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AUTHORITY

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RCS MEDDH - 288 (R1)

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

Including

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

AD 863623

ANNUAL PROGRESS REPORT

1 July 1968 - 30 June 1969

VOLUME I

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

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RCS MEDDH-288 (R1)

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES, INCLUDING BIOCHEMISTRY, COMMUNICABLE DISEASES AND IMMUNOLOGY, INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE

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

Annual Progress Report
1 July 1968 - 30 June 1969

Volume I

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

Each transmittal of this document outside the agencies of the U. S. Government must have prior approval of the Commanding Officer, U. S. Army Medical Research and Development Command, Washington, D. C. 20314, or the Director, Walter Reed Army Institute of Research.

Destroy this report when no longer needed. Do not return it to the originator.

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

SUMMARY

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

FOREWORD

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

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PROJECT 3A061101A91C
IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00
In-House Laboratory Independent Research

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6477	69 07 01	DD-R&E (AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 01 31	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	095
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code): (U) Biochemical Action of Trace Substances-Effects of Trace Metals on Hormone and Enzyme Activity (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 002300 Biochemistry 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 09	NA	DA	C. In-House

17. CONTRACT/GRANT: NA
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	2	90
FISCAL YEAR CURRENT 70	2	25

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

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Division of Biochemistry
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Hertz, Dr. W.
TELEPHONE: 202-576-3528
SOCIAL SECURITY ACCOUNT NUMBER: [redacted] (PII Redacted)

ASSOCIATE INVESTIGATORS
NAME: Roginski, E. R.
NAME: DA

21. GENERAL USE
Foreign Intelligence Considered

22. KEYWORDS (Precede each with Security Classification Code): (U) Chromium; (U) Metals; (U) Diabetes; (U) Glucose; (U) Arteriosclerosis; (U) Insulin; (U) Fatty Acids; (U) Metabolism; (U) Nutrition.

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To define the mode of action of Chromium/III/ in metabolism, to establish the nature of its interaction with insulin and related substances, to determine the metabolic defects in chromium deficiency in animals and man and to correct such defects by adequate supplementation.

24. (U) Aspects of glucose, protein and fat metabolism are investigated in chromium-deficient rats and in diabetic patients, in collaboration with Schroeder, Dartmouth, and with 3 other medical schools. In Vitro tests are performed for biological activity of specially prepared complexes of chromium with biological materials to determine the influence of chemical structure on Biological activity.

25. (U) 69 01 - 69 06 Since the need for pilot plant size quantities of the natural chromium complex in yeast became apparent, the effort was concentrated on the development of simple batch procedures as the first steps of purification, which are suitable for pilot plant operation. It was found that prolonged autolysis of the yeast cells releases a considerable proportion of the active chromium complex (glucose tolerance factor) into solution. This activity can be further purified by a simple charcoal treatment followed by phenol extraction at acidic PH. It is expected that this method will be applicable to pilot plant operation. The resulting fractions were routinely tested in the biological assay for potentiation of the action of insulin on glucose oxidation. They predictably stimulated the effects of the hormone at least fivefold. The studies of intestinal absorption were concluded, fractions extracted from chromium-51 labeled yeast were administered to pregnant rats, and the radioactivity was accumulated by the fetus. Concentration of chromium-51 in fetal liver exceeded that of maternal liver by a factor of three. Chromium-51 as yeast extract also labeled the specific, biologically important chromium pool of rats which regulates plasma chromium levels after glucose or insulin injection. These two latter observations emphasize the importance of chemical binding of chromium, as it occurs in glucose tolerance factor. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 095, Biochemical action of trace substances -- effects of trace metals on hormone and enzyme activity.

Investigators:

Principal: Walter Mertz, M.D.
T. K. Li, MAJ MC
Associate: Edward E. Roginski, GS-11

Description:

The objective of this research includes the following goals: To define the mode of action of chromium in metabolism; establish the mode of interaction on the insulin and related substances; determine metabolic and anatomic defects resulting from chromium deficiency; study the long-term effects of chronic chromium deficiency in animals and man and to correct existing deficiencies by adequate supplementation. To define the organic, active complex in which chromium is present in biological systems; to study its effects in various in vivo and in vitro systems; to devise methods for large scale production of sufficiently prepurified preparations for testing in patients. To define the biochemical function of trace metals in biological membranes.

Progress:

1. Laboratory research. The work on the purification of the natural organic chromium complex, glucose tolerance factor, was continued.

a. Chemical: A method to obtain extracts with consistently high biological activity was developed. It consists of autolysis of the yeast cells, grown in a medium, with repeated additions of glucose. While the cell residue also contains chromium with biological activity, the main activity is found in the supernatent. It can be further purified by absorption on charcoal, relatively selective elution, and subsequent separation between phenal and water. The following chemical properties have been established: The factor is water soluble, has acidic properties, is easily dialysable and remarkably heat stable, even under acidic or alkaline conditions. The factor appears to combine with insulin, upon standing, with a resulting product of greatly increased insulin activity. This latter activity was associated with the ^{131}I-insulin peak, upon gel filtration, suggesting that both are closely associated.

b. Biochemical: The production of sufficient quantities of chromium-51 labeled glucose tolerance factor has made possible the testing for biological activity in various systems. The stimulation of insulin activity in epididymal adipose tissue was used as the basal assay. Tissue from chromium deficient rats that was almost completely refractory to insulin, responded to the hormone when small amounts of glucose tolerance factor were added. It was also shown in collaborative studies with others that a tissue described as absolutely refractory to insulin exhibited a near perfect dose-response curve to the hormone in the presence of GTF.

The studies on placental transport of chromium were continued. Whereas simple chromium salts were not transported into the fetus, the chromium in form of glucose tolerance factor was accumulated against a gradient. The concentration of chromium-51, given to the pregnant mother in this form, was approximately three times higher in the fetal than in the maternal liver. Similar observations were made with non-radioactive chromium. The element when fed as part of a natural high-chromium diet, accumulated on the fetus, whereas inorganic chromium salts grew in drinking rate. These observations strongly suggest a vitamin-like nature of GTF-chromium, i.e. an essential compound that cannot be synthesized in the organism. However, the essentiality of chromium for the rat has not yet been proven unequivocally.

Similar observations with GTF were made in another system: There is a specific body pool of chromium which releases the element into the blood when peripheral glucose utilization is increased, either by a glucose load or by insulin injection as injection of chromium-51-chloride does not label this pool, except perhaps after a lag period of several weeks. Chromium-51 in form of glucose tolerance factor does: Animals stomach tubed or injected with GTF, respond to insulin with a very pronounced rise of radioactivity in the blood.

Absorption of inorganic chromium salts from everted intestinal sacs in vitro is poor, but is enhanced by a variety of liganda (amino acids, amines, etc.). Chromium in form of GTF is absorbed at a greater rate than any of the other combinations tested.

2. Clinical studies: Collaborative clinical and metabolic studies are reported separately. A number of additional trials have been initated, results are not yet available.

Summary and conclusion:

The properties of the naturally occurring, biologically highly active chromium complex, glucose tolerance factor were further defined. GTF is a water soluble, chromium containing organic complex of relatively

low molecular weight. It is heat stable and has acidic properties. While it is relatively ineffective in the absence of insulin, it greatly potentiates the action of the hormone. It is transported into the rat fetus, it is absorbed in the intestines better than inorganic chromium salts and it labels the specific chromium pool which is responsible for increasing the effectiveness of insulin *in vivo*.

The chemical structure of this substance has not been identified.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 095, Biochemical action of trace substances -- effects of trace metals on hormone and enzyme activity.

Investigators
Principal: Walter Mertz, M.D.
T. K. Li, MAJ, MC
Associate: Edward E. Roginski, GS-11

Publications:

1. Mertz, W. Problems in Trace Element Research. Proc. 2nd Annual Conference on Trace Substances in Environmental Health, Columbia, Mo. (1968) p. 163-169.

2. Roginski, E. E. and Mertz, W. Intestinal absorption of chromium(III). Fed. Proc. 28: 299, (1969).

3. Mertz, W. and Roginski, E. E. Effects of chromium(III) supplementation on growth and survival under stress in rats fed low protein diets. J. Nutrition 97: 531-536 (1969).

4. Roginski, E. E. and W. Mertz. Effects of chromium(III) supplementation on glucose and amino acid metabolism in rats fed a low protein diet. J. Nutrition 97: 525-530 (1969).

5. Mertz, W. Chromium: Occurrence and function in biological systems. Physiol. Reviews 49: 165-239 (1969).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6480	69 07 01	DD-R&E (AR) 636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. GROUP MBTRS	9A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM	9. WORK UNIT
68 10 31	D,CHANGE	U	U	NA	NL	☒ YES ☐ NO		

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	098
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with security Classification Code)

(U) Chromium Complexes of Insulin and Related Compounds (21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

008300 Inorganic Chemistry; 02300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 06	CONT	DA	B. CONTRACT

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 66 07 EXPIRATION: 69 08

b. NUMBER: DA49-193-MD-2244

c. TYPE: S,CT d. AMOUNT: 41,322

e. KIND OF AWARD: EXT f. CUM. AMT. 71,322

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PREVIOUS 69	1	13
FISCAL YEAR CURRENT 70	0.2	b

19. RESPONSIBLE DOD ORGANIZATION

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TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

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TELEPHONE: 301-927-3800

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Mertz, W.

NAME: 1

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with security Classification Code)

(U) Chromium; (U) Insulin; (U) Chelating Agents; (U) Mineral Metabolism

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with security Classification Code)

23. (U) To study the interaction of trivalent chromium with biological materials, particularly with nutrients which compete for chromium in the GI tract, with carrier substances which bind the element in the blood and with insulin for which chromium is a co-factor, to determine chemical parameters which are essential for biological activity and to synthesize compounds for biological testing.

24. (U) The influence of PH on the degree of olation and of various potential chelating agents on the solubility of chromium is determined using a membrane dialysis technique. New approaches are in process for synthesis of new chromium complexes with ligands of biological interest, in aqueous and non-aqueous systems.

25. (U) 68 07-69 06 A process of sequential dialysis has been developed which has provided a laboratory system to study the mechanism of the chromium in reaction with biological substances. This process has been used to evaluate and compare many biological substances including phosphates, anions of organic acids, Krebs cycle components, bases and Lormone activities. A comparison of oxalate complexes of trivalent chromium with urea disclosed that urea forms coordination complexes but does not form a stable chelate Oxalate showed a much stronger potential for chelate formation. The biological polyphosphates (adenosine phosphates and thiamine pyrophosphate) showed strong coordinating affinity for chromium as did the citrate and isocitrate compounds of the Krebs cycle. In the absence of such agents the rate of diffusion of chromium was decreased.

For technical reports, see Walter Reed Army Institute of Research, Annual Progress Report, 1 July 1968 - 30 June 1969.

*Available to contractors upon originator's approval.

DD, FORM 1 MAR 66 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Independent Laboratory Research

Work Unit 098, Chromium complexes of insulin and related compounds

Investigators.
Principal: Carl L. Rollinson, Ph.D.
Associate: Walter Mertz, M.D.
Eleanor Rosenbloom, Ph.D.

Description

Chromium is the most recent addition to the list of the essential trace metals. This element has a role in glucose, protein and lipid metabolism in enhancing the action of insulin. In attempting to establish the chemical mechanism responsible for the biological activity of chromium, we have applied the principles of coordination chemistry to the study of the reactions of Cr(III) with many biological substances.

For both theoretical and practical reasons, it is important to establish the mechanism of the biological effects of chromium and this requires a knowledge of the reactions of Cr(III) with biological substances. What must be investigated is the fascinating and complicated chemistry of Cr(III) in basic media containing many substances capable of reacting with Cr(III). More specifically, what is needed is a procedure for determining the relative coordinating tendencies of biological substances in media of a pH at which the most probable reaction of Cr(III) is polymerization due to bridging OH^- groups.

The "method of sequential dialysis" meets this requirement. This method was described in detail in our previous report which contains numerous tables of results demonstrating the utility of the procedure. In the meantime, we have used this method to evaluate and compare many more biological substances, including compounds of the Krebs cycle, glycolytic chain, phosphates, bases, and anions of organic acids.

Chromium(III) reaction mixtures are charged into the two compartments of the dialysis cells (5 ml. into each compartment); the reaction mixtures are identical except that the Cr(III) in the "back" compartment of the dialyzer is labelled with ^{51}Cr. The dialyzers are agitated in a temperature-controlled shaker at a standard rate. Samples are removed at intervals from front and back compartments and counted with a scintillation counter. For each time interval a separate dialyzer is used; otherwise errors are introduced because of the change in ratio of solution volume to membrane area. Each reaction mixture is dialyzed at least

twice. The first dialysis is started one hour after preparation of the reaction mixture. After the reaction mixture has aged 24 hours, the dialysis of another sample is started, etc.

Progress

The relative effectiveness of the ligands can be expressed numerically as follows:

R'_I, R'_{II}.....percent attainment of dialysis equilibrium, 24 hours' dialysis, for the successive dialyses.

R', the percent attainment of dialysis equilibrium, will increase from 0 to 100 if all the Cr(III) remains diffusible in the 24 hour dialysis period and from 0 to less than 100 otherwise. With the most effective ligands, the successive values of R' will remain 100% or not much less. Even if the successive values are less than 100% for a given ligand, it may be considered quite effective if the value remains constant.

Q'_I, Q'_{II}.....percent decrease in area under the dialysis curve as the reaction mixture ages, always calculated on the basis of the area under the curve for the 1-hour old mixture.

The areas under the dialysis curves are proportional to the rates of diffusion of the Cr(III) species. The coordinating tendency of a ligand is reflected in its ability to keep the rate of diffusion of Cr(III) from decreasing. The most effective ligands are characterized by zero or low values of Q' over the longest aging periods.

In Table I are listed numerical results for the most effective ligands so far encountered in comparison with results for representative examples of substances having little coordinating tendency.

Effect of Concentration and Chelation:
Comparison of Urea and Oxalate

Oxalate is a powerful chelating agent whose complexes with Cr(III) are well known. Urea forms coordination complexes with Cr(III) but does not chelate nor are the complexes formed by direct action of this ligand on Cr(III) in aqueous solution. The difference in coordinating tendency is demonstrated by the relative abilities of these substances to compete with the hydroxide ion.

The curves for the two concentrations of urea are identical and essentially no different from those for the phosphate buffer. It is apparent that urea does not coordinate with Cr(III) under the experimental conditions. On the other hand, oxalate allows only minor changes over a period of a week although at 10^{-3} M it is no more effective than urea. Thus oxalate, which is present in some foods, is far more likely to combine with Cr(III) than is urea, which is abundant in animal organisms.

Sequences of Related Compounds

In the following series of compounds (in order of increasing coordinating tendency), the slant line separating the series into two segments indicates a large difference between the last compound of the first segment and the first compound of the second segment. The numbers indicate the range of values of Q' (for 24 hours' aging of reaction mixture).

Structure:		
"glutarate"	glutarate < glutamate <α -Ketoglutarate	(32.3 - 17.0)
"succinate"	succinate < fumarate/< aspartate < oxalacetate < malate < tartrate	(38.5 - 1.1)
"propane-tricarboxylate"	1,2,3-propane-tricarboxylate <aconitate/ <isocitrate < citrate	(56.7 - 1.0)
3-carbon mono-carboxylate	lactate < pyruvate/ < 3-phosphoglycerate	(52.7 - 0.0)
α-amino acid	pro < glu < lys < leu < gly < asp < met < ser	(31.3 - 7.3)

Metabolic Cycles and Chains

Among the most effective ligands investigated are citrate and isocitrate (Krebs cycle) and 3-phosphoglycerate and fructose-1,6-diphosphate (glycolytic chain):

succinate < fumarate < aconitate <α -ketoglutarate/ < oxalacetate < malate < isocitrate < citrate < (38.5 - 1.1).

glucose-6-phosphate < lactate < glucose-1-phosphate < pyruvate < glucose < α-glycerophosphate < fructose/< 3-phosphoglycerate < fructose-1,6-diphosphate (60 - 0.0).

Biological Polyphosphates

ATP, ADP and thiamine pyrophosphate are equal in coordinating ability to inorganic pyrophosphate and triphosphate. This is an exciting finding and certainly warrants more detailed investigation.

The related nucleoside (adenosine), the base (adenine) and monophosphate (AMP) have essentially no coordinating tendency. Adenine has considerable coordinating ability but it is nowhere near as effective as ADP and ATP (Q' for the series of four compounds; 43.9 - 2.0).

Bases

Adenine is far more effective in stabilizing the Cr(III) reaction mixture than uracil and thymine (Q' for the series of three compounds, 36.5 - 20.0). It would be interesting to extend this study to other purine and pyrimidine bases to determine what correlation exists between structure and coordinating tendency.

Bile Salts

Preliminary results indicate that oleate reacts with Cr(III) to form products having low rates of diffusion. We plan further investigation of the reactions of fatty acids with Cr(III) both with and without the bile salts that are involved in the absorption of fats from the intestine. We have found that glycholate and taurocholate alone are ineffective (Q', 57.0 - 38.9).

Summary and Conclusions

Many questions exist concerning the metabolism of the essential metals. A promising approach to these problems is to investigate the reactions of the essential metal ions with other substances in the biological system. Competition for metal ions by such substances will determine the chemical forms of the metal compounds and consequently the availability of the metals. In particular, irreversible incorporation of a metal into non-diffusible compounds would render it unavailable. Determination of the abilities of biological substances to produce or prevent this result is thus a requirement for establishing the mechanisms by which the essential metals perform their biological functions. We have developed a method (sequential dialysis) by which biological compounds can be efficiently evaluated in this respect and have applied it to the study of the reactions of chromium, a metal essential to the functioning of insulin. Citrate, isocitrate and the biological polyphosphates (e.g., ATP, ADP, thiamine pyrophosphate) are especially effective in forming compounds with chromium that have a high constant rate of transport for days in media at physiological pH; in the absence of such agents, the rate of diffusion of chromium(III) progressively decreases. We propose: to extend the application of the method to iron and other transition metals; to test numerous refinements designed to make the method more precise, rapid, versatile and sensitive; to investigate additional compounds as carriers of chromium; to investigate the effect of chemical form on absorption of metal ions by microorganisms. Any compound or reaction mixture presumed to have biological activity will be subjected to appropriate tests.

TABLE I

MOST EFFECTIVE BIOLOGICAL LIGANDS*
(COMPARISON WITH SOME INEFFECTIVE SUBSTANCES)

compound	$f^{R'}_{I}$ †	$f^{R'}_{II}$ †	$f^{R'}_{III}$ †	$Q^{'}_{I}$	$Q^{'}_{II}$
fructose-1,6-diphosphate	91.8	95.5	95.5	0	1.1
tartronate	91.7	92.7	91.9	0	1.3
3-phosphoglycerate	81.1	79.7	79.6	0	0
citrate	100.0	100.0	100.0	1.1	1.0
tartrate	96.7	95.4	-	1.1	-
ATP	98.8	100.0	100.0	2.0	3.0
isocitrate	100.0	99.9	99.5	2.1	3.9
oxalate	97.3	100.0	98.1	2.1	1.7
ADP	99.0	100.0	96.0	2.6	1.5
malate	87.5	86.4	90.0	2.8	0
thiamine pyrophosphate	99.5	99.6	98.9	2.6	1.5
pyrophosphate	99.3	100.5	99.4	3.1	0.7
triphosphate	100.0	99.3	100.0	3.4	3.5
oxalacetate	100.0	99.1	100.0	5.4	6.6
serine	95.7	94.0	-	7.3	-
methionine	96.1	92.1	-	8.1	-
malonate	93.9	85.7	78.5	11.0	21.6
fructose	82.3	53.2	-	19.1	-
proline	90.8	70.1	-	31.3	-
glucose	87.4	65.4	-	35.3	-
AMP	93.6	60.2	54.3	41.3	51.8
urea	85.9	58.1	-	44.1	-
standard phosphate buffer only	86.0	59.5	47.8	44.3	54.5
glucose-1-phosphate	99.9	52.3	29.7	52.5	63.8
lactate	87.1	46.4	42.4	52.7	59.2

* based on reaction with Cr(III); standard reaction conditions.

† for reaction mixtures aged 1 hour, 24 hours and 1 week respectively.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Independent Laboratory Research

Work Unit 098, Chromium complexes of insulin and related compounds.

Investigators.
Principal: Carl L. Rollinson, Ph.D.
Associate: Walter Mertz, M.D.
Eleanor Rosenbloom, Ph.D.

Publications:

None

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68 10 31	D. CHANGE	U	U	NA	NL	X	
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(U) Metallic Micronutrients and Intermediary Metabolism (30)

012900 Physiology

64 07	CONT	DA		B. CONTRACT

FISCAL YEAR 69	1	17
70	0.8	17

a. DATES/EFFECTIVE: 69 07 EXPIRATION: 70 03
b. NUMBER: DA 49-193-MD-2595
c. TYPE: S.CT d. AMOUNT: 17,000
e. KIND OF AWARD: EXT f. CUM. AMT.: 98,584

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NAME: Mertz, W.
1

(U) Chromium; (U) Vanadium; (U) Mineral Metabolism; (U) Nickel; (U) Germanium

23. (U) To study the effects of chronic nutritional deficiency of metallic trace elements in animals, with emphasis on detection of long-term degenerative processes. Elements include chromium, vanadium, nickel, germanium, nideium, zirconium, arsenic, antimony and tin.

24. (U) Animals are raised in a special laboratory allowing strict control of trace metal contamination. Diets, deficient in a selected element, but supplemented with all other essential dietary factors, are fed to animals during their life span. Chemical, pathological, historical examinations and functional tests are made on deficient animal groups receiving deficient, normal, excessive and toxic amounts of the element.

25. (U) 68 07 - 69 06 Mice and rats were exposed for their lifetimes to small doses of essential and abnormal elements in drinking water. Growth rates, survival and longevity, tissue pathology, tissue concentrations of trace elements and blood pressure, cholesterol, glucose and uric acid, aortic plaques, lipids and tumor rates were evaluated. Chromium deficiency induced elevated cholesterol and glucose levels on both regular diet and on one containing white sugar. Selenite was toxic, selenate not. A zinc chelate of CDTA abolished cadmium hypertension in rats. Cadmium feeding or injection raised blood pressure and diminished responses to norepinephrine and angiotensin; these changes were reversed by the zinc chelate. Preliminary conclusions are that renal cadmium is an accessory factor in hypertension; the mechanism differs from renal ischemic hypertension; chromium deficiency may be an accessory factor in atherosclerosis, other trace elements are either inert or exert vague toxicity not associated with any demonstrable disease. A model for human arterial hypertension has been developed in rats fed cadmium.

For technical reports, see Walter Reed Army Institute of Research, Annual Progress Report, 1 Jul 68 - 30 Jun 69.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 105, Metallic micronutrients and intermediary metabolism

Investigators.
Principal: Henry A. Schroeder, M.D.
Associate: Walter Mertz, M.D.
D. F. Frost, Ph.D.
A. P. Nason, B.S.

Description:

Certain trace elements are essential for survival, growth and optimal function of mammals. Other trace elements may have metabolic roles. Others, which can be called "abnormal," have no known functions, some of them may accumulate in mammalian tissues with age and exert innate toxic effects, especially under present environmental exposures which have increased as a result of modern industrial practices.

The general purpose of this research is to ascertain whether or not any common chronic diseases of industrialized man result from a) accumulation of a specific abnormal trace element, or b) from marginal deficiency of an essential trace element. To approach this problem, one needs to know whether or not tissue deficiency of an essential trace element occurs in man, especially as a function of aging, and which abnormal elements accumulate in tissues and which do not. The next step is to expose animals to each element and observe effects.

Weanling mice and rats are born in our laboratory specially equipped to minimize external trace element contamination. Groups of approximately 100 each are fed diets which are low in trace elements, with controlled additions to or omission of elements from deionized drinking water and are observed for their lifetimes.

Functions measured in mice are growth rates, weights, survival and longevity, microscopic pathology and incidence of tumors; these are measured in rats, as well as urinary protein and glucose, blood pressure, lung, kidney, spleen and liver are ashed and analyzed for the metal given. An equal number of controls are treated identically. Analyses are made by microchemical, fluorimetric and atomic absorption spectrophotometric methods.

Progress:

The most significant research during the past year was the reproduction in rats of the two major biochemical alterations found in human atherosclerosis and believed to be associated with this prevalent disease in some causal manner. By feeding rats a diet low in chromium, relative hypercholesteremia and hyperglycemia were regularly induced. When the diet was composed of torula yeast, lard and sugar, which was even lower in chromium, the same abnormalities were induced when the sugar was refined white, but when it was brown,both serum cholesterol and glucose were low. White sugar contained 0.03 ug/g chromium and 0.11% ash; brown sugar 0.24 ug/g chromium and 3.2% ash. A "raw" sugar, 0.06 ug/g chromium and 0.32% ash, produced intermediate effects. The addition of 5 ppm chromium (III) to drinking water resulted in low serum cholesterol in both sexes and low glucose levels in females; male levels responded to brown sugar (189). We have demonstrated that males require more chromium than do females for reduction of glucose levels, whereas females require more chromium than males in order that serum cholesterol levels be depressed. As an additional hypoglycemic agent in the rat is nickel, and as brown sugar contained five times as much nickel as did white, it is possible that this differential effect on glucose was due to nickel.

Similar rats deficient in chromium have been shown previously to exhibit an increased incidence of aortic plaques and a high concentration of aortic lipids. These animals will be examined for such pathological changes when they die a natural death in about two years time.

Further implication of chromium in the human disease resulted from the finding that aortas of persons dying of coronary occlusion were usually wholly deficient of chromium, whereas those of subjects dying accidentally contained chromium. The tissues of foreigners contained much more chromium than did those of Americans, the latter having only 1.7 mg as their body burden. We have been unable prolongedly to affect glucose tolerance in five diabetic patients ingesting chromium as the chloride, acetate and oxalate for two years or longer. The feeding of manganese at 10 mg/day affected it temporarily, for three months only, in three patients. We have been able to affect favorably the serum cholesterol levels of five persons institutionalized for mental disease, without affecting another five. No significant effects occurred until the fifth month of treatment. The dose was 2.0 mg/day as the acetate. Because of the poor and erratic absorption of chromium, a readily absorbable form in quantities sufficient for clinical trial is urgently needed.

In respect to cadmium hypertension in rats, either the feeding of cadmium in water or the intraperitoneal injection was followed by a significant rise in blood pressure when measured directly. This change was accompanied by a decreased vascular reactivity to norepinephrine and angiotensin. The intravenous injection of Na_2Zn CDTA, which has a greater affinity for cadmium than for zinc and removes renal and hepatic cadmium, lowered blood pressure and restored vascular reactivity to norepinephrine. Life-term exposures of rats to germanium, arsenic and tin have been published.

A survey of environmental sources of magnesium has been completed. Partitioning of foods into their component fractions (oils or fats, refined sugar and molasses, refined flour, coarse flour and germ) does not mean that the inorganic nutrients necessary for the metabolism of each fraction stay with the fraction. Thus oils and fats are virtually devoid of magnesium which stays with the skim milk, defatted residue of corn or flour, or in the muscle of meat. Refined flour has little magnesium compared to whole wheat, refined sugar is virtually devoid of it, molasses and raw sugar having much. These circumstances require a "balanced" diet, other sources of magnesium supplying the deficit. Man and his animals are the only living things requiring a balanced diet; wild animals have a naturally balanced diet. This idea applies to other essential micronutrients, which are not replaced in the fractions of food resulting from refining.

Concentrations of eight trace metals in normal human hair from 160 subjects aged 1-102 years have been determined.

Summary

In order to evaluate biological effects of trace elements, mice and rats were exposed for their lifetimes to small doses of each of many essential and abnormal elements in drinking water, in a laboratory and on a regimen designed to avoid environmental contamination. Growth rates, survival and longevity, microscopic pathology of tissues, concentrations of trace elements in tissues, and in rats, blood pressure, serum cholesterol, glucose and uric acid, aortic plaques and lipids and tumor rates were measured or examined. Surveys of the human environment for two elements in foods, water, vegetation, wild animals were also made, by trace element analysis, and human tissue concentrations for four. Chromium deficiency induced elevated cholesterol and glucose levels both on our regular diet and on a diet containing white sugar. Selenite was toxic, selenate not. A zinc chelate of CDTA abolished cadmium hypertension in rats and removed some renal and hepatic cadmium. Cadmium feeding or injection raised blood pressure and diminished responses to norepinephrine and angiotensin; these changes were reversed by the zinc chelate. Chromium declined in Americans but not foreign human tissues with

age. Normal dietary intakes of selenium and magnesium were ascertained. Preliminary conclusions from this and previous work are that renal cadmium is an accessory factor in human hypertension, its mechanism differing from that of renal ischemic hypertension, that chromium deficiency may be an accessory factor in atherosclerosis, that other trace elements may suppress spontaneous tumors in mice and that a number of other trace elements are either inert or exert vague toxicity not associated with any disease. As a result of this work, two prevalent human diseases have been reproduced in rats: (1) A model for human arterial hypertension has been developed in rats fed cadmium. The pathological and physiological criteria are similar: e.g., hypertension, cardiac enlargement and renal arteriolar hypertrophy and early sclerosis. The hypertension can be controlled by removing the renal cadmium by chelation and replacing it with zinc in the chelate. (2) A model for human atherosclerosis has been developed in rats deficient in chromium and fed refined white sugar. The physiological criteria are similar: e.g., relative hypercholesteremia which increases with age, and mild to moderate hyperglycemia. The pathological manifestations were an increased incidence of aortic plaques and increased aortic lipids; this aspect is being intensively restudied. These three changes were prevented by the feeding of trivalent chromium.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 105, Metallic micronutrients and intermediary metabolism

Investigators.
Principal: Henry A. Schroeder, M.D.
Associate: Walter Mertz, M.D.
D. F. Frost, Ph.D.
A. P. Nason, B.S.

Publications

1. Schroeder, H.A. Serum cholesteral levels in rats fed thirteen trace elements. J. Nutrition 94: 475-480 (1968.

2. Schroeder, H.A., Kanisawa, M., Frost, D.V. and Mitchener, M. Germanium, tin, and arsenic in rats. Effects on growth, survival, pathological lesions and lifespan. J. Nutrition 96: 37-45 (1968)

3. Schroeder, H.A. and Tipton, I.H. The human body burden of lead. Arch. Environ. Health, 17: 965-978 (1968)

4. Schroeder, H.A., Nason, A.P and Tipton, I.H. Essential metals in man: Magnesium. J. Chron. Dis. 21: 815 (1968).

5. Schroeder, H.A. Serum cholesterol and glucose levels in rats fed refined and less refined sugars and chromium. J. Nutrition 97: 237-242 (1969).

6. Kanisawa, M. and Schroeder, H.A. Life-term studies on the effect of trace elements on spontaneous tumors in mice and rats. Cancer Research 29: 892-895. (1969)

			DA 01 6488	69 06 30			
68 07 01	B.Termination	U	U	NA	NL	NO	
	6XXXXX	3A061101A91C	00	208			

(U) Physicochemical studies of the biologic significance of antiviral antibody (09)

CLO100 Microbiology

64 07	CONT	DA		B. Contract

	68	2	49
	69	2	65

a. Effective 68 09 a. Expiration 69 06
b. Number DA-49-193-MD-2633
c. Type 8. CT c. Amount $12,376
d. Kind of Award SUP d. Cum Amt $248,618

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(U) Viral antibody; (U) Immunoglobulin; (U) Attenuated virus; (U) Local antibody

23. (U) To obtain definitive information on biologic characteristics of various types of antiviral antibody so as to determine their significance in immunity in man.

24. (U) By study of the physicochemical characteristics of antibody in serum and nasal secretions using gel filtration, ultracentrifugation, radioautography and electrophoresis to determine specific differences and sequential appearance following immunization and infection in animals and man.

25. (U) 68 07 69 06 Immunochemical studies of serum and nasal secretions obtained from individuals and recruits undergoing natural infection with adenovirus type 4 and other adenoviruses have been studied utilizing radioimmunodiffusion. These studies have shown that this technique allows the characterization of the various classes of antiviral antibody with a degree of precision in clarity not possible by other techniques.

Studies of the immune response following measles immunization of children have been completed and have shown the importance of viral-anti-viral complexes in the pathogenesis of untoward reactions.

This work unit terminated due to transfer of support to a regularly funded program (3A061102B71Q) For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69)

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task (M), In-House Laboratory Independent Research

Work Unit 106, Physical-chemical Studies of the Biologic Significance of Anti-Viral Antibody

Investigators.
Principal: Joseph A. Bellanti, M.D.
Associates: Malcolm S. Artenstein, M.D.
Edward L. Buescher, COL, MC

Description.

The biologic significance of anti-viral antibody is studied to define the significance of various immunochemical classes of antibody resulting from immunization or infection. By understanding these mechanisms improved procedures for prevention of viral illnesses and the development of biological markers of immunity will become evident.

Progress.

1. In our previous work with characterization studies of anti-viral antibody utilizing physical-chemical techniques of separation, we were not able to detect with precision the relevant classes of antibody associated with anti-viral antibody free of contamination. However, work over the past year utilizing radioactively labeled adenovirus type 4 has shown such characterization of anti-viral antibody in serum and secretions can be achieved. The specificity of the adenovirus type 4 RID antigen reaction was evaluated using acute and convalescent sera from patients with illnesses from which adenoviruses other than type 4 were isolated (Table I). An AV-4 infection was included for comparison. One individual with a type 6 and one with a type 7 infection showing no evidence of recent type 4 infection (both had low levels of type 4 N antibody), revealed evidence of antibody rise using the RID test. These data indicate that the antigen employed is group and not type specific. Of interest, only one individual (AV-1 infection) had no preexisting N or CF antibody to the agents tested, and his acute serum revealed no binding of radioactive virus. Thus, the γG activity in the other three acute sera probably represent low level antibody activity due to previous infection with one or another type of adenoviruses.

The sensitivity of the RID test is also illustrated by Table I. It can be seen that serum RID titers may reach levels 10-100 times greater than those measured by neutralization or complement-fixation tests.

Further definition of the group specificity of the test was provided by means of blocking reactions. Concentrated unlabeled AV-4 and AV-7 antigens were prepared as crude cell harvests; polio type 2 and uninfected cultures served as blocking materials. Blocking was performed in two different fashions: 1) by flooding the micro-plates with unlabeled virus prior to the addition of radioactive antigen and, 2) in other experiments by adding unlabeled virus to serum dilutions prior to immunodiffusion. Both methods gave almost identical results. RID titers of the poliovirus 2 and control sera were identical. RID antibody responses in patients with adenovirus types 1, 4, 6, or 7 infections were all reduced 4 to 32-fold by blocking with unlabeled antigens of either type. Antibodies of γA, γM and γG specificity all showed the blocking phenomenon.

Table II illustrates the various antibody separation techniques used to study one patient. It is quite apparent that the centrifugation and gel filtration techniques failed to separate completely the antibody activity of the individual immunoglobulins which was so readily accomplished by the RID assay.

The development of nasal secretory antibody is shown in Table III, which gives the frequency with which antibody was detected each week in serum and nasal washings of recruits naturally infected with AV-4 virus. Of five recruits with serologic evidence for adenovirus infection, but from whom no virus was isolated, all developed nasal antibody. There were four recruits who were lost to the study and four subjects who escaped infection. Of the latter, three had antibody in the first serum obtained and two of three showed nasal antibody when first tested. RID assay of NW and sera from three recruits is shown in Table IV. In these subjects γA and γG activity were noted with the serum frequency in NW specimens but γM was not detected. N antibody resided in both γA and γG since specimens which contained only one active immunoglobulin by RID showed N activity.

The advantages of RID assay over other methods of immunoglobulin antibody separation are the simplicity and clarity of the separations achieved. Furthermore, the sensitivity of the procedure makes feasible studies of fluids such as nasal secretions which are usually available in only small amounts with low activity compared to serum. Although the antigen used here is group specific, it seems probable that type specificity of the radioactive antigen could be achieved by physicochemical isolation of radioactive adenovirus subunits.

In the present studies of natural adenovirus infection of the respiratory tract, nasal antibody was detected in all patients within one or two weeks of onset. This antibody response was sustained for at least six

weeks, the longest period tested. Characterization of this activity by RID, indicated that it was associated with both the γA- and γG-immunoglobulins. Many studies have now shown that although γG-immunoglobulins may be present in nasal secretions, secretory γA is the predominent immunoglobulin in these specimens. Further, protection from infection with parainfluenza type 1 correlates better with the presence of local antibody than of serum antibody. Since reinfection by specific adenoviruses is unusual this must be related to the type of immunity produced by previous natural infection. Therefore, if immunization procedures are to be effective, they should simulate the antibody response seen following natural infection, namely the development of local respiratory tract antibody as well as serum antibody.

2. Studies of serum and local antibody responses in children following immunization with attenuated and inactivated measles vaccines have been completed. These studies have indicated that in 24 children immunized with inactivated attenuated measles-virus vaccine, responses of serum antibody were comparable both in peak titers and in immunoglobulin contribution. In eight of nine children immunized with attenuated measles-virus vaccine, nasal antibody associated primarily with the γA and less often with γG-immunoglobulins developed; nasal antibody was detected in only three of seven immunized with inactivated measles-virus vaccine, and in these both γA and γG activities were observed. Of eight children with preexisting serum antibody, six had nasal antibody in the preimmunization specimens. It was speculated that the differences in ability of these vaccines to stimulate local respiratory tract antibody may in part explain the greater clinical effectiveness of the attenuated vaccines as well as the occurrence of altered reactivity of the host who received inactivated vaccine.

Studies over the past year have been concerned with the mechanism of untoward reactions seen in recipients of inactivated measles vaccine. There have been several reports of unusual systemic and local reactions occurring in children previously immunized with inactivated measles vaccine who later are exposed to natural measles or who receive live measles vaccine. These reactions consist either of a <u>generalized</u> response consisting of high fever, edema atypical exanthemata and severe pneumonia, following exposure to measles or local response consisting of erythema and induration with regional lymphadenopathy at the site of subsequent live measles immunization. A variety of mechanisms have been suggested to explain the pathogenesis of these reactions. These include: 1) delayed hypersensitivity to measles virus antigen(s), 2) sensitization to tissue components derived from the host cell in which the measles vaccine virus is propagated, 3) an Arthus-like phenomenon due to the rapid recall of antibody interacting with measles virus.

Studies over the past year have been conducted in six children who previously have received inactivated measles vaccine some one to three years previously who were reimmunized with live measles vaccine (Table V). All had very low or almost negligible neutralizing antibody at the time of revaccination.

Results of these studies are summarized in Table V and indicate that at the time of inoculation the serum titers were either quite low or absent. Following immunization a local reaction consisting of local erythema and induration developed within 24 hours following immunization.

The antibody responses in serum and nasal secretions following attenuated vaccine are summarized in Table VI. It can be seen that prior to immunization serum antibodies were low; in one case D.J. antibody had fallen from a titer of 1:64 approximately 294 days prior to immunization to <1:4 at the time of immunization.

Immunopathologic studies conducted by Dr. Peter Ward were performed on frozen section of biopsy material obtained by skin biopsy. Direct immunofluorescence was performed on frozen sections to determine the presence of human γG, γA and $\beta_1 C$ globulin and measles antigen. These results indicate that γG, $\beta_1 C$ globulin and measles antigen are localized in deposits in walls of blood vessels in the dermis and subcutis. By light microscopy, mixed cellular infiltrates were present consisting of mononuclear cells, (including lymphocytes), polymorphonuclear leukocytes and eosinophils in and around walls of blood vessels.

These findings present direct evidence for the role of immediate hypersensitivity in the induction of the atypical skin reaction to measles antigen. The data also suggest that injury of other tissues may be mediated by virus-antibody complexes. Since many recipients of inactivated measles-virus vaccine appear to be unable to produce significant amounts of local γA antibody, although the serum antibody response is adequate, exposure to natural measles would result in viral replication in the respiratory tract. An accelerated response of serum antibody would result in viral replication in the respiratory tract. An accelerated response of serum antibody would follow, thus providing the conditions for formation of immune complexes in the lung, with subsequent tissue injury. Chanock et al. have speculated on this mechanism in the pathogenesis of disease due to respiratory syncytial virus. This is not to dismiss the participation of cell-mediated mechanisms but only to underscore the role of immediate hypersensitivity.

These observations have direct relevance to the problem of immunoprophylaxis of respiratory disease and suggest that vaccines that

stimulate serum antibody selectively without local respiratory tract antibody should not be used. First of all, they fail to produce local protective antibody, and secondly, they may create an immunologic imbalance that, by the aberrant nature of the response, can lead to a condition of hypersensitivity rather than protective immunity.

3. Studies over the past year of serum and local responses seen following immunization with bacterial vaccines have included studies in the human salmonella, immunization of children. These studies have indicated that a significant amount of serum γM-immunoglobulin is specific antibody following immunization of children with inactivated vaccine.

Studies over the past year have been conducted in rabbits immunized with Escherichia coli utilizing electrochemical techniques comparing isolated 19 S and 7 S antibody to the electrochemical events of E. coli. Female albino New Zealand rabbits weighing two to three kilos were used in these studies. The animals were bled prior to immunization in order to assay their natural antibody levels to E. coli, the animals had been immunized with 1×10^8 heat-killed E. coli strain L113 obtained from Dr. W.P. Weidanz. On day 5 the animals were bled at the height of the 19 S response actually received additional thrice-weekly injections of 1×10^6 heat-killed E. coli cells intravenously and were bled at approximately weekly intervals.

Antibody determinations were performed on sera and serum fractions using a passive hemagglutination technique employing E. coli 0113 lipopolysaccharide (LPS) coated sheep erythrocytes and sera were also checked by a standard tube bacterial agglutination method employing 1×10^9 heat-killed cells at 56°C for 18 hours.

Selected sera were fractionated by gel filtration with Sephadex G 200. A 5.0 ml serum sample was applied to a column 70 x 2 cm eluates collected in 5.0 ml fractions using phosphate buffered saline, pH 7.3 . Protein concentrations in the Sephadex fractions were determined by the Lowry Method and the antibody profile of the chromatogram determined by the passive hemagglutination method.

Isolated 19 S and 7 S antibody pools were prepared by combining fractions 33 to 40 to 57, respectively, concentration to 5 ml by positive pressure dialysis and standardization to a protein concentration of 350-370 mg%. The saline titer of both pools were adjusted to the same titer of 1:32 to 1:64.

The E. coli 0113 used to study the electrochemical effects of the 19 S and 7 S antibody fractions were prepared by inoculating 400 ml of

a trypticase soy broth (Baltimore Biological Labs.) 2.7% in pH 6.7 KH_2PO_4 (0.1M) buffer containing 1% glucose and incubating for 16 hours at 37°C. The suspended organisms were collected by centrifugation (500 x g, 4°C, 15 minutes Servall RD-2B) and resuspended in 50 ml of the pH 6.7 phosphate buffer (0.2M). The process was repeated a second time and the sedimented bacteria were suspended in a quantity of 0.2M phosphate buffer sufficient so as to give for a dilution of 1:20 an absorbance of 0.67 at 625 nm (Gilford Model 300 microspectorhotometer). This is equivalent to approximately 10^9 bacterial/ml of the undiluted sample.

Prior to electrochemical measurements, two tubes each containing 10 ml of the buffered E. coli suspensions were mixed respectively with an equal volume of either a buffered 1:50 dilution of 19 S or 1:25 dilution of 7 S antibody. These dilutions were based on prior determination so as to achieve equivalent amounts of antibody based upon agglutination titers. Controls consisted of tubes containing 10 ml of the E. coli suspension mixed with 10 ml of the phosphate buffer. All tubes were incubated for 16 hours at 37°C, followed by chilling in an ice-bath and centrifugation for 15 minutes at 5000 xg at 4°C. The sedimented cells were suspended in 20 ml of the pH 6.7 (0.2M) phosphate buffer. This process was repeated a second time prior to electrochemical studies.

The anolytes consisted of 5 ml of bacterial suspension, and 5 ml of pH 6.7, 0.2M phosphate buffer. The cathode compartment contained 40 ml of 0.1M potassium ferricyanide in phosphate buffer. This established the cathode as a nonpolarizable counter-electrode. After equilibration of the electrochemical cells to bath temperature (37°C) for 10 minutes, a 1,000 —∿— resistance was inserted in parallel with the cell and the background current (I = 4-5 ua) recorded (Multriter II, Texas Instrument Company) for a period of 5 minutes. At this time 2.5 ml of 0.473 M glucose was rapidly added in the pH 6.7 buffer. The rate of increase of current with time (dI/dt), the maximum current (I max), and the coulombic outputs were obtained. Each series of experiments was performed in triplicate.

The current time curves representing each experiment, i.e., control antibody and 7 S antibody, are illustrated in Figure 1. The various electrical parameters obtained from the current/time curves are described in Table VII.

These results indicate that the coulokinetic behavior of glucose metabolizing E. coli 0113 after exposure to 19 S and 7 S antibody show differences. It was found that the more potent 19 S fraction had the maximum inhibitory effect on the rate of electron donation by the bacterial to the electrode as indicated by the lower maximum current (I max) attained by

the electrochemical system as compared to the control. In addition, the rate of transport of the metabolizable substrate to the sites of enzymatic activity within the cell was also depressed. This was suggested by the lower rate of initial current increase (dI/dt) after introduction of the substrate into the system. The less potent 7 S fraction had a relatively smaller effect on the above parameters. Neither the 19 S or 7 S antibody fractions had any apparent effect on the microorganisms' ability to metabolize the total quantity of substrate as indicated by the coulombic outputs being essentially the same for the control as for those E. coli previously exposed to the antibody.

Summary and Conclusions.

1. Immunochemical studies of serum and nasal secretions obtained from individuals and recruits undergoing natural infection with adenovirus type 4 and other adenoviruses have been studied utilizing radioimmunodiffusion. These studies have shown that this technique allows the characterization of the various classes of antiviral antibody with a degree of precision in clarity not possible by other techniques.

2. Studies of the immune response following measles immunization of children have been completed. These studies have been extended to include investigations of untoward vaccine reactions seen in prior recipients of inactivated vaccine who are subsequently exposed to live virus either in nature or as a consequence of live measles immunization. These studies have shown the importance of viral-antiviral complexes in the pathogenesis of untoward reactions.

3. Studies in the human and in the experimental animal utilizing Salmonella and E. coli, respectively, have been completed. In the experimental animal these studies have been extended to include studies of the electrochemical events following interaction of purified 19 S and 7 S antibody on the metabolism of E. coli.

Table I

Specificity of Adenovirus Type 4 Radioimmunodiffusion Antigen

Infection sero-type and clinical disease	Serum date	Neutralizing titer					AV CF	Radioimmunodiffusion with type 4 adenovirus		
		AV-1	AV-3	AV-4	AV-6	AV-7		γA	γM	γG
AV-6 (conjunctivitis)	7/15/65	< 5*	NT	10	< 5	20	NT	≥ 32	0	64
	8/ 3/65	< 5	NT	20	≥ 80	20	NT	≥ 32	1	≥ 2048
AV-1 (conjunctivitis)	11/20/67	< 5	< 5	< 5	< 5	< 5	0	0	0	0
	12/ 4/67	10	< 5	80	< 5	< 5	≥ 160	≥ 32	4	≥ 2048
AV-7 (ARD)	9/29/67	10	< 5	5	< 5	< 5	< 5	0	0	64
	10/13/67	5	< 5	10	< 5	10	20	≥ 32	0	≥ 2048
AV-4 (ARD)	2/ 2/65	40	10	< 5	< 5	10	5	0	0	64
	3/15/65	40	10	≥160	< 5	20	40	0	0	≥ 2048

*Reciprocal of serum dilution.
NT = Not tested.

Table II

AV-4 Antibody in Serum Following Infection (# 208 FD)

Interval after onset of illness (days)	Serum titer[1] (untreated)	Antibody[1] in sucrose density gradient[2]										Titer by radio-immunodiffusion[3]		
		1	2	3	4	5	6	7	8	9	10	γA	γM	γG
3	< 1:5	-	-	-	-	-	-	-	-	-	-	0	0	0
11	1:20	-	-	+	+	+	+	-	-	-	-	16	$\bar{>}$ 4	$\bar{>}$ 2048
53	1:80	-	-	+	+	+	+	+	+	-	-	$\bar{>}$ 32	1	$\bar{>}$ 4096

[1]Neutralizing antibody.
[2]γM usually in tubes 1-4, γA and γG in tubes 5-8.
[3]Reciprocal of serum dilution.
+ = positive, - = negative for antiviral antibody.

Table III

Frequency of N Antibody by Week in Recruits Naturally Infected with AV-4

	Before	Week following infection					
		1	2	3	4	5	6
Nasal	2/35*	11/23	15/19	12/15	16/16	13/14	7/9
Serum	1/35	15/16	9/9	8/8	11/11	14/14	9/9

*No. pos/No. tested

Table IV

Development of Adenovirus Type 4 Antibody in Serum and Nasal Secretions Following Infection

Subject	Specimen date	Virus isolated	Serum specimens					Nasal secretions			
			Titer[1]		Radioactive binding			Titer	Radioactive binding[2]		
			N	CF	γA	γM	γG	N	γA	γM	γG
65-5	1/22/65	0	< 5	10	< 2	< 2	32	< 1	-	-	-
	2/8	Adv 4	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2/15	Adv 4	10	10	8	<2	128	1	-	-	1
	3/2	0	40	40	8	<2	≥ 512	4	2	-	2
	3/15	0	ND	40	8	<2	32	8	+	-	+
	3/22	0	64	ND	ND	ND	ND	8	2	-	-
65-1	1/22	0	< 5	< 5	0	0	≥ 32	2	-	-	-
	2/15	Adv 4	< 5		0	0	128	< 1	-	-	-
	2/22	0	≥ 80	40	0	4	≥ 2048	ND	-	-	1
	3/22	0	≥160	40	0	4	512	8	+	-	-
65-3	1/22	0	< 5	5	0	0	64	< 1	ND	ND	ND
	2/22	Adv 4	< 5	ND	0	≥4	≥ 256	< 1	ND	ND	ND
	3/1	0	5	ND	0	2	256	2	1	-	-
	3/8	0	20	20	0	0	≥ 1024	8	ND	ND	ND
	3/22	0	40	20	10	0		≥ 32	≥32	-	2

[1]Reciprocal of serum dilution

[2]+ = Antibody present, not titrated.

- = No antibody in undiluted specimen.

ND = Not done.

Table V

Clinical Data in Individual Children Previously Immunized with Inactivated Vaccine (K-MV) Subsequently Immunized with Live, Attenuated Measles Vaccine (L-MV)

Subject	Age (yrs.)	Race/Sex	Immunizations			Serum N Titer	Clinical Reaction*		Biopsy/ time (hrs)
			K-MV	L-MV	Interval (mos.)		Severity	Time (days)	
S.B.	55/12	N/F	9/20/66 10/ 8/66 11/ 1/66	3/ 1/68	18	4	+2	(1)	+(48)
V.M.	5	W/F	2/25/65 3/26/65 4/24/65	6/ 3/68	40	4	+2	(1)	0
A.M.	6 5/12	W/F	2/22/65 3/26/65 4/28/65	6/ 3/68	40	< 4	+2 +4	(1) (5)	0
D.J.	3 11/12	N/M	6/ 6/67 7/ 3/67 8/ 8/67	6/27/68	12	< 4	+2	(1)	+(24)
M.E.	2 3/12	W/F	4/29/66 6/17/66 9/12/66	10/28/68	18	< 4	+	(1)	0
J.F.	5 7/12	N/M	11/ 1/66 11/29/66 12/27/66	4/30/69	29	< 4	+2	(1)	+(30)

* +1 Local erythema + induration < 5 x 5 cm
+2 Local erythema < 5 x 5 cm + fever 101°F
+3 Local erythema and induration < 5 x 5 cm, + fever 101°F, + regional adenopathy

Table VI

Development of Measles Neutralizing (N) Antibody in Serum (S) and Nasal Secretions (NS) of Children Previously Immunized with Inactivated Measles Vaccine (K-MV) Following Immunizations with Live Attenuated Measles Vaccine (L-MV)

Subject	Specimen	Measles N Antibody Titer on Indicated Day Prior to or After Immunization with L-MV						
		Pre	0	3	5	7	14	Follow-up
S.B.	S	4 (-20)*	4	4	-	-	512	-
	NS	< 1	< 1	< 1	-	-	< 1	-
V.M.	S	4 (-33)	4	4	4	-	-	64 (103)*
	NS	-	< 1	< 1	< 1	-	-	8
A.M.	S	< 4 (-33)	< 4	< 4	< 4	-	-	128 (103)
	NS	-	-	< 1	< 1	-	-	2
D.J.	S	>64 (-294)	< 4	< 4	4	-	-	256 (47)
	NS	< 1	< 1	< 1	< 1	-	-	< 1
M.E.	S	< 4 (-21)	< 4	< 4	< 4	< 4	32	128 (11)
	NS	< 1	< 1	< 1	< 1	< 1	< 1	2
J.F.	S	32 (-394)	NT	NT	NT	NT	NT	NT
	NS	< 1						

*Number in parentheses indicates the day prior to or after administration of L-MV vaccine
NT - Test not completed

Table VII

Comparative Electrical Behavior of Escherichia coli 0113 after Exposure to 19 S and 7 S Antibody Fractions When Metabolizing Glucose

	dI/dt (uA/min)	I max (uA)	Coulombs
Control	8.19 ± .09	705 ± 5	8.3 ± 0.3
19 S	3.19 ± .07	530 ± 5	8.1 ± 0.2
7 S	4.17 ± .12	565 ± 2.5	8.6 ± 0.2

Project 3A061101A91C - IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 - In-House Laboratory Independent Research

Work Unit 106 - Physical-chemical Studies of the Biologic Significance of Anti-Viral Antibody

Publications

1) Bellanti, J.A., Sanga, R.L., Klutinis, B., Brandt, B.L. and Artenstein, M.S.: Antibody Responses in Serum and Nasal Secretions of Children Immunized with Inactivated and Attenuated Measles-Virus Vaccines. New Eng. J. Med. 280:628, 1969

2) Allen, M.J., Bellanti, J.A. and Jackson, A.L.: Cellular Electrophysiology. XVII. Effect of Specific Antibody on the Coulokinetic Behavior of Escherichia coli. Currents in Mod. Biol. 4:1969

				DA 0A6490	69 07 01		
68 10 31	D. Change	U	U	NA	NL	X	

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23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number, procedure, test of each with Security Classification Code)

23. (U) To investigate genetic and metabolic alterations in cell populations surviving injury. Among such alterations, those responsible for the progressive deterioration observed in individuals exposed to ionizing radiation and for the difficulties encountered in the use of frozen cells and tissues for grafting have military significance and, accordingly, constitute the specific objectives of this project.

24. (U) To maximize experimental control, in vitro cultures of partially synchronized fibroblastic cells were frozen in the period before (G1) and during (S) the synthesis of deoxyribonucleic acid (DNA) and the injury thus induced was analyzed in terms of post-thaw viability, chromosomal complement and division cycle kinetics.

25. (U) 68 07-69 06 While there has been extensive investigation into mechanisms controlling cell growth and regeneration following injury, this work unit is concerned with cytogenetic and metabolic alterations of cell populations which survive injury. Such alterations assume obvious importance in terms of progressive deterioration observed in individuals exposed to ionizing radiation and the problem involved in use of frozen cells and tissues for grafting. In order to maximize experimental control, a well defined in vitro culture system is used to study fibroblastic cell populations following freezing and radiation injury. Experiments on growth kinetics of synchronous populations of cells frozen in G1 (pre-DNA synthesis phase) and S (DNA synthesis phase) support the evidence of our previous studies that pre-DNA synthetic cells are highly sensitive to conventional methods of freeze preservation. The effect of freezing is lethal for 50-60 percent of these populations. However, S phase cells are much less sensitive to the freezing process exhibiting a survival of approximately 85 percent of the controls. The lag to log to stationary transition observed in these cell cultures are being investigated to relate the parameters of normal growth kinetics and metabolic events to events seen in cell injury, survival and repair. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 108, Cytogenetic and metabolic determinants in the evolution of cell populations following injury

Investigators.
Principal: Andre D. Glinos, M.D.
Associate: Robert I. Werrlein, M.S.; Edwin M. Bartos, PhD; James M. Vail, PhD.

The Problem.

Clinical observations and animal experimentation have shown that the sequence of changes in the myelocytic and the erythrocytic series after whole body exposure to ionizing radiation is the same in principle for all species although dose and time relationships vary markedly among species. These changes are shown schematically in Figure 1. Four phases are distinguished:

1. The degenerative phase, during approximately the first five days for man, characterized by a rapid decline of the peripheral blood count from the normal level A to level B. The degree of depression and the time of its minimum value are species-dependent and they are also dose-dependent within a species. At very high doses no efforts at regeneration are seen and the probability of infection and death in this phase are high. At doses in the lethal range, however, the degenerative phase is terminated by:

2. The phase of abortive rise, approximately from day five to day seven for man, characterized by the temporary rise of the blood count from level B to level C. The time of the presence of the abortive rise is species-dependent and its magnitude is a function of the dose. The abortive rise is responsible for delaying significantly radiation death, the probability of which is highest during:

3. The phase of marked and prolonged depression of the blood count to level D. The height of this level and its duration are also species- and dose-dependent; in man it lasts approximately from day seven to day thirty. In survivors this phase is succeeded by:

4. The final regenerative phase, when the blood count returns to the normal level A and may transiently overshoot.

The peripheral blood count represents a steady state equilibrium between the continuous production of cells and their maturation, ageing and death. Consequently, the sequence of events just described may be best

explained on the basis of the injury sustained by the blast cells of the hemopoietic centers. Three types of such injury are recognized. In order of decreasing severity these are: a. immediate cell death; b. delayed cell death, occurring after the cells complete a limited number of post-irradiation divisions; cells sustaining this type of injury are referred to as inactivated cells. c. temporary division delay, immediately after irradiation, affecting inactivated cells as well as those which have escaped permanent injury altogether.

Following irradiation the cell population density in the peripheral blood decreases from its normal level A to level B as a result of the first and the third types of injury. - The temporary increase of the cell density to level C, referred to as abortive rise is due to the limited number of divisions which inactivated blast cells undergo when the temporary delay effect wears off. The subsequent decrease to level D would be due to the death of these cells and the depletion of the hemopoietic centers except for the small number of blasts without injury. If level D is compatible with life the gradual repopulation of the hemopoietic centers by the blasts which escaped injury results in the eventual restoration of the normal peripheral cell population density during the final regenerative phase.

As it was stated previously, the abortive rise C is responsible for delaying significantly radiation death, the probability of which is highest during the ensuing depression to level D. It follows that if inactivated cells could be induced to perform a greater number of divisions, thus maintaining the blood count at level C until the advent of the final regenerative phase (see dotted line C-c in Figure 1), radiation death would be prevented.

Accordingly, the objective of this task is the study of the mechanisms allowing inactivated cells to perform a limited number of post-irradiation divisions and of ways that could be used to increase that number. It is expected that this in turn will lead to the development of <u>therapeutic</u> methods for radiation injury, i.e. treatment <u>after</u> exposure, in contrast to the presently available methods of limited prevention through treatment before exposure.

<u>Approach</u>.

The ability to perform a limited number of divisions after inactivation is not limited to the blast cells of the hemopoietic system but is shared by all irradiated cell systems <u>in vivo</u> as well as <u>in vitro</u>. The latter allow maximal experimental control and analytical resolution. A number of important cytogenetic and metabolic parameters of such a system, involving large populations of mouse fibroblastic cells in suspension, are under intensive study in connection with the mechanisms involved in freezing injury (cf. Walter Reed Army Institute of Research Annual Progress Report, this Work Unit, 30 June 1968) and wound healing

(cf. Walter Reed Army Institute of Research, Annual Progress Report, Work Unit 075, Cell Growth and Regeneration, 30 June 1969).

The advantages of utilizing the same system to study radiation injury are obvious, provided that early findings on post-irradiation division obtained through experiments involving small numbers of cells seeded on glass (Glinos, A.D. and North, H.H. Trans. N.Y. Acad. Sci. II, 26: 145, 1963) are shown to be applicable to large suspension cultures. This change of scale in the experimental system is essential since it is most likely that the desired increase of the number of post-irradiation divisions performed by inactivated cells will involve their supplementation with essential metabolites, the identification, extraction and purification of which will necessitate large numbers of cells as source material.

Results.

In the aforementioned early experiments, replicate cultures on glass were prepared using small cell inocula originating in suspension cultures which were either in the low density logarithmic phase of growth or in the high density stationary phase. These replicate cultures were irradiated and the analysis of the dose-response curves obtained indicated that inactivated cells originating in the logarithmic phase of growth were capable of performing a larger number of post-irradiation divisions than similarly irradiated cells originating in the stationary phase.

During the period covered by the present report, it was sought 1. to develop a method for detecting the number of post-irradiation divisions performed by inactivated cells following a single radiation dose delivered directly to a large suspension culture; and 2. to investigate possible differences in the chromosomal complement of the cells in the logarithmic and the stationary phases which could be involved in the different response to irradiation suggested by the early experiments.

1. In the case where continuous cell division balanced by cell death results in a steady state population such as the one shown in Figure 1, it is to be expected that the loss of the ability of a part of the population to divide either immediately (cell death), or following a few divisions (cell inactivation), will be reflected by a decrease in the steady state level, regardless of whether there is immediate disintegration of the dead or inactivated cells, or not. It is precisely this process which is illustrated by the changes AB and CD of the blood count in Figure 1. In contrast, when continuous cell division without cell death results in a logarithmically increasing cell population, radiation induced cell death or cell inactivation will result in actual decrease of the cell count only if followed by immediate disintegration. When disintegration is delayed, as is often the case with ionizing radiation in the lethal range, dead and inactivated cells will continue to

be counted and it may be predicted that the population curve will show a declining rate of growth or a plateau and only a minimal decline in cell number. In such a case, the number, the time of appearance, and the duration of the plateaus in relation to the radiation dose would allow the recognition, in the post-irradiation population curve of a logarithmically growing suspension culture, of the four phases shown in Figure 1. This in turn would provide the means for a comparative estimate of the number of post-irradiation divisions performed by inactivated cells.

