents the decline of succinate oxidation by preventing the accumulation of oxaloacetate. It does so either by preventing the formation of oxaloacetate or removing it before it can enter the Krebs cycle.

Endotoxin is inactivated by liver fractions. The activity of these fractions can be stimulated by malate and ATP indicating oxidation of the lipid moiety of the endotoxin. Endotoxin is not destroyed by takadiastase, a polysaccharide destroying enzyme.

Countercurrent distribution systems have been developed to purify alanine and tyrosine transfer RNAs from yeast. Structural differences have been shown between the two RNA types. Studies of species specificity for amino acids showed some striking differences between species, although many common factors relating to 14 amino acids studied were observed.

The factor found in liver of rats which inhibits the activity of tyrosine-α-ketoglutarate transaminase has been partially separated from the enzyme by chromatographic adsorption on DEAE-cellulose. Kinetic studies have shown that the inhibitory factor combines with enzyme and substrate to form an enzyme-inhibitor-substrate complex.

Initial studies designed to produce a selenium-free diet has demonstrated a favorable comparison for three commercial salt mix additives. A synthetic diet (WRAIR) which is sulfate-free does not compare favorably with growth rate of commercial diets containing sulfate. Copper toxicity in this diet appears important.

Electroanalytical methods have been successfully employed for demonstration of interaction between insulin, chromium, and the mitochondrial membrane. A hanging-drop electrode has allowed determination of submicrogram amounts of selenium from tissue digests. Coulometric generation of titrants appears feasible for determination of calcium, magnesium, ferrous iron, and ammonia. The lower limits of detection restrict the applications of orthophosphate determination from biological specimens.

Publications:

1. Seward, C. R. and Corwin, L. M.

In Vitro Studies on The Fate of α-Tocopherol in Rat Liver Homogenates, Arch. Biochem & Biophys 101, 71 (1963)

2. Corwin, L. M. and Schwarz, K.

Relation of Tocopherol to Enzyme Sulfhydryl Sites, Arch. Biochem & Biophys 100, 385 (1963).

3. Seward, C. R. and Corwin, L. M.

Studies On The Fate of α-Tocopherol in Rat Liver Homogenates, Fed Proc. 22, 319 (1963).

4. Doctor, B. P. and Connelly, Carol M.

Nucleotide Sequence Variations in Alanine and Tyrosine-RNA from Yeast, Fed Proc 22, 230 (1963).

5. McElroy, O. E., Tetreault, C., Knoblock, E. C.

Purification of Tyrosine-α-ketoglutarate Transaminase (In preparation)

6. Christian, G. D., and Purdy, W. C.

The Polarography of Copper (II) in Orthophosphate Medium. J. Electroanal. Chem., 5, 85 (1962).

7. Christian, G. D., Knoblock, E. C., and Purdy, W. C.

Polarographic Study of Mitochondrial Suspension, Biochim Biophys Acta, 66 (1963), 415-419.

8. Christian, G. D., Knoblock, E. C., Purdy, W. C. and Mertz, W.

A Polarographic Study of Chromium-Insulin-Mitochondrial Interaction. Biochim Biophys. Acta, (In Press).

9. Christian, G. D., Knoblock, E. C., and Purdy, W. C.

A Direct Argentometric Titration of Orthophosphate; Application to Coulometric Titrations. 772nd Meet. Wash. Chem. Soc., May 3, 1963, College Park, Md.

10. Christian, G. D., Knoblock, E. C., and Purdy, W. C.

Coulometric Titration of Ammonia With Hypobromite Using Direct Amperometric End-Point Detection, 722nd Meet. Wash. Chem. Soc., May 3, 1963, College Park, Md.

11. Christian, G. D., Knoblock, E. C., and Purdy, W. C.

Electroanalytical Determination of Selenium in Biological Materials, 722nd Meet. Wash. Chem. Soc., May 3, 1963, College Park, Md.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 B 813, Army Medical Basic Research in Life Sciences

Task 05, Immunology (Immunohematology)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Immunochemistry
Division of Communicable Diseases and Immunology

Period Covered by Report: 1 July 1962 - 30 June 1963

Principal Investigators: Mary B. Gibbs, M.S.
Frank R. Camp, Maj, MSC

Assistants: Louis Aguilu, Sfc.
Joseph Dreyfus, Sp4

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 B 813 Title: Army Medical Basic Research In Life Sciences

Task 05 Title: Immunology (Immunohematology)

Reporting Installation: Walter Reed Army Institute of Research
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A method has been developed to utilize the model B Coulter counter to provide free cell data of hemagglutination applicable in the log probit assay method of Wilkie and Becker. Studies on the use of instrument total particle counts of agglutinated samples have shown that such data can not be employed in quantitative hemagglutination methods based on a linear relation between agglutination response and serum concentration. Moreover, total particles are an unreliable index of hemagglutination reactions since the total particle to free cell relationship differs with the mode of aggregation of hemagglutinating systems. Thermodynamic studies of an immune anti-A serum with cells of various A subgroups has demonstrated the heterogeneity of binding affinities of the isoagglutinin and qualitative differences among the A antigens of A_1, A_2, A_1B and A_2B cells. Experimental data in support of a proposed hypothesis defining the mathematical relation of the antigen-antibody reaction and equilibrium constant of hemagglutination revealed that a more sophisticated hypothesis must be evoked to conform with experimental findings. The conditions required for reproducibility and maximal reactivity of saline agglutinating anti-C, anti-E and incomplete anti-C sera were established and the log probit assays of these sera validated. Quantitative hemagglutination studies of cells from AB individuals has demonstrated a reduction in A and B activities of these cells from that of the antigens on group A and group B reference cells. Differences in slopes and positions of log probit assay curves of the antigens of AB cells has permitted definition of four subtypes of A_1B and five subtypes of A_2B cells.

BODY OF REPORT

Project 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task 05 Title: Immunology (Immunohematology)

Description: The purpose of this task is to study the phenomena involved in the agglutination reaction, normal and abnormal, of the blood group systems.

Progress:

1. Application of electronic cell counting technics to quantitative hemagglutination studies.

Investigations on the application of the Coulter Model B counter to quantitative hemagglutination studies of blood group systems were continued. Because of the overlap in size distribution of singlet and doublet cells in agglutinated cell populations, it was necessary to locate, empirically, an upper threshold on the instrument which would eliminate counts of aggregates of two or more cells from the count of single cells. This was accomplished by relating instrument counts taken at various threshold settings to the mean of several hemocytometer free-cell counts. To eliminate hemocytometry from the method, the following technique was developed. The threshold for a given test cell appearing to be related to the size distribution of the cell, empirical thresholds and size distribution curves of approximately 200 A,B and AB cells reacted with anti-A and anti-B sera were determined. The results of this study established that the upper threshold for free cell counting can be determined from the size distribution curve of the unagglutinated cell population. The method has been tested and found valid with numerous avid and non-avid anti-A and anti-B sera reacted with A_1,A_2, A_x, B, A_1B and A_2B cells.

2. Demonstration of the limited applicability of total-particle counts to quantitative hemagglutination studies.

An extension of an earlier investigation on the use of total particle measurements, i.e., counts of agglutinates and free cells, in the log probit assay of Wilkie and Becker was prompted by recent publications of quantitative hemagglutination methods based on instrument total-particle counts. In these publications the contention has been made that free-cell counts are not as reproducible as total-particle counts. However, data have been obtained demonstrating that the contrary is, in fact, true. Free-cell counts are remarkably reproducible whereas there is a distinct non-reproducibility of total particles when agglutination tests are performed under varying experimental conditions. Additional information has been obtained on the limited applicability of total particle data in quantitative hemagglutination methods based on a linear relation between agglutination response and serum concentration. Probit log and logit log assay curves derived from such data are not linear over a sufficiently wide range of agglutination to validate estimation of an Hd. It was also established with anti-A, anti-B, anti-M and anti-D sera, that total particles are an unreliable index of hemagglutinating systems since they reflect the mode of aggregation rather than the numbers of cells actually involved in agglutinate formation.

3. Thermodynamic Studies on the A-anti-A system.

Given concentrations of A_1, A_2, A_1B and A_2B cells were reacted to equilibrium with varying concentrations of a high-titeres anti-A serum and the antibody remaining free in the supernatants measured. This information was used to construct the thermodynamic plots described by Scatchard and Klotz. The results of this study confirmed the earlier findings that the A isoagglutinin is heterogenous in respect to its binding affinities with A sites. The Scatchard curves consist of two slopes; an initial steep slope indicating the presence of antibodies with strong binding affinities and, as the antibody concentration increases, the slope flattens indicating that iso-antibodies with weaker binding affinities are reacting with antigenic sites. These findings are in good agreement with those of Dr. Hugh-Jones et al with I^{131}-labelled anti-C sera and those of the Wurmser's with immune anti-A sera and A_1 cells. The earlier findings that cells of the different A subgroups give thermodynamic curves of differing slopes was also confirmed. These observations lend support to our previous contention of qualitative differences among A subgroup antigens based on changes in slope of log probit assay curves. It was also shown that A_1 and A_2 cells exhibiting equal agglutinating strengths remove comparable amounts of antibody activity upon absorption and that A_1 cells of unequal strengths remove differing amounts of antibody activity in accordance with their agglutinating strengths. The fact that the Scatchard thermodynamic curves for the latter cells is parallel but shifted in position provides evidence that these cells possess differing numbers of antigenic sites.

4. Attempts at a mathematical theory describing the hemagglutination reaction.

In collaboration with Dr. C. Patlak of the Institute of Mental Health, NIH, an investigation was initiated on the derivation of a mathematical expression for the interaction of hemagglutinins with red cell antigens. It is hoped that such an expression will lead to a definition of the relationship of the equilibrium constant of the agglutinating system to the slope of the log probit assay curve. Based on assumptions that all cells are identical in numbers of independently acting combining sites, and that a bond between two cells is formed by one antibody moelcule, an hypothesis was proposed to define the equilibrium status of agglutinated cells. In testing this hypothesis, data was obtained on the maximum amount of antibody capable of being bound to a cell, and the ratios of doublet, triplet and quadruplet to singlet cells formed at various levels of agglutination. The results of these experiments did not support the original hypothesis, therefore, a new hypothesis must be derived in accordance with experimental findings.

5. Studies on the interaction of Rh-Hr genes as evidenced by the phenotypic expression of their red cell antigens.

To provide confirmatory evidence in support of observations made with Rh-position cells of a Japanese family that the presence of a postulated Cde gene in Rh (D)-positive individuals inhibits expression of the Rh (D) antigen in a predictable manner discernible by the sensitive log probit assay method, a collaborative project was initiated with Dr Richard Rosenfield of

the Mt. Sinai Hospital, New York City. Samples of blood have been obtained from the parents and thirteen offspring of a Rh(D)-positive family with a weak D^u antigen. These valuable blood samples will be subjected to quantitative studies of the interaction of the rh'(C),rh"(E), hr"(c) and Rh_o(D) antigens, as expressed by inhibition or enhancement of companion antigens. It was, therefore, necessary to establish the conditions required for reproducibility and maximal reactivity of the saline agglutinating anti-C, anti-E and the incomplete anti-c sera to be employed. It was found that the original method of assay, used by Silber et al with anti-D sera, had to be modified as follows: (1) unbuffered saline was found essential for adequate agglutinate formation with anti-E serum; phosphate buffered saline being found inhibitory, (2) papainized c-positive cells were found to give a more reproducible assay than trypsinized cells, and (3) the time required for equilibration of agglutination with all Rh-Hr sera tested was decreased when constant agitation was used without the initial 45 minute water bath incubation period.

6. Quantitative studies of the hemagglutination of erythrocytes of the AB blood group.

A quantitative hemagglutination study was made of the A and B antigens of 86 AB erythrocytes. Group AB cells of equal freshness were compared with standard A,B,A_1B and A_2B cells using immune anti-A and anti-B sera. AB cells exhibited lesser A and B activities than the group A and Group B reference cells and variations in slopes and positions of the A-anti-A and B-anti-B log probit assay curves of A_1B and A_2B cells gave indication of subtypes within each of these subgroups of AB cells. By means of these differences, it has been possible to categorize A_1B cells into four subtypes and A_2B cells into five subtypes. Since previous work had shown that the activities of cells from homozygous BB and AA individuals do not differ from those of cells from heterozygous BO and AO individuals, the reduction in activity observed with the A and B components of AB cells can be attributed to an interaction of these blood group genes at the ABO locus resulting in a suppression of A activity by the companion B antigen and vice versa.

Summary and Conclusions:

1. Studies on the use of the model B Coulter counter for enumeration of free cells in agglutinated cell populations has been completed. A method has been developed which gives log probit assay curves by instrumentation identical in slope and position to those obtained by hemocytometry. The method has been found valid with numerous anti-A and anti-B sera reacted with A_1,A_2, A_x, B,A_1B and A_2B cells.

2. Studies were completed on the use of total particle (agglutinates and free cells) measurements of agglutination in evaluating hemagglutinating systems. Log probit assay curves obtained from total particle data are not sufficiently linear over the region of partial agglutination to justify interpolation of an HD_{50} and the relationship of total particles to free cells

differs with the avidity of the antiserum, the strength of the antigen on the red cell, and the type of antiserum employed. These findings indicate that total particles are an unreliable index of hemagglutination reactions.

3. Thermodynamic studies of an immune anti-A serum with cells of various A subgroups has demonstrated the heterogeniety in binding affinities of the A isohemagglutinin. Differences in slope of the thermodynamic plots gave indication of qualitative differences among the antigens on A_1, A_2, A_1B and A_2B cells. The reliability of the method was shown by the finding that A cells of equal agglutinating strengths produce identical thermodynamic curves while cells of differing strengths produce parallel curves shifted in position.

4. Experimental data on the maximum numbers of A_1 sites on red cells and the equilibrium distribution of singlet, doublet, triplet and quadruplet cells in agglutination has not supported a proposed hypothesis defining the mathematical relation of interaction of antibody and red cell antigen to the equilibrium constant of hemagglutination. A more sophisticated hypothesis must be evoked to conform with experimental evidence.

5. The conditions required for reproducibility and maximal reactivity of saline agglutinating anti-C, anti-E and incomplete anti-c sera were established and the log probit assay of these sera validated. A large family study was initiated on the interaction of the C,c and E antigens on the expression of the D antigens employing these antisera.

6. Quantitative hemagglutination studies of 80 cells from AB individuals has made possible the categorizing of A_1B cells into four subgroups, designated infratypes, and A_2B cells into five infratypes. An interaction of the A and B genes has been suggested for the variations in A and B activities of the AB infratypes, as demonstrated by differences in slopes and HD_{50}'s of their log probit assay curves.

Publications:

1. Gibbs, M. B. and Becker, E. L. A Change in Character of the Anti-A Iso-Antibody Following Immunization with Blood Group A Substance. Paper Presented at the IX Congress of the International Society of Blood Transfusion, Mexico City, Sept. 1962.

2. Camp, Frank R. Jr., Gibbs, Mary B. and Aguilu, Luis A. Evidence for Gene Interaction at the ABO Locus as Demonstrated by Quantitative Studies of the Erythrocytes of the AB Blood Group. Paper Presented at the IX Congress of the International Society of Blood Transfusion, Mexico City, Sept. 1962.

3. Camp, Frank R. Jr., Gibbs, Mary B. and Aguilu, Luis A. Demonstration of the Interaction of the A and B Genes by the Quantitative Hemagglutination Method. Paper Presented at the 15th Annual Meeting of the American Association of Blood Banks, Memphis, Tenn., Nov. 1962.

4. Gibbs, M. B. and Becker, E. L. Quantitation of Hemagglutination by Enumeration of Free Cells. Nature 198: 90, 1963.

5. Gibbs, M. B. Evaluation of an Electronic Counter for Quantitative Hemagglutination Studies. Fed. Proc. 22: 673, 1963.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 B 813, Army Medical Basic Research in Life Sciences

Task 05, Immunology (Mechanism, Pattern, and Specificity of the Immune Response)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Serology
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

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ABSTRACT

Project 3A 0 12501 B 813	Title: Army Medical Basic Research in Life Sciences
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Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
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Allergic encephalomyelitis (guinea pigs) and runt disease (albino rat) were studied as possible laboratory models of autoimmune response. Allergic encephalomyelitis was influenced by the organ, and species of animal providing tissue for antigen. Runt disease produced a marked reduction of C' levels in affected animals.

Studies of antibody-complement bactericidal reactions on gram negative organisms revealed differences for even closely related organisms (P. ballerup, S. typhimurium, and S. paratyphi C 32V). For example, different incubation times were required for bactericidal activity.

Serum protein fractions from normal and S. dysenteriae immunized rabbits both contained bactericidal activity in Cohn's Fraction III-0.

Effect of quinacrine and chloroquine on in vitro antigen-antibody systems, and complement (in vitro and in vivo) was studied. In vitro incubation of quinacrine with whole complement selectively inactivates the second and fourth components. Administration of anti-malarials to guinea pigs produced no changes in C' titers determined by R-reagent assay.

Normal antibody titers against sheep erythrocytes, S. typhosa "O" and extracts of normal and irradiated rabbit testes first declined, then rose to higher than normal levels in whole body X-irradiated rabbits. Antigens from rat and rabbit testes prepared at various intervals after irradiation showed both chemical and serological differences.

BODY OF REPORT

Project 3A 0 12501 B 813 — Title: Army Medical Basic Research in Life Sciences

Task 05 — Title: Immunology (Mechanisms, Pattern and Specificity of the Immune Response)

Description: This task is concerned with aspects of the mechanism and patterns of the immune response. Immunological reactions directed against antigens foreign to the host, represented by bacteria and viruses, and antibodies against constituents of normal mammalian tissue were studied. The effect of anti-malarial drugs on in vitro antigen-antibody systems and upon complement (in vitro and in vivo) were investigated. Other studies included the mechanism of the neutralization of coliphage of the T series by "normal" and immune sera, the influence of cultural conditions on the susceptibility of some gram-negative bacteria to the action of the antibody-complement system, and the ability of "autoimmune" antibodies to react with substances isolated from both homologous and heterologous tissue sources. Modification of the immune response was studied in x-irradiation of the animals. Immunological and chemical differences between antigens obtained from normal and irradiated tissues were investigated.

Progress:

1. Studies on allergic encephalomyelitis in the guinea pig

This study is concerned with the mechanisms of immune response, with particular reference to the production of autoantibodies and their possible role in autoimmune disease. The present investigations were initiated in an effort to develop a laboratory model that could be used to elicit basic information concerning factors that mediate autoimmune responses under experimental conditions, and thereby provide some insight on the predisposing causes of autoimmune disease in humans. One of the models investigated thus far have been allergic encephalomyelitis in the guinea pig.

It has been known for some time that guinea pigs receiving a single intracutaneous injection of a rabbit brain-Freund adjuvant homogenate often develop encephalomyelitis accompanied by ascending paralysis. The present report deals with studies to determine whether the paralytic response in the guinea pig is influenced by the organ and/or species of donor animal used in preparing the homogenate, whether the production of tissue antibodies can be correlated with the appearance of paralysis, and whether the immune response affects circulating complement (C') levels.

Homogenates containing 20% rabbit brain, rabbit liver, or fish brain in complete Freund adjuvant and tissue culture medium #199 were prepared in a Teflon-pestle tissue grinder. These preparations, including controls wherein tissue or adjuvant were omitted, were inoculated intracutaneously into the shoulder area of male guinea pigs and albino rats. Each animal was examined daily for the appearance of a skin lesion at the site of injection and for evidence of paralysis. In addition, the animals were weighed at four-day intervals, were bled at weekly intervals, and the sera assayed for C' levels and complement-fixing tissue antibodies.

Six of the eight guinea pigs inoculated with rabbit brain-adjuvant homogenate developed ascending paralysis within 12-15 days, and in each case death occurred 18-24 hours following the onset of paralysis. In spite of the severity of these reactions, C' levels were not altered and no weight loss was observed. Nevertheless, all animals receiving this homogenate, even those without paralysis, developed tissue antibodies that reacted in complement fixation tests with guinea pig brain antigen.

None of the guinea pigs receiving rabbit brain homogenate without Freund adjuvant developed paralysis, nor did they develop skin lesions at the site of injection. Moreover, C' levels were unaltered and anti-brain antibodies were not detected in complement fixation tests. Animals receiving rabbit liver-Freund adjuvant homogenate likewise did not experience paralysis and tissue antibodies (guinea pig anti-brain) were not detectable. C' levels again corresponded closely with those of the normal controls but skin lesions did develop at the site of injection. The only observed response of guinea pigs receiving non-tissue homogenate (Freund adjuvant alone) was the development of skin lesions at the injection site.

Guinea pigs inoculated with a fish brain-adjuvant homogenate did not develop paralysis. In fact, the only observed response was the appearance of skin lesions at the site of injection 7-8 days post-inoculation. Control animals receiving fish brain alone exhibited no detectable immunological or clinical response.

In contrast to the response observed with guinea pigs, none of the rats receiving the rabbit brain-Freund adjuvant homogenate developed paralysis although skin lesions and anti-brain antibodies did appear. However, as before, C' levels were unaltered and there was no demonstrable weight loss. The rats were completely refractory to a homogenate containing rabbit brain alone (no adjuvant). On the other hand, rats receiving rabbit liver-adjuvant homogenate developed skin lesions and anti-brain antibodies, but showed no other response to this inoculum. Finally, the only observed reactions of rats inoculated with Freund adjuvant alone were the development of characteristic skin lesions at the site of injection 7-8 days after inoculation.

2. Tissue antibody and complement levels in runt disease

Runt disease, a syndrome induced by injection of immunologically-competent lymphoid cells from a homologous donor into an immunologically-tolerant host, generally is regarded as the result of immunological reactions of the donor cells that have proliferated in the lymphoid tissues of the host. It was considered likely that the grafted donor cells would produce antibodies against host tissues and the present report deals with studies to determine whether tissue antibody levels were abnormally elevated during the course of the disease. Therefore, this syndrome was also investigated for use as a possible laboratory model. In addition, the effect of the disease on complement (C') levels also was investigated.

Newborn albino rats were inoculated intraperitoneally with approximately 10^6 lymphoid cells obtained from the spleen of an adult male Canadian hooded rat. The inoculum was prepared by suspending the spleen in Tissue Culture Medium #199, homogenizing in a Teflon-pestle tissue grinder for 30 seconds and filtering the homogenate through two layers of gauze before use. Each litter of rats arbitrarily was reduced to ten animals of which eight received 0.1 ml. of the cell suspension; the two control litter mates each received 0.1 ml. of Medium #199. Prior to exsanguination (25-35 days post partum), inoculated animals of a given litter were segregated according to size. Those significantly smaller than the control litter mates were designated "small runts" and those that approached the size of the controls were categorized "hemi-runts". Sera from a given group were pooled and stored at -35°C until used in tests. Complement fixation tests employing antigens derived from organs and tissues of various animal species were used to assay tissue antibody levels in the serum pools and C' assays were performed at the time that the specimens were thawed.

All animals of a given litter did not experience the disease with the same degree of severity. Nevertheless, one or more of the classic symptoms of the runt disease syndrome usually was evident. Most striking of these was the relatively small size of the runted animals. In addition, some developed alopecia around the eyes and nose, and the more severely affected walked with the mincing gait characteristic of the disorder. Two periods of crisis were observed, the first at about the tenth post-inoculation day, the second at about day 20. Although some deaths occurred during each crisis, the majority of animals surviving the second crisis lived for at least two weeks, and some appeared to recover completely, eventually attaining the weight of the control litter mates.

Neither liver extract antigens of albino rat, rabbit, or chicken origin, nor albino rat spleen antigen revealed a significant increase of complement-fixing tissue antibody levels in the runted animals. In addition, tests with calf thymus nucleoprotein antigen were uniformly non-reactive. Tests with liver antigen from runted animals likewise showed no increase of tissue antibody, and intradermal inoculations with normal rat

liver antigen failed to elicity detectable immediate or delayed reactions. Sera from one group of animals with acute runt disease, on the other hand, reacted with antigen prepared from newborn albino rat skin.

The level of circulating C' was markedly decreased in animals suffering from runt disease, and the degree of C' reduction appeared to be directly related to the severity of the disorder. Particularly significant was the 4- to 5-fold decrease of C' titer that was observed in the small runt groups.

3. Bactericidal reactions of the antibody-complement system against gram negative organisms

Bactericidal reactions of the antibody-complement system against gram negative organisms have been performed using the spectrophotometric growth assay technic developed by Muschel and Treffers for the titration of bactericidal antibody. One of the limitations of this technic is the requirement of a relatively large inoculum of the test organism (about 10^7 organisms/ml.) which contributes to the decrease in sensitivity of the technic. Therefore, methods with greater sensitivity and using a smaller inoculum of test organism was required to study gram negative organisms which have resisted the action of antibody and complement, for example, P. ballerup, S. typhimurium, and S. paratyphi C (32V). The plate count method was evaluated and methods were developed for using the Coulter counter to study the more complex systems involved with resistant organisms.

a. Bactericidal action of normal and immune rabbit sera against P. ballerup and S. paratyphi C (32V) in presence of active absorbed guinea pig complement - P. ballerup and S. paratyphi C (32V), which resisted the action of antibody and complement were streaked on meat extract agar (MEA) plates and incubated at 37°C for 16 hours. The organisms were washed off the plates with normal saline, and the suspension was adjusted to an optical density (O.D.) of 0.08 on the Coleman Model 14 Spectrophotometer. Ten-fold dilutions of these suspensions were made in saline, and the following final dilutions were made for each organism.

P. ballerup: 1/3 dilution of 10^{-4} dilution of original suspension (about 1140 organisms/ml.)

32V: 2/3 dilution of 10^{-4} dilution of original suspension (about 950 organisms/ml.)

The following protocol was followed for each organism:

Tube 1	Normal rabbit serum (½ dilution) Absorbed C' + organisms (1:1)	1.0 ml.
Tube 2	Immune rabbit serum (1/100 dilution in normal rabbit serum) Absorbed C' + organisms (1:1)	1.0 ml. 1.0 ml.
Tube 3	Absorbed C' (active) + organisms(1:1) Saline	1.0 ml. 1.0 ml.
Tube 4	Absorbed C' (inactive) + organisms Saline	1.0 ml. 1.0 ml.
Tube 5	Organisms + saline (1:1) Saline	1.0 ml. 1.0 ml.

The tubes were placed on a 37°C water bath and at hourly interval 0.1 ml. samples were taken from each tube and streaked on MEA plates using sterile glass beads for spreading. The results are shown in Table I.

Using the plate count method it was possible to demonstrate that both organisms were sensitive to the action of antibody and complement. However, the reaction did not follow that of the typhoid organisms. As indicated below, in the typhoid bactericidal action, as much as 50% of the test inoculum was killed in the first 1/2 hour. In the case of P. ballerup and 32V, the bactericidal action was not observed until after the third hour of incubation during which time there was multiplication of the test organism. There is no explanation for the lack of a bactericidal effect of the immune serum against 32V. However, a prozone effect may be a reason. Saline was definitely toxic to both organisms, indicated by the decreased number of survivals with the lapse of time in the saline control tubes. At the concentration of organisms used in the test, the amount of antibody in the normal serum appeared to be near optimum because additional amount or an excess represented in the immune serum tube did not increase the bactericidal potency of the system.

b. Bactericidal action of normal and immune rabbit sera against S. typhi strain Ty2 in presence of endogenous complement - A S. typhi strain Ty2 was grown on MEA plate at 37°C for 16 hours. The organism was washed off the plate with saline, and the suspension was adjusted to an O.D. of 0.085. The organism was diluted in saline so that an amount containing about 6000 organisms/ml. was added to each test. Complement was present in the undiluted

normal rabbit serum used. Immune serum was diluted 1/100 in normal rabbit serum so both normal and immune sera tubes contained essentially equal amounts of complement. The mixtures were incubated at 37°C and 0.1 ml. samples were removed and spread on MEA plates with the aid of sterile glass beads. The results obtained are shown in Table II.

In contrast to the reaction of serum against P. ballerup and 32V, as shown in Table I, in which bactericidal activity was not observed until three hours after incubation, the activity against S. typhi strain Ty2 appeared to be immediate because as much as 50% of the initial inoculum was killed in the first 1/2 hour. It is interesting to note that the presence of antibody in excess (represented by the immune serum tube) does not increase the bactericidal potency of a serum as evidenced by the almost identical response in the normal and immune sera tubes. This probably points to the fact that the damage to the bacterial cells is effected early in the reaction with an amount of antibody which is at an optimum.

c. Bactericidal action normal guinea pig serum against S. typhimurium The other "resistant" bacterium, S. typhimurium, was tested against active normal guinea pig serum only because rabbit antiserum against this organism was not available. S. typhimurium was prepared in the similar manner as described earlier for other organisms tested. The final filution of S. typhimurium in each test numbered about 750 organisms/ml. One ml. of 1/2 dilution of sterile guinea pig complement was mixed with 1 ml. of the diluted test organism and incubated at 37°C. The results obtained are shown in Table III.

It was noteworthy that guinea pig serum in presence of complement appears to impose a bacteristatic effect on S. typhimurium. The serum appears to be incapable of killing the test organism, but it prevents it from multiplying during the time tested. However, it must be noted, also, that in the first three hours, some multiplication is observed. After the third hour, the number of bacteria remains essentially the same as that of the initial inoculum. This effect may be merely a reflection of lack of an optimum concentration of antibody in the normal guinea pig serum.

d. Investigation of the bactericidal reaction of P. ballerup - Since learning that P. ballerup is susceptible to the action of antibody and complement after an incubation period of 2 to 3 hours, it seemed pertinent to find out what the roles of the antibody and complement were during this period. The organism was grown at 37°C for 16 hours, A known concentration of P. ballerup was then sensitized with heat inactivated normal and immune rabbit sera respectively which were diluted 1/20 at 37°C for 1 hour. The suspension was then centrifuged and the supernatant sera discarded and replaced with an equal amount of heat-inactivated guinea pig serum absorbed

with the homologous antigen (1/3 dilution) as growth medium. After thorough mixing, 1 ml. each of these suspensions was distributed into 4 different tubes and incubated at 37°C. Tubes 1 were taken out after 1 hour incubation, tubes 2 after 2 hours incubation, tubes 3 after 3 hours incubation and tubes 4 after 4 hours incubation. Tubes were kept in the refrigerator until the last of the tubes had finished incubating. Simultaneously, a constant amount of active absorbed guinea pig complement was added to each tube and placed in the 37°C water bath. Samples were removed from each tube at certain time intervals. The time intervals and the results obtained are shown in Table IV.

The results indicate that complement is not required during the first three hours of incubation, since the tube incubated for 1 hour prior to the addition of complement took 2 additional hours even in presence of complement before bactericidal effect was observed. The tube incubated for 2 hours prior to the addition of complement took 1 additional hour in presence of complement before a bactericidal effect was observed. However, the tubes which were incubated for 3 and 4 hours prior to the addition of complement showed bactericidal effect in the first 1/2 hour after complement was added, demonstrating the immediate bactericidal nature of the reaction. An interesting observation in this experiment was in control tubes sensitized with normal rabbit serum, The dilution of normal rabbit serum used to sensitize P. ballerup was 1/20, which was thought at the time to be adequate to dilute out the normal antibody. However, the results indicate that there was still sufficient amount of antibody present to render the system bacteristatic but not enough to render it bactericidal as shown by the immune serum.

When it was observed that complement was not required during the first few hours of incubation, it was necessary to find out if antibody was necessary or whether a mere multiplication of the test organism was sufficient. P. ballerup was grown for different lengths of time in presence of 1/10 dilution of heat-inactivated absorbed guinea pig sera as nutrient. The samples represent 0, 1, 2, 4, and 6 hours incubation at 37°C. Simultaneously, equal amounts of an optimum mixture of antibody and complement was added to each tube and 0.1 ml. samples were removed at different time intervals, spread on MEA plates and the colonies counted. The results are shown in Table V.

It is quite evident from the results obtained that antibody must be present during the first 3 hours of incubation before complement becomes effective, since P. ballerup grown in nutrient serum lacking antibody for 4 to 6 hours requires additional three hours in presence of antibody and complement before bactericidal effect can be observed. The reason for the requirement of antibody during the first 3 hours of incubation of P.ballerup before complement can act can not be explained at the present time. Whether the possibility of the antibody directing the organism to expose bactericidal antigens not previously detectable must be explored. Further studies are necessary to answer various questions which may lead to an answer in solving the problem of the bactericidal reaction of serum against gram negative organisms.

Table I. Colony counts of P. ballerup and 32V tested against normal and immune sera in presence of complement and controls.

	P. ballerup					32V				
Time (hrs)	Normal	Immune	C'	C'Δ*	Saline	Normal	Immune	C'	C'Δ*	Saline
0	88	117	99	101	96	85	81	51	68	73
1	139	118	138	136	117	51	81	69	76	67
2	304	278	224	230	105	99	85	101	95	65
3	172	260	213	336	104	130	107	**	96	48
4	10	62	45	>400	96	20	106	99	124	22
5	0	5	30	>400	75	10	138	21	129	0
6	0	2	14	>400	41	7	155	5	203	0
7	0	0	12	>400	17	2	144	5	199	0

*Δ = heated at 56°C for 1 hour
** = contaminated plate

Table II. Colony counts of S. typhi strain Ty2 tested against normal and immune rabbit sera in presence of endogenous complement.

Time (hrs)	Normal	Immune	Normal Δ	Immune Δ	Saline
0	554	589	550	610	553
1/2	308	335	558	634	585
1	191	181	622	639	631
2	24	26	>700	>700	529
4	0	0	>700	>700	520
6	0	0	>700	>700	484

Table III. Colony counts of S. typhimurium tested against normal guinea pig serum in presence of endogenous complement.

Time (hrs)	Normal	Normal Δ	Saline
0	69	79	79
½	75	79	73
1	77	91	76
1½	104	108	82
2	109	145	68
2½	137	154	70
3	89	174	73
4	83	468	63
5	85	> 500	56
6	44	>500	70
7	73	> 500	50

Δ = heated at 56°C for 1 hour.

Table IV. Colony counts of P. ballerup subjected to complement after incubation with antibody for various lengths of time.

Time (hrs)	N-1	I-1	N-2	I-2	N-3	I-3	N-4	I-4
0	115	71	102	91	183	161	241	211
½	135	146	152	190	152	80	275	83
1	168	157	206	201	171	72	285	35
2	293	327	161	137	101	29	213	4
3	174	146	103	108	101	25	138	7
4	124	75	61	24	70	3	107	0
5	96	30	89	10	51	7	116	0
6	94	8	81	7	60	3	94	0
8	117	1	96	2	76	0	101	0

N = sensitized with normal serum I = sensitized with immune serum
Numbers after N and I indicate the length of time (hrs) the sensitized P. ballerup was incubated at 37°C before complement was added.

Table V. Colony counts of P. ballerup subjected to antibody and complement after various growth periods.

Time (hrs)	A-0	I-0	A-1	I-1	A-2	I-2	A-4	I-4	A-6	I-6
0	4	4	7	7	**	**	33	33	46	46
1	4	4	9	10	**	**	51	46	59	77
2	6	6	19	12	**	**	104	103	105	107
3	10	8	6	16	**	**	108	110	141	150
4	2	7	1	17	**	**	30	115	39	218
5	1	7	0	25	**	**	3	159	3	239
6	0	6	0	32	**	**	0	135	0	215
7	0	5	0	36	**	**	0	140	0	308

**= Sample lost
A = active absorbed guinea pig C' + antibody
I = absorbed guinea pig C' heated at 56°C for 1 hour + antibody
Numbers after A and I represent hours of incubation prior to addition of antibody + C'

e. Use of the Coulter Counter for measuring bacteria. - Having learned that the turbidimetric method of measuring bactericidal reaction is not the most sensitive method available, and the plate count method, although sensitive enough, lacked accuracy and was time-consuming, a need was felt for a sensitive but rapid and accurate method of determining the number of bacterial survivals. Since a number of investigators had already achieved success in counting bacterial particles with the Coulter Counter, investigations were initiated to determine if methods employing this instrument would be advantageous to study of the bactericidal action. Preliminary results showed that an orifice of 30, the threshold setting of 15, the aperture current at 0.707 and the amplification at 1/4 gave satisfactory results and the instrument was used to establish the growth curve of one of the test organisms, S. typhi strain 0901. Strain 0901 was placed on an MEA plate and incubated at 37°C overnight, following the organism was washed off the plate with broth, and an appropriate dilution was made so that the initial count would be about 2000. The suspension was then placed in a 37°C water bath and at a definite time interval an aliquot of the suspension was removed and counted. The latent period of growth at which time no multiplication is observed lasted for 2 hours. It is generally accepted axiom that bactericidal action of serum components against gram negative organisms requires actively growing cultures for bactericidal effect to take place. In other words, the test organism should be in the log phase of the growth curve before the bactericidal effect could be instituted. In the case of the typhoid organisms as much as 50% of the bactericidal effect is observed in the first 1/2 hour of the reaction. This means that, according to the growth curve obtained on the Coulter Counter, the bactericidal effect on the typhoid organisms is completed during the latent phase, since in 2 hours, almost no survivors are detected. From the curve obtained the generation time was calculated. For strain 0901, it was 36 minutes. The identical value was obtained in another experiment when the starting number of organisms was about 13,000, so the accuracy of the counts was confirmed.

4. Comparison of the bactericidal activity against Shigella dysenteriae in normal and immune serum protein fractions

This study was conducted to further characterize the similarities or differences between normal and immune antibodies against Shigella dysenteriae. Rabbits were prebled, then immunized with a single intravenous injection of killed Sh. dysenteriae. Each rabbit was bled 6 to 8 hours after the injection and then on the 1st, 2nd, 3rd, 7th, 14th, 21st, and 28th days after injection. Serums were fractionated by the procedure in Method 10 of Cohn et al and the precipitates were dissolved in magnesium-saline to the original serum volume. Bactericidal tests according to the photometric growth assay method of Muschel and Treffers were performed on the serum fractions together with the original serums. Titers expressed as the dilution of serum or serum fraction which kills 50% of the assay organisms are presented in Table VI where it may be seen that the bactericidal activity of normal, early immune and late immune rabbit sera was present in Cohn's fraction III-0. These findings constitute an additional criteria for demonstrating the similarity of normal and immune bactericidal antibodies against Sh. dysenteriae.

Table VI. Bactericidal Activity of Normal and Immune Serum Protein Fractions Against Shigella dysenteriae

Rabbit # 3 Serums taken:	Bactericidal Assay Titers I: Original Serum	Fraction III-O	Fraction II
Pre-immunization	67	64	<2.5
6-8 hrs after injection	64	54	<2.5
1st day " "	100	91	<2.5
2nd day " "	143	132	<2.5
3rd day " "	1869	1418	26
7th day " "	27778	24691	156
14th day " "	20000	14545	267
21st day " "	15385	10811	109
28th day " "	7477	5000	167

5. Effect of Anti-malarial Drugs on Immune Mechanisms

Evidence from clinical literature shows that quinine, quinacrine or chloroquine administered in high dosage over a period of several months ameliorates rheumatoid arthritis, discoid lupus erythematosus and other diseases of supposed "autoimmune" nature. The possible effects of these drugs on the various parameters of the abnormal and normal immune mechanism such as complement, natural antibodies, and antibody formation remains unknown. Previous unpublished work using egg albumin and rabbit antibody to egg albumin showed that quinacrine and chloroquine had no effect on the in vitro combination of antigen with its homologous antibody. However, significant anticomplementary activity was noted at drug concentrations of 1×10^{-3} M to 4×10^{-2} M. Additional studies are concerned with the determination of the specific complement components inactivated by the drugs, the associated electrophoretic changes in quinacrine treated serum, and the effect of the drugs on whole complement and complement component titers in guinea pigs.