In order to test the validity of this concept, suspension cultures of the L strain mouse fibroblasts were set up in Eagle's minimum essential medium (sMEM) supplemented with 10 per cent horse serum. Initial total suspension volume and population density were 200 ml and 3,5 to 4,5 x 10^5 cells per milliliter respectively. The temperature of incubation was 35°C and the gas phase 5 per cent CO_2 in air. The medium of the culture was changed daily. Under these conditions growth was logarithmic with the population density of the culture doubling approximately every 24 hours. When a density of 10^6 cells/ml was reached, the cultures were divided into 4 equal parts of 45 ml each, which were then centrifuged for 30 min at 800 rpm. Each cell pellet obtained was resuspended in 100 ml of fresh medium placed in a 250 ml Erlenmeyer flask. Three of the flasks were then irradiated with progressively increasing doses, using a Co^{60} gamma ray source delivering 200 R/min, the fourth serving as control. Following irradiation all four flasks were incubated under identical conditions. The media were renewed daily by centrifuging the cultures and the cell populations diluted whenever densities exceeded 10^6 cells/ml. Cell counts were performed at the end of each 24 hour incubation period, just prior to medium renewal, and again 2 hours later. The latter delay was necessary in order to obtain a proper dispersion of the cells from the centrifugation pellet.

The results of such an experiment are shown in Figure 2, where the logarithmic increase of the population density is indicated on the left ordinate while the corresponding number of population doublings is indicated on the right ordinate. It can be seen that following irradiation on day 0, there is a decline in the rate of growth indicated by a decrease of the slope of the cell population curves of all three irradiated flasks compared to the control. There are no plateaus in the growth curves of the flasks irradiated with 300 R and 400 R and therefore it is not possible to identify discrete phases in the post-irradiation kinetics of these cultures. In contrast, in the case of the flask irradiated with 500 R, the initial decline of the slope of the cell population curve leads to a plateau, followed by a period of growth from the fourth to the ninth day at which time a second plateau of one day's duration is succeeded by the resumption of logarithmic growth.

By comparing this curve with the curve shown on Figure 1 and on the basis of the analysis of cellular radiation injury made previously,

it is suggested that the first four days after irradiation with 500 R correspond to the degenerative phase. This would be caused by temporary division delay and cell death followed by some cell disintegration, hence the negative slope of the curve from day 3 to 4. The phase of the abortive rise, due to the proliferation of inactivated cells together with uninjured cells, would be represented by the growth period between day 4 and day 9 while the second plateau would indicate the end of this process and the initiation of the regenerative phase on day 10.

2. A quantitative analysis of the chromosomal complement of the cells used in the system and of the alterations induced by freezing injury has already been given (Glinos, A.D. and Hargrove, D.D. Exp. Cell Res. 39: 249, 1965, and Walter Reed Army Institute of Research Annual Progress Report, this Work Unit, 30 June 1968). Using these previous data as a baseline, the frequency distribution of the chromosomes of cells in the logarithmic and the stationary phases of growth was compared and the results are shown in Table 1. A significant difference was found in regard to the per cent of cells having 40-49 chromosomes, i.e. being just hyperdiploid, which decreased and the per cent of cells having 80 chromosomes or more, i.e. being tetra- and hypertetraploid, which increased in the stationary phase.

Discussion, Conclusions and Recommendations.

1. As it was mentioned earlier, the current objective of this task is the development of a therapeutic method for radiation injury based on maintaining the blood count at the level of the abortive rise until the advent of the regenerative phase (cf. Figure 1, line C-c). To do so, it will be required that the process of post-irradiation division of inactivated cells, which results in the abortive rise, be prolonged. Inactivation involves genetic injury rendering the cells unable to synthesize the RNA messengers and protein factors necessary for cell division. Their ability to perform a limited number of divisions under the circumstances, therefore, indicates a. that these division factors are stored in the cells in quantities sufficient for the initiation of several divisions, and b. that division stops when these factors are diluted below a minimum essential level by the very process of division which they induce.

The problem of prolonging division of inactivated cells, therefore, has two parts: first, identification, extraction and purification of the division factors, and second, supplementation of inactivated cells with these factors.

In turn, the problem of the identification of the division factors would be greatly facilitated if it could be demonstrated that under rigidly controlled experimental conditions the concentration of these factors in a given cell system may be made to change in a predictable,

reproducible way. This appears to be precisely the case in the cell culture system described, where the fact that cells inactivated in the stationary phase perform fewer divisions than cells inactivated during the logarithmic phase, suggests that the former contain a lower concentration of the division factors than the latter.

The sequence of events following irradiation described in detail in the section on results suggests further that in suspension cultures the number of divisions performed by inactivated cells is indicated by the height of the second plateau. In the case of the logarithmically growing culture irradiated with 500 R shown in Figure 2, this plateau is at the height of just under four population doublings. This is in excellent agreement with the figure of just under four divisions, indicated by the dose-response curves obtained earlier with cultures growing on glass (cf. Elinos, A.D. in Control of Cellular Growth in Adult Organisms, Figure 3. Academic Press London, 1967). Accordingly, it is proposed to:

a. Continue the work on irradiated logarithmic cultures by additional experiments of the type described in Figure 2, including additional radiation doses and using plating efficiency viability tests and H_3 thymidine labelling to obtain detailed information on the relative contribution of temporary division delay, cell death and cell inactivation to the sequence of events following irradiation. This in turn will allow the determination of the number of divisions performed by inactivated cells with a far greater accuracy than it was hitherto possible.

b. Repeat the experiments described above, with irradiated stationary suspension cultures. In this case growth will be induced by dilution immediately after the exposure to radiation. By taking into consideration the kinetics of the lag phase following such dilution it will be possible to evaluate the number of divisions performed by inactivated cells and to determine whether the difference between cells originating in the logarithmic and stationary phases, found previously with cultures grown on glass, holds also true for suspension cultures.

c. In case this difference is confirmed, to begin RNA and protein analysis of cultures in the logarithmic and the stationary phases. In this fashion, concentration differences found between these two types of cells will lead to an early distinction between molecular species which are likely to be involved in the initiation of cell division and those which are not.

d. Test the ability of the fractions thus obtained to increase the number of post-irradiation divisions of inactivated cells through addition to the media of suitably irradiated cultures.

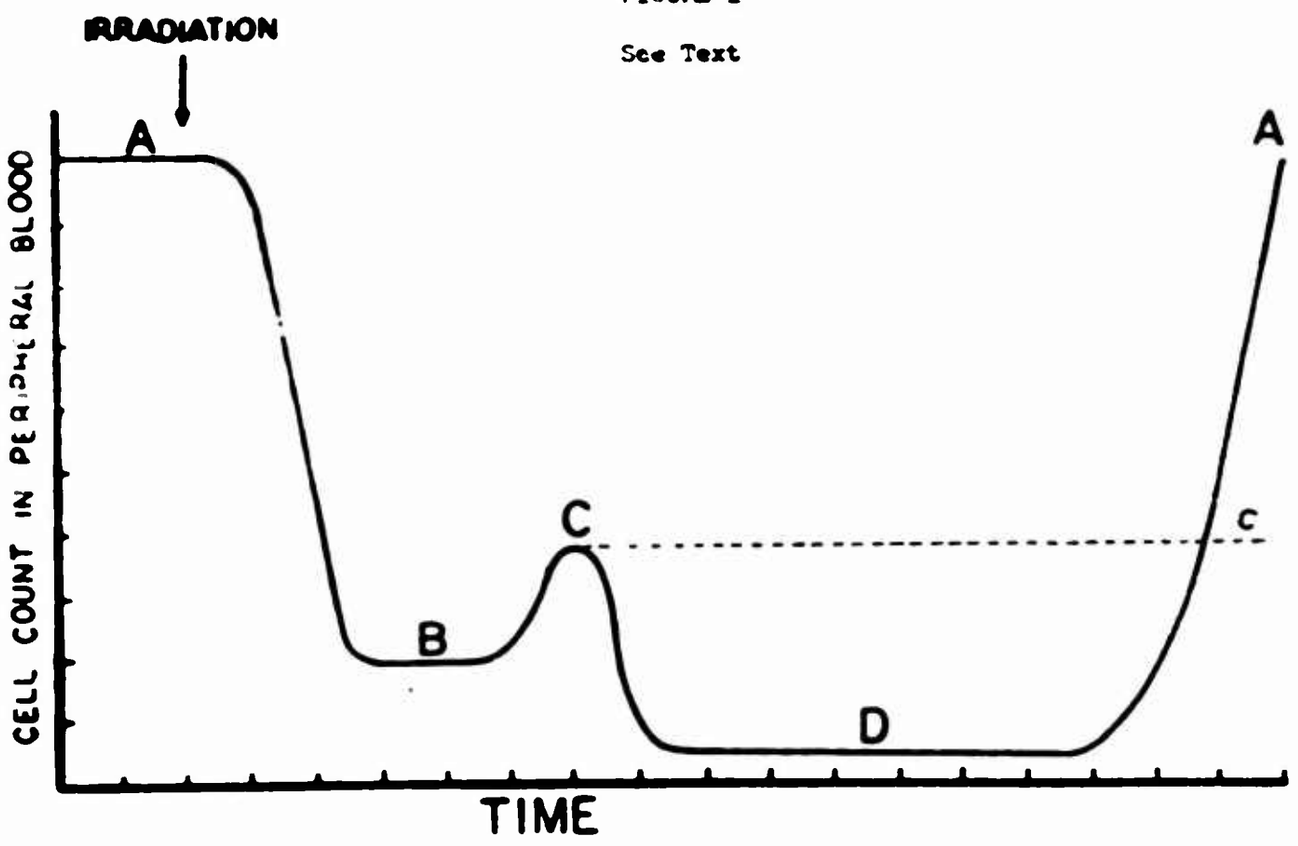
2. An alternative interpretation of the finding that inactivated stationary phase cells are capable of fewer post-irradiation divisions than similarly inactivated logarithmic phase cells would be that the chromosomal complement of the cells undergoes such changes during the stationary phase that it sustains greater chromosomal injury.

In Table 1 the eleven per cent of cells with tetra- and hypertetraploid chromosome numbers (> 80 range) in the stationary phase is compensated by a corresponding decrease of the diploid and hyperdiploid cells (40-49 range). This indicates that the process responsible for the increase of the cells with more than 80 chromosomes is endomitosis which results in chromosome redundancy. In turn, this increases radiation resistance in the sense that it enhances the capability of the cells to sustain a certain amount of chromosomal injury without untoward effects on cell life and function.

These data then would tend to exclude chromosomal injury as a factor contributing to the reduced number of post-irradiation divisions that cells inactivated during the stationary phase are able to perform. It is proposed to test the validity of this concept by direct analysis of chromosomal injury during the first four divisions of cells irradiated in the logarithmic and the stationary phases.

FIGURE 1

See Text



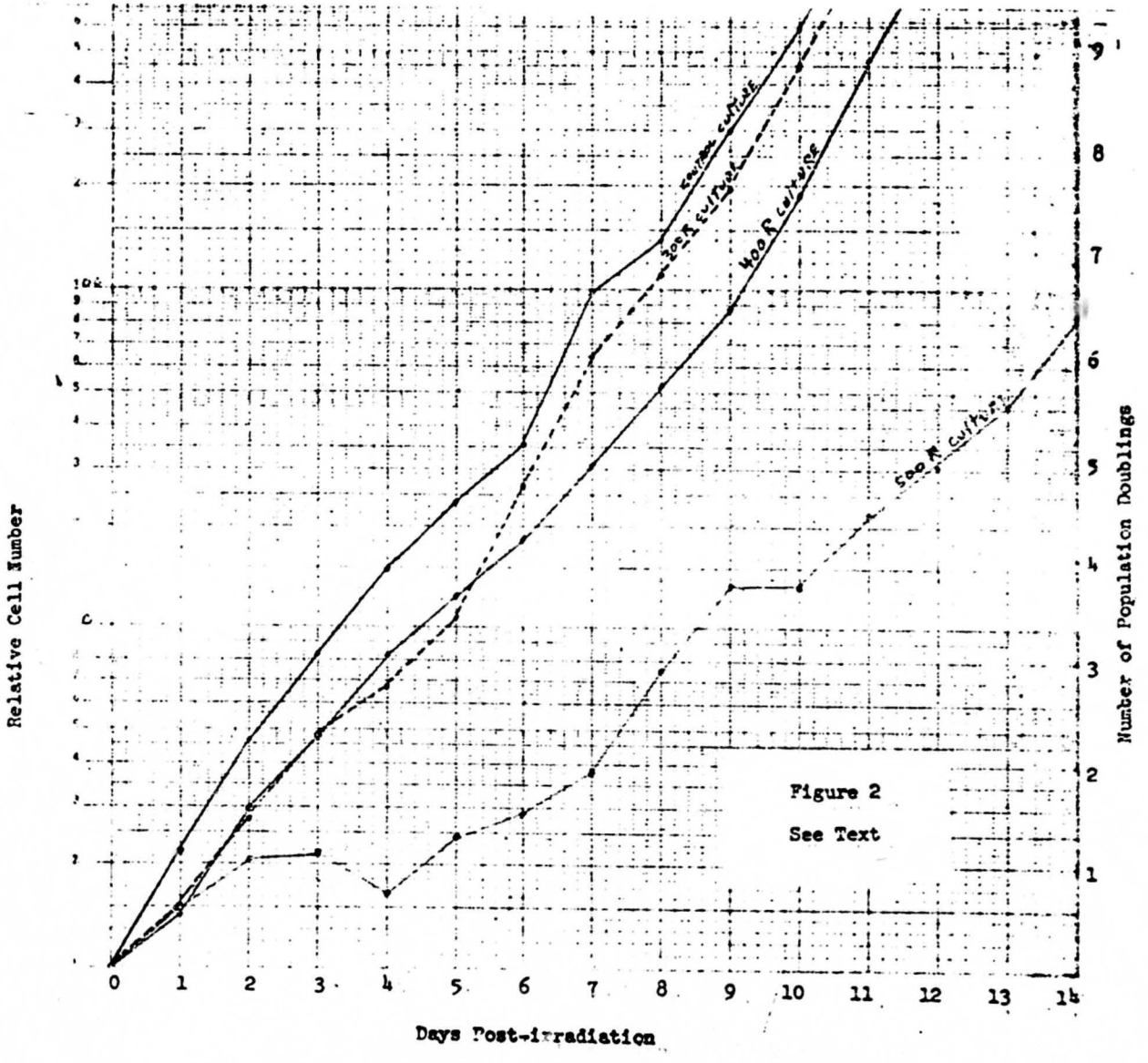


Figure 2

See Text

COMPARISON OF THE FREQUENCY DISTRIBUTION OF CHROMOSOMES

IN LOGARITHMIC AND STATIONARY CULTURES

Chromosome Distribution Range	Per cent of Cell Population	
	Logarithmic	Stationary
<30	1	6
30 - 39	2	3
40 - 49	76	53
50 - 59	21	24
60 - 69	0	1
70 - 79	0	2
>80	0	11

TABLE 1

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 108 Cytogenetic and metabolic determinants in the evolution of cell populations following injury

Publications.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

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69 01 31	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

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c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Effects of Physiological and Psychological Stress upon Infection and Disease (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 003500 Clinical Medicine 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
10 64	NA	DA	C. In-House

17. CONTRACT/GRANT: Not Applicable

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
PRECEDING 69	2	75
CURRENT 70	2	65

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Res

ADDRESS: Division of CD and I
Washington, DC 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Air Force installation)

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3552

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Mason, Dr. J. W.

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Respiratory Infection; (U) Stress; (U) Endocrine Response; (U) Hormones; (U) Viruses; (U) Sociology; (U) Personality Type; (U) Human Volunteer.

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Definition and evaluation of various environmental and personal factors which contribute to physical and psychological stress experienced by military personnel, and determination of how these affect the overt clinical manifestations of naturally acquired infections. When factors are defined, efforts to modify clinical manifestations by modification of environment or human response to it are made.

24. (U) Endemic overt diseases in military populations are identified and studied for microb. etiology, variation in clinical manifestations. Environment in which they occur is defined. These findings are correlated with patient /s immunological susceptibility, physiological responses to environment and its stresses, and with personality types, and social backgrounds. Factors suspected of influencing disease severity are evaluated in controlled experiments.

25. (U) 69 01 - 69 06 All hormonal and statistical analyses on this project are now complete. Because of several striking pre-illness hormonal differences in the sick and control groups, it is felt that the findings of this study merit publication and preparation of the manuscript is now in progress. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 65 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 113, Effect of physiological and psychological stress upon infection and disease

Investigators.

Principal: John W. Mason, M.D.; COL Edward L. Buescher, MC
Associate: Robert M. Rose, M.D.; CPT Richard O. Poe, MC; CPT Marvin S. Wool, MC; Edward H. Mougey, M.S.; Frances E. Wherry, A.B.; David R. Collins, B.S.; Elizabeth D. Taylor, M.S.; Percy T. Ricketts, B.S.; Norman Krasnegor, M.S.

Description.

This study was designed to explore the possibility that stress-related, pre-illness changes in hormonal levels may play a contributory role in the pathogenesis of acute respiratory infections. The feasibility of the study was suggested by the high incidence of acute adenovirus infections in Army recruits during basic training in the winter months at Ft. Dix, New Jersey. Furthermore, the great majority of such illnesses usually are clustered during the third and fourth week of basic training. It was, therefore, possible to study a population in which a very high incidence of respiratory illness could be predicted within a designated two-week period.

Progress.

Hormonal Balance in Medical Illness.

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

Summary and Conclusions.

All hormonal and statistical analyses on this project are now complete. Because of several striking pre-illness hormonal differences in the sick and control groups, it is felt that the findings of this study merit publication and preparation of the manuscript is now in progress.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 113 Effect of physiological and psychological stress upon infection and disease

Publications.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6500	69 07 01	DD-R&E (AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
68 05 31	D. CHANGE	U	U	NA	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	611UIA	3A051101A91C	00	118
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Electron Microscopy of Intestinal Epithelium (33)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
65 02	CONT	DA			B. CONTRACT

17. CONTRACT/GRANT

a. DATE/EFFECTIVE: 69 06 EXPIRATION: 70 05

b. NUMBER: DA-49-193-MD-2705

c. TYPE: J. FFP d. AMOUNT: None

e. KIND OF AWARD: CON f. CUM. AMT. $44,390

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	1	1
FISCAL YEAR CURRENT 70	1	3

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: New York Medical College

ADDRESS: New York, N. Y. 10025

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Hartman, R. E.

TELEPHONE: 212-TR6-5500

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Conrad, COL M. E.

NAME: 1

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Hepatitis; (U) Sprue; (U) Electron Microscope; (U) Virus; (U) Intestine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To characterize the intestinal lesions of tropical sprue and infectious hepatitis and to attempt to identify virus particles in specimens from patients with these diseases.

24 (U) By electron microscopy intestinal specimens from U. S. soldiers with infectious hepatitis and tropical sprue are being studied to show the changes that occur, relate them to physiologic abnormalities and attempt to demonstrate viral particles.

25 (U) 68 05 - 69 06 No progress was achieved during the period of this report because of the unavailability of a functioning electron microscope. Project is being continued for one year without additional funds.

*Available to contractors upon originator's approval.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 118, Electron microscopy of intestinal epithelium

Investigators.
Principal: Roberta Hartman, Ph.D.
Associate: Richard Hartman, Ph.D. and COL Marcel E. Conrad, MC

No work was performed under this contract during this fiscal year for technical reasons beyond the control of the investigators. Project to be continued without additional funding through the next fiscal year for completion.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 118, Electron microscopy of intestinal epithelium

Publications.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6501	69 07 01	DD-R&E (AR) 636

3. DATE PREV SUM'RY	4. FND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 01 31	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	119
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procedure and Security Classification Code)
(U) Cytochemical Analysis of Growth of Malarial Parasites (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
002300 Biochemistry 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 07	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE/EFFECTIVE: NA
b. NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AGENCY:
f. CUM. AMT.
EXPIRATION:

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	2	43
FISCAL YEAR CURRENT 70	2	55

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC 20012
RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION
NAME: Armed Forces Institute of Pathology
ADDRESS: Washington, DC 20305
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Bahr, G. F.
TELEPHONE: 202-576-2915
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Stenn, CPT K.
NAME: Meszoely, CPT C. DA

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code)
(U) Malaria; (U) Plasmodia; (U) Metabolism; (U) Electron Microscopy

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with its Security Classification Code.)

23. (U) To determine the quantitative aspects of growth of malaria parasites in terms of rate of synthesis of protein, lipids and nucleic acids.

24. (U) By examination of the cellular and subcellular entities comprising malaria parasites with cytospectrophotometry, interference microscopy and quantitative electron microscopy.

25. (U) 69 01 - 69 06 The increase in red cell volume from time of entry of a merozoite has been measured. Cytofluorometric measurement of total DNA, RNA, and protein has been done. Preparations of disrupted parasites have made possible measurement of nuclear contents. The structure and organization of the nucleus is being studied. The protein components are preferentially susceptible to digestion by pronase and the effect is enhanced by a combination of pronase and RNA-ase. Instrumentation is completed for diagnosis by changes in nuclear patterns in electron micrographs. Technique for automated screening of malarial blood smears is not significantly better than standard methods. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.

* Author's or contractor's address

DD FORM 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 119, Cytochemical analysis of growth of malarial parasites

Investigators.
Principal: Gunter F. Bahr, M.D.
Associate: CPT Kurt Stenn, MC: CPT Charles Meszoely, MSC

This is a continuing project whose objective is an in depth elucidation of the structure and composition of the malaria parasite, with particular reference to its nucleus, and a study of the synthetic mitotic activities of the nucleus.

Initial studies were concerned with red cell volume changes in infection. These have been reported previously, but briefly with the penetration of the merozoite there begins a slow but exponentially accelerating volume increase of the red cell which is accompanied by a decrease in its bouyant density and at higher parasitemias, increased osmotic fragility.

Recent studies have been concerned with the parasite nucleus. Cytofluorometric measurements of total DNA, RNA, and protein content have been made. Preparations of disrupted parasites reveal in the nuclei an assembly of bumpy 350-500 (mean of 420) Angstrom unit thick chromatin fibrils. Calculation of the total length of fiber and of its DNA content indicates that the nuclear DNA of plasmodia is tightly packed in a coiled-coil fashion, analogous to the DNA in mammals and insects. Other calculations give a total double helix length of 15,340 microns, or in other words, 11 microns of DNA are packed into 1 micron of 420 Angstrom fiber.

Although the DNA content suffices for the coding of about 300-400 different proteins, it is not likely that all of the parasite genome is active at any time. Assuming that most gene functions at any one time are suppressed by bonding and/or steric mechanisms, there would be only limited stretches of DNA helix accessible to DNA-intercalating drugs in the non-replicating chromatin.

No conclusion can be reached as yet on how the chromatin fibrils are organized into chromosomes and into how many. There are indications of two relatively loosely organized chromosomes which tend to have a close relationship to nuclear pores. Several electron microscopic studies have shown the convergence of spindle fibers at nuclear pores as well as cell membrane condensations outside these, leading eventually to schizogenous buds.

Measurement of DNA, RNA, and nuclear proteins in the parasite nucleus indicates that nuclear composition is rather analogous to that of the host.

In other ultrastructure studies treatment of thin sections with pronase resulted in most of the cytoplasmic elements of parasite and red cell being unaffected. This is interpreted to mean that the protein components of the nucleus are preferentially susceptible to digestion. A combination of RNA-ase and pronase digestions enhanced the effect on the nucleus. It also eliminates the numerous RNP particles from the parasite cytoplasm. Since nuclear DNA is intimately associated with proteins in stranded structures, it can be assumed that the removal of these proteins has led also to the release of the majority of the DNA from the section. Some electron dense blotches remain in the nuclei of mature schizonts. These are most likely DNA condensations in an organism that is about to shut-off its major metabolic activity.

Preliminary studies had indicated that fluorescence techniques when applied to scanning of malaria blood smears had greater diagnostic sensitivity and accuracy than conventional techniques. A larger series of tests revealed that fluorochromed preparations scored only insignificantly higher diagnostically than the proven stains. Only speed of count would justify the use of fluorochromes, but this is probably not sufficient for support of this procedure.

Efforts in the immediate future are to be concentrated on further study of plasmodial nuclear organization using three avenues of approach. These are: enzyme extraction of thin sections; autoradiography; and study and measurement of chromatin fibrils in various stages of parasite growth and after treatment with antimalarials.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 119 Cytochemical analysis of growth of malarial parasites

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6505	69 06 30	DD-R&E (AR) 636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
68 10 31	K. COMPL.	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	611101A	3A061101A91C	00	120
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Provide with security classification code):
(U) Behavioral Baselines for the Experimental Study of Uremia (21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS:
012900 Physiology; 003500 Clinical Medicine; 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
65 11	CONT	DA			B. CONTRACT

17. CONTRACT/GRANT

a. DATE/EFFECTIVE: 67 11 EXPIRATION: 68 10
b. NUMBER: DA-49-193-MD-2819
c. TYPE: U. CPFF d. AMOUNT: $32,410
e. KIND OF AWARD: EXT f. CUM. AMT. $82,910

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 68	1	30
FISCAL YEAR CURRENT 69	0.3	11

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Institute for Behavioral Research

ADDRESS: Silver Spring, Md. 20910

PRINCIPAL INVESTIGATOR (Provide name if U.S. author institution)
NAME: Findley, J. D.
TELEPHONE: 301-587-2909
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Teschan, COL P.E.
NAME: 3

21. GENERAL USE

22. KEYWORDS (Provide KEY WORD security classification code):
(U) Kidney; (U) Body Fluids; (U) Hemodialysis; (U) Anuria; (U) Uremia

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with security classification code.)

23 (U) To elucidate the pathogenesis of the uremic syndrome, to identify chemical factors presumably responsible for uremic symptomatology.

24 (U) Quantitative techniques of experimental psychology are employed in operant-conditioned rhesus monkeys to trace behavioral decrements (1) in the course of acute and chronic renal failure and (2) in response to infusion of discrete chemical substances or of materials derived from uremic patients. Selected subjects are also studied by means of electroencephalograms.

25 (U) 68 07 - 68 10 The uremic syndrome has been experimentally induced in rhesus monkeys. Technique of peritoneal dialysis has been successfully employed to reverse the behavioral changes associated with uremia. Thus a model is now available to study the selective effects of various compounds.

*Available to contractors upon established need-to-know approval

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 120, Behavioral base lines for the experimental study of uremia

Investigators.
Principal: COL Paul E. Teschan, MC
Associate: Dr. J. D. Findley; CPT Richard M. Finkel, MC; CPT Paul B. Lamborn, Jr., VC; G. J. McCormick, Ph.D.; LTC Coy D. Fitch, MC

Description.

This project is an attempt to develop a behavioral model for the bioassay of uremia. Briefly, it consists of training rhesus monkeys to perform certain counting tasks by operant conditioning behavior techniques, followed by experimental induction of uremia by ureteral ligation. A measurable and reproducible decline in counting accuracy following development of uremia is seen.

Progress.

The behavioral program has been improved by the addition of counting requirements. In one preparation it has been possible to demonstrate a measurable decline in counting accuracy before clinical uremia was evident and to reverse the decline in behavior by peritoneal dialysis. This suggests that the behavioral change was due to uremia and that this model is a workable bioassay for uremia.

In addition the techniques of peritoneal dialysis in an alert, sitting rhesus monkey has been improved.

Recommendations.

A behavioral bioassay for uremia has been successfully developed. This model may now be utilized as a test system for specific suspected uremic toxins. It should be possible to maintain elevated concentrations of the suspected toxin while correcting all other biochemical abnormalities of uremia by selective alterations of dialysate fluid.

Research will continue under this same Project 3A061101A91C, but under Work Unit 188 entitled as above.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 120 Behavioral base lines for the experimental study of uremia

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6508	69 06 30	DD-R&E (AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
68 11 30	H. TERM.	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	122
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Antigenic and Biologic Classification of Dengue Viruses (07)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 11	CONT	DA	B. CONTRACT

17. CONTRACT/GRANT

a. DATE/EFFECTIVE: 67 11 EXPIRATION: 68 10
b. NUMBER: DA-49-193-MD-2846
c. TYPE: S. CT
d. AMOUNT: $60,263
e. KIND OF AWARD: EXT
f. CUM. AMT. $168,808

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 68	3	61
	CURRENT 69	0.3	20

19a. RESPONSIBLE DOD ORGANIZATION

a. NAME: Walter Reed Army Institute of Research
b. ADDRESS: Washington, DC 20012

c. RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W.H.
TELEPHONE: 202-576-3551

d. GENERAL USE

Foreign Intelligence Not Considered

20. PERFORMING ORGANIZATION

NAME: Yale University
ADDRESS: New Haven, Conn.

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Downs, W.G.
TELEPHONE: 203-777-2435
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Halstead, LTC S.B.
NAME: 1

21. KEY WORDS (Procede each with Security Classification Code)

(U) Dengue; (U) Viruses; (U) Antigens; (U) Biological Properties

22. TECHNICAL OBJECTIVE, 23. APPROACH, 24. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

a. (U) Antigenic analysis of dengue viruses, with particular reference to the classification of wild viruses recovered in various parts of the world; characterization of biological properties of wild dengue viruses.

b. (U) Conventional methods for antigenic analysis will be compared with novel systems employing cell culture techniques and partially pure lines of viruses. Cross protection studies in rhesus monkeys using selected wild dengue 1-4 strains will be undertaken to determine the degree and duration of cross protection between dengue types.

c. 68 10 - 68 11. Effective with renewal on 68 11 01 project was transferred to regularly funded 3A061102B71Q program. Future reports will be prepared by appropriate project officer, HQ, USAMRDC.

DD FORM 1498-1 (FOR ARMY USE)

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 122, Antigenic and biologic classification of dengue viruses

Investigators.
Principal: W. G. Downs, M.D.
Associate: LTC Scott B. Halstead, MC

Description.

In order to augment dengue studies at WRAIR, arrangements were made with Yale University whereby antigenic analyses and biological characterization of dengue virus strains were undertaken with support from ILIR funds. These studies were intended to gain more insight on cross-protection between dengue strains.

Progress and Results.

Support of the research from ILIR funds was continued only from 1 July - 31 October 1968 when the project was transferred to a regularly funded program. This report, therefore, covers only that period. Analyses of a number of dengue strains from dengue, hemorrhagic fever, and mosquito isolations were continued. In addition, observations were continued on monkeys which had been infected initially with dengue 4 and challenged 3 months later with dengue 2. Additional animals were infected with dengue 4 in an attempt to determine the factors affecting reproducibility of abnormal host reactions and the mechanism of this reaction.

Recommendations.

In view of the military interest in dengue in general and this research in particular, it was recommended that the project be continued. Consequently, effective with renewal of the contract on 1 November 1968, support was transferred to a regularly funded program (3A061102B71Q). The present work unit is therefore terminated.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 122, Antigenic and biologic classification of dengue viruses

Investigators.
Principal: W. G. Downs, M.D.
Associate: LTC Scott B. Halstead, MC

Publications.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6509	69 07 01	DD-R&E (AR) 636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP'S INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
69 01 31	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10a. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	123
b. [illegible]				
c. CONTRIBUTING				

11. TITLE (Include Security Classification Code): (U) Population Genetics of Hemoglobin E. Thalassemia and Related Genetic Polymorphisms in Thailand (TH) (23)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD
65 11	CONT	DA		B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 69 05 EXPIRATION: 70 04
b. NUMBER: DA-49-193-MD-2847
c. TYPE: S.CT
d. AMOUNT:
e. KIND OF AWARD: CON
f. CUM. AMT.: $109,367

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PREVIOUS: 69	1	3
FISCAL YEAR CURRENT: 70	1	3

19. CONTRACTOR/GRANTEE

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Medical School
University of Michigan
ADDRESS: Ann Arbor, Mich 48105

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. academic participant)
NAME: Rucknagel, D. L.
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Frick, COL L. P.
NAME: 1

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide Publication Code Word)

(U) Hemoglobin E; (U) Thalassemia; (U) Genetics; (U) Population

23. TECHNICAL OBJECTIVE; 24. APPROACH; 25. PROGRESS (Furnish individual paragraphs identified by number. Provide end of each with security classification code.)

23. (U) To perform detailed epidemiologic studies in Thailand of certain traits, including various hemoglobins and blood enzymes, as they may relate to susceptibility or resistance to selected epidemic diseases.

24. (U) Examination of specimens collected from family and tribal groups in Thailand. Serologic and biochemical studies to be performed mainly in the U.S.

25. (U) 69 01 - 69 06 Contract has been extended without additional funds so as to permit further analysis of a great amount of data. Results of the first analysis have been published. In brief, a unique rapidly migrating inherited variant of ceruloplasmin has been detected. The variant is as common as HGE and its frequency varies with HGE in the country. Thalassemia is most common in Northern Thailand. Significant differences in blood group gene frequencies are also evident in the analysis but they do not correlate well with the above system. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.

*Available to contractors upon originator's approval

DD FORM 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 123, Population genetics of Hemoglobin E, thalassemia and related genetic polymorphisms in Thailand.

Investigators.
Principal: Donald L. Rucknagel, M.D.

The purpose of this research is to perform detailed epidemiologic studies in Thailand on genetic blood polymorphism in order to obtain information on their relationship to susceptibility or resistance to epidemic diseases.

Blood specimens were collected from over 3000 persons in 10 provinces throughout Thailand. These have been treated with standard procedures according to the type of polymorphism under consideration. Since then various analyses have been performed.

In an analysis that has been completed recently the investigator reported detection of a unique, rapidly migrating inherited variant of ceruloplasmin which is described as the Thai variant. This variant is as common as Hemoglobin E and its frequency varies with HGE in Thailand. These, plus the A form of 6-phosphogluconate dehydrogenase, constitute a system of coordinated clines with a maximum frequency in Northwestern Thailand and progressively lower frequencies in North, Central and Southern regions. Thalassemia is most common in Northern Thailand. Significant differences in blood group gene frequencies are also evident in the analysis. However, they do not correlate well with the above systems. Rh gene frequencies are different in Southern Thailand from the remainder of the country, e.g., blood group O is more frequent in Northern Thailand than elsewhere, but in Surin Province it is lower than elsewhere in the Northeast. The frequency of M is also lowest in Surin Province.

The data thus far indicate strong migrational effects and ethnic differences. The question of selection must be deferred to a later analysis.

Further analyses are in process and will be reported as completed.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 123 Population genetics of Hemoglobin E, thalassemia and related genetic polymorphisms in Thailand

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION* DA 0A 6510	2. DATE OF SUMMARY* 69 07 01		REPORT CONTROL SYMBOL DD-R&E (AR) 636
3. DATE PREV SUM'RY 68 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY* U	6. WORK SECURITY* U	7. REGRADING* NA	8a. DISP'N INSTR'N NL	8b. SPECIFIC DATA-CONTRACTOR ACCESS ☒ YES ☐ NO	9. LEVEL OF SUM A. WORK UNIT
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	61101A	3A061101A91C		00	124		
b. CONTRIBUTING							
c. CONTRIBUTING							

11. TITLE (Procede with Security Classification Code)*

(U) Encoding of Planar Graphs for Chemical Structure Retrieval (CN)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

009700 Mathematics and Statistics

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 11	CONT	DA	A. GRANT

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 68 08 EXPIRATION: 69 09

b. NUMBER:* DA-MD-49-193-66-G9218

c. TYPE: G. GRANT d. AMOUNT: None

e. KIND OF AWARD: CON f. CUM. AMT. $15,710

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 69	1	1
	CURRENT 70	0.3	1

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Walter Reed Army Institute of Research

ADDRESS:* Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME:* University of Toronto

ADDRESS:* Ontario, Canada

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME:* Gottlieb, C. C.

TELEPHONE: 416-928-2986

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Jacobus, D. P.

NAME: 5

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Matrix Theory; (U) Algebra; (U) Mathematics

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) A method of encoding planar graphs which is minimal in length has previously been described by list coding as ideal from a computer point of view. It is not recognizable to a chemist. The technical objective of this work is to develop a mechanism for decoding this computer notation.

24 (U) The parenthesis code is to be translated back into coordinates so as to constitute a "picture". The technique involves the routing of the chemical graph in a plane starting from an edge rather than from the center or centers of the graph.

25 (U) 68 07 thru 69 06 The contract with the University of Toronto is concerned with the problem of isomorphism with the specific objective of attempting to stay within the parenthesis-bracket code for representation of chemical structures and yet so manipulate the cord as to obtain a tree permitting easy coding and decoding of the notation to conventional chemical projections. The theoretical study on the mathematical properties of chemical diagrams continues to be of profound importance for the computer programs involving the manipulations of chemical structures. Some of these techniques are to be incorporated in the program for the new WRAIR computer. During the past year, significant progress has been made on the problem of graph isomorphism and it is expected that this work will culminate in a Masters Thesis during the coming year. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report for 1 Jul 68 - 30 Jun 69.

*Available to contractors upon originator's approval.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 124, Encoding of Planar Graphs for Chemical Structure Retrieval

Investigators
Principal: Dr. C. C. Gottlieb
Dr. David P. Jacobus

Description

The purpose of this work is to determine the theoretical considerations in the computer encoding of chemical structures. This work is most important in order to be sure that we do not build a chemical information system which is mathematically unsound. There are several technical areas in which problems exist. One area involves the development of a relatively short notation from the representation of chemical structures. This means the development of a code which is close to the theoretical limits previously determined to be necessary for the unequivocal representation of structures. The code presently under development is the "parenthesis-bracket code".

The second problem is then to determine if one graph is isomorphic with another, i.e., in terms of chemistry, is one molecule the same as another.

The third problem is a piece of one graph embedded in a second graph. This is known as the problem of inclusion. Appreciable progress in the mathematical aspects of this problem continues to be made. The problem has been translated to the series of Ph.D. and Masters candidates who have produced studies on various portions of the work. The most recent contribution is work by Dr. Derek Corneil on the problem of graph isomorphism. This program which is mathematically sound may well be useful in determining chemical identity.

Progress

The contract with the University of Toronto is concerned with the problem of isomorphism with the specific objective of attempting to stay within the parenthesis-bracket code for representation of chemical structures, and yet to manipulate the code as to obtain a tree permitting easy coding and decoding of the notation to conventional chemical projections. The theoretical study on the mathematical properties of chemical diagrams continues to be of profound importance for the computer programs involving the manipulations of chemical structures. During the past year, significant progress has been made on the problem of graph isomorphism and it is expected that this work will culminate in a Masters Thesis during the coming year.

Summary and Conclusions

The development of sound procedures for the handling of chemical

structures on the new machine is of fundamental importance. During the past year significant progress has been made in developing an alogrithm which will be useful for the programming connected with chemical identify.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 124, Encoding of Planar Graphs for Chemical Structure Retrieval

Publications

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6528	69 07 01	DD-R&E (AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
68 10 31	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMAR	61101A	3A061101A91C	00	127
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE: (U) Machining the Tropical Diseases Bulletin for use on Search Services to WRAIR and USAMRDC Personnel

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 002600 Biology; 004200 Computers

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 08	CONT	DA	B. CONTRACT

17. CONTRACT/GRANT
a. DATE/EFFECTIVE: 68 07 EXPIRATION: 69 07
b. NUMBER: DA-48-193-MD-3039
c. TYPE: J. FFP d. AMOUNT: $32,627
e. KIND OF AWARD: EXT f. CUM. AMT.: $178,064

18. RESOURCES ESTIMATE	PROFESSIONAL MAN YRS	b. FUNDS ($ in thousands)
FISCAL YEAR 69	1	71
FISCAL YEAR 70	0.1	6

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012
RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3061

20. PERFORMING ORGANIZATION
NAME: Biological Abstracts
ADDRESS: Philadelphia, Pa. 19104
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. academic institution)
NAME: Parkins, P. V.
TELEPHONE: 215-L09-1100
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Jacobus, D. P.
NAME: 3

21. GENERAL USE
Foreign intelligence not considered

22. KEYWORDS (Provide KWIC index security classification code): (U) Medicine in Literature; (U) Abstracting and Indexing; (U) Information Processing; (U) Information Retrieval

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Provide individual paragraphs identified by number. Provide end of each with security classification code.)

23 (U) To provide, in a readily accessible form, all data contained in the Tropical Diseases Bulletin, Volumes 1 through 63.

24 (U) Several approaches are being made to determine how this data can be most economically obtained, i.e., from existing indexes, from keywords in test and title, etc.

25 (U) 68 07 01 to 69 06 30 A data base in searchable format was completed for volumes 45 - 63 of the Tropical Diseases Bulletin. The subject, title and author indices for these volumes were also generated. Pull text from volumes 1 - 44 of the Tropical Diseases Bulletin remains to be processed. The subject, title, and author indices from volumes 1 - 63 are now stored on Data Cells to allow computer assisted searching. This implementation has resulted in a much greater coverage for each search question. For technical reports, see Walter Reed Army Institute of Research Progress Report, 1 Jul 68 - 30 Jun 69.

*Available to contractors upon originator's approval.

DD FORM 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

TASK 00, In-House Laboratory Independent Research

Work Unit 127 Machining the Tropical Diseases Bulletin for use on search services to WRAIR and USAMRDC personnel

Investigators
Principal: Mrs. P. Parkins
Associate: Miss L. Schultz

Description

The purpose of this investigation is two-fold. The first is to put into digital form all the information contained in the Tropical Disease Bulletin, Volumes 1 through 63, so that digital searching and digital display of appropriate abstracts can be achieved. The second objective is to develop capabilities for the handling of full text on a file of limited but yet significant size.

Progress

A data base in searchable format was completed for volumes 45 through 63 of the Tropical Disease Bulletin. The subject, title, and author indices for these volumes were also generated. Full text from volumes 1 through 44 of the Tropical Disease Bulletin remains to be processed. The subject, title and author indices from volumes 1 through 63 are now stored in Data Cells to allow computer assisted searching. This implementation has resulted in a much greater coverage for each search question.

Summary and Conclusions

This work is proceeding well. The speed of input has been such that the building of the full text system rather than an abbreviated text system appears feasible as a result of the detailed instructions generated for the typists. Further work on input variations is expected to lead to additional improvements. The availbility of the Tropical Disease Bulletin in digital form is expected to improve WRAIR's coverage of both tropical diseases and geographic areas.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 127 Machining the Tropical Diseases Bulletin for use on search services to WRAIR and USAMRDC personnel

Publications

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6529	69 06 30	DD-R&E (AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
68 10 31	H. TERM.	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	128
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code): (U) Transmission, Control and Treatment of Infectious Diseases of Military Importance in Equatorial Asia (MF)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS:
010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 10	68 09	DA	A. GRANT

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: 67 10 EXPIRATION: 68 09
b. NUMBER: DADA17-C-68-G9253
c. TYPE: G. GRANT d. AMOUNT: 10,000
e. KIND OF AWARD: EXT f. CUM. AMT. 10,000

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 68	1	10
FISCAL YEAR CURRENT 69	0.3	3

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC 20012
RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W.H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION
NAME: Institute for Medical Research
ADDRESS: Kuala Lumpur, Malaysia
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Omar-Ahmad, U
TELEPHONE: NA
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Rapmund, COL G.P.
NAME:

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Infectious Diarrheas; (U) R-Factor; (U) Enteropathogens; (U) Aborigines

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To facilitate studies on certain diseases in Malaysia.

24 (U) Through conventional lab procedures, more particularly for isolation and characterization of enteric pathogens.

25 (U) 68 07 - 68 09 Serum and other examination material were collected from an additional 21 patients in the Aborigine Med Ctr. A possible etiologic agent was identified in 18 of these cases. Other data are being accumulated regarding the relative reliability of obtaining fecal specimens for bacteriological examination by different techniques. R-factor containing entero bacteriaceae have been obtained in a pre-antibiotic community and are undergoing genetic study. Significant segregation has not been observed in these strains. Segregation following transfer of R-factor to other bacteria is being studied. Work unit has been terminated for administrative reasons. The study will be supported from a regularly funded program (3A062110A811) and reported under DAOA 7413. For technical report see Walter Reed Army Institute of Research, Annual Progress Report, 1 Jul 63 - 30 Jun 69.

*Available to contractors upon originator's approval.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 128, Transmission, control, and treatment of infectious diseases of military importance in equatorial Asia

Investigators.
Principal: Ungku Omar-Ahmad, M.D.
Associate: COL Gary P. Rapmund, MC; MAJ C. E. Davis, MC

Description.

This work unit was established in order to facilitate studies of certain diseases being conducted as a part of a larger program. The main effort has been directed toward an investigation of diarrhea in aborigines in Malaysia, with special reference to the detection of antibiotic resistant enterobacteriaceae.

Progress and Results.

During the period of 1 July - 30 September 1968 to which this report applies, an additional 21 patients hospitalized in the Aborigine Medical Center were added to the study. All were given a complete physical examination and routine laboratory tests. Acute and convalescent sera were taken and stored at -20°C.

A possible etiologic agent was identified in 18 of the 21 patients (86%). In 13 cases the sole agent was Entamoeba histolytica, in 3 others only enteropathogenic bacteria were found, another had only Trichuris trichiura and the other E. histolytica and enteropathogenic bacteria. These findings correlate well with those from 50 other patients.

Genetic studies of R factor-containing (R+) enterobacteriaceae in a pre-antibiotic community in Sabah (north Borneo) are continuing. Significant segregation (loss of resistance determinants RD in serial subculture) has not been observed in R+ Esch. coli from Sabah. However, if the R factor is transferred to Salmonella typhimurium LT-2, rapid segregation occurs during serial subculture. In the case of one of these R factors which contains resistance determinants to ampicillin, tetracycline, sulfonamides, and streptomycin, the pattern of loss is different from that reported previously. Tetracycline resistance is segregated simultaneously with sulfonamide and streptomycin resistance. Ampicillin resistance, however, is stable throughout multiple subcultures.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 128, Transmission, control, and treatment of infectious diseases of military importance in equatorial Asia

Investigators.
Principal: Ungku Omar-Ahmad, M.D.
Associate: COL Gary P. Rapmund, MC; MAJ C. E. Davis, MC

Recommendations.

It is recommended that support to this project be continued. However, for reasons of administrative simplification, its support was transferred to a regularly funded program (3A062110A811). Future reports will be made by the appropriate project officer, Headquarters, U.S. Army Medical Research and Development Command, under DAOA 7431. This work unit is therefore terminated.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 128, Transmission, control, and treatment of infectious diseases of military importance in equatorial Asia

Investigators.
Principal: Ungku Omar-Ahmad, M.D.
Associate: COL Gary P. Rapmund, MC; MAJ C. E. Davis, MC

Publications.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6532	69 07 01	DD-R&E (AR) 636

3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DOWN GRADE	9. SPECIFIC DATA FOR CONTRACTOR ACCESS	10. LEVEL OF EFFORT
69 01 31	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. MAN UNIT

11. PERFORMING ORG	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	170
b. CONTRIBUTING				
c. CONTRIBUTING				

12. TITLE (Precede with Security Classification Code)

(U) Trace Metal Concentrations in Biological Matrices (09)

13. SCIENTIFIC AND TECHNOLOGICAL AREAS

008300 Inorganic Chemist 003500 Clinical Medicine 014000 Radio and Radiation

14. START DATE	15. ESTIMATED COMPLETION DATE	16. FUNDING AGENCY	17. PERFORMANCE METHOD
10 66	NA	DA	C. In-House

18. CONTRACT/GRANT: Not Applicable
a. DATE/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

19. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 69	3	50
FISCAL YEAR CURRENT 70	3	55

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W.H.
TELEPHONE: 202-576-3551

21. GENERAL USE
Foreign Intelligence Considered

22. PERFORMING ORGANIZATION
NAME: Walter Reed Army Inst of Res
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Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Army duty position)
NAME: Mahin, LTC, D. T.
TELEPHONE: 202-576-2211
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS
NAME: Bass, GS-14, B. G.
NAME: DA

23. KEYWORDS (Precede each with Security Classification Code)

(U) Trace Elements; (U) Homeostasis; (U) Metabolism

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To establish modern methods of qualitative and quantitative analysis for trace metals in health, disease and toxicology within the Army Medical Service.

24. (U) Utilizing atomic absorption spectrometry and neutron activation analysis, concentrations of trace elements will be defined in terms of sites of action and catalytic interaction with substrates. Emphasis will be placed on antagonistic or additive effects with related groups of elements. Initially, efforts will be limited to the following block of elements - manganese, copper, zinc, selenium, vanadium, cobalt and iodine.

25. (U) 69 01 - 69 06 Investigations in the role of trace metals in Wilson's Disease, viral hepatitis, cystic fibrosis and other pathological states have been conducted. The metals included copper, zinc, chromium, manganese and gold. Procedures have been developed for analysis of vitreous to determine metallic content of ocular fluid due to an intraocular foreign body. Support of the Opthamology Clinic of WRGH in these analyses continues. A procedure for analyzing trace quantities of arsenic in biological specimens has been established, and a collaborative program with AFIP personnel is under consideration to implement trace element procedures in the Armed Forces forensic pathology effort. Other investigations of trace metal metabolism and new procedure development are continuing. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.

Available to contractors upon additional approval

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task (X) In-House Laboratory Independent Research

Work Unit 170, Trace metal concentrations in biological matrices

Investigators.
Principal: LTC Dorsey T. Mahin, MC
Associate: Billy G. Bass, B.S.; Elvio A. Levri, M.S; MAJ Thomas E. Runyon, MC (Ophthalmology Svc, WRGH); Robert T. Lofberg, Ph.D.

Description.

The objective of this work unit is the quantitative determination of trace elements in defined biological systems. Comparative studies with other biochemical moieties and the development of state-of-the-art instrumentation and methodologies are integral parts of this work unit.

Progress.

1. Methodology.

Several techniques are employed in performing the substasks of this work unit. Neutron activation analysis and atomic absorption analysis are the principal techniques employed. In neutron activation analysis, samples are irradiated in the Walter Reed Research Reactor and the resulting gamma-ray activity is measured to obtain a quantitative and qualitative assay of the elements contained in the specimen. In atomic absorption analysis, the sample is aspirated into a flame and burned. The resulting characteristic photoemission in the flame is detected by an appropriate photosensitive device, and the elemental composition is quantitatively determined by the intensity of the characteristic color of the flame. These two methods have been highly complementary and provide the installation with a unique capability to perform routine screening of biological samples as well as precise measurements on micorgram quantities.

2. Biological studies.

a. Vitreous evaluation in globes containing intraocular foreign bodies.

Ocular response to the presence of a metallic intraocular foreign body (iofb) varies with the type of metal present within the eye. Some metals such as gold, silver, platinum, and tantalum are relatively inert. If the intraocular location of this type of metal were such that attempted removal constitutes a grave risk to the eye, a clinician might decide to

leave it in place and follow the patient, the premise being that leaving the object within the globe offered less risk than surgical intervention. On the other hand, metals such as copper, iron, zinc, aluminum, and mercury cause severe reactions and present a hazard to retention of vision, or to the eye itself, and attempt at removal is almost always indicated. In the past there was no practical method of determining the type of metal within a globe containing a metallic iofb except for history, which is often inadequate, and the magnet test. The magnet test is ineffective when the iofb is so encapsulated in vitreous fibrosis that deflection becomes imperceptable. Neutron activation analysis has proved to be an effective, safe way to analyze vitreous for metallic content. Metal concentrations in the vitreous reflect the nature of the metallic iofb.

Pilot experiments were performed on adult albino rabbits. Sterile copper, pure iron, brass and steel implants were made in the eyes of the rabbits and the vitreous copper and iron concentrations were measured in the treated animals and in control animals with similar surgery but no implants. Some results are given in Tables 1, 2 and 3.

TABLE 1

VITREOUS COPPER CONCENTRATIONS OF GLOBE CONTAINING COPPER IOFB (PPM)

AS A FUNCTION OF TIME POST IMPLANTATION

2 Days	1 Week	4 Weeks	Baseline (Control)
2.60 - 6.36	2.50 - 11.48	2.80 - 17.61	0.04 - 1.20

Copper values in globes containing iron iofb: 0.2 - 1.66 ppm
The range of values observed in several animals is shown.

TABLE 2

VITREOUS IRON CONCENTRATIONS OF GLOBE CONTAINING IRON IOFB (PPM)

AS A FUNCTION OF TIME POST IMPLANTATION

2 Days	1 Week	4 Weeks	Baseline (Control)
3.72 - 6.28	3.94 - 20.64	3.84 - 10.88	0.22 - 1.48

Iron values in globes containing copper iofb: 0.2 - 5.50 ppm
The range of values observed in several animals is shown.

TABLE 3

VITREOUS ANALYSIS OF GLOBES CONTAINING ALLOY IMPLANTS

	Copper (ppm)	Iron (ppm)
Brass	6.60	0.11
	7.16	0.78
Steel	0.37	10.48
	1.80	13.88

Values given are actual measurements from two samples of each animal.

Analysis was made of vitreous from two patients who were combat casualties with iofb's. The results of the analyses are shown in Table 4.

TABLE 4

VITREOUS ANALYSIS OF TWO PATIENTS WITH IOFB'S

	Copper Concentration (ppm)	Iron Concentration (ppm)
Patient A	0.66	10.26
Patient B	0.60	6.00
Human Baseline	0.28	0.53

Patient A IOFB Analysis: 0.15% Cu; 98% Iron
Patient B IOFB Analysis: Trace Cu; 99.9% Iron

The tests demonstrated the applicability of trace metal analysis to military clinical opthalmology.

b. Analysis of serum and urine of patients with serum hepatitis.

The analysis of zinc concentrations in serum and urine has been shown to be useful in the diagnosis of serum hepatitis. Work at this laboratory has shown that zinc concentrations in serum of patients with serum hepatitis ranges from 50-64 micrograms/100 ml. Zinc concentrations

in urine from these patients range from 700-2000 micrograms/day. Control values for normal individuals range from 85-130 micrograms/100 ml in serum and 400-600 micrograms/day in urine.

c. Study of trace metal concentrations in patients with Wilson's disease.

A study has been made of the role of copper in the diagnosis of Wilson's disease. The results of the study showed that these patients manifest high liver copper concentrations and low serum copper concentrations. Liver copper concentrations in these patients ranged from 100 micrograms/gram dry weight to 1500 micrograms/gram dry weight. Liver copper concentrations in normal individuals range from 10-30 micrograms/gram. The serum copper of patients with Wilson's disease ranges from 30-60 micrograms/100 ml. Normal values of serum copper established at this laboratory are 75-140 micrograms/100 ml.

d. Arsenic concentrations in biological matrices.

For the past several years forensic medical pathologists have used neutron activation analysis to determine trace metal concentrations. This laboratory has established procedures for assaying trace quantities of arsenic in biological specimens. This technique is useful to the forensic pathologists because of its simplicity and the small quantity of sample required for analysis. The latter requirement is highly important in the forensic science field. In cooperation with personnel at the Armed Forces Institute of Pathology, specimens from an individual who died from arsenic poisoning were analyzed by this laboratory. The results showed elevated arsenic concentrations in brain, liver, kidney, fingernails, and hair samples. Further studies in collaboration with the Armed Forces Institute of Pathology are planned.

e. Cystic fibrosis screening by activation analysis.

The analysis of fingernail clippings for sodium content has been suggested as a diagnostic test for cystic fibrosis. Experience at this laboratory does not confirm its value. Quantitative determination by neutron activation analysis of sodium in fingernails of patients with cystic fibrosis show normal values after simple washing with glass distilled, deionized water. Normal values of sodium concentration in fingernails determined in this laboratory fall within the following ranges:

17-33 meq/kilogram (cleaned)

19-116 meq/kilogram (uncleaned)

The ranges of sodium concentration in fingernail clippings from patients with cystic fibrosis were:

10-30 meq/kilogram (cleaned)

19-353 meq/kilogram (uncleaned)

These results suggest that the activation analysis method is merely a sophisticated and expensive sweat test. More analyses are being made as samples become available to substantiate these findings.

3. New methodology.

An activation analysis procedure has been developed to determine rapidly with good precision manganese and copper serum values in one milliliter or less of serum. The sample is irradiated in a neutron flux of 10^{12} cm^{-2} sec^{-1} for two hours following nitric acid digestion. The activity plus carrier is absorbed from saturated lithium chloride solution on Dowex 1-8 contained in a syringe. After washing the resin with more lithium chloride, the syringe is counted in a 5-inch diameter by 5-inch thick sodium iodide well crystal which is connected to a 400-channel pulse height analyzer. The carrier is washed off the syringe by dilute acid and analyzed. The sample concentration is determined by comparison to irradiated standards, correcting the unknown for decay and chemical yield. This procedure allows a substantial reduction in analysis time for these elements.

4. New instrumentation.

A high resolution gamma-ray spectrometry system has been designed and installed for use in activation analysis. This system consists of a 20 cc Li-Drifted Germanium Detector connected to a low noise amplifier system (Tennelec TC 135-200 combination). The data are stored in a 400-channel pulse height analyzer. This spectrometer system has been useful in arsenic analysis. Other types of analyses appear promising and will be investigated. The principal advantage of the system is the reduction of post-irradiation chemical separation required for the analysis. This reduction in analysis time provides a greater operational efficiency within the laboratory.

Summary.

A method has been developed for identifying the metallic nature of intraocular foreign bodies (iofb). Analysis of vitreous can assist in determining the potential hazard of the iofb, and, in conjunction with other diagnostic techniques, may substantiate the necessity for surgical removal of the iofb. Trace metal concentrations of iron and copper in vitreous for normal and iofb containing globes have been established at this laboratory.

The trace metal analysis of serum and urine in patients with infectious hepatitis has continued. The ranges of zinc and iron concentrations in normal and infected individuals have been established.

Trace metal analysis studies have demonstrated a consistent pattern of elevated copper concentration in the livers of patients with Wilson's disease. Serum copper concentrations are depressed in these patients. The ranges of serum and liver copper concentrations have been determined for normal and diseased individuals.

A method has been developed for analyzing trace quantitites of arsenic in biological specimens. The range of arsenic concentration in normal specimens has been determined. Specimens of brain, liver, kidney, bile, fingernails and hair taken from a patient who died from arsenic poisoning have been analyzed. Elevated arsenic concentrations were found. A collaborative program is planned with AFIP personnel to utilize neutron activation analysis of trace metals in forensic pathology.

A high resolution Lithium-Drifted Germanium gamma-ray spectrometry system has been installed and has been effective in simplifying trace metal analysis.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 170, Trace metal concentrations in biological matrices

Publications.

1. Levri, E. A., Angel, C. R., Mahin, D. T. and Boyce, W. "Are Sera and Urinary Levels of Certain Trace Elements a Meaningful Measure of Trace Element Balance." Proc. Univ of Missouri, 2nd Ann. Conf. on Trace Substances in Environmental Health, pp. 207-221, 1968.

2. Battistone, G. R., Levri, E. A. and Lofberg, R. T. "A Combined Nuclear, Electrochemical and Atomic Absorption Technique for Determining Zn, Cu and Mn." IADR Abstract No. 237, 1968.

3. Donati, R. M., McLaughlin, M. M., Levri, E. A., Berman, A. R. and Stromberg, L.W. R. "The Response of Iron Metabolism to the Microbial Flora: Studies on Germfree Mice." Proc. Soc. Exp. Biol. Med. 130 (2), 920-922 (1969).

4. Runyon, T. E. and Levri, E. A. "Vitreous Analysis in Eyes Containing Copper and Iron Intraocular Foreign Bodies." Amer. J. of Ophthalmol. In press (1969).

5. Lofberg, R. T. and Angel, C. R. "A Simplified Neutron Activation Analysis Method for Copper and Manganese." Anal. Letters 2 (5), 239-245 (1969).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	FOR OFFICIAL USE ONLY (Mark)
DA 0A6538	69 06 30	

3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SECURITY CLASSIFICATION		6. TYPE OF PLAN	7. UNDER REVIEW BY	8. APPROVED FOR PUBLIC RELEASE	9. LEVEL OF SUMMARY
69 04 30	K. Completion	U	U	NA	NL	[X] Yes [ ] No	

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	171
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)* (U) The Use of Aotus trivirgatus as a Tool for Studies on the Therapy of Infections with Plasmodium falciparum (05)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 07	CONT	DA	B. Contract

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: 68 07 EXPIRATION: 69 06
b. NUMBER:* DADA17-67-C-7176
c. TYPE: S.CT d. AMOUNT:* $178,402
e. KIND OF AWARD: EXT. f. CUM. AMT. $358,772

18. RESOURCES ESTIMATE	PROFESSIONAL MAN YRS	FUNDS (In Thousands)
FISCAL YEAR 68 PRECEDING	2	180
FISCAL YEAR 69 CURRENT	1	178

19. RESPONSIBLE DOD ORGANIZATION
NAME:* Walter Reed Army Institute of Research
ADDRESS:* Washington, DC 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION
NAME:* University of California
ADDRESS:* Davis, Calif. 95616

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Schmidt, L. H.
TELEPHONE: 916-752-0420
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Frick, COL L. P.
NAME: 1

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Malaria; (U) Primate; (U) Aotus trivirgatus; (U) Plasmodium; (U) Antimalarials

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To establish Aotus trivirgatus (night monkey) as a laboratory primate and to characterize infections with P. falciparum in this host.

24. (U) Establish requirements for maintaining healthy animals, infect by sporozoites or blood injection, develop methods of assessment of antimalarials.

25. (U) 69 01 - 69 05 Eight experiments with 5 strains of P. falciparum and involving 354 Aotus were performed. A comparison of the response of Camp strain and the CH/Q variant to quinine shows that difference in the two in man also carries over to the monkey. The Cambodian I, Vietnam, Monterey, and Malayan IV strains have been characterized for their response to antimalarials. The Vietnam Oak Knoll P. falciparum is being adapted to the intact Aotus. Contract was terminated 30 Apr 69 due to relocation of the investigator. Work will be continued under a new contract to be supported from 3A663713D829 funds and reported under Agency Accession No. DA 0E7590. Property purchased under old contract will be transferred to new contract. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.

*Available to contractors only on "not to exceed" request.

DD, FORM 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 171, The use of Aotus trivirgatus as a tool for studies on the therapy of infections with Plasmodium falciparum

Investigators.
Principal: Leon H. Schmidt, Ph.D.

The purpose of this work unit is to develop the A. trivirgatus-P. falciparum infection model into a usable system for the evaluation of antimalarial activity of chemical compounds and for various biologic studies on human plasmodia. To this end work was continued on standardization and characterization of P. falciparum infections in the Aotus. Concurrently, observations on husbandry of the monkey also continued.

To date, establishment of the Aotus in the laboratory has presented no serious problems. The monkeys do well in groups of 5 or 6 in wire mesh cages 32 X 32 X 32 inches in dimensions. They accept commercial monkey chow, lightly soaked in reconstituted milk, and green peanuts as their basic diet. Rigid temperature control, however, is required; the optimum for the genus is 82 to 86°F. Temperatures below 76°F usually lead to serious respiratory infections. The monkey will accept temperatures over 86°F if the relative humidity is kept above 60%.

The single most serious health problem encountered to date has been the susceptibility of the Aotus to Herpes simplex. Two outbreaks--both among stock monkeys--accounted for the loss of 45 of 50 in one shipment and 182 out of 340 making up three other shipments. All indications point to aerogenic dissemination of the virus. Measures for protection of the Aotus against Herpes will be required if the animal is to have full utilization in the laboratory.

Respiratory diseases of bacterial origin can be a serious problem but if diagnosed promptly yield to specific therapy. In order of prevalence, these agents are Pasteurella multocida, Klebsiella pneumoniae (type 2), Bordatella bronchisepta, and Diplococcus pneumoniae.

Enteric infections with Shigella and Salmonella have been sporadic in occurrence. In one outbreak 66 deaths occurred from Shigella sonnei before being brought under control by chloramphenicol. Actual infections with Salmonella have been few, however, up to two-thirds of some shipments have yielded Salmonella B/i isolates. But symptoms appear in very few animals, and the infection clears spontaneously.

Very few helminth and protozoan infections have been observed. About 3% of the Aotus exhibit a microfilaremia but without symptoms.

Trypanosomes resembling T. cruzi were seen in 2 of 2300 animals.

Substantial progress has been enjoyed in the standardization of plasmodial infections in the Aotus and in the characterization of the infections in terms of pathologic features and susceptibility to antimalarials.

Up to the end of March 1969 attempts have been made to establish 13 different strains of P. falciparum and one of P. vivax. Five of the P. falciparum strains (Malayan Camp, Uganda Palo Alto, Vietnam Monterey, Vietnam Oak Knoll, and Malayan IV) had been passaged successfully in Aotus before receipt in the laboratory, as had the single P. vivax strain (Vietnam Palo Alto). Of the remaining 8 P. falciparum strains, passages were effected with the Chicago quinine tolerant variant of Malayan Camp (Malayan Camp CH/Q) and Cambodian I. Efforts to establish McLendon, Uganda I, Malayan Camp KC, Thailand Manoon, Malayan Pooley, and Malayan Taylor have not been successful to date. Further attempts will be made to establish the McLendon and Uganda strains since they are reported to be wholly drug sensitive. The former is especially interesting because it was isolated before World War II and the advent of modern antimalarials.

Studies have been made on the course of infection with each of the 8 successfully passaged strains. The route of inoculation of the parasite is a relatively unimportant determinant of the course of infection once parasitemia is established. Whether given IV or IP, from 10^3 to 10^4 parasites must be inoculated to produce infection. However, parasitemia occurs 2 to 4 days earlier when the inoculum is given IV.

The course of infection evoked by a standard inoculum (e.g. 5×10^6 parasites) varies markedly according to strain. The Malayan Camp, its CH/Q variant, and the Uganda Palo Alto strains of P. falciparum all produce fulminating infections which usually progress to death. In fatal cases 70 to 90% of red cells usually are parasitized, and actual counts of parasites range from 12,000 to 14,000 per 10^4 red cells. With these strains there are no cyclic undulations of the parasitemia, and all stages of asexual parasites occur in the peripheral blood. An occasional Aotus develops a low grade infection which becomes chronic. The reason for this has not been determined.

Infections produced by the Cambodian I and Vietnam Oak Knoll strains proceed on a benign course. Both strains cause low grade chronic infections with parasitemias rarely exceeding 10% and are usually much lower. Moreover, infections with these strains are characterized by cyclicity of parasitemia corresponding to the absence of larger parasites, especially segmenters, from the peripheral blood.

The course of infection with either the Malayan IV or Vietnam Monterey strains is intermediate between the extremes described above.

Parasitemias peak at between 15 and 30% and such infections are occasionally fatal. Sequestration of the larger parasites occurs but is not so marked as in infections with the Cambodian I and Vietnam Oak Knoll strains.

The response of infections with Malayan Camp, Malayan Camp CH/Q, and Uganda Palo Alto strains to chemotherapy has been defined precisely. The Malayan Camp and Uganda Palo Alto strains respond in an almost identical manner to treatment with chloroquine, pyrimethamine, or quinine. Maximum tolerated doses of pyrimethamine have no effect. Well tolerated doses of chloroquine and quinine are curative. Thus these strains are classified as pyrimethamine resistant and chloroquine and quinine sensitive. Infections with the Malayan Camp CH/Q variant are also pyrimethamine resistant and chloroquine sensitive. However, they are partially suppressed but not regularly cured by maximum tolerated doses of quinine.

The Malayan IV and Vietnam Monterey strains are fully resistant to pyrimethamine, are more resistant to quinine than the Malayan Camp CH/Q, and have a considerable resistance to chloroquine. The Vietnam Monterey strain is more resistant to chloroquine than is the Malayan IV, but maximum tolerated doses did not eradicate infection with either strain.

Evaluation of the chemotherapeutic characteristics of the Vietnam Oak Knoll P. falciparum and Vietnam Palo Alto P. vivax is still underway.

The results of these studies have shown that infections with P. falciparum can be established in the Aotus and that these infections can be used to assess differences in strain susceptibility to chemotherapeutic agents. Moreover, there are now available model infections with different orders of susceptibility to drugs such as chloroquine, quinine, and pyrimethamine. Although the spectrum of drug susceptibility is not yet complete, in that a totally susceptible strain is lacking, it should be possible to proceed with the search for new drugs with the use of strains already available.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 171 The use of Aotus trivirgatus as a tool for studies on the therapy of infections with Plasmodium falciparum

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6539	69 07 01	DD-R&E (AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. RE-GRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
68 10 31	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	172
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Migratory Animal Pathological Survey (JA)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 07	CONT	DA	C. IN-HOUSE

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	3	60
FISCAL YEAR CURRENT 70	3	65

19. RESPONSIBLE DOD ORGANIZATION

NAME:
Walter Reed Army Institute of Research
ADDRESS:
Washington, DC 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME:
USA Rsch and Dev Grp (Far East)
ADDRESS:
APO San Francisco 96343

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)
NAME: McClure, H. E.
TELEPHONE: NA
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Ornithology; (U) Migration; (U) Vectors

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Particular interest is in the role of migrating animals in the transport of disease.

24 (U) Major effect will be on bird banding and recovery in various areas of SEA. Ectoparasites will be collected, blood and tissues will be examined. Area supervision will be from Bangkok.

25 (U) 68 10 - 69 06 Current report of activities is not yet available from FERO.

*Available to contractors upon originator's approval.

DD, FORM 1 MAR 68 1498-1 (FOR ARMY USE)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6430	69 06 30	DD-R&E (AR) 636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 01 31	H. TERM.	U	U	NA	NL	☑ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	173
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with security classification code)

(U) Thermal Properties of Liquid Water (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

016700 Thermodynamics

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 06	CONT	DA	C. IN-HOUSE

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

	18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUND. (In thousands)
FISCAL YEAR PRECEDING	68	1	12
FISCAL YEAR CURRENT	69	0.5	6

19. RESPONSIBLE DOD ORGANIZATION

NAME:
Walter Reed Army Institute of Research

ADDRESS:
Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W.H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME:
Walter Reed Army Institute of Research

ADDRESS:
HQ, WRAIR
Washington, DC 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)

NAME: Bach, Sven A

TELEPHONE: 202-OX4-3346

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

DA

22. KEYWORDS (Precede each with security classification code)

(U) Water; (U) Water Structure; (U) Water Anomalies; (U) Differential Calorimetry; (U) Vapor - Pressure Osmometry

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security classification code.)

23 (U) To elucidate the temperature dependence of the physical properties of liquid water and their relationship to living systems.

24 (U) Research will proceed from the hypothesis that water structure changes discretely into specific structural forms at various temperatures and that the structure dominating the various ranges constrain living processes because of long association during evolution of the macromolecules and membranes with structured water.

25 (U) 68 12 - 69 02. Work unit terminated at latter date due to reassignment of investigator. Latest work was directed to the properties of water films which were laid down in the calorimeter by warming and then cooling a piece of water-moistened filter paper. The exchange of water between the calorimeter wall and the sample cup produced easily measurable signals. The maximum delta T was a function of preparation temperature but the plotted data points appear to show discontinuities at 15, 30 and 46 degrees C. When the calorimeter wall was coated with stearic acid, this effect disappeared. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 173, Thermal properties of liquid water

Investigators.
Principal: COL Sven A. Bach, MC

Description.

The objective of this research is to elucidate the temperature dependence of the physical properties of liquid water and their relationship to living systems. The research proceeds from the hypothesis that water structure changes discretely into specific structural forms at various temperatures and that the structures dominating the various ranges constrain living processes because of the long association during evolution of the macromolecules and membranes with structured water.

Progress and Results.

During the six months preceding termination of this project and which is the subject of this report, the principal effort was directed to the properties of water films which were laid down in the calorimeter by warming and then cooling a piece of water-moistened filter paper. The exchange of water between the calorimeter wall and the sample cup produced easily measurable signals. The maximum delta T proved to be a function of preparation temperature. Preparation temperatures were at 1° increments between 8° and 50°C with 10 to 20 runs per point. However, the delta T's were not a smooth function of preparation temperature since discontinuities were shown at 15°, 30° and 46°C. When the calorimeter wall was coated with stearic acid, this effect disappeared.

Recommendations.

None. Due to reassignment of the principal investigator, active research has ceased and a final report is in preparation. This work unit is therefore considered to be terminated effective 2 February 1969.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 173, Thermal properties of liquid water

Publications.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6431	69 07 01	DD-R&E (AR) 636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP/SCHED	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
68 10 31	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	174
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with security classification code)

(U) Applications of Electroanalytical Techniques to Biochemistry and Clinical Chemistry(21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry

13. STUDY BEGIN	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 06	CONT	DA	B. CONTRACT

17. CONTRACT/GRANT

a. DATES/EFFECTIVE 69 06 EXPIRATION 70 05
b. NUMBER DADA 17-67-C-7161
c. TYPE S.CT
d. AMOUNT 28,706
e. KIND OF AWARD EXT
f. CUM. AMT. 81,932

18. RESOURCES ESTIMATE

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS ($ Thousands)
69	1	28
70	1	25

19. PERFORMING ORGANIZATION

NAME: University of Maryland
ADDRESS: College Park, Md. 20740

PRINCIPAL INVESTIGATOR (Provide name if D.A. contract performing)
NAME: Purdy, W.C.
TELEPHONE: 301-454-2619
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Knoblock, COL E.C.
NAME: 1

20. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W.H.
TELEPHONE: 202-576-3551

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide keywords and security classification code)

(U) Clinical Chemistry; (U) Analytical Chemistry; (U) Electrochemical; (U) Biochemistry

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Provide end of each with security classification code.)

23. (U) To extend the precision of electroanalytical chemistry techniques to clinical chemistry analyses and to investigative biochemistry, to provide primary reference methods for evaluation of clinical and investigative chemistry methology.

24. (U) Electroanalytical procedures, including coulometry, polarography, chronopotentiometry and associated techniques, will be applied to analyses of biochemical compounds. All details of the reactions will be studied in order to develop highly precise and specific analyses which will be available as primary reference methods against which other methods and procedures can be compared.

25. (U) 68 07 - 69 06 Atomic absorption spectroscopy, coulometry, polarography and chronopotentiometry have been applied procedures of clinical chemistry. Coulometric analyses have been developed for the phosphatases, for serum cholesterol, salicylate and phenobarbital derivatives with good precision. A coulometric procedure for uric acid has been developed which exceeds the precision of spectrophotometric methods. Polarographic study of tryptophane and phenylalanine in non-aqueous media has been accomplished. A very sensitive method for vanadium in biological samples using atomic absorption has allowed measurement of parts per billion of this trace metal. Additional work with these methods promises to produce highly accurate reference methods for clinical analysis processes.

For technical reports, see Walter Reed Army Institute of Research, Annual Progress Report, 1 Jul 68 - 30 Jun 69.

26. Available to contractors upon originator's approval.

DD FORM 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 174, Applications of electroanalytical techniques to biochemistry and clinical chemistry

Investigators.
Principal: William C. Purdy, Ph.D.
Associate: Marvin Brooks
A. James Engel
Caroline Huheey
Charles Manning
Joseph Monforte
John Moody
Henry Nipper
Robert Troy

Description.

The object of this program has been to extend the precision of electroanalytical chemistry techniques to the analyses of clinical chemistry with the expectation that the precision and specificity of some analyses could be improved over the conventional spectrophotometric analyses. The electroanalytical procedures, including coulometry, polarography, chronopotentiometry and associated techniques have been applied to assay of biochemical compounds. Details of reactions have been studied in order to develop highly precise methods which will be available as primary reference methods against which other methods and procedures can be compared.

Progress.

Four general techniques have been employed during this study, atomic absorption, coulometry, polarography, and chronopotentiometry. These techniques have been applied to several different systems and these systems will be discussed in turn.

Atomic Absorption. A satisfactory calibration curve for trace quantities of vanadium has been obtained by atomic absorption using the nitrous oxide-acetylene flame. The method is that of Crump-Wiesner. Aqueous solutions containing 0.0, 0.020, 0.040, 0.060, and 0.080 ppm vanadium were adjusted to pH 1.0, and the vanadium extracted with 5% cupferron into methyl isobutyl ketone. The organic layer was aspirated and the vanadium absorption determined using a Perkin-Elmer Model 303 with a Perkin-Elmer Intensitron vanadium lamp and a nitrous oxide-acetylene flame. Methyl isobutyl ketone was used to set zero absorption, and 10X scale expansion was employed. The experimental work consisted mainly of adjusting the instrument for maximum sensitivity so as to duplicate the work reported by

Crump-Wiesner. Results show that about 4 ppb V was present as contamination from the deionized water or from other sources. Although the blank had a small absorption, the 5% cupferron in methyl isobutyl ketone (MIBK) had no absorption when the zero point was set with MIBK.

An attempt was made to determine vanadium in urine. The dry ashing method for the determination of vanadium in urine was adapted for atomic absorption. The results indicate the presence of up to 0.04 ppm vanadium in the urine sample, a value which is suspiciously high. It was found by Heydorn and Lukens that dry ashing in porcelain can introduce significant amounts of vanadium into the sample. Since this may have been the case here, and because the dry ashing method was found to be extremely slow, a wet ashing method will be substituted in subsequent investigations.

Coulometry. The determination of acid and alkaline phosphatases: Determination of phosphate in serum has many inherent problems in that phosphate from other sources is present in high concentrations and interferes with the quantitative determination of the enzyme-released phosphate. As a result, most work has been done determining the substituted phenol formed.

Most clinical methods consist of hydrolysis of the ester and measuring the absorbence due to formation of the free substituted phenol. The absorbence is measured using a visible, ultraviolet, or fluorescent spectrometer. The literature cites no electrometric method applied to the determination of the phosphatases.

The method of electrical determination chosen for phosphatases was constant current coulometry. Since most of the work in the literature has been done with sodium beta-glycero-phosphate, phenyl phosphate, and p-nitrophenyl phosphate, it was decided that one of these methods should be adapted to coulometry. For these substrates there is a large body of information on activators, inhibitors, pH- and temperature conditions. Other substrates were discounted because they were simply chosen for their absorptive properties and not their redox possibilities.

Based on the work of Van Zyl and Murray, Lichtenstein, and Cuta and Kucera, the coulometric bromination of the phenol following enzymatic hydrolysis looked very promising. Significant problems were encountered using phenol in that standard solutions were difficult to prepare. As a result, a change was made to the p-nitrophenol system.

No success was encountered with direct titration by bromine since the reaction did not proceed as rapidly as the addition of bromine. A back titration of potassium thiocyanate with bromine was used after adding a two-fold excess of bromine and allowing a reaction of ten minutes. Sufficient KSCN was added in excess of the bromine. The excess of KSCN was then titrated. At the present time reproducible results are being obtained, but the stoichiometry is questionable. It appears that more work is needed to determine if changes in bromination rate, amount of excess bromine added, or pH will give more meaningful results.

Once a procedure to determine p-nitrophenol is established, another problem of considerable importance will be studied. Whatever method of determination is chosen, i.e., bromination or reduction, the reaction will occur equally well with the unreacted p-nitrophenyl phosphate. Thus, a quantitative separation of the unreacted material must be found. Except for these modifications, the coulometric method should parallel the normal method for the determination of the phosphatases.

The determination of cholesterol: The clinical analysis of cholesterol is usually based on the measurement of unstable colors formed with either the Lieberman-Burchard reaction or the $FeCl_3$-H_2SO_4 reaction, and no effort is made to isolate cholesterol from its analogs in serum. These analogs frequently react with color reagents and have different absorptivities than cholesterol.

The lack of selectivity suggests that an effective separation of cholesterol from its analogs should be initiated to improve the accuracy of the reference methods. Koeller and Hill have indicated that 7-dehydrocholesterol is the major interference in normal serum. Others have suggested that this analog and many other analogs of cholesterol can be separated from each other by means of thin-layer chromatography on silica gel plates which have been impregnated with silver nitrate. Studies with this technique in which a Mallinckrodt ChromAR sheet was impregnated with silver nitrate showed that 7-dehydrocholesterol could be effectively separated from cholesterol. These plates have the advantage of easy elution of the separated materials due to their method of construction. Separated materials may be cut out of the chromatogram for the elution of the sample as in paper chromatography. After cholesterol has been isolated from the thin layer chromatogram, almost any sensitive method of analysis may be employed for its determination.

The application of coulometric titrations was studied in a variety of solvents in which bromine was generated at a platinum anode. With such a system, materials like cyclohexene could be titrated directly with 99.5% titration efficiency. Cholesterol titrated too slowly as the end point was approached, so a direct titration proved to be inadequate with this solvent system. Lathosterol and 7-dehydrocholesterol also titrated too slowly to be analyzed directly in this system. A macro-cell residual titration (50 ml) of cholesterol was examined at 200 and 400 µg levels, where the excess bromine was consumed by a standard solution of arsenite. Results indicate that a precision and accuracy within 1% are possible with standard solutions of cholesterol. Micro-cell titrations (5 ml) indicated that cyclohexene can be titrated with 100% titration efficiency. This suggests that cholesterol may be titrated at the 20-µg level in the micro cell with the same accuracy obtained in the macro-cell titration.

The determination of creatinine: Creatinine represents one of the ten most run clinical tests. Normal values vary widely according to diet, but range from 1.0 to 1.8 grams excreted per 24 hours.

The most common detection system for creatinine is that of Folin using the Jaffe reaction. The reaction is between an alkaline solution of picric acid and creatinine. A similar reaction using 3,5-dinitrobenzoate shows no advantage. The drawback to the Jaffe reaction is its non-specificity. It has been established that 90% of the Jaffe positive material in urine is creatinine and about 70% in blood.

To increase specificity, Lloyd's reagent is sometimes used as a specific adsorbing agent and the creatinine is then desorbed with alkaline picrate solution. Ion exchange chromatography has been used successfully also. Other methods to improve specificity include prior oxidation with ceric sulfate or with iodine.

Another detection method in use is that of Van Pilsum. Creatinine is oxidized in alkaline solution with o-nitrobenzaldehyde. The final product of the reaction is methyl guanidine. The methyl guanidine is detected colorimetrically after use of the Sakaguchi reaction. The method is reported to be fairly specific for creatinine.

Attempts to reduce creatinine at the dropping mercury electrode (DME) failed. Both rotating platinum and wax-impregnated graphite electrodes were used in an attempt to oxidize creatinine and creatine gave negative results.

Various oxidizing agents were tried using the Jaffe reaction as criteria. Positive results were obtained with O-nitrobenzaldehyde and $Hg(OAc)_2.ClO^-$, while BrO^- interfered with the Jaffe method, making impossible interpretation of the results. Nitrite ion in base and ceric ion did not react.

Coulometrically generated silver(II)-ion was tried and found to react slowly with creatine but not at all with creatinine. Hypobromite ion was generated and found not to react. Since mercury(II)-ion reacted, mercury(I)-ion was generated and tested; it did not react. At present mercury(II)-ion is being tested by using cyanide ion to obtain a potentiometric end point. Amperometric end points fail to detect mercuric ion because of the formation of mercuric oxide.

It seems that the reaction of creatinine with oxidizing agents is more than just an electron transfer but must involve a coupling of some kind in the transition state. Thus far only o-nitrobenzaldehyde and mercuric ion have been shown to react. Both these systems are being explored further with mercuric ion apparently having the best chance of success.

The determination of sodium phenobarbital: The final set of standard conditions for the coulometric titration of sodium phenobarbital with electrogenerated mercury(II) has been established. The titration is performed in a total volume of 2.5 ml. One ml of 0.67 M $NaClO_4$ and 0.5 ml of acetone are added to the cell in addition to 1 ml of a standard sodium phenobarbital solution. The final concentrations are 0.3 M $NaClO_4$ and 20% acetone

by volume. The cell is thermostatted at 37°C. A representative set of data is given in Table II. The numbers in parentheses are the number of duplicate trials made at each concentration. It is concluded that approximately 75 to 250 micrograms of sodium phenobarbital can be titrated with an error of about 1 to 7%.

Since the reaction is a precipitation, an effort to reduce the solubility of the precipitate by lowering the temperature of the reaction was made. It was thought that this would increase the sharpness of the titration curve. The temperature was lowered to 4°C, but the titration curves obtained were very poor. Only a gradual increase in current was observed, making the titration curves totally unusable. Apparently the reaction rate was affected to a larger extent than was the solubility of the mercury phenobarbital precipitate. When the gradual increase in current was observed, it was at a time that was much longer than the theoretical time.

There is good evidence that Hg(II) is not generated directly under the conditions described above. At high generation currents, a gray to black precipitate forms in the titration cell; upon close inspection, finely-divided elemental mercury can be seen mixed with the precipitate. The interpretation of this observation is that Hg(I) is initially generated, and this disproportionates to form Hg(II) and elemental mercury. The Hg(II) then reacts with the barbiturate. Since the Hg(II)-barbiturate complex is very stable, the disproportionation equilibrium is in favor of the HG(II). This same observation has been made in the coulometric titration of thiourea with electrogenerated Hg(II) (42).

Studies have also been made to apply the above coulometric titration of sodium phenobarbital to the analysis in blood serum. The barbiturate was extracted from the serum using 50 ml of chloroform. The barbiturate was then extracted from the chloroform with 5.0 ml of 0.45 N NaOH. One ml of the NaOH extract was acidified and added to the coulometer cell where the coulometric titration was performed under the same conditions as previously described. It was necessary to acidify the NaOH extract because of the possibility of mercuric oxide formation at pH values of 10 or higher. The results of these titrations were unsatisfactory and very high blank values were obtained.

Methylene chloride was used to extract the barbiturates from serum. This compound has been shown to be satisfactory for nearly all the common barbiturates at a pH of about six. Initially only blood serum without added barbiturate was used so that the selectivity of the methylene chloride extraction could be examined. The procedure employed was to take 1.0 ml of serum and add to this 4.0 ml of pH 6 phosphate buffer. Two 15-ml portions of methylene chloride were used for the extraction, and the extracts were combined and extracted with 5.0 ml of 0.45 N NaOH. One ml of the NaOH extract was then acidified and added to the coulometer cell. The coulometric titration was performed as above. A negligible titration value of 0.05 μeq was obtained. The methylene chloride extraction is therefore more selective than the chloroform extraction and this compound is presently being used for the extraction of sodium phenobarbital from serum.

To determine the optimum conditions for the recovery of sodium phenobarbital from serum, a standard ultraviolet method was employed. A known amount of standard sodium phenobarbital was added to 1.0 ml of serum, 4.0 ml of pH 6.15 phosphate buffer was added followed by 15.0 ml of methylene chloride. The extraction was performed and the barbiturate was back-extracted with 5.0 ml of 0.45 N NaOH. Two 2.0-ml aliquots of the NaOH extract were taken and each was separately diluted to 10.0 ml with water. The absorption of these solutions was measured at 256 mμ on a Beckman Model DB spectrophotometer. This single extraction with methylene chloride yielded recoveries ranging from 75 to 80%.

Two 15.0-ml portions of methylene chloride were then used, the extracts combined, and the NaOH extraction was performed on the combined extracts. The remainder of the procedure was identical to that described above. Recoveries ranged from 91 to 95%. The results were consistently within this range and this procedure is now being used exclusively.

When the methylene chloride extraction is performed, an emulsion is formed. The two most common methods for treating an emulsion are centrifugation and filtration. Centrifugation was not successful in completely breaking down the emulsion, so several methods of filtration were investigated. The most satisfactory method is to use a millipore pre-filter (essentially a glass fiber filter disc) in combination with one piece of Whatman No. 1 filter paper. Using this combination, the recoveries range from 91 to 95%. Some adsorption on the filter paper does occur, making the recoveries slightly on the low side, but still acceptable.

After the optimum conditions for the recovery of sodium phenobarbital from serum were established, comparisons of this method with the coulometric method were made. One of the critical factors affecting the coulometric method is the pH. If the pH is less than 6, there is an immediate rise in current indicating that the Hg(II) is not reacting with the barbiturate. Therefore the pH must not fall below 6. Several buffer systems were investigated in the pH range between 7 and 8. Among these were ammonia-boric acid, carbonate, and citric acid-sodium dihydrogen phosphate. When generation of the reagent was carried out in these buffer solutions, no increase in current occurred. This indicated that the generated reagent was reacting with the buffer solution.

Since a satisfactory buffer system could not be found, the pH of the sodium hydroxide extract was adjusted with 6 N nitric acid. A pH-combination electrode was used to measure the pH since only 1.0 ml of the NaOH extract was being added to the coulometer cell. The adjustment and control of the pH to about 7 was rather difficult, even when the HNO_3 reagent was added with a syringe. When the pH was finally adjusted to about 7.0 to 7.5, and the titration performed, only 51% recovery was obtained. This same procedure was given additional trials, but the results were very random and recoveries were never better than 51%.

The determination of salicylates: Work has continued on the development of a coulometric titration which is suitable for the determination of salicylates in clinical samples. This work has been aimed at a method based on generation of an excess amount of bromine, followed by (i) back-titration with coulometrically generated Cu(I), or (ii) addition of a standard solution of KSCN to react with the remaining bromine, followed by titration of the KSCN with generated Br_2.

The cell used in these studies is similar to those described in Lingane. The titration vessel is fashioned from an inner 40/35 ST joint which has been sealed at the lower end to form a beaker-like container. The vessel can accommodate conveniently volumes from 15 to 30 ml. A cap for the vessel has been made from the corresponding outer joint. Three tubes are sealed into the top to allow intrusion of three Sargent Pt electrodes into the cell, and an isolation tube with a fine porosity frit is ring-sealed to allow a fourth Pt electrode to be isolated from the other three. It was found that a small ventilation hole must be provided in the cap. Otherwise, the tight fit of the joints caused a pressure differential inside the cell when the cap is placed atop the lower part. This pressure forces liquid from the cell up into the frit and allows loss of sample, causing erroneous results. Much time and effort was spent in the search for this source of error.

In our first investigatory titrations, Br_2 was generated at a fast rate, then it was back-titrated with Cu(I) to check the reproducibility of the system. The generating current was 10 mA and the solution used was 1.0 M NaBr, 0.067 M $CuSO_4$, and 0.33 M H_2SO_4. End points were obtained as described above and some typical data are quoted in Table III.

In addition, a salicylate solution was titrated to evaluate the reproducibility of that part of the procedure. The titration was not reliable, and after some deliberation, it appeared that the indicator electrodes were unable to follow the Cu(II)/Cu(I) couple at high titration rates. It was found that a generation rate of 1 mA was satisfactory from this standpoint. Unfortunately, this is unsatisfactory from another standpoint.

The reason for back-titration of salicylate is that the reaction with bromine is slow. When unreacted salicylate and bromine remain in the cell, the back-titration with Cu(I) must be fairly rapid so that differences in titration time are not too large. Large variations in titration time can cause varying amounts of reaction between Br_2 and salicylate while the back-titration step is being carried out. This seems very probable with a generation rate of 1 mA.

At this point, a study of both the influence of the amount of excess Br_2 generated and the reaction times was in order. It was realized that in order to properly investigate the latter, some means to quench the bromination reaction was needed. KSCN was chosen as the quenching agent.

The preliminary studies of the Br_2-salicylate-KSCN system have begun.

Although somewhat tentative, results suggest that more Br_2 is consumed in the reaction with salicylate than has been reported in the literature. Further work is anticipated on this aspect of the problem.

Polarography. Polarography of amino acids in dimethylsulfoxide: Polarographic studies of amino acids in dimethylsulfoxide (DMSO) solvent were continued. The polarographic characteristics of DL-tryptophan, and partially, of DL-methionine and DL-phenylalanine, have been elucidated.

The reduction waves for all three amino acids occur at approximately E 1/2 = -2.39 V, beyond the half-wave potential for the reduction of water (-2.07 V). (All potentials are measured vs. an aqueous SCE.) It has been observed that if the water concentration increases, the height of the amino acid wave decreases (measured as the height above the plateau of the water wave). Due to this anomalous behavior, to be discussed later, it was considered critical that the water concentration remain constant throughout all runs.

To insure this, the procedure for purifying and handling the DMSO was changed. J. T. Baker reagent grade DMSO distilled over Fisher 8-14 mesh indicating activated alumina was found to be free of polarographic impurities and to have a constant, low water content. This solvent was stored no longer than two days in a sealed flask, under dry, pre-purified nitrogen. The supporting electrolyte, tetraethylammonium perchlorate, after recrystallization from water was dried in a vacuum oven at 60°C for about 24 hours, and stored over P_2O_5 desiccant. This procedure has yielded very reproducible water waves for solutions over a large number of runs.

Polarography of vanadium in DMF: This study of the polarography of vanadium has diverged into two segments. One segment is concerned with the polarographic wave forms of vanadium in dimethylformamide (DMF) and the other with a DMF reference electrode. The second grew out of a need developed in the first.

Previous studies were made on the vanadium wave which occurs at about -0.38 V vs. the cadmium reference electrode. The results are difficult to interpret because the E 1/2 varies as much as 20 mV and does not vary in a consistent pattern. Because of this, the reference electrode was checked.

The reference electrode used for the above studies was a massive $Cd_{(Hg)}$, $CdCl_2$ electrode described by Marple. The electrode needed testing for consistency with and without a load. This testing had been done by Marple and the electrode's characteristics had been found to be satisfactory. The only difference in the make-up of our cells was that Marple had a specific $CdCl_2$: $CdCl_2.2\text{-}1/2H_2O$ ratio in his cell while we did not. However, with a ratio varying from 20:1 to 20:6, the maximum difference was only 1.6 mV and our electrode seemed to vary more than that.

The first test performed was to run a series of metals in water and compare the obtained E 1/2 values with those in the literature. The values were corrected for iR drop, and the resistance was determined in two ways, with a conductance bridge, and by varying the concentration of cadmium ion and calculating the R by $R = (\Delta E / \Delta i_d)_2$. The second method gave the many varied results shown in Table XVII.

There seemed to be two possible reasons for the large discrepancy in E 1/2. One was the inconsistency of the reference electrode. The other was a large and changing junction potential developed between two different solvents. The junction potential would not be a problem when running vanadium in DMF; it would be a problem when there was a water-DMF interface, as when running Cd and Zn to test the electrode.

Li, Na, and K have been run polarographically in DMF using a Hg pool reference electrode. These were run in DMF to compare the Cd reference electrode with the Hg pool. The results were again poor, and since there is no significant junction potential here, it would indicate that the reference electrode is not acting well.

The junction potential was tested by building an SCE in the opposite compartment of the cell and comparing the SCE with our standard reference. Readings were taken at regular intervals after the center compartment of the cell was filled. The potential vs. time was recorded. The shape of the curve obtained was not consistent from one run to another but the curves did show that a considerable potential was built up between the two solutions. When a 46,000-ohm resistor was connected between the electrodes, the potential dropped sharply; the potential dropped slowly as the current flowed. When the resistor was removed, the potential rose sharply.

Up to this point there was no agar plug between the SCE and the sample compartment. A plug was added in the hope that this would stop the transference sufficiently to prevent a significant junction potential. The resistance was also increased to allow only 1 μA of current. The potential changed only 3.4 mV in 15 minutes of operation. With the agar plug still in place, the Cd and Zn aqueous solutions were run again and the results are given in Table XVII.

Now that it was possible to impose conditions to maintain a constant potential reference source, we tried to make up a series of reference electrodes in 15-ml vials, each electrode containing varying amounts of water. These were equilibrated over the weekend at 25°C and then tested by placing them in one side of the H-cell and comparing them with the SCE while 1 μA of current was flowing. The results were random, even when long equilibration times in the H-cell were employed. The standard deviation was 11.3 mV between the electrodes with the longest equilibration time in the H-cell.

It was obvious that if one wanted to use the $Cd_{(Hg)}$, $CdCl_2$ reference electrode, certain restrictions would have to be placed on its use. It would be best if a small electrode that carried no current were used in place of the massive electrode which carried a current. For the constant potential coulometric work an electrode with a thirsty-glass plug was employed. It carried no current but used the same $Cd_{(Hg)}$, $CdCl_2$ half-cell. The first electrode of this type was not completely satisfactory because the zinc oxide-sodium silicate used to seal the thirsty glass to the glass tube was slowly dissolved by the DMF. Many modifications of this electrode were tried until a satisfactory one was found.

The electrode is formed out of a 3" piece of 1/4" polyethylene tubing. In one end of this tubing is placed a 1/4" piece of thirsty glass which has been cut off flush with the end of the tubing. On top of the glass is placed a layer of glass wool followed by a 1/2" layer of $Cd_{(Hg)}$, NaCl, and $CdCl_2$, all mixed intimately so as to give a shiny granular mixture. This is covered with a mixture of NaCl and $CdCl_2$ in DMF. The DMF used in the fabrication of this electrode was distilled twice over P_2O_5 to remove water. In handling, a little water was undoubtedly introduced. A glass tube with a platinum wire sealed in one end formed the electrical connection, and the top of the polyethylene was melted around the glass tube at the top to form an air-tight seal. This keeps atmospheric water from the electrode contents and, because the air pocket inside the tubing is small, it keeps the volume in the tubing from changing when the external DMF level is different from the internal level. Marple states that it is important to have the amalgam in the form of a slurry. With the electrode described here the opposite is true. It is important that no liquid mercury flow down and cover the top of the thirsty glass.

Six of these electrodes were made up at one time and they showed only 2.8 mV standard deviation after two weeks of use. When not in use, they are stored in tetraethylammonium perchlorate (TEAP) solutions of DMF and are not protected from the atmosphere.

This electrode seems to be reliable and reproducible. Some studies on the effect of water have begun using this electrode. Because of the resistances involved, however, it was necessary to modify the Sargent Model XXI polarograph so that it could function as a three-electrode instrument.

Chronopotentiometry. The cause and solution to one problem has been found. When using differential inputs to operational amplifiers, one must consider the possibility that the two inputs do not have the same gain. This was discovered in connection with the Scaling amplifier, and it was found that to correct for it a variable resistance from the non-inverting input to ground was needed to adjust the gain. The fixed 250 K resistor was replaced with a fixed 215 K and a variable 50 K resistor. The inputs are shorted to see the same signal and the amplifier balanced. Then with normal signal through the Sensing resistor, the voltage output of the Blank Amplifier is made to swing through a 2 to 5 common mode voltage range (expected in the cell) and the 50 K resistor is adjusted until the output of the Scaling amplifier remains constant.

The P85AU amplifier has a high (20,000/1) common mode rejection ratio and can be used as the above-described Scaling amplifier. After proper adjustment it gave constant output with constant current input over 2 to 10 volts common mode range. Attempts to use a 10-K potentiometer as the current sensor and eliminate the gain of 10 at the Scaling amplifier led to instability and oscillations. The 1 K with gain of 10 is satisfactory.

The Scale-inverting amplifier was rewired with a switch to give a choice of compensation methods: (i) addition of extra current to the test cell to give 100% efficiency or (ii) subtraction of the blank voltage from the set voltage and integration to give an effective time for τ. It was found that the integrating circuits are critical to transient switching pulses even in the timer circuits. It was necessary to put capacitors across the timer brake coils to lower transients in order to get good integration values.

Attempts to use the Photovolt Integraph as a digital readout were discontinued after preliminary calibration. The Integraph drifts over long-term integration and does not count fast enough to give enough significant figures. Also, it counts at a different rate with + inputs than with - inputs. A simple voltage-to-frequency converter may work better to give digital readout. Such a device can be built around a uni-junction transistor acting as a saw-tooth generator, if an input-conditioning circuit is used. The putput pulses can be read on Decimal Counting Units.

A test to compare the area of the Blank and Test cells was conducted with the same 1 mM cadmium solution in both cells. The same 200 μA current through both cells gave 10% greater area in the Blank cell. Coating the cells with Dessicote to prevent the solution from penetrating the glass-mercury surface helped somewhat. A new cell setup was constructed by machining a block of Lucite. The cell diameters were machined and polished to less than 1/1000th inch. Neither mercury nor water wet plexiglass and thus give a more reproducible surface interface. It is found that careful cleaning, filling, and nitrogen purging is necessary for the two cells to give the same τ-value with the same 1 mM cadmium solution.

Rodgers (65) gives computational procedures to show the effect of the cell double-layer charging on the shape of the chronopotentiogram. He makes corrections by obtaining an estimate of the capacitance effect and from a graph obtains a correction value for a parameter related to τ. He also observes that the best τ-values are obtained by reading the time between set intervals either side of E 1/4. This is the procedure we have been using with the auto-switching mode.

Recent attempts to determine the coulombs used in charging the double layer and capacitor with a 0.1 M KCl solution in the test cell, gave a wide range of values. The poising of the cell before electrolysis is quite critical. Also, there may be electroreducible impurities in the solution as the coulombs passed, varying the current. The previous runs on a given mercury pool also affect the coulombs used. Attention is being given to the use of graphite electrodes. A means of trimming them to a reproducible area is being sought.

Summary:

The work covered by this report has been carried out by four analytical techniques: atomic absorption spectroscopy, coulometry, polarography, and chronopotentiometry.

A satisfactory calibration curve for trace quantities of vanadium has been obtained by atomic absorption using the nitrous oxide-acetylene flame. Contamination from the deionized water and other sources was found to be about 4 p.p.b. of vanadium. Following a dry-ashing procedure, the vanadium in urine samples was measured. Although the results are, for the moment, tentative, they indicate the presence of up to 0.04 p.p.m. vanadium in the sample.

Coulometric titrations have been applied to the determination of acid and alkaline phosphatases. Following hydrolysis of the phosphate ester by the enzyme, phenol or a substituted phenol, and phosphate are released. The phenol is then titrated with generated bromine.

The same titrant is employed for the titration of cholesterol. Interferences from 7-dehydrocholesterol, desmosterol, and dihydrocholesterol can be removed by thin-layer chromatography. Results suggest that cholesterol may be titrated at the 20-μg level with a precision and accuracy within 1%.

Coulometrically generated mercury(II) is being used for the titration of creatinine and sodium phenobarbital. The determination of glucose via glucose oxidase has been modified in an attempt to extend it to the determination of maltose through maltase. Once maltose can be determined, it should be possible to develop a procedure for amylase.

Standard salicylate samples have been assayed by an indirect titration following bromination of the benzene ring. The excess of bromine is then determined either with copper(I) or potassium thiocyanate.

The coulometric determination of uric acid has been compared with colorimetric and uv-enzymatic methods. The data indicate that the coulometric procedure is much superior to either of the other methods. Considerable attention has been given to the study of the titration reaction and to the nature of intermediates formed under the conditions of the determination. Of prime importance is the isolation of the uric acid from all potential interferences in the determinative step.

The polarographic behavior of DL-tryptophan in dimethylsulfoxide has been elucidated. Partial studies have been undertaken on the characteristics of DL-methionine and DL-phenylalanine in the same solvent. For the polarography of vanadium in dimethylformamide, the need for a stable reference electrode was recognized. A cadmium-amalgam reference electrode has been developed which maintains a stable potential in this solvent, even after several weeks of use. Additionally, the Sargent Model XXI polarograph has been modified to accommodate a three-electrode capability.

Final modifications on the differential derivative chronopotentiograph have not made it possible to determine what we consider to be the best estimate of the transition time. This is crucial for the kinetic studies which we intend to make.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 174, Applications of electroanalytical techniques to biochemistry and clinical chemistry

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1. Moody, J. R., Christian, G. D., and Purdy, W. C. The coulometric titration of tetraphenylborate ion with electrongenerated silver ion and a biamperometric end point. Application to the determination of potassium and polyethyleneglycols. Anal. Chim. Acta, 42: 153, 1968.

2. Troy, R. J., and Purdy, W. C. Analysis of uric acid in biological media using coulometrically generated iodine. Paper #8, Analytical Division, 4th Middle Atlantic Regional Meeting, Washington, D.C., February 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
68 10 31	D. CHANGE	U	U	NA	NL :	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	175
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11. TITLE (Precede with Security Classification Code)*

(U) X-Ray Diffraction Studies of Biological Interest (21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

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22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Chemistry; (U) Analytical Chemistry; (U) Biochemistry; (U) X-Ray; (U) Pharmacology

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To study chemical structure and analogues of chemical compounds and the products of the interaction of these compounds in biochemically important systems, to learn more regarding the specificity of chemical structure in treatment of disease.

24. (U) To relate x-ray structure analysis to specific configurations of the chemical molecule which enhance protective capacity of the chemical.

25. (U) 68 07 - 69 06 Accurate structural data have been derived on a series of different molecular systems which show activity in control of malaria. A data base now consists of crystal structural data on chlorguanide, daraprim, folic acid, chloroquine, quinacrine, acridine, quinine, and a series of related compounds. Data from these studies will be used for further evaluation of structure-activity relationships of a series of potential antimalarial agentr.

For technical report seeWalter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.

*Available to contractors upon originator's approval.

DD, FORM 1 MAR 66 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 175, X-ray diffraction studies of biological interest

Investigators.
Principal: James M. Stewart, Ph.D.
Associate: Edward C. Knoblock, COL MSC
Herman Ammon
Ellco Boonstra
Roger Chastain
Wilson DeCamp
Charles Dickenson
Harold Marr
Linda Plastas
Hugh Preston
Plato Watts
Jean Willis

Description

The object of this study was to study the chemical structure of a series of chemical compounds of biochemical interest in order to learn more regarding the specificity of the chemical structure of some of the biologically active chemicals in the treatment of disease. Due to the military importance of the agents used for treatment of malarial infections, emphasis has been placed on the compounds which are primarily effective against this disease and their kindred agents in order to ascertain the locus of specificity and enhancement of effect. It is expected that x-ray structure analysis depicting specific configurations within the chemical molecule will aid in evaluations of the chemical structure which may lead to enhanced protective capacity of this chemical against the infectious agent.

Progress

1. Maintenance of the WRAIR XRD-6 Diffractometer.

During the second year of the use of the WRAIR Diffractometer, a number of compounds have been studied. We have had continuing maintenance problems with the General Electric-Datex equipment. The major problem of reliability is focused on the card reader. It is very difficult to keep it synchronized properly with the DATEX "logic". In addition, during this period, the "chi" circle worm drive gear became so worn that replacement was required. This was accomplished through the excellent machine shop facilities of WRAIR. The overall performance of this device

is still only about 40% of its potential despite our efforts. The University of Maryland, Department of Chemistry, is obtaining a computer controlled instrument to be housed in the same room with the WRAIR instrument and it will serve to back up and supplement our data gathering ability. We look forward to this installation in September of 1969.

The overall quality of the data processed from the WRAIR XRD-6 cannot be faulted. The x-ray diffraction data gives "state of the art" crystal structures. In section three are summarized the results obtained to date.

2. Crystallization of Compounds

Crystallization of quinine sulfate was accomplished by allowing a saturated solution of quinine in chloroform to evaporate very slowly in a vacuum desiccator. Crystallization occurring over a period of several months resulted in platelets. The largest crystals obtained were approximately .50 x .26 x .06 mm.

The crystallization of chloroquine phosphate was attempted in several solvents. The best solvent appears to be 95% ethanol. Several stages of recrystallization from a saturated solution started from seed crystals was carried out. Each solution was seeded with crystals obtained from previous recrystallizations. This technique has resulted in crystals which appear suitable for x-ray diffraction studies.

Crystallization of folic acid was attempted in a number of ways. The most successful method to date has beenslow evaporation of a solution of folic acid in dilute aqueous HCl. Crystals in the form of needles resulted. Their cross section appears to be too small for use in x-ray diffraction studies. Subsequent recrystallization using these needles as "seeds" will probably result in suitable crystals.

3. Status of Computing

The current version of x-ray-67, our crystallographic computing monitor, is in excellent condition. We have used it consistently to solve and refine the compounds listed below in Part 4 of this report. Dr. Chastain has supplied us with other links of the system and our collaboration on the automatic centrosymmetric phase determination programs is a success. A number of the compounds listed below were determined by the use of these programs. The papers for a number of these compounds are in preparation.

4. Structures Completed and Under Study and Their Status

The following table is designed to give a capsule view of the crystalline materials which we have treated by means of the WRAIR XRD-6 Diffractometer. It is divided into three distinct categories: (1) those compounds solved and being prepared for publication, (2) those substances in the

process of solution, and (3) those materials for which we propose to establish precise structural information.

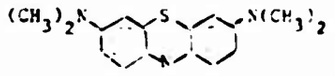
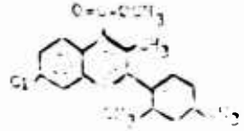
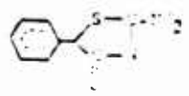
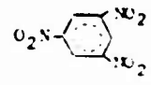
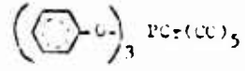
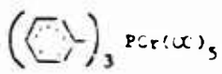
Summary and Conclusions

At the present time, there is practically no detailed molecular geometry established for any of the known antimalarial compounds. In fact, even the simpler moieties of these compounds have not been determined. We have, therefore, at this point set out to gather accurate structural data on many different molecular systems which show activity in malaria control. Our first objective is to establish a sound experimental basis for making comparisons of the various drugs and other reactive compounds involved in the malaria parasite's metabolism. We are using as a guide in this program the biochemists at WRAIR, Bergers "Medicinal Chemistry" compendium, the recent review article (Science, 162, 1346) "Malaria and Victory in Vietnam" by W. Modell and information of the type contained in Chemical and Engineering News of April 28, 1969 on page 42 on a new antimalarial.

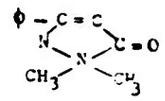
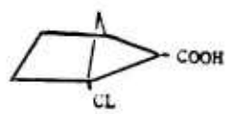
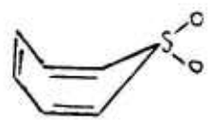
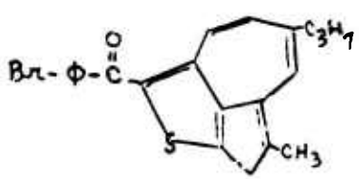
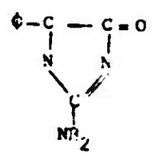
Practically every structure we undertake, in addition to building our data base concerning the malaria problem, has interesting structural features of interest to chemists. Tautomeric forms are resolved, postulated resonance systems are checked, and accurate bond lengths and angles are established for previously unknown molecular configurations.

Single crystal x-ray diffraction is now moving into a time when it can be used easily and quickly to establish accurate molecular configurations. We hope to take advantage of this fact to elucidate the structures of antimalarial drugs. In this way, we hope to get fundamental data that will help further understanding of the control of the disease.

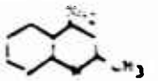
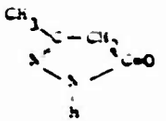
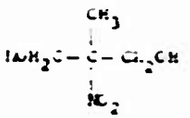
SOLVED STRUCTURES

	COMPOUND	FORMULA	CODE NAME	SOURCE	COMMENTS
1.	Methylene Blue (hydrated)		Blue	M. Parr	Antimalarial
2.	2-(2,4-dimethyl phenyl-3-methyl-6-chloro-7,8 benzoquinoline-4-carboxcyclic acid methyl ester		[illegible]	Rhumatra	Antimalarial Precursor
3.	5-Phenylpseudohydantoin		Hydant	L. Plastea	Antiarrhythmic effects
4.	Ethyl-5-phenyl-2-imino-4-oxo-1-imidazolidine carboximidate	$C_{12}H_{14}N_4O_2$	Imidaz	L. Plastea	Antimalarial [illegible]
5.	Picryl Chloride		Piccl	J. Willis	[illegible]
6.	Sodium chloride	NaCl	NaCl	Sorrento	[illegible]
7.	Pentacarbonyltriphenyl-phosphitechromium(0)		Moritz	M. Plastea	[illegible]
8.	Pentacarbonyltriphenyl-phosphinechromium(0)		Cride	M. Plastea	[illegible]

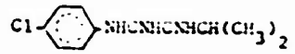
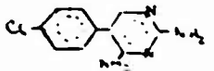
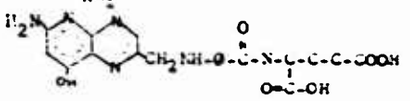
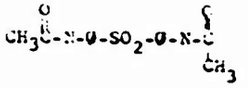
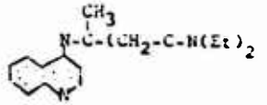
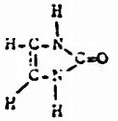
SOLVED STRUCTURES

	COMPOUND	FORMULA	CODE NAME	WORKER	INTEREST
9.	1,1-dimethyl-3-phenyl-pyrazolium-5-oxide		Freddy	DeCamp	Antipyrine structural isomer
10.	Exo-1-chloro (2.1.1) bicyclohexane-5-carboxylic acid		Clod	P. Watts	Parent (2.1.1) bicyclic compound
11.	thiepin-1,1dioxide		Thipin	Ammon & Watts	Aromatic character determination
12.	3-methyl-6-isopropyl 5'-parabromobenzoyl 8H-azuleno(1,8-bc) thiophene		Brsazu	Ammon & Watts	
13.	5-phenyl-2-imino-4-oxo-1-imidazolidine		Dimegu	Chastain	Blocking agent in protein degradation

STRUCTURES UNDER INVESTIGATION

	COMPOUND	STRUCTURAL FORMULA	CODE NAME	WORKER	INTEREST
1.	2-methyl-4-amino quinoline		Aniq4	F. Hahn	Antimalarial Precursor
2.	Quinine Sulfate	$(C_{20}H_{23}O_2N_2)_2 \cdot H_2SO_4$		F. Hahn	Antimalarial
3.	tetracarbonylbistri-phenylphosphie chromium(0)	$[(\phi\text{-}C)_3P]_2\text{-}Cr\text{-}(CO)_4$	Cr4e	Fenton & H. Plastas	Coordination Backbonding theories
4.	3-methyl-pyrazolin-5-one		Pyraz	DeLamp	parent of many steroids
5.	1,3-dihydroxy-2-CH_3 2-nitropropane		Diol	Marr	Geometry in di-alcohols
6.	bis(triphenylphosphine) diphenylacetylene platinum (II)	$(\phi_3P)_2Pt(\phi\text{-}C\equiv C\text{-}\phi)$	Ptolan	H. Plastas	Coordination geometry
7.	diphenyl-dimethyl phosphine chloride	$[(\phi)_2P(CH_3)_2]^+Cl^-$	Dicl	H. Plastas	Coordination geometry

PROPOSED STRUCTURES

	COMPOUND	STRUCTURAL FORMULA	CODE NAME	MODEL	INTEREST
1.	Chlorguanide		B.guan	L. Plastae	Antimalarial
2.	Daraprim		Daprim	L. Plastae	Antimalarial
3.	Folic acid		Folic	Freisen & Matts	Metabolite important in malaria
4.	4',4'''-sulfonylbis-acetanilide		DmSDS	Dickerson	Antimalarial
5.	4,4'-sulfonyldianaline	NH_2-Ø-SO_2-Ø-NH_2	DDS	Dickerson	Leprosy Drugs
6.	Chloroquine		Chlgin	Watts & Stewart	Antimalarial
7.	2-imidazolone		Imdone	DeCamp	Parent bond lengths & angles
8.	Quinacrine (Atebrin)	$C_{23}H_{30}N_3$ OCl	Atbrin	Watts	Antimalarial
9.	Acridine		Acdine	Watts	Antimalarial Precursor

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 175, X-ray diffraction studies of biological interest

Investigators.
Principal: James M. Stewart, Ph.D.
Associate: Edward C. Knoblock, Col MSC
Herman Ammon
Alico Boonstra
Roger Chastain
Wilson DeCamp
Charles Dickenson
Harold Marr
Linda Plastas
Hugh Preston
Plato Watts
Jean Willis

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				DA 08 6433	69 06 30	
68 10 01	H. TERM	U	U	NA	NL	
a. PRIMARY	61101A	3A061101A91C		00	176	
b. CONTRIBUTING						
c. CONTRIBUTING						

(U) Field Epidemiological Survey Team (VN)

001500 Clinical Medicine, 005900 Environmental Biology

68 01	CONT	DA	C. IN-HOUSE

FISCAL YEAR	68	2	110
FISCAL YEAR	69	2	110

a. CONTRACT/GRANT: NA

b. NUMBER:

c. TYPE:

d. ORG OF GRANT:

e. CUM AMT.

a. PERFORMING ORGANIZATION

Walter Reed Army Institute of Research

Washington, DC 20012

a. PRINCIPAL INVESTIGATOR

Meroney, COL W. H.

202-576-3551

a. PERFORMING ORGANIZATION

Walter Reed Army Institute of Research

Med Rsch Team, Vietnam

Washington, DC 20012

a. PRINCIPAL INVESTIGATOR

a. GENERAL USE

Foreign Intelligence Not Considered

a. KEYWORDS

DA

(U) Epidemiology; (U) Malaria; (U) Tropical Diseases; (U) Vectors

23 (U) To determine the feasibility and the productivity of a Special Forces medical epidemiological team working in a forward combat area.

24 (U) Train, equip and deploy such a team of 8 Medical Officers, 3 MSCs and 16 EM to Vietnam.

25(U) 68 10 - 69 04 FEST team has been disbanded and the work unit therefore terminated. Effort of the team has been made a part of the Med Rsch Team (VN) program.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

68 10 30

(U) Tropical Disease [illegible]

002600 Biological [illegible]

67 08

Walter Reed Army [illegible]

Washington, D. C.

Computer Applications, Inc.

Silver Spring, Maryland 20910

Foreign Intelligence [illegible]

(U) Information Retrieval;

(U) Malaria; ( ) [illegible]; ( ) Abstracts; (U) Computer Programming

23 (U) The objective is to develop a computer system to create, maintain and index the abstracts from the Tropical Disease Bulletin. Since the size of the field of each item in the Tropical Disease Bulletin source file varies, the result of this objective will be a Tropical Disease Bulletin master file in a format compatible with other existing retrieval systems. A second objective will be to develop manipulative capabilities such as the updating of the master file and sorting on certain fields within the file.

24 (U) The problem oriented FIRST language will be used because of the ability to handle variations in spelling of words and punctuation.

25 (U) 68 10 - 69 06 Software development for processing input has been completed. These programs create the title, author and journal records from volumes 45 - 62 of the Tropical Disease Bulletin (TDB) in accordance with specifications from Biological Abstracts. Programs to create a packed data base from volumes 45 - 63 of TDB have been completed. This file contains all special characters from the original flexowriter input tapes. A second file with special characters deleted has also been created and is used to expedite searching. Search techniques to handle full text and fraction of words were also developed and tested. These techniques provide the present capability to retrieve information in our files. Full time production work to incorporate information from volumes 1 - 44 of the TDB remains to be completed. Further analysis is needed to develop techniques which expedite search time and manipulation procedures involved in discovery of synonyms without use of a thesaurus. Contract was terminated 69 06 30 due to relocation of investigator. Work will be continued under a new contract which is to be negotiated in the near future. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report for 1 Jul 68 - 30 Jun 69.

*A verbatim to contractors upon originator's approval

Project 3A061101A91C IN HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 177, Tropical Diseases Bulletin information retrieval system

Investigators:
Principal: Harry W. Voccola

Description

The purpose of this effort is two-fold. The first objective is to develop the software capable of handling the input created by the encoding of the Tropical Disease Bulletin and to manipulate it so as to correct errors, build the file, reformat index tapes suitable for handling by the regular Biological Abstracts system. The second objective is to develop a search technique capable of handling full text, fractions of words within text, and ultimately, manipulation procedures involving the discovery of synonyms without the use of a thesaurus.

Progress

During the past year programs in TEMAC and MAP have been completed in order to build and correct the file created in the Tropical Disease Bulletin project. These programs have involved detection of errors and insertion of corrections as well as compiling the input tapes into an appropriate master file. In addition, programs have been completed for converting to Biological Abstracts the subject, title, and author indices so that the existing Project EXPERT system can handle the Tropical Disease Bulletin. In addition, search questions indicating words or parts of words have been successfully run on either the full text or the titles of Tropical Disease Bulletin. The future work visualizes compressing the existing file to reduce the search time, the provisions of OR questions, and the development of a routine inquiry system capable of being operated by a non-programmer.

Summary and Conclusions

This work has made good progress in that there is now a searchable file using relatively simple search strategies. The introduction of more sophisticated techniques is to be expected during the forthcoming year. These techniques are expected to lead to a general searching capability capable of handling relatively unpurified text. Such a capability will be of broad interest to the Army Medical Service.

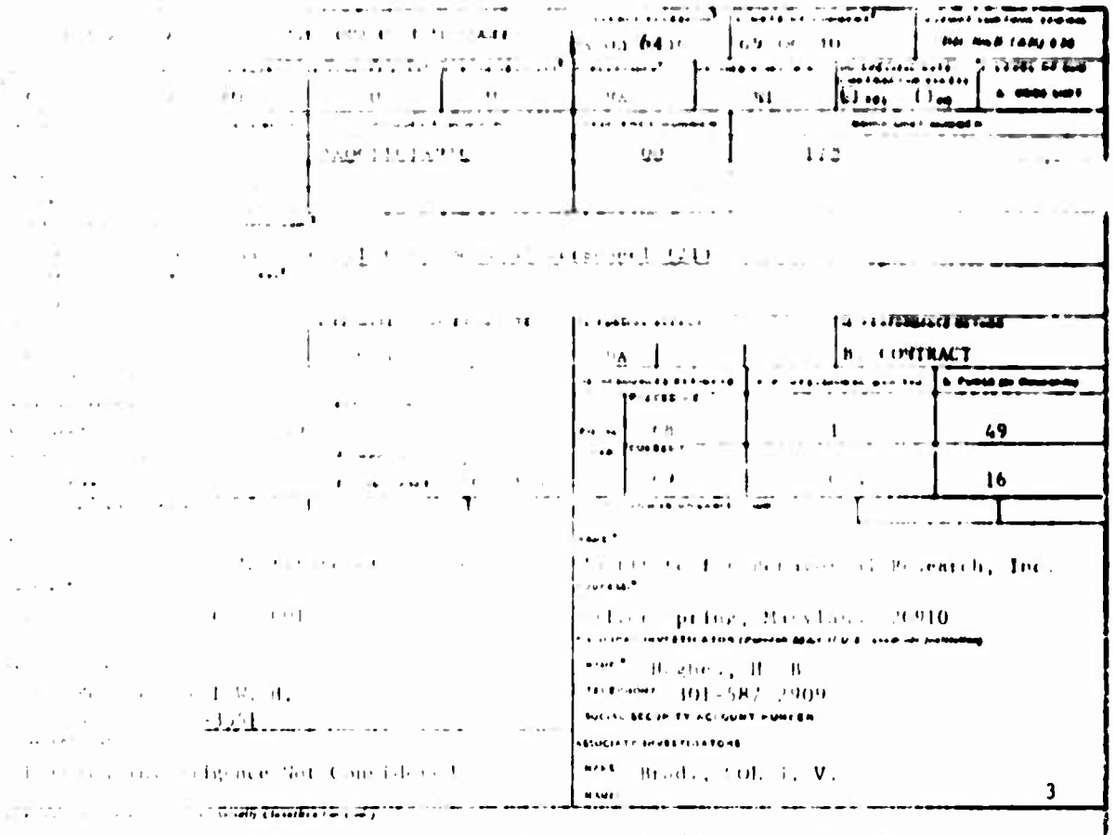
Project

Task

Work Unit III, Tropical

Publications

None



(U) Programmed Thai; (U) Psychol Research; (U) Psychol Behavior

ABSTRACT, IF THIS IS A CONTINUATION SHEET, (Indicate classification of abstract)

(U) The objective is to improve the effectiveness of an experimental course in Programmed Thai for military medical personnel by means of revision in technical features and content that will be identified, systematic behavioral analysis of verbal interaction between military medical personnel and their Thai counterparts.