Using R reagents for complement component analysis, it has been found that incubation of whole guinea pig serum with chloroquine resulted in non-selective inactivation of all the complement components as determined by R reagent assay. After incubation of whole serum with 2×10^{-2} M quinacrine there was a 90% reduction in C'2 activity and a 72% reduction in C'4 activity, with no detectable loss of the other complement components. Dialysis, with subsequent removal of the drug failed to reverse the anticomplementary action of quinacrine.

Electrophoretic changes in quinacrine treated serum were investigated using 0.7% agar (Ionagar) gel on microscope slides. Veronal buffer pH 8.2, ionic strength .025, gave best results. Addition of the test sample mixed with an equal volume of molten agar at 40° into a 1 mm. X 15 mm. trough, allowed separation of guinea pig serum into eight distinct components. Following fixation and staining with Amido Black, qualitative changes were observed in the Alpha$_1$, Beta$_1$, and Beta$_2$ globulin regions, with the appearance of a new band in the Beta$_1$ region. The relationship of these changes to the inactivation of C'2 and C'4 by quinacrine is currently being evaluated. Accurate sectioning of the agar gel into 1 mm. segments and elution of material allows localization of peak component activity to areas 1 mm. in width. The combination of gel diffusion methods using rabbit antibody to whole guinea pig serum, staining, and assay for functional activity on each segment, allows excellent correlation of functional activity with the corresponding precipitin and electrophoretic band.

In vivo drug anticomplementary activity was checked as follows. Four groups of six (500 gm.) male guinea pigs were bled by cardiac puncture and four aliquots of each animal's serum stored at minus 60°. Quinacrine dihydrochloride 20 mg./kg./day, chloroquine dihydrochloride 20 mg./kg./day and primaquine phosphate 1 mg./kg./day in saline solution and a saline control were then administered intraperitoneally for five weeks to animals in each of the four respective groups. Following this, the animals were again bled and the sera stored as previously described. Whole complement and complement component assays were done on successive days; the titration for a single component from one-half the animals both predrug and postdrug treatment being done in a single assay. The titration for the remaining half of the animals was done on the following day. No significant drop occurred in the whole complement or component titers after five weeks of drug treatment.

6. Natural antibody against tissue in x-irradiated animals

Whole body radiation (WBR) of animals is known to cause a decline in levels of natural hemolytic antibody (Talmage, et al., J. Infec. Dis., 99:241, 1956) and normal bactericidal antibody for Escherichia coli (Kornfeld, et al., J. Immunol. 84:77, 1960). Sera from normal rabbits have been shown to possess natural antibodies against many rabbit tissues (Kidd and Friedewald, J. Exp. Med. 76: 543, 1942). It was found that WBR of rabbits resulted in a decline of normal complement fixing (CF) antibody against antigens extracted from normal and irradiated rabbit testicular tissue. This decline was observed 9 to 14 days following a 500 r dose of x-rays and resulted in a 50% reduction of preirradiation levels. Subsequently, a two-fold increase in normal antibody titers was observed in rabbits 2 to 4 months after 500 r WBR. This rise in tissue antibody levels suggested an autoimmune response against radiation modified tissue. However, a concommitant rise in normal antibodies against antigens of heterologous origin such as Salmonella typhosa "O" and sheep erythrocytes was also detected. Thus, it appeared that a "rebound" of the normal antibody synthesizing mechanism caused elevated titers after antibody production had been

temporarily inhibited. No evidence was obtained suggesting that serum from irradiated animals reacted better or more specifically with antigens from irradiated animals than with antigens from non-irradiated tissue.

To further test the hypothesis that autoantibody formation might result from x-ray damage of tissue, rabbits were given a 2000 r dose of irradiation to the testes only with the remainder of the animal shielded by lead. Sera were collected periodically for 3½ months after irradiation and tested for CF antibody using antigens from normal rabbit testes and testes obtained 2, 10, and 42 days after 2000 r. No rise was seen in normal antibody levels toward any of these antigens. A two-fold drop was observed two months after irradiation and this did not return to normal during the term of the experiment. This suggested that the 2000 r x-ray dose caused the tissue to degenerate sufficiently so that it lost its ability to sustain normal antibody levels.

7. Antibody response resulting from deliberate immunization with testes antigens

To learn if antibody response to testes antigen was inhibited by prior WBR a single iv. injection of rat testes antigen was given to rabbits 1 day after they had received 500 r WBR. Taliaferro and Taliaferro (J. Infect. Dis. 95: 134, 1954) have shown rabbits to be unresponsive to sheep erythrocyte antigen given during this "radiosensitive" stage of antibody formation. We found that in a similar manner testes antigen of a heterologous species failed to evoke an antibody response when injected 1 day after irradiation. This might explain why no evidence of autoantibody production is observed following irradiation although autoantigen might be present. These experiments are being expanded to learn how long the period of immunologic unresponsiveness to testes antigen persists following irradiation of an animal.

Normal and irradiated rat and rabbit tissue antigens combined with Freund's adjuvant were used to immunize rabbits by repeated injection. Antisera were collected at regular intervals following the final injection in order to compute mean peak antibody titers from groups of three rabbits each. Sera was tested for antibody by complement fixation, Ouchterlony plates and immunoelectrophoresis slides. The anticomplementary activity of each antigen was quantitatively measured. It was found that the immunogenicity, complement fixing reactivity and anticomplementary activity of the irradiated tissue antigens were in all cases less than that of the non-irradiated control antigens. The 42 day post 2000 r antigen was markedly less active in all respects than 10 day antigen, and the 10 day antigen less reactive than the 2 day material. With Ouchterlony plates as many as 6 bands of precipitation were found between normal rat testes antigen and its antiserum, whereas at most 2 weak bands were seen between normal rabbit testes

and its antiserum. With Ouchterlony plates some of the bands overlapped and there were no clear examples of a unique antigen-antibody system occurring between the irradiated antigen and antiserum. On the other hand, immunoelectrophoresis experiments with 42 day post 2000 r rat testes and its antiserum revealed the existance of one more arc of precipitation than was seen with the same antiserum and normal rat testes antigen. This suggested the presence of an antigen in the irradiated tissue not found in the unirradiated tissue. As expected, the antiserum to normal tissue produced stronger precipitation and a greater number of arcs with both irradiated and normal testes antigens.

8. Chemical differences between antigens prepared from normal and irradiated testes

Antigens were prepared in the cold from freshly obtained tissue by homogenizing at high speed with 4 parts of 0.85% NaCl solution and centrifuging twice at 700 g to obtain the supernatant fluid containing mitachondria, microsomes and soluble material. The sediment containing nuclei, debris and spermatazoa was not used. Tissue was obtained 2, 10 and 42 days after rabbits or rats were given a 2000 r dose of x-rays to the testes only with the remainder of the animal shielded by lead. Chemical tests of the antigens have indicated only minor differences inthe dry weights of whole tissues and extracted antigens from normal and irradiated animals. Minor chemical differences were observed between the second day post 2000 r and non-irradiated extracts. However, in all experiments the 10 day and 42 day post-irradiation antigens contained noticeably less total nitrogen, protein, carbohydrate, DNA, and phosphorous than non-irradiated controls. The opposite was found in the case of total lipid determinations where all of the irradiated testes extracts contained more lipid than the normal tissue extracts.

Summary and Conclusions:

1. Experimental findings indicate that allergic encephalomyelitis in the guinea pig is profoundly influenced by the organ as well as species of animal providing the tissue for the antigen. Moreover, complete Freund adjuvant appears to be an essential component of the inoculum. Paralysis occurred only with rabbit brain-adjuvant homogenates; inoculation with rabbit brain alone, or with rabbit liver or with fish brain, either with or without adjuvant failed to elicit the paralytic syndrome. Paralysis, moreover, did not appear to be associated with the production of anti-brain antibodies. Skin lesions at the site of inoculation apparently were due to the adjuvant rather than to tissue components of the inoculum. It is noteworthy that C' levels remained within normal limits even among the acutely affected paralyzed group. The albino rat proved to be refractory to inoculation with the rabbit brain-adjuvant homogenate; none developed paralysis even though the anti-brain antibody levels were relatively obscure at the present time.

2. The most striking immunologic change observed in runt disease was the marked reduction of C' levels in the affected animals; the magnitude of C' reduction appeared to be directly related to the severity of the disorder. None of the animals developed detectable complement-fixing antibodies against a variety of organ-extract antigens. However, one group of severely affected animals showed significant elevation of titers in tests with antigen prepared from the skin of newborn albino rats. It is suggested that this latter observation may be significant in view of the marked pathologic alterations of the skin that are associated with this disease syndrome.

3. The results to date show that apparently there are different modes of action of antibody and complement involved with their bactericidal action on even relatively closely related gram negative bacteria. Some difficulty is encountered, however, with conventional methods of bacterial growth assay. Preliminary investigations on the use of the Coulter Counter for bactericidal assay appear to be promising.

4. Normal antibodies against Sh. dysenteriae in rabbits and antibodies produced by artificial immunization of rabbits with the organism behave in a similar manner when fractionated by Cohn's Method 10.

5. The lack of in vivo anticomplementary activity indicates that at the concentrations studies, the striking in vitro effects of quinacrine and chloroquine on the complement system bear no relationship to their in vivo effects. As quinacrine remains the only chemical compound known to date which specifically inactivates C'2, the value of this drug in the study of immune hemolysis and other complement dependent reactions becomes obvious. Studies now in progress will allow possible correlation of the physical changes observed in drug treated serum, and the associated loss of complement activity. Studies on the possible effect of quinacrine and chloroquine on antibody formation and natural antibody are planned.

6. A "rebound" of normal antibody synthesis followed a temporary depression caused by 500 r whole body x-irradiation of rabbits. After irradiation, levels of natural sheep erythrocyte hemolysin, S. typhosa "O" agglutinin, and normal complement fixing antibody for rabbit testes antigens declined within two weeks. This was followed by a return to normal levels,and several months later a rise to above normal. No auto-antibody formation specific for radiation modified tissue was observed.

7. Tissues from irradiated animals extracted by saline were found to be generally less immunogenic, less serologically reactive as antigen and less anticomplementary than control antigens from non-irradiated animals. The presence of a single unique antigenic component in 42 day 2000 r rat testes was suggested by immunoelectrophoresis. However, Ouchterlony plates and immunoelectrophoresis studies showed fewer bands of precipitation between irradiated tissue antigens and their antisera than were found between non-irradiated tissues and their antisera.

8. Chemical analyses of antigens from rat and rabbit testes obtained 10 and 42 days after 2000 r revealed less total nitrogen, protein, carbohydrate, DNA, and phosphorous in the extracts than were found in non-irradiated controls. This appeared to be compensated for, in part, by increased amounts of total lipid in the irradiated tissue. The water content of the whole tissue or of the extracts was essentially the same in irradiated and normal tissues.

List of Publications:

1. Toussaint, A. J., and Muschel, L. H.: Studies on the bacteriophage neutralizing activity of serums. I. An assay procedure for normal antibody and complement. J. Immunol., 89: 27, 1962.

2. Muschel, L. H., and Toussaint, A. J.: Studies on the bacteriophage neutralizing activity of serums. II. Comparison of normal and immune phage neutralizing antibodies. J. Immunol., 89: 35, 1962.

3. Fife, Jr., E. H., Hook, W. A., and Muschel, L. H.: Tissue antibody and complement levels in runt disease. Proc. Soc. Exper. Biol. Med. 110: 524-526, 1962.

4. Polish, Edwin, and Muschel, Louis H.: Rheumatoid Factor and Complement-fixing Tissue Antibodies in Patients with Liver Disease. Am. J. of Digestive Disease, 7: 677-686, 1962.

5. Hook, W. A., Toussaint, A. J., and Muschel, L. H.: Antibody, lysozyme and complement levels in animals chemically protected against x-irradiation. J. Immunol., In press.

6. Sleeman, H. K.: Studies on soluble antigen substances isolated from human liver. Proc. Soc. Experi. Biol. Med., In press.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 B 813, Basic Research in Life Sciences

Task 05, Immunology (Pathogenesis and Immunity in Cholera)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Applied Immunology
Division of Communicable Disease and Immunology

Department of Experimental Pathology
Division of Special Activities

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Richard A. Finkelstein, PhD
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project 3A 0 12501 B 813 — Title: Basic Research in Life Sciences

Task 05 — Title: Immunology (Pathogenesis and Immunity in Cholera)

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1. Chick embryos become resistant to cholera (and other) endotoxins between the 11th and the 15th day of incubation. Like insulin, endotoxin causes a severe hypoglycemia in the younger susceptible embryos, but the mechanism of the two agents appears to differ.

2. Cholera can be induced in infant rabbits by the administration per os of sonically disrupted vibrios and other cell-free fractions or by intraintestinal inoculation with live vibrios. The morphological, physiological, and immunological attributes of this experimental disease are under investigation.

3. Cholera endotoxin in filtrates of the intestinal contents of human cases was identified and titrated by means of the Vibriocidal Antibody Inhibition Test and inoculation of chick embryos.

4. El Tor vibrios may be differentiated from V. cholerae by means of a simple, rapid hemagglutination test using chick erythrocytes.

5. Cholera vibrios do not penetrate the intestinal epithelium of starved guinea pigs suffering from a fatal enteric infection with this organism.

BODY OF REPORT

Project 3A 0 12501 B 813 Title: Basic Research in Life Sciences

Task 05 Title: Immunology (Pathogenesis and Immunity in Cholera)

Description: The purpose of this task is the study of the pathogenesis of cholera to develop better methods of immunization and treatment of this disease.

Progress:

1. The effect of age on susceptibility to cholera in embryonated eggs.

a. In previous reports, the age-associated development of resistance of chick embryos to an experimental cholera infection has been described and an operational concept of the "resistance" mechanism has been presented (Finkelstein and Ramm, 1962). However, an associated phenomenon, the development of resistance to endotoxins derived from V. cholerae and other gram-negative organisms was not resolved and has been the subject of intensive investigation. At 11 days of age chick embryos succumb to the intravenous inoculation of the order of 0.01 micrograms of purified endotoxin. However, on day 15 embryos tolerate 100 ugs of the same products administered by the same route. Carbon clearance studies have demonstrated that the function of the reticulo-endothelial system is not greatly increased in the older eggs. It was decided to undertake a survey of the effects of pharmacologically active compounds known or suspected to be liberated in or to affect endotoxin shock in other systems on chick embryos of both ages. The results are summarized in Table I. Of the compounds tested, only epinephrine, norepinephrine and bradykinin were toxic for the younger embryos in reasonably low doses. The magnitude of increase in resistance to these compounds in older embryos did not parallel that to endotoxin. (Bradykinin was not available in sufficient concentration to result in toxicity to the older embryos).

b. Dibenzyline at a level of 20 ug was found to protect embryos against the lethal activity of 50 ug of either norepinephrine or epinephrine, but no consistent protective effect either alone or in combination with nethalide against the toxicity of endotoxin could be demonstrated.

c. In several experiments, the blood levels of the catecholamines were determined at intervals following inoculation with endotoxin. Although rises in catecholamine levels were detected in some experiments the results were erratic on repetition.

d. The most striking results were obtained when chick embryo blood glucose levels were determined following administration of lethal doses of endotoxin. These results suggested some similarity between the effect of endotoxin and those of insulin on blood sugar. Accordingly the effect of insulin was similarly investigated in 11 and 15 day embryos. The results are summarized in Table II. While both endotoxin and insulin cause severe hypoglycemia in the younger embryos, there are two notable differences in the activities of the two agents. Whereas insulin causes an immediate decline in blood sugar, the effect of endotoxin is delayed for approximately two hours. In the older embryos, the hypoglycemia induced by endotoxin is

gradual and not marked, whereas insulin causes a severe decrease in blood sugar, similar to that in the younger embryos, but the embryo is apparently able to compensate and recovers. There is a marked increase in tolerance to the lethal activity of insulin with increasing age. Less than 0.2 u of insulin (= less than 8 ug) is lethal for 50% of 11 day embryos while 15 day embryos tolerate 20 units (the most concentrated solution tested), an increase in resistance or more than 100 fold. It was shown previously that cortisone and related drugs protect chick embryos against endotoxin. The adrenal corticosteriods have a regulatory action on carbohydrate metabolism and it is possible that the development of resistance could be associated with maturation of the adrenal cortex. However, administration of metopirone or amphenone, which block synthesis of these hormones, to chick embryos on day 11 failed to depress the resistance of the embryos to challeng with endotoxin on day 15. The observations on the effect of endotoxin on blood glucose levels suggest that a primary locus of action of endotoxin in this experimental system may be in inducing an alteration of carbohydrate metabolism. This could result from an alteration of cell membrane permeability and will be the subject of continued investigation.

TABLE I

Toxicity of Endotoxin and Other Compounds for 11 and 15 Day Old Chick Embryos Inoculated Intravenously

Compound	Approximate LD_{50} (ug/embryo) 11 day embryos	15 day embryos
Endotoxin	0.01	100
Epinephrine	10.0	100
Norepinephrine	20.0	200
Acetylcholine	1000	--
Heparin	500	--
Serotonin	> 100	--
Bradykinin	10-20	> 20
Kallikrein	> 10	--
Histamine	100	> 500
Isuprel	> 20	--
Angiotensin II	50	--
Insulin	8	> 800
Marsilid	> 100	> 200
JB516	> 100	> 200
Nethalide	100	--
Dibenzyline	> 20	--
DCI	--	≥ 1000
48/80	> 100	--
Amphenone	> 1000	--
Metopirone	> 100	--

TABLE II

Effect of Endotoxin or Insulin on Blood Glucose Levels in Chick Embryos

Age of Embryos	Treatment	Experiment Number	Time (hours) 0	2	4	8	20
11 days	Saline Controls	1	100#	98	102	-	-
		2	96	-	94	-	-
	Endotoxin*	1	104	91	21		
		2	-	84	11	Death	
	Insulin**	2	-	29	2	Death	
15 days	Saline Controls	3	112##	84	86	-	-
		4	111	112	118	115	111
	Endotoxin*	3	115	127	76	-	-
		4	-	119	101	97	84
	Insulin**	4	-	49	10	7	71

Blood glucose levels, mg %: each value represents pooled blood of 20 embryos.

* 0.1 ug S. marcescens endotoxin in 0.1 ml saline, I. V.

** 0.4 u insulin in 0.1 ml saline, I. V.

Each value represents pooled blood of 8 embryos.

2. Pathogenesis and immunity to experimental cholera in infant rabbits.

The suckling rabbit is the only laboratory animal which reproduces the characteristic signs and symptoms of cholera. In earlier experiments (WRAIR Annual Report 1960-61), it was possible to produce experimental cholera in a substantial proportion of infant rabbits by administration of cholera vibrios per os, but the results were variable and unpredictable. More reproducible results have been obtained using techniques recently described by Dutta et al. An active infection, giving rise to the characteristic rice water diarrhea, can be induced by the intra-intestinal inoculation of a moderate number (10^4) of rabbit-passed cholera vibrios. This experimental model has been used to study the histological alterations in the gut at intervals in experimentally infected and sham operated animals. Preliminary attempts to protect animals against this infection by prior intraperitoneal administration of rabbit antiserum against live vibrios were unsuccessful in our hands. Cholera can also be experimentally induced in this host by feeding a filter sterilized "cholera toxin" composed of the cell sap of ultrasonically disrupted highly concentrated vibrio

suspensions, in multiple doses following gastric lavage. This "cholera toxin" is a mixture of a variety of substances including cholera endotoxin and other components normally present in the vibrios. It is specific in that similar preparations from Shigella flexneri 2a fail to cause the characteristic disease. Purified cholera endotoxins likewise fail to cause diarrhea and death in infant rabbits. The choleragenic activity is destroyed by heating at 60 C for 60 minutes which causes obvious physical changes in the appearance of the material. It has been found possible to separate the cholera toxin into two fractions, neither of which alone has activity, but which regain activity when recombined. However, the complexity of the material has led us to seek simpler methods of preparing/more refined product with greater choleragenic activity which might be more easily concentrated, isolated, purified and identified. Some progress in this area has made using filtrates of young cultures in complex media and, more recently, in a modification of the simple defined medium of Finkelstein and Lankford. In addition to histological study, some of the physiological parameters of the experimental disease are also under investigation and it has been demonstrated that infant rabbits with cholera diarrhea have an accelerated intestinal transit time coupled with a marked decrease in gastric emptying. It has also been shown that the animals may be protected against "cholera toxin" by the use of rabbit antiserum against that product. Further study in each of these areas will be accelerated by the development of more purified preparations. The crude sonicate is lethal for 11 day chick embryos at doses of 0.0001 ml while the lethal dose in 15 day embryos is 0.1 ml, a thousand-fold difference. Purified cholera endotoxin preparations, however, exhibit a ten-thousand-fold difference under the same circumstances. This suggests that the crude sonicates contain, in addition to endotoxin, a substance which is toxic for 15 day embryos. However, Shigella sonicate exhibited the same pattern. The cholera sonicate also contains a disproportionately large amount of vibriocidal antibody inhibiting activity relative to its toxicity in comparison with purified endotoxin. Thus there is probably a considerable amount of non-toxic haptene or other moiety of endotoxin in the crude material.

3. Demonstration and quantitation of cholera antigen in cholera stool filtrates.

Recent studies have demonstrated that all of the components necessary to reproduce the signs and symptoms of cholera in infant rabbits are present in cell-free preparations from cholera vibrios and also occur in the human gut in the course of the disease. Numerous investigators who have speculated on the mechanism of pathogenesis of cholera have cast the cholera endotoxin in either a primary or a supporting role. However, there has been no conclusive demonstration of cholera endotoxin in the intestinal tract of humans during the disease. In fact, a recent publication indicated that the endotoxin content is below detection in mouse toxicity tests and implied that little or no lysis of the vibrios and/or release of endotoxin occurs in the gut. Since the presence or absence of cholera endotoxin, conclusively demonstrated, might be of considerable importance in defining the pathogenic mechanism, the problem was re-investigated using the Vibriocidal Antibody Inhibition Test, a sensitive technique for the specific recognition and assay of cholera antigens, which was described recently. Stool filtrates were prepared from patients with acute clinical cholera during the 1962 Inaba serotype cholera epidemic in Calcutta. The results are summarized in Table III. Thirty-four of the 46 stool filtrates tested

able
were from bacteriologically positive cases. Of these, 27 had titrat/ concentrations of antigen of the Inaba type. Three of the 12 bacteriologically negative cases also had demonstrable antigen content. Vibriocidal antibody inhibiting activity was present in amounts equivalent to between 10 and 100 ug of purified endotoxin activity in 7 of the 34 positive stool filtrates although the majority had less activity. The limited amounts of the filtrates which were available precluded an accurate titration of the toxicity for the chick embryo. As an expedient, groups of 8 or 10 11-day chick embryos were inoculated intravenously with 0.1 ml amounts of 1:10 dilutions of the sterile stool filtrates. Each embryo thus received 0.01 ml of filtrate. The results included (in parentheses) in Table III, indicate that 11 of the 46 stool filtrates tested proved to be toxic. Of these, 7 were from bacteriologically positive stools, six of which had an endotoxin equivalent content of 10 ug/ml or greater. Since 0.01 ug of purified cholera endotoxins is toxic for chick embryos, one might expect all the stool filtrates with titratable antigen content of 1 ug/ml or greater to be toxic in this test. This was not the case although there was a general association of toxicity with high antigen content. Apparently a portion of the titratable antigen activity is in the form of a non-toxic haptene or moiety of endotoxin as is the case with Dutta's "cholera toxin." Quantitative counts of the viable vibrios in the stools from which the filtrates were derived also correlated with the antigen content and toxicity results. While this study demonstrates there there is indeed a substantial amount of cholera endotoxin in the intestinal contents of the human cholera case, this demonstration alone is not sufficient to establish a relationship to pathogenesis of the disease.

TABLE III
Distribution of Cholera Antigen in Cholera Stool Filtrates

Bacteriological Diagnosis	Antigen Content (ug/ml)*				
	<0.25	0.25-1.0	>1.0-<10.0	10-<100	Total
+	7+ (0)‡	13 (1)	7 (0)	7 (6)	34 (7)
-	9 (2)	2 (1)	1 (1)	0	12 (4)
Total	16 (2)	15 (2)	8 (1)	7 (6)	46 (11)

*As determined in the VAIT (Finkelstein, 1962) in comparison with the activity of purified Inaba endotoxin, D344.

+Number of specimens with indicated range of antigen content.

‡Figure in parenthesis indicates the number of filtrates which were toxic for 11 day chick embryos inoculated I.V. with 0.1 ml of 1:10 dilution.

4. Differentiation between Vibrio Cholerae and El Tor Vibrios.

At present, El Tor vibrios are differentiated from Vibrio cholerae in the laboratory primarily by their ability to hemolyze goat or sheep erythrocytes. However, the results of this test have been extremely variable depending on the modification of the test used and the nature of the strains being tested. Although the recent cholera outbreak in the Philippines and surrounding areas has destroyed the textbook dictum that El Tor is not associated with explosive epidemic cholera, it is still of importance to be able to differentiate the two groups of "agglutinable" vibrios for epidemiologic and other considerations. Preliminary observations in this laboratory indicated that freshly isolated agar-grown El Tor strains were directly hemagglutinative for chick erythrocytes while V. cholerae strains were negative in this test. These observations were extended with over 600 strains of El Tor vibrios and V. cholerae. The results are summarized in Table IV. In no cases, in the series examined, were V. cholerae strains found to be hemagglutinative while 100% of the El Tor strains tested had this property. The identity of strains was verified by serological tests, hemolysis tests with sheep erythrocytes and reaction to Group IV cholera-phage. We had previously found variants of V. cholerae capable of causing hemagglutination and expected some exceptions to this rule, but this did not occur in the large number of freshly isolated and laboratory strains examined. The results suggest that direct hemagglutination with chick erythrocytes in a slide test using agar-grown vibrios is a simple, rapid and reliable method for differentiating El Tor vibrios from V. cholerae. The test may be applied at the time of primary isolation in parallel with slide agglutination tests with specific antisera for most rapid identification.

TABLE IV
Hemagglutination Reactions of Vibrio Strains

Vibrio cholerae					
Isolated				Hemagglutination	
Place	Year	Source	No. of strains	+	-
Calcutta & Howrah	1955-62	Humans	197	0	197
Bihar	1961	"	7	0	7
Orissa	"	"	7	0	7
Uttar Pradesh	"	"	21	0	21
Madras	"	"	7	0	7
Thailand	1959-60	"	15	0	15
Dacca	1960-62	"	33	0	33
Total			287	0	287

TABLE IV (Cont'd)
Hemagglutination Reactions of Vibrio Strains

Isolated Place	Year	Source	No. of strains	Hemagglutination +	-
El Tor vibrios					
Middle East	Unknown	Unknown	2	2	0
Indonesia	1938-62	Humans	42	42	0
		Water	14	14	0
		Flies	3	3	0
Thailand	1959-60	Humans	2	2	0
Hong Kong	1961	"	64	64	0
Macao	"	"	1	1	0
Philippines	"	"	193	193	0
	1962	"	20	20	0
Calcutta	1958	Water	8	8	0
Total			349	349	0

5. It was previously observed that Shigella flexneri transmigrated across the intact intestinal epithelium of starved guinea pigs within six hours after oral challenge. This was considered to be the first step in the process of ulcer formation which is so characteristic of this disease Similarly starved guinea pigs suffer a fatal enteric infection if challenged with V. cholerae. The purpose of the present work was to study the distribution of the vibrios in the intestinal tract of these animals and to determine if these organisms penetrated the intestinal epithelium. As in our previous work the fluorescent antibody technique was employed. At 2 hours and 4 hours post-challenge few vibrios were found in the lumen of the ileum. At 8, 12 and 16 hours post-challenge large numbers of cholera organisms were observed in the intestinal lumen of most animals; they were also seen between the villi and in the crypts of Lieberkuhn. In many areas the vibrios appeared to adhere to the mucosal surface. At 24 hours animals which appeared sick or were moribund had tremendous numbers of V. cholerae in the intestinal lumen; large clumps of organisms adhered to the epithelial surface and extended deep into the crypts. Guinea pigs which appeared well at 24 hours after challenge had few organism in the lumen and only occasionally were vibrios seen in the crypts. The numbers of V. cholerae in the caecum and colon of these animals were much fewer in number and more randomly distributed than observed in the small intestine and were rarely seen in the crypts. Quantitative bacterial counts confirmed the observations made with the fluorescent antibody procedure. At no time during the process of infection were V. cholerae cells seen to penetrate the intestinal epithelium and enter the lamina propria. Sections of ileum processed by routine procedures and stained with hematoxylin and eosin indicated that the mucosa remained intact even in moribund animals.

Summary and Conclusions:

1 The embryonated hen's egg, exquisitely susceptible to cholera and other endotoxins administered by the intravenous route on day 11 and virtually completely resistant on day 15, is a unique tool for the study of the mode of action of endotoxin. Endotoxin causes a severe hypoglycemia in susceptible embryos, like insulin, but differs from insulin in the manner by which it accomplishes this.

2. Cholera may be produced in infant rabbits by the oral administration of cell-free products of the cholera vibrio or by intra-intestinal inoculation of living vibrios. The cell-free choleragenic activity is inactivated by heat and its action can be prevented by antiserum. Associated with the choleraic diarrhea in infant rabbits is a decrease in transit time and an inhibition of gastric emptying. It is accompanied by slight alterations in intestinal morphology.

3 Cholera endotoxin has been demonstrated in the filtrates of the intestinal contents of human cholera cases. A substantial amount is present in the gut during the course of the disease but its association with the pathogenesis in a causal manner remains unresolved.

4. El Tor vibrios may be differentiated from V. cholerae by means of a simple, rapid slide hemagglutination test using chick erythrocytes and suspensions of agar-grown vibrios.

5. Cholera vibrios do not penetrate the intestinal epithelium of starved guinea pigs suffering from a fatal enteric infection with this organism.

List of Publications:

1. Finkelstein, R. A. and Ramm, G. M. 1962. Effect of age on susceptibility to experimental cholera in embryonated eggs J. Infect Dis 111: 239-249.

2. Finkelstein, R. A. 1962. Vibriocidal antibody inhibition (VAI) analysis: a technique for the identification of the predominant vibriocidal antibodies in serum and for the recognition and identification of V. cholerae antigens. J Immunol 89: 264-271.

3. Finkelstein, R A and Gomez, C. 1963. Comparison of methods for the rapid recognition of cholera vibrios. Bull. Wld. Hlth. Org. 28: 327-332.

4. Finkelstein, R. A. and Mukerjee, S. 1963. Hemagglutination: A rapid method for differentiating Vibrio cholerae and El Tor vibrios. Proc. Soc. Exp. Biol. Med. 112: 355-359.

5. Finkelstein, R. A., Mukerjee, S. and Rudra, B. C 1963. Cholera antigen in stool filtrates from cholera patients. Bact. Proc. 1963: 62 (abstract).

6. Finkelstein, R. A., Mukerjee, S. and Rudra, B. C. 1963. Demonstration and quantitation of antigen in cholera stool filtrates. J. Infect. Dis. In press.

7. Norris, H. T., Dutta, N. K., Finkelstein, R. A., Formal, S. B. and Sprinz, H. 1963. Morphologic alterations of the intestine of ten day old rabbits given intact and ultrasonically disrupted cholera vibrios or cholera endotoxin. Fed. Proc. 22 (No. 2, Part I) 512 (abstract).

8. Formal, S. B. Experimental cholera infections in laboratory animals. CRL Technical Advisory Committee. Feb. 1963.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 B 813, Army Medical Basic Research in Life Sciences

Task 05, Immunology (Mechanism of immune hemolysis and anaphylaxis)

Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
	Department of Immunochemistry Division of Communicable Disease and Immunology
Period Covered by Report:	1 July 1962 through 30 June 1963
Principal Investigators:	B. H. Alexander, PhD J. F. Barbaro, PhD E. L. Becker, PhD, MD L. Haffner, MS L. Kagen, MD C. Punte, PhD* P. Schur, MD S. Tawde, PhD**
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Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task 05 Title Immunology(Mechanism of immune hemolysis and anaphylaxis)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

A factor from rabbit plasma similar to the 11S component of hemolytic complement has been shown to participate in histamine release from rabbit platlets by an antigen antibody reaction. 11S component is present in guinea pig and rabbit complement. 5S antibody is capable of fixing a fraction of complement. A methanol soluble hemolytic factor can be released by an antigen-antibody reaction from guinea pig serum; complement is not involved. Kallikrein and PF/dil can be isolated from human serum as two distinct and different permeability globulins. They are both inhibitable by C'1 esterase inhibitor. Guinea pigs are less susceptible to the toxic action of phosphonates than are rabbits. This is probably due to the lesser susceptibility of guinea pig acetylcholinesterase to inactivation by these compounds.

BODY OF REPORT

Project No. 3A 0 12501 B 813 — Title: Army Medical Basic Research in Life Sciences

Task 05 — Title: Immunology (Mechanism of immune hemolysis and anaphylaxis)

Description: The purpose of the work of this task is to provide an explanation of the events in the acute, antibody mediated allergic reactions in terms of the reactions of the enzymes involved. For this purpose the immune hemolytic reaction is being used as a model for the more complex allergic reactions.

Progress:

1. Purification of Isolated Components of Guinea Pig Complement. Work on this project was stopped when Dr. George Wirtz left the Department last August and no further work is contemplated until and if a replacement is found.

2. Role of Histamine in Anaphylaxis.

a. In the preceding Annual Report we concluded that the release of histamine from rabbit platlets by an antigen antibody precipitate and rabbit plasma required a factor(s) from plasma adsorbed on the precipitate and factor(s) which remained in the supernatant. The factor(s) adsorbed on the precipitate was similar to the 11S component of Muller-Eberhard required in complement action, insofar as it, too, could be adsorbed on the precipitate in the presence of EDTA. Therefore, rabbit plasma was treated with immune precipitates in the presence of EDTA, washed with Tyrodes solution, and then extracted with phosphate buffer pH 5.30, M=0.3. This is the same buffer shown by Muller-Eberhard to remove 11S component from aggregated gamma globulin containing adsorbed 11S component. The extracted immune precipitate was not able to restore histamine release when added to the supernatant plasma and platlets. This demonstrates that something was extracted from the precipitate. However, the ultimate proof that this was the 11S component is still lacking, since isolated human 11S has not been able to restore histamine releasing activity to supernatant plasma. The isolated human 11S would restore hemolytic activity to the same supernatant.

b. As an offshoot of the above experiments, attempts have been made to determine if serum from other than human species possess hemolytically active 11S component. Rabbit and guinea pig, as well as human serum could be rendered hemolytically inactive by absorbing them with immune precipitates in the presence of EDTA. Inactive human serum (R11S) had 75 percent of its hemolytic activity restored by a human 11S; guinea pig R11S had 63 percent and rabbit 100 percent of its hemolytic activity restored by the same 11S preparation. The 11S component activity was not inhibited by pre-incubation with 50 Mg of soybean trypsin inhibitor/ml of 11S preparation. The C'1 activity of the human, rabbit and guinea pig R11S was determined using guinea pig R1. The rabbit showed full C'1 activity; whereas, the human contained 77 percent of the original C'1 activity, and the guinea pig only 50 percent of the C'1 activity of the serum from which it was prepared.

3. Permeability Factor Studies.

a. Attention was turned from attempts to fractionate Cohn Fraction III obtained commercially by ethanol fractionation of whole human plasma to the fractionation of whole human serum. Two factors with permeability increasing activity were separated from whole human serum by means of ethanol precipitation, starch block electrophoresis and DEAE-cellulose chromatography.

b. The first of these, globulin A, is apparently serum kallikrein. It is a gamma globulin, not bound to DEAE-cellulose, with a sedimentation constant of 11S. It is an esterase, inhibited by DFP, soy bean trypsin inhibitor and human serum C'1 esterase inhibitor. Preparations of globulin A can produce a smooth muscle contracting agent (kinin) from unheated plasma or plasma heated to 56 or 63°.

c. Globulin B, the second fraction obtained, is apparently PF/dil. It is a beta globulin, bound to DEAE-cellulose. It has a sedimentation constant of approximately 6S. It too is a serum esterase, inhibited by DFP, SBTI and human C'1 esterase inhibitor. Its preparations can produce kinin from unheated plasma but not unheated plasma.

4. The Complement Fixing Activity of 5S Antibody From Rabbits and Sheep.

a. Precipitating and complement fixing rabbit and sheep anti egg albumin and anti human serum albumin with sedimentation coefficients of 7S (7S antibody) were isolated by means of sodium sulfate precipitation. They were digested, as described by Nisonoff with pepsin to yield a fragment with a sedimentation coefficient of 5S (5S antibody). In conformity with the results of others the specific precipitating ability of the 5S antibody with its homologous antigen was unimpaired. However, contrary to the conclusion of others the 5S antibody was found to specifically fix both guinea pig and rabbit complement. Washed 5S antibody containing specific precipitates when added to complement were 300 times more effective on a weight basis than the same antibody to which antigen was added in the presence of complement. The 5S antibody containing preformed immune precipitates were capable of only fixing up to 40 percent of the added complement, no matter how much precipitate was added. This fixation was not due to contamination with undigested 7S antibody, since treatment of the 5S precipitates with cysteine or 2 mercapto-ethanol destroyed all of its complement fixing ability.

b. Absorption of guinea pig complement overnight in the cold with 5S immune precipitates removed 20 to 40 percent of the guinea pig complement. The complement remaining could not be fixed by fresh 5S immune precipitates, but the 7S containing immune precipitates fixed it as effectively as unabsorbed complement. The presence of 0.001 MEDTA prevented the absorption of complement by 5S immune precipitates.

c. Work in progress seems to indicate that 5S antibody is capable of causing both Arthus reactions and systemic anaphylaxis in rabbits.

5. The Liberation of a Methanol Soluble Hemolytic Factor by Antigen Antibody Precipitates Acting on Serum.

a. Fischer and Haupt (Zeit. f. Naturforschung 16:321, 1961) reported that antigen antibody reaction occurring in the presence of fresh serum would produce a methanol soluble hemolytic substance. They attributed this to a phospholipase present as one of the components of complement being activated to produce lyso-lecithin. Because of the obvious importance of this claim the following investigation was initiated.

b. Antigen antigody precipitates prepared at equivalence from rabbit anti-egg albumin and anti-human serum, as well as sheep, erythrocytes sensitized with rabbit antibody were incubated with guinea pig serum at varying temperatures for varying times. The precipitates or cells were centrifuged off and to the supernatant was added seven volumes of methanol. Following removal of the precipitate the methanol was taken off, and the residue tested for hemolytic activity against rabbit red cells.

c. Methanol soluble hemolytic activity was obtained as described by Fischer and Haupt. No activity was produced when antigen-antibody precipitates were added to midpiece or an R4. Unimpaired activity was found with endpiece and an R3 even though in the mere preparation of an R3 some hemolytic activity was produced.