24. (U) Application of a psychological principle whereby speaking and writing skills are taught by shaping, listening and viewing discriminations, through a completely automated method involving coded film strips and synchronized audio-tapes presented with an audio-visual device that also permits electronic recording of responses.

25. (U) 68 10-68 11 Contract terminated 68 10 30 and a final technical report has been submitted by the investigator. Course materials have been turned over to Div of NP, WRAIR. Observations and findings of PI and Project Officer at SEATO Lab will be basis of an extensive revision of the programmed language course to be carried out as an in-house effort by Div of BP. For technical report see Walter Reed Army Institute of Research, Annual Progress Report, 1 Jul 68 - 30 Jun 69.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 178, Programmed Thai for military medical personnel

Investigator.
Kenneth Stuart, LTC, MSC

Description.

The investigations conducted in conjunction with this work unit were concerned with the experimental testing and evaluation of an automated instructional program in Thai language skills for military medical personnel, developed under contract with the Institute for Behavioral Research, Heidi B. Hughes, Ph.D., Principal Investigator.

Progress.

The program in its final form as it was received from IBR for testing consisted of the following:

A. A prototype audiovisual device (including a control unit);

B. Subject materials divided into 40 sections, or lessons;

1. Audio materials presented on continuous tape cartridges;

2. Visual materials presented on black and white continuous film strips;

3. A student manual, divided into 40 sections, or lessons;

4. A three-part word book, or dictionary;

5. Audiotapes of the practice sections of the student manual;

C. Edward M. Anthony's A PROGRAMMED COURSE IN READING THAI SYLLABLES, Ann Arbor, The University of Michigan Press, 1962.

These materials were completed and delivered to WRAIR for testing in December 1967. The completed program had not been administered to subjects at IBR prior to delivery.

Observations and data based on the administration of the program to 17 volunteer subjects in Thailand indicate that the program fell far short of its stated objectives: performances fell below the S-1, S-2 levels; subjects did not acquire even modest reading or writing skills; subjects were unable to apply learned material in basic conversation situations, the prototype audiovisual device was inadequate for the purpose; the program contained much poor, incorrect, or unacceptable Thai, and at least some of the materials were culturally incorrect or actually offensive to the native Thai; the student manual presented arbitrary and often incorrect explanations.

At the same time that the program was being administered and tested in Thailand, the content was reviewed in detail, and a complete critique was prepared, with suggested revisions. The student manual was similarly treated. Incorrect statements concerning structure, pronunciation, usage, etc., were also noted. A supplemental (advanced) 20 lesson program was prepared, using audiotapes and a workbook, and additional textual material acquired for supplementary use. These materials have been incorporated into the entire "package" which now constitutes the program.

The mission of this investigator was to administer and evaluate the IBR program, and to identify problem areas. Following submission of an Interim Report in September 1968, it was considered feasible to revise the program, select a suitable audiovisual device from among commercial products available, and prepare new softwear for further testing in Thailand. It has become evident that current funding and personnel limitations preclude the level of commitment of resources which an adequate remake of the program requires. In order therefore to put to use the materials on hand, and the experience and information gained, the program will be turned over to the Defense Language Institute, for whatever use they may be able to make of it in connection with Thai language course development and the development of their own audiovisual program in Thai.

Summary and Conclusions.

An experimental course in the Thai language, presented by means of an audiovisual device and based on the principles of successive approximation, developed at the Institute for Behavioral Research (IBR) was taken to Thailand for evaluation and further development. The program was administered to seventeen volunteer subjects, selected from among personnel at the USA Medical Component, SEATO. At the same time, the program was subjected to detailed review. Results of this study indicated that the program failed to achieved its

terminal behavior goals, and that an extensive revision of all parts of the program was indicated. Data and critique were presented to Director, WRAIR, in an interim report in September 1968. At that time, it was considered feasible to attempt an in-house revision of the program. This revision, begun in Thailand, was to have been put into final form at WRAIR by the end of June 1969. A careful review of additional costs, personnel and time requirements, indicates that limited resources in all three areas preclude further development of this program at WRAIR at this time.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 178 Programmed That for military medical personnel

Publications.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6438	69 07 01	DD-R&E (AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
68 10 31	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	179
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Novel Synthesis of Organophosphonates (31)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012100 Organic Chemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
68 04	CONT	DA	B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 68 04 EXPIRATION: 70 03
b. NUMBER: DADA17-68-C8098
c. TYPE: U.CPFF
d. AMOUNT: $48,504
e. KIND OF AWARD: NEW
f. CUM. AMT.: $48,504

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PRECEDING 69	1	24
	CURRENT 70	1	18

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W.H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Esso Rsch & Engr Co
ADDRESS: P.O. Box 172
Linden, N.J. 07036

PRINCIPAL INVESTIGATOR (Provide Rank or Grade and Complete Address)
NAME: Brois, S.J.
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Becker, E.L.
NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Organophosphonates; (U)Antiensyme Activity; (U) Alkylphosphonates (U) O-Aryl-OAlkyl; (U)Aminoalkylphosphonates

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by numbers, precede each with appropriate Security Classification Code.)

(U) To prepare a wide spectrum of O-aryl-O-alkyl alkylphosphonates and O-aryl-O-alkyl aminophosphonate esters where aryl designates β-nitrophenyl and alkyl specifies ethyl, cyclopentyl and cyclohexyl groups.

(U) Through procedures available in the chemical literature, with modification as required.

(U) 68 10 - 69 06 The convenient and reproducible synthetic pathway to the mixed ester, diethyl p nitrophenyl phosphite was devised. Complete spectral and analytical data confirmed the structure. The Arbusov reaction of 3 phthalimidopropyl bromide with the diethyl p nitrophenyl phosphite to form the p nitrophenyl ethyl 3 phthalimidopropyl phosphonate was attempted. The nuclear-magnetic resonance study revealed that the mixed ester failed to react with the nitrogen protected amino alkyl bromide. Apparently the strong electron withdrawal effect of the p nitro group decreases the nucleophilic strength of the phosphite ester dramatically. Work is now in progress to synthesize the compound utilizing the Tjioe procedure. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 July 68 30 June 69.

DD FORM 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 179, Novel syntheses of organophosphonates

Investigators.
Principal: Stanley J. Brois, Ph.D.
Associate Elmer L. Becker, Ph.D., M.D.

Description.

The purpose of this task is to prepare a wide spectrum of O-aryl-O-alkyl alkylphosphonate and O-aryl-O-alkyl aminoalkylphosphonate esters where aryl designates p-nitrophenyl, and alkyl specifies ethyl, cyclopentyl and cyclohexyl groups.

Progress.

1. Two approaches are being used for the preparation of the desired ethyl p-nitrophenyl ω-aminoalkylphosphonates. The first approach involves preparing and protecting the ω-amino group with phthalimido group, and then cleaving this intermediate product. The requisite N(ω-Bromoalkyl) phthalimides where alkyl corresponds to propyl, butyl, pentyl and hexyl have been prepared. The second approach involves the reaction of a ω-bis (trimethylsilyl) aminoalkane halide with the appropriate phosphite ester. The formation of the bis (trimethylsilyl) amide potassium salts has enabled the preparation of 6-(bis-trimethylsilyl) aminohexyl bromide via displacement on 1,6-hexanedibromide by potassium bis-(trimethylsilyl) amide. The homologous reagents, i.e. N(bis trimethylsilyl) aminoalkyl bromide (alkyl = propyl, butyl and pentyl) have been prepared. Spectral and analytical data confirm their structure.

2. A convenient and reproducible synthetic pathway to the mixed ester, diethyl p-nitrophenyl phosphite was devised. The Arbuzov reaction of 3-phthalimidopropyl bromide with the diethyl p-nitrophenyl phosphite to form the p-nitrophenyl ethyl 3-phthalimidopropyl was attempted in the temperature range 125-170°C. A systematic nuclear magnetic resonance study revealed that the mixed ester failed to react with the nitrogen protected amino alkyl bromide. Apparently the strong electron withdrawal effect of the p-nitro group decreases the nucleophilic strength of the phosphite ester dramatically. In the light of these observations, the use of mixed ester as a route to the desired p-nitrophenyl ethyl ω-amino-

alkylphosphonates has been abandoned. Work is now in progress to synthesize the compounds utilizing the Tjioe procedure.

Summary and Conclusions.

1. The requisite N(w-Bromoalkyl) phthalimides where alkyl corresponds to propyl, butyl, pentyl and hexyl have been prepared.

2. The formation of the bis (trimethylsilyl) amide potassium salts has enabled the preparation of 6-(bis-trimethylsilyl) amino-hexyl bromide via displacement on 1,6 hexanedibromide by potassium bis-(trimethylsilyl) amide.

3. A convenient and reproducible synthetic pathway to the mixed ester, diethyl p-nitrophenyl phosphite was devised.

Project 3A061101A91C [illegible] LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 179, Novel syntheses of organophosphonates

Publications.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6439	69 07 01	DD-R&E (AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
68 10 31	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	180
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with security classification code)

(U) The Importance of Chromium in Disorders of Carbohydrate Metabolism (33)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry; 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
68 05	CONT	DA			B.CONTRACT

17. CONTRACT/GRANT

a. DATE/EFFECTIVE: 68 05 EXPIRATION: 69 07
b. NUMBER: DADA 17-68-C-8119
c. TYPE: S.CT d. AMOUNT: 30,351
e. KIND OF AWARD: NEW f. CUM. AMT. 30,351

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	1	24
FISCAL YEAR CURRENT 70	0.1	1

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: State University of New York
ADDRESS: Syracuse, New York 13210

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic participation)
NAME: Doisy, R. J.
TELEPHONE: 315-473-5128
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Mertz, W.
NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with security classification code) (U) Absorption; (U) Chromium; (U) Diabetes Mellitus; (U) Glucose Tolerance; (U) Insulin antibodies.

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security classification code.)

23. (U) To study chromium metabolism in normal, diabetic and elderly human subjects and to biochemically define defects in absorption, handling and excretion of chromium associated with impaired glucose metabolism. To identify the nature of the circulating chromium complex which appears in the plasma in response to glucose ingestion.

24. (U) Oral 51-chromium will be administered and plasma and urinary 51-chromium concentrations will be determined at intervals for three days. The same measurements will be made following intravenous injection of 57-chromium. Comparison of experimental data will allow assessment of intestinal absorption, plasma half-life and excretion in all three types of subjects. Studies of the nature of circulating chromium will initially use animals. Electrophoretic patterns of chromium-containing plasma fractions, before and after a glucose load, will be compared and the eluted fractions tested for biological activity in the epididymal fat pad assay.

25. (U) 68 07 - 69 06 Research performed furnishes further evidence that insulin-requiring diabetics display an abnormal metabolism of chromium. The following specific observations were made: (1) Insulin-dependent diabetics had two or three times greater excretion of chromium in comparison with normal or oral-agent diabetics. (2) Plasma evaluations of normal and insulin-dependent diabetics gave an indication that chromium immediately enters tissues and returns to the plasma after three or four days. (3) Highest excretion of chromium occurred within the first 24 hours with more gradual release on second and third days. (4) In all geriatric subjects studied the plasma levels of chromium were abnormally low.

For technical report see Walter Reed Army Institute of Research Annual Technical Progress Report, 1 Jul 68 - 30 Jun 69.

*Available to contractors upon originator's approval.

DD, FORM 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 180, The importance of chromium in disorders of carbohydrate metabolism

Investigators:
Principal: Richard Doisy, Ph.D.
Associate: Walter Mertz, M.D.

Description:

The importance of trace minerals in human and animal metabolism cannot be overestimated. The evidence supporting the role of chromium in metabolism, and the occurrence of a deficiency state in humans has been documented previously.

Our present knowledge concerning the biological role and function of chromium is woefully inadequate. The extent and degree, and the reasons for the deficiency states in the adult population are completely unknown at this time. After certain basic information is obtained, we can hopefully find the answers to these questions. This report summarizes the data we have gathered over the past year concerning chromium and its role in metabolism.

The object of this research is to study chromium metabolism in normal, diabetic, and elderly human subjects and to biochemically define defects in absorption, handling, and excretion of chromium as associated with impaired glucose metabolism. The nature of the circulating chromium complex which appears in the plasma following glucose ingestion will be studied in detail. Oral 51-chromium will be administered and plasma and urinary 51-chromium concentrations will be determined at three-day intervals. Similar measurements will be made following intravenous injection of 51-chromium. Comparison of experimental data will allow the assessment of the importance of intestinal absorption, plasma half-life, and excretion in all three subject groups.

Progress:

A total of eighty-two subjects have been studied in S.U.N.Y. Upstate Medical Center. Twenty-four were normal individuals aged 21 - 70. Seventeen were elderly normal subjects aged seventy and over. Twenty-two were insulin-requiring diabetics aged 21 - 70. Ten were diabetics controlled by oral agents or diet, and nine were diabetics whose requirement for exogenous insulin was questionable.

Subjects ate their meals in the hospital and ingested their dose of ^{51}Cr with their meals. The chromium preparation consisted of either 100 microcuries of $^{51}CrCl_3$ or 100 microcuries $Na_2{}^{51}CrO_4$ reduced to trivalent Cr with ascorbate.

Blood samples were taken at zero hour, one hour, two hours, four hours, 24 hours, 48 hours, and 72 hours, after ingestion of the dose. Urine collections were for 72 hours (i.e., in three separate 24-hour samples).

The results show that in all individuals the plasma levels of radioactive chromium are very low. In only two individuals did the plasma level reach 1%. In normal subjects under 70 years of age plasma levels changed only slightly over the first 24 hours, but rose to higher levels at 48 and 72 hours. In contrast the elderly normals (> 70) show a peak at two hours and a steady decline thereafter. The oral agent diabetics (maturity-onset) displayed a pattern very similar to the elderly.

The insulin-dependent diabetics showed an elevation of plasma chromium levels two-and-a-half - seven times higher than normals. The plasma levels are significantly different ($p < 0.05$) at two and four hours when compared with all other individuals. Other than the increased concentrations, the insulin-requiring diabetics are rising at 72 hours, while all other groups are falling.

It would appear there are three separate and distinct patterns in terms of the plasma Cr levels. The similarity of the elderly normal and maturity onset diabetics curves is of interest. It raises an interesting question. Is the maturity-onset diabetic prematurely aged with respect to chromium metabolism? Conversely, we know elderly normal people tend to abnormal glucose tolerance tests and appear to be diabetic as judged by their GTTs.

The plasma curves of the normals and insulin requiring diabetics both give an indication that Cr leaves the plasma compartment and presumably goes into the tissues, and comes back out after three or four days. A few diabetics were studied at 96 hours and had higher levels at 96 hours than at 72 hours. Future studies will be carried out over a longer duration. Table II records the urinary excretion of chromium for the same groups as shown in Table I. For all individuals the highest excretion occurred during the first 24 hours with a gradual decline on the second and third days.

The insulin-dependent diabetics had two to three times higher total excretion of ^{51}Cr than did normals or oral agent diabetics. The difference was significant at the 1% confidence level. The first and third day collections were also significantly higher ($p < 0.05$).

TABLE I

Plasma ^{51}Cr Concentration (% of original dose

Subject	Time after administration of ^{51}Cr					
	1 hour	2 hours	4 hours	24 hours	48 hours	72 hours
Normal subjects age 21-69	099(11)	082(.3)	073(20)	060(11)	101(9)	.139(9)
SEM	011	021	015	020	104	.067
Normal subjects age 70 and over	021(5)	060(16)	062(10)	030(10)	.021(9)	.017(7)
SEM	009	010	009	005	.005	.006
Insulin dependent diabetics	209(16)	198(18)	169(16)	105(16)	.052(1?)	.125(11)
SEM	160	181	128	112	.016	.044
Diabetics controlled by oral agent or diet	no values	038(9)	060(9)	026(7)	.022(7)	.014(4)
SEM		009	008	005	.004	.003

Numbers in parentheses indicate numbers of patients studied

TABLE II

Mean [illegible] Output [illegible] of Original Dose

	Time after administration of [illegible]			
	[illegible] Hours	[illegible] Hours	[illegible] Hours	Total
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	.008	.067
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	.096
[illegible] Dependent Diabetics	[illegible]	[illegible]	.210(18)	1.383(18)
	[illegible]	[illegible]	.063	.169
Diabetics controlled by oral agent or diet	.553(10)	.107(10)	.038(9)	.636(9)
	.104	.016	.005	.121

Mean values which are significantly different ($p < 0.05$) from means in normals under 70 years are underlined

Numbers in parentheses indicate numbers of patients studied

Five subjects received ^{51}chromium labeled albumin in an effort to learn more about the metabolism of chromium. External monitoring over the liver, spleen, and precordial areas was done daily in some of the subjects. An increase in counts over the liver occurred at approximately seven-eight days. Plasma levels and urinary excretion data were also collected.

One insulin-requiring diabetic of long standing who had previously displayed marked ketosis was given a single i.v. tracer dose of $^{51}Cr\ Cl_3$. The dose was small (i.e., 1 microgram, 63 microcuries). Electrophoresis was not practical to ascertain localization of the radioactivity. Thus, we resorted to ammonium sulfate fractionation of the sera.

It has been shown by others in rats, and we have observed that small i.v. doses of Cr are transported in the globulin fraction of serum (i.e., on transferrin).

Serum was collected daily and subjected to ammonium sulfate fractionation. An equal volume of serum was mixed with saturated $(NH_4)_2SO_4$. This would precipitate the globulins. The tubes were counted and the supernate decanted. Most (70 - 80%) of the ^{51}Cr remained in the albumin fraction for the twelve days during which the counts were followed.

As yet we have not studied normal subjects "in vivo" in this regard, but the results suggest the possibility that there is an alteration of the binding sites in the globulin fraction in diabetics. We have incubated Cr "in vitro" with normal and diabetic sera. The normal sera have more counts in the globulin fraction than do the diabetic sera (i.e., 50% vs 30% approximately).

Our most recent efforts have been devoted to a preliminary study of the binding of ^{51}Cr "in vitro" by gastric juice. Since the insulin-requiring diabetic apparently absorbs more Cr, an investigation into binding by gastric juice seemed warranted. Briefly, we have incubated gastric juice with ^{51}Cr and then separated the bound radioactivity on Sephadex. The Cr appears to bind to a low molecular weight molecule. Preliminary evidence suggests the diabetic gastric juice does not bind Cr in the same fraction as does the normal subject. Further studies are now in progress. Such differences may help explain why absorption is varied. It is not known whether binding is a prerequisite to absorption, or whether binding prevents absorption. It is clear, however, that gastric juice binds copious amounts of trivalent chromium.

Summary and conclusions:

The results described above are further evidence that insulin-requiring diabetics display an abnormal metabolism of chromium. Coupled with the

knowledge that adequate chromium stores are required for normal carbohydrate metabolism, the implication is that chromium may be involved in the etiology of diabetes. Studies such as we have described will help elucidate the physiological role and importance of chromium for normal metabolism. Biochemically speaking, alterations in carbohydrate metabolism lead to concomitant alterations in lipid metabolism. Thus, in the overall picture, chromium is probably involved not only in diabetes, but also in arteriosclerosis and atherosclerosis as well. The health and longevity of a large segment of our population certainly depends on an increased knowledge of the role of trace minerals in metabolism. Studies such as these are an obligation of the scientific community.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

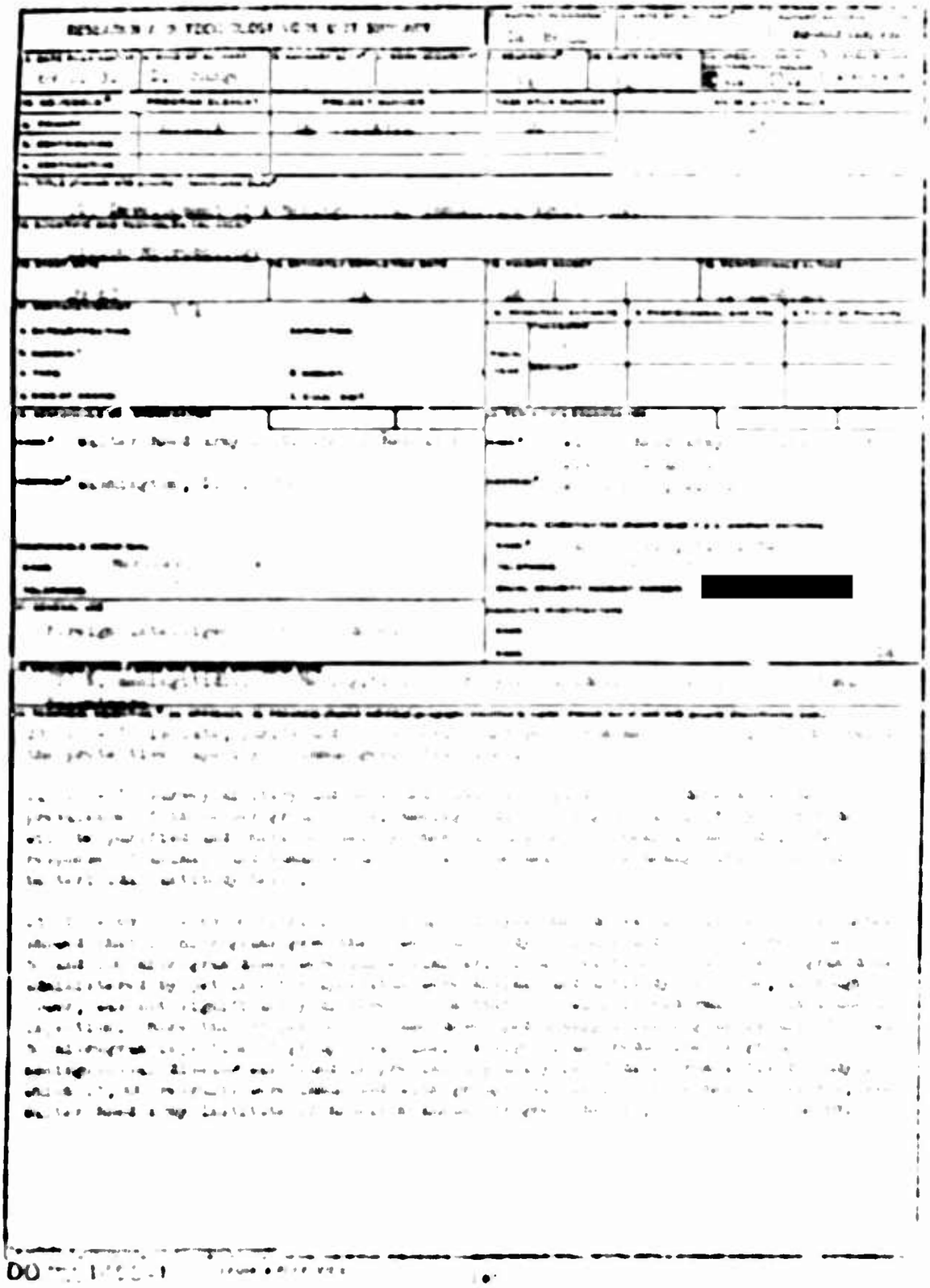
Work Unit 180, The importance of chromium in disorders of carbohydrate metabolism

Investigators:
Principal: Richard Doisy, Ph.D.
Associate: Walter Mertz, M.D.

Publications:

1. Doisy, R. J., Streeten, D. H. P., and Chodos, R. B. Effects and metabolism of chromium in normals, elderly subjects and diabetics. Proc. 2nd Annual Conf. on Trace Substance in Environmental Health. Columbia, Mo. University of Missouri (1968)

2. Doisy, R. J., Heitzman, R., Doisy, R. J., Chodos, R. B., Rehani, S. and Streeten, D. H. P. Chromium absorption and excretion studies in diabetic and normal subjects. Diabetes 18 (Suppl 1) 351 (1969)



PII Redacted

Table 1. Consent form for volunteers.

Meningococcal meningitis is a disease which occurs in Army personnel as well as in civilians. The problem has been under study at the Walter Reed Army Institute of Research and a vaccine has been prepared to combat this disease. After completion of safety tests in cultures and in animals the vaccine preparations have been inoculated into workers in the laboratory and in Army recruits and were found to be safe and potent.

In order to document the ability of the vaccine to prevent infection by meningococci it is necessary to further define the dose which is effective. A single injection of a small quantity will be made in the skin of the forearm. The only reaction anticipated is an area of redness about the size of a fifty cent piece which may be slightly tender and disappears in 36-48 hours. Serum samples and throat cultures will be obtained several times during the basic training cycle, as part of the usual meningococcal surveillance program.

Without duress and of my own free will I do hereby consent to participate in this research study.

Date _____ Signature _____

Date _____ Signature _____

Table 2. Number of volunteers in each platoon receiving each dosage of meningococcal group C polysaccharide.

	Dose in micrograms			
Platoon	10	50	100	Unvaccinated
1	11	5	2	30
2	10	15	16	7
3	9	10	12	18
Totals	30	30	30	55

Serum specimens and pharyngeal cultures: Serum was obtained by venipuncture prior to vaccination and two and seven weeks later and was stored frozen prior to testing. Pharyngeal cultures were obtained prior to vaccination and at intervals of two weeks throughout the duration of basic training. Chocolate agar containing lincomycin and polymixin was used to isolated meningococci which were then identified by fermentation reactions and serogrouped using antisera prepared in this laboratory.

Results:

1. No immediate reactions occurred. No adverse reactions were noted. Vaccination sites were observed after 24 and 48 hours. The skin reactions seen were those previously noted, ie, erythema which was maximum at 24 hours and gone by 48 hours. An occasional individual had slight swelling or tenderness of the site at 24 hours.

The size of the skin reaction was measured in centimeters for each axis and the mean of these two measurements was utilized to compare reactions to each dosage level. Seven of 28 recruits who received 10 micrograms failed to show a skin reaction; all men who received 50 or 100 micrograms developed skin reactions. From Table 3, which compares the mean size of the skin reaction at each dosage level, it is apparent that size of the skin reaction is directly proportional to the dose of antigen administered.

Table 3. Size of local skin reaction at varying doses of vaccine.

Dose	No. of men	Mean skin reaction (cm)	95% confidence limits
10 micrograms	28	0.6	0.3-0.8
50 micrograms	27	1.4	1.1-1.8
100 micrograms	26	2.6	2.1-3.1

2. Antigenicity was measured by hemagglutination (HA) response. Four fold or greater titer rises were found within two weeks of injection in all 58 men tested who received either 50 or 100 micrograms. In the 10 microgram group three of 28 tested had no titer rise; titers were 8, 8, 8; 8, 16, 16; and 32, 32, 16 in the 0, 2 and 7 week sera. It may be of significance that all three individuals who failed to respond to 10 micrograms of vaccine had elevated HA titer prior to receiving the vaccine. Summaries of mean number of tubes increase (2-fold dilutions) and geometric means (Table 4) of HA titers in the various dosage groups show

a trend for increasing titers with increasing dose of vaccine. Although the differences are not statistically significant, the combination of lowest titers and presence of three immunization failures with the 10 mcg program dose indicate that a larger dose should be administered in subsequent studies.

Table ... Hemagglutination antibody response to graded doses of group C polysaccharide vaccine ...

Dose mcg/program	No. of men	Mean titer rise at ... weeks	Titer range	Geometric mean titers No. with ... wks.	... wks.	... wks.	No. with ... rise
...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...

... Comparison of skin reaction and serum antibody titers. The presence and size of the skin reaction showed no correlation with pre-immunization HA titer (Table ...).

Table ... Comparison of pre-immunization antibody titer and skin reaction to group ... vaccine.

HA titer	No. of men with skin reaction: Negative	Positive
...	...	...
...	...	...

Furthermore, there was no correlation between skin reaction and the post-HA antibody response. Those men with no visible skin reaction ... had ... and high HA antibody titers whereas men with very large areas of skin erythema and induration ... the highest titers.

Thus, the two parameters [illegible] seems probable that the [illegible] remaining in the predominantly polysaccharide vaccine.

4. Carrier studies: Table 6 shows the results of carrier studies performed at two week intervals. Transmission was [illegible] (34, 27, 29 and 42 percent) and only 4 percent of men carried a group meningococcus in the final survey. Group B was the most frequently identified serogroup; 29 percent were [illegible] in the last survey. Although the number of group C strains was too small to provide useful data on vaccine prevention it is of interest that only two men receiving the 50 microgram dose acquired a group C strain and lost it before the next survey. No group C acquisitions occurred in the 100 microgram group. Two men in the 50 microgram group acquired a C strain; six naive control recruits acquired a C organism. No meningitis cases occurred in this company.

Carrier studies [illegible] compared with [illegible] carrier status at the time of the 3rd and 4th surveys (Table 7).

The percentage of men carrying group [illegible] groups and was 50 percent of naive control men in the 4th survey in Company B-1-1). Thus, although 42 percent of the men in Company B-1-1 were immunised the reduction in group transmission occurred primarily in those men receiving the largest doses of vaccine and transmission among the naive control men was not significantly different from that seen in the non-immunised [illegible] companies.

5. Effect of vaccine on respiratory illness: Thirty-five men were hospitalised with URI during the last training period; six were meningococcal illnesses. The number of men with URI by vaccine group was as follows: naive control - [illegible]; 50 microgram group - [illegible]; 100 microgram group - [illegible]; 200 microgram group - [illegible]. Thus, vaccine did not predispose to nor prevent URI.

[illegible]

[illegible]	[illegible]	Relative Bo.			
		[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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Table 7. Carrier rates for meningococci in three cohort companies

Company	No. of survey	No. men tested	% Pos. Mgc	% group C carriers
C-3-3	3	97	30	10
	4	84	32	17
B-3-3	3	97	36	5
	4	79	63	11
B-3-3	3	138	30	2
	4	138	42	5

2. Comparison of three routes of meningococcal group C polysaccharide vaccine in man.

All previous studies of meningococcal polysaccharide vaccines have utilized the intradermal route of injection. Since future large scale studies would be simplified by use of the jet injector apparatus this mode of immunization was tested in recruit volunteers. Comparison was made with needle injection of the same amount of polysaccharide (50 micrograms) given intradermally (ID) and subcutaneously (SC)

Preliminary tests were carried out on five laboratory volunteers given group C vaccine in 0.5 ml volume by the jet injector apparatus (jet gun) into the deltoid region. Local reactions to the injection were minimal. Two individuals had no visible reaction; three had an area of erythema measuring one cm 24 hours after injection which disappeared by 48 hours. No systemic reactions occurred. All five developed significant HA titer rises in two weeks.

Recruit volunteers (Company E-5-3, Fort Dix) were randomly divided into three groups: ID, SC and jet. Lot C-5 vaccine was used; all volunteers receiving a dose of 50 micrograms. ID and SC groups received 0.2 ml volume; the "jet" group was given 50 micrograms in 0.5 ml volume to minimize leakage losses.

Serological and carrier state studies were performed as before.

Results: ID and jet groups comprised 30 men each; SC group had 23 men; 14 men were not vaccinated. Another 20 men who were not present initially were added to the control group later. No adverse reactions occurred. Although some men in the SC and jet groups developed erythema at the site of injection, these were much less noticeable than in the ID group in which the typical reaction was seen.

Every man who received vaccine showed significant HA antibody response within two weeks. Table 8 shows the mean number of tube rises (2-fold dilutions) and geometric mean titers. Although the jet injected group had the lowest mean, the differences are not statistically significant.

Carrier studies in this company are shown in Table 9. The number of group C meningococcal nasopharyngeal acquisitions was too small for valid comparisons: ID = 1/28, SC = 4/23; jet = 3/30, controls = 4/31. Only 6 percent of men were carriers of group C organisms at the time of the final survey. Two other companies in the same battalion, A-1-3 and B-1-3, in the same week of training had group C carrier rates of 14 percent and 31 percent respectively. This wide variation in group C prevalence in different companies makes it difficult to ascribe to the vaccine the low (6 percent) rate in company E-5-3 in which 70 percent of men were vaccinated.

Table 8. [illegible] polysaccharide [illegible]

Route	Peak rise*	[illegible]	[illegible]
Nonvaccinated	-	-	[illegible]
ID	5.0	[illegible]	[illegible]
SC	5.0	[illegible]	[illegible]
Jet	5.0	[illegible]	[illegible]

*No. of tubes (2-fold dilution)

Thus, booster doses do not always stimulate HA antibody production in those few men who fail to develop antibody after primary injection, and booster doses do not produce a secondary response in men who developed antibody rise to the primary inoculation. Further studies of the nonresponders are being considered to detect possible immunoglobulin deficiencies or to measure immunologic response by some other antibody test.

4. The protective effect of group C polysaccharide vaccine.

Previous studies have shown the general safety and immunogenicity in man of the meningococcal group C polysaccharide. In a small study in recruits it was shown that a local pharyngeal immunity develops following vaccination which protects against acquisition of the carrier state. To demonstrate the ability of the vaccine to protect against overt meningococcal disease was the goal of the present investigation.

Design of the study: A number of factors were considered important in the design of the experiment. The relatively low attack rate of disease, even during epidemics, required a very large sample size in order to demonstrate significant protective effect. Thus, if the attack rate was 4/1000/8 weeks 3,500 men would have to be immunized to show a 90 percent protection and 35,000 vaccinated recruits would be required if the disease rate was 0.4/1000/8 weeks.

Another constraint was that a limit be placed upon the proportion of a given population which would be vaccinated. If more than 20 percent of a group was immunized it seemed probable that a herd immunity would result whereby there might be too few susceptibles to maintain high rates of transmission. A third major factor was the inability to predict when or where an epidemic would occur; thus, several basic training centers would have to be studied simultaneously. Random selection of vaccinated individuals was assured by offering the vaccine to every fifth man on the reception roster.

Description of the study: Five recruit training centers were selected (Fort Dix, Fort Polk, Fort Knox, Fort Bragg and Fort Lewis) and the program began in late January 1969. Lot C-6 vaccine was used initially and was replaced by Lot C-7 at each post except Fort Dix. Immunizations were given by jet injector apparatus; occasionally subcutaneous (SC) administration by needle was used. At Fort Knox only SC injection was used. Following a period of briefing concerning the vaccine, volunteers were immunized with the polysaccharide vaccine. They then received the mandatory poliovirus, tetanus-diphtheria and influenza virus vaccines along with the recruits who refused the experimental vaccine and those who were not offered it. Training cadre and dispensary personnel were alerted to report any adverse reactions to the physician in charge of the program.

Records: An alphabetical file card was prepared on each vaccinated recruit. In addition, reception rosters were marked to indicate those who were immunized. A control group equal in size to the number of men immunized was obtained by selecting the name which appeared immediately following those who were offered the vaccine. This 20 percent control group received no placebo injection; the remaining men were considered a 60 percent control group. Individuals who failed to enter basic training because of administrative reasons or because of AWOL were subtracted from the totals.

To determine the percentage of vaccinated men in training companies, rosters from companies were examined at intervals during the study period.

Meningococcal illnesses were diagnosed by hospital physicians who were not involved in the vaccine study and who were unaware of the individual's vaccination status. Diagnoses were confirmed by routine bacteriological cultures of blood and cerebrospinal fluid, the meningococcal isolates being sent to Walter Reed Army Institute of Research for confirmation. Serologic diagnoses were performed by passive hemagglutination tests on acute and convalescent serum specimens.

Results: Tabulation of the total number of men in the vaccine group for each post is given in Table 12. These numbers have not been adjusted for losses, which were small. No adverse reactions were noted in the group receiving purified group C polysaccharide. Immediate effect of the meningococcal vaccine was slight local discomfort, which was much less severe than that due to the influenza vaccine.

Table 13 shows the percentage of vaccinated men in a sample of 11 training companies at Fort Dix. The percentage of immunized men varied from 13.5 to 24.

The number of confirmed meningococcal illnesses by post and vaccination status are given in Table 14.

These data are incomplete in that a large number of men are still at risk (ie BCT not completed). Fort Lewis had no cases in the group under study at the time of this report and, therefore, is omitted from the table. Assuming a distribution of 20 percent of men vaccinated there is a significant protection against group C disease in the immunized group.

The specificity of the group C polysaccharide vaccine is evidenced by the fact that four group B illnesses occurred in the vaccinated men. Of interest, group B illnesses were more frequent in vaccinated men than in controls. A possible explanation is that carrier studies have shown that total meningococcal carrier rates are similar in

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Table [illegible] Effect of group C vaccine upon meningococcal infections in Army recruits.

Vaccine status	Confirmed cases[1] by serogroup: Fort Dix B	Fort Dix C	Fort Knox B	Fort Knox C	Fort Bragg B	Fort Bragg C	Fort Polk B	Fort Polk C	Totals B	Totals C	Suspect cases[2] total-group C
Group C vaccine	0	0*	2	1	2	0	0	0	4	1**	0
Controls 20%	0	4	0	2	1	0	0	1	1	7	1
80%	0	20	0	7	0	0	1	0	1	27	3
Total control	0	24*	0	9	1	0	1	1	2	34**	4
Total cases	-	24	2	10	3	0	1	1	6	35	4

[1] Blood or CSF - positive culture

[2] Clinical course = gpC HA rise and/or isolate from other source.

* and ** = <0.02

control and vaccinated groups but that group C carrier rates are reduced in those receiving group C vaccine. Thus, these men are more likely to become exposed to group B strains and may, therefore, have a higher risk of group B disease.

Summary and conclusions:

Comparison of 10, 50 and 100 microgram doses of lot C-4 vaccine showed lack of HA antibody response in 11 percent of men who received the low dose; geometric mean titers were also lowest in this group, although the figures were not statistically significant. Since the difference between 50 and 100 micrograms was minimal the 50 microgram dose will be used in subsequent studies. Size of skin reaction following intradermal injection did not correlate with HA response and probably represents a reaction to some material other than polysaccharide.

When administered by jet injector apparatus local reactions to the group C vaccine were minimal and antibody response, although lower, was not significantly different from that following intradermal or subcutaneous injection.

Serologic studies on three lots of group C vaccine show that more than 95 percent of immunized men develop serum HA antibody rise. Booster doses do not appear to effectively stimulate the development of significant antibody increases.

Preliminary results of a large scale field study involving more than 13,000 vaccinated recruit volunteers in five Army basic training centers indicate a significant reduction in meningococcal disease in men receiving group C vaccine. There was no protection against group B disease.

The participation of the following personnel in the field study is gratefully acknowledged: Preventive Medicine Office, OTSG - COL R. Singer and LTC J. Gauld; Dept. of Biologics Research, WRAIR - Dr. J. Lowenthal, Dr. S. Berman, Mrs. P. Altieri; Fort Dix, New Jersey - LTC J. Smith, COL F. McCaleb, and CPT E. Allen; Fort Polk, Louisiana - CPT L. Stickley; Fort Knox, Kentucky - LTC I. Hernandez-Fragoso, CPT R. Motly and CPT T. Moll; Fort Bragg, North Carolina - LTC K. Runcik; and Fort Lewis, Washington - CPT C. Schultz and CPT C. Weidmer.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 181, Development of a meningococcal immunizing agent

Publications.

Goldschneider, I., Gotschlich, E. C., and Artenstein, M. S. Human Immunity to the Meningococcus. I. The Role of Humoral Antibodies. J. Exptl. Med. 129:1307-26, 1969.

Goldschneider, I., Gotschlich, E. C., and Artenstein, M. S. Human Immunity to the Meningococcus. II. Development of Natural Immunity. J. Exptl. Med. 129:1327-48, 1969.

Gotschlich, E. C., Liu, T. Y., and Artenstein, M. S. Human Immunity to the Meningococcus. III. Preparation and Immunochemical Properties of the Group A, Group B and Group C Meningococcal Polysaccharides. J. Exptl. Med. 129:1349-66, 1969.

Gotschlich, E. C., Goldschneider, I., and Artenstein, M. S. Human Immunity to the Meningococcus. IV. Immunogenicity of Group A and Group C Meningococcal Polysaccharides in Human Volunteers. J. Exptl. Med. 129:1367-84, 1969.

Gotschlich, E. C., Goldschneider, I., and Artenstein, M. S. Human Immunity to the Meningococcus. V. The Effect of Immunization with Meningococcal group C Polysaccharide on the Carrier State. J. Exptl. Med. 129:1385-95, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. DATE PREV SUMMARY	2. KIND OF SUMMARY	3. SUMMARY SCTY	4. WORK SECURITY	5. FUNDING	6. DISCIPLINE	7. TYPE OF CODE	8. LEVEL OF SUM
68 11 30	D. CHANGE	U	U	NA	NL	X	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	182
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code) (U) Correlation of Aggression, Dominance, Stress, Combat Defeat with Testosterone, Corticosteroids and Performance. (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
68 08	CONT	DA			B. CONTRACT

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: 68 08 EXPIRATION: 69 07
b. NUMBER: DADA 17-69-C-9014
c. TYPE: S.CT d. AMOUNT: $69,256
e. KIND OF AWARD: NEW f. CUM. AMT. $69,256

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. PROFESSIONAL MAN MONTHS
FISCAL YEAR PRECEDING 69	1	63
FISCAL YEAR CURRENT 70	0.1	6

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
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TELEPHONE: 202-576-3551

PERFORMING ORGANIZATION
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Bernstein, I.S.
TELEPHONE: 404-963-6281
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Rose, R.M.
Levine, M.D.

20. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Stress; (U) Aggression; (U) Testosterone; (U) Performance; (U) Rhesus Monkey

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To conduct a longitudinal examination of social experiences and genetic background that will produce information on crucial variables operating to influence future performance and endocrinological responses to stress.

24. (U) Through a multidisciplinary study by three collaborating laboratories of the endocrine response and performance capabilities of a natural population of rhesus macaques with known genetic and behavioral histories and subjected to programmed stress.

25. (U) 68-08-69 06
Completion on or about 31 July 69 of the four compounds and observation area; training of technicians in behavioral observations; initiation of computer program for longitudinal data analysis.

*Available in contractor's proposal or estimator's approval.

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Project 3A061101A91C INHOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, Inhouse Laboratory Independent Research

Work Unit 182, Correlation of Aggression, Dominance, Stress, Combat Defeat with Testosterone, Corticosteroids and Performance

Investigators.
Principal: Irwin S. Bernstein, Ph.D.
Associate: Robert M. Rose, M.D., Murray D. Levine, Ph.D.

Description.

This study is designed to correlate endocrine responses with behavioral measures of stress in non-human primates. Social groups of non-human primates are to be established in large outdoor compounds with provision for undisturbed observation and captured as required. This project combines the skills and experiences of the Department of Psychiatry, NP Division, Walter Reed Army Institute of Research, the Behavioral Research Laboratory of the Human Engineering Laboratory, Aberdeen Proving Grounds and the Yerkes Regional Primate Center to provide a multidisciplinary study of the variables contributing to the performance and hormonal responses to stress. The Yerkes Center will provide a population of rhesus monkeys (macaca mulatta) with known behavioral histories from the time of birth to fully mature adults. This population will be developed over several years and will be studied periodically at Yerkes, at Aberdeen and at Walter Reed, where appropriate. In addition a population of adult monkeys with known social status and quantitatively measured social relationship will be available to correlate these measures with operant performance, and endocrinological response data. This will be of particular importance regarding hypothetical relationships between hierarchiacal position, aggression, testosterone and future performance during stress. Manipulation of these social groups will be also used to obtain depressed individuals and individuals of markedly changed social status to test for the influence of these variables on endocrine activity.

Progress.

During the first year facilities for conducting this research were designed and construction will be completed by 31 July 1969. Simultaneously with the provision of physical facilities, a behavioral batteryhas been developed to collect the desired data, and technicians trained in the use of this battery. In addition, computer programming was initiated to organize the very large amount of longitudinal data that will be collected on over 100 animals. At Aberdeen design and construction of special metabolism cages was completed which permit simultaneous observation of the aggressive interactions of large rhesus monkeys with continuous collection of urine. At the Department of Psychiatry, NP Division, WRAIR a biochemical laboratory has been completed which perform analysis of plasma and urinary samples from the animals under study for testosterone and cortisol levels.

Summary and Conclusions

This summer the first group of 40 males will be established and after the group has stabilized, the most and least dominant animals will be removed for study of testosterone and cortisol responses during stress. In addition during the course of this coming year two groups of animals with a normal sex and age distribution will be established. This will provide the infants with known behavioral and genetic history to be studied endocrinologically during their response to programmed stress.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 162 Correlation of Aggression, Dominance, Stress, Combat Defeat with Testosterone, Corticosteroids and Performance

Publications

None.

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	611BA	Rabbitology	BA		181	

(U) Diseases of Military Animals in Southeast Asia

010100 Microbiology 005900 Environmental Sc

87 10	BA	BA	C In House

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Russell, MAJ B L

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[redacted]

PII Redacted

Foreign Intelligence Considered

(U) Canine hemorrhagic syndrome (U) Canine encephalomyelitis (U) Filariasis (U) Diarrhea (U) Rabies (U) Brucellosis (U) Anaplasmosis (U) Babesia (U) Leptospirosis

23. (U) To define, study, diagnose, and control known and potential infectious diseases of military dogs in Southeast Asia. Initial consideration will be given to infectious agents known to exist in Southeast Asia. Studies will include the epidemiology and control of canine viruses, bacteria, leptospira, mycoplasma, and parasites.

24. (U) Conventional epidemiologic and microbiologic techniques are employed and new procedures will be developed as needed.

25. (U) 00 01 - 00 06. After successful completion of the preliminary test of diethylcarbamazine and styrylpyridinium to control heartworm, hookworm, and roundworm in stray dogs at Ft Benning, GA, all dogs have been placed on the drugs. Using whole blood shipped to the laboratory we met the two presumptive idiopathic hemorrhagic syndrome (IHS) isolates from Southeast Asia have been made. These isolates have undergone four successive passages in laboratory beagles. Dogs given either passage material develop similar clinical signs following an incubation period of approximately 10 days. These signs include elevation of rectal temperature, high sedimentation rate, and anemia. Two reactive isolates were recovered during enabling mouse passage of IHS materials. These two agents are currently believed to be mouse agents as dogs inoculated with the same material did not develop antibody. In addition, suckling mice, hamsters, ferrets, and a variety of cell cultures have been exposed to material of known infectivity. To date, 120 fatal cases of IHS have been reported from Southeast Asia. All suspects are currently being followed. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 68-30 June 1969.

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Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task (s) In-House Laboratory Independent Research

Work Unit 183, Diseases of military animals in Southeast Asia

Investigators
Principal MAJ D. L. Huxsoll, VC
Associate Dr. L. N. Binn, Ph.D., MAJ P. K. Hildebrandt, VC; COL R. M. Nims, VC, COL S. G. Asbill, VC; CPT J. C. Lewis, VC, MAJ D. C. Zeller, VC, I. E. Hemelt, A.B.; A. R. Werner, Jr.

Description

To define, study, diagnose and control known, potential, and unknown infectious diseases of military dogs in Southeast Asia. Initial consideration has been given to investigation of infectious diseases affecting military animals in Southeast Asia. Studies include the epidemiology, pathogenesis, and control of infectious diseases of known and unknown etiology.

Progress

1. Hemorrhagic Disease of Military Dogs in Southeast Asia. In 1968 an epizootic of a highly fatal hemorrhagic disease occurred in U. S. military dogs in Southeast Asia. To date, over 100 fatal cases are known to have occurred. The disease appears to be the same as that observed in British military dogs in Singapore. The disease has been called "Trecker Dog Disease", "Idiopathic Epistaxis", "Canine Hemorrhagic Fever" "Tropical Canine Pancytopenia", and "Idiopathic Hemorrhagic Syndrome". A hemorrhagic disease resembling the disease in Southeast Asia has been observed also in French military dogs in Tunisia.

- The Disease in Singapore. The disease was first encountered late in 1963 at the R.A.F. Station, Changi, Singapore, and in 1964 at the 1 Gurkha Dog Company, Singapore. The mortality rate was high in Army and R.A.F. dogs during 1964. In the early part of 1965, there was an apparent decrease in the morbidity of the condition, but this was followed by an acute flare-up in September 1965. Between September 1965 and June 1967, a large number of dogs died or were destroyed because of the disease (Wilkins, J. N., et al. Vet. Rec., p.57, July 8, 1967). Both military and civilian dogs have been affected.

For the most part, the clinical and pathological features of the disease in Singapore are the same as those observed in U. S. military dogs in Southeast Asia. Mac Yean (Malaysian Kennel Review No. 66, December 1968) has reported that the incidence of disease among Alsatians appears to be higher than for any other breed of dogs and purebred dogs seem to be affected more often than local mixed breeds

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Singapore ... reported to ... 100 fatal...

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Based ... to Mad Medani
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1967). ... reported
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an infected case ... sible to transmit
the disease ... and filtered
through a ... From these
findings, ... found

British ... to 30-40 days
after ... ted dogs, however
they ... doubling mice,
rabbits ... through experimental
inoculation ...

The disease ... act since normal
dogs sharing ... do not develop
the disease ... transmitted by an
arthropod ... can be controlled
through ... ticks (Mac Vean,
D. W. ...

... hemorrhagic disease
described by ... 31-40 1961)
and ... 117-136) in French
military ... disease observed
in dogs ... seems to affect
only ... rich in the pre-
dominant ... seem have been
observed ... recognized in
Tunisia ... occurred sporad-
ically ... in 1958 nearly

half of a total population of 140 military dogs from France and Germany developed the disease. The most spectacular sign was epistaxis which occurred in approximately 30% of the cases. Other prominent signs were fever, hepatic and renal pain, albuminuria, anorexia and intense thirst, congestion of mucous membranes which later became pale, loss of weight, and dull hair coat. Erythrocyte counts were highly variable. Leukocytes increased at first, but as the disease progressed a severe leucopenia developed. In terminal stages the number of lymphocytes far exceeded that of neutrophilic polymorphs. Affected dogs had a thrombocytopenia. Coagulation and prothrombin times were normal but bleeding time was increased.

Post mortem examination revealed widespread hemorrhages. In some cases, hemorrhage was found in nearly all organs. Histologic lesions of the kidney were consistent. These included thickening of Bowman's capsule with cellular proliferation, thrombosis of numerous arterioles and capillaries, endothelial and perithelial proliferation, and lymphocytic or plasmacytic infiltration around arterioles. Thrombosis of arterioles was also found in liver, lungs, spleen, and myocardium.

Outbreaks of disease corresponded to periods of infestation with ticks. Elimination of ticks controlled the disease.

Numerous attempts were made to isolate the etiologic agent. Since leishmania was found in a large number of cases and is known to attack the vascular and reticuloendothelial system, this organism was considered the possible etiologic agent of the disease.

The French observed that in order for a dog to contract the disease it must live in Tunisia for a given length of time, usually six months to one year but sometimes longer.

c. Disease in U. S. Military Dogs. During late 1966 and early 1967 the U. S. Army purchased approximately 30 Labrador breed "Tracker" dogs from the British Army in Malaya. These dogs were shipped to South Vietnam for military use. Within a few months after their arrival in South Vietnam, several of these dogs developed disease characterized by epistaxis, lymphadenopathy, and corneal opacity. Laboratory tests revealed anemia and elevated blood urea nitrogen.

The first recognized deaths in American origin German Shepherd type military dogs occurred in mid-September 1968. Retrospective studies of case reports show that there was a death strongly suspicious of the hemorrhagic disease on 26 July 1968 and another on 23 August 1968. The disease has occurred in at least 25 Army units. Air Force and Marine units are also involved.

The disease was reported to have been found in the non-military dog population in Vietnam starting in January 1969. Cases have also been reported in ARVN (Army of Republic of Vietnam) military dogs with several deaths to date.

The tick appears to be intimately associated with the disease. Outbreaks and peak incidence have corresponded to periods of severe tick infestations.

In most instances, the dog is presented with unilateral or bilateral epistaxis with no report of prior clinical signs. The dog may die within one day of the onset of hemorrhage, although the average is five to seven days. Some dogs survive this phase and become "chronic" cases.

Based on studies of normal dogs in Vietnam, an arbitrary rule of thumb has been used to classify any dog with a WBC of less than 7,000 and/or a hematocrit of less than 40% as a "suspect". A high percentage of dogs which consistently exhibited such findings have succumbed to the disease.

d. Clinical Findings. The most frequently observed signs are epistaxis, loss of weight, posterior weakness, vomition and dehydration in some chronic cases, lethargy, ecchymotic hemorrhages especially on the abdomen and between the toes, anemia, edema of limbs and scrotum, and petechial hemorrhages on the penis.

Hematologic examinations have revealed a normochromic, normocytic anemia. In many instances, there is also a severe leucopenia with white blood cell counts falling below 500. In chronic cases, BUN and creatinine levels may be elevated. Also, some dogs have shown an increase in SGOT and alkaline phosphatase levels. Coagulation time is normal, but bleeding time is greatly prolonged. The sedimentation rate is accelerated in some cases.

Examination of clinical records has revealed that the disease may commence with a febrile episode of one to four days duration accompanied by a moderate leucopenia. At this time, the dog may be lethargic and lack stamina. In most instances, there is also an unexplained weight loss. Following this the dog becomes clinically normal, although hematologic examination discloses a variable WBC and anemia. Epistaxis and severe anemia or leucopenia appear approximately 90 days following the initial febrile episode, although this interval may vary greatly.

Plasma specimens from cases in Southeast Asia have been examined for all known clotting factors by LTC Tuthill of the Coagulation Laboratory, Department of Hematology, WRAIR. No abnormalities were observed.

Serum electrophoresis studies were made on sera from affected dogs by Dr. Beisel at the U. S. Army Medical Unit, Fort Detrick, Maryland. In general, the electrophoretic patterns were characterized by an increase in gamma-globulins and a decrease in alpha-1-globulins and albumin. The beta and alpha-2 fractions were usually normal, but in a few cases were increased. Serum glycoprotein determination revealed an increase in gamma-glycoglobulin and a decrease in the alpha-1-glycoglobulin and albumin fractions. The increased gamma-globulin levels suggest that

the disease was diagnosed after the acute immunologic response had occurred. Based on serum electrophoretic studies made in human infections, Dr. Beisel suggested that the disease might be viral as opposed to bacterial.

Sera from affected dogs have been serologically tested with numerous rickettsial antigens by Dr. Elisberg of the Department of Rickettsial Diseases, WRAIR. Antibody was not demonstrated in any of the sera examined.

Serum specimens are also being tested serologically for antibody to numerous viruses. These tests have not been completed. Antibody to Group B arboviruses has been demonstrated in some sera with the HI test (Brandt, W., Department of Virus Diseases, WRAIR); however, these results may not be significant, since Japanese encephalitis virus which is found in this area is known to infect dogs.

e. Pathology Studies. In January 1969, the Walter Reed Army Institute of Research was requested to send a team to Southeast Asia to investigate this outbreak. This provided an opportunity to make intensive post mortem examinations and collect specimens for histopathological examination. In addition, tissues from other cases in Southeast Asia were collected at necropsy, fixed in 10% formalin and shipped to the Department of Veterinary Pathology, Division of Veterinary Medicine. To date, over 50 cases have been examined and macroscopic and microscopic findings have generally been consistent.

There is usually evidence of weight loss, rough hair coat, and a focal area of moist dermatitis maybe present somewhere on the body. There may be swelling of some portion of one or more limbs. Blood often oozes from nostrils. A few cases have corneal opacity.

On primary incision, thin watery blood will ooze from cut vessels. Subcutaneous edema and hemorrhage account for the swelling noted on limbs.

Lymph nodes throughout the carcass are somewhat swollen and rusty to dark brown in color, the color change being more prominent in the medullary areas. Some nodes are hemorrhagic and the hemorrhage appears to be of recent origin.

There are petechial to ecchymotic hemorrhages in various organs, most consistently on serosal and mucosal surfaces of the urinary bladder, the prostate, and the testicle. The gastrointestinal tract often has small focal hemorrhages. In some, these may be rather diffuse (paintbrush hemorrhage). The colon usually contains dark bloody feces.

The spleen is normal in size and color; however, the consistency is somewhat firmer than usual.

The liver is light tan in color. Size is within normal limits. Lobules are often more discernable than usual.

Kidneys are pale to tan in color, often with petechial hemorrhages near the cortico-medullary junction.

A common finding in the thoracic cavity is petechial hemorrhages on the parietal pleura and a few petechiae on the surface of the lungs.

There are often hemorrhages in the tonsils and the mucosa of the nasopharynx.

In dogs with epistaxis, a large blood clot is present in and among the nasal turbinates.

f. Microscopic Pathology. The most prominent and consistent change is a plasmacytosis in most organs, the extent and intensity increasing with a prolonged course of illness.

There is a varying degree of hemorrhage in the subepicardial and endocardial areas of the heart. Occasionally, a few extravasated red blood cells are noted between myocardial fibers. Small blood vessels are cuffed with mononuclear cells, the majority being plasma cells. This cuffing is more common near the endocardial and epicardial surfaces. In one case, a focal disseminated non-suppurative myocarditis was observed. Numerous small foci of necrotic and degenerated myocardial fibers with aggregates of mononuclear cells were scattered throughout the myocardium.

There have been few changes observed in the lung, although focal areas of hemorrhage have been observed in some cases and also focal areas of vasculitis. This vasculitis consists of focal subendothelial infiltration of mononuclear cells mixed with dead and degenerated polymorphonuclear cells. Occasionally, this inflammatory focus extends into the media and is associated with a fibrinoid necrosis. There may be a mononuclear cell accumulation about small and medium sized blood vessels. The congestion and edema seen in some lungs may well be a post mortem change.

The liver has a rather consistent change of varying intensity in the central lobular area. In its mildest form it consists of hepatic cell degeneration. In more severe cases, the hepatic cell population in the central lobular area is reduced, the space occupied by dilated sinusoids and a condensation of residual reticular tissue or an increased amount of fibrous tissue. There may be a superimposed acute central lobular coagulative necrosis involving 1/4 to 1/2 of the lobule.

The Kupffer cells may be moderately increased in number and often contain a yellowish-brown pigment interpreted as hemosiderin. There may be a mild periportal infiltrate of plasma cells

In only one case were changes observed in the adrenal glands. In this case, a plasma cell infiltrate was present in the subcapsular area and several focal areas of necrosis were present in the cortex.

The kidneys have a varying degree of mononuclear cell infiltrate consisting primarily of plasma cells. This is most intense in the cortico-medullary area surrounding blood vessels and subcapsular veins. This infiltrate partially or completely surrounds many glomeruli.

No ante mortem changes have been noted in tubular epithelial cells. Many tubules often have a proteinaceous appearing material in the lumen.

Occasionally, glomeruli appear to have thickened capillary tufts. This is more prominent in chronic cases. The tracker dogs which have been ill for several months have severe glomerulosclerosis.

The gastrointestinal tract has varying degrees of hemorrhage in the lamina propria of the mucosa or in the submucosa. Occasional small vessels will have a small aggregate of plasma cells at their periphery.

The pancreas is not remarkable. In an occasional dog, the supporting connective tissue stroma will be infiltrated with plasma cells.

The urinary bladder often has focal areas of hemorrhage in the submucosa, sometime extending between fibers of the muscular layer. Blood vessels will occasionally have a cuff of plasma cells.

The testicle and prostate often have small focal hemorrhages confined to the connective tissue stroma. Plasma cell infiltrates are present in the stroma only and more numerous about blood vessels. No changes of the glandular parenchyma have been observed.

No parenchymal changes have been noted in the thyroid and pituitary glands; however, plasma cell infiltration may be present in the connective tissue capsule and stroma.

In the spleen, splenic corpuscles are present but not numerous and are not particularly active. Few mitotic lymphocytes are observed. There is an increased number of plasma cells at the periphery of the corpuscle and throughout the pulp. There may be moderate hemosiderosis.

Most lymph nodes have dilated medullary sinuses with evidence of active phagocytosis of lymphocytes. At the periphery of the node, follicles though present, are not particularly numerous nor particularly active. There are few mitotic lymphocytes. Many plasma cells are present, especially in the para-cortex and medullary areas. Hemosiderosis is quite prominent in most nodes. The amount of blood is variable in each case and with each dog.

Although the lesions observed in the liver are minimal, a consistent finding is a plasmacytosis of the portal areas. This may be quite subtle and only around blood vessels in other cases, it is quite heavy and will include cuffing of vessels and the parenchyma. Several cases have focal areas of inflammation and necrosis of the parenchyma not associated with blood vessels. These areas consist of small nodules

and malacia with gitter cells and are found most often in the mid-brain and brain stem.

There is often a cuff of plasma cells surrounding vessels in the retina and sclera of the eye. A few eyes have had posterior retinal hemorrhages.

The subcutaneous lesions consist essentially of edema and extravasated red blood cells and a few mononuclear inflammatory cells.

The cell population of the bone marrow is depressed with the myeloid elements most severely affected. Megakaryocytes are reduced in numbers and in some cases are not recognized.

Muscular tissue is consistently normal.

g. Transmission Studies. Tissues for transmission studies were received from eight dogs which died in Southeast Asia with typical signs of the hemorrhagic disease. These tissues were collected aseptically during necropsy examinations and were shipped on dry ice or in liquid nitrogen. Tissues were also received from one dog with a fever of undetermined origin (FUO). In addition, fresh whole blood specimens from 11 dogs with hemorrhagic disease were shipped from Southeast Asia at 4° C. One of the blood specimens was collected from a dog (Honda) which developed signs of the disease after being inoculated intravenously with blood from a natural case.

Twenty percent tissue suspensions were prepared in veal infusion broth with 0.5% bovine albumin. Antibiotics were not used. Suspensions were cultured; however, no significant bacterial isolations were made. All whole blood specimens shipped at 4° C were inoculated into dogs immediately upon receipt in the laboratory.

Beagles 14 to 16 weeks of age were used for transmission studies. All materials were inoculated intraperitoneally with the exception of fresh whole blood which was inoculated intravenously. To facilitate examination of large numbers of tissues, some inocula consisted of pools of tissue suspensions. Two dogs were inoculated with each inoculum. Following inoculation, the dogs were examined and rectal temperatures recorded twice daily. Hematologic examinations including hematocrit, hemoglobin, wbc, differential, and erythrocyte sedimentation rate were made daily. Twice weekly, throat, nasal and rectal swabs, and blood were collected and preserved for virus isolation. Other tests performed twice weekly included BUN, SGPT, coagulation time and prothrombin time. Serum specimens were collected and frozen weekly. In later studies, the daily hematologic examinations were reduced to three per week.

Isolation attempts were also made in suckling mice less than 48 hours old. The mice were inoculated intracerebrally with 0.02 ml of inoculum and intraperitoneally with 0.05 ml. Two litters were inoculated with the 20% tissue suspension and to overcome any possible interference, two litters were also inoculated with a 1% suspension. If no signs of

disease occurred, several mice from each litter were harvested 10 days post inoculation for blind passage. All mice which died or developed signs of disease were harvested and passed. All suckling mice were observed for 21 days.

Weanling mice were also inoculated with 20% and 1% tissue suspension. Each of five mice was inoculated intracerebrally with 0.03 ml and intraperitoneally with 0.1 ml of inoculum. Weanling mice were observed closely for signs of disease for 30 days.

Twenty percent tissue suspensions containing 1,000 units of penicillin and 1 mg of streptomycin per ml were inoculated into cell cultures of primary dog kidney and thymus, Walter Reed continuous canine cell line, the Madin-Darby canine kidney cell line, baby hamster kidney (BHK21) and Vero monkey kidney cell line. The cultures were examined for 14 days at which time they were passed.

To date, there has been no evidence of transmission of the disease to laboratory beagles inoculated with tissue suspensions prepared from frozen tissue shipped to the laboratory. This is based on the lack of any evidence of disease in inoculated dogs during a 60 days observation period and the failure to demonstrate any lesions in the inoculated dogs at necropsy.

In addition, no agent has been recovered in cell cultures inoculated with tissue suspensions prepared from frozen tissue.

Two isolations have been made in suckling mice inoculated with tissue suspensions prepared from frozen tissues of two different dogs from Vietnam. Both isolates produced cytoplasmic inclusions in neurons of mice inoculated intracerebrally. The agents produced CPE in primary dog kidney and KHK21 cells with large cytoplasmic inclusions which fluoresced green when stained with acridine orange and examined in U.V. light. The two isolates hemagglutinate human "O" cells and are resistant to ether and chloroform. These agents had the chemical and biological characteristics of Reovirus and Reovirus type III antiserum inhibited hemagglutination of the isolates to one-fourth the homologous titer. No cross reactions were observed with Reovirus type I and II.

The two suckling mouse agents were probably recovered from the mice used in the isolation studies. This is based on the fact that antibody to the agents and Reoviruses type I, II, and III could not be demonstrated in the sera of dogs inoculated with the same tissue suspensions used to inoculate the mice. Furthermore, dogs which have developed signs of disease following inoculation with fresh whole blood specimens and are described below did not develop antibody to the suckling mouse agents or Reoviruses type I, II, and III.

Transmissible agents have been recovered from fresh whole blood specimens from two cases in Southeast Asia. One specimen was collected from a dog (Honda) which had been previously inoculated intravenously with blood from a natural case in a sentry dog. Approximately five days post inoculation, Honda became febrile and a drop in white blood cells was noted. The blood specimen submitted to this laboratory was collected in citrate 39 days post inoculation. One hundred ml of blood was inoculated intravenously into dog A25. Five days post inoculation, dog A25 became febrile and a drop in packed cell volume and leukocytes was observed. This was accompanied by an acceleration in sedimentation rate. The dog then appeared to return to normal; however, the signs reappeared approximately 12 days post inoculation and persisted for three weeks (Fig 1).

During this time, the dog showed a marked loss of weight. Temperature and sedimentation rate then returned to normal although the dog remained anemic. At 50 days post inoculation, the dog appeared to be recovering from the anemia. This dog has been followed for 120 days. At 65 days post inoculation, it appeared to relapse; however, at present it is clinically normal and hematologic studies reveal no abnormalities. Babesia was identified in the blood of the dog.

At 33 days post inoculation, 40 ml of blood was collected in citrate from dog A25 and each of four dogs was inoculated intravenously with 10 ml of the blood. All dogs became febrile and hematologic studies revealed an accelerated erythrocyte sedimentation rate, a drop in leukocytes, and anemia. This is illustrated in Fig 2 using dog A47 as a typical example.

Since Babesia was identified in dog A25 and subsequent passages, the effect of an antibabesial drug on the course of the disease was examined. Four dogs were used in the study. Two dogs were given Berenil (Farbwerke-Hoechst Ag) at the rate of 0.25 mg per kg of body weight two days preinoculation and continued seven days post inoculation. All four dogs were inoculated with 10 ml of blood collected 55 days post inoculation from dog A25. All four dogs developed the usual signs of disease including fever and marked loss of weight. Laboratory studies revealed an elevated erythrocyte sedimentation rate and a drop in leukocyte and anemia. However, the onset of signs differed in the treated and untreated groups. The untreated group became febrile three to four days post inoculation while in the treated group no evidence of disease occurred until 15 days post inoculation. Babesia were identified in the blood of the untreated dogs; whereas, Babesia could not be demonstrated in the treated dogs. These findings served to suggest that an agent other than Babesia may be involved.

The Honda isolate which was first recovered in dog A25 has undergone four successful passages in laboratory beagles. Signs of disease and laboratory findings have been similar in all passages.

Fig. 1 Fluctuations in body temperature, leukocyte count, packed cell volume, and erythrocyte sedimentation rate of dog A-25 following inoculation.

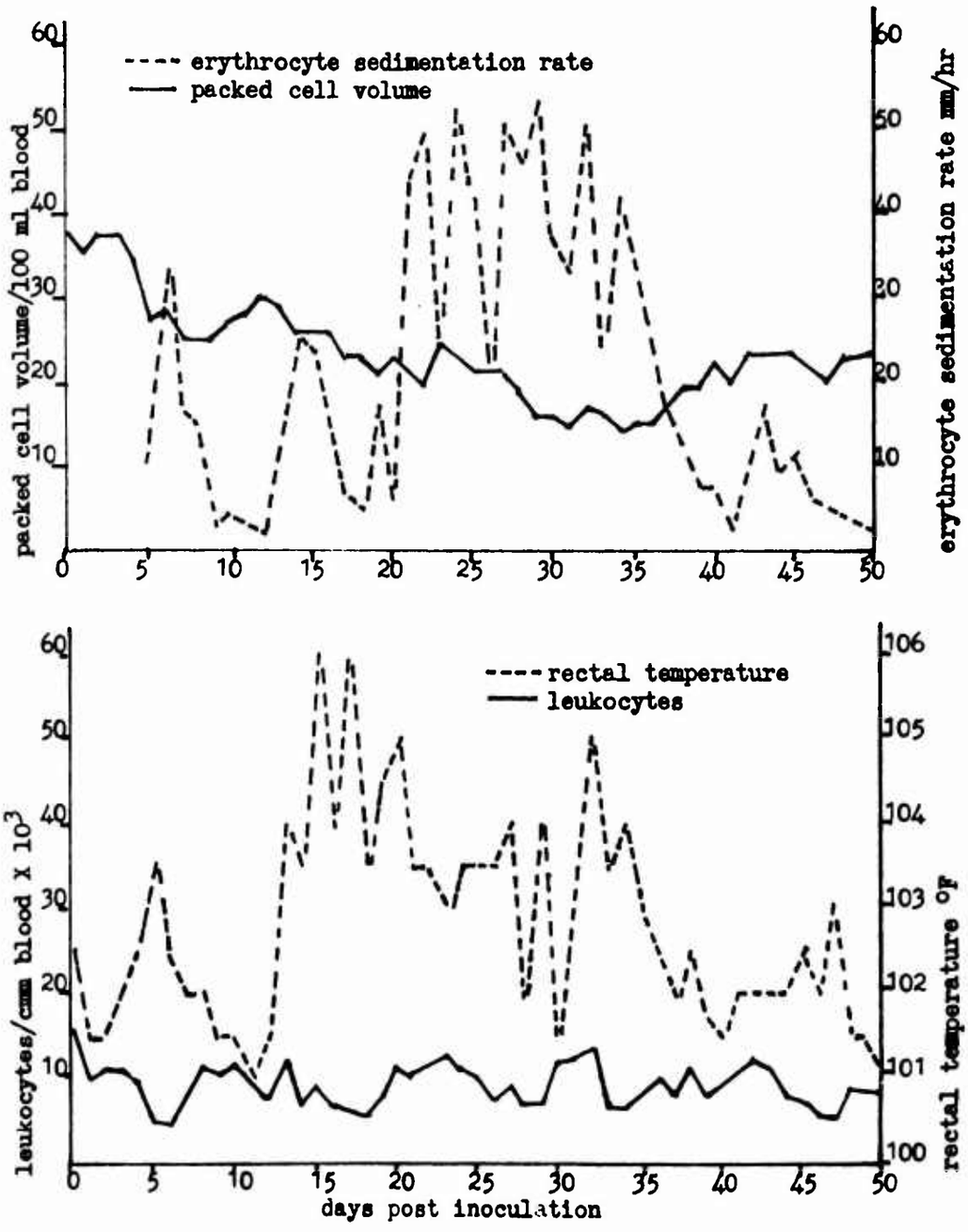
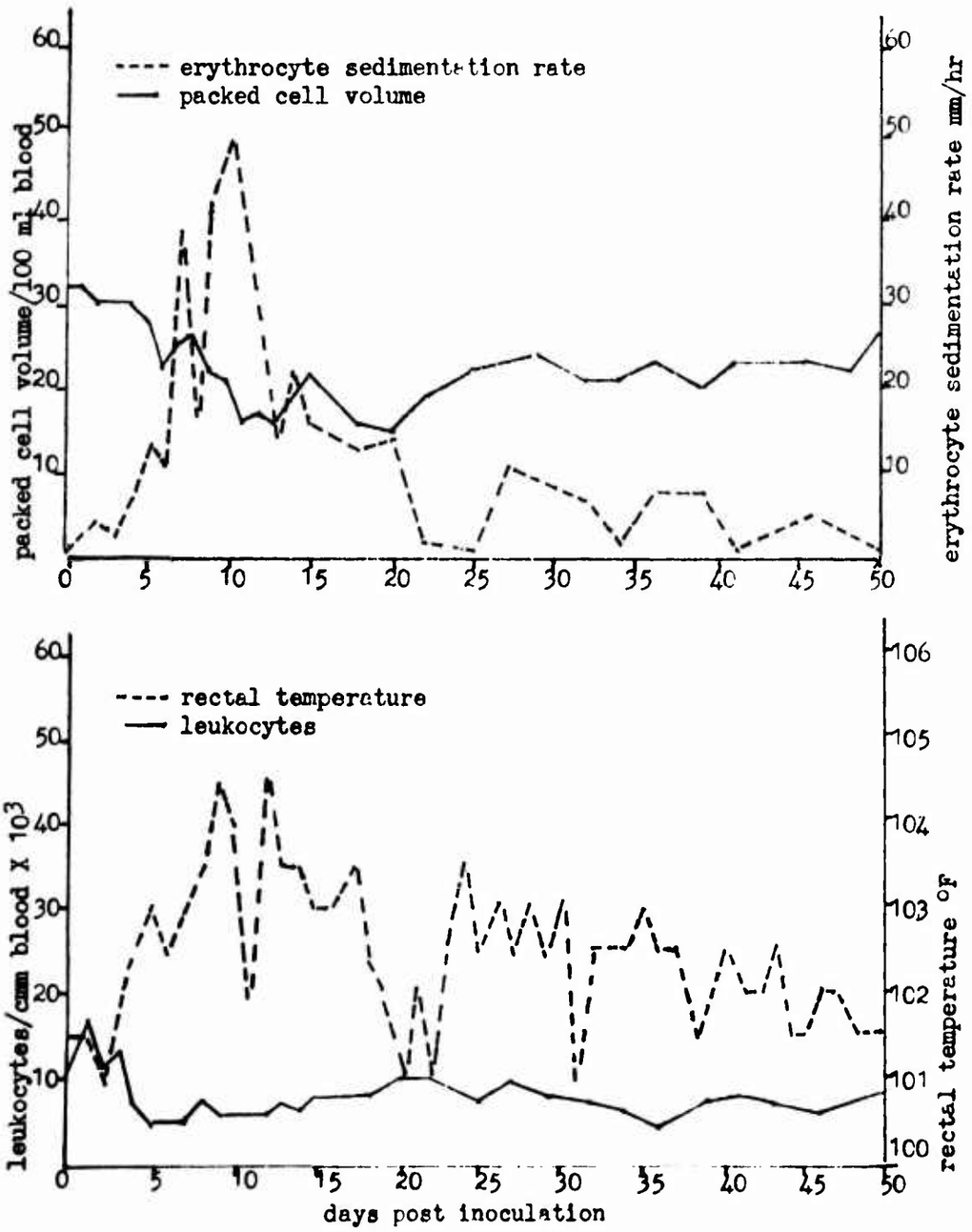


Fig. 2 Fluctuations in body temperature, leukocyte count, packed cell volume, and erythrocyte sedimentation rate of dog A-47 following inoculation.



A disease similar to that produced by the Honda isolate has also been produced in a laboratory beagle inoculated with whole fresh blood from a chronic bleeder (Schatzie 2A69). The 10 ml blood specimen was shipped at 4° C and upon receipt in the laboratory was inoculated intravenously into a laboratory beagle (A39). Twenty days post inoculation, this dog became febrile and laboratory studies revealed an accompanying acceleration in erythrocyte sedimentation rate and a drop in leukocytes and packed cell volume (Fig 3). The dog remained febrile for approximately seven days and at 50 days post inoculation there was evidence that the dog was recovering from the anemia. This isolate has also been successfully passed four times in laboratory beagles. On second passage, the incubation period was reduced from 20 to 10 days (Fig 4); however, a further reduction in incubation did not occur in third passage (Fig 5). The signs of disease and hematologic findings in second, third, and fourth passages were essentially the same as in the original passage.

Babesia have not been observed in blood smears of any dogs inoculated with the Schatzie isolate, although numerous blood smears stained with Giemsa and Acridine orange have been examined.

The Honda isolate has an incubation period of five days; whereas, the incubation period of the Schatzie isolate is ten days. The presence of Babesia in the Honda material may account for the difference in incubation period. The signs of disease produced by the two isolates are essentially the same.

Impressions made from lung tissue at post mortem examination of dogs inoculated with either the Honda or Schatzie isolates contained mononuclear cells with large cytoplasmic inclusions. These inclusions appear to consist of aggregates of small elementary bodies and are also present in lesser numbers in mononuclear cells of the spleen and kidney. Attempts to transfer material of known infectivity to other host systems are still in progress.

2. Evaluation of Chemoprophylactics for Control of Heartworm and Hookworm in Military Dogs. Anclyostomiasis (hookworm infestation) and Dirofilariasis (heartworm infestation) are serious problems in military dogs in CONUS and Vietnam. At Fort Benning, Georgia, where scout dogs are trained for duty in Vietnam, hookworm ova have been demonstrated in as many as 70% of the dogs. The incidence of microfilariasis varies. In some instances, microfilaria of Dirofilaris immitis have been demonstrated with a Knotts' test in 10% of the dogs.

Treating dogs for hookworm and heartworm infestations poses several problems. Animals to be treated may be debilitated and may succumb to secondary bacterial or viral infections. Damage caused by heartworms to the heart and pulmonary vessels may be irreparable, and the treatment itself may require four to six weeks. Prophylaxis seems to be the best answer to the problem.

Fig. 3 Fluctuations in body temperature, leukocyte count, packed cell volume, and erythrocyte sedimentation rate of dog A-39 following inoculation.

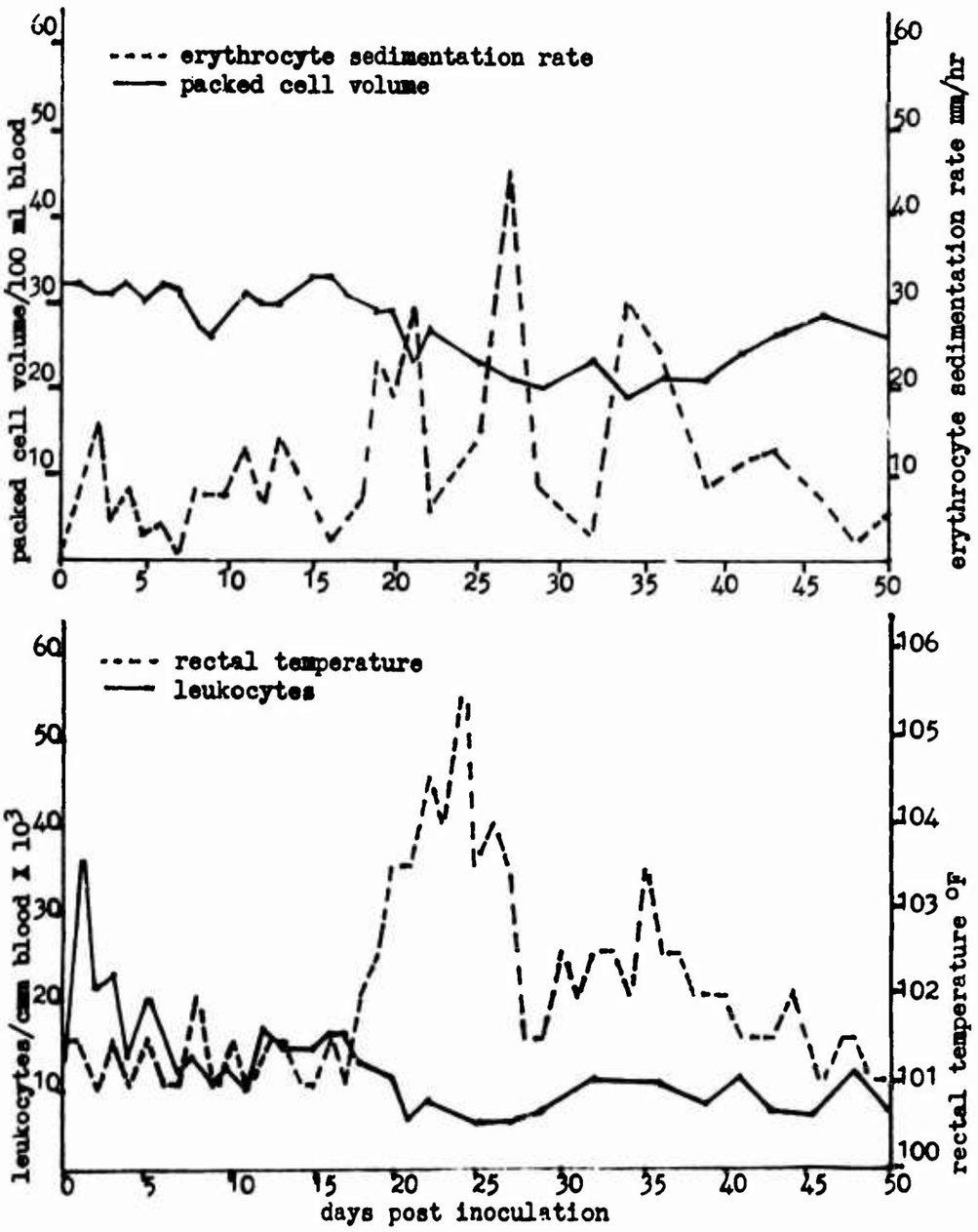


Fig. 4 Fluctuations in body temperature, leukocyte count, packed cell volume, and erythrocyte sedimentation rate of dog A-40 following inoculation.

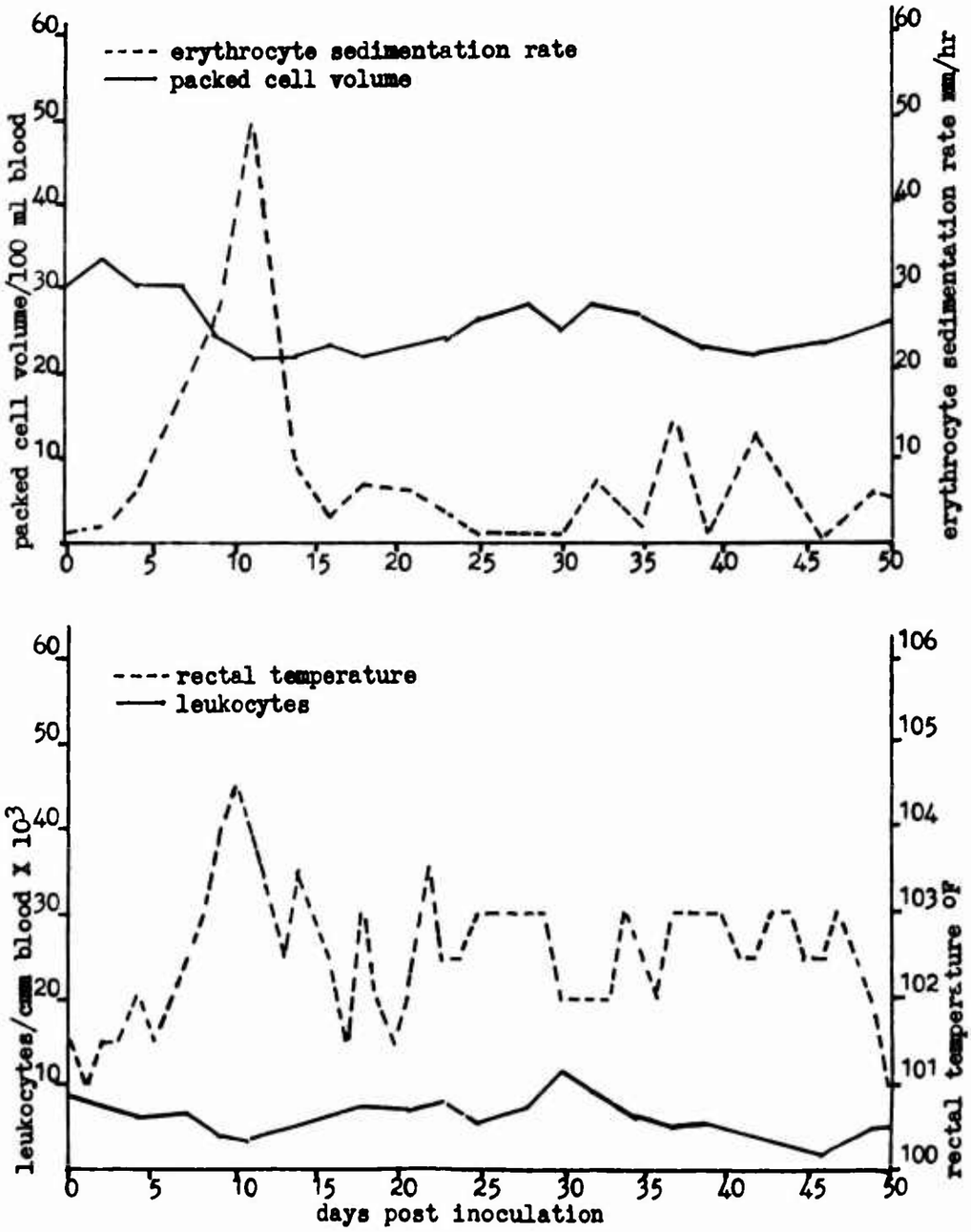
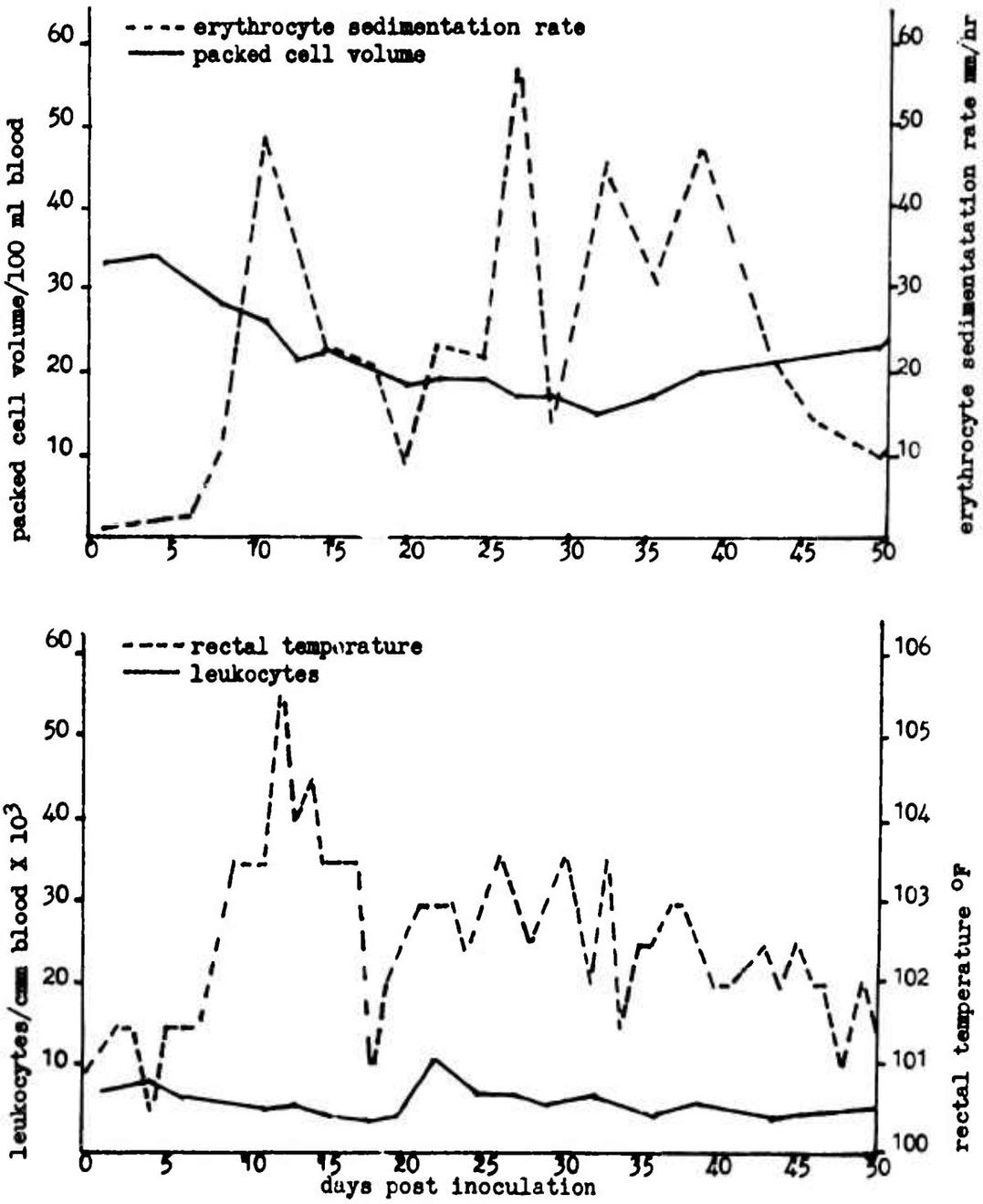


Fig. 5 Fluctuations in body temperature, leukocyte count, packed cell volume, and erythrocyte sedimentation rate of dog A-69 following inoculation.



Diethylcarbamazine (CARICIDE) has been shown to be an effective prophylactic for heartworm infestation (G. S. Tullock, et al. Report from School of Aerospace Medicine, Brooks Air Force Base, 1968). Styrylpyridinum (STYRID), developed by the American Cyanamid Company, has been shown to be effective in control of hookworm infections and can be used prophylactically by daily administration. A STYRID-CARICIDE combination is available from the American Cyanimide Company in limited quantities for experimental studies and field trials. This combination is purported to control hookworms, roundworms, and heartworms. Cooperative studies were established at Fort Benning, Georgia to test the efficacy of STYRID-CARICIDE in military dogs.

One training unit of 88 dogs was divided into two groups. Forty-two dogs were placed on daily doses of 200 mg STYRID and 400 mg CARICIDE (diethylcarbamazine citrate) in tablet form. The remaining 46 dogs in the unit were not treated.

Another unit of 50 dogs was divided into two groups to evaluate the efficacy of the drugs when administered in liquid form daily in the feed. Of the 50 dogs, 30 were treated daily with 7 cc of STYRID-CARICIDE Oral Liquid, which contained 524 mg styrylpyridinum and 210 mg diethylcarbamazine base. Twenty dogs were used as controls and were not treated.

Body weight and results of fecal examinations, hematocrit, and Knotts' tests were recorded at two week intervals during the duration of the field trial. In addition, the performance of the dogs was rated and recorded using the systems under which all dogs at the training center are rated.

The results of fecal examinations are summarized in Table 1. Parasite ova were not found in any of the treated dogs during the 10 week period.

In the early part of the trial period, it became evident that dogs receiving the drug daily in a liquid form in the feed did not gain weight as readily as the controls in the same unit, Table 2. This difference was not as readily apparent at the end of the 10 week trial period. The oral liquid has a very bitter taste and when a dog is placed on the drug the total food intake may be initially reduced. As the dog becomes accustomed to the taste, food intake is not impaired.

Hematocrit values of all dogs remained in the normal range and no significant difference in performance was noted between treated and untreated dogs.

Microfilaria were not found in the blood of any dog during this period. It was impossible to evaluate the effectiveness of the drug for control of Dirofilarisis immitis.

It is apparent from preliminary observations that the drugs provide effective control of intestinal parasites, cause no side effect, and do not impair performance.

TABLE 1

Results of Fecal Examinations of Dogs on Styrid-Caricide Field Trial

At Ft. Benning, Georgia

Number/Percent*

Group	Initial	2 Weeks	4 Weeks	6 Weeks	8 Weeks	10 Weeks
Tablet Control 46 dogs	15/32.6	7/15.2	5/10.9	6/13.0	2/4.3	1/2.2
Tablet Treated 42 dogs	7/16.6	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
Liquid Control 20 dogs	0/0.0	6/30.0	1/5.0	2/10.0	0/0.0	0/0.0
Liquid Treated 30 dogs	4/13.3	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0

*Number/percent of dogs positive for hookworms and/or roundworms

TABLE 2

Variations in Body Weight of Dogs on Styrid-Caricide Field Trial

At Ft. Benning, Georgia

Gained/Lost*

Group	2 Weeks	4 Weeks	6 Weeks	8 Weeks	10 Weeks
Tablet Control 46 dogs	3/0	3/1	5/1	5/0	3/4
Tablet Treated 42 dogs	1/0	2/0	3/2	5/3	2/4
Liquid Control 20 dogs	1/0	6/0	7/0	15/0	6/0
Liquid Treated 30 dogs	0/1	1/2	1/1	11/1	4/0

*Expressed as number of dogs gaining or losing more than 5 lbs. as compared to their initial weight

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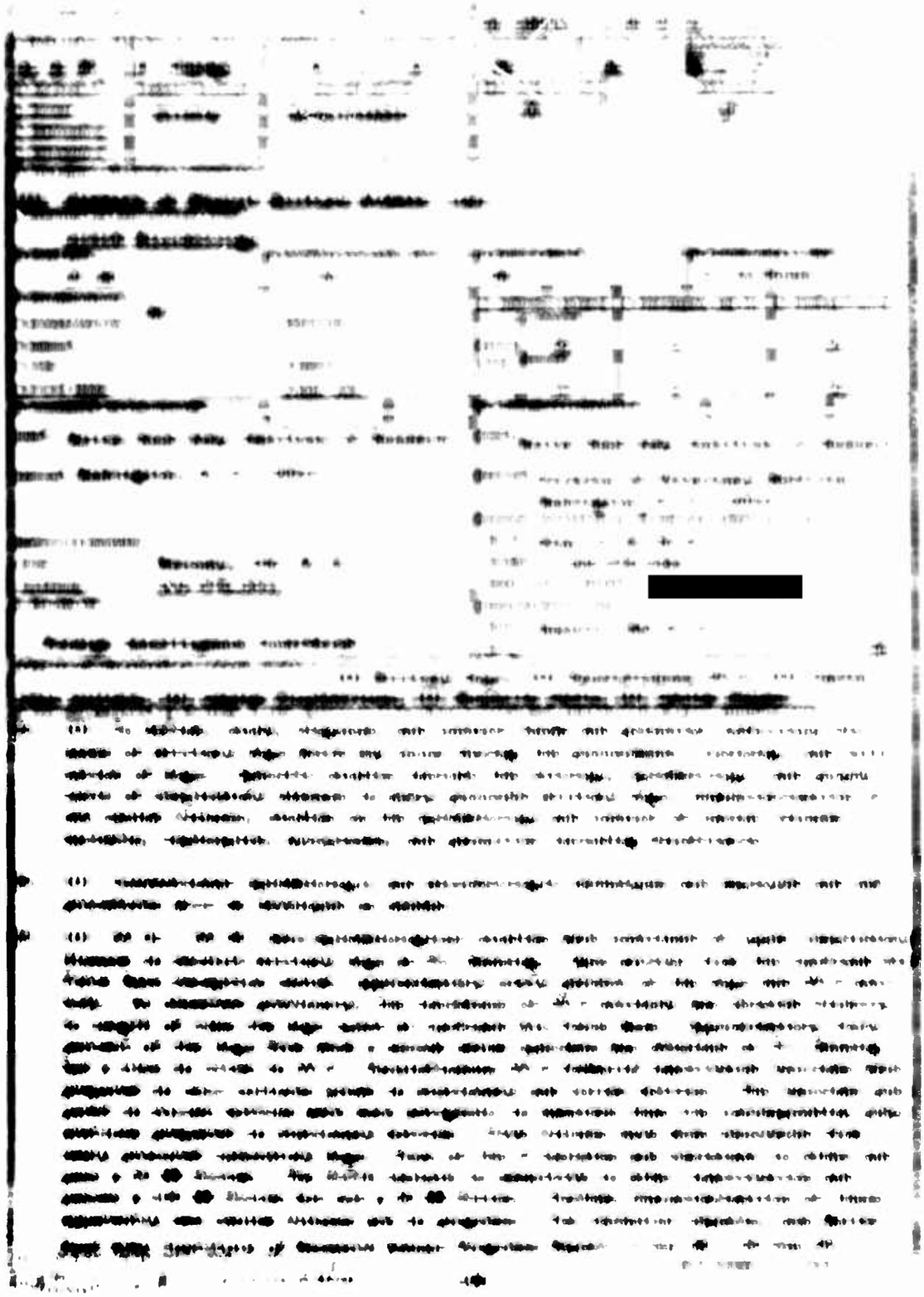

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Table 1 [illegible]

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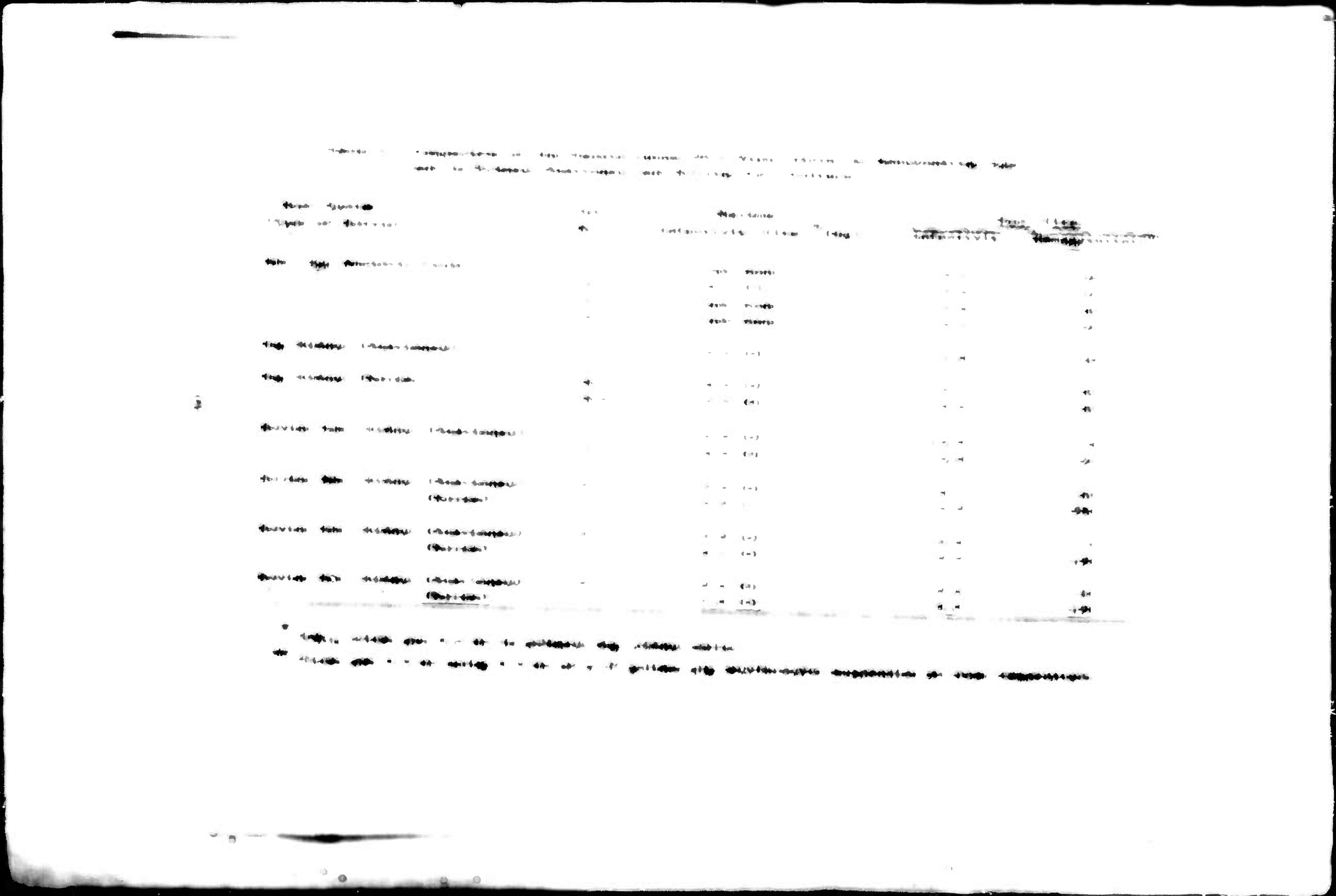

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Table 4. Antibody Response of Hamsters Given Formalin-Inactivated Rabies Vaccine*

Vaccine Tested	Lot No.	No. of Doses	No. Seropositive/No. Tested (Geometric Mean Titer)	
Embryonic Egg Amniotic Fluid	1	1	7/8	(8)
		2	8/8	(256)
	2	1	1/6	(16)
		2	6/7	(64)
	3	1	5/8	(16)
		2	6/6	(64)
	5	1	7/7	(64)
		2	8/8	(256)
Dog Kidney Cell (Roller)	2	1	2/7	(8)
		2	8/8	(64)
Bovine Embryonic Kidney Cell				
(Stationary)	3	1	6/6	(16)
(Roller)		1	3/3	(256)
(Stationary)	4	1	3/5	(4)
(Roller)		1	3/3	(16)
(Stationary)	5	1	6/6	(16)
(Roller)		1	4/6	(32)

* All hamsters were given an intraperitoneal inoculation of 1.0 ml on day 0. Those hamsters given 2 doses, received the second dose on day 7.

** Geometric mean titer.

Table 5. Development of Neutralizing and Hemagglutination-Inhibition Antibody in Dogs Vaccinated with a Formalin-Inactivated SV-5 Vaccine*

Test	No. Dogs with Antibody on Day (GMT)**/Total							
	0	8	10	15	18	22	36	43
Neutralizing	0/8 (<4)	5/8 (<4)	7/8 (4)	7/8 (4)	7/8 (8)	7/8 (8)	7/8 (4)	8/8 (8)
Hemagglutination-Inhibition	0/8 (<20)	8/8 (20)	8/8 (40)	7/8 (20)	7/8 (20)	5/8 (20)	7/8 (20)	Not done

* Lot #1 of the amniotic fluid type vaccine inoculated subcutaneously on day 0 and 10.

** Neutralizing titer of 1:4 or>; hemagglutination titer of 1:20 or>; GMT = Geometric Mean Titer.

Table 6. Results of Parainfluenza SV-5 Challenge of Vaccinated Dogs

Vaccine Status	Challenge Route	No. Dogs	No. Dogs Shedding Virus/Total (Aver. No. Days Virus Recovered)	No. Dogs with Neut. Antibody on Day (GMT)*			
				0	7	14	28
+	Intranasal	4	4 (3.75)	4 (4)	4 (32)	4 (≥1024)	4 (≥1024)
	Contact	4	3 (1.75)	4 (8)	4 (4)	3 (> 64)	4 (≥ 64)
0	Intranasal	4	4 (3.5)	0 (<4)	2 (<4)	4 (64)	4 (64)
	Contact	3	3 (3.3)	0 (<4)	0 (<4)	1 (< 4)	3 (64)

* Geometric Mean Titer.

anamnestic type antibody response; i.e., developing antibody more rapidly and to higher titers than the control dogs. Three of the 4 vaccinated dogs exposed by contact had a similar anamnestic response but occurring 1 week later. In summary, the formalin inactivated SV-5 vaccine produced from infected amniotic fluid produced low levels of antibody in dogs without any untoward reactions. On challenge, the vaccinated dogs were infected and developed an anamnestic antibody response.

3. Recovery and Characterization of a Minute Virus of Canines (MVC).

In comparison to other species, comparatively few new canine viruses have been described. This may be a reflection of the limited number of studies conducted and the limited number of virus indicator systems employed. Therefore, attempts were made to develop new continuous canine cell culture lines. This report summarizes the use of a canine cell line developed in the laboratory for the isolation and characterization of a hitherto undescribed canine virus.

The cell culture used for the recovery of this virus was derived from a subdermoid cyst of an irradiated dog. In the first 7-8 cell passages, the cells were fibroblastic and then became epithelial. At present, this cell line designated Walter Reed canine cell line has been subdivided over 185 times.

In June 1966, rectal swab specimens were obtained from normal military dogs at Ft. Benning, Ga. The swabs were placed in broth to which antibiotics were added. The supernatant fluids of the centrifuged broth were inoculated into the Walter Reed canine cell line, primary dog kidney, human embryonic kidney, and monkey kidney cell cultures. Only in the Walter Reed canine cell line was a cytopathic effect seen. The infected cells round up, the cell membranes become distinct, protoplasmic strands are evident, and the cells detach from the glass. On continued incubation the cytopathic effect progresses until all the cells detach from the glass. Large intranuclear inclusions were evident in hematoxylin and eosin stained infected cells. A clear zone can be seen between the inclusion and the nuclear membrane. The nuclear inclusions fluoresce green when stained with acridine orange. No such inclusions are present in control cells. The isolate could not be related by means of neutralization tests with known or reported canine viruses; e.g., canine adenoviruses, canine herpes, canine distemper, rabies, SV-5, reovirus type 1 and Echo virus 6.

The production of cytopathic effects could be observed in successive subcultures of infected Walter Reed canine cells but not in primary dog kidney or thymus cell cultures or in the Madin-Darby continuous canine kidney cell culture. The isolated agent, 2 X 66, did not produce cytopathic effects in cell cultures of a wide variety of

primary and continuous cell cultures derived from different species, nor did the cytopathic agent cause overt disease in suckling and weanling mice, hamsters, guinea pigs, and rabbits.

Observations on the isolation of the virus and the specific serologic reactions are summarized in Table 7. The first cytopathic effect was noted on the 6th day of cultivation for specimens from 2 dogs, 2 X 66 and 7 X 01. The remaining 2 isolates were evident on the 4th and 9th day of the first subculture. In each instance the cytopathic effect was similar and each agent was reisolated from the original rectal specimen. The 2 X 66 isolate was selected as a reference strain and after 3 terminal dilutions virus pools were prepared and antiserum was made in rabbits. The antiserum neutralized all the original isolates and repeat isolates. A rise in serum neutralization antibody titer was demonstrated in 3 dogs. The serum specimens were obtained at the time the rectal specimens were obtained and 30 days later. Sera from the 4th dog were not available. j The virus hemagglutinated monkey red blood cells at 4° C and it was possible to demonstrate a rise in antibody titer in hemagglutination-inhibition test with the same serum samples. These findings are excellent evidence of the canine origin of the virus.

Table 7. Recovery of Minute Virus of Canines (MVC)

Dog Number	Day CPE Noted (Passage)	Antibody Titer	
		Neutralization	Hemagglutination Inhibition
2 X 66*	6 (Original)**	64/256***	40/160
7 X 01	6 (Original)**	< 16/256	20/80
5A37	4 (Subpassage)**	< 16/64	< 10/40
6 X 54	9 (Subpassage)**	not available	not available

* Reference strain.
** Agent reisolated.
*** Serum specimen on day rectal specimen obtained. / Serum specimen approximately 30 days later.

Studies on the chemical and physical properties of the reference virus are summarized in Table 8. The virus was resistant to chloroform and ether treatment. At pH 3.0 the virus was stable for 3 hours at room temperature. The virus had a remarkable degree of stability at 60° and 70° C. At 80° C in one of 2 experiments, the virus survived 1 hour; in the second experiment the virus was recovered after 30 minutes. To determine the type of nucleic acid, the effect of 5-iodo 2-deoxyuridine on the growth of the virus was measured.

[illegible]

[illegible]

[illegible]

for an improved plaque test. Recently, Leibovitz (Am. J. Hyg. 78: 173, 1963) has described a highly enriched medium for cell cultures, designated L-15, which does not utilize the usual bicarbonate buffer system. Preliminary experiments were carried out using the L-15 medium enriched with 10% fetal bovine serum. In most instances good monolayers of DK cells were formed in petri dishes. However, consistently good monolayers were formed when 0.5% lactalbumin hydrolysate was added to the growth medium. For plaquing, the overlay medium consisted of equal parts of (a) 2X-L-15 medium without phenol red, 20% fetal bovine serum, 2% L-glutamine (200 mm), antibiotics and 5% of a 1:1000 neutral red stock, and (b) 1.4% purified agar. The 2 media were mixed in equal volume before overlaying.

Comparative titrations of several strains of canine herpes in tube cultures and in dishes indicated that both methods gave similar titers and the number of plaques was inversely related to dilution. Comparative titrations of rabbit anti-canine herpes sera in tubes and dishes indicated in many instances the plaque test gave 2 to 4-fold higher titers, and low levels of neutralizing activity in several canine sera were detected which were negative in the tube test. Thus, the canine herpes plaque reduction test improves the sensitivity and accuracy of the neutralization test.

Antigenic differences have been noted for various members of the herpes virus group, i.e., herpes simplex, herpes simiae, and equine herpes. Canine herpes strains isolated at WRAIR or Cornell were antigenically similar. However, the plaque reduction test made possible a precise determination. Antisera to canine herpes viruses were prepared in rabbits and plaque reduction tests carried out (Table 10). No significant antigenic differences were noted among the viruses from WRAIR, Cornell, NIH, and Richmond, Va. Therefore, it appears for diagnostic or epidemiological purposes neutralization tests could be carried out with either the WRAIR or Cornell isolates.

Table 10. Results of Cross-Neutralization Tests of Canine Herpes Viruses

Rabbit Serum Vs.	1/Titer Against Indicated Virus (origin)				
	F205 (Cornell)	4-63 (Walter Reed)	D004 (Walter Reed)	34-66 (Richmond, Va.)	Stewart (NIH)
F205	4*	16	16	16	not done
4-63	64	64	256	256	not done
D004	64	64	64	64	64
34-66	4	4	4	4	not done

* Highest dilution causing 80% or greater plaque reduction.

5. Virus Studies of Newly Procured Laboratory Dogs.

In cooperation with the Department of Laboratory Animals, studies were initiated to define the possible viral etiology of respiratory disease in newly procured "conditioned dogs." In contrast to previous experience, conditioned dogs from a vendor in Texas had an unusually high incidence of respiratory disease during their quarantine period. Accordingly, appropriate blood, throat and rectal specimens were obtained from these dogs for virus isolation and serological tests. In July 1968, of 35 dogs included in the study, 13 developed signs of respiratory disease and 4 dogs died. Seven transmissible agents were recovered from throat and rectal specimens of 4 sick dogs. The agents included 2 parainfluenza SV-5 isolates, 1 canine herpes virus, 2 minute viruses of canines, and 2 unknown agents. The 2 unknown viruses, throat (L198T) and rectal (L198R) isolates, were recovered from dog L198 with fatal respiratory disease. Although convalescent serum was not available from this dog, rises in titer to both viruses were demonstrated in other dogs of this group. L198T virus is a small, acid labile, ether resistant RNA virus, and L198R appears to be a middle-sized ether sensitive agent. L198T can be propagated in the Walter Reed canine cell line but not in primary dog kidney. L198R virus multiplies in primary canine kidney and thymus cells. Detailed characterization of these 2 viruses are in progress. In addition sero-epidemiological tests are being carried out to determine the spread of these 2 viruses and the other canine viruses in the conditioned dogs.

Summary and Conclusions.

1. Respiratory Disease in Scout Dogs.

Further studies were conducted to define the etiology and epidemiology of respiratory disease in scout dogs. Previous studies had resulted in the recovery of a highly communicable parainfluenza SV-5 virus. During this investigation, 23% (81/346) of the dogs developed respiratory disease and 2 dogs died. The signs of disease occurred in dogs within 2-to 3-months of entry into service. A relationship between length of service at the Lackland Air Force Base recruitment center and the incidence of SV-5 antibody was demonstrated. At Ft. Benning approximately 40% (50/133)of the dogs had a rise in SV-5 antibody titer including 14 of 34 dogs with signs of disease. Over 90% of the dogs had SV-5 antibody upon completion of training. Parainfluenza SV-5 virus infections continue to be associated with a significant percentage of respiratory disease in military dogs.

2. Development and Evaluation of a Formalin-Inactivated Canine Parainfluenza SV-5 Vaccine.

Parainfluenza SV-5 virus was recovered from military dogs with upper respiratory disease in 1966. Subsequent studies showed the virus was

highly communicable among military dogs and associated with respiratory disease over a 2-year period. Therefore, studies were begun to develop a formalin-inactivated SV-5 vaccine. Experimental vaccine lots were prepared from the amniotic fluid of embryonating hen's eggs and from primary dog kidney and embryonic bovine kidney cell cultures. Formalin at 1:4000 concentration at 36 C inactivated the amniotic fluid preparations in 8 hours and the cell culture preparations in 24-48 hours. One dose of the experimental vaccines produced neutralizing antibody in hamsters. Two doses of the amniotic fluid type SV-5 vaccine produced neutralizing and hemagglutinating-inhibiting antibody in dogs but did not prevent infection on challenge. Further studies with more potent vaccines should be carried out in dogs as well as studies on the role of "local antibody" in immunity to respiratory SV-5 infection.

3. Recovery and Characterization of a Minute Virus of Canines (MVC).

Recently dogs have become important in military operations in Southeast Asia and the Division of Veterinary Medicine is providing laboratory support in the diagnosis and control of infectious diseases. In studies of canine virus diseases, few canine cell lines are available and therefore, studies were carried out to initiate new canine cell cultures. This report describes the utilization of a canine cell line developed in this laboratory for the isolation and characterization of a hitherto undescribed virus. Four antigenically related transmissible viral agents were recovered from fecal specimens of 4 asymptomatic military dogs. The agents produced a cytopathic effect (CPE) in a continuous dog cell line. Antibody titer rises were shown in serum samples from 3 of the 4 dogs. The virus has produced CPE in this cell line but not in primary kidney or thymus cell cultures, nor in cells of human, simian, porcine, bovine, feline, and murine origin.

The agent is resistant to ether, chloroform and heat treatment. Infected cell cultures stained with acridine orange contain intranuclear green fluorescent inclusions. By electron microscopy the intranuclear particles measure 20-21 mu. The buoyant density in cesium chloride is approximately 1.40. These properties are consistent with the members of the picodnavirus group. By hemagglutination inhibition tests MVC could be readily distinguished from H-1, Rat Virus and MVM. Canine gamma globulin contained high titers of neutralizing antibody and antibody was frequently found in military dogs and in beagles of a breeding colony.

4. Development of a Simple Plaque Procedure for Canine Herpes Virus and its Utilization for Antigenic Comparisons of Canine Herpes Viruses.

A simplified technique for plaquing viruses in a modified Leibovitz L-15 media has been developed. The procedure will enable the plaquing

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

Publications.

1. L. M. Binn, E. C. Lazar, G. A. Eddy, and M. Kajima. Minute Virus of Canines. Bact. Proc. 161, 1968.

2. L. M. Binn, E. C. Lazar, M. Rogul, V. M. Shepler, L. J. Swango, T. Claypoole, D. M. Hubbard, S. G. Asbill and A. D. Alexander. Upper Respiratory Disease in Military Dogs: Bacterial, Mycoplasma and Viral Studies. Am. J. Vet. Res. 29: 1804-1815, 1968.

				DA 08 6446	69 07 01		
01 01 69	D. Change	U	U	NA	NL		

	61101A	3A061101A91C	00	185

(U) Veterinary Combat Surgery (09)

012900 Physiology 003500 Clinical Medicine

07 68	NA	DA	C. In-House

a. DATE EFFECTIVE: NA	EXPIRATION:		
b. NUMBER:			
c. TYPE:	d. AMOUNT:		
e. KIND OF AWARD:	f. CUM. AMT.		

10. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (in thousands)
FISCAL YEAR 69 (PRECEDING)	1	25
FISCAL YEAR 70 (CURRENT)	1	25

11a. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Veterinary Medicine
ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Goodwin, CPT B. PII Redacted
TELEPHONE: 202-576-5222
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:
NAME:

9a. ASSIGNABLE SUBORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL, W. H.
TELEPHONE: 202-576-3551

12. GENERAL USE

Foreign Intelligence Considered

DA

13. KEYWORDS (Select from AMSC Publication Security Classification Code)

(U) Cyanoacrylates; (U) Veterinary surgical equipment

14. TECHNICAL OBJECTIVE, 15. APPROACH, 16. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To adapt newly developed surgical procedures to the combat situation and to develop additional surgical techniques and procedures as required.

24. (U) To evaluate usefulness of new hemostatic techniques such as cyanoacrylate sprays, to evaluate the usefulness of means of controlling wound infection and to investigate the role of hemorrhage shock in canine combat casualties.

25. (U) 69 01 - 69 06 A prototype canine surgical table for combat use has been fabricated and awaits field testing. Field testing of pneumatic splints, topical screwworm medication and cyanoacrylic hemostatic sprays are planned in Southeast Asia. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1968 - 30 June 69.

Available to contractors only on individual request

DD FORM 1 MAR 63 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 185, Veterinary Combat Surgery

Investigators.
Principal: CPT B. S. Goodwin, VC
Associate: COL R. M. Nims, VC

Description

Major objectives are to adapt newly developed surgical procedures to combat situations and develop additional surgical techniques and procedures as required.

Progress

1. A prototype canine surgical table for field use is being fabricated. This table is designed to be light weight, easily transportable and with a trough-shaped table surface to conform more closely to the anatomical configuration of the canine. The surface consists of two identical halves, hinged with a flexible material which also serves as a drainage trough. This also permits infinite variation in table surface configuration, so that either half of the table can be positioned from horizontal to 45° toward vertical, increasing its versatility for different surgical procedures. The surface is also capable of being tilted along its long axis. Removable extensions will adapt it to special situations, such as orthopedic manipulations. The surface of the table will be slotted to improve drainage. Being removable from the base, it is potentially useful as a stretcher or litter.

The table base, of light-weight metal, will collapse for crating and movement.

Dimensions and configuration of the table will permit the surgeon closer proximity to the surgical field. The trough configuration of the surface will minimize the need for restraining straps and sandbags which are necessary on conventional flat surfaced operating tables.

The prototype table will be tested in actual use situations and it is hoped to be able to test it under field conditions.

2. Cyanoacrylic Hemostatic Sprays

These sprays, which were developed using the canine as the experimental subject, have proven useful for hemostasis of solid organ and soft tissue trauma. Their adaptability for field surgical procedures will be studied. The feasibility of incorporating antibiotics for control of bacterial wound contaminants will be investigated.

3. Pneumosplint

A commercially available inflatable splint (Redisplint; Parke-Davis) was investigated for possible application as a field first aid item. Fixation proved inadequate for transporting canine fracture cases over rough terrain by vehicle. Also, the device is subject to failure from tears and punctures. A desirable feature of the device is the hemostatic effect of the uniformly applied pressure.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 185 Veterinary Combat Surgery

Publications

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6447	69 07 01	DD-R&E (AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP'G HISTORY	8b. SPECIFIC DATA RESTRICTION ADDED	9. LEVEL OF EFF
68 10 31	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	611101A	3A061101A91C	00	186
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Production of Meningococcal Polysaccharides, Types A and C (31)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMING DIVISION
68 10	CONT	DA	B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 68 10 EXPIRATION: 69 09
b. NUMBER: DADA 17-69-C-9029
c. TYPE: U. CPFF d. AMOUNT: $164,955
e. KIND OF AWARD: NEW f. CUM. AMT.: $164,955

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR: PREVIOUS 69	2	122
FISCAL YEAR: SUMMARY 70	0.5	41

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL, W.H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Squibb Institute for Medical Research
ADDRESS: New Brunswick, NJ 08903

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Brown, W.E.
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Artenstein, M.S. 3
NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code) (U) Meningitis; (U) Meningococcal Polysaccharide A; (U) Meningococcal Polysaccharide C; (U) Neisseria meningitides

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)

23. (U) To develop procedures and methods for scaling-up production of meningococcal polysaccharides Types A and C to 20 gram lots.

24. (U) Modification of small-scale procedures and adaptation to quantity production.

25. (U) Two lots of group C meningococcal vaccine (C-7 and C-8), totalling 37,750 doses, have been produced which meet the required chemical and purity specification and which have proved to be safe and immunogenic in volunteers. Lot C-7 has been given to 5292 and lot C-8 to 665 recruits. For technical report see Walter Reed Army Institute of Research Annual Progress Report,(1 Jul 68 - 30 Jun 69.)

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498-1 (FOR ARMY USE)

[illegible]

[illegible] OD [illegible]

[illegible]

Investigators.
Principal: [illegible]
Associates: [illegible]

[illegible] Command, [illegible] group C [illegible] Colorado [illegible] are on file at [illegible] Board.

[illegible] Lot Cm, [illegible] Both lots have [illegible] 95 percent of [illegible]

Lot Cm [illegible] Fort Bragg and Fort Lewis and on the [illegible] Lot Cm [illegible]

One systemic [illegible] but no other [illegible] fever, [illegible] vaccine. All [illegible] explain this. [illegible] the possible [illegible] significance [illegible]

				DA 0B6449	69 07 01		
01 01 69	D. Change	U	U	NA	NL	60	

PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
61101A	3A061101A91C	00	187

(U) Computer Aided Printing of Chemical Structures (09)

012100 Organic Chemistry 019600 Display Devices and Equipment

START DATE	ESTIMATED COMPLETION DATE	FUNDING AGENCY	PERFORMANCE METHOD
01 69	NA	DA	C. In-House

	RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (in thousands)
FISCAL YEAR	PRECEDING 69	0	5
	CURRENT 70	0.1	5

PRIOR EFFORT	NA
EXPLANATION	
a. GRANT OR CONTRACT NUMBER	
b. AMOUNT	
c. TYPE	
d. CUM. AMT.	
e. END OF CONTRACT	

PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Division of Medicinal Chemistry
Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Feldman, A. P. (M.S.)
TELEPHONE: 202/576-3168
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:
NAME:

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

Meroney, COL W. H.
202/576-3551

Foreign Intelligence Considered

DA

PII Redacted

(U)Typesetting; (U)Computers; (U)Publishing

23.(U) To set chemical structures and associated information directly into type, from coded files, accomplishment of this objective will allow the Government to distribute to the multiple groups participating in the Army Malarial and Radiation Drug Development Programs information needed to encourage meaningful participation.

24.(U) The chemical structures on file were entered by means of chemical typewriters, which code the actual layout of the structures typed. Therefore, it is possible to display these structures on demand. Currently, this is done by means of high-speed printers having a modified font. It should also be possible with tape-driven photo-typesetting equipment.

25.(U) 69 01 - 69 06 A preliminary program has been written to test the logic to be used in converting typewriter to typesetter font. Funds are being transferred to the Government Printing Office for the use of their typesetting machines and for the purchase of a chemical font to be designed according to specifications developed in this project. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 187, Computer aided printing of chemical structures

Investigators.
Principal: David P. Jacobus, M. D.
Associate: Alfred P. Feldman

1. General

The Division of Medicinal Chemistry to date has accumulated large files, consisting of both chemical and biological information and coded for computer searching (1). While this information is accessible through computer searching, there is also an urgent need for the preservation of this information for archival purposes in a non-coded, legible format.

Because all the material is already coded, there exists the possibility of preserving it, not as a roomful of data sheets, nor as voluminous computer printouts, but in manageable book form, set in high quality type, well indexed, and readily distributed. Because the material is encoded, the high costs of manual typesetting and proofreading are avoidable.

It is the object of the work described here to make encoded chemical structures amenable to typesetting in the above fashion. Because such diagrams are two-dimensional, and because existing typesetting machines operate on a line-by-line basis, such diagrams, in the past, had to be printed from "cuts" prepared from hand-drawn originals. A novel method of encoding chemical structures used in this division (2), is capable of feeding two-dimensional chemical diagrams line-by-line to a high speed printer, and hence also to a tape-driven typesetting machine (3). The method is of general applicability.