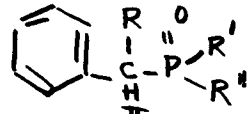
d. Heating serum at 56° or 61° in the absence of antigen-antibody aggregates causes an increased production of hemolytic factor. This was markedly enhanced by heating in the presence of EDTA or phosphate. Temperatures above 61° caused a decreased production.

e. The hemolysis of red cells by the methanol soluble factor increases with increasing length of time the factor is in contact with the cells. The hemolysis by lyso-lecithin, on the other hand, as shown by Gorter, Rapport and confirmed by ourselves is independent of the time of contact with cells after the first 10 minutes or so. This indicates that the methanol soluble hemolytic activity was not caused by lyso-lecithin. This was confirmed by showing that the major hemolytic activity produced by heating serum had different adsorptive behavior on thin layer chromatography than lyso-lecithin.

6. Mechanism of In Vivo Anaphylactic Reactions.

a. Work on the synthesis of organophosphorus inhibitors has continued. The compounds in Table I were synthesized and their structures proved. They are undergoing in vitro and in vivo testing now.

TABLE I



Empirical Formula	R	R'	R''	M.P.	Boiling Point °C	mm	$N_D^{25°}$	Analyses C T	C F	H T	H F	N T	N F	P T	P F
$C_{17}H_{18}NO_7P$	$-OC-CH_3$	$-OC_2H_5$	$-O-C_6H_3(NO_2)$	104-6°	-	-	-	53.8	53.8	4.8	4.9	8.1	8.9	4.0	4.1
$C_{16}H_{26}NO_5P$	$-N(C_2H_5)-C-OC_2H_5$	$-OC_2H_5$	$-OC_2H_5$	-	144	0.05	1.4911	56.0	55.2	7.6	7.6	4.1	4.1	9.0	8.5
$C_{13}H_{22}NO_3P$	$-NH-C_2H_5$	$-OC_2H_5$	$-OC_2H_5$	-	132	0.9	1.4926	57.6	57.3	8.2	8.3	5.2	5.3	11.4	11.6
$C_{14}H_{24}NO_3P$	$-NH-C_3H_7$	$-OC_2H_5$	$-OC_2H_5$	-	114-125	0.05	1.4893	58.9	59.5	8.5	8.4	4.9	5.0	-	-
$C_{12}H_{20}NO_3P$	$-NH-CH_3$	$-OC_2H_5$	$-OC_2H_5$	38-9°	106	0.11	1.4971	56.0	56.5	7.8	7.8	5.5	5.2	12.0	12.1
$C_{15}H_{24}NO_5P$	$-N(CH_3)-C-OC_2H_5$	$-OC_2H_5$	$-OC_2H_5$	-	180°	2.7	1.4936	54.7	54.7	7.4	7.5	4.3	4.3	9.4	9.1
$C_{16}H_{26}NO_5P$	$-N(C_2H_5)-C-OC_2H_5$	$-OC_2H_5$	$-OC_2H_5$	-	156°	0.4	1.4906	56.0	55.6	7.6	7.5	4.1	4.1	9.0	8.6
$C_{20}H_{26}NO_3P$	$-N(C_2H_5)-C-C_6H_5$	$-OC_2H_5$	$-OC_2H_5$	-		-	-	-	-	-	-	-	-	-	-
$C_{11}H_{18}NO_5P$	$-N(C_2H_5)-C(=NO)-CH_3$	$-OH$	$-OH$	165-6	-	-	-	51.4	51.5	6.3	6.3	5.4	5.9	12.0	11.5
$C_{16}H_{18}NO_4P$	$-N(C_2H_5)-C(=NO)-C_6H_5$	$-OH$	$-OH$	163-5	-	-	-	60.2	59.7	5.7	5.3	4.4	4.6	9.70	9.3

b. The enzyme responsible for the breakdown of the phenylalkyl and alkyl phosphonates described in the preceding report is not the same as "para-oxonase," the enzyme responsible for hydrolyzing para-oxon. It has a pH optimum around 10.3, and is not inhibited by Ba^{++} or EDTA to anywhere near the same degree as para-oxonase.

c. The guinea pig is significantly less susceptible to the acute toxic effects of the phenylalkyl and alkylphosphonates than is the rabbit, despite the fact that the rate of breakdown and inactivation of these compounds by the enzymes of the guinea pig is distinctly less than that of the rabbit enzyme. The susceptibility of red cell cholinesterase from the rabbit to be inhibited by these compounds, however, is much greater than the corresponding enzyme from the rabbit. This suggests that this difference in acetyl cholinesterase from the two species is responsible for the species difference in susceptibility to the anticholinesterase agents.

Summary and Conclusions:

1. The precipitate absorbable factor(s) in rabbit plasma which participates in histamine release from rabbit platlets by immune precipitates has a number of the properties of 11S. However, definitive proof of this is lacking.

2. Guinea pig and rabbit complement require the 11S component for their hemolytic activity.

3. Kallikrein and pF/dil have been isolated from normal human serum and shown to be distinct from each other.

4. C'1 esterase inhibitor inactivates both kallikrein and pF/dil.

5. 5S rabbit and sheep antibody fixes a fraction of whole guinea pig complement. The fixation is much more evident when the antibody is contained in preformed immune precipitates.

6. Guinea pig serum treated with antigen-antibody aggregates in the presence or absence of chelating agents produces a methanol soluble hemolytic substance. This substance is not lysolecithin.

7. The enzyme responsible for the breakdown of phosphonates in rabbit and guinea pig plasma differs from any heretofore described.

8. Guinea pigs are less susceptible to the toxic action of phosphonates than are rabbits, which mirrors the lesser susceptibility of guinea acetyl cholinesterase to inactivation by the same compounds.

Publications:

1. J. F. Barbaro and E. L. Becker. Comparison of the complement fixing potency of antiserum from various species. J. Infect. Dis. 111:175, 1962.

2. J. P. Leddy, G. L. Freeman, A. Luz, R. H. Todd. Inactivation of skin sensitizing antibodies of human allergy by thiols. Proc. Soc. Exp. Biol. & Med. 111:7, 1962.

3. E. L. Becker, T. R. Fukuto, B. Boone, D. Canham and E. Boger. The relationship of enzyme inhibitory activity to the structure of n-alkylphosphonate and phenylakyl phosphonate esters. Biochemistry 2:72, 1963.

4. Kagen, L. J., Leddy, J. P. and Becker, E. L. Isolation of two permeability globulins from human serum. Nature 197:693, 1963.

5. Kagen, L. J., Leddy, J. P. and Becker, E. L. The presence of two permeability globulins in human serum. J. Clin. Investig. (In press).

6. Kagen, L. J., and Becker, E. L. Inhibition of permeability globulins by C'1 esterase inhibitor. Fed Proc. 20:13, 1963.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 B813 Army Medical Basic Research in Life Sciences

Task 05, Immunology (Characterization, Identification and Purification of Antigens and Antibodies

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D.C.

Departments of Virus Diseases and Immunochemistry
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project 3A 0 12501 B 813 — Title: Army Medical Basic Research in Life Sciences

Task 05 — Title: Immunology (Characterization, Identification and Purification of Antigens and Antibodies

Reporting Installation: Walter Reed Army Institute of Research
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Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

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1. Studies were undertaken to study in detail factors in the specific immune response of the guinea pig to infection with Japanese B Encephalitis virus. These studies have included the determination of the time of onset of appearance of complement fixing, hemagglutination inhibition, and neutralizing antibodies following inoculation of virus by various routes and on different dosage schedules. Detailed studies are currently in progress to determine the relationship between the physico-chemical characteristics of these antibodies to their appearance in time following inoculation. The data thus far suggests the earliest antibody, of all three types, belong to the 19S class of gamma globulins, and the late antibody to the 7S class.

2. Preliminary studies were undertaken to define and demonstrate viral antigens and antibodies by means of the agar double-diffusion technique of Ouchterlony. Prototype strains of vaccinia and adenovirus 3, 4 and 7 were used in the study. Studies are now in progress utilizing the gel-filtration principle of Sephadex, for the purification of viral antigens.

3. The addition of fresh normal serum to the antiserum agar layer of an Oudin tube increases the precipitate density of the leading edge of the precipitate band. Heating the normal serum to 56°C causes a loss of this enhancing effect, and if sufficiently prolonged, addition of such heated serum leads to an actual decrease in the density of the precipitate band. Increasing the salt concentration in the Oudin tube causes an increase in the density of the precipitate band when chicken antisera are used.

BODY OF REPORT

Project 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task 05 Title: Immunology (Characterization, Identification and Purification of Antigens and Antibodies

Description: The present emphasis of the work being carried out under this task is on the development of gel-precipitin methods for the quantitative determination in absolute weight units of antigen and antibody concentrations which can be applied to mixtures of antigens, and to antisera containing multiple antibodies.

Progress:

1. Characterization of Arbovirus Antibody.

a. The study was undertaken to examine in greater detail factors in the specific immune response of the guinea pig to infection with Japanese B Encephalitis virus with reference to the time of onset of appearance of antibody. Secondly, the study was designed to ascertain if the observed antibody response was dosage-dependent on antigenic mass or on the route of inoculation. Lastly, it seemed of interest to determine if the previously observed serologic differences between "early" and "late" antibody could be explained by physico-chemical differences in the respective antibodies.

b. Preliminary experiments involving experimental infection of the guinea pig with herpes simplex, parainfluenza III, and with Japanese B Encephalitis viruses, agents which are known to produce infection in this host, revealed similar time relationships of antibody production. Japanese B Encephalitis virus was subsequently chosen as a model for the present study.

c. The virus strain used for all guinea pig inoculations was the JBE virus, Nakayama strain, in the 45th mouse passage, and 1st hamster passage, with an LD_{50} of 10^{-7}/0.03 cc in mice. Neutralizing, HI, and CF antibody determinations were performed with the M1/311 strain. Evidence for infection in the experimentally inoculated guinea pigs was the presence of viremia on the 3rd day following inoculation.

d. Four groups of 48 guinea pigs (Table I) were immunized on single and multiple dosage schedules by the following routes: intracerebral, intraperitoneal, intradermal, and subcutaneous. The material used for inoculation was diluted to contain an LD_{50} of $10^{-5.8}$/0.03 cc. The animals were prebled initially, and then bled sequentially on days 3, 7, 10, 14, 21, 28, and 31. Neutralizing, CF, and HI antibody determinations were performed on each serum, and viremia studies on the 3 and 7 day bleedings.

Table I

Group	Route	No. of Animals	Controls	Dose Virus(LD_{50});Frequency
I	i.c.x1	4	2	10^7 x 1
	i.c.x3	4	2	10^7 x 3
II	i.p.x1	4	2	10^8 x 1
	i.p.x3	4	2	10^8 x 3
III	i.d.x1	4	2	$10^{6.6}$x1
	i.d.x3	4	2	$10^{6.6}$x3
IV	s.q.x1	4	2	$10^{7.6}$x1
	s.q.x3	4	2	$10^{7.6}$x1

e. Following a single inoculation of virus by any route, CF antibody was detected as early as the 7th day reaching a maximum by the 14 to the 21st day, gradually receding thereafter. Also following a single inoculation by any route, the earliest that HI antibody could be detected was on the 10th day, reaching a maximum by the 21st day and it gradually receded. Three multiple injections spaced at 7 day intervals appeared to have little effect in bolstering the total antibody response. Neutralizing antibody determinations are now in progress. Viremia studies were positive in all cases on the 3rd day and negative on the 7th.

f. Characterization of these antibodies has been studied by means on the Waugh-Yphantis moving partition cell in the Model E ultracentrifuge, and by sucrose density centrifugation in the Model L. These studies reveal that the earliest antibody to be synthesized, in every case, appears to belong to the 19S macroglobulin class of gamma globulins, and the later antibodies to the 7S class.

g. The significance of these findings would suggest that the specific antibody response in the guinea pig to this virus is not dependent on dose, or route of inoculation. The heterogeneity of the early vs. late antibody response is consistent with other reported systems, and may offer a clue to the serologic differences in early and late convalescent responses following natural infection in the human.

2. Purification of Animal Viruses.

a. Preliminary studies using chick embryo propagated vaccinia virus were performed to standardize as well as purify the virus. It was hoped this technique would provide a simple method of field diagnosis for adenovirus disease in recruits.

b. Studies were conducted with tissue culture adapted strains of adenovirus types 3, 4 and 7, and human acute and convalescent sera for these viruses. Pre- and post-immunization sera from experimental animals were used for vaccinia and the adenoviruses. Normal tissue culture was used as a control.

c. The test appears to be of little value for the identification of adenovirus tissue culture isolates, because uninfected tissue culture homogenates show numerous precipitin lines with hyperimmune animal sera thus obscuring any specific lines due to virus. On the other hand of six cases recovering from known infections due to adenovirus 3, 4 or 7, one case recovering from a type 7 infection showed, in his convalescent serum, a precipitin line only against type 7 virus, but not against types 3 or 4. His acute serum showed no lines against any of the three types.

d. The findings would suggest the potential use of the method in the serologic diagnosis of adenovirus disease as an adjunct to the presently available methods. The main limitation of the method is the requirement for a high titered type specific antigen.

e. Attempts were also made to adsorb viruses differentially on Sephadex columns. These studies arose from a need for newer methods of purification of viruses for use in the agar double-diffusion method, and a need for purer viral antigens for use in the production of hyperimmune sera in experimental animals.

f. Preliminary studies were conducted utilizing adenovirus type 7, utilizing Sephadex G-50, in a 1x10cm column; the virus was eluted with phosphate buffered saline, ph=7.36, u=0.01M, and 10 1.0cc fractions were collected. HI, CF, and infectivity studies were performed on each fraction.

g. The results thus far indicate that some purification had occurred, Table 2. This could be visualized by the delayed appearance of phenol red indicator, and presumably other low molecular weight substances, in tubes 7 and 8. CF, HI, and infectivity were recovered in earlier tubes.

Table 2

Distribution of Antigen Activity and Infectivity in Eluates from Sephadex Columns.

Fractions	CF	HA	Infectivity	Phenol Red
1	0	+	0	0
2	++++	++++	+	0
3	+	++	+	0
4	0	+	±	0
5	0	±	0	0
6	0	0	0	0
7	0	0	0	+
8	0	0	0	+
9	0	0	0	0
10	0	0	0	0

3.

a. Preceding Annual Reports and publications have described a technique for the determination of antibody concentration in absolute weight units. The method utilizes the determination in an Oudin gel-precipitin reaction of the optical density of the leading edge of the precipitate band. The effect on the optical density of such non-specific factors as the concentration of normal serum in the antiserum layer, the temperature at which agar-antiserum layer is held before adding it to the Dudin tube etc., have been investigated, so as to enable the technique to be used for the routine determination of the concentration of antibodies in mixtures.

b. The addition of fresh normal rabbit serum to the antiserum agar layer increased the optical density of the leading edge of the precipitate. This enhancing effect could be abolished, and an inhibition induced by sufficiently prolonged heating of the serum at 54-56°C.

c. The effect of increased salt concentration on increasing the amount of specific precipitation with chicken antiserum has been generally confirmed. However, there are conflicting reports on the effect of such increased salt concentrations when the precipitin reaction with chicken antiserum is carried out in a gel medium. Using quantitative measurements

of precipitate density in Oudin tubes we have shown that increased salt unequivocally increases the optical density of the leading edge of the precipitate band. However, surprisingly, and anomalously the migration rate of the precipitate band also increases in increased salt. The same effect on the density of precipitate bands was noted employing Ouchterlony plates.

Summary and Conclusions:

1. Studies were undertaken to study in detail factors in the specific immune response of the guinea pig to infection with Japanese B Encephalitis virus. These studies have included the determination of the time of onset of appearance of complement fixing, hemagglutination inhibition, and neutralizing antibodies following inoculation of virus by various routes and on different dosage schedules. Detailed studies are currently in progress to determine the relationship between the physico-chemical characteristics of these antibodies to their appearance in time following inoculation. The data thus far suggests the earliest antibody, of all three types, belong to the 19S class of gamma globulins, and the late antibody to the 7S class.

2. Preliminary studies were undertaken to define and demonstrate viral antigens and antibodies by means of the agar double-diffusion technique of Ouchterlony. Prototype strains of vaccina and adenovirus 3, 4 and 7 were used in the study. Studies are now in progress utilizing the gel-filtration principle of Sephadex, for the purification of viral antigens.

3. The addition of fresh normal serum to the antiserum agar layer of an Oudin tube increases the precipitate density of the leading edge of the precipitate band. Heating the normal serum to 56°C causes a loss of this enhancing effect, and if sufficiently prolonged, addition of such heated serum leads to an actual decrease in the density of the precipitate band. Increasing the salt concentration in the Oudin tube causes an increase in the density of the precipitate band when chicken antisera are used.

Publications: None

ANNUAL PROGRESS REPORT

Project: 3A 0 12501 B 813	Basic Research in Life Sciences
Task: 08 - Neuropsychiatry	(Anatomical and Physiological Substrata of Behavior)
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
	Departments of Neurophysiology, Experimental Psychology, and Neuroendocrinology Division of Neuropsychiatry
Period covered by report:	1 July 1962 to 30 June 1963
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ABSTRACT

Project: 3A 0 12501 B 813 — Basic Research in Life Sciences

Task: 08 - Neuropsychiatry — (Anatomical and Physiological Substrata of Behavior)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Depts. of Neurophysiology, Experimental Psychology, and Neuroendocrinology
Division of Neuropsychiatry

Period covered by report: 1 July 1962 to 30 June 1963

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1. Anatomical studies of the mammalian nervous system have been continued and amplified. The main activity has been concerned with neural mechanisms of behavioral and general neurological interest: (a) the limbic system; (b) efferent connections of the neocortex; (c) the histological characteristics of retrograde axon degeneration, and (d) electron-microscopic studies of the central nervous system. In addition, anatomical investigations of the submammalian nervous system have been initiated with a study of spino-bulbar connections in the bird. Research in Stain Technology was continued successfully.

2. Physiological studies in behavior have been continued or initiated in the following areas: (a) binaural localization of sound sources in space; (b) circadian rhythms in hibernating mammals; (c) homeostatic mechanisms in hemorrhagic hypotension; (d) intracranial self-stimulation (ICS) and behavior; (e) neuroendocrine changes associated with ICS, and, (f) neurochemical and neuroendocrine studies of limbic system function.

BODY OF REPORT

Project No. 3A 0 12501 B 813 Title: Basic Research in Life Sciences

Task No. 08 - Neuropsychiatry Title: (Anatomical and Physiological Substrata of Behavior)

Description and Progress:

1. The Limbic System. The anatomical analysis of the limbic system has during the past year been continued with a study of the efferent connections of parts of the hippocampal gyrus, specifically the presubiculum and entorhinal area in the cat. Both of these allocortical regions have long been known to emit massive projections to the hippocampal formation proper, but no data were available regarding their possible further connections. The data obtained to date have shown that such additional connections include: a. a projection from the presubiculum via the fornix bundle to the septal region, thalamus (paratenial nucleus), and hypothalamus (mammillary body), and b. a pathway from the entorhinal area to the basolateral cell groups of the amygdaloid complex. The latter connection is of particular interest, as it identifies the entorhinal area as a cortical region with a dual efferent affiliation, viz. with two major, and basically antagonistic, structures in the limbic system: the hippocampus and amygdala. A paper describing these findings has been read before the 75th Annual Convention of the American Association of Anatomists, held in Washington, D.C., April 1963.

2. Efferent Connections of the Neocortex. This study has been concerned mainly with the subcortical projections arising in the frontal and parietal association areas. The analysis of the frontal projections in the monkey has been amplified and is now nearing its completion. The complex neural pathways originating in the frontal association cortex indicate a close liaison of this major cerebral area with the limbic system. This is suggested by the finding of pathways to the cingulate gyrus and presubiculum, to the temporal neocortex, the hypothalamus, the habenula, and medial regions of the mesencephalic tegmentum. A preliminary report of these findings was contributed to the Pennsylvania State University Symposium on The Frontal Lobe held in August 1962 and shortly to be published in book form.

Efferent connections of the parietal association cortex are being studied in the cat, with particular emphasis on cortico-thalamic connections. This study is still in an early phase and more experimental material is needed before any conclusions can be drawn.

3. A Study of the Histological Characteristics of Retrograde Axon Degeneration has been initiated during the past year. The problem is of considerable practical importance especially in neuropathological studies of brains containing lesions of long standing. It is widely assumed, but by no means proven, that retrograde axon changes are similar to the well-known (Wallerian) drop-like disintegration observed in anterograde axon degeneration. This notion, which has long affected the interpretation of observations in human postmortem and animal experimental material, is contested by the findings made to date. In several cases of surgical transsection of the medial lemniscus in the cat, no fragmentation of axons was found proximal to the lesion as late as 4 months following surgery; although the lemniscal axons distal to the lesion exhibited drop-like disintegration within 10 days. The findings made so far indicate that retrograde axon degeneration is characterized by a slow atrophy without drop-like disintegration. Because of the need for long survival times (up to 1 year), these experiments are being continued.

4. Electron-Microscopic Studies of the Central Nervous System. The original intent of this program has been to study the ultra-structure of cerebral edema and the effects of the various clinical therapeutic procedures on it. The program, since its initiation in November 1962, has thus far been devoted to familiarization with the many techniques employed in electron microscopy. These include learning to operate the electron microscope itself, carried out in the Department of Biophysics, AFIP, and at the RCA Corporation, Camden, New Jersey; studying the effects of various fixatives on the brain and attempting to establish the optimum method of fixing rat brain showing cerebral edema; learning the techniques of embedding and ultrasectioning of the tissues, and establishing controls of normal rat brain tissue.

For the most part the program has progressed at a steady pace. The main difficulty encountered thus far has been in obtaining satisfactory fixation of brain tissue and especially pathological brain tissue. This is, however, a universally recognized problem encountered by all investigators in this field and is currently being worked out in several laboratories over the country. The recent introduction of various aldehyde fixatives used in intracardiac perfusion appears promising and is being tried in this laboratory with some success.

It is proposed to subject rats to various noxious procedures designed to produce cerebral edema and ascertain the individual ultra-structural characteristics of these various procedures, if there be any, and if possible to establish a prototype of injury and response. Subsequently, it is planned to study the effects of various agents employed clinically to combat cerebral edema such as hypertonic solutions, cortisone derivatives, hyperventilation and hyperthermia.

5. Experimental-Anatomical Research in the Submammalian Nervous System has been initiated during the past year with a study of pathways ascending from the spinal cord to the brainstem in the bird (pigeon). This study was intended to extend the recently completed study of such connections in mammals. In the pigeon, an ascending fiber system corresponding to the mammalian spinothalamic tract could be traced to various levels of the brainstem reticular formation. Of particular interest are the findings concerning the distribution of some such fibers in the thalamus. These observations may lead to more reliable identification of homologies between mammalian and submammalian thalamic nuclei, a problem heretofore approached exclusively by the much less reliable way of normal-anatomical studies.

6. Stain-technological Research during the past year has resulted in the development of a method of counterstaining brain material previously impregnated by the Golgi-Cox technique. This method represents a significant technical advance. The Golgi method, unique in its capacity to provide extraordinarily detailed and complete pictures of individual neurons, suffers from the handicap that it does not usually furnish enough random landmarks to afford accurate cytoarchitectonic identification of cell-groups. Despite many previous efforts, no reliable method of Nissl-type counterstaining was heretofore available. The present technique, based upon Neutral Red staining in a medium of carefully controlled pH, has been accepted for publication by the journal, Stain Technology.

7. Neural Mechanisms of Auditory Discrimination. This study is now in a final phase and a comprehensive account of the observations made during the past years is in preparation. Briefly, the results of the study indicate a specific role of the superior olivary complex in the mechanism of binaural spatial localization of sound sources. In the current stage of the experiment, a detailed study is made of the lower limits of the cat's sound-localizing capacity, using sounds of short duration and various frequencies, with close spacing in the dual speaker assembly. This study is performed both before and after the placement of small focal lesions in various subdivisions of the superior olivary complex.

A further study initiated during the past year, concerns bilateral cortical and bilateral superior olivary responses before and after the placement of small lesions localized in the brain stem auditory nuclei. This study is in an initial phase and no conclusions can be drawn to date.

8. Long Term Studies on Hibernating Animals. In this study, in progress since 1961, the role of the environmental temperature and illumination in the circadian activity rhythm of several species of hibernating ground squirrels has been evaluated further. Techniques used include continuous lead recording of brain temperature by means of implanted miniaturized thermoprobes coupled to a low-weight counterbalanced cable by a "rotary contactor" device. The arrangement leaves the animals complete freedom to move. Animals belonging to three species of Citellus (C. beecheyi, C. lateralis, and C. tridecemlineatus) are kept under constant conditions of cold and illumination. It is found that even under these constant conditions, all three species emerge from hibernation in the Spring and re-enter hibernation in the Autumn. During the hibernation season, the extent of time spent in hypothermia slowly increases to a maximum and then decreases. The hibernation/arousal ratio during the hibernation season can be computed from the recordings, and shows species-characteristic differences. The observations made to date suggest the existence of neural mechanisms, which "gate" or program the entire process of hibernation. The periodicity of onset of discharge of these mechanisms is considered endogenously determined and to be of approximately one year's duration.

In a concurrent study, an attempt was made to correlate the behavioral and thermoregulatory aspects of the hibernation cycle with the total quantity of electrical activity occurring in the amygdaloid complex. The latter is a component of the limbic system and stands in close association with the hypothalamus, a structure known to be fundamentally involved in thermoregulatory processes as well as in the sleep-wakefulness cycle. The integral of the electro-encephalogram recorded from the amygdaloid complex was found to evolve in remarkable parallel with the temperature plot, and it has become clear that in at least one species (C. beecheyi) arousal from hibernation occurs in synchrony with one of the bursts of increased activity recorded from the amygdala.

9. Effects of Intracranial Self-stimulation (ICS) upon Heart Rate in Rats. This study was undertaken in order to investigate the recent contention that the "rewarding" effect of electrical stimulation of certain brain regions (the Olds-Milner phenomenon) accrues to activity of the parasympathetic nervous system. The region from which the "rewarding" effect can be elicited extends from the septal area caudalward through the hypothalamus into the midbrain. Continuous records of the heart rate in self-stimulating animals with the intracranial electrode implanted in various loci within this region indicate a pronounced absence of positive correlation of the rewarding effect with activation of either the sympathetic or parasympathetic components of the autonomic nervous system. In agreement with other investigations, self-stimulation through electrodes in the septal region was generally accompanied by cardiac deceleration. However, animals with electrodes in certain hypothalamic loci (dorsomedial and ventromedial nuclei) showed increased heart rates when self-stimulating. These findings make general propositions relating rewarding effects of brain stimulation to parasympathetic "quieting" reactions of the autonomic nervous system untenable.

10. Intracranial Self-stimulation and Behavior. The experimental analysis of motivational properties related to rewarding intracranial stimulation has focused upon the study of two choice preference behavior in animals with multiple rewarding electrodes in several different brain loci. In an investigation of the stimulus duration parameter under these conditions, it has been found that animals show a consistent preference for the longer of two stimulus durations suggesting that the self-regulation effects which have been previously reported in which the animal terminates the stimulus after a given period cannot be accounted for on the basis of punishing effects alone. Additional experiments in this area have indicated that the reward strength of intracranial stimulation increases with prolonged durations up to 10 and in some cases 20 seconds with further increases in duration adding no increment in reward strength. This same two choice preference method is also being used to study differences in the locus of intracranial self-stimulation as a function of food deprivation. Previous studies in this area have suggested that the increases in rate of self-stimulation under conditions of food and water deprivation may be due primarily to a change in general activity level. The analysis of differential effects of such deprivation operations in relation to different rewarding self-stimulation placements in the brain provides a technique for separating those effects related to activity changes from those presumed to be a direct function of the deprivation operation upon neural tissue.

An investigation of the effects of intracranial self-stimulation upon heart rate has demonstrated that the cardiac rate during intracranial self-stimulation is strongly dependent upon the locus of the stimulating electrodes. These studies have shown that acceleration was obtained during self-stimulation of the hypothalamus while deceleration of the heart rate was obtained during stimulation of septal sites. Variability of the heart rate was also found to decrease markedly during the intracranial self-stimulation periods regardless of the direction of the change in rate. The results of these studies call into serious question those over-generalized theoretical accounts of the brain stimulation phenomena in terms of parasympathetic dominance.

Additional studies have focused upon an analysis of stimulus generalization gradients during self-stimulation of the brain and upon the analysis of more specific stimulus control phenomena under conditions of electrical self-stimulation. The effects of brief post-reinforcement delays upon self-stimulation thresholds have also been analyzed and the relationship of seizure activity in various brain loci to the thresholds of electrical self-stimulations are under analysis. The location of various rewarding electrode placements in the dog brain have also been evaluated and impedence changes from an initially high value of 100,000 ohms to a rapidly declining value of 15 to 20,000 ohms after repeated stimulation have been under investigation with this same organism.

Summary:

Studies on the behavioral effects of intracranial self-stimulation have continued to explore the anatomical, physiological, and environmental factors which determine the characteristics of performances maintained by electrical self-stimulation of the brain in experimental animals. Studies in this area have been concerned with the motivational properties of rewarding brain stimulation and the interaction between neuroanatomical locus and such environmental manipulations as food deprivation. In addition, the differential effects of intracranial self-stimulation upon the heart rate as a function of locus of stimulation has also been investigated.

11. Neural Regulatory Mechanisms in Hemorrhagic Hypotension. A study of neural control of cardiovascular and respiratory function in the course of hemorrhage-induced hypotension by monitoring of certain physiologic variables has been initiated. Data have been collected on 13 dogs during acute exsanguination under (25-28 mg/kg) Nembutal anesthesia. New techniques and devices for measurement of further variables have been tested and modified during the course of these initial experiments. The findings made in 10 dogs indicate a stereotyped temporal sequence of physiological events accompanying exsanguination. After loss of 45-50% of the blood volume which

the animal will eventually shed, a bradycardia suddenly appears against the background of a more gradually developing fall of the pulse pressure to 50-40 mm Hg. This bradycardia can be prevented by bilateral section of the vago-sympathetic trunk in the neck.

Plans for the future include amplification of the present project, 1) by a study of the effect of anesthetics on the phenomenon; 2) by simultaneous recordings from various vascular beds/brain, liver, kidney, etc.), using optical-density plethysmography and concentrating on variables such as pulse propagation time, to be used as criteria for selective differential bloodflow; 3) by the development of data-processing techniques permitting more adequate read-out and correlation of findings; 4) by monitoring of variables in waking animals subjected to chronic controlled hypotension, and 5) by use of a transmural valve for a study of the effects of respiratory rate and amplitude upon the venous return.

12. Neuroendocrine Changes Associated with Intracranial Self-Stimulation. A series of experiments has been completed in seven monkeys with plasma and urinary hormone measurements before, during and after one to five-day periods of self-stimulation in the pre-optic region. Lever responses are very high, ranging as high as 23,000 responses/24 hour on a continuous reinforcement schedule. In all experiments marked urinary 17-hydroxycortico-steroid increases, ranging from two to sixfold, have been observed in association with such preoptic self-stimulation. Preliminary results with other hormonal measurements in these animals indicate that epinephrine and norepinephrine are also markedly increased. Urinary estrogen levels, on the other hand, appear to drop during the stimulation but rise above normal afterwards, in the recovery period. In three experiments urinary androgen levels become elevated but it is not yet clear whether or not this is preceded by a brief initial decrease in levels. Plasma BEI levels rise slowly with self-stimulation indicating an increase in thyroid activity. It appears so far that the general pattern of hormonal response is very similar to that observed during conditioned emotional stress in the monkey with the possible exception of the androgenic component which may be uniquely dissociated.

13. Neurochemical and Neuroendocrine Studies of Limbic System Function. The serotonin concentration in approximately ten selected brain areas has now been measured in six monkeys following six hours of electrical stimulation of the amygdaloid complex. Multiple correlations appear to be emerging from these experiments relating anatomical, neurochemical, electro-physiological and neuro-endocrine factors. During the six-hour stimulation period, plasma 17-hydroxy-corticosteroid levels initially rise and then fall, often reaching a level below normal by the end of the six-hour period. When such a hormonal pattern is observed, electrophysiological changes, consisting of after discharges in the hypothalamus, are consistently observed and extremely low serotonin concentrations are found in the septal area and the hypothalamus. When stimulation fails to elicit the characteristic plasma 17-hydroxycorticosteroid response, neither the electrical changes in the hypothalamus nor the serotonin changes in septal and hypothalamic regions are observed. These findings may indicate a regional neuro-chemical basis for the secondary suppressive effect of limbic system stimulation upon the pituitary-adrenal cortical system.

In related experiments, substantial increases in plasma aldosterone levels have been observed following stimulation in that portion of the central gray substance in the reticular formation of the midbrain which appears to articulate with the limbic system. An attempt is being made at present to see whether or not such plasma aldosterone responses can be explained on the basis of ACTH release.

Summary and Conclusions:

1. Anatomical studies of the mammalian nervous system have been continued and amplified. The main activity has been concerned with neural mechanisms of behavioral and general neurological interest: (a) the limbic system; (b) efferent connections of the neocortex; (c) the histological characteristics of retrograde axon degeneration, and (d) electron-microscopic studies of the central nervous system. In addition, anatomical investigations of the sub-mammalian nervous system have been initiated with a study of spino-bulbar connections in the bird. Research in Stain Technology was continued successfully.

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Hodos, W. and Brady, J. V. Brain stimulation. International Encyclopedia of the Social Sciences (in press).

_____ and Valenstein, E. S. An evaluation of response rate as a measure of rewarding intracranial stimulation. J. comp. physiol. Psychol., 1962, 55, 80-84.

Karten, H. J. 1963 Projections of the parahippocampal gyrus of the cat. (Abstr.) Anat. Rec. 145:247-248.

Kling, J. W. and Beer, B. 1962 (April) Timing behavior as a function of current and locus of ICS. Address at Eastern Psychological Association, Philadelphia, Pa.

_____ and Beer, B. (in press) Timing behavior as a function of current and locus of ICS. J. exp. Anal. Behav.

Mason, J. W., Nauta, W. J. H. and Cain, C. E. (in preparation) Serotonin Concentrations in Selected Brain Areas in the Normal Rhesus Monkey.

McHugh, P., Black, W., Tolson, W., Taylor, E., Mougey, E., Brady, J. V. and Mason, J. W. (in preparation) Patterns of Hormonal Change Associated with Preoptic Self-stimulation in the Monkey.

Moushegian, G. and Rupert, A. 1962 Binaural Interaction in the Superior Olivary Complex of Cat. J. Acoust. Soc. Am. (In the Press).

Nauta, W. J. H. 1963 Central Nervous Organization and the Endocrine Motor System. 2nd International Congress of Neuroendocrinology. (In the Press).

_____ 1963 Some Efferent connections of the Prefrontal Cortex in the Monkey. In: Warren-Akert (Ed.): The Granular Frontal Cortex and Behavior. (In the Press).

Perez-Cruet, J.; Black, W. C. and Brady, J. V. 1963 Differential effects of hypothalamic and septal self-stimulation on heart rate. Science. (In the Press).

Perez-Cruet, Jorge; Black, William C., and Brady, Joseph V. Differential effects of hypothalamic and septal self-stimulation upon heart rate. Address at Eastern Psychological Association, April 1963, New York City.

Perez-Cruet, Jorge, Black, William C., and Brady, Joseph V. Differential effects of hypothalamic and septal self-stimulation on heart rate. Science, 1963 (in press).

Ramon-Moliner, E. 1963 Dendritic patterns of the cat's brain stem. (Abstr.) Anat. Rec. 145:366

Strumwasser, Felix; Gilliam, J. J. and Smith, J. L. 1963 Long-term studies on individual hibernating animals. Proc. Finn. Acad. Sciences. (In the Press)

_____ Smith, J. L., Gilliam, J. J., and Schlechte, F. R. 1963 Identification of Active Brain Regions involved in the processes of hibernation. Proceedings of the XVI International Congress of Zoology. (In the Press)

Valenstein, E. S. and Beer, B. 1962 Reinforcing brain stimulation in competition with water reward and shock avoidance. Science. 137, 1052-1054.

Vane, M. A., Ramon-Moliner, E. and Fletcher, G. 1963 Counterstaining of sections impregnated by the Golgi-Cox method. Stain Technology, In the Press.

ANNUAL PROGRESS REPORT

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TASK: 08 Neuropsychiatry
(Electrophysiological Studies of the Nervous System)

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WALTER REED ARMY MEDICAL CENTER
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PERIOD COVERED BY REPORT: 1 July 1962 through 30 June 1963

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ABSTRACT

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The present report is essentially a continuation of studies reported last year, i.e. they are concerned with electrophysiological correlates of behavior. Emphasis is on the nature of neural coding and information processing in the peripheral and central nervous systems. Of particular interest is the relation of neural coding to sensory input and discrimination. The use of computer techniques has been expanded to include more sophisticated kinds of data analysis as well as continued investigation of biological problems revealed by electrical recording.

BODY OF REPORT

Project No. 3A 012501 B 813, Basic Research in Life Sciences

Task No. 08 Neuropsychiatry
(Electrophysiological Studies of the Nervous System)

Description:

The intent of this task is to correlate neurophysiological structure with categories of behavior and their assessment by means of electrical recording. Where applicable, behavioral problems are designed as corollary experiments. Emphasis is on sensory information processing, discrimination and learning, and central integrative neural networks.

Progress:

I. STUDY OF SENSORY PROCESSES

A. Auditory System - Single Unit Analysis

Superior Olivary Complex (Centripetal Neurons). The superior olivary complex is currently recognized to be made up of at least five major subdivisions. The major nuclei are: 1) the superior olive proper, 2) the medial superior olive, 3) the internal pre-olive, 4) the external pre-olive, and 5) the nucleus of the trapezoid. Afferents to these nuclei come mainly from the ventral cochlear nucleus although it is said that the nerve and dorsal cochlear nucleus innervate them as well. The nucleus of considerable interest for binaural investigators is the medial superior olive, also known as the accessory nucleus. The cells of this nucleus receive bilateral innervation; and electrophysiological measures of their response characteristics show that both excitatory and inhibitory interactions, sensitive to changes in frequency, intensity, and time contribute to the processing of auditory stimuli.

A click delivered to one ear produces a response which may be inhibited by a click to the opposite ear. This inhibition is effective for time differences between the binaurally presented clicks of as little as 8 microseconds.

Many units of this nucleus exhibit spontaneous activity and may be driven by tones. Although a tone excites a unit when it is delivered to one ear, the same tone to the opposite ear inhibits it. Units isolated and studied from other nuclei of the superior olivary complex show, in the main, only monaural responses. Furthermore, the configuration of the slow-wave response evoked by a click stimulus varies considerably in the various nuclei of this complex, and may be used to predict reliably the locus of the electrode tip during the experiment.

Latency measures of some units in the accessory nucleus show that their spike latency parallels the latency of N_1 (measured at the round window) as intensity is varied. This indicates a constant delay between the N_1 response and

the spike latency. Other units in the accessory, however, do not show parallel latency shifts with intensive changes; these units instead exhibit a considerably greater dynamic range than the evoked N_1 latency. These facts make it obvious then that N_1 latency measures must be made or account taken of it.

There are essentially four types of response areas for accessory units. These types were ascertained by stimulating left and right ears independently with tones and noting whether the unit's spontaneous rate was increased or decreased. Type 1 units were excited by tones presented to the left ear and to the right ear. Type 2 units were excited over a wide range of intensities and frequencies, but inhibited punctately by tones to the opposite ear. Type 3 units exhibited both excitatory and inhibitory response areas to tones delivered to one ear. Type 4 units were excited by one ear and inhibited by the other ear over wide combinations of frequency and intensity.

All the units studied show a remarkable sensitivity to frequency, intensity, or time and show that the processing of neural information at this cellular level is delicately tuned to small changes in the parameters of sound.

Superior Olivary Complex (Centrifugal Neurons). The efferent component of the cochlear nerve has its origin in cells of the superior olivary complex. These fibers pass upward to the floor of the fourth ventricle, decussate, join the bundle of the opposite side, and travel through the internal acoustic meatus and make intimate contact with the hair cells in the cochlea. Electrophysiological recordings from single units of this centrifugal system in the region of the superior olivary complex reveal considerable differences from the centripetal auditory units in their responses to tones. These units were established as olivary cochlear units by 1) their location in the brain stem, 2) their long latency to sounds, 3) their low, regular, spontaneous activity, and 4) their driven activity was low and regular, rarely exceeding 50 pulses per second.

All results on both the centripetal and centrifugal units point to excitatory and inhibitory processes as mechanisms of responding to tones. Though it is not yet clear how these processes may be incorporated into a model that would shed some light on fundamental questions of hearing, it is fairly certain that both are equally important.

Eighth Nerve (Efferent component). Efferent units in the cochlear nerve exhitit inhibitory characteristics as do the afferent, however, the time course of inhibition (and recovery) is longer for the efferents. This fact, as well as some others, necessitates the postulation of at least two separate classes of inhibitory fibers in the auditory nerve - one initiated by the efferent olivochochlear system, the other probably by processes within the cochlea itself.

Eighth Nerve Different Component. A paper on this problem has been published in the Journal of Neurophysiology entitled, Unit Responses to Sound from Auditory Nerve of Cat.

Eighth Nerve Anatomy. An attempt has been made to trace the course of the VIII nerve from the brain stem into the interval acoustic meatus and to the hair cells of the cochlea. Several staining procedures have been tried successfully. This material has proven invaluable in current electrophysiological experiments where it is important to ascertain the location of particular components of the auditory nerve.

B. Visual System

1. Human Visual System

An experiment on the spectral sensitivity of the human electroretinogram in early dark adaptation has been completed. High luminance pre-exposures of yellow, blue, and green light were employed. By this procedure the scotopic contribution to the spectral curves was greatly reduced and the maximum sensitivities occurred in the photopic region. For low criterion a-waves the peak sensitivity was near 555 mu, while low criterion b-waves tended to have highest sensitivity in the red region of the spectrum. Selective effects from the chromatic pre-exposures were small for the b-wave and not apparent for the a-wave. Comparison of the a- and b-wave sensitivities showed that the b-wave retained more responsiveness in the blue region of the spectrum.

A study of the effect of various light adaptation levels upon the human electroretinogram is in progress. Adaptation levels ranging from complete dark adaptation to high luminance white adaptations are being used. The effects of various broad-band colored test flashes and various durations of test flash are being investigated. Preliminary results suggest that the differential threshold, as measured by a constant criterion amplitude of b-wave, give results comparable to psychophysical thresholds. The a-wave appears to show a different slope as a function of adaptation level. The implicit time (latency to peak) of the b-waves seems to be relatively independent of adaptation level and to depend mainly on test flash luminance. Apparently, various measures of the ERG apparently are differing functions of adaptation level. The results are of interest in relation to theories concerned with rhodopsin concentration and visual sensitivity.

2. Frog Visual System

On- and off-components of the electroretinogram (ERG) were investigated as functions of intensity and wavelength of light stimuli. The relative spectral sensitivities of the two components were used as a general index to what role on- and off-mechanisms might play in wavelength-dependent behavior.

The experiments were performed on intact, immobilized Rana catesbiana. Light flashes of calibrated wavelength, intensity, and duration were delivered in Maxwellian view to the frog's eye. In addition, the same retinal area was illuminated by an adapting light in the light-adapted experiments. The ERG was picked up with wick-electrodes on the cornea. Repetitive stimulation and averaging techniques were used. This technique increased the reliability of the

measures and allowed recording responses to relatively low-intensity stimuli. Responses have been followed down to below 2 uV in amplitude. Light flashes of 3.2 sec duration were presented every 5 sec. Preliminary experiments indicated that these stimulating conditions elicited clearly distinguishable on- and off-responses of about the same order of magnitude. Data were collected from a number of preparations under three levels of light adaptation and dark adaptation.

For the quantitative treatment of the data, amplitude measurements were made of the on- and off-responses. The on-response was measured from peak to peak i.e., bottom of a-wave to top of b-wave. Two amplitude measures were obtained from the off response; (1) the voltage difference between the baseline and the initial positive peak, and (2) the voltage difference between the baseline and the later negative peak. The two measures of off-response generally yielded the same spectral sensitivity curves, the results therefore are reported for the former measure only.

For each wavelength the response measures were plotted as a function of light intensity. Generally the amplitudes of both on- and off-components increased as the intensity of the stimulus increased. However, when a wide range of intensities were used, these response-intensity curves were not always monotonic. Curves were fitted to the response-intensity data by inspection.

Relative spectral sensitivity curves were derived from these response-intensity functions by determining the relative energy at the various wavelengths required to produce a criterion response measure. The criterion response measures were selected for the on- and off-components so that they involved the same light energy in the middle of the spectrum.

Under some conditions the off-response is relatively more sensitive to short-wavelength light (blue) than the on-response under the same conditions. The conditions that tend to reveal this differential spectral sensitivity are: (1) low background illumination, i.e., when the eye is dark-adapted or weakly light-adapted; and (2) low luminance test flashes, i.e., when relatively small responses are used as criteria. These findings suggest the action of the retinal green rods which absorb blue light. The findings also support the hypothesis that the on- off-mechanism of the retina could form the basis of the neural code of color.

3. Turtle Visual System

Electrical Recording. Work has continued on the analysis of spectral curves in the turtle tectum. The evidence for a 500 - 540 mu process was confirmed. When compared to spectral curves obtained from electroretinograms recorded simultaneously, this process shows a criterion-height dependence not seen in the peripheral record. Matched curves from the eye and tectum recorded to the same low-level stimulus shows this difference most dramatically. The electroretinogram curve shows a characteristic red peak near 640 - 650 mu and a secondary shoulder near 575 mu. The curve then falls off abruptly at either end. The tectum curve, on the other hand, is somewhat broader and shows two well-defined peaks. The long wavelength peak is near 660 mu while the short wavelength peak is near 520 540 mu.

Behavior. In an endeavor to assess the role of color vision in this animal's behavior, an experiment was designed and set up during the past year. Turtles are currently being conditioned to withdraw their heads to supra-threshold light to avoid a short-duration shock. The animals master the task to 90% correct criterion quite easily, usually within 80 trials. They are then subjected to a series of trials employing dimmer and dimmer luminances. The animal makes a conditioned withdrawal response whenever he sees the light stimulus. The point at which he no longer withdraws his head is taken as threshold. Experiments are currently in progress to investigate several wavelengths in order to describe a spectral sensitivity curve.

A second behavioral set-up utilizes a free-operant situation. The animal is placed in a darkened tank of water and can raise a trap door permitting a view of the "outside world". The animal appears willing to work on an imposed schedule to satisfy this "curiosity" drive. The long range plan is to get a stable level of performance and then subject the animal to a discriminative set of stimuli. Problems under consideration include color, movement, and acuity.

II. CENTRAL INTEGRATIVE PROCESSES

A. Organization of Parieto-Visceral Ganglion in Aplysia.

In the parieto-visceral ganglion of Aplysia californica it is possible to label certain nerve cells and to be assured that one is following the same type of nerve cell from preparation to preparation. The labeling is based on the constancy of the pattern of results of several tests. This paper deals with three particular cells, A, B and C. Cells B and C receive direct input from cell A; each time cell A discharges a single impulse (spontaneously or directly evoked) a unitary IPSP (inhibitory post-synaptic potential) appears in cell B and an EPSP (excitatory post-synaptic potential) in cell C. The EPSP in cell C does not represent an inverted IPSP since depolarization of the cell beyond the spike threshold does not cause reversal. It is particularly significant that acetylcholine released by electrophoresis from a micropipette, applied to the soma of cell B causes hyperpolarization while application to cell C causes depolarization. In addition the membrane potential at which the acetylcholine-induced hyperpolarization of cell B disappears corresponds with the membrane potential at which the IPSP's due to input from cell A also disappear, close to -60 mV. The findings indicate that the same transmitter is liberated at the different branches of cell A and that the membrane of cells B and C are probably uniformly differentiated over most of their surfaces to allow response in one of two ways. The response in cells B and C to cell A impulses are further differentiated, in part due to particular processes occuring in the terminals of cell A.

Two identifiable neurons, in the parieto-visceral ganglion of Aplysia californica emit spike output patterns dependent on clock time. One of these neurons becomes active between 1700 and 1900 hours EST, maintains its activity for approximately 8 continuous hours, and is inactive for the remainder of the day. The rhythm occurs in the ganglion, isolated from all peripheral receptors,

in a sea water or artificial medium. The activity pattern can be shown to be endogenous to that cell. No patterned synaptic influx can be demonstrated when the cell is hyperpolarized thus blocking the spike output. Furthermore, hyperpolarization phase-shifts individual bursts. Subsequent burst size is remarkably independent of how many 'expected' bursts are suppressed or the stage of the burst at which hyperpolarization is applied. A model can be developed to account for these observations. It includes the production on a clock schedule of a depolarizing substance by the cell. The 'instructions' for the temporal schedule of this substance are considered built into the cell and no different in principle than those which are executed during the multifarious processes of cellular development. The further pursuit of the analogous processes in such nerve cells may give insight into those 'instructions' which are laid down within single neurons as the result of past experience.

B. Computer Analysis of Neural Responses.

1. Visually Evoked Potentials in the Nervous System

The digital computer placed in operation during the period covered by the previous report continues to prove its value. The original configuration of data processing equipment and computing equipment has been maintained. In addition several elements have been added: (1) a high speed punch tape reader which is used to load programs and data at rates up to 300 characters per second, (2) an off-line flexwriter for preparing punch tape without tying up the computer, and (3) 500 words of additional memory. A main application of the computer has been that of averaging signals which are hidden by noise, however, a number of other valuable uses have been found. Statistical programs have been developed for carrying out analysis of variance, Fourier analysis, curve fitting, multivariate analysis and correlation analysis. Programs have also been developed for smoothing evoked potentials and for automatically measuring some of their principal characteristics. Methods by which the system could be improved continue to be considered. It has become apparent that still more could be accomplished with a noncirculating type of computer.

Studies of the relation between visually evoked potentials in the retina and the unexposed cortex of the intact human subject have continued. Experiments dealing with the adaptational effects of flickering light have been completed. It has been found that potentials at both locations show the most significant changes in their waveforms and amplitudes when a flickering light is first presented. A uniform response to a flickering light is rapidly achieved. Current studies deal with the spectral sensitivity of the retina and the occiput. Response sensitivity is being mapped for stimuli positioned at various spots on the retina. Studies of normal subjects are being supplemented by studies of subjects having lesions in the retina or in the optic nerve. It is now possible in the best cases to detect lesions of the central retina which have an area of one square millimeter. It has been found that the occipital response becomes larger if both eyes rather than one eye alone is stimulated. In addition, it is

sometimes possible, when using occipital potentials as a measure, to detect lesions of the optic nerve and to estimate whether such lesion occurs before or after the optic chiasm. Experimentation along these lines is continuing.

2. Aurally Evoked Potentials in the Nervous System

Computer extraction of non-random stimulus-linked responses reveal that very little of the brain is silent to moderate intensity (60 db) clicks. The entire cerebral and cerebellor cortex has been mapped in 1/4 cm^2 to 1 cm^2 areas in the normal sleeping and waking cat. A technique of chronic implantation of 4 to 8 bipolar electrodes has been used. Analysis thus far has been to compute a factor expressing the amplitude-frequency change of the raw EEG in sleep-wakefulness and comparing it with a change in the evoked response. The correlation has not been striking. A more clear picture begins to emerge regarding regional cortical responsiveness to clicks in sleep changes. Subcortical effects of sleep appear to be more clearly related to amplitude-frequency changes in spontaneous activity of the EEG. It is planned to map subcortical areas more extensively, to study paradoxical stages of sleep, and to study the effects of lesions and drugs on these phenomena.

3. EEG and the Development of Cortical Evoked Potentials in Human Infants

Study of the development of the cortical electrical responses to light and sound stimuli in the child has been continued this year. The data is stored on magnetic tape or run "on line" using a general purpose digital computer programmed to average responses to repetitive stimulation.

Several of the babies whose first EEG was obtained in early weeks after birth have been followed for almost two years. Their changing responses to the same set of stimuli has been mapped. Onset latency of the responses to both auditory and visual stimuli decreases with age and the wave forms become more complex. One hundred and one records on 85 children have been performed to date, including 45 sets of data on 40 neonates recorded at 45 minutes to 9 days of age. This last group of babies was studied using a portable tape recorder and recording unit in the newborn nursery of Walter Reed General Hospital. Several abnormal children including a child with expressive aphasia and a mongoloid baby have been followed.

A study of the effects of changing the intensity of auditory stimuli on the evoked response, the heart rate, and babies' observable behavior has just been completed and a report is being prepared. Twelve babies, 2, 3 and 4 days old from the Walter Reed Army Hospital newborn nursery, were studied. There were 4 babies at each age level: 2 boys and 2 girls. Criteria for choosing these babies as a normal population included a history of normal pregnancy, labor, and delivery; birth weight of 6 - 9 lbs; Apgar Score of 8 or better; and a normal physical examination.

Bipolar recordings from 3 electrode scalp placements were obtained. Heart rate was obtained with precordial electrode. The babies were observed by the psychologist for periods before, during, and after the stimuli and behavior was recorded. Trains of 250 clicks from a loud speaker 24 inches from the subject's head were delivered at a rate of 1 per second. After an interval of 2 minutes, a train of clicks attenuated 5 db from the preceding set was given. This was repeated attenuating the click 5 db each time, the softest click being about 30 db above normal adult threshold. At this point the procedure was repeated increasing the intensity in 10 db steps to the loudest click, about 65 db above adult threshold. Average evoked responses were displayed using the X - Y plotter of the digital computer, and various components of the response were measured. Heart rate was counted for periods before, during, and after the stimulus.

All of the babies studied showed evoked responses of a characteristic wave form with the positive peak of greatest amplitude for the loudest sound at about 260 msec and an earlier positive peak at about 65 msec. Often other components were distinguishable. Response amplitude diminished as the sound was attenuated and a response to the softest click was not discernable in all the subjects. When the sound intensity was increased, the response amplitude grew although usually not to the previous size. Heart rate changes do not appear to be as reliable, but there is some evidence of acceleration with the stimulus if there is a startle or movement and a rather rapid habituation of both heart rate and behavioral response.

C. Experiments Relating the Alpha and Kappa Rhythms of the Electroretinogram to Behavior

Work was continued on the kappa and alpha EEG rhythms and its relation to behavior and other variables. A study was made examining in detail the effects of problem solving activity on the alpha and kappa EEG rhythms.

The problem solving was carried out with a highly simplified form of the concept problems, introduced by Smoke and used by others. Extensive previous work with these problems has led to specification of the type of processing carried out by the subject during discrete stages of the problem solving. This work also led to the specification of the "information load" or "cognitive strain" imposed on the subject at each stage. It therefore became possible to correlate changes in EEG with point to point changes in mental performance and with variations in the load imposed on the subject at each point.

In addition to the conventional recording, a dual channel scoring apparatus, described in detail by Kropfl, Chapman and Armington (cf. ref.), was used to score the presence of kappa and alpha rhythms objectively.

The problems the subject solved may be briefly described as follows. After a preparatory signal the subject is shown a horizontal array of eight figures. Each figure is either black or white on a background of blue. This example is

then removed and a second example is shown containing another array of eight figures. In the second array four of the eight figures have the same color as in the preceding slide. The subject's task is to specify those figures that remained constant in color over the slides. From the point of view of logical operations, a subject is required to determine the intersection of the two arrays or carry out a logical "and" operation on them. During the first slide, the subject is required to store the information. During the second slide the subject selects out the relevent information for his answer. It was possible on the basis of preliminary measurements to construct problems that placed either high or low storage requirements, or high or low selection load on the subject.

Preceding the problem presentation, a blank slide and a warning signal is given; following the problem presentation, the subject is required to call out his answers and then a feedback slide with the correct answer is given. A comparable set of control slides is given which require the subject to count aloud (silently) at appropriate times. The EEG rhythms are scored during each phase of the control and experimental tasks. In general the EEG data show a high degree of intra-subject reliability for both alpha and kappa and for both the control and experimental tasks. The alpha control curve remained relatively steady across the six slides. The alpha experimental curve however, shows a pronounced dip during the first problem example and during the second problem example. High storage load gives greater depression than low storage and high selection load greater depression than low selection load. The effects of storage load are consistently stronger than those of selection load. Although the kappa experimental task curves are reliable within a subject, they are not consistent from subject to subject. In some of the subjects the kappa curves rise during the problem examples. In the majority of the subjects the relation between kappa and alpha was positive.

These findings are a marked contrast to the effects of mental addition tasks on alpha and kappa (cf. Chapman, Bragdon and Armington). Mental addition tasks resulted in marked and general increases in kappa, but less marked and less general depression in alpha. Since the problem-solving task was a visual one while the mental-addition tasks are primarily auditory, the differences may stem from the modality involved. This hypothesis is currently being investigated.

D. Electromyographic Studies of Paretic Muscles During Sleep

The purpose of this study is to investigate the action potential changes which occur in muscles of a paretic limb during the different stages of sleep as compared to the muscles of the contralateral side in hemiplegic patients.

The records for this investigation are being obtained from 50 hemiparetic patients who are available on Neurology Wards at WRGH. A Grass Model III-D eight channel EEG machine is utilized to obtain the records. The recording machine is run continuously throughout the night session (approximately 6 hours). All electrical activity is recorded by means of surface electrodes. The EMG values for the spastic muscle as well as the normal muscle during each of the EEG stages of sleep is computed by means of an integrator unit which resets automatically every 20 seconds.

A three way analysis of variance (AxBxS design) will be computed from the mean EMG values. This will permit assessment of two main aspects (stage of sleep, and neurological involvement) over the 50 subjects as well as assessment of possible interaction effects (stage of sleep and neurological involvement). The appropriate test will then be applied to specific differences between means for the involved and noninvolved muscles.

The electrical activity of a paretic spastic muscle has been a controversial issue, specifically the conditions of the muscle of a spastic limb during sleep, in terms of its action potential, has never been presented in the literature. Changes in heart rate throughout the recording and any basic differences in the spontaneous sleep EEG activity between the affected and normal hemispheres of the brain in hemiparetic conditions should be of clinical and academic interest. Some of the records obtained in preliminary pilot studies show that there was intermittent electrical activity in the spastic muscle throughout the different stages of sleep while the normal muscle was electrically silent for the most part. Furthermore, the EMG activity of the spastic muscle showed an increase during the light stages of sleep and a decrease during the deep stages.

Summary and Conclusions:

Much of the work discussed here is a continuation of work reported last year. The auditory system continues to unfold as an extremely complex system with subtle interactions. The accessory nucleus shows an interesting response pattern dependent on bilateral innervation. A click to one ear may be inhibited by a click to the other. The time discrimination between clicks is as little as 8 microseconds, an extraordinary short time which points to the fantastic efficiency of the sensory input processing system. Of additional interest is the fact that a unit driven by a tone presented to one ear may be inhibited when that same tone is presented to the other.

In human vision, high luminance chromatic adaptation has been used to investigate color processes by means of electrical recording. The effects are quite small and mostly evident in the b-wave. Spectral curves derived from b-waves matched to a constant height of a-waves, at different wavelengths, show the b-wave to be more sensitive in the blue region of the spectrum. This observation fits in well with classical notions of the b-wave being relatively more scotopic than the a-wave.

In the frog visual system comparison of spectral curves obtained from low intensity on- and off- responses point to the off-response being relatively more sensitive to blue light. The differential sensitivity appears to be dependent on factors such as low level background illumination and weak test flashes. These findings may play a role in explaining the action of retinal green rods present in the frog eye.

The change in relative spectral sensitivity, when comparing tectal spectral curves to curves derived from the ERG in the cone eye of the turtle, suggests the presence of a possible coding system. The tectal curves show a prominent process near 540 mu not apparent in the peripheral record. It is evident for low intensity flashes in the dark-adapted eye, and appears to be absent when bright lights are used or when the visual system is light adapted. To ascertain whether this blue sensitivity is important to the animal, a behavioral experiment for determining thresholds is currently in progress. The results should be especially interesting in view of much anecdotal evidence that young turtles use this blue sensitivity to find their way to the water after being hatched.

Investigation of the parieto-visceral ganglion in aplysia has continued. The capability of a single cell to excite one cell and inhibit another has been implemented with further work. Acetylcholine applied to the cells receiving input causes hyperpolarization at one cell and depolarization at the other. It appears that the liberated transmitter is the same at different branches of the evoking cell and that the cells receiving input are uniformly differentiated so as to allow one of two responses. Further differentiation occurs as a function of the particular terminal process of the evoking cell involved. Evidence has been uncovered for a fairly precise spontaneous discharge pattern from two neurons in aplysia. The discharge is dependent on clock time and would imply production of an instructional code probably by some kind of temporal scheduling.

Operation of the digital computer reported last year has been considerably expanded. Evoked potentials in the occiput have been compared to the ERG by means of this technique. The transitional state of the responses to flickering light stimuli is of great interest. Most of the changes in potential occur at first presentation of the stimuli followed by rapid adaptation to some stable level. This is not surprising as it confirms various theories that view the nervous system as a detector of input changes. Further use of the computer to detect low level signals hidden in noise has been used in audition, in cortically evoked potentials in children, and in visually evoked potentials in adults. Its use has been also applied to data analysis utilizing various statistical techniques.

List of Publications:

1. Armington, J. C., & Biersdorf, W. R. Color Vision. Ann. Rev. of Psychol., (1963), 14, 93-114.

2. Biersdorf, W. R., & Granda, A. M. Effects of stimulus duration upon spectral sensitivity of the human electroretinogram. J. Opt. Soc. Amer., (1962), 52, 1402-1406.

3. Biersdorf, W. R. Effects of light adaptation levels upon the human electroretinogram. Paper presented at Vision Research Conference, Columbus, Ohio, May 20, 1963.

4. Biersdorf, W. R. Electrical responses of the human eye following intense chromatic pre-exposures. Paper presented at Eastern Psychol. Assoc. New York City, April 13, 1963.

5. Chapman, R. M., Armington, J. C., & Bragdon, H. C. A quantitative survey of kappa and alpha EEG activity. Electroenceph. clin. Neurophysiol., (1962), 14, 858-868.

6. Chapman, R. M. Comparison of spectral sensitivities of the on and off components of the electroretinogram. Paper presented at the Eastern Psychological Association Meeting, New York City, April 13, 1963.

7. Glanzer, M., Chapman, R. M., Clark, W. H., & Bragdon, H. C. Changes in two EEG rhythms during a problem-solving task. Paper presented at the Third Annual Scientific Meeting of The Psychonomic Society, St. Louis, August 1962.

8. Granda, A. M. Electrical responses of the light- and dark-adapted turtle eye. Vision Research, (1962) 2, 343-356.

9. Granda, A. M. & Biersdorf, W. R. Electrical responses of the human eye following intense chromatic pre-exposures. Vision Research, (submitted)

10. Granda, A. M. & C. E. Stirling. Spectral sensitivity of the turtle tectum. Paper presented at Eastern Psychological Association, New York City, April 13, 1963.

11. Kropfl, Walter J., Chapman, Robert M., & Armington, John C. Apparatus for scoring selected electroencephalographic rhythms. Electroenceph. clin. Neurophysiol., (1962), 14, 921-923.

12. Moushegian, G., Rupert, A. & Galambos, R. Micro-electrode study of ventral cochlear nucleus of the cat. J. Neurophysiol., (1962), 25, 515-529.

13. Moushegian, G. & Rupert, A. Binaural interaction in the superior-olivary complex of cat. J. Acoust. Soc. Amer., (1962), (Abstr.), 34; 1988.

14. Moushegian, G., Rupert, A., & Whitcomb, M. A. Response patterns of superior olivary units in cat to sounds. J. Acoust. Soc. Amer., (1963), (Abstr.) 35, 782.

15. Moushegian, G. Some parameters of unit response patterns in cochlear nerve of cat. J. Acoust. Soc. Amer., (1963), (Symposium) 35, 794.

16. Rupert, A., Moushegian, G. & Galambos, R. Unit responses to sound from auditory nerve of cat. J. Neurophysiol., (1963), 26, 449-465.

17. Rupert, A., & Moushegian, G. Olivocochlear and auditory unit responses in brain stem of cat. J. Acoust. Soc. Amer., (1963), (Abstr.) 35, 783.

18. Sheatz, G. C. An inclined plane magnifying ruler for EEG Y-axis measurement. Electroenceph. clin. Neurophysiol., (Submitted).

19. Strumwasser, F. Some operational principles in the central-nervous-system of Aplysia. Paper presented at Symposium on Comparative Neurophysiology, Leiden, Holland, 1962.

20. Strumwasser, F. A circadian rhythm of activity and its endogenous origin in a neuron. Fed. Proc., (1963), 22, 220.

21. Strumwasser, F. Post-synaptic inhibition and excitation produced by different branches of a single neuron and the common transmitter involved. XXII Int. Physiol. Congr., (1962), 2, 801.

22. Tepas, D. I., Armington, J. C. & Kropfl, W. J. Evoked potentials in the human visual system. Biological Prototypes and Synthetic Systems., (1962), 1, 13-21. New York: Plenum Press, Inc.

23. Tepas, D. I., & Armington, J. C. Properties of evoked visual potentials. Vision Res., (1962), 2, 449-461.

24. Tepas, D. I. & Armington, J. C. Electroretinograms from noncorneal electrodes. Investigative Ophthalmology, (1962), 1, 784-786.

ANNUAL PROGRESS REPORT

PROJECT: 3A 012501 B 813	Basic Research in Life Sciences
TASK 08	Neuropsychiatry (Analysis of the Structure of Behavior and Correlated Somatic Events)
REPORTING INSTALLATION:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D.C. Department of Neurophysiology Division of Neuropsychiatry
Period covered by report:	1 July 1962 through 30 June 1963
Principal Investigators:	Malvin Cole, Captain, MC Raymond Johnson, Ph.D.* Sidney Marvin, Lt Col, MC Miles Miller, Captain, MC Robert E. Robinson III, E.E.* Dr. Edwin A. Weinstein**
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ABSTRACT

Project: 3A 012501 B 813 Task 08	Basic Research in the Life Sciences. Neuropsychiatry (Analysis of the Structure of Behavior and Correlated Somatic Events)
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D.C. Department of Neurophysiology Division of Neuropsychiatry
Period Covered by Report:	1 July 1962 through 30 June 1963
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Further studies on behavioral responses to neurological lesions have been carried out. Changes in patterning mechanisms of language in patients with left brain lesions were compared to those associated with right brain damage. A failure of patients with lesions of the dominant hemisphere to use metaphorical speech was found. Also it was found that hallucinations are particularly apt to appear when there is a shift from one symbolic system to another. These studies have been extended to relationships of patients with delusional systems, the current stress and their socio-cultural background. Studies in Developmental Dyslexia and related syndromes and investigations of the breakdown of the learning process and its relationship to operant conditioning are being conducted. Conditioned emotional physiological response is being further explored to determine whether under increasing stress or motivation, generalization will be increasingly restricted or facilitated and whether some measureable dimensions of stimulus similarity are more vulnerable than others. A data processing system for physiological variables has been developed for displaying measurements in digital form for "on line" readout and for storeage in appropriate format for subsequent computer processing.

Body of Report

Project No. 3A 012501 B 813
Task 08

Basic Research in Life Sciences
Neuropsychiatry
(Analysis of the Structure of Behavior and Correlated Somatic Events)

Description

Behavioral effects of brain damage have been studied over the past several years in these laboratories. The changes in patterning mechanisms of language in patients with left sided brain lesions were compared to these associated with right brain damage by analysis of recorded interviews and special tests. Testing included the naming of several categories of objects, the ability to spell, calculate and rhyme and the use of idiom, synonyms, antonyms, homonyms and humor. The results (References 2 and 4) indicate the failure of (aphasic) patients with lesions of the dominant hemisphere to use metaphorical speech. They were unable to establish the limits of phonetic and semantic classes, to match and differentiate among the elements of a class, and could not use a stimulus element in a number of nominal categories. These forms of patterning were done accurately by patients with right brain lesions. Here the changes appeared in the interaction in the total environment, which involved the way the patient defined the situation in terms of his disability and cultural role.

A series of delusions expressed by patients admitted to Walter Reed General Hospital are being collected in screening interviews and classified to study symbolic patterning of delusional systems, current stress and socio-cultural background. These data will then be correlated with clinical evaluations of the patient's premorbid personality, cultural background, social class and the current stress leading to hospitalization in an attempt to determine the effect environmental forces and linguistic antecedents have had on the delusional content.

Studies are being conducted in problems of learning such as in Developmental Dyslexia and related syndromes. Also arrangments are being made and pilot studies have been started for further investigations of the breakdown of the learning process and its relationship to operant conditioning.

The experimental assessment of the effects of stress on speech is being conducted in studies in semantic conditioning. This data is then used in attempts to predict and interpret language changes. Under increasing stress or motivation the generalization of a conditioned emotional-physiological response may be increasingly restricted or facilitated. Also some measurable dimensions of stimulus simularity may be more vulnerable to stress than others.

Development of polygraph equipment into a data processing system for physiological variables has been accomplished. Parameters that are

currently processed by this system include the electrocardiogram for heart rate, ballistocardiogram, temperature, tissue optical density for pulse volume, respiration and pulse propagation time.

Progress

1. Studies of Behavioral Effects of Brain Lesions.

The changes in patterning mechanisms of language in patients with left brain lesions were compared to those associated with right brain damage by analysis of recorded interviews and special tests. Testing included the naming of several categories of objects, the ability to spell, calculate and rhyme and the use of idiom, synonyms, antonyms, homonyms and humor. The results (References 2 and 4) indicate the failure of (aphasic) patients with lesions of the dominant hemisphere to use metaphorical speech. They were unable to establish the limits of phonetic and semantic classes, to match and differentiate among the elements of a class, and could not use a stimulus element in a number of nominal categories. These forms of patterning were done accurately by patients with right brain lesions. Here the changes appeared in the interaction in the total environment, which involved the way the patient defined the situation in terms of his disability and cultural role.

Patients with disturbances of interaction in the environment, mainly those who had sustained acute head injuries and rupture of brain aneurysms, were studied in recorded interviews throughout the course of recovery. The behavioral sequelae were analyzed from the standpoint of metaphorical speech. In the stage of greatest disturbance of brain function, patients represented their disabilities in fictitious designations of events (confabulation) and place and time (disorientation, reduplication). With improvement of brain functions, the same problems appeared in more conventional idiom. For example, a patient with an ataxic gait originally could recall nothing of the events leading up to his accident. Subsequently, he said it had occurred when his car was "weaving back and forth" across the road. Patients also represented their problems in relationships with people involving the use of admonitions, threats, jokes, vows, clowning, imitation and expressions of worry, sympathy and affection. Many of the symbolic themes that appeared initially in confabulations and delusions, such as family, children, work and death, persisted in altered language patterns. Clinical descriptions of behavior such as "paranoid," "concrete," "obsessive" and "hostile" are being classified from the standpoint of alterations in metaphorical language -- that is words and gestures -- involving the correlation among milieu of brain function, circumstances of external stress and cultural role.

Aphasic patients do not use metaphor in a reorganization of their environment and adapt poorly to stress as in the "catastrophic reaction." The apparent exceptions are those patients with jargon and verbal stereotypy, and these forms of language may be regarded as attempts at metaphor analogous to confabulation (Reference 3). These observations support the need for a new classification of aphasia, abandoning the expressive-receptive models.

Analysis of the patient's account of the onset of his disability showed significant correlations with subsequent behavior. For example, patients who described their accidents in the idiom of physical violence had many somatic symptoms and frequently went on to develop conversion hysteria, in contrast to those who used other forms of language. The relationship of hallucinations to delusions was studied. The occurrence of hallucinations was found to depend not only on the location of the lesion, but on the rate of recovery of brain function and the rapidity of shifting from one symbolic system to another. Analogies to the occurrence of hallucinations when drugs such as barbiturates are withdrawn from an addicted person are suggested.

The correlation of the content of highly condensed metaphorical systems such as delusions and the premorbid social background has been carried out in another 40 brain injured subjects, and also in patients hospitalized for psychotic reactions. Along with the determination of the symbolic values, i.e., preferred channels of communication in the particular family, the variables of cultural group and social class are being used. In collaboration with Dr. Roy Eck, Psychologist at the Roanoke Veterans Administration Hospital, a group of 50 male patients with hallucinations and delusions was selected on the basis of low income, below eighth grade education and rural residence. The content of their delusions will be compared to that of high income, urban residence, college educated patients.

2. Symbolic Patterning of Delusional Systems, Current Stress and Socio-cultural Background.

A series of delusions expressed by patients consecutively admitted to Walter Reed General Hospital are being collected in screening interviews and classified according to their symbolic themes. These data will then be correlated with clinical evaluations of the patient's premorbid personality, cultural background, social class and the current stress leading to hospitalization in an attempt to determine the effect environmental forces and linguistic antecedents have had on the delusional content.

On approximately 50 selected delusional patients two types of verbatim interviews will be obtained emphasizing different descriptive areas. In one interview the events leading to hospitalization will be discussed to obtain a sample of the patient's delusional speech patterning. The second interview will be a routine discussion of the events of his past history to obtain a sample of his nondelusional speech patterning. One or more of the patient's close relatives will also be interviewed to obtain samples of the language patterning used in describing the patient's illness, background history and personality characteristics. These three interview samples will then be analyzed for common recurring themes and predominent areas of "symbolic valence" to determine if recurrent language patterns and symbolic themes in the speech of the relative and non-delusional speech of the patient are also reflected in his delusional productions.

In the analysis of the interviews for recurrent themes and patterns the data will be transferred to IBM punch cards and analyzed in an IBM 7090 computer in collaboration with Dr. Joseph Jaffe of Columbia University. These computed data will contain the incidence and location of each word used in the interview and will facilitate comparing word usage in the family group and between delusional and non-delusional productions of the same patient. These data will then be used in the designing of other computer programs to obtain further correlations using other elements and combinations of language patterning.

3. Studies of Problems in Learning.

Studies are being conducted in Developmental Dyslexia and related syndromes. Arrangements have been made to do psychological testing, neurological examinations, and electroencephalograms and skull X-rays where indicated. These tests are done on school children with these difficulties who are referred to an out patient clinic. These data are not yet complete and conclusions are premature at this time. Arrangements are also being made and pilot studies have been started for further investigations of the breakdown of the learning process and its relationship to operant conditioning.

4. Studies in Semantic Generalization.

In the long history of the experimental and clinical study of the brain damage and its behavioral consequences, it has been frequently easier to observe gross alterations of motivation or drive states than it has been to detect more subtle cognitive defects. This fact has prompted the suggestion that in trying to specify relationships between brain injury and performance decrements, motivation will be often an intervening variable of central importance. To explore more fully this involvement an attempt is being made to experimentally assess the effects of stress on speech and then to use this data in predicting and interpreting language changes as a function of brain damage. Specifically, a study is being conducted to determine whether, under increasing stress or motivation, the generalization of a conditioned emotional-physiological response will be increasingly restricted or facilitated and whether some measurable dimensions of stimulus similarity are more vulnerable to stress than others.

Stimulus words under current study were selected to represent three dimensions: 1) Similarity of Connotative Meaning. The Semantic Differential seven point scale of 20 paired adjectives provided a quantitative means of selecting words with near identical profiles on the scale. The degree of similarity was systematically varied; 2) Polarity of Connotative Meaning. The profiles of words, the points of which fall toward either extreme of the seven point Semantic Differential scale, are an index of the loading of meaningfulness. Words with a high degree of polarity tend also to be words which evoke many responses in a free association test; 3) Associative Clustering. Lists were compiled to include Kent-Rosanoff

stimulus words and the responses which those words had a high probability of eliciting in a free association test.

The following modification of a classical conditioning procedure is employed: 1) Preliminary Habituation Period. The stimulus list containing experimental and control words is presented to obtain base levels of physiological responsiveness (EKG, finger pulse, respiration, and GSR); 2) Conditioning Trials. Each word is then repeated and the subject asked to free associate to each one for 15 seconds. If the word to which the subject is responding is the excitatory stimulus (eventual CS), he receives a shock stressor (US) at the end of the free association period. The excitatory stimulus occurs several times in the list; 3) Recall Test. The subject is requested to recall as many of the list words as he can; 4) Extinction Trials. Each word is again repeated and the subject free associates to each one, but the shock US is omitted. It is during this set of trials that CRs and generalization are observed in the physiological response records; 5) Recall Test. The subject is once more requested to recall the list of stimulus words.

A balanced factorial design will permit an assessment of each of the three variables independently and in interaction among themselves at graded levels of experimental stress. To date, enough subjects have been tested to fill the cell representing the main effects of profile similarity, and data is currently being collected on the polarity x profile similarity interaction.

Technical Advances.

Development of polygraph equipment into a data processing system for physiological variables has been accomplished. Parameters that are currently processed by this system include the electrocardiogram for heart rate, ballistocardiogram, temperature, tissue optical density for pulse volume, respiration and pulse propagation time. Time of day and identification codes are added to these data. These measurements are displayed in digital form for "on line" readout and also are stored in appropriate format for subsequent computer processing.

Summary

Further studies on behavioral responses to neurological lesions have been carried out. Changes in patterning mechanisms of language in patients with left brain lesions were compared to those associated with right brain damage. A failure of patients with lesions of the dominant hemisphere to use metaphorical speech was found. Also it was found that hallucinations are particularly apt to appear when there is a shift from one symbolic system to another. These studies have been extended to relationships of patients with delusional systems, the current stress and their socio-cultural background. Studies in Developmental Dyslexia and related syndromes

and investigations of the breakdown of the learning process and its relationship to operant conditioning are being conducted. Conditioned emotional physiological response is being further explored to determine whether under increasing stress or motivation, generalization will be increasingly restricted or facilitated and whether some measureable dimensions of stimulus similarity are more vulnerable than others. A data processing system for physiological variables has been developed for displaying measurements in digital form for "on line" readout and for storeage in appropriate format for subsequent computer processing.