2. Progress.

Contact was established with the Government Printing Office to explore the feasibility of the typesetting programs discussed above. Following an affirmative answer, test programs were written, and an experimental grid was designed, compatible both with the chemical typewriter

font used for input, and the requirements of the Government Printing Office typesetting machines (Linofilm and Linotron).

References:

1. D. P. Jacobus, D. E. Davidson, A. P. Feldman and J. A. Schafer: "Experience With the Mechanized Chemical and Biological Information Retrieval System". J. Chem. Documents; in press.

2. A. Feldman: "Two Dimensional Structure Encoding Typewriter", U. S. Patent 3,358,804 issued 19 Dec. 1967.

3. A. Feldman: "A Proposed Improvement in the Printing of Chemical Structures, Which Results in Their Complete Computer Codes", Am. Document, 15 205 (1964).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 08 6451	69 07 01	DD-R&E (AR) 636

3. DATE PREV SUM	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. FOREIGN MKT'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 03 31	D. CHANGE	U	U	NA	NI.	☒ YES ☐ NO	A. WORK UNIT

10. KID CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	611101A	3A061101A91C	00	188
b. SECONDARY				
c. SECONDARY				

11. (U) Behavioral Baselines for the Experimental Study of Uremia (21)

12. 012900 Physiology; 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 02	CONT	DA	B. CONTRACT

17. CONTRACT/GRANT
a. CURRENT FY 69 02 EXPIRATION 70 01
b. NUMBER DADA 17-69-C-9094
c. TYPE U.CPFF CURR AMT. $43,629
d. ORIGINAL NEW ORIG AMT. $43,629

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PREVIOUS 69	0.5	18
FISCAL YEAR CURRENT 70	0.5	25

19a. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

19b. PERFORMING ORGANIZATION
NAME: Institute for Behavioral Research
ADDRESS: Silver Spring, Md. 20910

20. REVIEWING OFFICIAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

21. PRINCIPAL INVESTIGATOR (Person most responsible for U.S. technical performance)
NAME: Taub, E.
TELEPHONE: 301-585-3915
SOCIAL SECURITY ACCOUNT NUMBER:

22. CLASSIFICATION
Foreign Intelligence Not Considered

23. ASSOCIATE INVESTIGATORS
NAME: Teschan, COL P. E.
NAME:

24. KEYWORDS (U) Uremia; (U) Behavioral Baselines; (U) Kidney; (U) Body Fluids; (U) Hemodialysis

25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Provide brief individual paragraphs identified by numbers. Precede text of each with security classification code.)

23 (U) Development and utilization of behavioral baselines for the experimental study of uremia in primates in a broader study of the uremic syndrome.

24 (U) Continuous assay of biochemical and behavioral changes associated with nephrectomy, ureteral ligation, and infusion of compounds relevant to experimental uremia.

25 (U) 69 02 - 69 06 Continued progress has been made in the techniques of peritoneal dialysis in alert, functioning uremic monkeys. This includes avoidance of infection and utilization of automated dialysis equipment. The previously developed model has served as an assay of the following compounds in the pathogenesis of uremia: guanidine succinic acid, urea, creatinine. The data obtained suggest that these compounds are not the cause of the behavioral changes in uremia. EEG analysis of uremic monkeys has revealed changes similar to those observed in man, reversible by dialysis, which support the above conclusions.

28. PUBLICATIONS, REPORTS, AND ARTICLES (NONE)

DD FORM 1498-1 (FOR A GUIDE USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 188, Behavioral base lines for the experimental study of uremia

Investigators.
Principal: COL Paul E. Teschan, MC
Associate: MAJ Charles B. Carter, MC; Mr. Edward Taub

Description: The clinical syndrome of uremia has been recognized for many years. The nature of the toxic substances present in uremia awaits development of sensitive methods of studying uremia in the laboratory.

Progress: Development of a sensitive bioassay system, utilizing operant-conditioned behavior patterns in monkeys in the uremic and nonuremic state offers opportunities for the study of the effects of uremia on cerebral function. Such a system has been developed at the WRAIR.

Utilizing this system it is possible to reverse the encephalopathic effects of uremia with peritoneal dialysis, indicating that the toxic substance or substances responsible for the encephalopathy are small, dialyzable molecules.

It has also been possible to study the effects of specific suspected toxic substances by allowing their concentration to remain elevated during treatment of uremia with peritoneal dialysis. This is readily accomplished by placing these substances in peritoneal dialysate at concentrations designed to maintain pathologic extracellular fluid concentrations.

Results: Thus far, urea and guanidinosuccinic (GSA) acid, both suspected uremic toxins, have been evaluated in this manner. They do not, separately, appear to be the toxic factors in uremia. We have been able to demonstrate normal operant conditioned behavior in the presence of marked elevations of GSA and urea.

Conclusions and Recommendations: It is possible, utilizing the present bioassay system, to study the effects of specific suspected uremic toxins on cerebral function. Studies of many remaining single toxins and combinations of toxins remain to be done.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 188 Behavioral base lines for the experimental study of uremia

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	DD-R&E (AR) 613
DAOB 6450	69 07 01	

3. DATE SUMMARY CHANGED	4. KIND OF SUMMARY	5. SECURITY CLASSIFICATION	6. DOWNGRADING	7. DISCIPLINE	8. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF EFFORT
69 03 31	D. Change	U U	NA	NL	☐ Yes ☐ No	A. Joint Unit

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	189
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Preparation of Tuberculin Fractions (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
03 69	NA	DA	B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 03 69 EXPIRATION: 02 70
b. NUMBER: DADA 17-69-C-9108
c. TYPE: J.C. d. AMOUNT: P$6,974
e. KIND OF AWARD: New f. CUM. AMT: P$6,974

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	0.3	2
FISCAL YEAR CURRENT 70	0.7	5

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Inst of Res
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RESPONSIBLE INDIVIDUAL
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PII Redacted

ASSOCIATE INVESTIGATORS
NAME: Fife, E. H. Jr.
NAME: 1

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Tuberculin fractions; (U) A-protein fraction; (U) C-protein fraction; (U) Polysaccharide fraction; (U) SAFA test.

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number, precede text of each with Security Classification Code.)

23. (U) To supply a specified quantity of the A- and C-protein tuberculin fractions monthly.

24. (U) By procedures described by the investigator in the scientific literature.

25. (U) 69 03-69 06 Regular delivieries of the A-protein and C-protein antigen fractions have been made according to the agreement in the contract. In addition, a new polysaccharide antigen prepared from the cell walls of tubercle bacilli was supplied in quantities sufficient for critical evaluation in the SAFA test. Findings thus far indicate that the polysaccharide antigen is superior to the A- and C-protein for detection of simian tuberculosis. For technical reports see Walter Reed Army Institute of Research Annual Report, 1 Jul 68 - 30 Jun 69.

DD FORM 1498 (FOR ARMY USE)

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 189, Preparation of tuberculin fraction

Investigators.
Principal: Lewis F. Affronti, Ph.D.
Associate: Earl H. Fife, Jr., M.S.

Description.

This work unit consists of a contract to prepare specific tuberculin antigen fractions and to supply the Department of Serology, WRAIR, with specified amounts of each antigen. Fractionation procedures developed by the principal investigator are used to isolate A-protein and C-protein antigens from the filtrates of M. tuberculosis cultures. These antigens are used by investigators in the Department of Serology in studies on the serodiagnosis of human and simian tuberculosis.

Progress.

Regular deliveries of the A-protein and C-protein tuberculin antigen fractions have been made to the Department of Serology, WRAIR, in accordance with the terms of the contract. In addition, a new polysaccharide antigen fraction prepared from the cell walls of the tubercle bacilli (Birnbaum, S. E. & Affronti, L. F., J. Bact. 95 : 559, 1968) is being supplied in quantities sufficient for critical evaluation in the SAFA test. Results of preliminary evaluations in the SAFA test indicate that the C-protein antigen is superior to the other fractions for the serodiagnosis of human tuberculosis, whereas the polysaccharide fraction is the most sensitive, specific antigen for simian tuberculosis.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

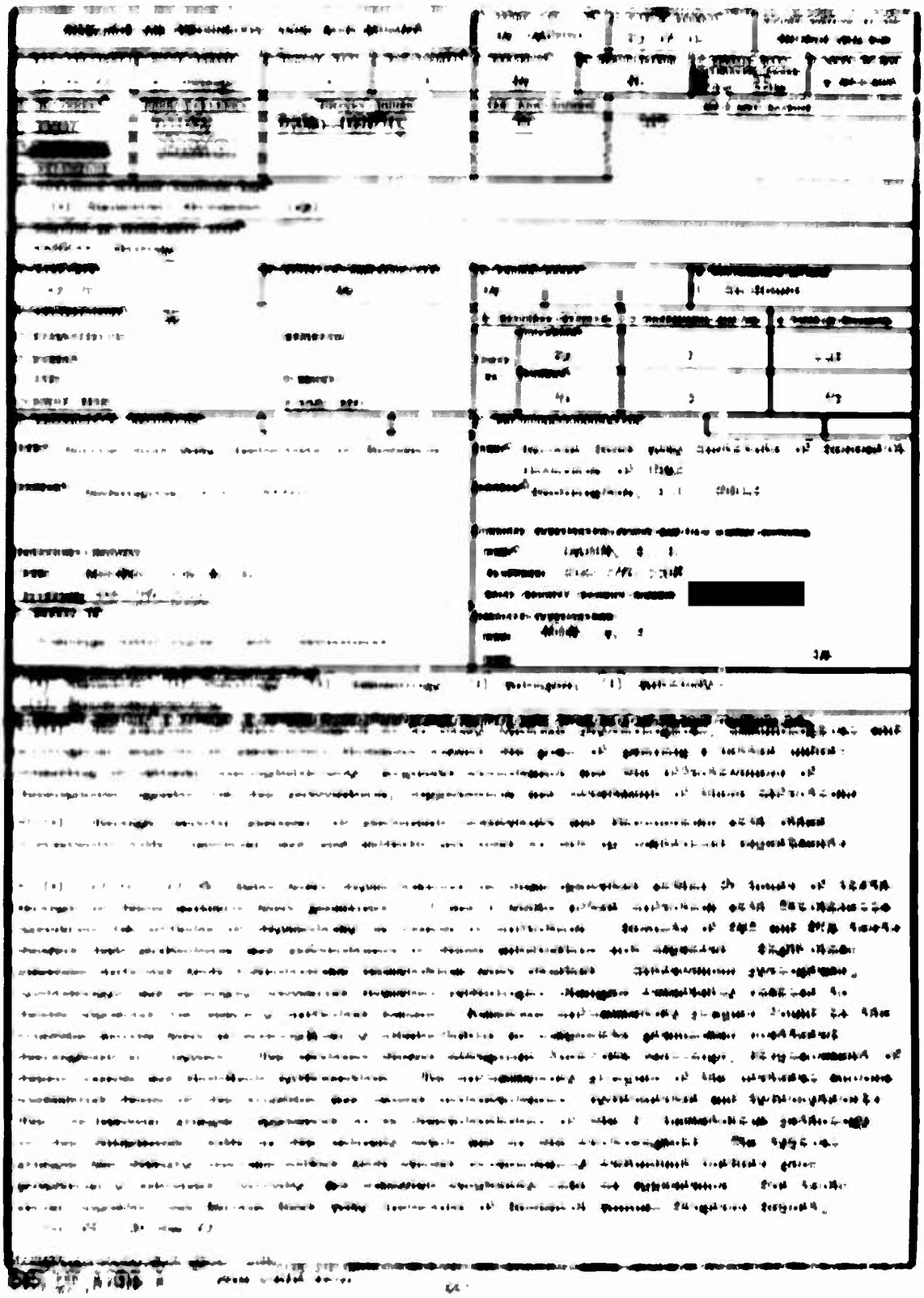
Task 00 In-House Laboratory Independent Research

Work Unit 189, Preparation of tuberculin fraction

Publications.

None.

[illegible]



PII Redacted

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 165, Parasitic diseases

Investigators.
Principal: Elvio H. Sadun, Sc.D., Lib. Doc.
Associate: John F. Barbaro, Ph.D.; Allen W. Cheever, M.D.; MAJ Edward J. Colwell, MC; Ralph E. Duxbury; MAJ Duane G. Erickson, MSC; Franz von Lichtenberg, M.D.; Ivan Mota, M.D.; Donald T. O. Wong, Ph.D.

1. Resistance produced in mice and rats by inoculation with irradiated Trypanosoma rhodesiense.

Attempts to produce immunity in animals against African trypanosomiasis have been undertaken by several investigators with varying degrees of success. The difficulties encountered in artificial immunizations were attributed to a number of factors such as the multiplicity of trypanosome species, variations in strain virulence, instability of trypanosome antigens and variant specificity of protective antitrypanosome antibody. Some of the problems that hinder progress in each of these areas have been discussed in recent review of this subject.

Active immunization was induced against African trypanosomiasis by the use of live parasites which had been attenuated by animal passage, by chemical treatment or by infecting animals with live virulent parasites and then curing the infection with trypanocidal drugs. Considerably less success was achieved by immunizing animals with parasites which had been killed either by chemical means or physical means.

Irradiation of parasites interferes with their physiologic processes and frequently inhibits their normal development and multiplication. Although vaccines consisting of irradiated helminth parasites have been reported against cestodes, nematodes and trematodes, only infrequent attempts to immunize against protozoan infections with ionizing radiation have been reported. Immunization by the use of irradiated malarial parasites has been accomplished in chickens and rodents.

Although ionizing radiation inhibits trypanosomes in their normal multiplication the reproductive processes are affected by a lower concentration of radiation than that required to kill the trypanosome. Halberstaedter found that with Trypanosoma gambiense a dose of approximately 12,000 roentgens (r) did not affect motility but interfered with normal division and infectivity. He determined that the direct lethal dose occurred between 100,000 and 600,000 r and corresponded roughly to that for nonparasitic protozoa (paramecia). Since the dose needed to suppress normal division and infectivity is only a small fraction of that required to kill the trypanosomes, irradiation may enable one to take advantage of the special immunological properties of living parasites.

with an unimpaired reproductive capacity would be obviated. To assess this hypothesis, Stubbs et al. inoculated mice with T. equiperdum which had been irradiated with high X-ray doses to render the parasites non-infective although viable. All the mice which did not develop infection following inoculation with irradiated trypanosomes died after they were given a challenge inoculation. This led the authors to conclude that "no immunological response followed the injection of the X-ray avirulented trypanosomes." Conversely, in experiments with one of the natural trypanosomes of rodents, T. lewisi, Sanders and Wallace found that rats immunized with irradiated blood forms produced a solid immunity to a challenge inoculation given 15 to 28 days after the first inoculation.

In view of these considerations, a series of investigations was undertaken to study the effect of ionizing radiation on the reproduction and survival of the African trypanosome, T. rhodesiense, and to determine the extent to which the resulting attenuated parasites could produce protective immunity in mice and rats. The immunizing effect of irradiated T. rhodesiense which had been frozen and stored was also investigated. In addition, cross protection studies were made using 3 species of African trypanosomes, T. rhodesiense, T. gambiense and T. brucei.

Male ICR strain mice and male WRCF rats derived from the Wistar strain were used throughout these studies. The mice weighed 20-25g and the rats 150-170g at the beginning of each experiment.

The experiments were conducted with the Wellcome strains of T. rhodesiense and T. gambiense and the Lugala I strain of T. brucei. Trypanosomes for irradiation were obtained from heavily parasitized stock animals placed under ether anesthesia. Boood was collected from the severed axillary vessels of the mice and from the heart in rats. A minimal amount of heparin was added to the syringe before the blood was drawn. For determining the numbers of trypanosomes present, a sample of the blood was first diluted 1:200 in a red cell pipette with physiological saline containing 1% formalin and 5% stock Geimsa's stain. The trypanosomes in this mixture were then counted with a hemocytometer to determine the amount of infected blood needed for immunization and control purposes.

The parasitized blood for irradiation was diluted in normal rodent serum, then placed in separate 10 x 75mm glass tubes for exposure to the various radiation doses used. The tubes were kept in an ice-water bath before, during and after irradiation.

Irradiation was accomplished with a Gammacell 220 cobalt-60 irradiator which delivered approximately 12,000 r/minute. The tubes containing the trypanosomes in diluted blood were placed in Dewar flasks to which ice had been added. The flasks were put into the holders of a circular rack fitted to a planetary gear system which allowed the holders to rotate on their axes while the rack rotated on its axis. This method insured a uniform exposure of the trypanosomes to irradiation. The irradiated blood was highly diluted in Locke's solution before the animals were inoculated. Immunizing inoculations were given intraperitoneally in

0.1 - 0.2 ml amounts containing the desired number of trypanosomes. In experiments with the effects of freezing irradiated T. rhodesiense for later use in immunizations, the method of freezing described by Ashcroft was followed. The irradiated, frozen trypanosomes were stored at -70°C until they were used. Nonirradiated inocula, processed in a similar manner, were administered intraperitoneally to the control animals immediately after completion of the experimental animal inoculations.

Tail blood was collected from the rats at regular intervals and the serum was separated and stored at -20°C for use in fluorescent antibody tests. These tests were conducted according to the method of Williams et al. for African trypanosomiasis. Direct examination of tail blood for the presence of trypanosomes was made daily on all animals during the first week after inoculation and at 3-4 day intervals thereafter. Animals surviving challenge were held for at least 30 days before they were considered to be free of infection.

A first experiment was designed to determine the effect of exposure to gamma irradiation on the infectivity of T. rhodesiense for mice. A total of 233 mice was separated into 19 groups. The mice of group I received intraperitoneal inoculations of 2,000,000 nonirradiated trypanosomes. Those in groups II through IX received the same number of trypanosomes which had been exposed to a single dose of irradiation varying from 10,000 to 1,000,000 r. Those of groups X through XIV received two doses and those of groups XV through XIV received three doses of irradiated trypanosomes at weekly intervals. The results, summarized in Table 1, showed that all animals inoculated with nonirradiated trypanosomes developed progressive infections and died 3-5 days after inoculation, with an average survival time of 3.6 days. Although no animals survived inoculation with trypanosomes irradiated at 10,000 r (Group II) their mean survival time was prolonged as compared with the controls. With the exception of 3 mice in group X and one in group XI, all mice survived inoculation with trypanosomes which had been exposed to doses of 20,000 or more roentgens. The trypanosomes exposed up to 160,000 r retained their motility and normal outline prior to inoculation even though their normal division and infectivity was apparently affected. Even at this high level of irradiation parasites could be observed in the blood of the recipient animals for at least 24 hours after inoculation. Most of the trypanosomes exposed to 320,000 r and all of those examined after exposure to 640,000 or 1,000,000 r had lost their motility.

A second experiment was undertaken to determine whether the surviving mice of Experiment 1 would exhibit a demonstrable resistance to a challenging infection with nonirradiated trypanosomes. As indicated in Table 2, the mice were divided into 18 groups. All animals were challenged with 1,000 nonirradiated T. rhodesiense 1 week after the last immunization. Twenty mice (Group Ia) were used as controls of the challenging doses.

A marked protection to a challenging infection was built up in the mice following exposure to irradiated trypanosomes. This acquired resistance was noticeable regardless of the level of irradiation at

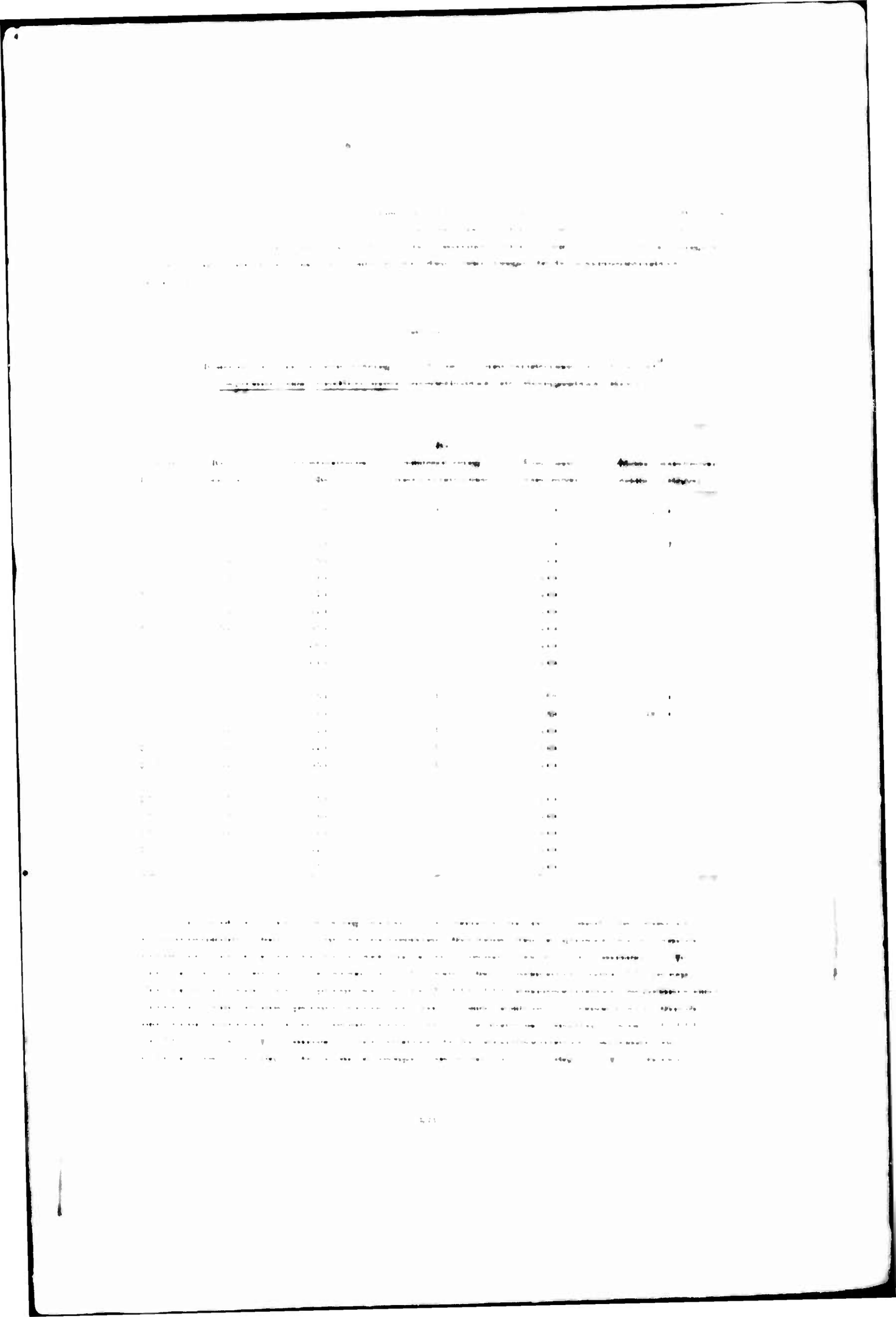


Table [illegible]

Number of rats surviving [illegible] inoculations of 4×10^6 *Trypanosoma rhodesiense* irradiated at designated doses

Group No.	No. rats	Irradiation (kr)	No. immunizing inoculations	Percent survival	Mean survival time (days)
[illegible]	[illegible]	[illegible]	0	0	4.1
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	
[illegible]	[illegible]	[illegible]	[illegible]	100	

[illegible]

[illegible]

received a second inoculation with [illegible]
challenged with nonirradiated trypanosomes [illegible]
immunizing dose and tested again at weekly intervals [illegible]
after challenge. The fluorescent antibody test, [illegible]
sera from the rats on each collection date, was negative
the first inoculation and 5 days later, but was positive
of 1:8, 7 days after the primary immunizing dose. It
at increasing titers which reached a peak (1:64) 7 w
At the end of the experiment, 18 weeks after challenge,
bodies at a titer of 1:64 were still detected.

Table 4

Development of immunity in rats to [illegible]
Trypanosoma rhodesiense and challenged with nonirradiated
(Immunizing doses 4 x 10^6; challenge dose 1

Group No.	No. rats	Irradiation (Kr)	No. immunizing inoculations	[illegible]
I-a	30	0	[illegible]	
II	5	20	1	
III	5	40	1	
IV	11	60	1	
V	5	90	1	
VI	5	120	1	
/II	5	180	1	
VIII	5	240	1	
IX	5	20	[illegible]	
X	5	40	2	
XI	11	60	2	
XII	5	[illegible]0	2	
XIII	5	120	2	
XIV	5	180	2	
XV	5	240	2	
XVI	[illegible]	[illegible]0	[illegible]	
XVII	[illegible]	[illegible]0	[illegible]	
XVIII	[illegible]	[illegible]0	[illegible]	
XIX	[illegible]	[illegible]0	[illegible]	
XX	[illegible]	120	[illegible]	
XXI	[illegible]	180	[illegible]	
XXII	[illegible]	[illegible]0	[illegible]	

Table 5

[illegible] with frozen irradiated (60 Kr) T. rhodesiense

Group	Percent survival	Mean survival time (days)
Immunization (4×10^6)		
[illegible], frozen	100	--
Non-irradiated, frozen	0	3.1
Challenge (2×10^3)		
[illegible], frozen	100	>30
[illegible]	0	4.0

[illegible] long immunity would last in rats which [illegible] irradiated trypanosomes and challenged 1 week [illegible] inoculation, another experiment involving 52 [illegible] were divided into 9 groups (Table 6) and [illegible] with 1,000 trypanosomes. Groups of [illegible] month, 3 months, 5 months and 7 months after [illegible] were inoculated with nonirradiated [illegible] immunity controls. As summarized in Table 5, [illegible] on previously immunized developed [illegible] survival time of 5.2 days. Conversely, [illegible] when given a second challenge 1 or 3 [illegible] and 13 of the 14 survived when rechal- [illegible]. However, only 1 of 16 rats survived a rechallenge [illegible] challenge.

[illegible] whether the observed immunity was indeed a [illegible] inoculations were made into normal rats with blood [illegible] from rats that had survived challenge. [illegible] 7 days after they had been rechallenged [illegible]). No trypanosomes could be found in [illegible] examined microscopically. Periodic exami- [illegible] with 0.4 ml of blood from a [illegible] and no evidence of infection [illegible] inoculation. Two rats were killed [illegible]. Spleen impression smears [illegible] trypanosomes. Emulsions of [illegible] grinder and approximately [illegible] (2 spleens and 2 livers) were [illegible]. No trypanosomes were seen in [illegible] and no evidence of in- [illegible] of 30 days.

Table 6

Rechallenge of rats immunized with 4×10^6 irradiated *Trypanosoma rhodesiense*

No. rats	No. immunizing inoculations	Time of rechallenge (month after 1st challenge)	Percent survival	Mean survival time (days)
12	0	-	0	5.2
2	2	1	100	>30
3	3	1	100	>30
2	2	3	100	>30
3	3	3	100	>30
8	1	5	88	8.0
6	3	5	100	>30
4	1	7	0	5.0
12	2	7	8	5.9

To determine whether the protection obtained by immunization with irradiated parasites is species specific, studies were designed in which mice were challenged with homologous and heterologous species of African trypanosomes. Three groups of mice were given inoculations of 4×10^6 irradiated *T. brucei*, *T. gambiense* and *T. rhodesiense*, respectively, on days 0, 7 and 14. On day 21 the groups were subdivided so that a homologous challenge and two heterologous challenges, all with 1000 trypanosomes, could be made for each of the 3 species. The results of homologous challenge showed that there was complete protection against *T. gambiense* and *T. rhodesiense* and that 60 percent of the mice were protected against *T. brucei*. None of the mice survived challenge with a heterologous species, although the mean survival time of the mice that died was prolonged as compared with that of the challenge controls (Table 7).

Analysis of the foregoing experiments indicates that, in general, a strong acquired resistance to infection to *T. rhodesiense* is produced in mice and rats by vaccin tion with irradiated trypanosomes of the same strain. The resistance developed is sufficient to inhibit greatly the multiplication of the trypanosomes and to permit the animals to survive otherwise lethal infections with nonirradiated parasites.

In view of this evidence, it is surprising that control of trypanosomiasis by artificial immunization has proved to be so difficult. The literature on the subject reveals that in all parasitic infections

attempts at immunization with various [illegible] extracts from them have given disappointing results when compared with immunity stimulated by experimental infections. It is very likely that immunizations with irradiated trypanosomes may give rise to a host response produced by infection with the living nonattenuated parasites. In this respect, the damage of irradiation to the trypanosomes can be compared to that resulting from drug action. Growing treated trypanosomes in vitro with parafuchsin and Roehl and Uhlig method in vivo with subcurative doses of parafuchsin.

Table 7

Percent and time of survival of mice after immunization with irradiated (60 Kr) trypanosomes (homologous and heterologous species)

(Immunizing dose: 4×10^6; challenge dose: 1×10^3 parasites)

		Percent Surviving Challenge					
		[illegible]		T. gambiense		T. rhodesiense	
No. mice	Group	[illegible]	MSTD*	Percent survivors	MSTD*	Percent survivors	MSTD*
26	Irradiated T. brucei	[illegible]	[illegible]	0	4.0	0	4.8
24	Irradiated T. gambiense	[illegible]	[illegible]	100	>30.0	0	4.0
25	Irradiated T. rhodesiense	[illegible]	[illegible]	0	4.4	100	>30
15	Non-irradiated [illegible]	[illegible]	[illegible]	0	4.0	0	4.0

*MSTD = Mean survival time, [illegible]

The only report we have in the literature on the use of irradiated pathogenic trypanosomes for inducing immunity was that of Stubbs et al. They found that mice which had been inoculated with irradiated T. equiperdum were successfully reinfected, indicating that no immunological response followed the injection of irradiated virulent trypanosomes. Our results showing that [illegible] immunity develops from those vaccination attempts, are in better agreement with the above observations, and seem to be more in line with observations reported by Fernandes et al. in American trypanosomiasis. They reported that when Actinomycin D was added to cultures of T. cruzi, the organisms retained their normal motility but lost the ability to multiply and were unable to incorporate radioactive thymidine into DNA. When these parasites were injected into mice, they appeared to be avirulent but retained their immunogenicity

[illegible]

[illegible]

[illegible]

the surface of the skin with a ruler. As shown in Table 8 absorption of mouse anti-T. spiralis sera with rabbit anti-mouse 7S γ1 completely suppressed the ability of the antisera to induce PCA reactions after a sensitization period of 2 hours but left intact the ability of the same antisera to induce PCA reactions after 72 hours.

Table 8

Effect of Absorbing Mouse Antiserum with Rabbit Anti-Mouse 7S γ1 on PCA Reactions Induced after a Sensitization Period of 2 or 72 Hours

	PCA Titer* After 2 Hours		PCA Titer After 72 Hours	
Antiserum+	Before Absorption	After Absorption	Before Absorption	After Absorption
TB2	400	0	400	400
TB3	150	0	150	150
TB4	200	0	200	200
TB6	200	0	200	200

*Reciprocal of the highest antiserum dilution giving a skin reaction. Each value is the mean of 6 animals.

+Antisera were first diluted 1 to 25 with saline and then absorbed.

In other experiments in which the antisera were absorbed with rabbit anti-mouse γ2a or γ2b, no decrease in the PCA activity at 2 or 72 hours was detected.

Fahey, Wunderlich and Mishell showed by immunoelectrophoresis that mice produce 4 major classes of immunoglobulins: 7S γ1, 7S γ2, IgM and IgA. The 7S γ2 includes 2 subclasses: the 7S γ2a and 7S γ2b. Although all five immunoglobulins have antibody activity, only the 7S γ1 was shown to have the ability to sensitize mice for passive cutaneous or systemic anaphylaxis. More recent studies have indicated that mice can also produce a reagin-like antibody when injected with antigen plus adjuvant or when infected with T. spiralis. Previous attempts to separate these antibodies by using gradient chromatography on DEAE-cellulose resulted in initial eluates able to induce PCA only after 4 hours and late eluates inducing PCA after 4 and 72 hours. Whether the last eluates had only one antibody causing both the 4 and 72 hour PCA or 2 antibodies, one responsible for the 4 hour PCA and another for the 72 hour PCA was still undetermined. Our present experiments strongly suggest that PCA reactions induced after a short sensitization period are due to 7S γ1 antibody whereas the 72 hour reactions are due to another antibody requiring a longer sensitization period to elicit an anaphylactic response. This observation implies that under the experimental conditions used, the reagin-like antibody was not able to induce PCA within 2 hours after

sensitization. However, this does not necessarily imply that a purified or more active preparation of the reaginic antibody would not be able to induce PCA reaction within a short sensitization period. The present results also provide an explanation for previous observations showing that heating of mouse antisera containing reagin-like antibody did not change the PCA titer when reactions were elicited 2 to 4 hours after sensitization although completely suppressing them when induced 72 hours later. If the present interpretation of our results is correct, mouse reagin-like antibody probably represents a new class of antibody comparable to that found in man, although further experimental data are required to to establish this point.

3. Separation of mouse homocytotropic antibodies by biological screening.

Mice produce 2 homocytotropic antibodies, a γ1 heat resistant antibody able to induce passive cutaneous anaphylaxis (PCA) after a short sensitization period and a heat labile antibody responsible for PCA reactions after a long sensitization period.

Recently we have shown that absorption of mouse antiserum with rabbit anti-mouse γ1 removes its ability to induce 2 hour PCA in mice without decreasing its PCA activity in rats. Treatment by heat, however, destroys completely its ability to induce PCA in rats without decreasing its 2 hour PCA activity in mice. Based on these results we suggested that the 2 hour homologous PCA activity of mouse antisera was due to mouse γ1 and that the rat PCA activity was probably due to mouse reaginic antibody.

Previous attempts to separate mouse reaginic antibody from mouse γ1 antibody by gradient chromatography on DEAE-cellulose and by block electrophoresis were only partically successful. Since reaginic antibodies have a remarkable affinity for tissue, we attempted to separate the two antibodies by injecting mouse antisera containing both antibodies into normal mice and observing their rate of disappearance from the recipients' sera. Under these conditions one would expect that the reaginic antibody would be rapidly taken up by the tissues of the recipient mice, but the γ1 antibody would remain in the serum for a considerably longer time. In addition these experiments were supplemented by studies of the effect of heating and absorption with rabbit anti-mouse γ1 on the homologous and heterologous (rat) PCA activity of these antisera.

Animals. Bagg and hairless male mice weighing 20-30g and Walter Reed strain albino male rats weighing 150-250g were used throughout.

Antigens. Five times crystallized ovalbumin (Ov) obtained from Pentex Inc., (Kankskee, Ill.) and an extract of Trichinella spiralis larvae was obtained at 4°C in Tris buffer, pH 7.4. Lyophilized aliquots of the finished product were sealed in vials and stored at 4°C. Solutions were made when necessary by dissolving the dry extract in saline (0.8 percent NaCl).

Antigens.

[illegible] serum
logical [illegible]
by injection [illegible]
containing [illegible]
additional intra[illegible]
Blood was obtained [illegible]
the last dose of [illegible]
other anesthetic and [illegible]
the blood to clot [illegible]
in a refrigerator [illegible]
vidual antisera [illegible]
sera were used: anti[illegible]
antigenic stimulation; anti[illegible]
stimulation; anti[illegible]
lation and anti-[illegible]
tions of antigen. [illegible]
with 100 washes. [illegible]
same number of [illegible]
fection the [illegible]
as described above.

Biological [illegible]
strain, [illegible]
mouse anti[illegible]
hours later the recipient [illegible]
blood was [illegible]
obtained by centrifu[illegible]
or kept at -20°C. [illegible]
activity of the [illegible]
using as a control [illegible]
1:5 with normal mouse [illegible]
which indicated that [illegible]
We calculated that [illegible]
for an additional [illegible]
after a few hours [illegible]
sera would be expected [illegible]
agree very well with [illegible]

Rabbit anti-mouse [illegible]
7S γ1 sera were [illegible]
of Dr. J. Fahey, National [illegible], Bethesda,
Dr. F. Hymer, Molper, [illegible]
immunoelectrophoretic [illegible]
antiserum produced [illegible]

Absorption of anti[illegible]
were first diluted with [illegible]
experiments were [illegible]
anti-mouse γ1 [illegible]
activity of [illegible]

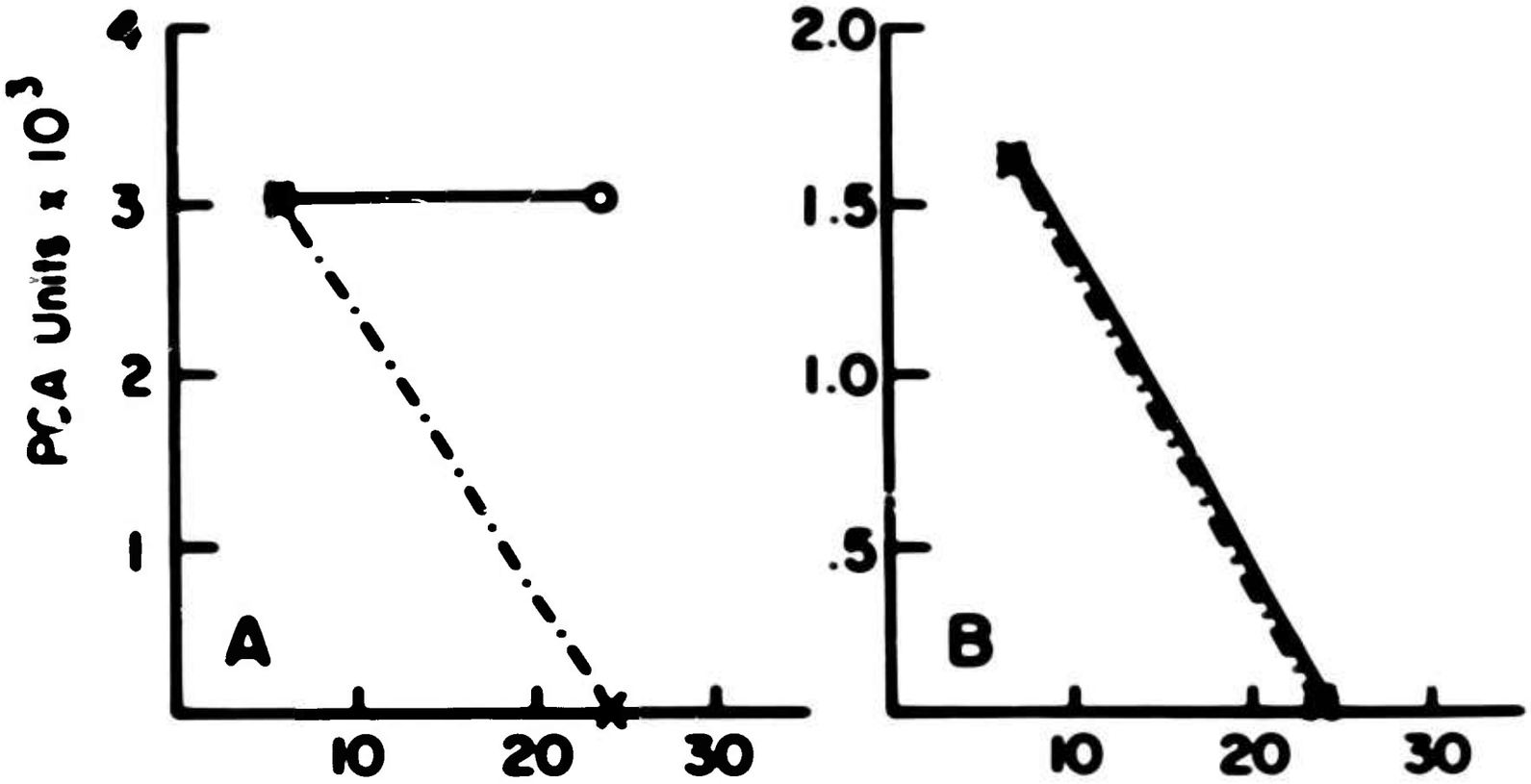

PCA Units x 10^3
4
3
2
1
A
10
20
30
2.0
1.5
1.0
.5
B
10
20
30
TIME OF SCREENING (Hours)

Table [illegible]

Effect of Absorbing Mouse Antiserum with Rabbit Anti-Mouse γ-
on Its Homologous and Heterologous (Rat) PCA Activity

Host	Antiserum Diluted At	Homologous PCA		Heterologous PCA	
		Nontreated Serum	Absorbed Serum	Nontreated Serum	Absorbed Serum
[illegible]	[illegible]	[illegible]*	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

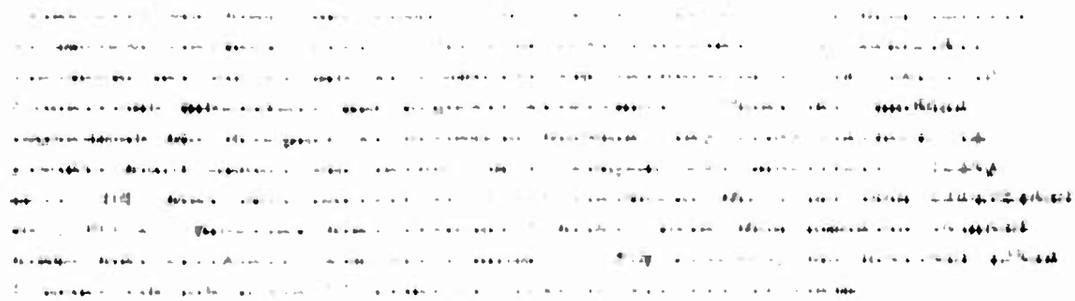
* [illegible]

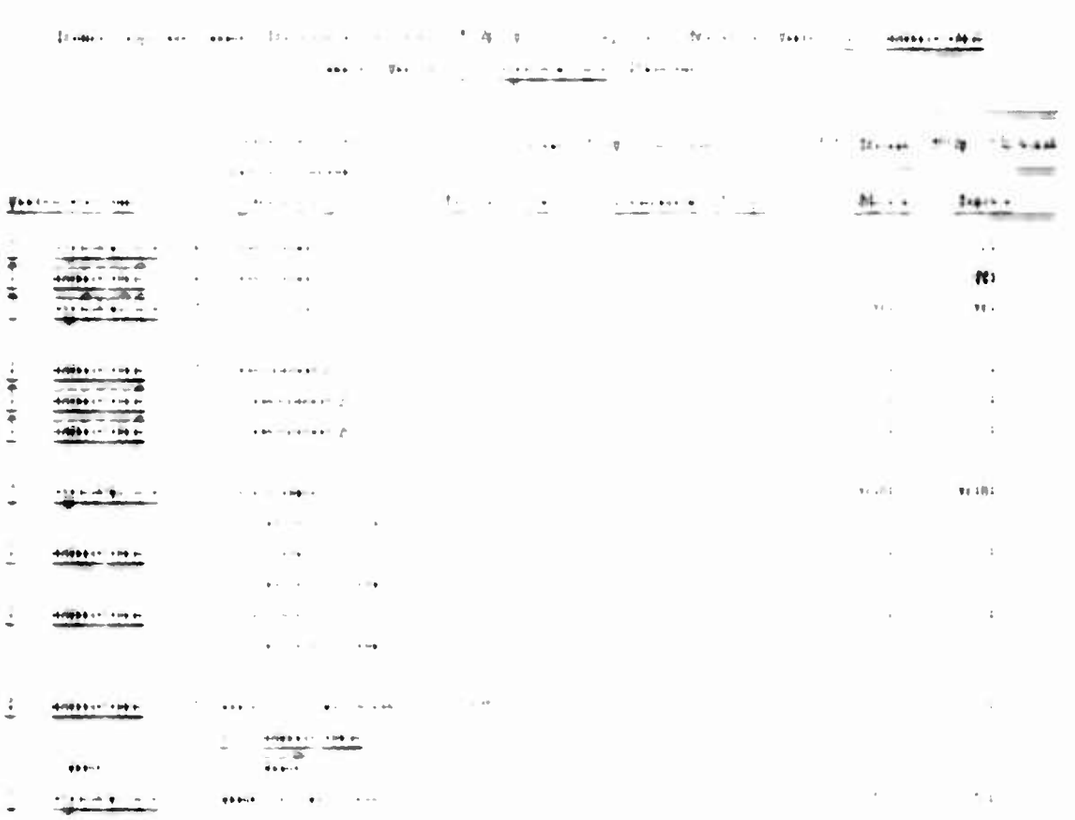
[illegible]

[illegible]

[illegible]

[illegible]





[illegible] antibody
[illegible] present in both pools against
[illegible] natural.

[illegible] PCA reaction induced with mouse anti-
[illegible] in mice are easily obtained when mouse
[illegible] In both cases very sharp blue areas
[illegible] challenging the animals with
[illegible] many hours. However, passive
[illegible] some serum resulted in PCA reactions
[illegible] sensitization and challenge was no
[illegible] differed very highly from the normal.
[illegible] were challenged 4 hours later
[illegible] intradermally, dis-
[illegible] the injected site, normally
[illegible] a high [illegible] PCA titer
[illegible] a generalized disease
[illegible] to account for the difference
[illegible] which is normally some
[illegible] cells, the determining
[illegible] in our challenge.

[illegible]

The failure of mice to produce reagin-like antibodies [illegible] could not be attributed to the passive transfer of antibodies from the possible presence of [illegible] factors, because [illegible] exposures to infection did not exhibit the production of [illegible] of reagin-like antibodies. In the other hand, [illegible] can be detected in sera of mice [illegible] actions are [illegible]

Table 5

Effect of Alcoholic Mouse Anti-[illegible] Sera on [illegible] Anti-Mouse [illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]				
[illegible]				
[illegible]				
[illegible]				
[illegible]				

[illegible]

[illegible]

Sixteen young chimpanzees (Nos. 1, 2, 4, 5, 7, 8, 9, 10, 12, 14, 15, 22, 38, 41, 42 and 59) were used in these studies. Their status of health at the time of exposure to infection with the Puerto Rican strain of S. **mansoni** has been described previously. Six animals (Nos. 4, 7, 9, 12, 14 and 59) were used as uninfected controls, six others (Nos. 1, 2, 5, 8, 10 and 38) were given a single exposure to 250, 1000 or 2000 cercariae and necropsy was performed after 7 to 36 months, and four others (Nos. 15, 22, 41 and 42) were exposed monthly to 100 or 250 cercariae and necropsy was performed 24 and 36 months after the first exposure. Five of the ten infected animals had been splenectomized previously. Frequent physical examinations included determinations of abnormalities in the feces, changes in appetite, alteration in behavior, temperature, weight and performance of abdominal palpation, ballottment and peritoneal aspiration for detection of ascites. In addition, esophagoscopy, liver biopsy and portal pressure determinations were conducted when indicated.

Parasitologic observations included stool egg counts (weekly, or bi-weekly for Nos. 1, 5, 8 and 10) by direct smear and AMS TII sedimentation technics and determination of egg viability and infectivity. At necropsy, worms were recovered and counted and the number of eggs per gram of tissue in various organs was determined by trypsin digestion.

Blood specimens were collected weekly during the first year and monthly thereafter. Hematologic procedures consisted of erythrocyte and leukocyte counts, differential enumerations and hematocrit determinations. Biochemical determinations included bromsulphalein clearance, serum transaminases (SGOT, SGPT), total serum protein and electrophoresis.

Microaggregated ^{125}I labeled human serum albumin was used to measure blood volume and hepatic blood flow on two occasions during the last month before the animals were killed. Four uninfected controls and four infected animals were studied. Doses of 0.025 and 0.050 mg albumin per kg body weight were used for the two sets of determinations. The batch of albumin used has been described in detail by Briner.

The portal pressure was measured under phencyclidine hydrochloride anesthesia. A 20 ga. needle connected by saline-filled tubing to a spinal fluid manometer was inserted into a branch of the inferior mesenteric vein. Duplicate readings obtained with the initial level in the manometer above and below the point of equilibrium were averaged for mean portal pressure values.

Serologic determinations by the fluorescent antibody test (FAT) were accomplished with strict adherence to the described method. Passive cutaneous anaphylaxis (PCA) reactions using cercarial extracts as antigen were studied as described previously.

The following reagents were prepared and tested for their sensitivity and specificity: 1) fluorescein labeled rabbit antibodies to chimpanzee gamma G globulin; 2) fluorescein labeled rabbit antibody to chimpanzee beta 1 C globulin; 3) fluorescein labeled globulin from chronically

infected chimpanzees for detection of schistosome antigens. Freshly prepared frozen sections of liver, lungs, kidneys and spleen (when available) from chimpanzees Nos. 2, 22, 38 and 42 were examined with these reagents in the direct immunofluorescent technic.

Pathologic studies were conducted by needle biopsy, surgical biopsy and necropsy. Since needle biopsies yielded only limited histopathologic information other than confirmation of active infection and, in some instances, of portal fibrosis, they were discontinued after two years. At necropsy, all major organs were dissected free and weighed, and specimens were collected for organ egg assays and for histopathologic studies. All tissue collected for histopathologic studies were fixed in buffered 10% formalin and stained with hematoxylin-eosin. Multiple sections for portions of the colon, small intestine, liver, lung and pancreas were prepared. Liver and lung specimens were also stained with Masson's trichrome, Wilder's reticulum and Verhoeff-van Gieson elastica stains.

1. Parasitologic observations. As reported earlier the onset of patency and fecal egg excretion varied individually and were somewhat related to the size of exposure. Each of the chimpanzees exposed to monthly infections gradually increased its egg output during the first 24 months and subsequently maintained reasonably steady average egg counts over the period of observation up to 36 months following the first exposure. By far the highest egg output was observed in chimpanzee No. 41. Although chimpanzees Nos. 22 and 42 excreted considerably fewer eggs than did No. 41, they passed many more eggs than did No. 38 which demonstrated only minimal clinical manifestations throughout the course of infection and the least degree of pathologic alterations. In all but two animals (Nos. 2 and 22) eggs appeared in the feces in greatest numbers between 22 and 27 months after exposure. There was little correlation between the number of cercariae to which the chimpanzees had been exposed, the number of worms recovered and the mean number of eggs per gram of feces (MNEPGF). Although fewer worms were recovered from No. 41 than from Nos. 22 and 42, the MNEPGF for No. 41 was considerably greater throughout the experiment.

In the post mortem studies, most of the schistosomes were found in the distal branches of the mesenteric veins and were active and well developed. Organ egg assays revealed a correlation between eggs in tissues, and extent of clinical and pathologic involvement. As in the previous experiments, most of the eggs were found in the large intestine and in the liver. The greatest number of eggs per gram of tissue digested was found in chimpanzee No. 41 while somewhat fewer occurred in Nos. 42 and 22. Relatively few eggs were found in the organs of animal No. 38. It is of interest to note that chimpanzee No. 41, which showed evidence of extensive portal-systemic collateral circulation, had 1598 eggs per gram of lung tissue whereas the other animals had considerably fewer eggs in this organ. No apparent effects of splenectomy on the parasitological findings were detected in these infections.

2. Clinical observations. The clinical manifestations of the chimpanzees exposed to a single cercarial dose and of those with multiple exposures up to 2 years following infection have been described previously. All animals had proportionate weight gains throughout the course of the infection with the exception of chimpanzees Nos. 41 and 42. Several months prior to necropsy and beginning at the time when marked hepatic decompensation was observed, chimpanzee No. 41 lost weight and showed increasing muscular wasting, jaundice and lethargy. During the last few weeks of his life this chimpanzee was obviously listless and gave evidence of marked loss of muscular tone. The abdominal distension apparent in this animal 5 months after infection increased up to time of death 26 months later. Marked superficial abdominal circulation was later evident. Some abdominal distension was also observed in chimpanzee No. 42 just prior to necropsy.

Hematologic observations in these animals were unremarkable except for an increasing reduction in hematocrit in No. 41 and to a lesser degree in No. 42. During the third year of infection, the hematocrit in animal No. 41 was 25 per cent and a reduction in erythrocyte count was noted. No intestinal bleeding and no marked changes in stool consistency were evident in any of these animals.

No evidence of esophageal varices was noted by endoscopic evaluations in most chimpanzees. However, varices 2 to 4 mm in diameter were seen in chimpanzee No. 41 beginning at about 26 or 27 cm from the upper incisors and continuing to the esophageal-gastric junction at 30 cm. In chimpanzee No. 42 the esophagus had prominent superficial veins 1 to 2 mm in diameter in isolated areas, and in No. 22 increased surface vascularity was also seen; however, no true varices were seen in these two animals. Mean portal pressure and hepatic blood flow determinations conducted in a number of animals near the end of the experiment failed to demonstrate significant differences.

3. Biochemical observations. Monthly serial biochemical tests were performed from the time of exposure until the end of the experiment. Irregular mild elevations in the percent bromsulfalein retention and in serum transaminase levels (SGOT up to 110 units per ml and SGPT up to 50 units per ml) were observed on some occasions in a few animals. These findings, however, were not consistent and did not correlate with the intensity of the infection. Striking and consistent increases in total serum proteins occurred. In one animal receiving a single exposure (No. 2) the peak increase in total serum protein occurred somewhat earlier than in the others. A good example of the relationship between worm burdens and elevation of total serum proteins is chimpanzee No. 38 which received a cercarial exposure identical to that of No. 2, but yielded a much lower number of adult schistosomes and demonstrated elevated total serum proteins much later. Greater and earlier increases occurred in chimpanzee No. 2 which harbored considerably more worms than No. 38. Among the animals given monthly exposures, the increase in total serum proteins was considerably greater in those chimpanzees with the greatest clinical and pathological abnormalities resulting from infection.

A proportional decrease in serum albumin levels was observed in all animals except Nos. 2 and 38. However, in most animals this decrease was accompanied by an increase in total serum proteins so that the absolute amount of serum albumin did not significantly diminish. In chimpanzee Nos. 41 and 42 there was a significant decrease in serum albumin values despite the increase in total serum protein, and values as low as 1.4g/100 ml were observed in these animals before necropsy.

No consistently significant differences were observed in the amounts of alpha-1, alpha-2 and beta globulins in any of these animals. However, marked increases in gamma globulin levels were observed after infection. This increase occurred earliest in chimpanzee No. 2. Elevated gamma globulin values were much more marked in the animals with heavier infections, reaching peaks of 8.1 and 8.2g/100 ml in chimpanzees No. 41 and 42, respectively.

4. Serologic observations. Fluorescent antibody tests and tests for the passive transfer of immediate-type hypersensitivity from chimpanzees to rhesus monkeys were carried out. The results of serologic findings on chimpanzees following a single exposure to S. mansoni cercariae during the first 7 months of infection have been reported. The time course of antibody development in animals with chronic infections was quite similar for all the chimpanzees. In most cases antibodies were detected after the second month following exposure, reached a peak at approximately 10 months following infection and remained at a high plateau throughout the course of the experiment. No significant differences in peak titers were observed between the chimpanzees given a single exposure and those with multiple exposures. However, the early heavy worm burden in chimpanzee No. 2 resulted in a high peak titer much earlier than in other animals. Relatively low titers were observed for chimpanzee No. 38 beginning 14 months after exposure. In all other animals, the titers remained relatively constant.

Cutaneous reactivity was successfully transferred by injection from all but one infected chimpanzee (No. 15) to recipient tuberculin-negative rhesus monkeys. Thirty minutes after intravenous injection of the dye and cercarial antigen, positive PCA reactions characterized by local edema and blue cutaneous coloration were observed in monkeys passively sensitized 72 hours previously. This reaginic antibody was largely destroyed by heating the serum from the chimpanzees at 56°C for one hour before passive transfer experiments. Uniformly negative results were observed when serum from uninfected chimpanzees was employed. The time course development of reaginic antibodies in the several chimpanzees tested varied considerably. In one animal (No. 2) dermal reactivity was present 8 months following exposure and persisted for the duration of the experiment. In another animal (No. 15), no reaginic antibodies were detected at any time. In the other 4 animals only transient reaginic activity was demonstrated. In none of these chimpanzees were reaginic antibodies demonstrated earlier than 6 months after the first exposure.

Immunofluorescent studies of tissue from chimpanzees infected with S. mansoni were conducted to determine whether an immunologic mechanism

could be associated with the tissue lesions and the development of pipe stem fibrosis. In the livers of 3 chimpanzees (Nos. 2, 22 and 42) the labeled globulin from a chronically infected animal caused the staining of eggs within the granulomas and the fibrous scars. However, fluorescent staining was detectable only in the eggs and their immediate surroundings as described earlier. There was no evidence of schistosome antigen beyond the confines of these granulomas. In addition, faint staining from gamma G and beta 1 C globulins were seen in some of the fibrous septa of livers. However, the stain was very faint and of questionable significance.

5. Pathologic observations. The pathology of a light schistosome infection (chimpanzee No. 1) and the early stages of liver pathology in more heavily infected chimpanzees at autopsy have been described. In addition, the development of fibrotic hepatic lesions, as seen during the first two years in successive liver biopsies of heavily infected animals with both single and multiple exposures, has been described in detail.

The present report deals with the post mortem findings in five chronically infected animals (Nos. 2, 22, 38, 41 and 42) and with comparisons with the corresponding earlier biopsy results. This presentation will be made in order of decreasing intensity of the liver pathology observed post mortem.

Chimpanzee No. 41 was necropsied after 30 months. Extensive development of collateral circulation was evident. Dilated superficial veins were visible on the abdomen and esophageal varices, observed earlier by esophagoscopy, were demonstrated by latex injection post mortem to consist of submucosal vessels attaining 0.1 cm in diameter and subserosal vessels up to 0.2 cm. The spleen, which had been palpable antemortem and extended to the level of the iliac crest was found at post mortem to be enlarged (102 g), hard and chronically congested. The hard, dark brown liver weighed 551 g and its left lobe extended 2 cm below the costal margin. The free margin of the liver was sharp and its surface showed a net-like accentuation of the lobular pattern and a slight nodularity. The cut surface of the liver, which was sectioned with some difficulty, revealed a classical pipe stem pattern with the larger portal fields thickened up to 1.4 cm in diameter around vein branches 0.2 - 0.3 cm wide. In cross section these fields appeared as scalloped, whitish islands. The lobular liver pattern was otherwise preserved and the gallbladder was normal. Histological examination demonstrated granulomatous involvement of the small, middle sized and large portal fields. In some of the small ones, there was substitution of portal radicles by granulomas with occasional endophlebitis. The large tracts, riddled with eggs and granulomas, showed profound alteration of the vascular pattern including virtual obliteration of some portal branches. Along the margins, these showed fibrous spurring in the direction of adjacent smaller fields and partial fusion with them, a pattern characteristic of human pipe stem fibrosis and designated as stage 4 in our classification. Those occasional large fields not involved by eggs and pseudotubercles showed that pattern of fibrous thickening around vein branches designated as stage 3 in our earlier work and described below in more detail. Piece-meal necrosis of

The liver trabecular terminal plates were normal, but the lobular architecture was otherwise intact. The liver cells showed focal non-specific necrosis and degenerative changes which were absent in the antemortem biopsies. The Kupffer cells and abundant pigment. No dead adult worms were seen in any of the liver sections.

The lungs were firm and riddled with partly granular confluent lesions 0.1 to 0.2 cm in diameter, visible on both the pleural and the cut surfaces. Histologically, these corresponded to large, destructive pseudotubercles involving the walls of practically all of the smaller arteries from which they bulged out in a leaf-like branching pattern. Many arterial lumina were obliterated, and their elastic layers disrupted. Occasionally, multiple venular channels were found in these lesions, but these were not sufficiently dilated to be called angiomatoids. The heart weighed 70 g and the right ventricle showed thickened trabeculae carnae. No worms were found in the random lung sections examined after perfusion.

The colon showed numerous nearly confluent, firm submucosal and occasional subserosal nodules. The former were associated with diffuse congestion and tiny areas of ulceration. These lesions were less marked in the rectosigmoid. In the ileum and distal jejunum there were only a few scattered lesions which histologically showed severe granulomatous bilharzial involvement affecting all layers of the gut wall. A considerable proportion of the eggs were calcified.

The pararectal and periaortic fibrous tissue was thickened, and contained abundant schistosome eggs and pseudotubercles. The lymph nodes of the mesocolon, porta hepatis and lung hylus were enlarged, with many plasma cells and with reticular hyperplasia. An oxyurid worm was found in the appendix. Sporadic Balantidium coli were present in the mucosa, but did not invade host tissues.

As suggested earlier, the post mortem examinations of the other chimpanzees demonstrated a wide range of lesions which can be considered as milder forms of pipe stem fibrosis or as its precursor stages. In animals Nos. 22 and 42, the liver surface grossly showed numerous subcapsular pseudotubercles, but no granularity or accentuation of the lobular septa. On cross-section, fibrosis of the portal fields was grossly visible, but was of a lesser degree than in chimpanzee No. 41. The fibrous pattern was most prominent in the middle sized and smaller portal fields and appeared as fine, spidery-greyish markings leaving the lobular architecture undisturbed. In the larger fields, increased cuffing around veins and greater density were evident, although the spectacular enlargement and interconnection of portal tracts noted in chimpanzee No. 41 was missing. These changes were substantiated by the histological appearance of the sections. Granulomatous involvement of the small portal tracts was heavy, and many vein radicles were replaced by pseudotubercles or had become unidentifiable. Most of the large and middle sized portal fields, surrounded or bordered by granulomatous lesions, were prominent, but were generally devoid of eggs and granulomas and showed only the alterations corresponding to stage 3. A few of these contained spotty infiltrates

composed mainly of lymphocytes and other mononuclear cells. In a few instances, these infiltrates surrounded narrowed portal radicles. The fibrosis seen in these fields was disproportionate to their spotty inflammatory infiltration, and was characterized by dense, nearly hyaline collagen fibers which tended to be heavier around the portal veins. A few of these veins were so narrow as to become unidentifiable without trichrome staines. In the post mortem material, collagen deposition had become heavier and denser than was noted in the biopsies. The collagen fibers followed an orderly, slightly wavy linear pattern and were not concentric. From the periphery of the portal fields, fine fibrous septa radiated in the direction of smaller triads, in some instances with continuous connections between two or more of them but without affecting the lobular pattern or the position of the central veins. The latter and especially the larger sublobular veins were sometimes thickened by collagen deposited in all three of their layers. Lymphatic channels were seen in considerable numbers, but were less distended and prominent than in the biopsy specimens.