Publications

1. Weinstein, E.A. and M. Cole. Concepts of Anosognosia chapter in Dynamic Neurology. ed. L. Halpern, in press.

2. Weinstein, E.A. Affections of Speech with Lesions of the Non-Dominant Hemisphere. Read at the Meeting of the Association for Research in Nervous and Mental Diseases, New York, December 1962.

3. Weinstein, E.A., M. Cole, and M.S. Mitchell. Anosognosia and Aphasia. Read at the meeting of the American Neurological Association, Atlantic City, N.J., June 1963.

4. Weinstein, E.A. and N.J.A. Keller. Linguistic Patterns of Misnaming in Brain Injury. J. Neuropsychologia, vol 1, 1963.

5. Robinson, R.E. and S.L. Marvin. A Data Processing System for Physiological Variables. Submitted for publication.

ANNUAL PROGRESS REPORT

Project: 3A O 12501 B 813, Army Medical Basic Research in Life Sciences

Task: 08, Neuropsychiatry (Measurements of Performance and of Decrement of Performance)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Clinical and Social Psychology
Division of Neuropsychiatry

Period Covered by Report: 1 July 1962 to 30 June 1963

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ABSTRACT

Project: 3A 0 12501 B 813, Army Medical Basic Research in Life Sciences

Task: 08, Neuropsychiatry (Measurements of Performance and of Decrement of Performance)

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Research was continued on the nature of performance decrement with brain injury, and sleep loss. New tasks requiring the processing of digital information permitted the study of information load, speed load and storage and retrieval functions. Performance was also studied during natural sleep, and it was shown that sleeping subjects could perform simple discrimination tasks without awakening. Work on problem solving was continued and several steps in the problem-solving process were elucidated. Studies of the electrophysiological correlates of problem solving demonstrated a relationship between electroencephalographic patterns and problem difficulty. Research continued on the learning of syntactical structure by young children and adults. Longitudinal studies permitted the construction of syntactical models, and miniature languages for the analysis of the development of language. New studies of sensory perception were begun to identify the role of peripheral variables such as eye movements, size of retinal image, convergence and accommodation in visual perception. Work continued on the development of rational curve fitting techniques and the analysis of longitudinal and repeated measurements experiments.

BODY OF REPORT

Project: 3A 0 12501 B 813, Army Medical Basic Research in Life Sciences

Task: 08, Neuropsychiatry (Measurements of Performance and of Decrement of Performance)

Description:

This sub task has two purposes:

a. To develop methods for the analysis of behavior, particularly vigilance, data-processing and problem solving.

b. To apply techniques developed in this analysis to studies of the effects of brain injury, sleep loss and other stress.

The analysis involves both behavioral and physiological measurement, and studies have been completed of normal, sleep-deprived and brain-injured subjects. A series of inter-related projects have been carried out analyzing the effects of information-load, uncertainty and monotony on human data-processing. Studies of the encoding of information have been expanded to include several projects on the development and learning of language skills in children and adults. Various aspects of recent mathematical models from the field of psycholinguistics have been put to experimental test. Electrophysiological studies during sleep and waking have been expanded to include investigations of the effects of arousal, distraction and reinforcement on sensory evoked responses in man. Studies in the mathematics of measurement were expanded to a general investigation of rational methods of curve fitting, appropriate for use in repeated measurement experimental designs.

Progress:

a. Studies of problem solving and data-processing, storage of verbal material, verbal learning, and cognitive processes in language learning.

(1) Problem Solving. The purpose of the project is to study information-processing aspects of problem solving, and related problems of encoding and storage. Over the past year, work has been carried forward in three areas: problem solving; encoding of perceptual information; and learning and storage of verbal materials.

(a) Work Completed:

1. Information Processing in Problem Solving. A final series of experiments on the information processing aspects of solving concept problems was completed. The experiments on the solving of concept problems have explored the following factors:

(a) Example sign: whether the examples are positive or negative instances.

(b) Concept size: the ratio of the number of relevant dimensions to the total number of example dimensions.

(c) Series complexity: the presence of superfluous information.

(d) Information order: the sequence of examples in series consisting of mixed positive and negative examples.

(e) Storage load: the amount of information that has to be stored at the beginning of a problem.

(f) Selection load: the amount of information required to sort the example dimensions into relevant and irrelevant.

(g) Information rate: the rate at which new information is presented within the example series.

The findings make it possible to control the probability of solution of the concept problems. They also make it possible to specify in considerable detail the systematic operations carried out by the subjects in solving the problems. The operations consist of two distinct stages: 1) specification and storage of dimension values, 2) selection of relevant dimensions on the basis of example information. A general summary of a series of seven experiments carried out under the project is now in press (Psychological Monographs).

2. EEG Concomitants of Problem Solving. Experimental work was completed in a study of the relations between stages of problem solving and characteristics of the EEG output. An extended series of concept problems and control tasks was given to 12 subjects while EEG recordings were made from both the occipital and parietal areas, to measure changes in the output of both alpha and kappa waves. The results show systematic and predictable changes in alpha for each stage of the problem solving. The results for kappa, however, show marked individual differences. These results contrast with the results of preceding work by Chapman, Armington and Bragdon, who, using somewhat simpler mental operations tasks--mental addition--found that kappa showed marked regularity and predictability while alpha showed relatively weak effects.

(b) Work in Progress:

1. EEG Concomitants of Problem Solving. Experimental work has been started aimed at analyzing the differences between the findings of the concept problems study (described above) and an earlier study by Chapman, Armington and Bragdon, using mental addition tasks. The hypothesis being tested is that the changes found in both kappa and alpha are a function of the sense modalities involved in the tasks given to subjects. (One of the differences between the concept problems and the mental addition problems is that the former were presented visually, while the latter were presented auditorily). To test this hypothesis a set of tasks--addition problems--has been constructed which can be presented either visually or auditorily and which have been calibrated at three difficulty levels. These problems

will be presented to subjects and both kappa and alpha recorded. It is hypothesized that clear differences in kappa as a function of problem difficulty will appear with auditory, but not visual presentation, while clear differences will appear in alpha with visual but not auditory presentation.

(2) Encoding in Perception. The work on problem solving underlined the importance of the subjects' encoding mechanisms in the storage and processing of information. In order to study the encoding mechanisms in the framework of relatively simple tasks, a series of studies was carried out on the role of encoding in perceptual recall. The main outcome of these studies is a radical simplification of the problem of form perception.

(a) Work Completed:

1. Experiments with Systematically Generated Stimuli of the Type used in Information Theory Studies. The first series of experiments was carried out to analyze the determinants of the difficulty of perceptual recall of a systematically varied set of stimuli. The stimuli were arrays of eight shapes that were each either black or white. First, the accuracy with which subjects could reproduce these arrays under 1/2 sec. exposure was determined. Then, another method involving discrimination between arrays was used to determine the difficulty of the individual stimuli. The discrimination method yielded a similar ranking of accuracy scores. This indicated that the accuracy scores obtained with the reproduction method were not a function of the particular method used. The accuracy scores were then subjected to various types of analysis. An analysis based on information measure showed only partial success in accounting for the difficulty of individual stimuli. Analysis based on gestalt theory was also found to be unsatisfactory. Another type of analysis was constructed, based on the hypothesis that the subjects' perceptual processing includes a covert verbal encoding and that the length of the verbal code determines the difficulty of the stimulus for perceptual tasks. This was labelled the verbal loop hypothesis. Empirically-derived measures based on this hypothesis were shown to account for a major part of the variance in stimulus difficulty. The relevance of the assumption and the findings to the general problem of perceptual organization and encoding is discussed. The verbal loop hypothesis is presented as an alternative to gestalt and information theory analyses of organization. A paper describing this work has been published (Journal of Verbal Learning and Verbal Behavior).

2. Experiments with Binary Numbers. Further experiments were carried out to test the generality of the verbal loop hypothesis--the hypothesis that the subject's perceptual processing includes a covert verbal encoding and that the length of the verbal code determines the difficulty of the stimulus. In one experiment, a relation was demonstrated between verbal code length and the accuracy for binary numbers over the range from 0 to 11111111 (decimal 255). Since these numbers varied in length from one to eight digits, an alternative explanation based on the physical length of the stimulus

was considered. Predictions on the basis of number of digits were almost as effective as predictions on the basis of verbalization length. In another experiment, it was demonstrated, using binary numbers that had all been converted into eight digit form (e.g., 0 becomes 00000000), that the relation between verbal code length and accuracy is the more general one. The relation between length of the verbal code and accuracy remains invariant even when the effect of the physical length of the stimulus is eliminated. A third experiment replicated the results of the preceding experiment. A paper describing this work is now in press (Journal of Verbal Learning and Verbal Behavior).

3. Experiments with Conventional Figures. The final set of experiments carried the work on the verbal loop hypothesis to conventional figures of the type used in the studies of gestalt factors in perception. Procedures similar to those used in work described above were used. In one experiment, a relation was demonstrated between verbal code length and the difficulty of such conventional figures. In another experiment, a relation was demonstrated between the verbal code length and the judged complexity of these figures. This last set of experiments completes the demonstration of the generality of the verbal loop hypothesis. The conventional figures that were used in these experiments have proved intractable to analysis by either information theory or gestalt theory. A paper describing this work has been accepted for publication (American Journal of Psychology).

(b) Work in Progress: Effects of Exposure Time and Delay on Perception. Several implications of the verbal loop hypothesis are now being tested in an experiment in which the following are systematically varied: exposure times of stimuli, the time the subject must wait before reporting what he has seen, the presence or absence of an interpolated task during this delay period. Measurements are being made of the effect of these variables on accuracy of report of stimuli (binary numbers) which have been calibrated with respect to the verbalization lengths (encoding difficulty) they generate.

(3) Learning and Storage of Verbal Materials (Adult Subjects). Work has continued on the analysis of serial position effects in both rote learning and free recall of verbal materials. The general hypothesis motivating the work is that serial position effects can be used as a lever to discover the inner structure of storage mechanisms in human subjects.

(a) Work Completed:

1. Serial Position Effects in Rote Learning. An experiment was carried out to evaluate the hypothesis that serial position effects in verbal learning are based on facilitative effects. This type of explanation is opposed to the classical explanations in terms of inhibitory or interference effects, e.g., the Hull-Lepley hypothesis. One hundred and forty college students were given serial learning tasks in which particular items were facilitated by pretraining.

The results of this and earlier experiments indicated that neither the facilitative nor the inhibitory explanations are correct. The results of the experiments indicate rather strongly that attempts to handle the serial position effect as a result of the interaction of items in the list is incorrect. It seems now that the serial position effect is more likely to be a result of a strategy that the subject adopts in dealing with the list. One version of this view is that the subject generates the serial position effect on the basis of instructions that he gives himself at the start of the learning task concerning the structure of the list. Following up this idea, another experiment was carried out in which an attempt was made to bring these hypothesized instructions under experimental control by having the experimenter indicate verbally the beginning and middle of the list. A group of 60 subjects was run under the following experimental conditions. For half the subjects, the verbal instruction agreed with the physical appearance of the list (i.e., the experimenter identified the first item that appeared on the memory drum as the start of the list). For the other half of the subjects, it was in opposition (i.e., the experimenter told the subject that the first item that appeared was the middle of the list). The results indicate that verbalization plays a major role in generating the serial position effect and that the explanation of this effect in rote learning must be sought in terms of self-instruction by the subject.

(b) Work in Progress: Serial Position in Free Recall. Previous work on the free recall of verbal material in which presentation rate and amount of delay before recall were systematically varied has indicated that the subject makes use of two types of storage--short term storage and long term storage. The nature of these two storage mechanisms was examined further in an experiment in which presentation rate and number of repetitions of each item in the recall list was systematically varied. Twelve groups of subjects with 20 subjects in each group were tested, each on a series of eight 20-word lists. The data of this experiment are currently being analyzed.

(4) Cognitive Processes in Language Learning. The work continues to be organized into two separate but complementary sets of investigations. The focus of one line of investigation is on grammatical structure; here the main goal is to identify the cognitive processes involved in children's learning of the grammatical structure of language. The other area is concerned with the semantic aspect of language development, and the ultimate goal is to describe some of the conceptual correlates of verbal behavior, particularly those which seem to relate to so-called "abstract" thinking.

(a) The learning of the structure of artificial languages. An article, which reports a series of experiments on the learning of miniature artificial languages and proposes a theory of the learning of grammatical structure, has been revised and is now in press (Psychological Review). A further series of experiments on the learning of miniature linguistic systems has just begun. These experiments explore the learning of the roles of "function" morphemes (i.e., short, frequently recurring

elements such as articles, auxiliary verbs, prepositions, conjunctions, etc.). It is hoped that these new experiments will permit the theory developed in the previous work to be extended to account for the learning of grammatical transforms (i.e., for example, such relations between sentences as active-passive).

(b) The gathering of data on the way English grammatical structure developes in a small sample of children between 18 and 30 months of age has now been completed. The data were gathered by extensive tape-recordings, supplemented by written records kept by the mother. An article describing the earliest phase of development has been published (Language). The data are in good agreement with the ideas developed from the experimental work on the learning of the miniature artificial languages, i.e., that "what is learned" may be the temporal positions of words within phrases, and of phrases within a sentence.

(c) The investigation of conceptual correlates of language development has concentrated on studying the development in children of the ability to make certain kinds of distinctions, which earlier work has suggested may be related to the development of the concept of a logical "type" or "class." One such distinction is that between "real" and "apparent" (i.e. phenomenal) attributes of objects. Three experiments have now been completed in which child subjects have to learn to respond to the true attribute (which has to be remembered or inferred), and ignore the phenomenal attribute; in half the trials the appearance of the test stimuli is distorted (e.g., the apparent size altered by a lens, or the phenomenal shape altered by part-immersion in water). The data indicate that the ability to acquire such distinctions is closely related to age, and that the average age at which the distinction between real and phenomenal attributes is made is independent of the attribute involved (e.g., size, or shape), and of details of experimental procedure. The ability may define an important "level" of intellectual development. Reports on these experiments are in process of preparation.

(d) The development of "abstract" thinking is also being investigated through study of children's conception of relations of sameness, difference, and similarity. The method is to train very young subjects to respond to an identical pair of figures and to avoid a nonidentical pair. When a reliable first-trial-correct response has been developed, the subject is tested with generalization stimuli which involves stimulus-dimensions not used in the learning, or in which the positive pair of figures is merely similar (i.e. not identical). Children appear to show a good deal of generalization as young as they are testable, and the data suggest that the difficulty of detecting similarities is a function of the number of stimulus-dimensions which the subject has to scan in order to detect the similarity. Age-differences in this task seem to be differences in ability to scan a number of dimensions, rather than differences in "abstract thinking." A paper which includes a previous experiment on this subject has been published (Child Development Monograph), and a more recent experiment is being prepared for publication.

b. Studies of performance in drowsy states, sleep and with brain injury. The general plan of these experiments is to measure changes in performance on behavioral and physiological variables during sleep deprivation, normal sleep and with brain injury. The investigation encompasses four experimental studies. The studies include analyses of basic sensory processes in normal states.

(1) The analysis of performance before, during and after acute sleep deprivation.

(2) The study of performance and physiological patterns during deep drowsiness or natural sleep in order to measure depth of sleep, and the limits of data processing capacity during sleep.

(3) The experimental analysis of decrement in performance due to brain injury.

(4) The investigation of the physiological and psychological bases of visual perception.

(a) Experiment 1 - The effects of Acute Sleep Deprivation on Performance. This investigation studies the effects of one night of sleep loss on several performance tasks, and measures certain electrophysiological correlates of decrement. Earlier studies had shown that speed-load (rate of information processing) interacted with sleep loss to produce maximal impairment of tasks which required continuous data processing. Current studies examine the effect of information-load (number of alternatives) during sleep loss. A self-paced serial reaction time test is used where the number of alternatives in the display is varied between 4 and 10. Earlier studies had shown that sleep loss causes impairment of recall, and that impairment persists even when input is under control. That is the deficit in memory is not simply a function of failure of the sleep deprived subject to see or hear the stimulus display. Current studies examine whether this decrement in memory is due to a failure of storage or retrieval operations. Pilot studies suggest that retrieval functions are largely intact during moderate acute sleep loss. Earlier studies showed that background EEG rhythms could be used to predict missed signals on auditory vigilance tasks. Current studies investigate evoked electrical responses as predictors of performance.

(b) Experiment 2 - Discrimination of Auditory Stimuli During Sleep. This experiment examines the ability of human subjects to perceive and discriminate among auditory signals during natural sleep. A pilot study evaluated the effect of reinforcement on perception and response to a single tone presented periodically throughout the night. Sleep was monitored continuously by the EEG. Reinforcement procedures which converted the tone into a warning signal were effective in inducing avoidance responding in all stages of sleep. The effect of reinforcement was greatest for the emergent rapid eye movement (REM) stage that has been associated with dreaming. Here, when reinforcement was added, the probability of responding rose from approximately 0 to approximately .80. At this point, the subject's task was complicated by the introduction of a second tone. The subject had to discriminate between two tones.

In the absence of differential reinforcement, probability of responding to the critical signal was a direct function of background frequency, and an inverse function of background amplitude. Differential reinforcement had the same effect as in the pilot study. Subjects were able to switch their responding from one tone to the other with facility.

(c) Experiment 3 - Acquisition and Control of Operant Behavior During Sleep. Previous studies showed that human subjects were capable of executing rather complex, well-learned response sequences during sleep. Current studies attempt to bring such performance under the control of new stimuli without awakening the subject. If this can be accomplished a type of learning during sleep will have been demonstrated. Another experiment attempts delayed classical conditioning during the various stages of human sleep. The initial focus of research concerns the temporal parameters necessary to accomplish conditioning using paired tone and shock as the dependent variables and finger flexion as the independent variable. Results to date indicate the appearance of conditioned responses during sleep but behavior is not as consistent as in the waking state.

(d) Experiment 4 - Sensory Evoked Responses in Sleep, Waking and Fasting Subjects.

1. Average evoked electrical responses to clicks show consistent changes in morphology and amplitude as a function of the background stage of sleep. The amplitude of early components increases and of late components decreases as a function of the background EEG amplitude. Localization studies showed that the click evoked response had maximal amplitude at the vertex, and a study of intensity showed that amplitude was a direct function of the loudness of the click. The late components of the evoked response show average latencies and wave forms which are similar to the K-complex described by the Davises. A study is being planned to establish this identity.

2. Studies of animals and humans with implanted electrodes have shown that the evoked response to a repeated stimulus is diminished upon the introduction of a second distracting stimulus. Our current studies show that the amplitude of the late components of the response to clicks is reduced when the subject is engaged in a continuous mental task. The response is enhanced when the clicks are treated as a vigilance task. Our present investigation compares two possible interpretations of these results, an "arousal" hypothesis and a "distraction" hypothesis. Another investigation studies the influence of fasting (blood sugar level) on the auditory evoked response. For one subject the amplitude of the late components of the evoked response was increased during fasting, and reduced to baseline levels after oral administration of dextrose. Studies of the evoked response of various states, and stimulus conditions are continuing.

c. Studies of Brain Injury. A pilot study of selected neurosurgical patients used a general performance test apparatus which measured speed and accuracy in the handling of digital information. Speed-load and information-load were varied independently. Each of these stress variables caused decrement in the performance of brain injured patients, and their combined effect was synergistic.

f. Measurement of Human Retinal Images. Previous experiments on the nature of light distributions in human retinal images were extended to include the use of monochromatic as well as white light. The image forming properties of the eye were found to be no better with monochromatic light, leading to the conclusion that the departure of the human optics from that of an equivalent ideal system is due primarily to spherical (or irregular) aberration. The suggestion, in the optical literature, that better images of points, grids and lines ought to be produced with annular as opposed to circular pupils was tested with negative results. These experiments revealed, however, that less light is reflected through the margin of the pupil than the center. This phenomenon, not in agreement with simple optical theory, but resembling certain psychophysical effects is being further investigated.

(1) Stabilized Images. An apparatus for nullifying the effects of small eye movements is being built. Experiments are planned in which visual acuity will be measured with the retinal image of the target essentially motionless on the retina and with artificially induced sinusoidal motions of various frequencies and amplitudes.

(2) Measurement of Human Photopigments. The photoelectric ophthalmoscope used for measuring retinal images is being modified so that it may be used for the measurement of human photopigments in the intact eye. The purpose of the planned experiments is to extend the findings in this area by applying the computer averaging techniques and equipment developed in this laboratory for the improvement of signal-to-noise ratio in electrical measurements.

g. Studies of Physiological Nystagmus. This research is designed to examine the hypothesis of correlation between visual stimulus patterns and fine eye movements. A reflecting surface attached to a contact lense is used to measure fine eye movements optically and electrically. During the past year major effort has gone into improving the sensitivity and reliability of the measuring system. Studies are planned involving comparison of eye movements to various stimuli in normal and schizophrenic subjects.

Summary:

In a new sleep deprivation project, using military volunteers, research has continued on the precise nature of performance decrement. The effects of information-load and speed-load are being tested, as well as electrophysiological correlates of decrement. The studies of sleep loss include a series of experiments on memory. Procedures were designed to reveal whether the memory deficit observed during sleep loss is due to impairment of storage functions, retrieval functions or both. A continuous adding test developed from earlier studies of sleep loss was administered to a group of patients with closed head injuries. The test was designed to permit variation of information-load and speed-load. In the brain injured group there was evidence of interaction between the effects of these two variables. Control subjects showed impairment only under increasing speed-load. Studies of performance with sleep deprivation were extended to the

(1) Japanese B Encephalitis Study. A collaborative study with the Veterans Administration has continued on the follow-up of Japanese B. Encephalitis. A small number of matched control subjects obtained from VA files were given the follow-up test battery. In both the Japanese B group and the control group, scores on Army tests were higher at this testing than they were at Army entry in 1948 to 1951. The Japanese B group had shown temporary decrement during the acute phase of the illness. The increase in scores of a 12 year period raises questions concerning the validity of cross-sectional data in the literature. All cross-sectional studies have reported a drop in intelligence scores over the age range represented here. On the other hand one other longitudinal study has shown an increase in individual scores.

d. Exploration of Optimal Test Scoring Methods and the Analysis of Repeated Measurements. In physiological and psychological experiments where growth and decay processes such as learning, habituation, extinction, etc. are involved, there is some evidence that asymptotic growth curves such as the logistic, Gombertz, Mitscherlich, and hyperpola can adequately represent the results. A computer program and tables for fitting the Mitscherlich have been developed. A modified form of the Mitscherlich was devised which will fit most learning and habituation curves. A program for fitting the logistic is being explored. In the past decade J. I. Lacey and J. Wilder have attempted to show that the Law of Initial Values (LIV) applied to all autonomic measurements. In cooperation with L. C. Johnson of the San Diego Neuropsychiatric Research Center we have made some theoretical and emperical studies which suggest that the LIV may apply to autonomic variables such as heart rate and breathing rate where there is a direct neural negative feed back, but probably does not apply to GSR and peripheral vasoconstriction where there may be no direct neural feedback mechanism. Some equations have been developed combining the effects of initial level and habituation.

e. Studies of Sensory Processes and Perception. Work is continuing on the project of relating judgments of apparent size and distance of objects to the physiological variables of accommodation and convergence of the eyes: Experimentation has proceeded with the use of a blacked-out four-meter "vision tunnel" within which the visual targets are located to minimize distance cues. Judgments of apparent size and apparent distance are obtained from subjects in monocular viewing under two types of instructions: "equidistant," and "objective." Performances of subjects thus far has fallen into two classes: a) subjects who produce the same size judgments under both sets of instructions, and who perceive relative distance significantly reversed, i.e., they perceive the farther of two targets as being closer to them; and b) subjects who produce different size judgments under the two sets of instructions, and who perceive relative distances in the correct order. All subjects produce judgments of apparent distance which are much foreshortened from the physical values.

analysis of performance during natural sleep. It was shown that sleeping subjects could perform simple discrimination tasks without awakening, and that reinforcement of performance was possible during the sleeping state. Attempts to demonstrate simple conditioning and instrumental-response learning during sleep have had limited success. Work has continued on the storage and processing of information during problem solving. Considerable evidence developed to show that human subjects normally transform perceptual displays into verbal codes as an initial step in problem solving. Further operations on these perceptual data are done on the verbal code rather than on the data. Studies of the electrophysiological correlates of problem solving demonstrated a relationship between electroencephalographic patterns and problem difficulty. Research has continued on the learning of syntactical structure and grammatical categories in the language of young children and adults. Collection of complete samples of language in a longitudinal study of young children permitted the construction of syntactical models for the speech of children which could be compared with current models of adult speech. On the basis of such models, miniature languages were devised to study acquisition of parts of speech and sentence structure. Work has continued on the development of rational curve fitting techniques and the analysis of longitudinal and repeated measurement experiments. New studies of sensory perception were begun. These included precise measurements of basic peripheral variables (eye-movements, size of retinal image, convergence, accommodation) and visual perception.

List of Publications:

1. Biersdorf, W. R., Ohwaki, S., and Kozil, D. J. The effect of instructions and oculomotor adjustments on apparent size. Amer. J. Psychol, 1963, 76, 1-17.

2. Braine, M. D. S. Piaget on reasoning: A methodological critique and alternative proposals. In W. Kessen and C. Kuhlman (Eds.), Thought in the Young Child. Monogr. Soc. Res. Child Develpm., 1962, 27, 41-63.

3. Braine, M. D. S. The ontogeny of English phrase structure: The first phase. Language, 1963, 39, 1-13.

4. Braine, M. D. S. On learning the grammatical order of words. Psychol. Rev., in press.

5. Gaarder, K. R. Relating a component of physiological nystagmus to visual display. Science, 1962, 132, 471-472.

6. Gaarder, K. R. Alternative physiological mechanisms of information transfer between the retina and the brain. British Journal of Physiological Optics, in press.

7. Glanzer, M. and Peters, S. C. Re-examination of the serial position effect. J. exp. Psychol., 1962, 64, 258-266.

8. Glanzer, M. Grammatical category: A note learning and word association analysis. J. verb. Learn. & verb. Behav., 1962, 1, 31-41.

9. Glanzer, M. Toward a psychology of language structure. J. speech hear. Res., 1962, 5, 303-314.

10. Glanzer, M. Experimental study of team training and team functioning. In R. Glaser (Ed.), Training Research and Education. University of Pittsburgh Press, 1962, Pp. 379-407.

11. Glanzer, M. and Clark, W. H. Accuracy of perceptual recall: An analysis of organization. J. verb. Learn & verb. Behav., 1963, 1, 289-299.

12. Glanzer, M., Huttenlocher, J. and Clark W. H. Systematic operations in solving concept problems: A parametric study of a class of problems. Psychol. Monogr., in press.

13. Glanzer, M., and Clark, W. H. The verbal loop hypothesis: Binary numbers. J. verb. Learn. & verb. Behav., in press.

14. Glanzer, M. and Clark, W. H. The verbal loop hypothesis: Conventional figures. Amer. J. Psychol., in press.

15. Lubin, A. and Lana, R. E. Chi square revisited. Amer. Psychologist, 1962, 17, 793.

16. Lubin, A. The effect of correlation on the repeated-measurements design. Educ. psychol. Measmt., in press.

17. Wilkinson, R. T. Muscle tension during mental work under sleep deprivation. J. exp. Psychol., 1962, 64, 565-571.

18. Wilkinson, R. T. Interaction of noise with sleep deprivation and knowledge of results. J. exp. Psychol., in press.

19. Wilkinson, R. T. After-effect of sleep deprivation. J. exp. Psychol., in press.

20. Wilkinson, R. T. Artificial "signals" as an aid to inspection work. Ergonomics, in press.

21. Wilkinson, R. T. Effects of up to 60 hours's sleep deprivation on different types of work. Ergonomics, in press.

22. Williams, H. L., Tepas, D. I. and Morlock, Henry, C. Jr. Evoked responses to clicks and electroencephalographic stages of sleep in man. Science, 1962, 138, 685-686.

23. Williams, H. L., Morris, G. O. and Lubin, A. Illusions, hallucinations and sleep loss. In L. J. West (Ed.), Hallucinations. New York: Grune & Stratton, Inc., 1962, 158-165.

24. Williams, H. L. Psychologic testing. In A. B. Baker (Ed.), Clinical Neurology. New York: Hoeber-Harper, 1962, Pp. 389-406.

25. Williams, H. L. and Loveland, N. T. Adding, sleep loss and body temperature. Percept. mot. Skills, in press.

26. Williams, H. L. Sleep and waking. International Encyclopedia of the Social Sciences, in press.

27. Williams, H. L., Morlock, H. C., Jr., Morlock, J. T. and Lubin, A. Auditory evoked responses and the EEG stages of sleep. Ann. NY Acad. Sci., in press.

28. Williams, H. L., Hammack, J. T., Daly, R. L., and Dement, W. C. Responses to controlled auditory stimuli and the EEG stages of sleep. Electroenceph. clin. Neurophysiol., in press.

Papers Presented:

1. Braine, M. D. S. Developmental aspects of syntactical relationships in verbal behavior. Paper read at Study Section on Verbal Behavior, American College of Neuropsychopharmacology, Washington, D. C., January, 1963.

2. Glanzer, M. Language structure: Experimental and Developmental Aspects. Paper read at the University of Michigan, Speech Clinic, Ann Arbor, Michigan, August, 1962.

3. Glanzer, M., Chapman, R. M., Clark, W. H., and Bragdon, H. R. Changes in two EEG rhythms during a problem-solving task. Paper read at Psychonomics Society, Washington University, St. Louis, Missouri, August, 1962,

4. Glanzer, M. Systematic operations in solving concept problems. Paper read at Symposium on Concept Attainment, American Psychological Association. St. Louis, Missouri, September, 1962.

5. Glanzer, M. and Clark, W. H. The verbal loop hypothesis: Binary numbers. Paper read at 34th Annual Eastern Psychological Association Meeting, New York City, April, 1963.

6. Morlock, H. C., Jr., and Williams, H. L. The reinforcement of performance during sleep. Paper read at 34th Annual Eastern Psychological Association Meeting, New York City, April, 1963.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 B 813, ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task 09 **Physiology (Wound Healing)**

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Surgical Metabolism and Pathology
Division of Basic Surgical Research

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project 3A 0 12501 B 813, ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task 09	Physiology (Wound Healing)
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
Period Covered by Report:	1 July 1962 through 30 June 1963
Authors:	Edwin J. Pulaski, Col, MC, Costan W. Berard, Capt, MC, John B. Herrmann, Capt, MC, Stephen C. Woodward, Capt, MC, Smith Shadomy, 1/Lt, MSC, John W. Diggs, B. S., Lee D. Lewis, B. S., SP4 Clifford G. Rea, PFC Wilbur Pumpaly, PFC George Nokes
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In the past year the Department of Surgical Metabolism and Pathology has extended and broadened its investigative approaches into the basic mechanisms of mammalian wound healing. An additional parameter of the healing of dermal incisional wounds, namely orientation, has been added to those previously defined (time, skin thickness, and age of the animal). A new microporous surgical adhesive tape has been evaluated for skin closure and found to be as satisfactory as conventional suture technique under only optimal circumstances of apposition and adhesion. The multiple disciplinary approach to wound healing developed in this laboratory has been modified and applied to intestinal healing; to the standard biochemical, physical, and histologic dimensions of this study have been added a microbiological parameter. Work has been initiated on an evaluation of the use and histotoxicity of adhesive polymers for restoration of tissue continuity after injury.

BODY OF REPORT

Project 3A 0 12501 B 813, ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task 09 **Physiology (Wound Healing)**

Description:

(1) Fractionation of mature mammalian collagens
(2) Role of ascorbic acid in collagen synthesis
(3) Healing of incisional wounds in rats
(4) Evaluation of surgical suture materials
(5) Studies in colon wound healing
(6) Adhesive polymers -- use and toxicity.

Progress:

(1) Fractionation of mature mammalian collagens: In previous progress reports of this department there have been reported the details of a chromatographic method by which mammalian collagens from widely differing sources can each be shown to consist of at least four major components. One of these components, moreover, has chemical and physiological characteristics consistent with the predicted qualities of a collagen precursor. Research into this area of collagen metabolism has since become intensive at other institutions and simultaneously work here has been directed at other facets of wound healing and collagenogenesis.

(2) Role of ascorbic acid in collagen synthesis: Although extensive clinical and experimental data exist to show that wound healing is impaired in scurvy, the pathogenesis of the defect has defied definition. It has long been established that a major component of the syndrome is failure to produce reparative collagen. Work of this department over the past several years has involved careful histologic, biochemical, and physical quantitation of the magnitude of the collagen defect in scurvy and its restitution to normal when ascorbic acid is supplied to previously depleted animals. It has been noted in the course of this work that ivalon sponges implanted into the abdominal wall of scorbutic guinea pigs and removed seven to twenty-one days later often seemed to be floating in a pool of fluid blood. It was hypothesized that an anticoagulant substance might be present and that such a substance, if present, might inhibit or break down reparative collagen in its earliest extracellular phase of formation. The hypothetical substance was initially sought by extraction of scorbutic sponges for heparinoid activity by the techniques of Monkhouse and Jaques. Several trials all failed to reveal such activity. As a second step, fresh frozen sections of fourteen and twenty-one day

polyvinyl sponge implants in young guinea pigs were incubated with fresh blood plasma from normal and scorbutic animals. There was no evidence of any collagenolysis in either system although such sections were totally devoid of reparative collagen after a brief incubation with collagenase from Clostridium histolyticum. It is thus concluded that neither heparinoid nor collagenolytic activity can be demonstrated in the plasma or extravasated blood of scorbutic animals and that the collagen defect probably reflects a basic failure in synthesis rather than an imbalance of anabolism and catabolism.

(3) Healing of incisional wounds in rats: This department has in previous progress reports presented extensive data on the strength and histologic appearance of healing dorsal skin incisions in rats. It has been shown that such incisions continue to increase in strength for a period in excess of three months and that the histologic appearance of the scar is not final even at one year. These investigations have established that one parameter of wound healing, namely, time, is greatly in excess of that previously considered as adequate for completion of the process.

Another parameter, thickness of the healing skin, was evaluated in a subsequent progress report and it was shown that in rodents the skin thickness is directly proportional to body weight. Furthermore, the breaking strength of a healing skin incision, as measured for a strip of standard width, is directly proportional to the thickness of the skin at the wound site. This is to be expected since the strength of any tissue (or other material for that matter) is a function of its cross-sectional area. Despite this fact, almost none of the published studies on healing of skin wounds consider skin thickness when comparing wound strengths between various groups of animals. Such studies therefore represent data on "breaking strength" and are frequently erroneously interpreted as "tensile strength," which is actually breaking strength/unit area.

Studies of both breaking strength and thickness of skin incisions for several months postoperatively made it apparent that such data must be considered in the light of known changes in normal skin with age. In the last progress report there was presented detailed information on the chemical and physical changes in unwounded skin of litter-mate rats at various ages and weights. These data reflect importantly on the influence of age on both normal skin and the healing of skin incisions.

Studies this year have been performed to define the importance of yet another factor in the healing of incisional wounds in rats. This factor is orientation (or direction) of the skin incision. Since

the earliest description of lines of skin tension by Dupuytren and Langer, no basic work has been done on the significance of such lines to wound healing. There are abundant clinical observations that incisions oriented parallel to such lines seem to heal "better" and result in a superior cosmetic result but none of these studies include characterization of either the histologic appearance, breaking strength, or thickness of such incisions when contrasted with surgically identical incisions perpendicular to the skin lines. To derive such information, this department has studied the effect of orientation upon these characteristics in both unwounded skin and healing incisions.

It was shown (Table I) that the tensile strength of normal rat skin is significantly greater parallel to skin lines than at right angles to such lines. This observation is consistent with the

Table I. Tensile Strength of Normal Skin

Orientation	No. of Animals	Mean ± S. E. Body weight grams	Thickness (mm.)	Tensile strength (g/mm^2)
Perpendicular to skin lines	10	337 ± 8	1.26 ± 0.06	777 ± 61
Parallel to skin lines	10	353 ± 14	1.24 ± 0.07	994 ± 80
P value		No difference	No difference	<.05

evidence from x-ray diffraction that normal dermal collagen is oriented predominantly parallel to such lines. It is generally accepted that collagen is primarily responsible for the tensile strength of tissue and it might thus be predicted that tensile strength would be greater parallel to skin lines. This study is, however, to our knowledge the first experimental corroboration of this fact and emphasizes the importance of orientation to statements of the tensile strength of tissue.

Subsequent experiments (Tables II and III) demonstrated that the healing dorsal skin incision varies physically and morphologically depending upon its orientation. At both fourteen and twenty-one days, incisions parallel to skin lines are significantly thicker than incisions oriented perpendicularly. This is surprising in view of the usual clinical impression that crossing of skin lines leads to a more

unsightly scar. It may be that the less satisfactory cosmetic result seen clinically is more a factor of scar width than of thickness, contrary to popular impression. It was similarly surprising to find that incisions parallel to skin lines are actually weaker at 14 and 21 days than incisions perpendicular to such lines. This finding suggests re-examination of the usual concept that parallel incisions heal "better." Certainly under the conditions of these studies, the incision parallel to a skin line had no advantage in thickness or strength in comparison to a perpendicular incision. The principal morphologic difference between the two types of incision was found at the junction of newly forming connective tissue and pre-existing dermal collagen. The thinner, stronger perpendicular incision showed predominantly smooth interdigitation between new and old fibers while the thicker, weaker parallel incision had a junctional area of rather abrupt, claw-like communications. These studies on orientation have since been extended to healing of Z-plasties and full-thickness and split-thickness skin grafts but progress has been slow because of difficulty in working out a suitable experimental model. Results are too preliminary for reporting at this time.

Table II. 14-Day Postoperative Dorsal Skin Incision

		Mean ± S. E.			
Orientation	No. of Animals	Body weight (grams)	Thickness (mm.)	Breaking Strength (g)	Tensile Strength (g/mm^2)
Perpendicular	10	538 ± 8	1.33 ± 0.03	625 ± 44	80 ± 7
Parallel	12	535 ± 6	1.47 ± 0.05	329 ± 17	38 ± 2
P value		No difference	<.05	<.001	<.001

Table III. 21-Day Postoperative Dorsal Skin Incisions

		Mean ± S. E.			
Orientation	No. of Animals	Body weight (grams)	Thickness (mm.)	Breaking Strength (g)	Tensile Strength (g/mm^2)
Perpendicular	10	331 ± 3	1.37 ± 0.05	1291 ± 85	160 ± 14
Parallel	10	332 ± 4	1.69 ± 0.05	872 ± 41	88 ± 9
P value		No difference	<.001	<.001	<.001

(4) **Evaluation of surgical suture materials:** In the previous progress report of this department the strengths at 21 days of wounds closed with stainless steel, silk, and nylon were compared and found to show no significant difference. Numerous recommendations have been made over the past twenty-five centuries to use adhesives and adhesive tapes for the closure of incised wounds. Recently it has been reported that a new microporous surgical adhesive tape (Steri-Strips(R), Minnesota Mining & Manufacturing, St. Paul, Minn.) is strikingly successful as a replacement for skin sutures in the apposition of wounds. Tape closure has been cited for its speed, its technical facility, its superior cosmetic result (no suture marks), and its lessened susceptibility to wound infection (no internal foreign body). Despite increasing use of tape, studies of the physical characteristics of wounds so closed have been lacking. In the past year this department has investigated the physical characteristics and morphology of incisions closed with tape and standard suture technique. Our standard 5 cm. dorsal skin incision in the guinea pig was used and thirty incisions of each type were compared.

It was found that 17 of the 30 tape-closed incisions had to be excluded because of shedding of tape and wound disruption within 48 hours post-surgery. Even with use of compound tincture of benzoin to aid tape adhesion, satisfactory wound approximation was maintained in less than 50 per cent of the animals. The 13 taped incisions intact at 48 hours healed uneventfully and the results presented are derived from these animals and their sutured controls. At sacrifice (14 days postoperatively), incisional scars in both groups were all grossly apparent as fine white lines. There was no hyperemia, elevation, or widening of incision with either type of closure. In the sutured group, cross-hatching attributable to suture retention was rare and barely discernible on close inspection. The taped area showed no evidence of skin reaction or irritation. In neither type of incision was there a measurable shortening from the original 5 cm. length. There were no significant differences in histologic appearance, skin thickness, breaking strength, or tensile strength between sutured and taped wounds (Table IV). The results clearly indicate that the tape-closed wound heals as well as the sutured wound provided grossly observable approximation is attained and maintained. Reports of clinical trials do not indicate that problems of tape loss and wound disruption occur in man with the frequency encountered in this study.

Table IV. 14-Day Postoperative Dorsal Skin Incisions

		Mean ± S. E.		
Group	No. of Animals	Tensile Strength (g/mm^2)	Breaking Strength (g)	Thickness (mm)
Sutured	13	80 ± 6	462 ± 32	0.98 ± 0.12
Tape	13	76 ± 8	412 ± 36	0.92 ± 0.05
P value		>.60	>.70	>.60

(5) Studies in colon wound healing: Studies of this department in the field of cutaneous wound healing have been expanded during the past year to encompass other more complex tissue systems. The multiple disciplinary approach to wound healing developed in this laboratory has been modified and applied to intestinal healing. The colon was chosen for initial study because of the difficulties encountered with healing of traumatic and surgical wounds of this organ. The colon is a highly functional and structurally complex organ in which healing must proceed in an environment of bacterial contamination, mechanical and chemical irritation, and functional activity -- all classically detrimental to optimal wound healing. Poorly understood and inadequately studied, the colon offers a unique opportunity for the development of methods to improve the healing process in contrast to skin, where optimal healing is the rule rather than the exception.

A. Techniques. A standard, reproducible, operative model consisting of resection of a segment of the left colon in the rat and an open end-to-end anastomosis has been perfected. Methods have been developed to test the physical strength of the resulting anastomosis in terms of resistance to increasing intraluminal pressure (bursting pressure) and linear force (breaking strength). Histologic techniques have been adopted which permit assessment of the functional restoration of epithelial elements and the role of bacterial invasion as well as the structural aspects of inflammation, repair and reorganization. Because of the obvious importance of bacteria in colon healing, bacteriologic techniques have been adopted which provide a systematic method for the study of the wide spectrum of aerobic and anaerobic flora of the colon. Biochemical techniques have been applied to the study of the local response of the colon to injury.

B. The normal healing process. The first phase in this project has been a characterization of the normal healing process in the rat colon. Pathogen-free white male rats closely matched for age and weight and kept under standard environmental conditions were operated upon following a 24-hour fast to reduce colon contents. These animals were sacrificed at intervals ranging from three hours to six months postoperatively. Table V shows the results of physical measurements on the anastomosis and a brief histologic description of the phases of healing. Noteworthy findings include the predominate pattern of an intense inflammatory reaction with tissue necrosis in the early phases confirming the impression of a less than ideal healing environment. Despite this, all animals survived without significant operative complications. Although physical measurements of the logarithmic phase of the healing process provide a more accurate index of healing, histologic examination in the so-called latent phase and the stationary phase provides more detailed studies of the morphological and functional changes in these extremely important periods. Important

functional and structural changes are still occurring six months postoperatively, far beyond the 10-day point of maximal healing previously assumed.

Table V. Healing of a Standard Colon Anastomosis in the Rat

Interval Postop.	Bursting Pressure (mm.Hg.)	Breaking Strength (gm.)	Gross and Microscopic Morphology	Phase of Healing
3 hours	66 ± 12	19 ± 5	Edema, interstitial hemorrhage, acute inflammation, necrosis, local fibrinous peritonitis and adhesions, ulceration and early granuloma formation.	Latent or Lag (Exudation Edema and Necrosis)
6 "	84 ± 14	7 ± 2		
24 "	112 ± 16	28 ± 4		
2 days	90 ± 16	23 ± 3		
3 "	80 ± 6	24 ± 4		
4 "	84 ± 18	17 ± 3		
5 "	144 ± 20	37 ± 3	Granuloma formation with new collagen. Completion of mucosal covering.	Logarithmic (Fibrous Repair)
6 "	202 ± 22	58 ± 6		
8 "	274 ± 16	82 ± 6		
10 "	274 ± 16	82 ± 6	Maturation and partial reabsorbtion of connective tissue. Muscle regeneration and differentiation. Maturation of mucosa. Gradual, although not yet complete restoration of normal architecture.	Stationary (Reorganization and remodeling)
13 "	254 ± 16	88 ± 4		
17 "	254 ± 14	97 ± 5		
20 "	226 ± 10	97 ± 5		
24 "	202 ± 18	97 ± 7		
27 "	240 ± 20	104 ± 12		
1 month	244 ± 14	96 ± 12		
3 "	Physical measurements, not performed			
6 "				

Values expressed as mean ± standard error. 10 animals at each interval.

Pilot studies concerning the relative effect of silk vs. catgut on the healing process in the colon anastomosis, and the relative strength of longitudinal vs. transverse colotomy incisions have shown no statistically significant differences at the intervals studied except that transverse incisions appear to be significantly stronger at 18 days.

Table VI. Effect of Silk vs. Catgut on the Healing of a Standard Colon Anastomosis in the Rat.

Interval	Bursting Pressure (mm. Hg.)		Breaking Strength (gm.)		Histology
	Catgut (5-0)	Silk (6-0)	Catgut	Silk	
21 days	157.4 ± 13.8	200.6 ± 23.8	94.3 ± 9.3	109.3 ± 15.7	No significant difference at any interval.
28 days	256.0 ± 20.4	264.6 ± 28.2	114.2 ± 6.6	114.5 ± 8.3	
Significance	Not significant at any interval.				

All values expressed as mean ± standard error. 6 animals in each group.

Table VII. Healing of Longitudinal and Transverse Colotomy Incisions in the Rat.

Interval	Bursting Pressure (mm. Hg.)			Breaking Strength (gm.)		
	Longitudinal	Transverse	P Value	Longitudinal	Transverse	P Value
4 days	93.5±15.6	108.7±21.3	N.S.	18.2±2.2	19.5±2.6	N.S.
11 days	210.6±10.9	195.8±14.9	N.S.	64.4±5.7	54.2±3.2	N.S
18 days	184.4±15.5	145.9±24.3	N.S.	64.8±5.9	97.9±5.9	>.001

Values expressed as mean - standard error. Ten animals in each group at each interval. N. S. - Not significant at the P = 0.05 level.

C. Biochemical characterization of the normal and healing large bowel of the rat. The combined dorsal skin incision-ivalon sponge implant technique developed by this department for study of dermal healing permitted simultaneous assay of the histologic, physical, and biochemical parameters of the wound. Translation of this technique to investigation of colon anastomosis involved simple adaptation of the histologic and breaking strength methodology to a new situation. In this setting, however, the ivalon sponge implant technique was totally inapplicable as a biochemical approach to the production of collagen (as measured by a unique amino acid, hydroxyproline). Work has thus been directed at providing and using a suitable assay procedure for this system.

At 4-18 days postoperatively, narrow segments of colon, including the anastomosis and 1 mm. of proximal and distal normal bowel, were excised and weights measured. Tissues were then dried and reweighed and hydrolyzed in 4N HCl. Hydroxyproline in the hydrolysates was quantitated by the method of Newman and Logan. It was found that edema is essentially confined to the first four days post-surgery. No differences in hydroxyproline content were found among the operated specimens or, in fact, between these and normal bowel. Apparently, the ratio of pre-existing collagen in adjacent normal bowel to the reparative collagen at the site of anastomosis is so great that it is impossible to follow the healing process quantitatively by this technique.

These findings, however, raised the question of the actual contribution of collagen to the strength of normal or incised bowel. It is generally accepted that collagen is primarily responsible for the tensile strength of tissue and in the skin work of this department the wound strengths correlated well with the hydroxyproline contents of implanted sponges. Bowel, however, could be quite different. By histologic examination, dermis is rich in collagen whereas in bowel collagen is apparently sparse and largely confined to the submucosa and serosa. Biochemically, skin contains about half of all the collagen in the body while in walls of hollow viscera, for example, gut, collagen comprises

only 2 - 3% of the fresh weight. Finally, in the healing colon anastomosis, collagenogenesis is relatively subtle compared to skin incisions. For these reasons it seemed essential to define the role of collagen in this tissue.

Normal and anastomosed gut segments were incubated at 37° and physiologic pH in appropriate media containing either trypsin or collagenase (from Clostridium histolyticum). Trypsin is unable to attack native collagen but digests effectively the other proteinaceous components such as muscle. Collagenase, on the other hand, specifically lyses collagen fibers and leaves other components intact. The media were assayed biochemically and the incubated tissues submitted to frozen section examination and measurement of physical strength. The specificity of the enzymes was corroborated by the biochemical examination of the media. Trypsin-containing media was rich in total amino nitrogen, comprised mainly of dipeptides, and contained no more hydroxyproline than samples incubated in media without any enzyme. Collagenase-containing media, conversely, contained less total amino nitrogen than did trypsin media but was composed mainly of tri- and quatra-peptides rich in hydroxyproline. On frozen section, the collagenolyzed specimens showed good preservation of structure and were essentially indistinguishable from control, media-incubated specimens. Trypsinized specimens, on the other hand, were morphologically almost totally devoid of structure except for the apparently delicate fibrous network of residual collagen. When strengths of such specimens were measured, the results were most surprising. Both normal and healing bowel incubated with collagenase was completely friable despite its apparent good preservation of structure; strengths were so low that specimens fragmented before registering any strength at all. Trypsinized specimens, however, although appearing filmy and structureless, showed less than 25% loss of strength and in some instances were equally as strong as normal bowel tested either fresh or after incubation with enzyme-free medium.

It is thus concluded that collagen is primarily responsible for the tensile strength of normal or healing bowel despite its relatively subtle presence. It is further concluded that a biochemical assay procedure to be correlated with tensile strength must measure the appearance of new collagen at the anastomosis. Since the quantities of reparative collagen are small relative to pre-existing normal collagen, and since it is technically impossible to dissect out the anastomotic ring free of adjacent unwounded tissue, one must probably employ isotopic methods. Tritiated proline, fed to an animal with a healing bowel, should be incorporated in collagen at the wound as both tritiated proline and tritiated hydroxyproline. These constituents could be both quantitated accurately and visualized by autoradiography. It is felt that such an approach would provide

valuable information about the dynamics of collagen production in normal bowel healing and in the abnormal situation of excessive scarring and a tight, napkin-ring deformity of the anastomosis.

D. Effect of fecal flora on colon wound healing. Reduction of the bacterial flora of the colon would appear to be a logical method for improving the healing process. Despite the widespread use of intestinal antiseptics, the value of these agents has not been clearly established either clinically or experimentally except in cases of gross fecal contamination or devascularization of the anastomosis. Current investigation in this laboratory is concerned with the effect of neomycin, the most commonly used intestinal antiseptic, on the fecal flora and the healing process in the rat colon. Contrary to popular belief, neomycin does not even approach "sterilization" of the colon. Although adequate control of the coliform organisms and the staphylococcus is achieved, with moderate reduction of the enterococci and anaerobic spore-forming organisms, the total number of anaerobic organisms, which comprise about 90% of the bulk of fecal organisms is unaffected. (Table VIII) With the exception of the significant increase in breaking strength of the 11-day anastomoses, no apparent physical or histologic difference has been demonstrated in the healing process in the colon between animals treated with neomycin and controls (Table IX). The significance of this one positive finding remains to be investigated.

E. Future Investigations: The above studies are at present being prepared for publication, and further studies, including the utilization of germfree animals, are presently planned. Expansion of activities into problems of healing in other tissue systems are currently under consideration and development.

Table VIII. Effect of Neomycin on the Fecal Flora of the Rat

Organism or Group of Organisms[1]	Viable Organisms/gram wet feces Control	Neomycin Treated[2]	Per cent Reduction
Total Aerobes	$3.7 \pm 2.4 \times 10^8$	$1.4 \pm 1.3 \times 10^6$	≈99%
Total Anaerobes	$2.8 \pm 4.1 \times 10^{10}$	$1.7 \pm 3.1 \times 10^{10}$	not significant
Anaerobic Spores	$2.7 \pm 2.3 \times 10^3$	$4.2 \pm 1.7 \times 10^2$	not significant
Coliforms: Lactose +	$1.0 \pm 1.5 \times 10^5$	less than 10^3	≈100%
Lactose -	$4.8 \pm 8.5 \times 10^4$	less than 10^3	≈100%
Gram Positive Aerobes	$4.7 \pm 1.4 \times 10^7$	$1.3 \pm 1.4 \times 10^6$	≈99%
Staphylococci	$9.3 \pm 4.2 \times 10^3$	less than 10^3	≈100%
Enterococci	$2.5 \pm 3.1 \times 10^6$	$2.8 \pm 1.7 \times 10^5$	≈90%
Fungi	variable	unchanged	not significant

[1] Accepted criteria for the definition of these organisms and groups based upon cultural characteristics have been used. Standard bacteriological media and tube dilution plating techniques were employed. The above figures were derived from numerous determinations on 30 animals in each group and are expressed as the mean ± standard error.

[2] Neomycin base 40 mg/rat/day mixed with diet. This is at the high level of accepted adult human dosage on a body weight basis.

Table IX. Effect of Neomycin on the Healing of A Standard Colon Anastomosis in the Rat.[1]

	4 days		11 days		17 days		Gross & Microscopic Pathology
	B.P.	B.S.	B.P.	B.S.	B.P.	B.S.	
Neomycin[2]	115.0 ±21.2	17.5 ±2.6	217.1 ±6.6	80.6 ±5.6	192.9 ±15.5	89.5 ±7.6	No significant difference at any level.
Control	120.2 ± 8.0	16.4 ±2.0	233.4 ±13.7	64.4 ±4.0	217.6 ±16.8	89.0 ±11.7	
P value	N.S.	N.S.	N.S.	<.05	N.S.	N.S.	

[1]Sixty animals, 10 in each group and at each interval, were used in this experiment. Animals were controlled as to age, weight and sex (male) and were kept under similar environmental conditions. The above values are expressed as mean ± standard error.

[2]Neomycin base 40 mg/rat/day mixed with diet.

B.P. - Bursting Pressure. B.S. - Breaking Strength. N.S. - not significant.

(6) Adhesive Polymers - use and toxicity. Pilot studies in the field of vascular wound healing are currently in progress. Because of the technical skill and time required for the repair of vascular injuries, procedures and techniques for implementing or supplanting current arterial suture techniques would be of great value in the management of battlefield injuries and mass casualties. Recently, the use of self-polymerizing plastic adhesives such as 2-methyl-cyanoacrylate (Eastman 910 adhesive) have been proposed as a substitute for tedious arterial suture techniques. Preliminary reports from other institutions have been favorable. The structural formula for this compound, however, would indicate a high level of tissue toxicity, a point not stressed in the literature. Current studies in which this material has been implanted in various body sites in the rat, using methyl-methacrylate (structurally similar but non-self-polymerizing and nontoxic) as a control, reveal a high level of tissue toxicity. These studies have been performed in close cooperation with the Army Prosthetic Research Laboratory, where longer-chain compounds of this type are being synthesized and which will hopefully provide less toxic but still useful vascular adhesives.

Summary and Conclusions:

In the past year the Department of Surgical Metabolism and Pathology has extended and broadened its investigative approaches into the basic

mechanisms of mammalian wound healing. It has been shown that skin is not structurally homogeneous and that its heterogeneity has important implications for both the tensile strength of normal tissue and the mode of healing of incised wounds. A new microporous surgical adhesive has been demonstrated to be as satisfactory as conventional skin suture technique under special circumstances. By extension of previously developed techniques to a study of healing in the colon, it has been possible to characterize the healing process in a sigmoid anastomosis in the rat. The influence of neomycin bowel preparation on the microbiological, physical, and histologic components of this system has been studied intensively. Work has been initiated on an evaluation of the use and histotoxicity of adhesive polymers for restoration of tissue continuity after injury.

List of Publications:

1. Furste, W. and Pulaski, E. J.: Tetanus prophylaxis. Summary of Discussion at the 1962 National Health Forum. Ohio State Med. J., 59: 386-7 (Apr) 1963.

2. Pulaski, E. J.: Should appendectomy be done at the time of right inguinal herniorrhaphy? If so, should the patient be given prophylactic antibiotics? Modern Medicine, 31: 208-210 (Mar. 4) 1963.

3. Levenson, S. M., Crowley, L. V., Rosen, H., Berard, C. W., and Geever, E. F.: A procedure to evaluate experimental wound healing. Presented at the Annual Meeting of the American Association for the Surgery of Trauma, Hot Springs, Virginia, Ott. 1962. J. Trauma (in press).

4. Berard, C. W., Herrmann, J. B., Woodward, S. C., and Pulaski, E. J.: Healing of incisions closed with surgical adhesive tape. Am. J. Surg. (in press).

5. Berard, C. W., Woodward, S. C. Herrmann, J. B., and Pulaski, E. J.: Healing of incisional wounds in rats: the relationship of tensile strength and morphology to the normal skin wrinkle lines. Ann. Surg. (in press).

6. Pulask, E. J.: Gas Gangrene. In TRAUMATIC MEDICINE AND SURGERY FOR THE ATTORNEY, Vol. 9 - Selected Infections. Butterworth, Inc. Washington, D. C. (in press).

ANNUAL PROGRESS REPORT

Project: 3A O 12501 B 813, Army Medical Basic Research In Life Sciences

Task: 09, Physiology (Pressor Substances)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Clinical Chemistry
Division of Biochemistry

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Benjamin Mehlman
E. C. Knoblock, Lt Col, MSC
John Morell, Pfc

Assistant: Silas Lee, Sp 4

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No: 3A O 12501 B 813 **Title:** Army Medical Basic Research In Life Sciences

Task No: 09 **Title:** Physiology (Pressor Substances)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Benjamin Mehlman
E. C. Knoblock, Lt Col, MSC
John Morrell, Pfc

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Some 50 specimens have been received requesting epinephrine and norepinephrine assays on blood plasma as an aid in the diagnosis of pheochromocytoma. One pheochromocytoma tumor was analyzed for epinephrine and norepinephrine.

Active collaborative studies have been done with investigators of the Department of Immunology and the Division of Nuclear Medicine involving plasma catecholamines, histamine and serotonin, and with the Medical Unit, Fort Detrick, involving plasma and urinary catecholamines, and VMA (3-methoxy 4-hydroxy mandelic acid). Plasma catechols were done for the Project Mercury program. Investigation of four different methods for VMA resulted in a simplified and reliable method to be incorporated into the research program. In addition a method for urinary epinephrine, norepinephrine and dopamine was evaluated and a satisfactory method for plasma histamine and serotonin from the same blood sample was adopted.

BODY OF REPORT

Project No: 3A O 12501 B 813 **Title:** Army Medical Basic Research In Life Sciences

Task: 09 **Title:** Physiology (Pressor Substances)

Description: Analyses of pressor substances and metabolites is being done to provide diagnostic assistance to service hospital whose facilities are not adequate for the performance of these complicated techniques. In addition the role of these pressor substances in various types of trauma is still not well known and collaborative studies with other investigators are being carried out. New analyses are developed and adapted to use as needed and as time permits.

Progress: The analysis of some fifty samples for possible cases of pheochromocytoma represents a further drop from previous years. This possibly reflects the use of simpler urinary methods such as VMA to assist in this diagnosis.

The major collaborative effort this year has been with Dr's Kalas and Jacobson of the Division of Immunology in a study of humoral factors in Endotoxin Shock, in which some 300 samples were analyzed for plasma epinephrine, norepinephrine, histamine and serotonin. Epinephrine and norepinephrine were done by the method of Weil-Malherbe; histamine and serotonin by method of Waalkes, J. Lab Clin Med 53 824 (1959).

The second collaborative effort in terms of man hours was support for plasma and urine catechol levels for a classified project with the Medical Unit, Fort Detrick.

Smaller studies were done with Drs. Finkelstein and Farrar, Department of Immunology and with Lt Col Mundy of the Division of Nuclear Medicine. All involved the determination of plasma catecholamines.

For evaluation of the stress of space flight, plasma epinephrine and norepinephrine determinations were done on the astronauts during the various Mercury-Atlas flights.

Summary and Conclusions: The complexity of adequate determination of plasma catechol amines offers a great opportunity for collaborative research between the Division of Biochemistry and others within WRAIR requiring such analyses for their research program.

Plasma catechols, histamine, and serotonin-plus urinary catechols and VMA determinations are now available for research and clinical purposes within limitations of facilities available. Additional work on other metabolites such as metanephrine and normetanephrine will be done as time permits.

Publications:

Sarcione, E. J., Bach, N., Sokol, J. E., Mehlman, B., and Knoblock, E. C., "Elevation of Plasma Epinephrine Levels Produced by Glucogon in Vivo", Endocrinology 72, 523 (1963).

ANNUAL PROGRESS REPORT

Project 3A 0 12501 B 813, Army Medical Basic Research in Life Sciences

Task 09, Physiology (Gastrointestinal physiology)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Experimental Pathology
Division of Special Activities

Department of Germfree Research
Division of Basic Surgical Research

Department of Applied Immunology
Division of Communicable Disease
and Immunology

Department of Neurophysiology
Division of Neuropsychiatry

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: David C. Biggers, Capt., MC
Jean R. Dupont, Capt., MC
Helen Roland Jervis, Sc.D.
John P. Kalas, Capt., MC
Thomas J. Magnani, Capt., MC
Thomas G. Merrill
Helmuth Sprinz, Col., MC
Akio Takeuchi, M.D.

Assistant: A. S. Maliney, M.D.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task No. 09 Title: Physiology (Gastro-intestinal physiology)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: David C. Biggers, Capt., MC
Jean R. Dupont, Capt., MC
Helen Roland Jervis, Sc.D.
John P. Kalas, Capt., MC
Thomas J. Magnani, Capt., MC
Thomas G. Merrill
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

The investigators listed have been engaged in the study of the vegetative nervous periphery of the gut of germfree and conventional rodents, the enzyme histochemistry of the intestine of conventional and germfree animals under baseline conditions and enteric infections. The response of goblet cells to stimuli and the effect of various experimental models on mucous production, the morphological effects on the vegetative nervous periphery of endotoxin and of various drugs affecting the autonomic nervous system and the ultrastructure of the intestine as studied by the electron microscope.

BODY OF REPORT

Project No. 3A 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task No. 09 Title: Physiology (Gastro-intestinal physiology)

Description:

Special staining techniques of the vegetative nervous periphery have been applied to the intestinal mucosa and wall.

Procedures to determine histochemically the presence of several enzyme systems in the intestinal tract have been developed.

The ultrastructure of the intestinal tract is being investigated by electron microscopic investigations.

Progress:

The technique has been applied to determine the neuro-anatomical basis of the difference in size of the cecum of germfree and conventional laboratory rodents. These specialized techniques have also been applied in a third experimental investigation in which the effect of endotoxin i.v. on the morphologic changes of abdominal sympathetic ganglia and the plexus of Auerbach and Meissner is being studied.

The enzyme histochemistry of the intestinal tract of rats, guinea pigs and rabbits has been studied and the topography of enzyme distribution determined. After establishing a baseline, enzyme distribution in enteric infections and in germfree animals are being studied. In a separate investigation intestinal mucin production is studied in an attempt to further elucidate its role in enteric infections.

An electron microscopic laboratory has been established and a baseline study of the ultrastructure of the small intestine of the prenatal, postnatal and adult guinea pig is being undertaken.

Summary and Conclusions:

Specialized neurohistological techniques are being applied in continued studies of problems of gastrointestinal physiology (Dupont, Kalas, Sprinz and Maliney). The methylene blue staining was found to be most applicable.

Histochemical techniques have been applied to the intestinal mucosa in our continued studies of the gastrointestinal physiology.

Electron microscopic studies of the intestinal mucosa have been initiated.

List of Publications:

1. Helen R. Jervis. Enzymes in the mucosa of the small intestine of the rat, the guinea pig and the rabbit. J. Histochem. Cytochem. In press.

ANNUAL PROGRESS REPORT

Project No. 3A0 12501 B 813 Army Medical Basic Research in Life Sciences

Task No. 09 Physiology (Metabolism in Radiation Injury)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D.C.

Department of Radiobiology
Division of Nuclear Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Captain D. E. Davidson, Jr., VC

Assistants: Lt. Col. Kent Woodward, MC
Miss Ann R. Berman

Report Control Symbol: MEDDH-288

Security Classification: Unclassified

ABSTRACT

Project No. 3A0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task No. 09 Title: Physiology (Metabolism in Radiation Injury)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Author: Captain David E. Davidson, Jr., VC

Report Control Symbol: MEDDH-288

Security Classification: Unclassified

Organ function and metabolic studies in dogs irradiated from one to eight years ago are in progress for the purpose of identifying and evaluating possible late effects of irradiation. The experimental subjects are dogs which have been irradiated at the University of Rochester or at WRAIR under a variety of experimental conditions. About 150 irradiated dogs are available for study, 45 of which have received pre-irradiation protective chemical agents. Ten unirradiated control dogs are also under study. Thyroid function studies have been completed on 30 irradiated and 10 unirradiated dogs. No impairment of thyroid function has been observed in the irradiated group. Total body water and muscle mass determinations by radioisotope tracer techniques are also in progress in an attempt to evaluate the aging process. Hematopoietic function studies using Iron-59 and Chromium-51 as tracers have been completed in 16 irradiated dogs. Incomplete recovery of erythropoiesis has been observed 14-15 months after 450-600r whole-body irradiation.

BODY OF REPORT

Project No. 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task No. 09 Title: Physiology (Metabolism in Radiation Injury)

Description:

Research effort under this project has been directed towards attempting to identify and quantitate the late effects of whole-body irradiation on the metabolism and vital organ function of dogs surviving the acute phase of radiation injury. The delayed effects of whole-body irradiation have been studied in the past principally in laboratory rodents. Very little is known about late functional changes due to irradiation in the larger experimental animals. Dr. S. J. Michaelson has observed hypothyroidism in dogs surviving upper-body irradiation. It was therefore important to evaluate thyroid function in whole-body irradiated dogs. Determinations of the muscle mass by the measurement of natural potassium-40 in the WRAIR whole-body counter are in progress in order to evaluate the aging process in irradiated versus unirradiated dogs. Total body water determinations using tritium as a tracer are also being performed in order to estimate the lean body weight as a function of age.

Progress:

Approximately 150 dogs are being housed in outdoor kennels at Forest Glen in conjunction with long-term radiation effects studies. Fifty of these dogs were irradiated at the University of Rochester during the period 1955-1957. This group of fifty dogs has received single or fractionated whole-body x or gamma radiation doses of 200 to 1400r and are being studied under project DASA RD 40-61 Subtask 03.037 (Life Span Studies in Irradiated Dogs). These dogs have been made available under the supervision of Dr. S. J. Michaelson (University of Rochester) for non-destructive metabolic studies. Also available for study are a group of approximately 50 dogs surviving supra-lethal x-irradiation (450 to 1500r) by virtue of having received chemical protective agents before irradiation and a group of 38 purebred beagles which have survived whole-body irradiation at low dose rates. In the beagle colony, an unirradiated control group is also available.

Thyroid function studies have been completed in the group of 38 purebred beagles at about one year post-irradiation. Blood cholesterol and protein bound iodine levels have been determined by standard methods. Five microcuries of carrier-free iodine-131 were administered to each dog intravenously, and the thyroid uptake of the isotope was measured at 24 hour intervals over a three to five day time period using a 3 inch collimated sodium iodide crystal detector. At the same time the ratio of the count

rate over the thyroid to the count rate over the thigh was determined. Thyroid uptake of I^{131} reached a maximum between the second to fourth day. So far no significant differences in thyroid function has been observed between the irradiated and unirradiated dogs. The preliminary results of these studies are presented in the following table:

	No. of Dogs	Radiation Dose	72 hr I^{131} Uptake %		Thyroid/Thigh Ratio	
			Mean	Range	Mean	Range
Group I(Irradiated) (10-12 mo. post-rad)	19	450-750r @ 1r/min	28.8%	17.5-45.5%	6.4	2.6-9.1
GroupII(Irradiated) (8-9 mo. post-rad.)	9	400-600r @ 10r/min	33.7%	23.7-42.9%	8.0	4.5-11.8
Group III (Un-irradiated control)	10	SHAM irradiated	34.5%	20.2-49.1%	6.6	3.0-9.5

	No. of Dogs	Serum Cholesterol (mg%)		PBI (Micrograms %)	
		Mean	Range	Mean	Range
Group I	19	130	73-243	1.32	0.38-2.3
Group II	10	117	76-154	2.31	1.4-3.4
Group III	10	130	98-204	2.10	1.5-3.6

*Questionable Experimental result

Determinations of total body muscle mass by measurement of natural potassium-40 are in progress using the 9 inch sodium iodide crystal detector and 100 channel analyzer in the steel shielded room in this laboratory. On the same day the lean body mass is determined from the gross body weight and by determination of the total body water using tritium as a tracer. Total body potassium content is expressed as grams of potassium per kilogram of lean body weight. Results obtained so far confirm that the lean body potassium content decreases with age, and that males have a higher potassium content than females. Counting times of at least 45 minutes are required to keep counting error below the 5% level. The presence of tracer doses of iron-59 from earlier studies has prevented

accurate measurement of potassium-40 in some of the dogs. At the present time calibration of the whole-body counting system with potassium 42 is in progress. Inadequate data is available at the present time to permit evaluation of the effect of previous irradiation on body potassium levels.

The status of erythropoietic function in dogs surviving irradiation is also being evaluated. Total red cell mass and whole blood volume is determined by calculation of isotopic dilution of a blood sample taken one hour following intravenous injection of autologous red cells tagged with 75 microcuries of chromium-51. Red cell survival time is determined by the rate of disappearance of the tagged cells from the circulating blood over a three week observation period. One day after the injection of chromium-51, 10 microcuries of iron-59 are injected intravenously. Hematopoietic function is determined from the rate of plasma clearance of iron-59 from the plasma and by the rate and degree of iron-59 uptake by the circulating red cells. Plasma iron levels are determined by the standard chemical method at the completion of each study.

Erythropoietic function studies have been completed in 16 whole-body irradiated dogs up to the present time. At 14 to 16 months after whole body irradiation evidence of incomplete recovery of erythropoietic function has been observed. In some of these dogs heavy hookworm infestation has influenced the erythropoietic function, but this situation has since been corrected. A group of 9 dogs surviving 450r whole-body irradiation at 1r/min was studied 14-15 months after exposure. Only one dog in this group exhibited low red cell mass and prolonged plasma iron clearance indicative of depressed hematopoietic function. A group of 7 dogs surviving 600r @ 1r/minute were also studied 14-15 months after exposure, and 4 of this group exhibited low red cell mass accompanied by slow plasma iron clearance. The data from the irradiated dogs will be evaluated in the light of the results from erythropoietic studies in unirradiated kennel-mates. These studies are in progress.

Summary and Conclusions:

Dogs surviving from one to eight years after whole-body irradiation under a variety of experimental conditions are being studied for evidence of residual radiation effects on metabolism and vital organ function. Thyroid function studies in 30 dogs one year after exposure to 450-750r indicate that there is no observable functional impairment attributable to irradiation at this time. Total body potassium determinations by whole-body counting of natural potassium-40 and total body water determinations using tritium as a tracer indicate, as reported in other animal species, that in the dog the lean body content of potassium, i.e. the muscle mass, is higher in the male than in the female, and that it decreases with age after maturity. To date not enough data has been accumulated to permit evaluation of the influence of irradiation on the total body potassium level. Erythropoietic function studies in irradiation dose utilizing iron-59 and chromium-51 as tracers are in progress. Limited data obtained so far indicate incomplete recovery of erythropoiesis at 15 months after 450 or 600r whole-body x-irradiation.

List of Publications:

None

ANNUAL PROGRESS REPORT

Project No. 3A 12501 B 813,	ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES
Task 09	Physiology (Physiology of cell growth and regeneration)
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
	Department of Cellular Physiology Division of Basic Surgical Research
Period Covered by Report:	1 July 1962 through 30 June 1963
Principal Investigators:	Andre D. Glinos, M. D. Robert B. Greer, III, Capt, MC
Assistants:	Warner T. Brown, M. S. Robert J. Werrlein, B. S. SP4 Stanley D. Hajkowski PFC Don D. Hargrove
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 3A 12501 B 813 — Title: ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task No. 09 — Title: Physiology (Physiology of cell growth and regeneration)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Andre D. Glinos, M. D., Robert B. Greer, III, Capt, MC, Robert J. Werrlein, B. S., Warner T. Brown, Jr., SP4 Stanley D. Hajkowski, PFC Don D. Hargrove

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

A tissue culture system has been developed in which logarithmic and stationary growth phases closely approximate the kinetics of the growth and resting states of body tissues. When a portion of cells in the stationary phase is removed, a growth response occurs until the original cell population density is restored. This system therefore responds to cell loss in a manner analogous with the regenerative response of body tissues to injury or partial surgical removal.

It has now been shown that this analogy is not limited to the apparent kinetics of cell populations but that it extends to the genetic level in terms of the modal range of the chromosomes and to the physiological level in terms of the biosynthetic activities and the energy metabolism of cells in the two phases of the system.

Using this information as a springboard, further work will be focused on the molecular systems controlling cellular growth during tissue regeneration and repair.

BODY OF REPORT

Project No. 3A 12501 B 813, ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task 09 Title: Physiology (Physiology of cell growth and regeneration)

Description:

It is well known that tissue loss in the body, whether caused by surgical removal, or by trauma, irradiation, burn, or other injury, is followed by a reparative process characterized by cellular proliferation and tissue regeneration. The basic mechanisms for induction and regulation of these repair processes for the most part have remained elusive.

Work in this department continues to explore the basic mechanisms controlling cellular growth during these repair processes. In the past we described a cyclical feedback system controlling regeneration of the liver after partial hepatectomy (A. D. Glinos, Environmental Feedback Control of Cell Division, Ann. N. Y. Acad. Sci., 90: 592, 1960). A fall in serum protein following partial hepatectomy stimulates an orderly sequential retrograde activation of cytoplasmic protein and RNA synthesis, followed by nuclear RNA and DNA synthesis culminating in hepatic cell division, which continues until the serum protein level and the cell population of the liver are restored to normal. A tissue culture "model" of such a self-repairing system utilizing L-strain mouse fibroblasts in suspension culture has been developed in which there are both logarithmic and stationary growth phases analogous to growth and resting stages in vivo. Thus, removal of part of the cell population and dilution of the cell density of such a culture results in active cellular proliferation which ends when the part of the original population which was removed is replaced.

Such a system allows far greater experimental control, and enables quantitative work to be done with much greater accuracy and reliability than in an in vivo system. In order for the information thus obtained to be truly meaningful it has to be shown that the analogy between the two systems extends beyond the apparent cell population kinetics to the genetic and physiological levels of cellular activity.

Progress:

I. With respect to the genetic level it has to be shown that stationary and logarithmically growing populations are genetically identical, thus excluding the possibility that the stationary state involves only a selected small fraction of the original cell population

which is genetically capable of survival without growth. This point has been investigated by using the new methods of mammalian cytogenetics after their adaptation to our culture system.

It is well known that instead of the species specific single chromosome number or mode which characterizes all tissue cells in vivo, mammalian cell populations in culture exhibit considerable variation in the chromosome numbers of individual cells. Cytogenetic characterization and identification of such cell populations must therefore be based on a characteristic modal range rather than a single mode.

After extensive analysis of the frequency distribution of L strain cells with different chromosome numbers, a system was devised whereby the individual members of a given cell population are grouped together in distinct classes with a width of 10 chromosomes per class. The characteristic modal range of a given cell population is then expressed by the chromosome class which contains over 50% of the cells of the population.

On the basis of this system the cytogenetic constitution of four logarithmically growing and five stationary L cell populations were analyzed and the mean values thus obtained are shown in the next table:

Table I

Type of cell Population	Per cent of cells with chromosome numbers: 30	30-39	40-49	50-59	60-69	70-79	80
Logarithmic	2	-	18.3	69.0	6.7	-	4.0
Stationary	0.4	0.8	8.8	82.4	7.2	-	0.8

It can readily be seen that the characteristic modal range is identical for both logarithmically growing and stationary cell populations and is expressed by the 50-59 chromosome class. Furthermore, there seems to be an even higher percentage of cells within the characteristic modal range in the stationary population and a corresponding decrease of cells on either side of this range, thus indicating that the only selection taking place concerns the elimination of nonviable cells deviating from the norm. The presence of these cells in the logarithmically growing cultures would be explained by the fact that they are produced continuously by a relatively small number of abnormal mitoses taking place in such cultures.

II. With respect to the physiological point of view it must be shown that a stationary cell culture is so because the metabolism of the cells, including DNA, RNA and protein synthesis, is reduced in comparison to metabolism in the logarithmic phase. The alternative explanation would be high cell turnover, with an equally large number of regenerating and degenerating cells yielding a "steady-state" situation. If this were the case, intracellular metabolism would be expected to be similar in both logarithmically growing and stationary cultures.

A number of biochemical analyses have been carried out to evaluate both biosynthetic rates and energy metabolism on a per cell basis in the system. Using a modification of the methods of Scott, Fraccastoro and Taft (J. Cytochem. Histochem., 4: 1, 1956) and of Oyama and Eagle (Proc. Soc. Exp. Biol. Med., 91: 305, 1956), it has been possible to assay for the DNA, RNA and protein content of individual cells in the logarithmic and stationary phases. In addition, simultaneous assays were carried out for glucose utilization, lactate and pyruvate production, oxygen utilization, and pH of the medium. The following table is a summary of a typical such experiment:

Table II

Growth Phase	Cell Count per ml	mg x 10^{-9} per cell DNA	RNA	Protein	Glucose uptake gm/ml	Pyruvate product. gm/ml	Lactate product. gm/ml	Oxygen medium mM/L	pH
log.	300,000	42	71	394	270	23	270	0.18	7.4
stat.	7,000,000	31	39	260	1000	45	700	0	6.9

It can readily be seen that there are considerable metabolic differences between the two growth phases. Not only are the parameters of energy metabolism different (glucose uptake, lactate and pyruvate production, oxygen content and pH), but also the parameters of biosynthetic activity differ (RNA, DNA and protein), indicating that there is a general slowing down of cellular metabolism in the stationary phase. These results, coupled with the mitotic index of logarithmic cultures (1.5 - 3.0%) as against that of stationary cultures (0 - 0.15%), and the similar number of necrotic cells in each growth phase (5 - 12%), strongly point to the conclusion that the stationary phase cell population is in a different physiologic state relative to similar cells in the logarithmic growth phase.