The colonic lesions were severe in both these animals. Grossly, the mucosa showed numerous sessile nodules and elevations which were partly confluent and of relatively even size ranging from 2 - 4 mm. There were petechiae and/or shallow erosions of the overlying mucosa, as well as diffuse mucosal hyperemia. The serosa of the most heavily involved portions showed numerous scattered whitish pseudotubercles. Thickening of the colonic wall was confined to the mucosa and submucosa. Histological examination revealed lengthening of mucosal crypts and dilation of some of them. There was also massive egg deposition in the colonic mucosa and submucosa, with some lesions also affecting the deeper layers. In addition to showing eggs and granulomas in various stages of evolution, the nodular lesions revealed diffuse inflammatory infiltration with lymphoid cells and eosinophils, as well as increased vascularity and vasodilatation, thus accounting for their polypoid character. The upper colon and small intestine showed much smaller and more sporadic mucosal lesions. The appendix was affected in animals Nos. 42 and 22. Balantidium coli were present in the submucosa of the colon in No. 22, but no difference was noted between lesions containing ciliates and those in which ciliates were absent. Many adjacent lymph nodes were markedly enlarged, some of them contained eggs and pseudotubercles, but showed reticular and lymphoid hyperplasia in excess of their granulomatous lesions.

The lungs of both chimpanzees showed only sporadic pseudotubercles. In chimpanzee No. 42, a few venules at the cardia were filled with contrast medium injected during necropsy, but these did not show varicose dilatation. The spleen (No. 42) and accessory spleen (No. 22) in these animals showed chronic congestion.

In chimpanzee No. 2 gross and microscopic pathology of the liver were similar in nature to that observed in the two preceding animals, although to a much lesser extent. There were fewer pseudotubercles visible through the capsule; the fibrous thickening of the larger portal fileds seen on cross-section was mild and more widely scattered, and the

fine spidery pattern of the small fields observed in Nos. 42 and 22 was absent. Nevertheless, the lesions were qualitatively similar histologically. Thus, this animal appeared intermediate in degree of hepatic pathology.

The colonic pathology of chimpanzee No. 2 was the most severe of the series. The rectum showed only moderate lesions. The mucosa of the large intestine from the sigmoid through the ascending colon was studied with inflammatory polyps, mostly sessile, but occasionally pedunculated ranging from 2 mm to over 10 mm in diameter. These were tannish pink in color after perfusion and were partly confluent. Smaller non-polypoid lesions were present in the ileum and jejunum. The inflammatory polyps differed from those observed in the preceding animals primarily in their greater redundancy and tendency to erode. Groups of Balantidium coli occasionally invading the submucosa were found interspersed among the schistosome lesions. The same histological features were evident and granulomas of the muscularis and subserosa were again present.

Pseudotubercles of the lung were observed only very rarely in chimpanzee No. 2.

The liver of chimpanzee No. 38 was almost normal in gross appearance and consistency, except for the presence of a few scattered subcapsular pseudotubercles and some excess pigmentation. The cut surface of the liver revealed only occasional portal accentuation which was confined to small fields and best seen under magnification. The portal veins were thin-walled and undistended. Histological examination demonstrated pseudotubercles, eggs or contracted fibrous scars with mild spurring and branching of the field, on the average, in every 5th small portal field. The middle sized and larger portal fields were infiltrated by lymphoid cells and occasional eosinophils. These showed normal amounts of connective tissue and their vessels, as well as the central veins, were thin walled. The lobular and trabecular liver architecture was undisturbed. The colon of chimpanzee No. 38 showed scattered tiny submucosa granules representing pseudotubercles, each surrounded by a fuzzy diffusely hyperemic halo without nodularity or polyp formation. Similar sporadic lesions were seen in the small intestine. Histological sections exhibited scattered pseudotubercles, granulomas and mixed inflammatory infiltrates with eosinophils which resembled those seen in chronic mild human schistosomiasis. The deeper colonic layers were largely uninvolved in the samples studied.

No pseudotubercles were observed histolocially in random lung samples of chimpanzee No. 2, although eggs were recovered by digestion. The spleen showed acute congestion. The esophageal submucosa veins were normal.

The organs of all chimpanzees not specifically described above were either unremarkable or showed minor and insignificant lesions, related mainly to the terminal state induced by anesthesia, bleeding and operative manipulation.

In summarizing our observations, primary emphasis is given to 3 aspects of the study: I) the establishment of infection and the relative numbers and location of eggs and adult flukes; II) correlation of the parasitologic findings with the clinical and pathological manifestations, including biochemical and serologic observations; III) interpretation of the data in the form of a working hypothesis for the probable mechanisms in the development of pipe stem fibrosis and suggested ways by which the chimpanzee model can be utilized to answer some of the remaining questions.

I. Studies on adults and eggs

Viable eggs containing miracidia infective for suitable snail hosts were excreted in the feces in a reasonably constant manner for the duration of the experiment. As the infection progressed the numbers of eggs produced by the schistosomes did not decrease significantly, nor was there any indication that the number of worms might be reduced by a process of self cure as described in rhesus monkeys. In fact, the highest percentage of recovery of adult worms compared with cercarial exposures was obtained in a chimpanzee (No. 2) necropsied 36 months after a single exposure. Similarly, there was no suggestion that acquired immunity, if present, interfered with the establishment of worms from subsequent exposures. Indeed, except in No. 2, a higher percentage recovery of worms was obtained from chimpanzees which were exposed to monthly infections (Nos. 15, 22, 41 and 42) than from the animals receiving a single exposure. Moreover, chimpanzee No. 42 yielded a higher percentage of worm recovery after 3 years and 36 monthly exposures than did No. 15 after 2 years and 24 equivalent exposures. Schistosome eggs were found in all organs studied, with heaviest concentrations occurring in the liver and large intestine. As anticipated, the animal which developed extensive pipe stem fibrosis and portal-systemic collateral circulation (No. 41) had very large numbers of eggs in the lungs. In general, there was some relationship between the number of eggs in the tissues, the number of eggs excreted in the feces and the number of adult worms recovered post mortem. For example, in the chimpanzees which developed the most severe pathological lesions (Nos. 22, 41 and 42), the number of worms recovered and the number of eggs deposited in various organs was considerably greater than in the other animals. The relatively low concentration of eggs in the liver of chimpanzee No. 41 may have been caused by failure of the trypsin digestion technic to completely free the eggs from the dense portal fibrous tissue. This belief was supported by the results of KOH digestion. After KOH digestion, 3081 eggs per gram of liver were found in chimpanzee No. 41 as compared with 1307 eggs per gram in No. 42 and 1020 eggs per gram in No. 22. The high concentration of eggs in the feces of chimpanzee No. 41 predicates against the theory that in cases of Symmer's fibrosis, eggs concentrate in the liver because of the inability to reach the intestinal lumen. It is noteworthy that in the animals with early or late pipe stem fibrosis, there was no evidence of increased deposition of eggs in the small intestine, as observed in human autopsies.

II. Clinical and pathologic observations

The serum biochemical findings in infected chimpanzees contrasted in some important respects with the biochemical observations reported for mice with schistosomiasis. For example, no consistent significant alterations in the percent of bromsulphalein retention or in the levels of serum glutamic pyruvic transaminase or serum glutamic oxalo-acetic transaminase were detected in chimpanzees. On the other hand, the significant increases in total serum protein and in gamma globulin concentrations recorded previously in mice were also observed in infected chimpanzees. These alterations were much more dramatic in the chimpanzees than they were in the mice and reached levels never reported previously for schistosomiasis in man or experimental animals. This was particularly evident in the two severely diseased chimpanzees (Nos. 41 and 42) in which total serum protein levels as high as 12.6 and 12.8 g/100 ml and gamma globulin values of 8.1 and 8.2 g/100 ml, respectively, were recorded. The great increase in globulin concentration in the absence of corresponding increases in albumin produced strikingly reduced A/G ratios. These were more evident in the animals with heavier infections and became more marked with passage of time. Ratios as low as 0.18 were observed in chimpanzee No. 41 and as low as 0.20 in chimpanzee No. 42.

The hyperglobulinemia found in most of the infected chimpanzees was accompanied by a striking degree of lymph node reticular hyperplasia and plasmocytosis, particularly of the mesenteric and retroperitoneal lymph nodes. This appeared disproportionate to the number of eggs deposited in such nodes. A tendency to extensive lymph node invasion and hyperplasia was also observed in a group of chimpanzees infected with S. hematobium (unpublished data).

Our studies revealed that the time-course development of antibodies detected by passive cutaneous anaphylactic reactions is unrelated to the magnitude or manner of exposure (whether single or repeated), to the intensity of infection as determined by worm burden, or to the evolution of the disease.

The liver histopathology of these chimpanzees was similar to that observed at autopsy in heavily infected humans. Certain lesions first categorized in the course of this experimental study have also been recognized previously in human specimens, particularly the satge 3 portal lesion, i.e. fibrosis of middle-sized and larger portal fields in the absence of local egg deposition. In some human livers examined in earlier studies, this lesion was persistent in a few fields; however, most showed advanced stage 4 portal lesions resembling those described in chimpanzee No. 41. This finding agrees with Raso's and Cheever's observations that eggs concentrate in the portal fields characteristically in the advanced stage of human pipe stem fibrosis. Both in the chimpanzee and in human autopsy specimens, there was marked individual variation in the degree of hepatic involvement and fibrosis.

According to a recent report by Neves and Raso pipe stem fibrosis and portal hypertension can occur as early as 130 days after the acute "toxemic" stage of human schistosomiasis. It is therefore not surprising that some of our chimpanzees began showing early liver fibrosis within 7 months of their first exposure, and that fully developed fibrosis occurred in approximately 2 years.

From the limited observations now at hand, portal-systemic collateral circulation seems to develop more promptly and effectively in the chimpanzee than in man. However, despite evidence of significant presinusoidal portal pathology in many of these animals, elevation of portal pressure was not detected. The demonstration of extensive connections between hemorrhoidal and intrarachidial veins in one of our animals suggests that the development of collateral venous systems in the chimpanzee deserves further study. The development of submucosal esophageal varices in chimpanzee No. 41 is of interest. Similar experiments might provide a method for the experimental production of this lesion without resort to major vascular surgery, and thus might help in arriving at a better understanding of the pathophysiology of this complication commonly found in various forms of human liver fibrosis.

One is impressed with the relative severity of bilharzial pathology in the colon of the chimpanzee. The extensive development of inflammatory pseudopolyps seen in Nos. 2, 22 and 42 resembled the human lesions reported from the Egyptian focus; such lesions appear to be very rare in the Brazilian focus, and in South Africa and Venezuela. In man they are generally less densely aggregated. Similar pathology was seen in a group of chimpanzees necropsied 7 months after their first exposure to S. hematobium in our laboratory.

III. The evolution of infection with S. mansoni

These studies on chronic schistosomiasis in a singly and repeatedly exposed chimpanzees have permitted observations analogous to those of a severely infected human population, but with the advantage that many of the variables which are always encountered in humans living in endemic areas, such as dietary deficiencies, concurrent infections and the time and size of exposures could be better controlled. These experiments have given further insight into the natural history of bilharzial liver disease and have suggested ways by which the pathogenesis of this infection occurs.

Serial biopsies showed that egg deposition in the liver began in the distal, small portal fields and vein radicles. In these areas much of the fibrous proliferation occurred around granulomas or in egg-related inflammatory infiltrates. Unexpectedly, the onset of fibrosis in the middle-sized and larger portal fields was found to precede, rather than follow, local egg deposition. This stage of pipe stem fibrosis, previously designated as stage 2 is of uncertain pathogenesis. Ultimately, however, even the larger and more proximal branches of the portal tree were affected so that the eggs became concentrated primarily in the

fibrous tracts of the liver. In the chimpanzee and in human material studied by Raso and Cheever, there was a lack of concentration of eggs in portal tracts prior to the development of pipe stem fibrosis.

A histological analysis of the stage 3 fibrotic portal change reveals primarily a paucity of lymphoid cell infiltrates accompanied by many dilated lymph vessels and a wavy, parallel arrangement of the collagen fibers whose initial predilection for the adventitia of the portal vein was first observed in biopsy material. This rather diffuse fibrotic lesion is reminiscent of the sclerosis seen in long standing passive congestion or in lymph stasis, although neither hemosiderin nor lipid-laden macrophages were seen here. The lesions appear to coincide with widespread granulomatous involvement of the smaller, satellite portal fields branching off the larger triads and with encroachment or obstruction of the smallest portal radicles by granulomas.

Hepatic blood flow in human cases of hepatosplenic schistosomiasis has been found to be normal by Ramos et al. and by Mousa et al., and to be decreased by Razzak. Portal pressure was not significantly elevated in these chimpanzees and total estimated hepatic blood flow was normal. Even when marked portal obstruction and portal hypertension are present, as in heavily infected mice, increased arterial flow apparently compensates for diminished portal flow. In the chimpanzee, it is possible that circulation in the intrahepatic sector of the portal tree proximal to the narrowed radicles might be sluggish enough to produce endothelial damage and increased venular permeability. Initially, this might be compensated by accelerated lymphatic flow, but it eventually would stimulate desmoplasia in the adventitia of the vein subsequently spreading to the entire portal field. In analogous situations, e.g. in stasis dermatitis or in elephantiasis, fibrosis is believed to be related to the seepage of macromolecular lipoproteins or chylomicrons into the extravascular interstitium over a long period of time. Several observers, including Bogliolo have noted dilated lymphatics in portal spaces in early human pipe stem fibrosis.

It seems unlikely that portal fibrosis during stage 3 is elicited by antigen-antibody complexes, since in contrast to the findings of Magalhaes Filho et al., we were unable to demonstrate diffuse antigen or significant fixed gamma globulin localization in these sites. Furthermore, the inflammatory infiltration was milder than that usually associated with "immune complex disease" and the fibrosis was relatively delayed.

Although delayed hypersensitivity in the mouse has been related to granuloma formation and the latter to periportal fibrosis, its role in the development of pipe stem fibrosis in the chimpanzee has not yet been demonstrated. In this animal, periportal fibrosis precedes the deposition of a large number of eggs in the portal areas and would appear to have a different pathogenesis.

In any case, none of the proposed explanations discussed above can be dismissed with the evidence available thus far, nor can alternative

new theories be refuted. It would be desirable, as a first step, to investigate the dynamics of intrahepatic portal blood flow in normal chimpanzees and in animals showing progressive stages of experimental bilharzial pipe stem fibrosis using selective modern catheterization technics which would permit the measurement of portal pressure in small size distal vessels. Studies of the portal lymph flow, of vascular permeability and further immunological and immunohistochemical studies would also be desirable.

6. The requirement for sensitized lymphocytes in one form of antigen-induced histamine release from rabbit platelets.

Barbaro and Zvaifler demonstrated that a well-washed mixture of platelets and leukocytes from rabbits immunized with Shistosoma serum albumin in Freund's adjuvant released histamine on the addition of specific antigen. This mode of allergic histamine release differed from those described previously for rabbit blood in that it did not require added antibody and plasma factors. Since the release of histamine could be correlated with the presence of rabbit anti-schistosomal antibody, and a major portion, if not all, of the histamine in these experiments came from the platelets, the authors argued that the platelets were sensitized by the antibody. Immunologic and tissue culture studies show that well washed platelets from rabbits infected with Schistosoma mansoni released histamine on the addition of extracts of the Schistosomes if leukocytes from infected were present, but not if the leukocytes were absent.

The studies described here have confirmed the observations of Bachelet and Sadun and showed that lymphocytes are necessary in the histamine release. This is a function of a specific subpopulation of immunologically competent lymphocytes.

The methods of preparing antigen, of separating the histamine released from cells, and of infecting rabbits with schistosomes are the same as previously described. The times of rabbit infection ranged from 6 weeks in the experiments most recently done to 8 weeks after the initial infection.

The total leukocyte counts were determined by standard hemocytometer techniques. The proportion of the various cell types was obtained from differential counts of stained smears. Platelet counts were performed under phase microscopy.

Preparation of platelet suspensions: Blood was drawn from the central artery of the rabbit's ear with a [illegible] syringe containing [illegible] of 0.1 M ethylenediaminetetraacetate (EDTA). The ratio of blood to EDTA was 9 to 1. The blood was centrifuged in the cold at 1000 rpm for 10 min, the plasma discarded, and the fluffy coat, composed of platelets and leukocytes, resuspended in Tyrode's solution containing 0.1 M EDTA and 0.1% glucose. The platelet rich suspension was then centrifuged at 1000 rpm for 10 min, the supernatant discarded, and the platelets resuspended in Tyrode's solution containing 0.1% glucose. The platelet suspension was then tested by being centrifuged at 500 g for 5 min. The platelet

some from the leukocytes. The latter was a consistent finding. This form of histamine release is apparently the same as recently reported to occur when rabbits are immunized with protein antigens in Freund's adjuvant, the cell source being presumably the basophil.

Table 14

The Requirement of Sensitized* Leukocytes for Antigen-induced Histamine Release from Platelets

	Platelets (10^8)	Leukocytes (10^6)	Histamine	
			Total (ng)	Antigen-induced release (ng)
Experiment A				
Homo. fatty rat	[illegible]	[illegible]	Lost	[illegible]
Homo. platelets	[illegible]	-	[illegible]	[illegible]
Homo. leukocytes	[illegible]	[illegible]	[illegible]	[illegible]
Homo. platelets + homo. leukocytes	[illegible]	[illegible]	[illegible]	[illegible]
Experiment B				
Homo. platelets	[illegible]	-	[illegible]	[illegible]
Het. platelets	[illegible]	-	[illegible]	[illegible]
Homo. leukocytes	[illegible]	[illegible]	[illegible]	[illegible]
Het. leukocytes	[illegible]	[illegible]	[illegible]	[illegible]
Homo. platelets + homo. leukocytes	[illegible]	[illegible]	[illegible]	[illegible]
Het. platelets + homo. leukocytes	[illegible]	[illegible]	[illegible]	[illegible]
Homo. platelets + het. leukocytes	[illegible]	[illegible]	[illegible]	[illegible]
Het. platelets + het. leukocytes	[illegible]	[illegible]	[illegible]	[illegible]

*In this experiment 'sensitized' means from infected rabbits, 'similar'), 'normal' means from uninfected rabbits.

Attention was then turned to attempts to discover which type of cell in the leukocyte fraction was sensitized. The leukocyte fraction was always contaminated with a certain proportion of red cells. When mixed with normal platelets, red cells from infected animals free of leukocytes gave no histamine release on the addition of antigen. These red cells were used at the same concentration as the times greater than the concentration present in the leukocyte preparation that did contain histamine release from the platelets in the same experiment.

In experiments preliminary to a study of the cell types in the blood, neutrophils from a pigeon-induced peritoneal exudate of an infected

rabbit were found unable to sustain antigen-induced histamine release when added to platelets from normal animals, though neutrophils were used in a concentration three times greater than the total blood leukocytes from the same animal. The blood leukocytes gave distinct histamine release when added to platelets from the same normal rabbit used with the peritoneal neutrophils. Macrophages from a mineral oil-induced peritoneal exudate of infected rabbits were also unable to substitute for blood leukocytes in giving antigen-induced histamine release from platelets.

Although these experiments made it clear that neither peritoneal exudate macrophages nor neutrophils from infected animals could interact with platelets, the possibility remained that the corresponding cells from the blood might be active. Therefore, the cell types in the leukocyte fraction of the buffy coat were separated and tested for their ability to interact with platelets from normal animals. The results of one such experiment are seen in Table 16.

The results in Table 16, which are similar to those in two other experiments, show that a mixture of neutrophils and monocytes was unable to sustain histamine release. The lymphocyte fraction, however, was capable of giving as good an antigen-induced histamine release from platelets as the whole leukocyte fraction. The lymphocyte fraction was contaminated with 4 percent neutrophils. It might be argued, therefore, that although neutrophils alone or with macrophages are inactive, the 4 percent neutrophils are required in order to allow the lymphocytes to exert their effect on the platelets. In order to eliminate this possibility, a pure lymphocyte population from blood of infected rabbits was isolated and tested for its ability to allow antigen-induced histamine release from normal platelets.

The results of one such experiment using pure lymphocytes are seen in Table 17. Here, no other kind of leukocyte contaminated the lymphocyte fraction. When added to platelets, the sensitized leukocytes from which the lymphocytes were obtained gave 0.40 μg of histamine release upon addition of antigen; the pure lymphocyte suspension added to platelets gave 1.06 μg. The great increase in histamine release afforded by the lymphocyte suspension can be ascribed to the larger number of lymphocytes compared to those in the total leukocyte fraction. This is borne out in subsequent experiments in which the amount of histamine release was found to be linearly related to the number of pure lymphocytes added to a constant number of platelets.

The initial observation by Schoenbechler and Sadun that a pure platelet suspension from rabbits infected with S. mansoni is unable to release histamine on the addition of antigen was confirmed. These results also confirm similar findings in rabbits immunized with ovalbumin, reported by Siraganian and Oliveira. Thus, the conclusion by Barbaro and Zvaifler that the platelet can be sensitized by heat-labile homocytotropic antibody can no longer be held.

Table 16

The Leukocyte Cell Type Required to Give Antigen-induced Histamie Release from Platelets

	Platelets (10^8)	Leukocytes (10^6)	Lymphocytes (10^6)	Neutrophils & Monocytes (10^6)	Histamine	
					Total (μg)	Antigen-induced Release (μg)
Nor. platelets	0.83				0.80	0.10
Sens. leukocytes		0.92			0.10	0.08
Sens. lymphocytes			0.80		0.20	0.20
Sens. neutrophils + monocytes				0.44	0.02	0.03
Sens. lymphocytes + sens. neutrophils + monocytes			0.80	0.44	0.28	0.21
Nor. platelets + sens. leukocytes	0.83	0.92			0.77	0.18
Nor. platelets + sens lymphocytes	0.83		0.80		1.02	0.68
Nor. platelets + sens. neutrophils + monocytes	0.83			0.44	0.77	0.10
Nor. platelets + sens. lymphocytes + sens. neutrophils + monocytes	0.83		0.80	0.44	0.92	0.57

The present findings also confirm the previous observation by Schoenbechler and Sadun that in order to obtain antigen-induced histamine from well-washed platelets from infected rabbits, leukocytes from infected rabbits must be present. Leukocytes from noninfected animals were inactive, although platelets from normal rabbits used with leukocytes from infected animals did sustain release of histamine. This indicates that it is only the leukocyte which is sensitized. The cell type found capable of interacting with the platelet in this manner was the specifically sensitized lymphocyte. The nature of this sensitization is completely unknown at present. One possibility is that the lymphocyte is passively sensitized by heat-labile homocytotropic or other kinds of antibody or that the antibody responsible is produced by the lymphocyte. A further possibility is that this interaction is another example of those capabilities of sensitized lymphocytes which many consider to be related to delayed rather than immediate-type hypersensitivity.

Table 17

The Ability of a Pure Lymphocyte Suspension to Give Antigen-Induced Histamine Release from Platelets

	Platelets (10^8)	Leukocytes (10^6)	Lymphocytes (10^6)	Histamine Total (ng)	Histamine Antigen-Induced Release (ng)
Nor. platelets	3.1	ND*	ND	1.9	0.0
Sens. leukocytes	0.06	0.06	0.60	0.0	0.0
Sens. lymphocytes	0.10	-	2.10	0.0	0.0
Nor. platelets + sens. leukocytes	3.2	0.06	0.60	2.0	0.0
Nor. platelets + sens. lymphocytes	3.2	-	2.10	2.9	1.0

*Not done

The nature of the antigen-induced interaction of lymphocyte and platelet which leads to histamine release from the latter is also unknown. Whether the release of histamine is due to the release of a factor from the lymphocyte or requires a cell-to-platelet contact is unknown and the subject of future work.

All attempts to obtain fractions which were either pure neutrophils or pure monocytes have failed so far. In several experiments not reported here, it was found that neutrophil-monocyte fractions contaminated with lymphocytes caused histamine release greater than could be accounted for by the number of lymphocytes present. This last result suggests the following possibilities: (1) although pure lymphocytes can interact with platelets, with resulting release of histamine, the

presence of neutrophils or monocytes enhances this interaction, (2) neutrophils or monocytes under some circumstances are capable of the same interaction with platelets as lymphocytes though not necessarily through the same mechanism, and (3) the lymphocytes contaminating the neutrophil fractions are much more active than those isolated with the lymphocyte fractions. Work is presently proceeding to define among these possibilities.

7. The nature of the reaction of antigen with sensitized lymphocyte in the lymphocyte-dependent release of histamine from rabbit platelets.

Schwartzbaum and Ishac first demonstrated that sensitized lymphocytes from the blood of rabbits infected with histoplasma released when reacted with specific antigen histamine from rabbit platelets. This was confirmed by Siraganian, Oliveira, and Hirsch using an entirely different antigen-antibody system. Recently, we have reported that the lymphocyte is the sensitized cell which interacts with the platelet

The overall reaction of lymphocyte, antigen, and platelet can be divided experimentally into two distinct steps. The first involves the reaction of sensitized lymphocyte with antigen and is the step with the interaction of these lymphocytes with platelets to cause histamine release from the latter. In many preparations on the role lymphocytes play in the first step it has been shown that addition of antigen to the sensitized lymphocytes results not only in activation of antigen with specific receptors, but also in the damage of the cell in such a way that it is incapable of reacting with the platelet to cause histamine release. This damage to the lymphocyte by antigen was termed "cytotoxic" lymphocytes and the major purpose of this work was directed to determine the nature of the activation process.

In this work, the lymphocytes from rabbits sensitized to pneumococcus exposed to living organisms were tested for their ability to give antigen-induced histamine release from platelets. The antigen was a soluble extract of the pneumococcus.

The lymphocyte suspension was layered over Ficoll and centrifuged in an attempt to separate the lymphocytes from the other cells. The lymphocytes were obtained as a fairly pure cell suspension. Electron microscopic examination of the cell layer revealed that the cells were predominantly lymphocytes. The lymphocytes exhibited a very low incidence of spontaneous release of histamine.

All experiments were conducted with washed lymphocytes and washed rabbit platelets in the presence of Tyrode solution. The buffer used throughout was Tyrode solution.

In previous experiments, histamine release was observed when antigen, sensitized lymphocytes, and platelets were all mixed simultaneously. In the first experiments reported here the lymphocytes were

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

Table 10

The Effect of Storage of Antibiotic and Non-Antibiotic Lactobacillus at [illegible] for 48 Hours

Treatment of the lactobacilli	Lactobacillus pre-treated [illegible] with antibiotic			Lactobacillus pre-treated [illegible] with no antibiotic added		
	Total histamine µg	µg histamine released	Percent released	Total histamine µg	µg histamine released	Percent released
Experiment 2						
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	1.20	[illegible]
Effect of time at [illegible]	[illegible]	[illegible]	[illegible]	[illegible]	1.70	[illegible]
Experiment 3						
[illegible]	[illegible]	[illegible]	[illegible]	1.71	0.74	43
Effect of time at [illegible]	[illegible]	[illegible]	[illegible]	[illegible]	1.05	43

the cells pre-treated at 0°C with antigen inactivated so that they are incapable of being activated if brought to 37°C and fresh antigen added? 3) Why was there less cytotoxic activity from those leukocytes "activated" at 44°C?

Experiments were set up to answer the first question, and it was found that low temperatures have a deleterious effect on the ability of leukocytes to be activated. This was true whether the leukocytes were incubated in the cold with buffer and then reacted with antigen and platelets at 37°C or incubated in the cold with antigen and then reacted with platelets and antigen at 37°C. Also, there was an increase in activation of leukocytes at 0°C when the time of activation with antigen was extended from 15 mins. to 180 mins. Due to the deleterious effects of cold, the absolute amount of activation at 0°C for 180 mins. was distinctly less than activation at 37°C for 15 mins., however, the percent activation of available sites at 0°C for 180 mins. does not differ from the percent activation of available sites at 37°C for 15 mins. Thus, there is a dual effect of cold on the activation process; one, is the destruction of available sites and two, the lowering of the rate of activation.

To test if the deleterious effects of exposure to cold was on steps prior to activation or on the activated sites the following experiment was conducted. Leukocytes were pre-treated with Tyrode's solution or antigen at 37°C for 15 mins., washed, and one aliquot of each was tested immediately while a second aliquot was stored at 0°C for 48 hours before testing. The results clearly established that leukocytes not "activated" with antigen lose all thier activity after 48 hours. However, those "activated" with antigen prior to storage retain all their cytotoxic activity towards platelets. Hence, the non-activated sites are labile to the cold if not reacted with antigen but once the process is completed the resultant activated state is quite stable at 0°C.

Several tentative conclusions can be drawn from the results just described. It is clear that as stated there are two definite, experimentally separable, steps in the overall reaction leading to histamine release. The first step involves the activation of the sensitized lymphocyte with antigen; the second, is concerned with the interaction of "activated" lymphocytes with platelets to release histamine from the latter. It is clear that the histamine release does not occur from the production of a soluble factor resulting from the reaction of antigen and lymphocyte, thus differing from some of the other cytotoxic lymphocyte reactions. Our experiments have not yet ruled out the possibility that during the interaction of the "activated" lymphocyte with the platelet there may be produced a soluble factor which is cytotoxic towards the platelets.

There are at least two possibilities as to the process by which the cells become activated by antigen. First, the antigen may merely react with the cell bound antibody which, when combined with antigen, becomes cytotoxic toward platelets. Second, the reaction of antigen

with cell bound antibody initiates changes in the cell, probably enzymatic and complex, which results in an "activated" cell. Preliminary experiments favor the latter hypothesis.

The effect of time and temperature on the rate of activation suggests a complex enzymatic process triggered by the antigen-antibody combination. The decrease in activity of the lymphocyte produced by treating the cell with antigen at 44°C as compared to treating it at 37°C, and the lack of such an effect if the cell is treated at 44°C in the absence of antigen, suggests either, 1) that there is an antigen-antibody triggered step that is heat labile or 2) the production of an inactivator at elevated temperatures, which is probably cell bound.

The effect of cold on the lymphocyte cell indicates there is a step in the reaction which is sensitive to cold, so that storage of the lymphocyte in the cold in the absence of antigen leads to progressive inability of the cell to respond to antigen at higher temperatures. The fact that after the antigen-antibody triggered activation process has occurred at 37°C, the cells are stable in the cold for at least 4 hours indicates that this cold sensitive step occurs prior to the final activation step. Thus, in the reaction of antigen and lymphocyte at low temperatures there are two reactions occurring, while antigen-antibody triggered activation is taking place, although at a lowered rate, the cell is becoming less reactive by destruction of the cold sensitive sites.

In summary, the evidence presented here suggest that the process of activation of the sensitized lymphocyte by antigen to give a cell capable of interacting with platelets is complex and probably enzymatic in nature in which temperature effects different steps in the reaction quite differently.

8. Epidemiologic and serologic investigations of filariasis in indigenous populations and American soldiers in South Vietnam.

Human filariasis is known to be endemic in Malaysia and Thailand, and in North Vietnam, surveys have revealed filaria infections due to Wuchereria bancrofti and Brugia malayi in 1 to 2 percent of individuals examined.

During the early 1960's, a syndrome consisting of hypereosinophilia, bronchial asthma and lymphadenopathy was observed in the French troops repatriated from the Dunia area of North Vietnam. The epidemiology of B. malayi were unknown, recruits from Hanoi were tested, and treatment with arsenicals or diethylcarbamazine was followed by prompt clinical improvement.

In 1970, Comar reported on the results of studies of filariasis in various ethnic groups residing in the Dak Song district of South Vietnam which is located 110 kilometers southwest of Saigon. Montagnards, migrated to this location from the Dunia area of North Vietnam were

[redacted]

[illegible] infection with B. malayi were frequently encountered in examined immigrants; however, none were observed [illegible]. Failure to demonstrate infections with the latter [illegible] appropriate mosquito vector or animal [illegible].

[illegible], bancroftian microfilariae were detected in peripheral blood obtained from 6 of 200 Saigon prostitutes. [illegible] explanation for the lack of demonstrabel microfilaremia [illegible].

[illegible] employed in the present study to detect circulating [illegible] antibodies, serological immunodiagnostic techniques for bancroftian [illegible] have generally yielded unsatisfactory results. [illegible] complement-fixation and indirect hemagglutination [illegible] poor correlation with the presence or [illegible] filariasis. Recent reports on the use of intradermal [illegible] have given encouraging results. Filariasis skin testing, using [illegible] prepared by Fukuda, was accomplished on [illegible] subjects. Positive results were obtained with this [illegible] with bancroftian filariasis; and neighboring [illegible] non-endemic for this illness all had negative [illegible]. [illegible] precipitin test, using a concentrated [illegible] microfilarial antigen was employed against [illegible] with bancroftian filariasis. Positive [illegible] demonstrated in only 10 of 43 infected [illegible]. [illegible] positive impermeability of the microfilarial sheath [illegible] impermeability of the microfilarial sheath [illegible].

[illegible] fluorescent antibody (FAFA) test of Duxbury [illegible]. [illegible] antigens were prepared from adult [illegible] as described by Chaffee et al. Eighty-[illegible] with bancroftian filariasis [illegible]. [illegible] percent of healthy North [illegible]. These results indicated a high [illegible] specificity. The application of this test in [illegible] findings. Positive results were obtained [illegible] with microfilaremia, compared with [illegible] occurring in an apparently non-endemic [illegible].

[illegible] Malayan filariasis by demonstrating [illegible] is relatively insensitive method. Moreover, [illegible], [illegible] practical circumstances pose further [illegible]. [illegible] single blood specimen can be obtained from [illegible] can be hazardous. In the [illegible] sensitivity rate of 61 percent in 137 native [illegible] microfilariae in a single blood specimen [illegible]. In view of the high degree of sensitivity and [illegible], these positive reactions most likely reflect

immune responses to either human or non-human Filarioidea. Moreover, as these subjects reside in areas endemic for bancroftian filariasis, it is quite possible that the high rate of seropositivity is causally related to the latter infections. Unfortunately, conditions did not permit sampling of native residents in non-endemic areas for purposes of comparison.

In the South Pacific theatre during World War II, filariasis resulted in the evacuation of thousands of troops, with the loss of tens of thousands of man-days. Symptoms often subsided spontaneously upon evacuation to non-endemic areas; however, in some individuals, clinical attacks recurred for as long as 16 years subsequent to the initial exposure.

In South Vietnam, the significant rate of seroreactivity to filarial antigens in U. S. soldiers, particularly those deployed on field duty in endemic areas, appears to indicate exposure to either human or non-human Filarioidea. Bancroftian microfilaremia in one soldier has already been documented. This is particularly noteworthy, as patent parasitemia is not usually present until 5 to 12 months after the initial exposure. At the present time, the rate and degree of clinical incapacitation from acute filariasis in U. S. soldiers deployed in endemic areas is unknown.

Project 3A0[illegible]

Task [illegible], Comm[illegible]

Work Unit 16[illegible], Para[illegible]

9. Publications

Gore, R. W. and Sadun, [illegible]
for cestodiasis. [illegible]

Gore, R. W. and Sadun, [illegible]
(SAFA) test for the [illegible]
mental animals [illegible]

Nota, I., Sadun, [illegible]
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Sadun, E. H. [illegible]
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reagin-like antibody [illegible]
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von Lichtenberg, F. and Sadun, [illegible]
hepatic pipestem [illegible]
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Williams, J. S., Sadun, [illegible]
city of the immunoelectrophoretic [illegible]
antigens and [illegible]
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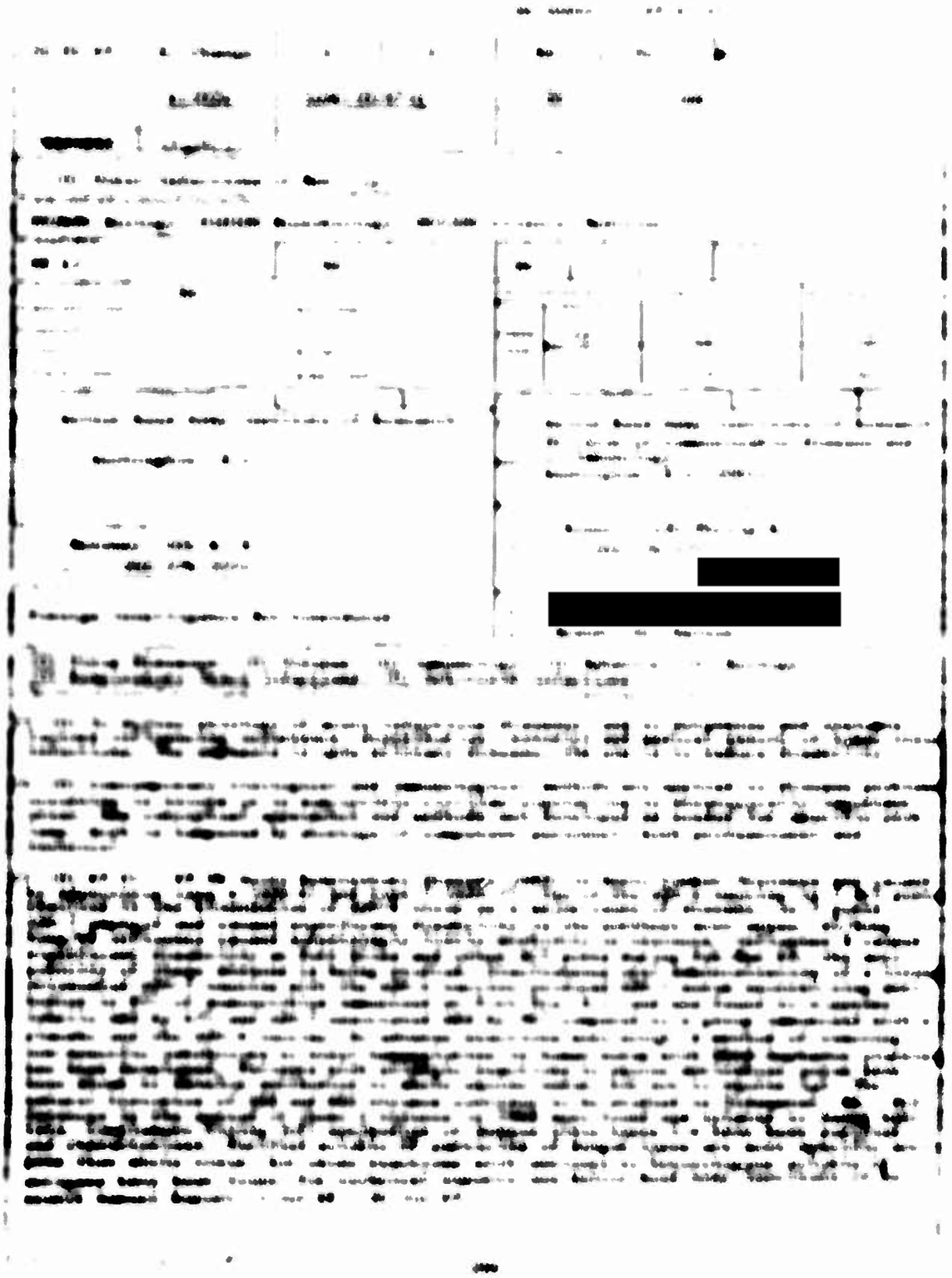

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survey of cancer patient sera and selected controls for possible reactions with adenovirus 7 antigens (supported by the Solid Tumor Virus group of the NCI, NIH) showed no difference in complement-fixing antibody activity to the human adenovirus 7 antigens between cancer patient or control patients. From these results, it was concluded that adenoviruses do not appear to be involved in production of significant numbers of human tumors, and permission to study efficacy and safety of living Ad7-7 vaccines in military personnel was granted by the Vaccine Development Branch of NIAID and the AMRDL, USA.

The following studies were designed and instituted to evaluate the safety and immunogenicity of L-Ad-7 administration to man, to evaluate the feasibility of simultaneous administration with L-Ad-4 and L-Ad-7 vaccines, and to evaluate the protective effect of L-Ad-7 on ARD in ECT's.

Study #1, Shipyard Volunteers, May-Jul 1968

Abstract

To determine whether L-Ad-7 vaccine could be safely administered orally to susceptible young adults, an experiment was undertaken in PROJECT [redacted] volunteers at Fort Detrick, Maryland in May 1968. Groups of susceptible volunteers were fed either $10^{7.5}$ TCID$_{50}$ of L-Ad-7 or placebo tablets. All men were followed daily for 21 days after exposure for evidence of respiratory disease, or other indisposition, and for evidence of infection by vaccine virus. Mild, afebrile respiratory disease was observed in two vaccinated and one control volunteer. In each instance, symptoms could not be related -- either pharyngeal or stool excretion of Ad7-7. Four vaccinated persons developed diarrhea, one of whom was febrile. These episodes may have been related, at least in part, to Ad7-7 infection of the GI tract. Shown of 10 infected volunteers shed Ad7-7 virus in stools (mean duration 13 days, range 8-18 days). No pharyngeal excretion of Ad7-7 was observed in any of these volunteers. Each of the eleven ultimately developed specific S antibody to Ad7-7. No evidence for person to person transmission of vaccine virus was obtained. The experiment establishes that L-Ad-7 can be safely administered orally to susceptible volunteers, and that in doses of approximately $10^{7.5}$ TCID$_{50}$/man, approximately 2/3 of susceptibles can be infected.

a. [redacted]

1. [redacted] Volunteers were chosen from enlisted personnel participating in [redacted]. A complete and comprehensive explanation of the study and its risks was given to the enlisted men by the Project Director in the presence of the principal investigators. Following this, each individual was interviewed personally and given an opportunity to ask additional questions and express their desire to participate. A consent statement, on file in the U.S. Army Medical Unit, Fort Detrick, Maryland, was signed by each volunteer.

The volunteer group consisted of 24 men found to be free of ADV-7 neutralizing antibody by tissue culture neutralization test. Sixteen volunteers received the adenovirus vaccine (L-AV-7) and eight volunteers received the placebo tablet on study day 0. Volunteers were housed on two closed wards for the duration of the study; each ward contained volunteers who received L-AV-7 and volunteers who received placebo enteric capsule. Detailed medical histories and physical examinations were performed on each volunteer on admission to the study wards. Complete hospital charts were initiated and maintained on each volunteer. Initial medical evaluation included an electrocardiogram, chest x-ray (PA and lateral), complete blood count, urinalysis and throat culture.

2. Vaccine Virus used for Immunization: L-AV-7 was derived from ADV-7 (strain 55142) propagated in human embryonic kidney (HEK) cells and was obtained by Wyeth Laboratories from Dr. Robert Chanock of NIAID. The strain was passaged three times in HEK, then through 12 passes in human diploid fibroblast cultures (WI-38), lyophilized, mixed with an inert vehicle, and prepared into enteric-coated capsules (Lot 16 CV-01001, Wyeth). The capsules were shown to contain an average of $10^{4.9}$ TCD_{50} upon titration of virus in HEK cell cultures. Virus obtained from the capsules was neutralized by hyperimmune ADV-7 antiserum in tissue culture neutralization tests. Volunteers not receiving L-AV-7 received an enteric-coated placebo capsule (enteric-coated press coat tablet Lot 350A-T-138, Wyeth); this preparation was shown to contain no cytopathogenic agent when a liquid suspension of it was inoculated into HEK tissue culture tubes.

3. Laboratory Studies: Blood was obtained at 0800 hours on study day -5, -3, 0 and daily through day 14 and then on day 18 and 21 for white blood cell and differential count, hematocrit and platelet count. Blood was obtained at 0800 hours on study day -5, -3, 0, 4, 7, 14 and 21 for total, direct and indirect bilirubin, SGOT, SGPT, alkaline phosphatase and BUN. Urinalysis was obtained on admission to the study and daily thereafter until day 14, then on days 16, 18 and 21. The above laboratory tests were performed by standard laboratory procedures.

Blood was obtained on day -5, 0, 7, 10, 14, 18, 21 and 35 for serologic studies. Serum neutralization tests were performed on serum samples from the volunteers in HEK tube cultures using ADV-7 strain A-3-143. Serum neutralization end-points were determined at a time when the test dose of virus showed 100 TCD_{50} in HEK tube cultures. A serum neutralization test utilizing 0 day and 35 day serum samples from each volunteer was also performed with 32 TCD_{50} of the vaccine strain 55142. A serum ADV-3 neutralization test was performed on samples of 0 day serum from each volunteer in HEK tube cultures against 100 TCD_{50} of strain 442. Adenovirus complement-fixation titers on 0, 21 and 35 day serum samples were determined by standard micro-titer procedures against an antigen prepared against ADV-4 strain RI-67.

Throat washings and stool [illegible] on each volunteer on study days -4, [illegible] 0.1 ml aliquots of each throat wash and 0.1 ml aliquots of a 10% suspension of each stool sample were inoculated into three WI-38 tube cultures. On study days -4, -1, -2, aliquots of throat washings were inoculated into WI-38 and Rhesus monkey kidney tube cultures and aliquots of stool suspension into Rhesus monkey kidney tube cultures in addition to the WI-38 cells. Tubes were incubated at 36°C and observed for cytopathic effect (CPE) every other day. Isolates exhibiting characteristic adenovirus CPE were typed in tissue culture neutralization tests in WI-38 tube cultures with hyperimmune ADV-7 antiserum and those exhibiting herpesvirus CPE were typed with hyperimmune herpesvirus [illegible]

B. Patterns of Virus Shedding

1. Stool Excretion (Figure 1): [illegible] of the 10 volunteers receiving L-AV-7 excreted ADV-7 in the stools. ADV-7 shedding was demonstrated first on study day 2 and last on study day 21. Duration of virus shedding varied between 0 days and 19 days with a mean of 13 days. None of the eight volunteers receiving the placebo tablet excreted ADV in the stool during the study. All strains isolated from stool specimens [illegible] other than ADV-7 was isolated from stool specimens [illegible] placebo volunteers.

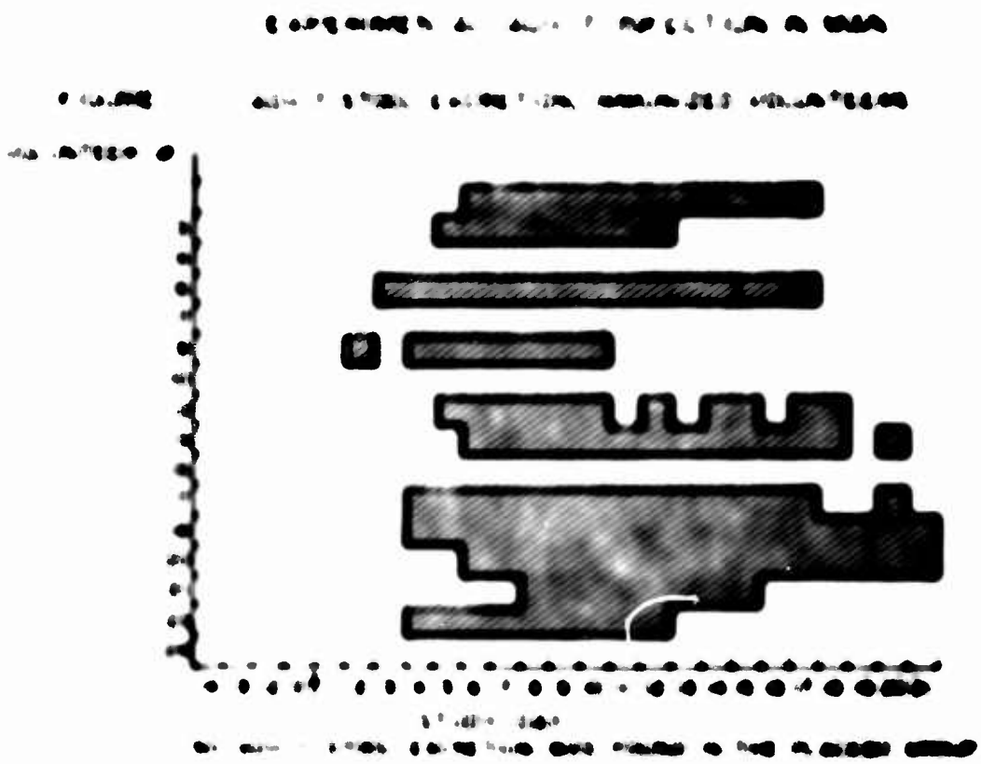
2. Nasopharyngeal Excretion: ADV-7 was not demonstrated in throat washings of either immunized or placebo volunteers. Herpesvirus hominis was found in throat washings of four volunteers, all in the immunized group - Volunteers 1 (day 9), 10 (day 6), 11 (days 10 and 18) and 18 (day 17).

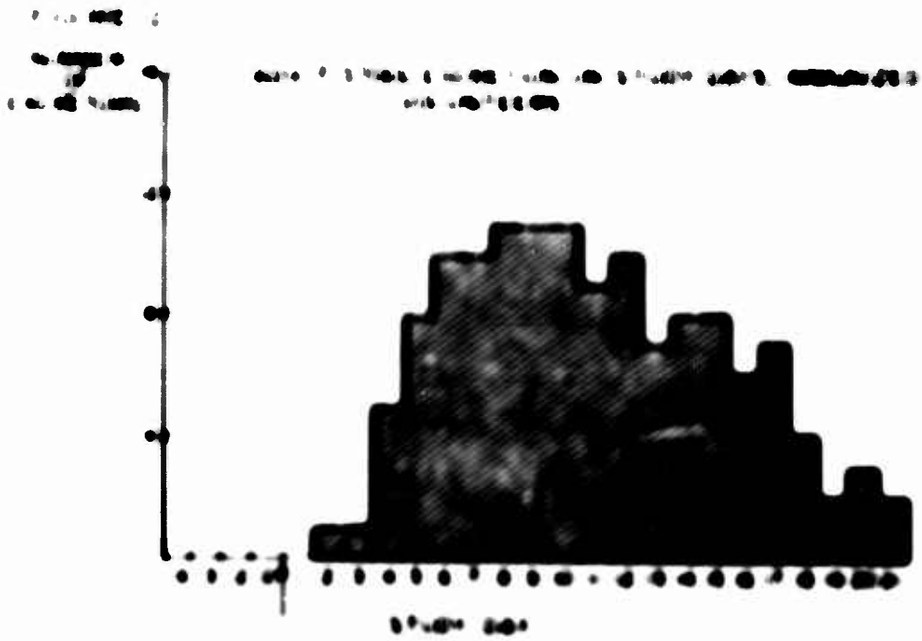
C. Antibody Responses

1. Immunized Volunteers: Table 1 details the ADV neutralizing (N) antibody titers of volunteers, immunized and placebo, to 0 day, 21 day and 35 day serum samples against 100 TCID50 of ADV-7 strain A 3 161. Neutralizing antibody was not detected in 21 day or 35 day serum of the eight placebo volunteers or the five immunized volunteers who failed to excrete ADV-7. Antibody responses of the 11 immunized ADV-7 excretors were as follows: two showed no detectable N antibody at 1:2 serum dilution and maximal titers were 1:16. A second ADV-7 neutralization test was performed with the 0 and 35 day serum with a smaller test dose (10 TCID50) of the vaccine strain 55162 as shown in Table 2. Eight ADV-7 N antibody titers were obtained in this test, and all [illegible] detectable ADV-7 N antibody.

D. Clinical Response to Immunization

Mild afebrile upper respiratory disease was noted in two immunized and one control volunteer. In all three [illegible] days, the symptoms did not be associated with pharyngeal [illegible] ADV-7 excretion.





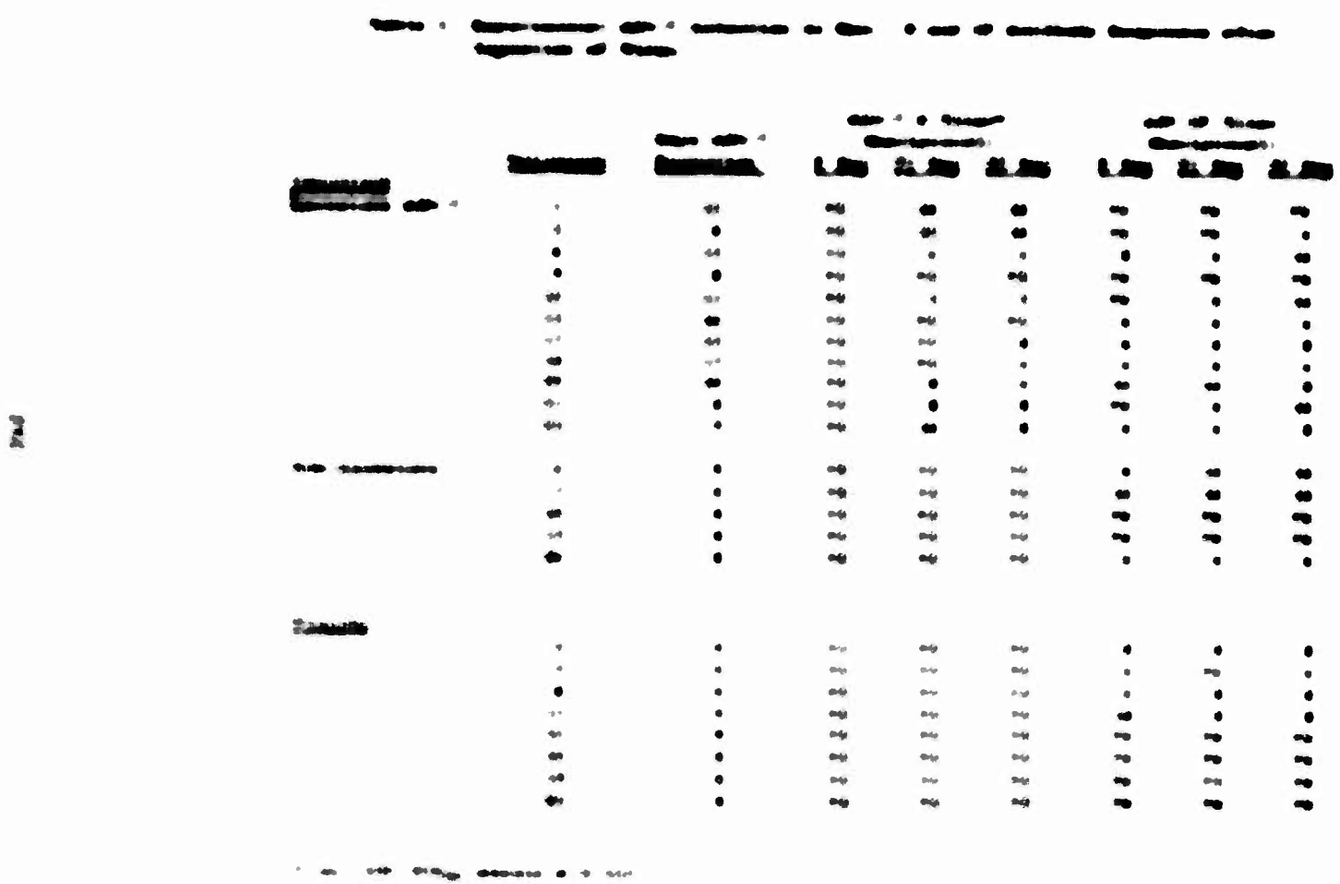


Table 1 [illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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1. [redacted]

[redacted]

2. [redacted]

[redacted]

L-AV-7 represents close to the human enteric infectious dose$_{50}$, and that further evaluation of L-AV-7 enteric vaccine should be performed with a higher dosage-$10^{5.5-6.0}$ TCD_{50}-of vaccine virus.

Table 4 Experimental ADV-7 Infection in Man: 0 Day ADV-3 N Antibody Titers in Immunized Volunteers

Group	Number	Serum ADV-3 Neut Titers (No.) <2	2	4	8	16
ADV-7 Excretors	11	7	1	1	2	
Non-Excretors	5	1		2		2

The pattern of stool ADV-7 excretion in immunized volunteers infected with L-AV-7 was found comparable to that of ADV-4 vaccine virus stool excretion as reported by Chanock, et al. (Table 5).

Table 5 Experimental ADV-7 Infection in Man: Comparison of Excretion of ADV-7 Vaccine Virus with that of ADV-4 Vaccine Virus after Enteric Immunization

Strain	ADV Type	No. Men Tested	No. Men Virus Recovered	No. of Days Virus Recoverable Range	Mean
55162	7	16	11	8-17	12
*CL86570	4	18	17	4-14	8

* From data of Chanock, et al JAMA 195:445, 1966.

ADV-7 was not recovered from the oropharynx of immunized volunteers. No evidence of communicability of the vaccine virus was found in that the eight placebo volunteers who also lacked detectable serum ADV-7 N antibody and who were housed together with the immunized group showed no virus excretion and did not develop ADV CF or N antibody rises during the course of the study.

In all immunized volunteers excreting ADV-7 vaccine virus in stool, serum N antibody (vs. 32 TCD_{50} of the vaccine strain 55162) was present in the 21 day serum. Titers of serum N antibody in these eleven men are

comparable to serum ADV-4 N antibody titers in volunteers immunized with live, enteric ADV-4 vaccine as reported by Chanock, et al (JAMA 195:445, 1966). Serum N antibody titers versus 100 TCD_{50} of a wild ADV-7 strain (A-3-143) were lower, and two of the eleven ADV-7 excretors lacked demonstrable N antibody at a 1:2 dilution in this test system.

Study #2, USAMEDTC Trainees, Oct-Dec 68.

Abstract.

A study designed to determine 1) the infectivity and immunogenicity of a living, enteric-coated adenovirus type 7 vaccine (L-AV-7) and 2) the infectivity and immunogenicity of this vaccine and a living adenovirus type 4 vaccine (L-AV-4) given simultaneously to volunteers was carried out in trainee volunteers at the USAMEDTC, Fort Sam Houston, Texas from Oct 68 - Jan 69. All volunteers were susceptible to ADV-4 and ADV-7 infections (serum N antibody <1:2). Thirteen volunteers received a placebo and L-AV-7 ($10^{5.4}$ TCD_{50}), thirteen received a placebo and L-AV-4 ($10^{4.0}$ TCD_{50}) and thirteen received both L-AV-7 and L-AV-4. No illnesses or adverse reactions attributable to immunization were found. Twelve of 13 volunteers receiving L-AV-7 and placebo excreted ADV-7 virus in stools and all 13 developed ADV-7 N antibody after immunization. One of these volunteers had pharyngeal ADV-7 excretion. All 13 volunteers receiving L-AV-4 and placebo excreted ADV-4 virus in their stool and developed ADV-4 serum N antibody post-immunization. Ten of the 12 susceptible volunteers given both L-AV-7 and L-AV-4 had stool ADV-7 excretion and developed ADV-7 serum N antibody. Eleven of the 12 had stool excretion of ADV-4 and eleven developed ADV-4 serum N antibody post-immunization. Coxsackie virus A-21 infections were detected in several of the volunteers near the time of immunization; coxsackie A-21 infection did not interfere with intestinal infection induced by either L-AV-7 or L-AV-4.

The experiment established that the L-AV-7 capsule containing $10^{5.4}$ TCD_{50} was comparable in safety, infectivity and immunogenicity to the L-AV-4 tablet containing $10^{4.0}$ TCD_{50} currently in military use. It also established that simultaneous administration of L-AV-4 and L-AV-7 was safe and led to the establishment of intestinal infection with both viruses as determined by viral isolation in nine of the 12 volunteers and the development of serum N antibodies to both viruses in ten of the 12 volunteers.

A. Design of Study:

1. The Study Group: Volunteers were chosen from enlisted trainees of Company A-3, the USAMEDTC, Fort Sam Houston, Texas. A complete and comprehensive explanation of the study and its risks was given to the enlistees by one of the responsible investigators. A consent statement, on file in the Department of Virus Diseases, WRAIR, was signed by each volunteer.

The volunteer group consisted of 39 trainees found to lack serum N antibody (serum dilution 1:2) to ADV-4 and ADV-7 by tissue culture

neutralization tests in serum obtained three months prior to commencement of the study. Trainees were quartered in barracks sharing restrooms and involved in the study and participated fully in training during the course of the study.

Although other respiratory illnesses were prevalent at the [redacted], all volunteers lacked respiratory symptoms when this study commenced. All volunteers were questioned daily in regards to respiratory illness or adverse reactions to immunization. Volunteers with illness were examined and treated by Dispensary physicians and throat washings for viral isolation were obtained.

1. Vaccine Viruses used for Immunization. T-80-7 was prepared into enteric-coated capsules (Lot No [redacted], Wyeth). The capsules were shown to contain $10^{3.6}$ TCID$_{50}$ on titration in MRC cell cultures.

T-AV-4 was prepared into enteric-coated tablets (Lot No [redacted], Wyeth). The tablets were shown to contain $10^{5.0}$ TCID$_{50}$ of AZP-4 by titration in MRC cultures.

Enteric-coated placebo coated tablets (Lot [redacted], Wyeth) were used as placebo tablets.

On study day 0, 11 volunteers received T-80-7 and T-AV-4 vaccines (Group A), 13 received T-80-7 vaccine and placebo (Group B) and 11 received T-AV-4 vaccine and placebo (Group C).

2. Laboratory Studies. Blood was obtained on study days 0 and 21 and six weeks post-immunization for serology. AZP serum 0 antibody tests were performed in MRC tube cultures and read at a time when the test dose of virus was 10 TCID$_{50}$. The vaccine strains of AZP-4 and AZP-7 were used in these tests. Comparable virus 6-21 0 antibody tests were performed in WI-38 tube cultures with a test virus dose of [redacted] TCID$_{50}$.