It now remains to be shown that the analogy between this suspension cell culture system and various in vivo preparations is valid with respect to the transition from the resting, stationary phase to the growth phase. For example, various specific biochemical events

are known to occur in the early hours and days following experimental partial hepatectomy. Within the first few hours cytoplasmic RNA increases rapidly in such a preparation, and approximately twelve hours later there is a striking increase in DNA activity. We are now in a position to study such events quantitatively *in vitro*, since we have already shown that the removal of cells from a stationary suspension culture causes a logarithmic growth response, analogous to post-injury tissue regeneration, until the previous stationary population is reached. By manipulation of various constituents of the growth medium, the rate of "repair" in tissue culture may be studied together with the sequence of biochemical events in the repair process. This information in turn may help to identify the extracellular conditions, limiting factors and specific stimuli responsible for initiating, controlling and arresting growth of cell populations both *in vitro* and *in vivo*.

Summary and Conclusions:

A tissue culture system has been developed in which logarithmic and stationary growth phases closely approximate the kinetics of the growth and resting states of body tissues. When a portion of cells in the stationary phase in removed, a growth response occurs until the original cell population density is restored. This system therefore responds to cell loss in a manner analogous with the regenerative response of body tissues to injury or partial surgical removal.

It has now been shown that this analogy is not limited to the apparent kinetics of cell populations but that it extends to the genetic level in terms of the modal range of the chromosomes and to the physiological level in terms of the biosynthetic activities and the energy metabolism of cells in the two phases of the system.

Using this information as a springboard, further work will be focused on the molecular systems controlling cellular growth during tissue regeneration and repair.

List of Publications:

None

ANNUAL PROGRESS REPORT

Project 3A 0 12501 B 813, Army Medical Basic Research in Life Sciences

Task 09, Physiology (Production of photopotentials by organic substances)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Division of Instrumentation

Period Covered by Report: 1 July 1962 -- 30 June1963

Principal Investigator: Irvin Levin, Ph.D.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task No. 09 Title: Physiology (Production of photo-potentials by organic substances)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 -- 30 June1963

Author: Irvin Levin, Ph.D.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Working space and apparatus were prepared and set up. Experience with the apparatus showed that an electrically-screened compartment, to shield the sensitive apparatus against electric-field transients, and a steady light intensity were needed to duplicate results from a single photo-active compound. The screened compartment was completed and installed; a special well-filtered power supply for a direct-current arc lamp is almost completed.

BODY OF REPORT

Project No. 3A 0 12501 B 813 Title: Army Medical Basic Research in Life Sciences

Task No. 09 Title: Physiology (Production of photo-potentials by organic substances)

Description:

The initial object of this investigation is to determine where the electric potential is produced in the photo-active organic molecule when irradiated. From this, the mechanism of the action of light on organic material should be uncovered. This work is based on the phenomenon that light affects certain organic structures to produce an electric potential which causes or indicates a primordial reaction.

Progress:

A suitable working area was prepared, and purchased and constructed apparatus were placed into operating position. Special optical cells, and supports for light source and cells, were designed and constructed. Fair electrical effects were obtained from representative organic compounds. As studies continued and more experience was gained with the apparatus, it was found that much electrical noise was present and that a given compound under investigation did not yield duplicate results. A screened chamber was then constructed to contain the sensitive apparatus; this removed the extrinsic electrical noise due to pick-up of body-capacitance changes and other related electric-field effects.

After continuing further with the study of one photo-active compound, it was discovered that only at frequent intervals was duplication of electrical behavior obtainable. Because this is intrinsic to the apparatus, the light source was checked and found not sufficiently constant in intensity but was varying randomly with the voltage of the electrical power line. An alternating-current arc lamp was being used. To obtain a more steady light intensity, a commercial direct-current arc lamp with large anode was chosen, and a well-filtered rectifying power supply was designed to provide a constant current to this lamp from the electrical power line. During the time this power supply was being fabricated in-house, one commercial

concern advertised a similar supply, and another concern, the lamp manufacturer, announced the availability of one in a few months. However, the present supply is almost completed and tests will be made with it and its lamp to determine whether this combination can produce the required duplication of results with one compound. After this step of apparatus development, comparison among organic structures will follow.

Summary and Conclusions:

At this time, the object is to improve the apparatus so as to obtain reproducible results with one photo-active compound before proceeding with the interrelationship of various compounds.

List of Publications:

(Nothing has been published by the author on the theoretical aspects of this phenomenon since 1953. The original work was done by him at the University of Maryland. The present project revives the original work and was authorized in October 1961 at WRAIR.) Recent publications by the author on a practical aspect of this phenomenon are:

Retinal Type of Photovoltaic Cell, NATURE 181, 832 (1958)

Photovoltaic Pile, NATURE 182, 44 (1958).

ANNUAL PROGRESS REPORT

Project: 3A 6 24101 A 816, AMEDS Development and Testing Program (Laboratory Modernization)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Clinical Chemistry
Division of Biochemistry

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: E. C. Knoblock, Lt Col. MSC
John R. Maher, Major, MSC
Larissa De Baare, M. D.

Assistant: Joseph Kelly, SFC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No: 3A 24101 A 816 **Title: AMEDS Development and Testing Program (Laboratory Modernization)**

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: E. C. Kneblock, Lt Col, MSC
John R. Maher, Major, MSC
Larissa De Baare, M. D.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

This program was devised as a means of systematic evaluation of new commerical equipment and procedures for possible inclusion into the Army Medical Laboratory. S series of commercial reagent assemblies have been examined and in most cases where bulk reagents were provided as a component the assemblies were not found to be satisfactory for inclusion in the Medical Supply System.

A sympesium was held in October 1962 entitled "Field Applications of Microtechniques". Publication of the proceedings is pending. Ultramicrochemical procedures have been used in evaluation of responses of the astronauts to space flight and in a series of collaborative studies on small laboratory animals.

Satisfactory methods include total protein, chloride, calcium, sodium, potassium, inorganic phosphorus, glucose, urea nitrogen, and cholesterol.

A field assembly set for microchemical determinations has been developed by contract. The prototype assembly, consisting of spectrophotometer, pH meter, centrifuge and titration apparatus is currently undergoing laboratory evaluation.

The new technique of disc electrophoresis has been added and is currently being evaluated. The preliminary information suggests increased resolution of protein components from the serum.

BODY OF REPORT

Project No: 3A 6 24101 A 816 **Title: AMEDS Development and Testing Program (Laboratory Modernization)**

Description: The purpose of this program is the systematic evaluation of commercial reagent assemblies, new analytical techniques, and new equipment for improving capability of the medical laboratory in support of military medical problems. Emphasis is on improved capability under field conditions.

Progress:

a. Evaluation of Chemistry Procedure Kits: Commercially prepared kits for a series of biochemical procedures were procured. These kits included procedures for amylase, transaminases, chloride, uric acid, cholesterol, blood urea nitrogen, alkaline phosphatase and glucose. In most cases the kits would produce satisfactory results if freshly prepared and properly stored. None are currently in properly packaged assemblies to find ready use in the system of military medical laboratories, where logistical problems involving stocking and refrigeration are factors for consideration. The prepackaging of enzyme determination assemblies offer promise for simplified and reliable procedures for these determinations.

Experience gained by these evaluations will serve as a basis for development of prototypes of reagent assemblies during the next year.

b. Symposium "Field Applications of Microtechniques": This symposium was held in October 1962 with many of the pioneer scientists in the field of ultramicrochemical analysis participating. During the course of the symposium a variety of techniques were explored. These included the analyses required for adequate handling of burns and traumatic injuries, the diagnosis of liver disease, the applications of enzyme and isoenzyme analyses to diagnostic problems, and application of the techniques for adequate sample preservation and transportation where reference laboratory work is required.

The general concensus of the meetings was that further development of microtechniques offers a definite possibility for simplification of the military laboratory service with considerable reduction in training requirements and logistical support.

The proceedings are currently being edited and will be published in the near future.

c. Laboratory Support - Project Mercury: The general laboratory support for evaluation of biochemical responses of the astronauts of Project Mercury during space flight was provided. These included the complete medical evaluation of the astronaut before, during, and after space flight. Determinations accomplished included complete blood and urine evaluations of electrolyte balance, calcium excretion, hemoconcentration, enzyme activity, blood catechol amine responses, and other factors. Despite considerable speculation as to the possible consequences of positive gravity forces and weightlessness, there was no parameter that would show the man paid a high biological price for his experience.

This special assignment required an aggregate of more than 1,200 individual determinations. Furthermore the problems of providing a portable laboratory for potential use led to a contract being let for a microanalytical laboratory system which provides for most clinical chemistry support work in a single package weighing approximately 90 pounds.

d. Field Laboratory Assembly: This assembly was developed by contract with Beckman Instrument Company and consists of five instruments. In any case where electrical power is required, self-contained and rechargeable (Nicad) batteries are utilized. Solid state (transistorized) circuitry is used to reduce size and electrical requirements and the instruments are "ruggedized" to withstand shipment. Emphasis was toward simplification and modular electrical components to overcome maintenance difficulties. Additional special characteristics of each component are as follows:

Spectrophotometer- A new concept using a probe-type light source enabling rapid determination of either micro or macro samples within the wavelength range of 365-700 mu. This instrument does not require expensive cuvettes and is completely self-contained in a shock resistant case.

pH meter and amplifier- An expandible scale meter with capabilities of using pH, pCO_2, pO_2, pNa, pCl electrodes for determination of acid-base balance rapidly. The instrument is packaged in a shock resistant case and has a laboratory life of approximately 20 hours between charging cycles. The amplifier circuit may be detached for electrochemical use.

Titrator- A precision instrument with no glass parts. Vibrator stirrer provides easy observation of indicator changes. Dial reads directly in milli-equivalents and is reset for each determination to reduce possibility of mathematical errors.

Centrifuge- Eight pounds in weight with hand operation at approximately 9500 rpm. Plasma samples in 0.25 ml tubes available in 1-2 minutes. Supplemental hematocrit head provides additional capability in field use.

Micromixer- A corrugated device which is hand operated to thoroughly resuspend centrifuged components when such procedures are required.

At the present the battery regeneration is from 110 volts, AC; however, a development is under way to provide a small assembly to allow regeneration from a variety of DC and AC sources.

The complete instrumental assembly has been delivered by the contractor. Following laboratory evaluations, additional prototypes will be procured for field testing by other units.

e. Protein Electrophoresis: Since moving boundary electrophoresis had previously indicated multiple components in low-density lipoproteins, further investigations have shown that calcium and cobalt precipitates of this protein fraction produce more distinct patterns than lipoprotein alone. The cobalt separation was superior to calcium in concentrating the material. Electrophoresis suggests that cobalt removes additional components not affected by calcium and in either case the appearance of several non-symmetrical peaks indicates the presence of additional components.

The use of disc electrophoresis for separation of the lipoprotein components shows many separations not previously observed distinctly. It is hoped that these components can be correlated with definite biochemical responses to injury and disease.

Summary and Conclusions: This year's work has indicated that further development of the system of ultramicro chemical procedures offers definite capability for simplifying the system of army medical laboratory support and at the same time extending the capabilities of the laboratory. Developments in electrolyte and acid-base analysis indicate both rapid and accurate analyses are practical for field use.

Advanced electrophoretic techniques have shown new separations of components in the lipoprotein moiety which previously were observed as responses to ionizing radiation injury and in cardiac disease.

List of Publications:

WRAIR Symposium: Field Applications of Microtechniques, October, 1962. In press.

ANNUAL PROGRESS REPORT

Project RD 40-61 BIOMEDICAL (NWER) (DASA) (03.008, Studies on Sensory and Motor Function in Monkeys)

Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C. Department of Experimental Psychology Division of Neuropsychiatry
Period Covered by Report:	1 July 1962 through 30 June 1963
Principal Investigator:	Joseph V. Brady, Lt Col, MSC
Assistants:	Joseph C. Sharp, 1st Lt, MSC Donald Daoust, BA
Reports Control Symbol:	MEDDH-288
Security Classification:	Unclassified

ABSTRACT

Project No.: RD 40-61	Title: BIOMEDICAL (NWER) (DASA) (03.008, Studies on Sensory and Motor Function in Monkeys)
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C. Department of Experimental Psychology Division of Neuropsychiatry
Period Covered by Report:	1 July 1962 through 30 June 1963
Authors:	Joseph V. Brady, Lt Col, MSC Joseph C. Sharp, 1st Lt, MSC
Reports Control Symbol:	MEDDH-288
Security Classification:	Unclassified

The completion of the first phase of this project yielded time-dose relationships that were necessary for the design of the second phase. The second phase, in accordance with the original proposal, attempts to study the effects of acute and massive doses of x-irradiation on sensory (visual) capacities and motor coordination before, during and after massive and acute doses of irradiation. The procedures and techniques for this second phase of the project have involved the establishment of complex and time-consuming behavioral performance baselines against which to evaluate radiation effects.

BODY OF REPORT

Project No.: RD 40-61 Title: BIOMEDICAL (NWER) (DASA) (03.008, Studies on Sensory and Motor Function in Monkeys)

Description:

The purpose of the experiments dealing with the effects of acute doses of x-irradiation on avoidance behavior in monkeys was to determine the latency and nature of behavioral changes after irradiation as a function of the dose. Once the time-dose relations of avoidance behavior had been worked out, the second phase was initiated. It was necessary to know how long avoidance behavior could be maintained under conditions of high motivation following x-irradiation before designing experiments utilizing complex behavior patterns. These complex patterns of behavior are a necessary component of the technique for assaying the sensory functions of animals. The utilization of operant conditioning techniques permit accurate and highly reliable assessment of sensory functions, hence, the use of a relatively small number of animals is justified.

Progress:

While operant conditioning techniques to assay the sensory capacities of animals have been used for some years now, the modification of these techniques to fit the radiation paradigm has required the development of special procedures. The desirability of using shock avoidance (as opposed to food reward, for example) and procedures for measuring various sensory capacities in a relatively short period of time has determined the direction of the technical developments presently in progress. Preliminary observations on four rhesus monkeys indicate that efficient and sensitive procedures for the evaluation of sensory capacities can be expected to emerge from progress on this behavioral development program.

Progress has also been made in the development of economical data collection and analysis procedures for extended performance samples during radiation exposure. A sequential event time analyzer and recorder has been developed utilizing a paper punch tape system compatible with available computer facilities. An exploratory study using rats, utilizing techniques applicable to monkey investigations, has demonstrated that the ability to discriminate between tones is not impaired by moderate doses of whole body x-irradiation. The result suggests, but does not conclusively prove, that the auditory modality is quite radio sensitive.

Summary and Conclusions:

Methods have been developed for the assessment of sensory capacities and motor coordination in primates. These methods will provide highly

reliable data from relatively few animals. Several components of sensory systems are being analyzed over short time periods in conjunction with functional assessments of motor coordination factors. A punch tape recording system compatible with existing computer facilities has been developed in conjunction with this project.

List of Publications:

1. Sharp, J. C. Tone discrimination by x-irradiated rats. Second International Congress of Radiation Research, Harrogate, England, 1962.

2. Brizzee, K. R., Jacobs, L. A., Kharetchko, X., and Sharp, J. C. Quantitative histologic and behavioral studies on effects of fetal x-irradiation in developing cerebral cortex of white rats. In R. Snyder (Ed.) Response of the Nervous System to Ionizing Radiation, Academic Press, Inc., New York, 1962.

3. Sharp, J. C., Ionizing radiation and primate behavior. Tenth V.A. Conference on Psychiatric Chemotherapy, Kansas City, Missouri, 1963.

ANNUAL PROGRESS REPORT

Project DASA RD 40-61, Biomedical (NWER)

Subtask 03.037 Life Span Studies in Irradiated Dogs

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Biophysics
Department of Radiobiology
Division of Nuclear Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: S. J. Michaelson, D.V.M.*
Capt D. E. Davidson, Jr., V.C.
Maj Robert W. Neidlinger, MC

Assistant: Minnie H. Davis

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

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ABSTRACT

Project DASA RD 40-61 Title: Biomedical (NWER)

Subtask 03.037 Title: (Life Span Studies in Irradiated Dogs)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Dr. S. J. Michaelson and Capt David E. Davidson

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

Long-term studies on a group of approximately fifty dogs irradiated at the University of Rochester during the period 1955-57 are being continued. These dogs have variously been exposed to 200r to 1400r of X or gamma radiation delivered to the whole body in single or fractionated doses. Semi-annual blood counts and clinical examinations have been conducted. Irradiation has induced excessive greying of the hair in many of the dogs. A 45 percent incidence of lens opacities and a 15 percent incidence of various benign neoplasms have been observed, however the incidence of these conditions in unirradiated dogs of similar age is not well known. Aside from the above mentioned defects the dogs in the colony remain in generally satisfactory physical condition. Two of the dogs--one with a benign tumor of the base of the tongue, and one with an inoperable papillomatous growth of the ear were sacrificed during the past year. Two additional dogs died during the course of the year--one of a septicemic disease of unknown etiology, and the other as the result of pyometra. Necropsy examinations were performed on all decedents. Histopathologic examination of tissues taken at necropsy is in progress.

BODY OF REPORT

Project DASA RD 40-61 Title: Biomedical (NWER)

Subtask 03.037 Title: Life Span Studies in Irradiated Dogs

Description: The effect of exposure to ionizing radiation on the life span has been studied in rodents in many laboratories. Data concerning the delayed effects of irradiation in other species is exceedingly scant. Information from the larger experimental animals is essential for any reasonable extrapolation to man. The dog as an experimental animal provides, in addition, the important capability for studying in somewhat greater detail the possible mechanisms of life span shortening, since many of the commonly used clinical laboratory determinations, organ function studies and radioisotope tracer techniques can be utilized in the dog.

Progress:

WRAIR has provided outdoor kennels at Forest Glen for housing fifty dogs for these long-term radiation studies. Feeding and routine care has been accomplished through the cooperation of the Division of Veterinary Medicine.

The dogs under study are survivors of various whole-body radiation exposures conducted at the University of Rochester under the supervision of Dr. Howland and Dr. Michaelson. The dogs were irradiated during the period 1955-57 with X-rays and/or Cobalt-60 gamma rays in single or fractionated doses. Cumulative doses of radiation of 200r to 1400r have been administered.

Semi-annual clinical examinations and routine blood counts have been continued as in the past. Urinalyses and blood chemistries have been performed on a less frequent schedule. Clinical examinations during the past year have revealed the progression of the radiation-induced greying of the hair in a large number of the dogs. Newly developed neoplasms were observed in six dogs. Of these, three were perianal adenomas, one a mammary fibroadenoma, one a benign papilloma of the base of the tongue, and one a papilloma of the external ear. The latter two dogs were sacrificed and necropsy examinations were performed. The mammary tumor was removed surgically and preserved for histologic diagnosis. One case of pyometra was successfully treated by ovariohysterectomy. Pyometra was diagnosed in one other dog at necropsy.

Ophthalmoscopic and slit-lamp examination has revealed a 45 percent incidence of lens opacities of various types and degrees. In nine dogs the cataracts were bilateral. The incidence of the lens opacities does not, however, correlate with the magnitude of the radiation dose, nor is the prevalence of cataracts in unirradiated dogs of similar age well-known.

Under the supervision of Dr. Michaelson, thyroid function studies and body water and muscle mass determinations by radioisotope tracer techniques have been carried out on a number of these dogs in conjunction with project No. 3A0 12501 B 813, Army Medical Basic Research in Life Sciences, Task No. 09 Physiology; Metabolism in Radiation Injury.

Summary and Conclusions:

Clinical and pathologic studies of a group of fifty dogs surviving whole-body radiation exposures is continuing. The semi-annual surveys indicate that the dogs in the colony continue to be in generally satisfactory physical condition, although histologic examination of tissues taken at death or sacrifice have frequently revealed degenerative lesions indicative of radiation injury. The incidence of cataracts and neoplasms in these dogs cannot be evaluated at the present time due to the lack of such information from unirradiated aging dog populations. Two of the dogs developing inoperable neoplasms were sacrificed during the past year. Two additional deaths were recorded--one, the result of an unidentified septicemia, the other, due to pyometra.

List of Publications: None

ANNUAL PROGRESS REPORT

Project DASA RD 40-61, Biomedical (NWER)

Subtask 03.038, Metabolism of Fission Products from Fallout

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Biophysics
Division of Nuclear Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Lt Col Kent T. Woodward, MC

Assistants: Major Henry T. Uhrig, MC
Major Richard C. Reba, MC
Captain S. N. Wampler, VC
Miss Ann R. Berman
Mrs. Mary K. Ellis

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project DASA RD 40-61 Title: Biomedical (NWER)

Subtask 03.038 Title: Metabolism of Fission Products from Fallout

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 July 1963

Authors: Lt Col Kent T. Woodward, MC
Maj Henry T. Uhrig, MC
Maj Richard C. Reba, MC
Capt S. N. Wampler, VC
Miss Ann R. Berman
Mrs. Mary K. Ellis

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. Eighty-five persons were monitored for evidence of increase in their body burdens of radionuclides above normally expected levels. No significant increase could be found.

Studies on the metabolism of the fission products, Xenon-133 and Krypton-85 are in progress. Investigations of radiostrontium excretion and methods to facilitate removal of this radionuclide were done.

2. The capabilities of Army area laboratories to detect single radioisotopic contaminants of subsistence were demonstrated in evaluation studies on standard methods adopted or developed in this laboratory. An improved method was developed for detecting P-32 in foods. Promising results were obtained on a new and simple method for detecting Zn-65 in subsistence.

BODY OF REPORT

Project DASA RD 40-61 Title: Biomedical (NWER)

Subtask 03.038 Title: Metabolism of Fission Products from Fallout

Description:

1. Personnel who are occupationally exposed to radionuclides are monitored by the whole body counting facility. Fission product metabolism studies on Xenon-133 and Krypton-85 are in progress. Radiostrontium was investigated, and attempts were made to facilitate the removal of radiostrontium after it had come into equilibrium with the bone calcium pool.

2. The evaluation of the hazards relating to fallout contamination of subsistence is contingent on the capability to identify and measure the fallout in subsistence. Currently available methods are cumbersome and complicated for routine use. Therefore, studies were conducted to develop simple quantitative detection methods for use with the U. S. Army Medical Laboratory system.

Progress:

1a. Gamma Radioactivity in Occupationally Exposed Subjects.

Corps of Engineers Reactor Group. During FY 63, 32 subjects of the Corps of Engineers Reactor Group were evaluated with the NaI crystal total body counting facility. No sign of increase of body burdens of radionuclides above the maximum permissible level was found.

Reactor Personnel, WRAIR. Base line studies have been made on WRAIR reactor personnel. No evidence of increase in body burdens above normal levels have been found. Periodic survey is being continued.

Army Chemical Center Personnel. Personnel potentially exposed to radiocontamination from the Army Chemical Center, Edgewood, Maryland, have been monitored with the NaI crystal. In FY 63, 43 subjects were studied. All evidence indicates levels of body burden of radionuclides well below maximum permissible levels.

1b. Fission Product Metabolism.

Xenon, Krypton Studies. Metabolic studies have been started on Xenon-133 and Krypton-85, produced as fission products. Xe-133 and Kr-85 have been administered by inhalation or intravenous injection in human subjects. Preliminary studies indicate that Kr-85 elimination can be expressed as a two component exponential equation with a t 1/2 of five

minutes for 95% of this gas and a smaller, 5%, component eliminated with a t 1/2 of approximately 45 minutes. For Xe-133 the elimination seems to be somewhat slower.

Radiostrontium studies. Methods to facilitate the removal of radiostrontium were studied. Sr-85 was given IV to a group of rats. Four tetracycline analogs were used. They were given in one instance prior to Sr-85 injection and in the other after Sr-85 had come into equilibrium with the calcium pool. Prior antibiotic treatment showed slight enhancement of elimination especially in the case of declomycin. Post-treatment showed no enhancement of elimination. Subsequent studies with prior treatment with declomycin have shown no such improvement in elimination. A review of the study is being made.

1c. Due to an extensive modification program, the liquid scintillation whole body counter system has not been in operation during the major portion of FY 63. These modifications include transistorizing of the electronics and installation of new photomultiplier tubes and scintillation fluid. Operation is anticipated shortly after installation of the new scintillation fluid.

2. Evaluation studies conducted in conjunction with seven Army Area Medical Laboratories to develop standard methods for analyses of radioactive contamination of foods were continued. During the past year, a high degree of accuracy was obtained amongst the participating laboratories in detecting single isotopic contaminants, e.g., Cs-137, Y-90-Sr-90, P-32, Zn-65, and S-35 in subsistence. Levels of isotopic contaminants employed were comparable to those that would be expected to be found under emergency conditions. Ninety-five per cent of a total of 77 distributed test samples were correctly identified. The quantitative analyses of these samples were within a 10% allowable error, which was within the measurement limitations of electronic equipment. Efforts are now being made to develop a proficiency to detect components of a mixture of isotopes in food.

A new and improved method was found for the detection of P-32. A modified procedure patterned after a method used at the Hanford Atomic Energy Project, Richland, Washington, was used. In the procedure, a sample of test food is ashed, dissolved in acid, diluted with water and successively treated with H_2SO_4 and ammonium molybdate. The P-32 is extracted as phosphyomolybdic acid using a solution of 20% butanol in ether. This procedure has now been standardized for use in Army laboratories. Further efforts were made to simplify methods to detect Zn-65. The use of resin columns to extract Zn-65 have been found to be unsatisfactory. Promising results have been obtained by ashing the food, either by heat or by use of HNO_3 and heat, with subsequent dissolution of the residue in acid. Further studies are being continued.

Summary and Conclusions:

1. Monitoring of 85 occupationally exposed personnel revealed no evidence of increase in body burdens above permissible levels. The whole body counting facility was used in the studies of fission product metabolism of Xenon and Krypton. Methods to facilitate the removal of radiostrontium were studied.

Resumption of operation of the liquid scintillation whole body counter system is expected shortly.

2. The capabilities of Army area laboratories to detect single radioisotopic contaminants of subsistence were demonstrated in evaluation studies on standard methods adopted or developed in this laboratory. An improved method was developed for detecting P-32 in foods. Promising results were obtained on a new and simple method for detecting Zn-65 in subsistence.

List of Publications: None

ANNUAL PROGRESS REPORT

Project DASA RD 40-61, Biomedical (NWER)

Subtask 03.074, Biological Effects at Cellular, Organ or Total Organism Levels

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Departments of Biophysics, Radiobiology, Veterinary Microbiology, Food Radionuclides, Veterinary Pathology, Clinical Chemistry, Germfree Research

Divisions of Nuclear Medicine, Veterinary Medicine, Biochemistry, Basic Surgical Research

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project DASA RD 40-61 Title: Biomedical (NWER)

Subtask 03.074 Title: Biological Effects at Cellular, Organ, or Total Organism Levels

Reporting Installation: Walter Reed Army Institute of Research
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. The influence of growth phase and DNA, RNA and protein syntheses on radiation killing and recovery of Escherichia coli.

2. Comparison of irradiation effects in germfree vs conventional mice to include: a) survival, b) age factors, c) chemical modifiers, d) thyroid function, e) monocontaminants.

3. Determination of the mechanism of bone marrow repopulation following irradiation and the relation of altered vasculature and stroma.

4. The role of Pseudomonas aeruginosa flora in modifying the extent of expected radiation injury or death.

5. Correlation of the alteration of serum protein electrophoresis patterns and immunoelectrophoresis studies following irradiation.

6. The response of irradiated dogs to vaccination with an attenuated strain of infectious canine hepatitis and rabies virus.

7. Determination of free radical production by electron spin resonance techniques following irradiation and correlation with the biological responses of irradiated living systems.

8. Evaluation of the protection afforded by grids from partial body irradiation and the role of healthy tissue surrounding irradiated areas.

9. Determination of sub-fractions of low density lipoproteins by complexes and fractionation and serum studies following irradiation. Development of a new technique of abnormal acid phosphatide determination following irradiation.

10. The early response of conjunctival cells to irradiation as studied by methylene blue uptake and decolorization techniques.

BODY OF REPORT

Project DASA RD 40-61 Title: Biomedical (NWER)

Subtask 03.074 Title: Biological Effects at Cellular, Organ, or Total Organism Levels

Description:

1. Studies of cell population survival kinetics after radiation are complicated because of 1) the physiological heterogeneity of even the most carefully isolated populations, and 2) the lack of information about specific molecular processes in normal cells. It is the intent of this research study to isolate and identify 1) effects produced by selective inhibition of specific molecular processes, i.e., syntheses of DNA, RNA, and proteins, in normal and irradiated bacteria; and 2) changes in radiation responses during different phases of the growth cycle in bacterial cell cultures.

2. Effect of the germfree state on the response to radiation.

3. A single hind extremity of a Lewis strain albino rat is being irradiated so as to include the distal half of the femur and the entire tibia. The radiation source is a 300 KEV G.E. Maxitron unit. Radiation doses to be studied are 2,000, 4,000, and 6,000 rads.

4. Rats have been given total body irradiation with 300 KV X-ray at mid-lethal levels. Cultures of tissue at autopsy for Pseudomonas aeruginosa have been correlated with cultures of water bottle drinking spouts.

5. Agar gel electrophoresis techniques have been used to study serum protein fraction changes following irradiation.

6. Response of X-irradiated puppies to vaccination with an attenuated strain of infectious canine hepatitis (ICH) virus and rabies virus was studied in two different groups of animals respectively. In the study conducted with ICH virus, serum neutralizing antibodies in low and high levels were found in retrospect, in serums obtained at onset of experiment. Irradiated animals were given 225 r X-radiation, which was equivalent to LD_{10}. Vaccination provoked no enhanced antibody response in controls or irradiated animals. Following vaccination and challenge with a virulent virus, no signs of disease were seen in irradiated or non-irradiated animals. Irradiated dogs with low initial antibody levels developed high titer neutralizing antibodies following challenge, whereas no remarkable response was seen in non-irradiated vaccinated animals. Animals that were irradiated four days prior to vaccination generally had lower antibody response than those irradiated four days after vaccination.

Vaccination provoked "secondary" antibody responses in animals with relatively high initial antibody titers. Tissue fluids collected during experiment are now being tested for presence of virus.

Dogs in the rabies experiment were exposed to 275 r x-radiation (LD_{56}) following vaccination with live virus. No untoward effects or signs of disease were produced in irradiated dogs. All vaccinated dogs were resistant to infection with a virulent rabies virus which was lethal for nine of 11 non-vaccinated dogs. Death occurred 8 to 13 days following challenge. Differences in susceptibility to rabies of non-vaccinated irradiated and non-irradiated dogs were not seen. Attempts to demonstrate a viremia in infected animals were unsuccessful. In rabid dogs, virus was demonstrated in brains and salivary glands, but not in liver, spleen, adrenal tissues, nor in saliva. Serological tests are now in progress.

Under test conditions, there was no evidence that irradiation of dogs effects the virulence of attenuated ICH and rabies virus, administered four days prior or after irradiation. There was evidence that the irradiation of dogs prior to vaccination (with ICH) depresses their antibody response. No differences were observed between irradiated and non-irradiated dogs in their susceptibility to infection to rabies virus. There was ancillary evidence based on serology that irradiated animals were susceptible to infection with ICH. Findings to date are consistent with those observed in studies in smaller animals with killed vaccines.

7. ESR studies with biological systems in the frozen state have lead to some conclusions concerning the relation between the ESR signal and the biological response to certain stimuli. The present studies are investigating simultaneously the biological response and ESR signal of biological systems in their natural state to irradiation.

8. The radiation protection afforded by sieves (grid) depends on the interaction between heavily irradiated tissue columns and practically non-irradiated tissue masses surrounding the damaged tissue complexes. According to previous findings the healthy tissue can exercise its beneficial effect only as long as the dose to the tissue under the bridged areas has not exceeded a certain percentage of the total incident dose. In order to get some more information on the latter point, special grids have been developed in which the radiation from the individual open areas tapers off to the unirradiated areas, thus producing a steady decrease from high to low dose values. This goal is achieved by use of spheres (shotgun pellets or bearings) in the most flexible way as to thickness of the absorbing shield and as to "open to close" areas ratio.

9. Research was continued on methods of resolving low density serum lipoproteins into sub-fractions. Work was also initiated on a method for the detection of a lipid component normally found in tissue but only in trace amounts in serum. The fractionation of low density lipoproteins

and the detection of abnormal serum lipids will have clinical application in the study of radiation injury as well as diseases in which there is a disturbed lipid metabolism. The metal-heparin complex method for lipoprotein determination as well as the newly developed method for serum acid phosphatides has been used in the study of the effects of whole body radiation in man.

10. This is an investigation of biomedical effects of ionizing radiation upon the conjunctival epithelium with emphasis on casualty producing phenomena and the estimation of the degree of injury.

Progress:

1. In E. coli 15 TAU, which requires thymine (T), arginine (A), and uracil (U) for normal growth, it is known that growth of a logarithmic-phase culture with thymine, but without arginine and uracil (+T-AU) for 90 min results in a 100-fold increase in multiplicity of UV (2537Å) survival curves (Hanawalt, P. C., Progr. in Photobiology, 305, 1961).

Examination of +T-AU cultures reveals that they are physiologically, morphologically, and radiologically indistinguishable from control (+TAU) cultures in the stationary phase. Blockage of protein and ribonucleic acid syntheses in log phase forces the cells into a premature but apparently genuine stationary phase, where they are more resistant both to UV and to ionizing radiation.

Comparing UV responses of sensitive (log phase) or resistant (stationary phase) cultures, it is found that (1) the final exponential portions of the survival curves are parallel, (2) the photoreactivable sectors of both cultures have the same constant value for all UV doses, and (3) the photoreactivation rates depend directly on UV dose and only indirectly on survival. It is concluded that, for the same UV dose, photoreactivable damage is qualitatively and quantitatively identical in the sensitive and resistant cultures. Various considerations lead to the tentative conclusion that the nonphotoreactivable damage is also identical. This suggests that both types of damage are subject to a "dark recovery" in the UV-resistant cells.

The increase resistance of stationary phase cells to ionizing (Cobalt-60 gamma rays) radiation is comparable to the increase observed for UV. This effect is being studied, since it appears likely that an analogous recovery capacity, rather than differences in primary lesions, also accounts for increased resistance of stationary phase cells to ionizing radiation.

The recovery capacity is believed to be related to the chronology and extent of specific synthetic processes occurring after irradiation.

Preliminary experiments have implicated DNA, and possibly RNA syntheses.

The physiological basis of the proposed recovery capacity is being studied.

2. The 30 day survival of germfree mice (ND-2 strain) after exposure to X-ray has been completed. The germfree state was found to provide increased resistance to radiation.

The same study is essentially complete for the new strain (ICR) which is now in use since it is commercially available. Again the germfree state produces an increase in tolerance to X-ray as compared to the conventional mouse.

The effect of the germfree state on survival after exposure to supra-lethal doses has been completed. The germfree mouse was found to survive at least twice as long as the conventional mouse after exposure to doses producing death from effects on the gastro-intestinal tract or doses producing death from central nervous system effects.

Sensitivity to radiation as a function of age is being measured by survival time after exposure to 1800 r. The germfree mouse exhibits a stable (9-10 day) survival time between 10 weeks of age and 1.5 years of age. In the normal mouse 1800 r produces death earlier (after approximately four days) and survival time varies with age being still less for younger (10 week old) and older (1 year) mice. Survival in younger age groups will be determined to complete this study.

Studies of the effect of radioprotective drugs on survival of mice in the germfree state were initiated. Two drugs have been used and were found to further enhance the protection already provided by the germfree state. This study is being continued with the use of other drugs.

Studies of the basic physiology of the germfree mouse were initiated by investigation of thyroid function as measured by retention of Iodine-131. Since this study is in an early stage no conclusive results are available.

The response of lymphoid tissues of the germfree mouse to irradiation has been completed in collaboration with Richard E. Horowitz, MD, Department of Pathology, Mount Sinai Hospital, New York, and Heinz Bauer, MD, Department of Germfree Research, WRAIR. This is reported in detail in the germfree department progress report, Division of Basic Surgical Research, WRAIR.

3. Studies are in progress to elucidate two major factors in marrow repopulation by irradiation. The first factor is the mechanism by which repopulation occurs. Three possible mechanisms are in situ regeneration,

infiltrative repopulation from contiguous non-irradiated healthy sites and metastatic repopulation via a vascular route from distant, healthy and non-irradiated sites. The second factor to be evaluated is the role played by altered stroma and fine vasculature in modifying the receptivity of the irradiated marrow to repopulating hemopoietic elements.

Studies are now in progress in which local X-irradiation is being administered at three levels to a single extremity of a female albino rat. After permitting the lesion to evolve for three months, isogenic marrow from non-irradiated donor male rats (pre-labeled with tritiated thymidine) will be given to the locally irradiated females by a parenteral route. This will provide a double label -- Y male chromosome and tritium -- allowing the detection of the donor marrow as it distributes through the host. Furthermore, it should be possible to distinguish between donor and host marrow elements.

An evaluation of alteration in bone marrow vascular architecture is being made by means of a microangiographic technique utilizing radio-opaque contrast media to fill the marrow vessels. Microradiography will be used to demonstrate the vascular architecture. Altered stroma will be evaluated with a variety of special histologic staining techniques.

4. The effect of Pseudomonas aeruginosa infection in rats exposed to gamma radiation has been investigated. Two hundred animals in groups of 20 were exposed to levels of radiation varying from 750 to 1000 Roentgens. Eighty three percent of those animals dying the first 12 days had positive spleen cultures for P. aeruginosa. Only 7% of those dying in the next 18 days were similarly positive. Water bottle drinking spouts were cultured three times in a second group of 200 animals singly caged. Forty two animals were selected at random from the ones positive on at least two occasions and 42 from those consistently negative. Twenty one rats from each group were irradiated to 850 Roentgens. Fourteen of the positives died and five of the negatives. Spleen cultures on 10 of the 14 yielded P. aeruginosa, while none of the five in the negative group did so. Survivors and controls were sacrificed. Throat and trachea, small and large bowel, and spleen were cultured. Thirteen of 28 positives (21 controls plus 7 survivors) yielded P. aeruginosa in the cultures, and only two of 37 (21 controls plus 16 survivors) of the negatives. A control program utilizing (1) chlorinated water and (2) water bottle sterilization and change three times weekly has been initiated.

5. Serum samples from rats, dogs, and monkeys which have been irradiated to lethal doses have been collected. Serum protein fraction changes have been observed using agar gel electrophoresis techniques. The changes appear to be an increase in one of the alpha globulin components in monkeys and dogs and one of the beta components in rats. The value of this change in evaluating prognosis has not been established at this time.

Rabbits have been hyperimmunized against serum from both irradiated and non-irradiated rats and dogs. Studies are underway which are intended to determine whether the changes mentioned above are the result of an increase in a normal component or whether they result from the appearance of an abnormal component which is antigenically different. Preliminary results are inconclusive at this time.

6. A study of the response of irradiated dogs to a live, attenuated vaccine was initially conducted with a modified infectious canine hepatitis (ICH) virus. Forty-eight beagle puppies, 5-7 weeks old, were obtained, and at time of receipt, randomly divided into six groups, as shown in Table 1.

Table 1

Treatment Groups for Studies of Response of X-Irradiated Dogs to an Attenuated Canine Hepatitis Virus

Group	No. of Dogs	Treatment
I	10	Vaccinated 4 days pre-irradiation
II	10	Vaccinated 4 days post-irradiation
III	10	Irradiated - Not vaccinated
IV	6	Vaccinated - Not irradiated
V	6	Challenge virus controls
VI	6	Experiment controls - no treatment

Dogs were given hyperimmune canine distemper serum (free of ICH antibodies), wormed and placed in quarantine for a period of 4 to 6 weeks. The passive immunization for distemper was continued at 10-12 day intervals throughout the experiment. Animals in Groups I, II, and III were given a commercially available ICH vaccine. The titer of the vaccine determined in dog kidney tissue culture (DKTC) was 10^4 per ml. It was administered subcutaneously in a 2.0 ml dose. Dogs in Groups I, II and III were given 225 r total body irradiation from a 2 MEV X-ray machine. On the basis of previous observations, an LD_{20-40} was expected with this exposure. However, the actual potency of the irradiation dosage was found to be LD_{10}. Dogs in all groups, except Group VI, were challenged 27 to 31 days post-vaccination with a tissue culture (DKTC) of virulent ICH strain, obtained originally from Cornell University. The LD_{50} of the challenge virus was 10^6 per ml. It was given subcutaneously in a 2.0 ml dose.

Blood, saliva, and feces for serological tests and/or virus isolation, were collected at onset of the experiment and periodically thereafter. Primary DKTC was utilized for viremia and serum neutralizing antibodies determinations. Constant virus (100 $TCID_{50}$) and varying serum dilutions were used for serum neutralization tests.

When dogs were procured, it was assumed that the ICH maternal antibodies would be present, but would have disappeared by the time the experiment was initiated. This assumption was incorrect, as disclosed by subsequent tests. In retrospect, serum neutralization titers at this time ranged from 1:4 to 1:126. Few dogs had titers at the highest level.

Irradiated animals gave characteristic lymphocytic dyscrasias, but otherwise displayed no abnormal signs. Following vaccination and challenge, no signs of disease were seen in any of the dogs. Determination of the presence of virus in saliva and feces are now in progress. The antibody response during the course of the experiment of dogs in the various groups that had a low (e.g., 1:4 to 1:8) and high (1:16 to 1:128) antibody titer is summarized in Table 2.

The vaccine provoked no rise in antibody titer in non-irradiated controls (Group IV). Presumably, on the basis of their poor antibody response after challenge with a virulent virus, these dogs were immune to infection. In contrast, comparable dogs in the irradiated groups (low pretreatment antibody level) gave no rise in antibody titer attributable to vaccination, but following challenge, relatively high titers were elicited. These rises may reflect greater susceptibility to infection attributable to irradiation stress. The antibody titers of animals in Group I (vaccinated and then irradiated) with significant initial antibody levels spiked rapidly following vaccination, and also following challenge, reminiscent of a "secondary" antibody response. Similar "secondary" antibody responses were seen in dogs of all groups with high initial antibody levels after challenge with virulent virus. Dogs in Group I with high initial antibody levels responded to the vaccine, whereas comparable dogs in Group II did not. These findings are consistent with other observations on the effect of irradiation on antibody response of vaccinated animals, depending on time irradiation stress was induced.

The difference between the responses of dogs with low and relatively high initial antibody titers may reflect a passive and active immune status.

In view of the difficulties encountered in the experiment with ICH virus, viz, the omnipresence of ICH antibodies in dogs, it was deemed desirable to repeat the experiment employing a rabies virus. The design of the first experiment was followed, except that the radiation dose was increased to 275 r. Forty-eight beagle puppies were obtained and randomly

Table 2

Serum Neutralizing Antibody Response in Dogs Following Vaccination and Challenge with Infectious Canine Hepatitis Virus

Group	Pretreatment* Antibody Level	Geometric Mean Antibody Titer								
		Days Post-Vaccination				Days Post Challenge				
		0	4	8-11	15-22	0**	3	5	12	19
I Vaccinated pre-irrad.	Low	-***	-	-	-	-	12	120	256	256
	High	32	103	78	256	144	256	256	256	256
II Vaccinated post-irrad.	Low	-	-	-	-	-	16	28	108	118
	High	28	48	34	40	82	184	256	256	256
III Irrad. - Not vaccinated	Low	-	-	-	-	-	22	94	140	86
	High	64	32	20	30	20	28	256	256	256
IV Vaccinated not irrad.	Low	-	-	-	-	-	-	-	16	24
V Challenge controls	Low					-	16	144	224	224
	High					126	168	256	256	256
VI Untreated not challenged	Low	-	-	-	-	-	-	-	-	-

* Low = Titers <1:16 (1:4 to 1:8), high = Titers 1:16 to 1:128
** 27-31 days post-vaccination
*** - = <1:16 (1:4 to 1:8)

placed in the six groups, vaccinated against distemper and ICH and held until they reached 12-14 weeks of age. A commercially available low egg passage Flurry rabies vaccine was employed; administered according to the manufacturers instructions. The challenge virus was a fox salivary gland virus (FSC) obtained from the New York State Department of Health. It was administered into the masseter muscle in 0.4 ml dose. Blood and saliva were collected at periodic intervals for serological or virological studies. Brain, salivary gland, adrenal, spleen, and liver were collected for virus isolation from dogs that died. Mouse inoculation, fluorescent antibody (FRA) and staining (Sellers) techniques were used to demonstrate virus in specimens. The presence of antibody was determined by mouse neutralization tests.

The irradiation dose of 375 r had a LD_{56} - approximately twice greater than the expected potency. Five animals each in Groups I and II and three in Group III died because of irradiation. Following vaccination, no untoward responses were noted in non-treated or irradiated dogs. On the 27th or 31st day following vaccination, a virulent rabies virus was given to all animals in Groups I through V. Two animals each in Groups I and II were not challenged, but were held six months to rule out or establish presence of rabies, attributable to the attenuated vaccine. The effects of the challenge amongst the five groups of dogs are summarized in Table 3.

Table 3

Susceptibility of Vaccinated & Non-Vaccinated
X-Irradiated Dogs to Rabies Infection

Groups	No. of Dogs Challenged	Deaths	Average Day of Death
I Vaccinated pre-irradiation	3	0	
II Vaccinated post-irradiation	1	0	
III Irradiated - not vaccinated	5	4	11
IV Vaccinated - not irradiated	6	1*	19
V Challenge virus controls	6	5	11

* Accidental death, following heart puncture on 19th day post-challenge. No rabies virus found in brain and salivary gland.

All vaccinated animals were protected against a virulent challenge of rabies. Deaths occurred in non-vaccinated animals from the 8th to 13th day post-exposure. The disease had an abrupt onset, terminating in death

in 24 hours. The "typical" clinical manifestation of rabies was seen in only one of the nine dogs that died. There was no early evidence of infection in sick animals. Difference in susceptibility to rabies between irradiated (Group II) and non-irradiated (Group V) animals was not demonstrated. Attempts to demonstrate viremia in rabid dogs, employing mouse inoculation techniques were unsuccessful. Employing mouse inoculation techniques, rabies virus was demonstrated in brain tissues of all nine and in salivary glands of five of nine dogs that died following challenge. However, no virus was detected in spleen, liver and adrenal tissues of infected dogs. Negri bodies were not seen in brain impression smears, but rabies virus was demonstrable by FRA techniques for all nine rabid dogs. Rabies was not demonstrated in the saliva of infected animals. Serological studies are now in progress.

7. On the basis of triphenyl tetrazolium chloride (TTC) viability tests, three kinds of seeds were selected for the studies: mustard, buck wheat, and winter rye. They were exposed to Cobalt-60 gamma radiation and their biological responses as well as the ESR signal changes were recorded. The biological tests followed the usual planting procedures and were supported by TTC viability checks. Both types of responses reported on in the literature could be confirmed. Depending on the system and the radiation mechanisms, the biological responses can be accompanied by an increase or decrease in the number of free radicals. In the latter case the damage would be correlated to the disappearance, that means the use of the free radicals in producing the damage. Influence of soaking and storage times on the effect are under way.

Smaller and Avery (Nature, 1959) demonstrated attenuation of the ESR signal associated with yeast upon addition of agents known to modify radiation response. Preliminary studies have shown that Smaller and Avery's technique of freezing the yeast results in 99+% kill. A method has been developed to slow freeze the yeast and reduce the kill by a factor of about 100, permitting survival studies to be accomplished. A haploid strain of yeast has been obtained to produce simpler survival curves.

ESR studies are proceeding to characterize and identify free radicals formed in frozen H_2O and D_2O.

Free radicals with an appreciable life time indicate a "triplet" state in which direct radiation of the excess energy is a "forbidden" transition. Irradiation of such a radical with broad band UV may raise the excitation level of the radical to a level from which it can spontaneously radiate the total excitation energy and return to its ground state. Measurements of half-life of such radicals produced by irradiation with and without UV treatment are being taken to confirm this hypothesis.

8. Two sizes of sphere grids were used in preliminary studies of the response to this kind of partial body shielding. The experiments were based on repeated exposure of Walter Reed mice to X-rays, using conventional grids (1.5 mm Pb, 2 mm and 10 mm diameter holes respectively, 52% "open to close" areas). Evaluation of the results of the experiment is under way.

9. Fractionation of low density lipoproteins. The previously reported method of isolating low density lipoprotein complexes and the sub-fractionation of these complexes on the basis of solubility in Na Cl was further studied. These sub-fractions display a variation in the phospholipid-cholesterol ratios as well as protein to cholesterol ratios. It has not yet been established that the above fractions exist as entities in the serum or artifacts resulting from the processing. To this end, a new technique, disc electrophoresis, has been introduced. Disc electrophoresis is more sensitive in the resolution of serum proteins than is paper or even conventional electrophoresis. In two attempts so far, redissolved low density lipoprotein complexes have displayed several bands. This is most encouraging since low density lipoproteins appear as a single band when subjected to paper electrophoretic techniques. It remains to be seen whether the sub-fractions based on Na Cl solubility display different mobilities by this new technique.

Abnormal serum lipids. Phosphatidic acids, the phospholipids in which the choline or other nitrogen-containing components are absent, are found in considerable amount in tissue, - especially heart tissue. The serum level of these compounds is normally quite low. It is conveivable that following radiation injury, these components may increase in serum. To this end, a method adapted to clinical investigation has been devised.

Serum is treated with acetone (1 ml serum to 10 ml acetone) and the precipitate is extracted several times with methanol. The methanol extract, containing phospholipids, is treated with a 10% $Ba\,Cl_2$ solution to precipitate the barium salts of acid phosphatides. The crude barium phosphatides are then ashed for two hours at 600° and phosphorous determined by the method of Fiske and Subbarow. Further purification steps are in process in order to identify the type of acid phosphatides present.

Most sera do not show much of this acid phosphatide component but occasionally high values have been encountered. No data are yet available to show any correlation between a high acid phosphatide level and diseased states.

Only two cancer patients who had received whole body radiation therapy have been available for study. In both cases, the low density lipoproteins increased. In one case, the value in terms of cholesterol rose from a pre-radiation level of 157 mg% to 202 mg% five days following the beginning of therapy. The low density cholesterol of the second

case rose from a pre-level of 147 mg% to 192 mg% in eight days. The medium and high density lipoprotein values fluctuated with an apparent pattern. A determination of acid phosphatides in the second case showed a change from 3.03 mg% to 18.25 mg%.

10. This experiment to determine the uptake and decolorization of methylene blue by irradiated conjunctival epithelial cells in situ was terminated after exposure of 18 dogs. Right eyes only were exposed to 100 r, 300 r, 900 r, or 2700 r. After 100 r or 300 r exposures, no increased uptake of dye was seen. After 900 r and 2700 r, there was increased uptake in terms of more cells being stained in the supra limbal region of the bulbar conjunctiva. However, the abrading effect of a foreign body beneath the eyelid produced staining which far exceeded that of any of the X-ray exposures that were used.

Summary and Conclusions:

1. A specific alteration in the metabolic state of cells prior to irradiation has been analyzed, and it was observed that the result of this alteration was to change the biological consequences, but not the nature of the radiation damage. It is concluded that 1) the radiosensitivity of cells at the time of irradiation is independent of their metabolic state, 2) the metabolic state immediately after irradiation determines the biological consequences of radiation damage, thereby influencing the shape of the experimentally measured dose-effect curve, and 3) differences in survival kinetics under different experimental conditions will be observed, even though the different kinetics arise from initially identical damage.

2. In all of the studies the germfree state has been shown to provide increased tolerance to radiation after exposure to radiation levels ranging from the minimum lethal dose to doses in the upper range of the CNS death. Exploratory studies on the ability of anti-radiation drugs to protect germfree mice against radiation - have already indicated that the effects are additive - the protective compound further increases the resistance to radiation provided by the germfree state.

3. Methods have been devised and studies are in progress to evaluate the role played by altered stroma and vasculature as they affect repopulation of radiation injured bone marrow.

4. Pseudomonas aeruginosa infection in rats used for radiobiology research will have an appreciable effect on results, if not controlled. Such infections can be reduced in an animal colony by the use of chlorinated drinking water.

5. Changes in electrophoresis pattern of serum proteins from irradiated rats, dogs, and monkeys have been observed. The value of this observation as a prognostic indicator has not been established at this time.

6. Response of X-irradiated puppies to vaccination with an attenuated strain of infectious canine hepatitis (ICH) virus and rabies virus was studied in two different groups of animals respectively. In the study conducted with ICH virus, serum neutralizing antibodies in low and high levels were found in retrospect, in serums obtained at onset of experiment. Irradiated animals were given 225 r X-radiation, which was equivalent to LD_{10}. Vaccination provoked no enhanced antibody response in controls or irradiated animals. Following vaccination and challenge with a virulent virus, no signs of disease were seen in irradiated or non-irradiated animals. Irradiated dogs with low initial antibody levels developed high titer neutralizing antibodies following challenge, whereas no remarkable response was seen in non-irradiated vaccinated animals. Animals that were irradiated four days prior to vaccination generally had lower antibody response than those irradiated four days after vaccination. Vaccination provoked "secondary" antibody responses in animals with relatively high initial antibody titers. Tissue fluids collected during the experiment are now being tested for presence of virus.

Dogs in the rabies experiment were exposed to 275 r X-radiation (LD_{56}) following vaccination with live virus. No untoward effects or signs of disease were produced in irradiated dogs. All vaccinated dogs were resistant to infection with a virulent rabies virus which was lethal for 9 of 11 non-vaccinated dogs. Death occurred 8 to 13 days following challenge. Differences in susceptibility to rabies of non-vaccinated irradiated and non-irradiated dogs were not seen. Attempts to demonstrate a viremia in infected animals were unsuccessful. In rabid dogs, virus was demonstrated in brains and salivary glands, but not in liver, spleen, adrenal tissues, nor in saliva. Serological tests are now in progress.

Under test conditions, there was no evidence that irradiation of dogs effects the virulence of attenuated ICH and rabies virus, administered four days prior or after irradiation. There was evidence that the irradiation of dogs prior to vaccination (with ICH) depresses their antibody response. No differences were observed between irradiated and non-irradiated dogs in their susceptibility to infection to rabies virus. There was ancillary evidence, based on serology, that irradiated animals were susceptible to infection with ICH. Findings to date are consistent with those observed in studies in smaller animals with killed vaccines.

7. The responses of seeds under different experimental conditions (water content, storage time) have been studied with conventional planting and the TTC viability test and have been correlated with ESR signal changes. While in certain systems the biological response is parallel with a decrease in the spin resonance signal, in other cases an increase of spin resonance signal is connected with the biological reactions. Quantitative studies, based on the use of TTC as an indicator of radiation damage, are encouraging and will be further developed. The other free radical studies as mentioned previously are also of a preliminary nature and no conclusions can be drawn from them at this time.

8. Following previous studies a new kind of grid was constructed using spheres to build the desired grid pattern. This method introduced an additional possibility to check the role of healthy tissue surrounding the radiation damaged tissue in the recovery from the radiation insult. The sphere grids produce a tapering off pattern of those underneath the grid and thus a different radiation burden which should change the radiation response of the exposed animals.

9. The increase in low density lipoproteins following radiation in man substantiated previous data in animals but not enough cases have yet been studied to make an adequate evaluation.

Low density lipoprotein complexes are being subjected to the new disc electrophoretic technique. A method for the detection of abnormal phosphatides in serum is being developed.

The use of acid phosphatide levels as an indication of radiation response shows considerable promise in the one case studied. The percentage increase over pre-radiation values is much greater than the lipoprotein response.

10. Staining of conjunctival cells following irradiation is related to absorbed dose, but conclusions are invalid in the presence of coexisting traumatic effects.

List of Publications:

1. Ginsberg, D. M., and J. Jagger, 1963, Photoreactivation and dark recovery of ultraviolet killing in Escherichia coli strain 15 TAU. Abstracts, 11th Annual Meeting, Radiation Research Society, Radiation Res., 19: 233.

2. Hightower, Dan, Uhrig, H. T., and Davis, J. I. Pseudomonas aeruginosa Infection in Rats Used in Radiobiology Research, to be presented at the 14th Annual Meeting, Animal Care Panel, October 1963.

ANNUAL PROGRESS REPORT

Project MD 40-61	BIOMEDICAL (MMER) (DASA)
WRP No.	
03.074	Biological effects at cellular, organ, or total organism level.
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C. Department of Cellular Physiology Division of Basic Surgical Research
Period Covered by Report:	1 July 1962 through 30 June 1963
Principal Investigator:	Andre D. Glinos, M. D.
Assistants:	Hanson H. North, B. S. PFC Don D. Hargrove Warner T. Brown, M. S. PFC Bruce R. Sather
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project RD 40-61	BIOMEDICAL (MEDR) (DASA)
WRAIR No.	
03.074	Biological effects at cellular, organ, or total organism level.
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
Period Covered by Report:	1 July 1962 through 30 June 1963
Authors:	Andre D. Glinos, M. D., Hanson H. North, B. S., PFC Don D. Hargrove, Warner T. Brown, M. S., PFC Bruce R. Sather
Reports Control Symbol:	MEDDM-288
Security Classification:	UNCLASSIFIED

I. Previous findings had suggested that the severe injury sustained by rapidly proliferating cell populations is not due to radiotoxins or particularly sensitive metabolic states at the time of irradiation but to the high incidence of post-irradiation cellular divisions resulting in the mitotic death of such populations.

This concept has now been firmly established by extending these observations to a far greater number of post-irradiation divisions than previously studied.

II. In preliminary experiments, irradiated cells growing in suspension have shown a higher rate of survival than when growing on glass either as large monolayer populations or as single, colony forming, cells. The conditions responsible for this finding, including the possibility of post-irradiation repair, are currently under investigation.

III. As the above findings suggest chromosome injury and repair as the key processes involved in cellular radiation death and survival, the development of a new system for analyzing the cytogentic constitution of mammalian cells in culture has been undertaken.

BODY OF REPORT

Project RD 40-61 Title: BIOMEDICAL (NWER) (DASA)

WER No.: 03.074 Title: Biological effects at cellular, organ, or total organism level.

Description:

It is well known that rapidly proliferating tissues such as the bone marrow and the intestinal epithelium are injured most severely by ionizing radiation, the acute radiation syndrome being an expression of this injury. Elucidation of the nature of the relationship between cellular proliferation and the degree of radiation injury sustained by a given cell population would seem therefore to be an essential prerequisite for the design of rational methods for the prevention and treatment of the acute radiation syndrome. In work previously reported the post-irradiation population kinetics of replicate cultures of the L strain mouse fibroblasts originating in the logarithmic and stationary phases of growth were compared. This comparison showed that, when induced to proliferate after being irradiated, both logarithmic and stationary origin cell populations decline rapidly, this decline being a linear function of the number of post-irradiation cell divisions up to the fourth division. Extrapolation of the dose-response curves obtained with these replicate cultures suggested that beyond the fourth division the irradiated cell populations reach a constant level identical for both logarithmic and stationary origin cells. If confirmed, these results would then indicate that the metabolic state of the cells at the time of irradiation, whether actively growing in the logarithmic phase or resting in the stationary phase, does not affect cellular radiosensitivity, death occurring predominantly during the first four post-irradiation divisions of the cells. Confirmation of these concepts would therefore require observations on irradiated cell populations of logarithmic and stationary phase origin beyond the fourth post-irradiation division. Since replicate cultures are impractical because of the very large cell populations involved, these observations were carried out on single cell colony-forming cultures.

Progress:

I. Cells from the logarithmic and stationary phases of suspension cultures of the L strain mouse fibroblasts were trypsinized and diluted serially to a concentration of 100-400 cells/ml of medium and inoculated into T-15 flasks. As soon as these dispersed single cells were

attached to the glass surface of the flasks they were irradiated with a Cs^{137} gamma ray source located in a special incubator with doses ranging from 17.5 r to 1000 r at a dose rate of 7 r/min. The irradiated flasks together with non-irradiated control flasks were then incubated for a period of 12 to 16 days in an atmosphere of 3% CO_2 in air to insure a constant pH. At the end of the incubation period the medium was discarded, the flasks fixed and stained, and the number of distinct colonies arising out of the original single cells counted. Since the objective of the experiment was to compare the survival of logarithmic and stationary origin cells beyond the fourth post-irradiation division, only colonies containing a minimum of 64 cells, indicating that the original cell and its progeny have undergone at least 6 divisions, were counted. These colony counts were then expressed as per cent of the counts obtained in the corresponding unirradiated controls. The mean value thus obtained in three experiments with logarithmic origin cells and three experiments with stationary origin cells are shown in the following table:

Table I

Donor cell state	Surviving Fraction at Corresponding Roentgen Dose							
	0	17.5	35	70	175	315	490	1000
LOG	1.00	.97	.90	.74	.44	.17	.07	.0039
STA	1.00	.95	.88	.71	.41	.175	.07	.0040

These results clearly indicate that beyond the first four post-irradiation divisions, survival reaches a constant level identical for both cells of logarithmic and stationary origin thus confirming the concept that the metabolic state of the cells at the time of irradiation does not affect cellular radiosensitivity and that cellular radiation death occurs predominantly during the first four post-irradiation divisions.

II. Although alteration of the conditions under which post-irradiation cell growth and division occurs are known to modify the survival of unicellular organisms, this has never been shown to occur with mammalian cells. Accordingly, an attempt was made in this direction by comparing the survival of irradiated cells growing in suspension with the survival obtained in the previous experiments where cells were grown as monolayers or as single cells, attached to glass surfaces. The number of post-irradiation divisions which can be observed in suspension cultures presents no particular problems because by suitable periodic dilution the total number of cells can be kept within certain limits thus allowing a follow-up of the cell population kinetics for

any number of post-irradiation divisions desired. In a preliminary experiment along these lines it was found that a cell population of logarithmic origin irradiated with 1500 r and followed in suspension culture up to the tenth post-irradiation division, was reduced after the first four post-irradiation divisions to a level corresponding to 0.36 of a non-irradiated control culture. From Table I it can be seen that on the basis of the previous experiments population level at 1500 r would be $\leq$0.004. The possible reasons for this unexpected high survival are currently under investigation with special attention focused on the possibility that metabolic conditions in suspension cultures, as contrasted to replicate or single cell cultures on glass, might be particularly conducive to repair of the injury sustained by key cellular structures.

III. As the experiments previously outlined suggest that the key structures involved in cellular radiation death and survival are the chromosomes, a new system for analyzing the cytogenetic constitution of mammalian cells in culture is currently under development. It is expected that this system will provide the basis for the subsequent analysis of (a) the relationship between the chromosomal constitution of mammalian cell populations in culture to the tissue of origin, (b) the relationship between chromosomal constitution and the metabolic state of logarithmically growing and stationary cell populations, and (c) the relationship between chromosomal injury and cellular radiation death and survival.

Summary and Conclusions:

I. Previous findings had suggested that the severe injury sustained by rapidly proliferating cell populations is not due to radiotoxins or particularly sensitive metabolic states at the time of irradiation but to the high incidence of post-irradiation cellular divisions resulting in the mitotic death of such populations.

This concept has now been firmly established by extending these observations to a far greater number of post-irradiation divisions than previously studied.

II. In preliminary experiments, irradiated cells growing in suspension have shown a higher rate of survival than when growing on glass either as large monolayer populations or as single, colony forming, cells. The conditions responsible for this finding, including the possibility of post-irradiation repair, are currently under investigation.

III. As the above findings suggest chromosome injury and repair as the key processes involved in cellular radiation death and survival,

the development of a new system for analyzing the cytogenetic constitution of mammalian cells in culture has been undertaken.

List of Publications:

Glinos, A. D.: Protein composition and growth-promoting activity of the serum following whole-body x-irradiation at different ages. Rad. Res. (in press).

ANNUAL PROGRESS REPORT

Project RD 40-61, Biomedical (Biological effects at cellular, organ, or total organism level)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Experimental Pathology
Division of Special Activities

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: D. C. Biggers, Capt., MC
H. Sprinz, Col., MC
L. M. Kraft, D.V.M.*

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

*Public Health Research Institute of the City of New York, Inc.
Otisville Branch, Otisville, New York.

ABSTRACT

Project No. RD 40-61 Title: Biomedical (Biological effects at cellular, organ, or total organism level)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: D. C. Biggers, Capt., MC
H. Sprinz, Col., MC
L. M. Kraft, D.V.M.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

The importance of epizootics of intestinal virus diseases in mice requires no elaboration. As this department only recently undertook to furnish additional support in pathology to the Division of Veterinary Medicine, it was felt best to join forces with an expert virologist, Dr. Lisbeth M. Kraft, who had first hand experience in this field and had made important contributions to the subject.

As the tissue changes are more severe in the disease caused by the virus designated as "Lethal Intestinal Virus of Infant Mice" (LIVIM), it was decided to study the pathology and pathogenesis of this infection first and then to broaden our investigation into other viral diarrheas of mice.

In the initial study, 50 infant mice of the C57BL strain were infected per os with a standard dose of LIVIM virus at ages varying from 1 to 14 days and sacrificed at intervals varying from 1 to 5 days. Twenty-five mice varying from 2 to 17 days of age served as controls.

The entire gastrointestinal tract was removed and fixed. Histologic preparations stained with H&E, Giemsa, and Laidlaw Inclusion Body Stain were made. Preliminary survey of the material from 9 infected animals and 6 controls has shown striking alterations in the mucosa. In the newborn through 6 days of age, the changes

affect the intestinal epithelial cells and are principally cytopathologic, i.e., syncitial formations, intracytoplasmic inclusion bodies, and intranuclear inclusion bodies with little or no change in the lamina propria or lymphoid tissues. However, in animals infected after 7 days of age, the mucosal response is principally proliferative with a marked increase in the crypt/villus ratio due to increased crypt length and shortening of villi. Indicative of altered cellular kinetics and increased turnover rate of the intestinal epithelium is a florid increase in mitotic activity in the crypts. This is combined with an increased cellularity of the lamina propria, and hyperplasia of lymphoid tissue with focal necroses.

BODY OF REPORT

Project No. RD 40-61 Title: Biomedical (Biological effects at cellular, organ, or total organism level)

Description:

Kraft has described an epidemic viral enteritis lethal to mice during the first 2 weeks of life. The disease is inapparent in adults although the virus can be recovered from the intestinal contents up to one year of age. Abnormal morphologic findings in the intestinal tract were reported in the spontaneous disease.

It is the purpose of this present investigation to critically analyze the histopathologic changes elicited by per os infection of mice at ages varying from 1 to 14 days and sacrificed after 1 to 4 days of infection. A pilot survey has shown striking alterations differing in pattern of response depending upon age of infection.

Progress:

Fifty C57BL mice were infected per os with a standard dose of 10^5 x ID_{50} of LIVIM virus at ages varying from 1 to 14 days. They were sacrificed at intervals varying from 1 to 4 days after infection. Twenty-five animals sacrificed at ages 2 to 17 days served as controls.

The entire gastrointestinal tract was removed and fixed in Helley's Solution. After overnight washing, the intestinal masses were stored in 70% ethanol.

In the pilot survey of this material, 9 infected animals at representative ages and 6 appropriate control animals were studied to determine, by a "screening" analysis, the proper mode of sectioning and what stains would best demonstrate the findings. In this survey, the following interesting alterations were noted. In the animals from 1 to 6 days of age the changes in the intestinal mucosa were principally cytopathologic consisting of syncytial formations ("balloon" cells), intracytoplasmic inclusion bodies and intranuclear inclusions with very little or no change in the lamina propria or regional lymphoid tissue. In animals infected from 7 to 14 days of age the mucosal response was more a proliferative change with a marked increase in the crypt/villus ratio, numerous

mitotic figures in the crypts, increased cellularity of the lamina propria, and increase in the local lymphoid tissue with focal areas of necrosis.

A problem was encountered in the control tissue in that many of the epithelial cells of these normal animals contained intracytoplasmic "bodies" of a slightly different textural quality and more irregular than the intracytoplasmic inclusion bodies in the infected animals, but of the same staining intensity with H&E and Giemsa stains. This problem was solved by applying a staining technique utilizing acid fuchsin and a phosphomolybdic acid mordant with Orange-G as a decolorizing agent. This stain colors the inclusion bodies in the infected animals brilliantly, while none of the intracytoplasmic bodies in the controls were stained.

The intestinal masses from all animals, infected and control, are now sectioned and stained with H&E, Giemsa, and Laidlaw's Inclusion Body Stain (acid-fuchsin, Orange-G, Hematoxylin). They will be analyzed with detailed recording of the following features: syncytial formation, intracytoplasmic inclusion bodies, relative numbers of villi, crypt/villus ratio, mitotic activity of crypts, number and location of goblet cells, integrity of brush border, cellularity lamina propria, cell population of lamina propria, state of submucosal blood vessels and connective tissue, state of intestinal lymphoid tissue and other pertinent observations.

Summary and Conclusions:

The histopathologic alterations in the gastrointestinal tracts of animals infected with LIVIM virus have been studied. This disease is one that lethally affects colonies of mice in the first few weeks of life, but exists as an inapparent infection in adult mice. In the initial pilot survey, a difference in the pattern of response of the intestinal mucosa was seen in the animals infected during the first 6 days of life as compared to animals infected during the period of 7 to 14 days of life.

Modes of sectioning and differential staining techniques have been worked out and the entire group of 50 infected and 25 control animals are ready at present for a critical analysis of the changes observed in the pilot survey.

List of Publications: None

ANNUAL PROGRESS REPORT

Project DASA RD 40-61, Biomedical (NWER)

Subtask 03.084, Dosimetry of Neutrons and Gamma Radiation

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Biophysics
Division of Nuclear Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Lt Col Dan Hightower, VC
Capt Harold M. Swartz, MC

Assistants: Mr. James B. Smathers
Dr. Milton Feldman
Dr. Robert T. Lofberg

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No DASA RD 40-61 Title: Biomedical (NWER)

Subtask 03.084 Title: Medical Aspects of Ionising Radiation (Dosimetry of Neutrons and Gamma Radiation)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Lt Col Dan Hightower, VC
Capt Harold M. Swartz, MC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Neutron flux and gamma dose measurements were made in the WRAIR reactor exposure facilities by the Nuclear Defense Laboratory. Initial routine dosimetry methods to be used in the research program were decided upon and procurement action initiated.

A technique is being developed which permits determination of neutron penetration in tissue without the addition of dosimetric devices or the use of "tissue equivalent" material. This technique is based on a determination of the Sodium-24 induced in tissue sections removed from animal cadavers which have been exposed to a neutron flux. The cadavers are frozen in liquid nitrogen prior to exposure to prevent diffusion of the Sodium-24. After the radioactivity has decayed to an insignificant level, the tissue sections are reactivated with appropriate standards to determine the total sodium content of the tissue and the original data corrected. A computer program has been written for processing the data.

BODY OF REPORT

Projects No DASA RD 40-61 Title: Biomedical (NWER)

Subtask 03.084 Title: Medical Aspects of Ionizing Radiation (Dosimetry of Neutrons and Gamma Radiation)

Description: This subtask is designed to correlate known biological endpoints with physical dose of mixed ionizing radiation with the view of eventually obtaining a dose-dependent endpoint which may be used as a measure of effective total body dose. Known biological endpoints will be used to correlate biological response to physical dose at the single organ and/or whole body level.

Progress: Subsequent to initial reactor criticality on 22 September 1962, considerable effort was exerted in bringing the Walter Reed Research Reactor into a fully operational and usable status. One aspect of this effort was the measurement of neutron flux and gamma ray dose at some sixty to ninety points in the reactor exposrue facilities. For the neutron flux measurements the threshold detector system consisting of the following detectors was utilized: Au, U-235, Pu-239, Np-237, S, Mg and Al. Glass rods and plates were used for the gamma measurements. Analysis of the results of these measurements indicate that the 1) experimental results are quite close to predicted values, 2) the intensity of radiations emitted from the operating reactor is quite linear with power, 3) the reactor, as built, is symmetric with respect to the center line.

During this reporting period, study was given to the entire radio-biological dosimetry program which would be required for the research program outlined for the Department of Biophysics. It was decided that knowledge of the following aspects of the exposure environment would be desirable:

1. Total absorbed dose
2. Dose as a function of depth in tissue
3. Total neutron dose
4. Total gamma dose
5. Neutron dose to gamma dose ratio
6. Neutron energy spectrum
7. Gamma ray energy spectrum
8. Total neutron flux
9. Dose rate

In addition, certain physical parameters applicable to tissue are needed. If available, these constants would permit a better theoretical approach to the many dosimetry problems facing an investigator. It was soon realized that all of the above information could not be readily obtained with the dosimetry systems now available. With this in mind, a combination of several

systems was chosen for use within the department, and procurement action was initiated. When the necessary supplies are received and the systems calibrated, they will be integrated into the initial, working dosimetry program of the department.

As a means of better determining and understanding neutron depth dose data, a technique utilising the activation of tissue sodium by neutrons was developed which enables one to determine neutron penetration into tissue by measuring the amount of radioactivity induced. This obviates the necessity of using flux perturbing dosimeters and/or "tissue equivalent" materials.

To prevent movement or diffusion during or after irradiation, the animal cadavers were frozen in liquid nitrogen.

The activated sodium-24 is used to measure neutron penetration. The frozen animal cadavers are exposed in the south thermal column of the Walter Reed Research Reactor. The total neutron flux used is approximately 3-4 x 10^{12} nvt with a cadmium ratio of approximately 10. Time of irradiation is fifteen minutes at a power level of 30 KW. After 30 minutes to permit the decay of short half life isotopes, the specimen is removed from the exposure chamber. Tissue sections are then removed and counted. Counting is accomplished in the "iron safe" which has an 8 x 4" NaI (Tl) crystal and a four hundred channel gamma spectrometer. Na-24 is measured from peak areas using the method of Covell. After the sodium has decayed, the tissue sections are reactivated with sodium standards. The sodium in the section is thus determined by comparing peak areas in the sample to those in the standard. The sodium content of the sample is then divided into the original data to obtain relative activity per gram of sodium. A plot of relative activity vs position of the sample yields a neutron penetration curve. A computer program has been written which permits one to feed the gamma spectrometer output tapes to the computer which then makes all computations and prints out relative activities. Plans have been finalised to make additional exposures with altered neutron spectra and exposures at room temperature. In addition, elements other than sodium will be analyzed in an attempt to detect changes in the neutron spectrum as the tissues are traversed.

Autoradiographic studies have been made on whole sections giving graphic visualization of the variation of neutron penetration with depth. The activity recorded by this technique is apparently due to activated isotopes of sodium, phosphorus and calcium. The applicability of microradioautographs to reveal microscopic variation of neutron penetration with depth is currently being investigated.

Summary and Conclusions:

1. Both neutron and gamma ray measurements in the Walter Reed Research Reactor exposure facilities have been made. Measurements indicate that the reactor is operating as predicted.

2. The dosimetry program for the radiobiology research projects of the Department of Biophysics has been determined, and the necessary procurement action initiated.

3. A technique for determination of neutron penetration into tissue is being developed. It is expected that analysis of the data so obtained will yield depth dose information superior to that now available.

List of Publications:

Hightower, Dan and Swarts, Harold M., "Measurement of Neutron Penetration By Tissue Activation" submitted for presentation at Symposium on Biological Effects of Neutron Irradiations, Brookhaven National Laboratory, 7-11 October 1963.

Kilminster, D., et al, "Gamma Dose and Neutron Flux Measurements of the WRAIR Reactor", Nuclear Defense Laboratory Publication, in press.

ANNUAL PROGRESS REPORT

Project DASA RD 40-61, Biomedical (NWER)

Subtask 03.085, Biological Effectiveness of Mixed Ionizing Radiation in Rodents

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Biophysics
Division of Nuclear Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Major John G. Maier, MC

Assistants: Lt Col Dan Hightower, VC
Mr. James B. Smathers

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project DASA RD 40-61 Title: Biomedical (NWER)

Subtask 03.085 Title: Biological Effectiveness of Mixed Ionising Radiation in Rodents

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Major John G. Maier, MC
Lt Col Dan Hightower, VC
Mr. James B. Smathers

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Studies of the biological effects of mixed ionising radiation on mammals are planned. Design and development of experimental protocols are in progress. These will begin upon completion of construction of the exposure facilities and correlative dosimetric studies.

BODY OF REPORT

Project DASA RD 40-61 Title: Biomedical (NWER)

Subtask 03.085 Title: Biological Effectiveness of Mixed Ionizing Radiation in Rodents

Description: Previously, predictions of the effects on man following exposure to the heterogeneous ionizing radiation produced by a nuclear weapon detonation have been largely based on extrapolation from data obtained in the laboratory. These data were obtained using fixed, single sources of radiation. It is the intent of this research subtask to achieve a more realistic basis for appraisal of responses to mixed radiations by 1) experimental determination of effects by mixed neutron-gamma exposures in rodents and larger mammals, and 2) comparison of these controlled results with existing data both from field weapons tests and from laboratory results with single sources.

Progress: Preliminary experiments are underway to develop dosimetry and exposure conditions in the WRAIR and DORF reactors. Special cages for rodent irradiation in the thermal column of the WRAIR reactor have been designed and are being constructed. The three-inch diameter circular port of the WRAIR reactor is being modified to provide either a collimated beam of relatively gamma-free fast neutrons or a collimated beam of thermal neutrons.

Summary and Conclusions: Since the preliminary experiments to establish experimental conditions are incomplete, no conclusions have been drawn. It is anticipated that these preliminary studies will be sufficiently complete to permit initiation of Phase I of the biological studies (single- versus fractionated-doses of total body irradiation using neutrons and gamma rays) during calendar year 1963.

List of Publications: None

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