Throat washings were obtained daily on all volunteers in Group B (T-AV-7 and placebo), 0.5 ml aliquots were inoculated into each of three MRC tube cultures. Throat washes were obtained on all volunteers who reported to the Dispensary with symptoms of respiratory infections. Aliquots were inoculated into MRC, WI-38 and Rhesus monkey kidney tissue culture tubes.

Stools or rectal swabs were obtained daily from all volunteers and specimens obtained on even study days (day 0, 2 etc.) were studied. 0.1 ml aliquots of a 10% stool suspension from volunteers of Group B and C were inoculated into each of three MRC tissue culture tubes. Separate aliquots of stool suspensions from Group B volunteers were treated with hyperimmune AZP-4 antiserum and with hyperimmune AZP-7 antiserum prior to inoculation. If no isolate was obtained from a stool suspension treated with either hyperimmune antiserum, repeat isolation attempts were made on an aliquot

[illegible] were observed [illegible]

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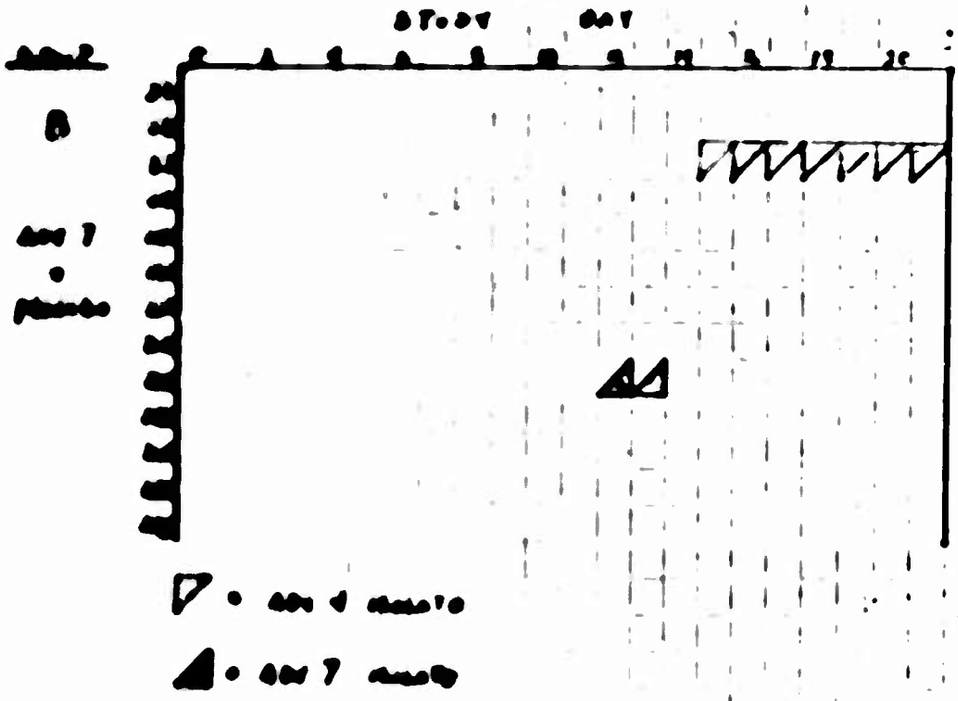
USAMEDTC Oct - Nov 1968

Fig 1 ADENOVIRUS PHARYNGEAL EXCRETION



ranged from one to 13 days with a mean of seven days. ADV-4 stool excretion was detected in 11 of the 12 susceptible volunteers. ADV-4 was first detected in stools obtained on study day 6; duration of ADV-4 stool excretion ranged from one to 11 days with a mean of 6.5 days. Stool excretion of both vaccine viruses was detected in nine of the 12 volunteers.

b) ADV-7 and ADV-4 N antibody titers: Ten of 12 volunteers (who lacked detectable serum N antibody to ADV-4 and ADV-7 at 0 week) developed ADV-7 N antibody three weeks post-immunization; the geometric mean titer was 15. At six weeks post-immunization, no further volunteers had developed ADV-7 N antibody and titers were similar (geometric mean 18) to those at three weeks after immunization. Eleven of the 12 Group A volunteers developed ADV-4 N antibody three weeks post-immunization with a geometric mean titer of five. At six weeks, ADV-4 serum N antibody was still not detected in the one volunteer found lacking antibody at three weeks; the geometric mean titer at six weeks was 13.

4. Illnesses and Adverse Reactions in Volunteers: Five volunteers reported to the Dispensary with respiratory tract symptoms during the course of the study. Clinical findings and isolation results of throat washings are shown in Table 6. No other illnesses, specifically diarrhea, or adverse reactions were noted in the volunteers.

5. Coxsackie Virus A-21 Infections in Volunteers: Stool excretion of Coxsackie A-21 virus and serum N antibody titers to this virus in study volunteers are detailed in Figure 5. It is apparent that the study was begun during an outbreak of Coxsackie virus A-21 in the volunteers; however, no illness necessitating sick call occurred in volunteers excreting this virus. As is shown in Table 7, infection with Coxsackie virus A-21 did not interfere with the acquisition of intestinal infection with either ADV-7 or ADV-4 vaccine virus and did not interfere with the development of specific ADV serum N antibody after immunization with either vaccine.

C. Discussion:

This study was conducted at the USAMEDTC, Fort Sam Houston, at a time when mild respiratory illnesses were prevalent among trainees. Coxsackie A-21 virus and a rhinovirus were isolated from study volunteers and it seems likely that wild ADV-4 was also present in the study population. No evidence for the presence of wild ADV-7 virus in the trainee population was found.

The Coxsackie virus A-21 infections in certain volunteers at the commencement of this study did not interfere with the establishment of intestinal ADV-4 vaccine virus in these volunteers. To our knowledge, the effect of existing infection by this virus, which is commonly isolated in military trainees, on the infectivity and immunogenicity of ADV-4 or ADV-7 living, enteric vaccines has not previously been determined.

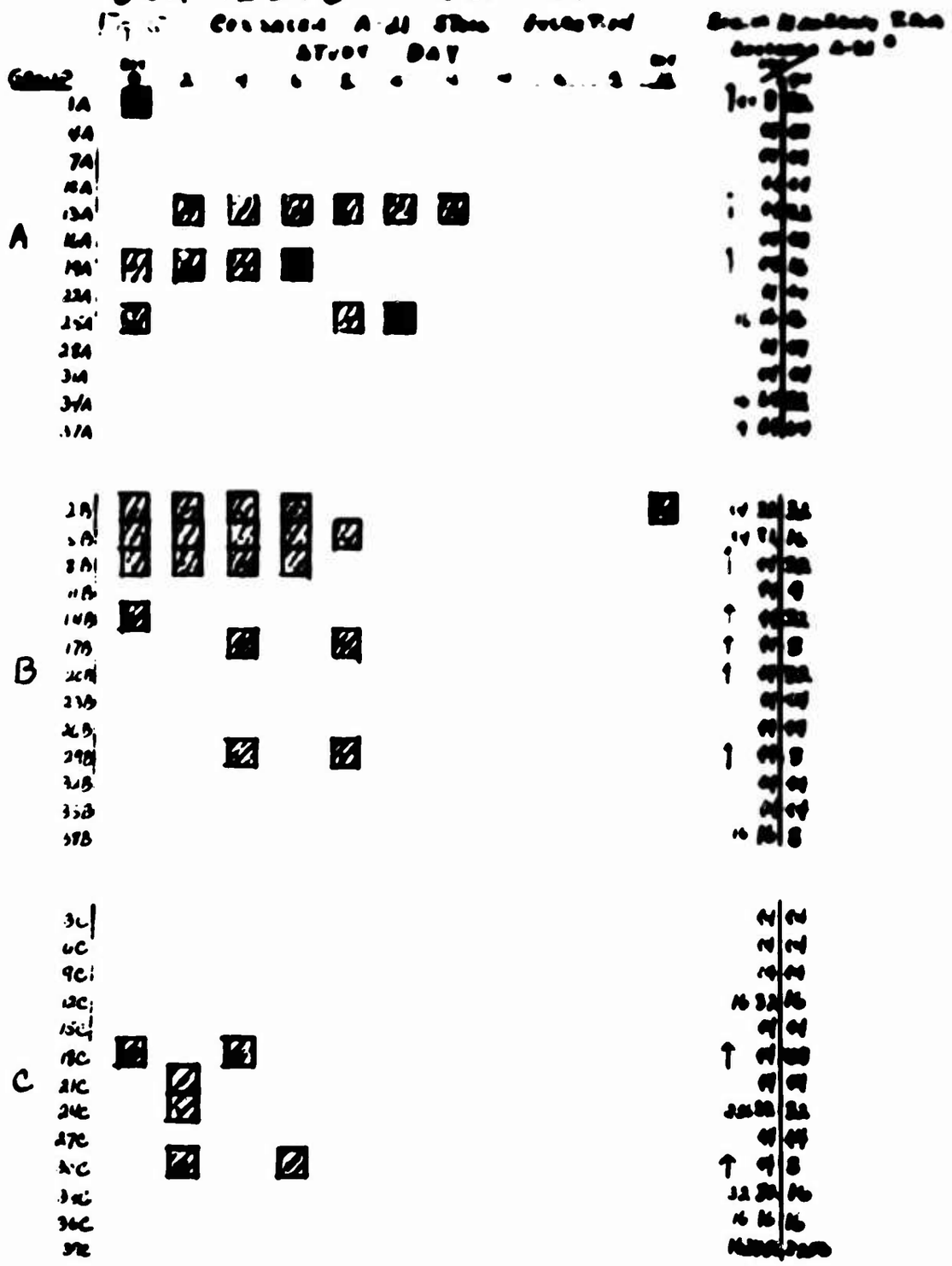
No adverse reactions were associated with the administration of either L-AV-4 or L-AV-7 singly or in combination to volunteers. Pharyngeal

Table 6

USAMEDTC Oct-Nov 1968

Clinical Findings and Isolates in Ill Volunteers

Volunteer	Vaccine	Study Day	Clinical Findings	Isolate
1A	L-AV-4, L-AV-7	12	Pharyngitis with exudate, cough, fever	ADV-4
11B	L-AV-7	4	Nasal congestion, headache, no fever	None
21C	L-AV-4	18	URI, cough, no fever	None
28A	L-AV-4, L-AV-7	8	Pharyngitis with exudate, no fever	None
37A	L-AV-4, L-AV-7	2	URI, no fever	Rhinovirus


USAMEDTC Oct - Nov 1968
A
B
C

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on post during the course of the study. A peak ADD rate of 0.4/100/week occurred on Post Day 20 for [redacted]. The rate remained above 0.2/100/week through mid-April and between 1.0-2.0/100/week until the end of the study.

2. [redacted] L-08-1 was prepared into enteric-coated capsules (Lot No [redacted], Upsher). These capsules contained [redacted] mg of L-08-1 as determined by titration in human albumin kidney (HAK) monolayer tissue culture tubes. L-08-4 was prepared into enteric-coated tablets (Lot No [redacted], Upsher) and contained [redacted] mg/tablet upon titration in HAK tissue culture tubes. Enteric-coated capsules containing lactose filler, supplied by Upsher Laboratories, were used as placebo capsules.

3. [redacted] BIT s were randomized within 72 hours after entrance on post. Those with social numbers ending in digits 0 and 9 were randomized with L-08-1 and L-08-4 and those with social numbers ending in digits other than 0 and 9 received a placebo capsule and L-08-4. L-08-1 and L-08-4 or placebo and L-08-4 were given simultaneously and at the same time the capsules received oral polio vaccine.

Eleven eight per cent of all incoming BIT s expected to enter the 8 receiving companies which were to begin basic combat training on D+1 the 00 were eliminated. 708 BIT s received placebo and L-08-4 (placebo group) and [redacted] BIT s received L-08-1 and L-08-4 (Type 1 group). Because of clinical-service problems in processing, about 5% of the remaining BIT s did not begin BCT in the 8 study companies. Together the type 1 and placebo groups comprised 95% of all recruits in the 8 study companies.

4. [redacted] All BIT s in the 8 study companies enlisted in Basic Unit Training (BUT) on the same day. Immunizations to the completion of Basic Combat Training were made by a member of the investigative team within 12 hours after hospitalization. A throat swabbing for stool specimens and an acute blood sample for serology were obtained on all hospitalized BIT s of the 8 study groups who had symptoms or findings of upper or lower respiratory tract infection. A convalescent blood sample was obtained three week of those recruits who made office hospitalization.

BIT s from the platoons of each of the 8 study companies were bled on the beginning of the first week and the fourth week of training. Blood obtained were used to determine antibody responses to immunization with L-08-1 and L-08-4.

B. [redacted]

1. [redacted] Aliquots of throat washings of hospitalized BIT s were mixed with an equal volume of 1.5% sodium containing 2% fetal bovine serum (FBS) and 1000 units of penicillin and 1000 ug/ml of streptomycin. After incubation at room temperature for 30 minutes, 0.1 ml of the throat wash mixture was inoculated into each of 3 HAK monolayer tissue culture tubes and into 1.5% sodium containing 2% FBS. All cultures were observed for cytopathic effect (CPE) on alternate days for at least 21 days.

[illegible]

Table 9 [illegible]

[illegible]	[illegible]		[illegible]	
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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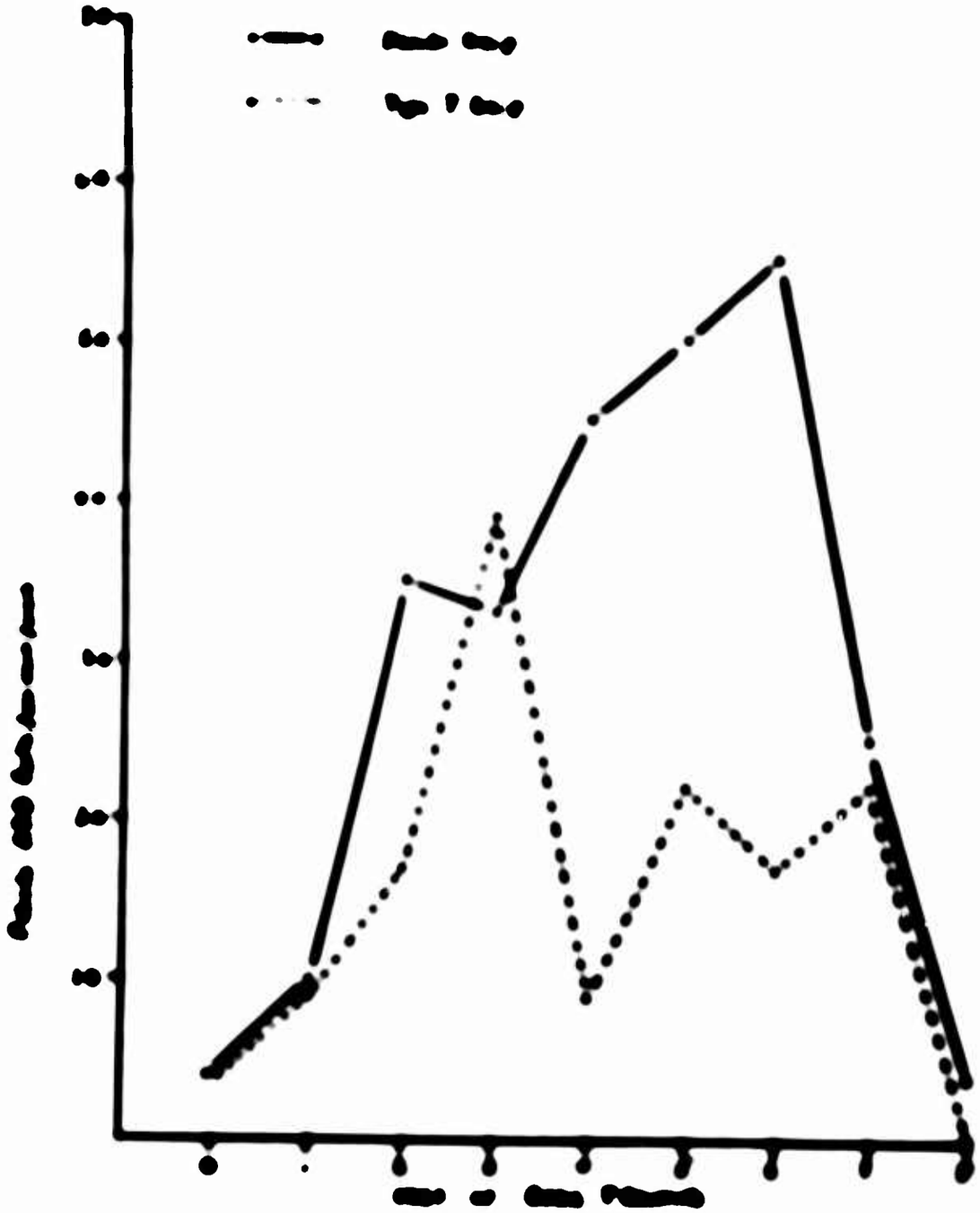


Table II Number of Hospitalizations and Rates of Hospitalization for Febrile ARD in Study Groups, Fort Dix 1969

Group	Total Recruits	Number ARD Hospitalizations	ARD rate/100/8 weeks
Placebo	499	240	26.1
Type 7	511	82	13.9*

* χ^2 = 11.1, p < 0.0005

to ARD-7 associated ARD rate of the two groups is highly significant (χ^2 = 20.0, p < 0.0005). The ARD-7 associated ARD rate by week for the two groups is shown in Figure 7.

ARD-4 associated ARD rates for the two groups are similar. Although the amount of ARD-4 associated ARD in post was insufficient to provide an adequate test of the protective effect of L-AV-4 on Type 4 associated ARD, the administration of L-AV-4 with L-AV-7 resulted in no greater rate of Type 4 adenovirus associated ARD than the administration of L-AV-4 with placebo.

B. Discussion

This study was designed to determine the safety, immunogenicity and protective effect of a live, enteric coated Adenovirus Type 7 oral vaccine (L-AV-7) administered together with the standard live Adenovirus Type 4 oral vaccine (L-AV-4) to Basic Combat Trainees. The safety of coadministered administration of both adenovirus vaccines was established as there was no increase in the Type 7 group (L-AV-7 and L-AV-4) developed gastrointestinal, cardiovascular, genito-urinary, or central nervous system disease during their basic training. Furthermore, ARD rates for the Type 7 and placebo group were similar during the first three weeks of BCT (the time of expected vaccine virus intestinal infection), and ADV-7 was not isolated from throat washings of Type 7 group recruits hospitalized in the three weeks after immunization. Thus, no ARD attributable to vaccine virus Type 7 was noted.

ADV-7 serum neutralizing antibodies were induced in 85% of Type 7 group recruits susceptible to ADV-7 infection (initial serum N antibody titers <1:4). The geometric serum N antibody titer was 20.3. Eighty-five per cent of Type 7 group recruits with initial low N antibody titers (1:4-1:16) had 4- to 8-fold or greater rises in Adenovirus Type 7 N antibody after immunization. The proportions of initially seronegative recruits who developed ADV-7 serum N antibody after immunization with L-AV-7 and L-AV-4 is similar to the proportion of susceptible recruits reported to

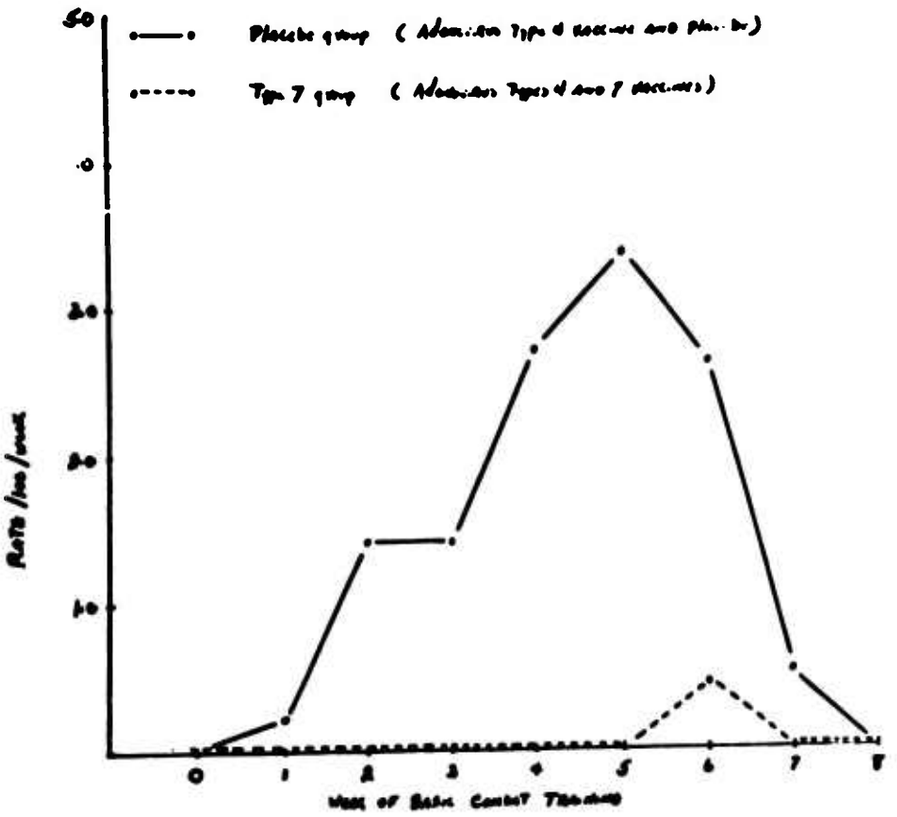
Table 11 Type-Specific Adenovirus ARD Hospitalizations in Type 7 and Placebo Groups, Fort Dix, 1969

		ADV-7-associated ARD		ADV-4-associated ARD	
Group	Strength	Number Hospitalized	Rate/100/8wks	Number Hospitalized	Rate/100/8wks
Placebo	920	113	12.3	7	0.9
Type 7	231	1	0.4*	2	0.9

* $x^2 = 28.8$, $p < 0.0005$

Fort Dix, N.J. Spring 1969

Fig. 7 Type 7 Adenovirus Associated Febrile ARD Rate, Study Groups



develop ADV-4 serum N antibodies after immunization with L-AV-4 alone (Van Der Veen, et al. J. Hyg, Camb 66:499, 1968). This study extended the observations reported in Study #2, this Report, that simultaneous administration of L-AV-7 together with L-AV-4 did not significantly reduce the immunogenicity of the L-AV-7 vaccine.

At a time when ADV-7 was associated with 47% of febrile ARD in the placebo group recruits, L-AV-7 immunization reduced the febrile ARD rate by 47% as compared to the rate in the placebo group. A 96% suppression of ADV-7 associated ARD was noted in the Type 7 group as compared to the placebo group, a reduction similar to that previously noted in ADV-4 associated ARD after immunization of recruits with L-AV-4 (Buescher, E.L., Med. Clin. N. America, 1968).

II. Adenovirus Surveillance Program Jul 68 - May 69.

Laboratory aspects of this program were performed under the direction of COL Albert Leibowitz, MSC, 6th USAML. Weekly ARD rates were calculated from data obtained from the individual posts by COL Ralph Singer, MC, Preventive Medicine Division, OTSG.

A. Patterns of Acute Respiratory Disease (ARD) on Individual Training Posts:

1. Fort Benning: ARD was mild at this southern post with a rate below 1.0/100/week except late December and in March when a peak rate of 2.2/100/week was recorded. Disease was due to both ADV-4 and ADV-7. ADV-4 vaccine was not given.

2. Fort Bliss: ARD rates were higher for more prolonged periods than in the past two years; and for the first time adenovirus morbidity was seen before the Christmas period. The ARD rate in late December exceeding 3.0/100/week and after Christmas gradually rose to a peak of 6.2/100/week on 15 March to decline below 3.0/100/week by the end of March. ADV-4 was the predominant pathogen isolated from 30 Nov 68 through 15 Mar 69. Although this virus was isolated from <50% of hospitalized Basic Combat Trainees (BCT's) during this period, 80% of BCT's sampled (24 of 30) showed ADV CF rises in February. In March, about one-third of hospitalized BCT's had ADV CF rises, while 40% had serologic evidence of Influenza B infection. ADV-4 vaccine was given to all incoming recruits beginning 8 Feb 68. By 22 Mar (when 75% of all recruits were immunized) ARD rates began to decline from a peak of 6.2/100/week on 15 Mar and thereafter ADV-4 was but rarely isolated from hospitalized BCT's at this post.

3. Fort Bragg: After mid-August 68, few adenovirus were isolated until Jan 69. The ARD rate remained below 1.2/100/week until Feb 69 despite isolation of A_2/HK/68 influenza virus from BCT's in Dec 68 and Jan 69 and serologic evidence of infection with this virus in

30% of hospitalized BCT's during this period. ARD rates rose to between 3 to 6/100/week between mid-February and May with a peak of 5.9 on 22 March. Disease was associated with both ADV-4 and ADV-7 during this period. Influenza B was isolated from BCT's in February, but accounted for <10% of all hospitalized ARD as determined by serology. The ADV-4 vaccine was not used on this post.

4. Fort Campbell: This post was unique in being the only post not using the ADV-4 vaccine in which ADV-7 was consistently the predominant respiratory pathogen. ARD rates of <2 were seen in Dec 68 associated with ADV-7; the ARD rate rose to >10.0/100/week on 8 Feb and declined to between 4.0 and 7.0 until April. During this period, both ADV-4 and ADV-7 were isolated with the latter strain predominant. Two isolations of ADV Type 21 occurred at Fort Campbell during winter 69.

5. Fort Dix: Although both ADV-4 and ADV-7 were present in July, ADV-4 became predominant from September-December with but rare isolates of ADV-7 during this period. The ARD rate rose to between 5.0-6.0/100/men/week in late November and December. A_2/HK/68 influenza was isolated in December, but this virus was associated with <10% of all hospitalized ARD (as determined by serology) in December and January and caused no disease thereafter. ADV-4 immunization of incoming BCT's was begun on 11 January, continued for 7 weeks, discontinued for 3 weeks (8 March - 22 March), and reinstituted for 4 weeks. Despite the fact that at no time were all BCT's immunized with ADV-4 vaccine, ADV-4 was uncommonly isolated after 11 Jan 69. ADV-7 caused significant amounts of ARD after 1 March and the ARD rate varied from 3.0-6.0 from then until mid-April. ADV Type 3 was isolated from a few BCT's in mid-February but did not cause disease thereafter.

6. Fort Gordon: Coxsackie virus A-21 was predominant from August-November 68 but as in past years, the ARD rate remained below 1.0/100/men/week during this period. The ARD rate rose to 3.0/100/week in late December associated with isolations of ADV-4, ADV-7 and A_2/HK/68 influenza virus. ADV-4 was predominant in Jan 69. ADV-4 immunization of incoming BCT's was begun on 8 February. The ARD rate peaked at 5.6/100/men/week on 1 March associated with equal proportions of ADV-4 and ADV-7 isolates. By the end of March 69 (when all BCT's had been immunized with the Type 4 vaccine), ADV-4 was rarely isolated and ADV-7 accounted for most ARD. The ARD rate dropped below 1.5/100/week by mid-April.

7. Fort Jackson: The ARD rate rose from <1.0/100/men/week in mid-Nov 68 to 3.1/100/week by mid-Dec 68, when ARD was associated with isolates of ADV-4 and ADV-7 in equal proportions. After Christmas, disease was due entirely to ADV-4 among adenovirus, and the rate rose to a peak at 6.2/100/week on 15 February. A_2/HK/68 influenza was isolated in Jan and Feb 69, but caused less than 20% of ARD (determined by serology) during these months. Incoming BCT's were immunized with ADV-4 vaccine

commencing 15 February. At a time when 75% of BCT's were immunized, ADV-7 supplanted ADV-4 and the ARD rate began a gradual decline to 2.0/100/week by 12 April, at which time ADV-4 was no longer isolated from hospitalized BCT's.

8. Fort Knox: ARD was virtually non-existent from Jul 68 through Feb 69 despite the sporadic isolation of ADV-4 and ADV-7 from BCT's. The ARD rate rose from 1.0/100/week on 8 March to 4.4/100/week on 22 March; this increase was predominantly associated with ADV-4, although some isolates of ADV-7 were obtained. Although the ADV-4 vaccine was not used, the ARD rate decreased to <1.0/100/week by mid-May. Two strains of ADV-21 were isolated in April.

9. Fort Lewis: ADV-4 and ADV-7 were isolated in near equal proportions on this post from Jul 68 through Apr 69. The ARD rate rose above 1.0/100/week in early November to 2.5/100/men/week in mid-December. Although A_2/HK/68 influenza was isolated in Jan 69, the rate remained below 2.0/100/week until mid-February when it rose precipitously to about 6/100/week and remained above 5/100/week throughout February and March. In April the rate gradually decreased from 3.0 to 1.5 by mid-May. ADV-4 vaccine was not used on this post.

10. Fort Ord: The ARD rate remained below 1.0/100/week until Nov 68 and thereafter gradually rose to a peak of 3.6/100/week in December; disease was then associated with ADV-4 and ADV-7 in equal proportions. Although A_2/HK/68 influenza was isolated in Dec 68 and Jan 69, less than 10% of BCT's hospitalized during these months had serologic rises to this virus. ADV-4 immunization of incoming BCT's was begun on 11 January. Thereafter the ARD rate remained between 1.0 2.0/100/men/week. ADV-7 became the predominant ADV isolated after mid-Feb 69 and ADV-4 was no longer isolated at the time when 100% of BCT's were immunized (15 Mar 69). A few serologic rises to influenza B were encountered.

11. Fort Polk: Despite the prevalence of Coxsackie virus A-21 infections in hospitalized BCT's from Jul-Oct 68, the ARD rate remained less than 1.0/100/men/week until 30 Nov 68 when ADV-4 disease was predominant. A_2/HK/68 influenza was isolated from BCT's in Dec 68 and Jan 69, but only a small proportion of hospitalized BCT's had serologic rises to this virus. ADV-4 vaccine was given to incoming BCT's commencing 11 January. ADV-7 appeared when 75% of BCT's were immunized, but ADV-4 remained a significant cause of ARD until 12 Apr 69, one month after immunization was achieved in 100% of BCT's. A peak rate of 2.4/100/week was encountered on 22 Mar 69 and the rate declined below 1.0/100/week by 5 Apr 69.

12. Fort Wood: Although both ADV-4 and ADV-7 were prevalent in Jul 68, ADV-4 was clearly predominant after August. The ARD rate rose above 1.0/100/week in mid-October to a peak of 4.5/100/week in December and disease was predominantly due to ADV-4. A_2/HK/68 virus

accounted for <10% of all ARD hospitalization in Dec 68 - Jan 69 ADV-4 immunization was begun in incoming BCT's on 11 Jan 69; thereafter, ADV-7 was the predominant pathogen while ADV-4 was but rarely isolated after 75% of BCT's had been immunized. The ARD rate rose gradually to 4.0/100/week in Mar; ARD was principally associated with ADV-7, although influenza B was associated with about 20% of ARD hospitalization in late February and early March. The ARD rate remained below 2.0/100/week after March.

B. Results of Enteric Adenovirus Type 4 Immunization on BCT Posts:

ADV-4 vaccine was given to incoming BCT's on 7 posts during 1969--Forts Bliss, Dix, Gordon, Jackson, Ord, Polk and Wood. In all 7 posts, ADV-4 was the predominant respiratory pathogen prior to immunization. In 6 of 7 posts, ADV-4 no longer represented the predominant respiratory pathogen 6 weeks later (after 75% of BCT's had been immunized), and within two weeks after all BCT's had been immunized, ADV-4 accounted for <5% of all ARD hospitalization. Only at Fort Polk was ADV-4 found to cause appreciable ARD after 100% immunization had been achieved; however, 5 weeks after complete immunization of Polk, ADV-4 was no longer isolated.

In 5 posts (Forts Dix, Gordon, Jackson, Ord and Wood) ADV-7 emerged 5 to 6 weeks after the beginning of ADV-4 immunization; in two (Bliss and Polk) only a few isolations of ADV-7 occurred after ADV Type 4 suppression. At Dix and Wood, ARD rates rose to above 6/100/week and 3/100/week respectively concomitant with the emergence of ADV-7 on post and rates of 1.5 - 2.0/100/week due to ADV-7 were evident into May. In the southern posts (Gordon and Jackson), ARD rates began to decline coincident with the emergence of ADV-7 and continued to decline throughout the Spring. At Ord, ADV-7 emerged in Feb 69 and was associated with ARD rates between 1.0 - 2.0/100/week throughout the winter and spring.

Thus, use of the ADV-4 vaccine resulted in the establishment of ADV-7 as the predominant pathogen in 5 of the 7 posts in 1969. In the northern posts with a prolonged ARD season, this resulted in outbreaks of ADV-7 disease. In southern posts with shorter ARD seasons, the emergence of ADV-7 coincided with the natural decline in ARD rates with the result that extensive outbreaks of ADV-7 did not occur. Similar patterns were seen in these posts after ADV-4 immunization in Winter-Spring 1967.

C. A_2/HK/68 Influenza:

Respiratory disease due to A_2/HK/68 influenza virus was distinguished by its lack of morbidity in BCT posts. Although the virus was isolated from BCT's at 8 of the 12 CONUS training posts in December and January, adenovirus was associated with the majority of ARD hospitalizations at that time. Serologic sampling of hospitalized BCT's at several posts confirmed that A_2 influenza resulted in little hospitalized ARD. In the 6 BCT posts for which serology was performed (Bragg, Dix, Jackson, Ord, Polk and Wood), only 10-20% of ARD hospital admissions tested had

significant antibody responses to A_2/HK/68 during the period of influenza prevalence (December and January).

The reasons for the low A_2/HK/68 morbidity are obscure but several factors may in part account for it:

1) Use of A_2/Aichi/68 monovalent vaccine in BCT posts commencing in mid-December 68.

2) Influence of Christmas leave (which occurred at estimated peak of outbreak) on the transmission of the virus among BCT's.

3) Mildness of A_2/HK/68 disease in young adults.

4) Protection afforded by military polyvalent influenza vaccine against A_2/HK/68 strain. (This factor seems unlikely - see succeeding section on A_2/HK/68 strains).

The relative role of the first three factors cannot be defined since they were all operating simultaneously during the appearance of the A_2/HK/68 strains in BCT posts.

D. Influenza B:

Strains of Influenza B were isolated at two posts (Wood and Bragg) and serologic evidence of Influenza B infection in BCT's was found at those two posts, Fort Bliss and Fort Ord. In all four posts, disease was limited to Feb or Mar 69. Influenza B was associated with about 40% of ARD at Fort Bliss, 20% of ARD at Fort Wood and less than 10% of ARD at Forts Bragg and Ord during its prevalence on these posts.

From mid-Dec 68 through Feb 69, incoming BCT's received monovalent A_2/Aichi/68 vaccine and hence, were not immunized against Influenza B. This likely accounts for the outbreaks of Influenza B recorded (which terminated shortly after polyvalent vaccine was reinstituted); however, representative Influenza B isolates are being characterized to explore the possibility of a significant new antigenic Influenza B variant.

III. Hong Kong Influenza.

Abstract.

An outbreak of influenza beginning in Southeast Asia in July 1968 prompted investigations into the characteristics, immunogenicity, and antigenic composition of the strains isolated. A_2/HK/68 influenza viruses were isolated efficiently in Rhesus monkey kidney monolayer tissue culture and in embryonated eggs. The serologic response to A_2/HK/68 infection in man resembled a primary antibody response. Major differences in antigenic composition of the 1968 strains and A_2 strains isolated from 1957-1967 were shown by HI and N tests with hyperimmune rooster antisera;

no antigenic similarity between the 1968 strains and the 1957 A_2 strain could be demonstrated. Recruits immunized with the 1968 military polyvalent vaccine had suitable N antibody responses to a 1965 A_2 strain, but only 16% developed N antibody titers of $\geq$1:10 to a 1968 strain.

Introduction.

An outbreak of influenza was first detected in Hong Kong in July 1968. Strains with characteristics of influenza were isolated from patients by the Hong Kong Government Virus Unit and preliminary characterization of them suggested that the outbreak was caused by a new variant of Influenza A. The following report describes investigations of the characteristics and antigenic composition of the A_2/HK/68 variants, an attempt to provide suitable 1968 strains for vaccine production, and an assessment of the potential impact of these strains in military personnel.

A. Methods:

1. Collections: Throat washings (in Hank's balanced salt solution containing 0.4% bovine plasma albumin) and acute and convalescent sera were obtained from airmen with influenza symptoms at Korat AFB, Thailand by LTC Thomas Smith of the SEATO Laboratory and shipped with dry ice to WRAIR. Throat washings were obtained from patients with influenza symptoms in Manila, the Phillipines by MAJ Lloyd Olson of the SEATO Laboratory and sent with dry ice to WRAIR. African green monkey kidney passages of strains isolated from patients in Hong Kong were forwarded to WRAIR by Dr. Ned Wiebenga of the 406th Medical Laboratory.

Serum was obtained from Basic Combat Trainees at Fort Dix, New Jersey eight weeks post-immunization with the 1968 military polyvalent influenza vaccine. Additionally, paired sera were obtained from an additional group of BCT's, prior to and four weeks after immunization with this vaccine.

2. Virus Isolation: Aliquots of throat washings treated with penicillin (1000 u/ml) and streptomycin (1000 u/ml) were inoculated into monolayer tissue culture tubes of primary rhesus monkey kidney (MK). The MK tissue cultures were washed twice with Hank's balanced salt solution and fed with medium 199 without serum prior to inoculation. MK cultures were observed daily for cytopathic effect (CPE) for 14 days and hemadsorption with guinea pig erythrocytes was performed when CPE was observed or at 7 and 14 days. Aliquots of throat washings were also inoculated into the allantoic and amniotic cavities of 9-10 day RIF-free embryonated eggs. After incubation of eggs at 35° for three days, hemagglutinins for human type O erythrocytes were sought by standard techniques. A second egg passage of allantoic and amniotic fluids which did not hemagglutinate on first passage was made.

3. Antisera: Rooster antisera were prepared to certain newly recovered viruses and certain older influenza strains. Pairs of 8-10 pound roosters were inoculated both intravenously and intraperitoneally with 5.0 ml of undiluted infected allantoic fluid. Sera were harvested 10 days after inoculation.

4. Serologic Procedures: Hemagglutination-inhibition (HI) and CF tests were performed as reported earlier (Weinberger, et al, Proc. Soc. Exper. Biol. Med. 114:413, 1963). After preliminary heating at 56°C for 30 minutes, human sera were treated with potassium periodate. Rooster sera were tested after treatment with receptor destroying enzyme (RDE). HI tests on human sera were performed with four HA units of A_2/HK/1/68 antigen and CF tests performed with four units of A_2/DC/327/63 antigen.

5. Neutralization Tests: Neutralizing antibodies in human and rooster sera were measured in MK monolayer cultures using a hemadsorption-inhibition technique. Serial 2-fold dilutions of serum previously heated to 56°C for 30 minutes were made in Hank's balanced salt solution containing 0.4% bovine plasma albumin. To 0.2 ml of each dilution of human serum was added an estimated 100-1000 hemadsorption doses (HAD_{50}) of A_2/Thai/303/68 (or A_2/HK/1/68) or A_2/Thai/379/65; after incubation at 25°C for one hour, 0.1 ml was then inoculated into each of two tube cultures of MK cells. In reciprocal quantitative N tests with rooster sera, serial 10-fold dilutions of each virus were mixed with equal volumes of 2-fold dilutions of rooster antiserum. Mixtures were incubated at 25°C for one hour; 0.1 ml was then inoculated into each of four tube cultures of MK cells. These cultures had been propagated with 10% calf serum with added antiserum to sv-5 virus, and were washed twice and maintained with medium 199 containing penicillin (100 u/ml) and streptomycin (100 ug/ml) prior to use. After stationary incubation at 37°C for three days, inoculated cultures were examined for CPE and tested for hemadsorption. Cultures were washed once with HBSS and 1.0 ml of a 0.1% suspension of fresh, washed guinea pig erythrocytes in HBSS was added. Erythrocytes were allowed to settle upon the cell sheets at 4°C for 30 minutes. Chilled tubes were examined immediately for hemadsorption. Titers of neutralizing antibody were expressed as the highest dilution of serum which completely inhibited growth of influenza virus in 50% of the inoculated tubes.

B. Results:

1. Recovery of Influenza Viruses: Influenza A viruses were isolated from 14 of the 19 throat washings received from Korat AFB, Thailand; in eggs 14/19, in MK monolayers 11/19. The isolation efficiency in eggs and MK monolayers from Korat airmen on whom paired sera were available is shown in Table 12. For practical purposes, the efficiency of isolation in MK monolayer tubes was comparable to that obtained in eggs. In addition, influenza A viruses were isolated in MK monolayers from 2 of 4 throat washings obtained in Manila, the Phillipines. Notable was the production of cytopathic effect by the 1968 strains in

MK monolayers. All strains isolated showed CPE on primary or second passage. Hemadsorption was no more efficient than CPE in detecting isolates in MK monolayers.

Table 12 Isolation Efficiency of Hong Kong Influenza Virus, Korat AFB Outbreak

Patient's Neutralizing (N) Antibody Rise	Number Patients	Isolation in	
		Egg	MK Monolayer
$\geq$ 4 Fold	12	11	9
< 4 Fold	4	1	0
Total	16	12	9

Despite the plethora of A_2/HK/68 virus strains sent from Hong Kong and Japan to CONUS for antigenic characterization, all but one, Aichi, was unsuitable as a potential vaccine candidate since they had been isolated in monkey kidney tissues and could conceivably contain adventitious monkey agents. Consequently, aliquots of several of the throat washings obtained from Korat and Manila were made and stored at WRAIR and as soon as A_2 influenza was detected in them, uninoculated throat washings and the primary RIF-free egg passages of two of the strains - A_2/Thai/303/68 and A_2/Phil/304/68 - were sent to the Division of Biologics Standards to be used as vaccine candidates should the Aichi strain prove unsuitable as a vaccine strain.

2. Serologic Responses in Influenza Patients: HI, CF and neutralizing (N) antibody responses in Korat AFB patients are shown in Table 13.

It is evident that both CF and HI tests were inefficient in detecting rises in 2 week convalescent sera. In contrast, 4-fold or greater rises in N antibody were detected in 2 week convalescent sera of 10/11 patients from whom A_2/HK/68 virus was isolated. Many of the 2 week rises in N antibody were minimal 4-fold increases, and were detected only because all patients had titers of $\leq$1:5 in acute sera. Acute HI titers were higher in these sera and probably the higher initial titers measured by HI masked rises at 2 weeks. Pretreatment of some pairs of Korat sera with DEAE-sephadex reduced HI titers in the acute sera to $\geq$1:10, and the proportion of HI rises after DEAE treatment approached the proportion of N rises seen in 2 week convalescent sera. The proportion of HI antibody rises at 4 weeks was more comparable to the proportion N antibody rises and 4 week convalescent titers were considerable higher than at 2 weeks. The slow HI and N antibody response

Table 13 Serologic Response to A_2/HK/68 Influenza Infection, Korat AFB Outbreak

Virus in TW	HI Antibody Rise[1/] by 2wk	HI Antibody Rise[1/] by 4wk	CF Antibody Rises[2/] by 2wk	N Antibody Rises[3/] by 2wk	N Antibody Rises[3/] by 4wk
Isolated	5/11*	8/10	5/11	10/11	10/10
Not Isolated	1/4	1/2	1/4	1/4	1/2

* Number with rise/Number tested.

1/ Tested with 4 HA units - A_2/HK/1/68

2/ Tested with 4 units - A_2/DC/327/63

3/ Tested with 10 HAD_{50} - A_2/Thai/303/68

seen after infection with A_2/HK/68 strains suggest that these strains were sufficiently different in antigenic composition from previous A_2 strains to elicit a primary type antibody response in man.

3. Antigenic Analysis of A_2/HK/68 Strains: The reactions of rooster antisera prepared to 1968 viruses with the hemagglutinins of both older prototype and newly isolated strains are shown in Table 14. It is evident that the antigenic composition of the 1968 strains is homogeneous. Antisera prepared to previous A_2 strains reacted weakly, if at all, with the hemagglutinins of the 1968 strains and no inhibition of 1968 strains was obtained with antiserum to the earliest prototype A_2 strain A_2/Form/313/57. Furthermore, antisera made to the 1968 strains reacted weakly with hemagglutinins of previous A_2 strains; no inhibition of A_2/Form/313/57 hemagglutinins was obtained with antiserum to any of the four 1968 strains tested. Similar findings are apparent when strains were characterized by neutralization reactions, as shown in Table 15.

To determine whether differences between the 1968 and older strains suggested by these HI and N tests were uniform and valid, the quantitative relationships between titers of rooster antisera and various viruses were studied. These quantitative tests were done with eight viruses (Table 16) and antisera prepared against six of the viruses. In the first series of tests, the ability of four antisera to neutralize five strains, one a 1957 strain and four 1968 strains was compared (Figure 8). These tests showed, first, that the 1968 strains behaved homogeneously; none was neutralized by potent antiserum to the 1957 A_2 strain and equivalent virus doses of each of the four 1968 strains tested was neutralized by individual 1968 antisera to a titer varying less than 0.5 log. Because homologous reactions of antisera to 1968 viruses were similar, it was concluded that these strains were antigenically similar.

Next, the reactions of antisera prepared against A_2/Form/313/57, A_2/Jap/170/62, A_2/Thai/379/65 and A_2/HK/1/68 were tested against the 1957, 1962, 1965, 1967 and two 1968 strains (Figure 9). Antiserum to the original A_2 strain appearing in 1957 (A_2/Form/313/57) neutralized A_2 strains isolated between 1957-1967 but did not neutralize the 1968 strains. Although minimal neutralization of the 1968 strains by antisera of the 1962 and 1965 strains was found, these antisera neutralized equivalent doses of the 1957-1967 strains to a much higher titer. Finally, antisera to one of the 1968 strains neutralized the 1968 strains to a much higher titer than any of the 1957-1967 A_2 strains and did not neutralize the earliest member of the A_2 strains, A_2/Form/313/57. Thus, by HI, N, or quantitative N tests, the A_2/HK/68 strains were found to be remarkably homogeneous, but readily distinct from A_2 strains isolated between 1957 and 1967.

4. Antibody Responses in BCT's Immunized with Polyvalent Influenza Vaccine: Since potent rooster antisera to A_2 strains isolated from 1957-1967 failed to neutralize significantly the 1968 isolates, neutralizing antibody titers to the HK 68 viruses were determined in recently immunized BCT's to investigate whether N antibodies to 1968

Table 14 Hemagglutination Inhibition (HI) Reactions of Rooster Antisera with A_2/HK/68 and Older Influenza Viruses

	Antigen									
Antiserum	A/PR/8/34	A_1/AA/56	A_2/Form/313/57	A_2/Jap/170/62	A_2/Thai/379/65	A_2/DC/303/67	A_2/HK/1/68	A_2/HK/19/68	A_2/Thai/303/68	A_2/Phil/304/68
A_2/Form/313/57	<10	<10	**1280**	1280	1280	160	<10	<10	<10	<10
A_2/Jap/170/62	<10	<10	40	**1280**	640	160	40	40	40	40
A_2/Thai/379/65	<10	<10	40	640	**1280**	640	20	20	20	20
A_2/DC/303/67	<10	<10	<10	80	320	**320**	<10	<10	<10	<10
A_2/HK/1/68	<10	<10	<10	40	40	20	**640**	640	640	640
A_2/HK/19/68	<10	<10	<10	40	80	40	640	**640**	640	640
A_2/Thai/303/68	<10	<10	<10	10	40	40	320	640	**640**	320
A_2/Phil/304/68	<10	<10	<10	40	40	40	1280	640	640	**640**

Table 15 Neutralization (N) Reactions of Rooster Antisera with Influenza Viruses

Antiserum	Virus: A/PR/8/34	A_1/AA/56	A_2/Form/313/57	A_2/Jap/170/62	A_2/Thai/379/65	A_2/DC/303/67	A_2/HK/1/68	A_2/HK/19/68	A_2/Thai/303/68	A_2/Phil/304/68
A_2/Form/313/57	<10	<10	640	640	320	160	<10	<10	<10	<10
A_2/Jap/170/62	<10	NT	10	80	40	40	10	<10	<10	<10
A_2/Thai/379/65	<10	NT	320	160	1280	640	10	<10	20	<10
A_2/DC/303/67	<10	NT	40	20	80	1280	20	10	<10	10
A_2/HK/1/68	<10	NT	<10	<10	20	20	320	320	640	320
A_2/HK/19/68	NT	<10	<10	<10	10	20	160	80	160	160
A_2/Thai/303/68	NT	<10	<10	<10	<10	<10	160	160	320	160
A_2/Phil/304/68	NT	<10	<10	<10	<10	<10	160	160	NT	160

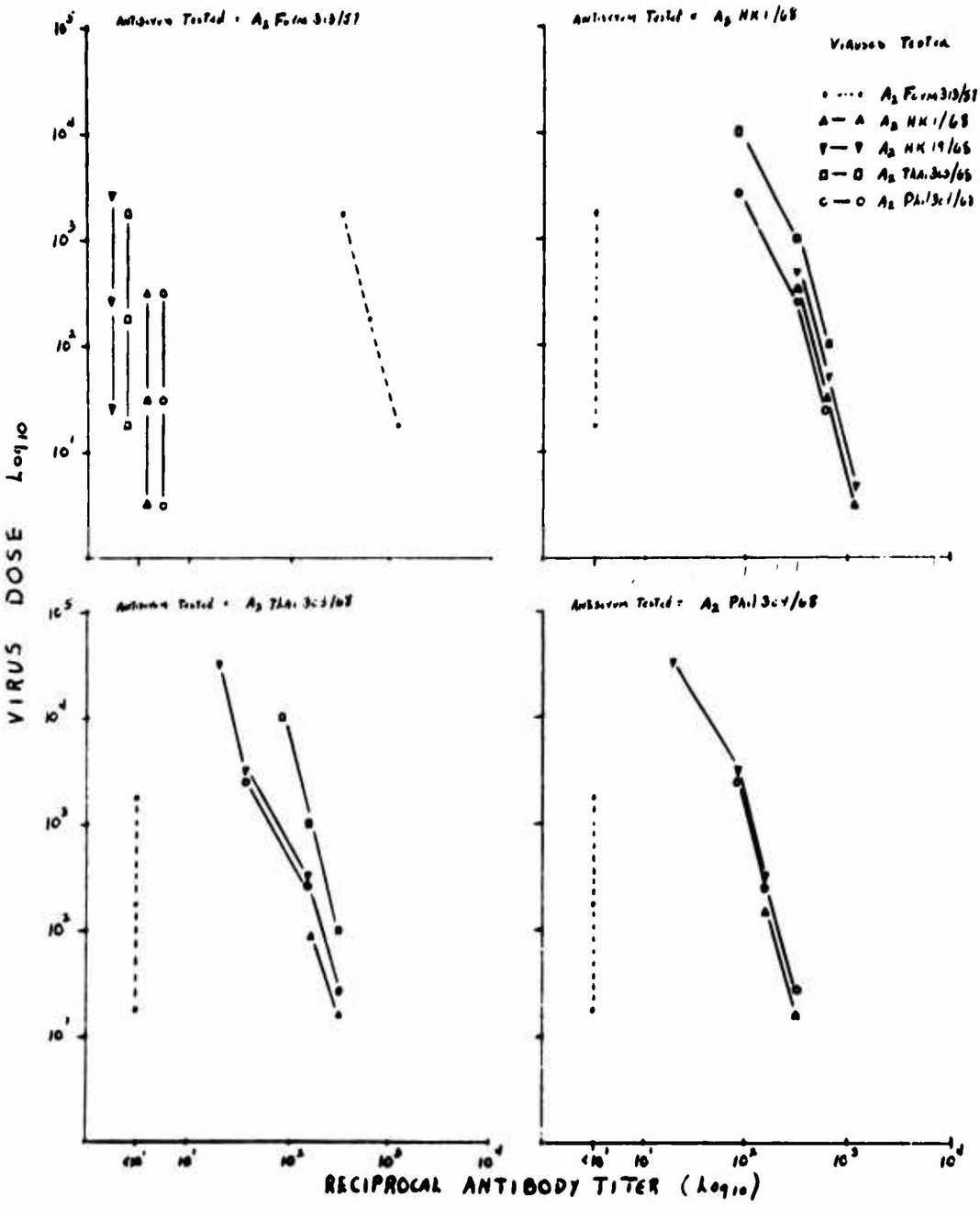


Fig 9 Quantitative relationships between A_2 strains: 1) comparison of N reactions of antisera to a 1957 and three 1968 strains.

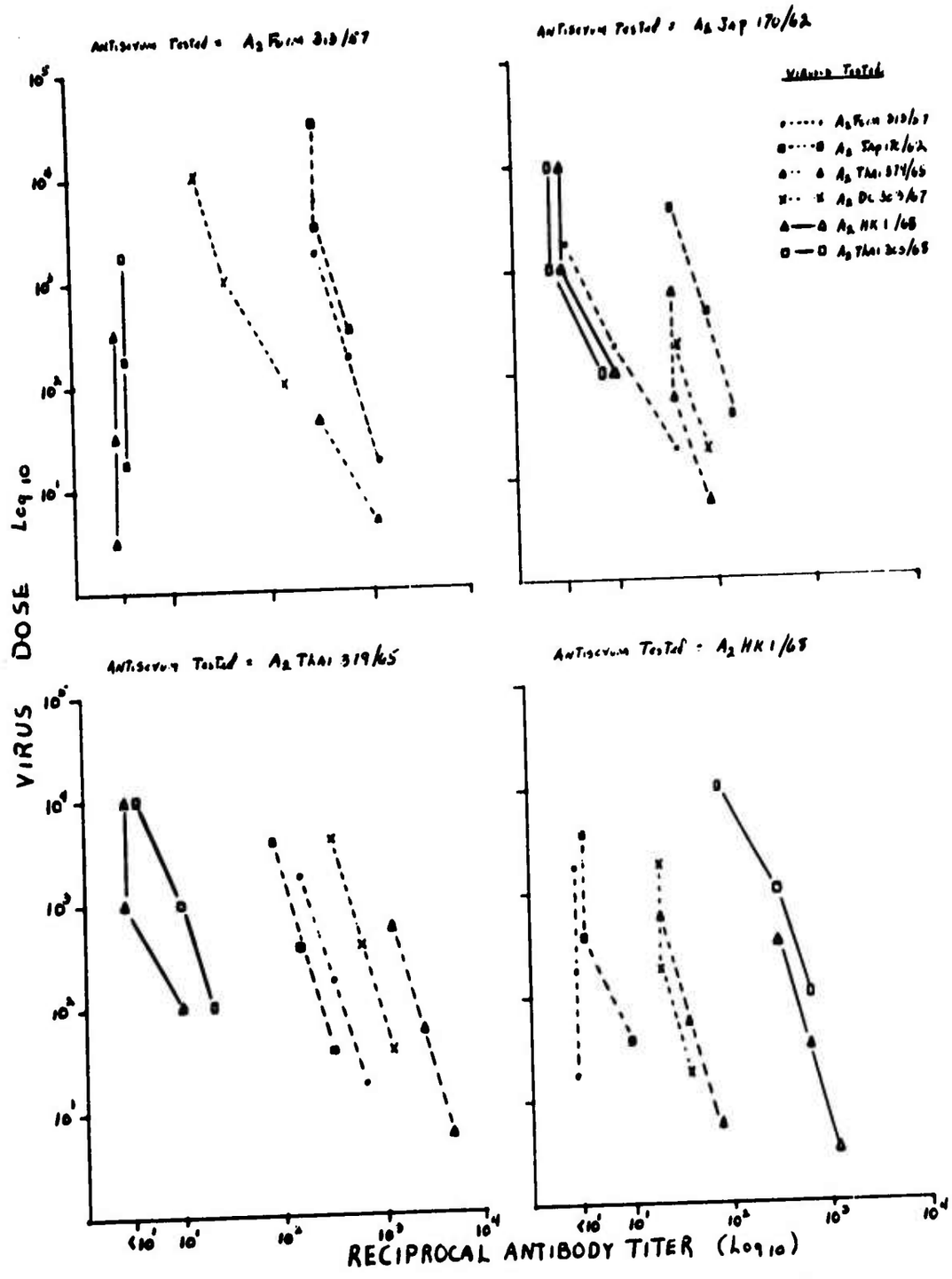


Fig 9 Quantitative relationships between A2 strains: 2) comparison of N reactions of antisera to 1957, 1962, 1965, and a 1968 strain.

Table 16 A_2 Influenza Virus used in Quantitative N Tests

Virus	No. Passages in Indicated Host	Antiserum Used
A_2/Form/313/57	E-6, MK-6	Yes
A_2/Jap/170/62	E-7, MK-6	Yes
A_2/Thai/379/65	E-4, MK-7	Yes
A_2/DC/303/67	E-2, MK-3, E-2, MK-6	No
A_2/HK/1/68	AgK? MK-3	Yes
A_2/HK/19/68	AgK? MK-3	No
A_2/Thai/303/68	MK-3	Yes

AgK = African green monkey kidney cell culture
MK = Rhesus monkey kidney cell culture
E = Embryonated egg

strains were induced by immunization with the 1968 military polyvalent vaccine whose major A2 antigen was A_2/Taiwan/64. The sera of 50 BCT's immunized 8 weeks previously were tested for N antibodies to 40 HAD_{50} of A_2/HK/1/68; only 12 were found to possess titers of ≥1:10 and but one ≥1:20. Subsequently, paired sera were obtained from a sample of BCT's prior to and 4 weeks after immunization and tested for N antibodies with 1000 HAD_{50} of A_2/HK/1/68 virus. As shown in Table 17, only 4% (2 of 50) BCT's had serum N antibody titers ≥1:10 prior to immunization and after immunization only 16% (8) possessed serum N antibodies titers ≥1:10. Paired sera of 20 of these BCT's were tested against 200 HAD_{50} of A_2/Thai/379/65 to determine their N antibody response to this virus after immunization. As shown in Table 17, all 20 BCT's had N antibody titers of ≥1:10 prior to immunization and N antibody titers of ≥1:40 after immunization.

Thus, although immunization of BCT's with polyvalent influenza vaccine produced high levels of serum N antibodies to the 1965 A_2 strain, it produced low or undetectable N antibody titers to the 1968 variant in the majority of BCT's.

C. Discussion:

As compared to some previous A_2 strains (notably the 1967 isolates), the A_2/HK/68 strains were easily isolated in embryonated eggs and MK tissue culture monolayers. Eggs were a slightly more efficient culture for isolation than MK monolayers, but difficulties in passaging the 1968 strains in eggs was encountered due to their lethal effect on chick embryos. A notable characteristic of the 1968 strains was their ability to grow on first passage in MK monolayers with the production of a distinctive cytopathic effect.

Table 17 N Antibody Response of Recruits to Influenza Virus Vaccine

	Incidence of Antibody to Indicated Virus			
	Pre-Immunization		Post-Immunization	
Antibody Titer	1965* %	1968** %	1965 %	1968 %
≥1:10	100	4	100	16
≥1:20	90	2	100	6
≥1:40	70		100	2
≥1:80	40		90	
≥1:160	25		85	
≥1:320	15		75	
≥1:640	15		65	

* A_2/Thai/379/65, 200 HAD_{50}
** A_2/HK/1/68, 1000 HAD_{50}

All BCT's were immunized with the military polyvalent influenza vaccine containing 100 CCA units of A/swine/1976/31, A/PR/8/34, A_1/Ann Arbor/1/57, and B/Lee/40, 200 CCA units of B/Mass/66 and 400 CCA units of A_2/Taiwan/64.

A considerable difference in antigenic composition of the 1968 strains compared to earlier A_2 isolates was found both by HI and N tests. Since the original A_2 influenza pandemic in 1957, 3 influenza A_2 outbreaks have occurred in CONUS, in 1963 (strains similar to A_2/Jap/170/62), in 1967 (strain A_2/DC/303/67), and in 1968 (strain A_2/HK/1/68). Strains isolated during the first two of these outbreaks were similar in their antigenic relationship to the strain causing the previous outbreak. Antisera raised to the earlier strain neutralized the succeeding strain to a titer comparable to the homologous strain, but antisera raised to the later strain but weakly neutralized the earlier A_2 variant. A_2/HK/68 strains were not similarly related to their preceding A_2 strain, A_2/DC/303/67; not only did 1968 antiserum but weakly neutralize the 1967 strain, but antiserum to the 1967 strain also weakly neutralized the A_2/HK/68 variants. Furthermore, although antiserum to the earliest A_2 strain A_2/Form/313/57 neutralized 1962-1967 strains; it failed to neutralize the 1968 strains, and although A_2/Form/313/57 was neutralized

to some degree by antisera raised to the 1962 and 1967 strains, it was not neutralized by antisera raised to the 1968 strains. Thus, the A_2/HK/68 strains represent a major shift in antigenic composition of A_2 strains. The primary type antibody response in patients following infection with the 1968 strains reflected this significant antigenic change.

Recruits commencing BCT almost uniformly lacked serum N antibody to the A_2/HK/68 strains. After immunization with the 1968 military polyvalent influenza vaccine containing 400 CCA units of A_2/Taiwan/64, only 16% of BCT's possessed serum N antibodies to the 1968 strains to a titer of $\geq$1:10. Thus, the standard military polyvalent vaccine was not expected to result in protection against disease caused by the 1968 strains.

Despite the marked difference in antigenic composition between the 1968 strains and preceding A_2 strains, despite the poor N antibody response to the 1968 strains produced by the military vaccine, and despite significant influenza morbidity in military personnel and civilians in Asia and in CONUS civilians, remarkably little morbidity due to A_2/HK/68 influenza was seen in CONUS Army Personnel (see adenovirus surveillance section, this Annual Report). The reasons for this lack of morbidity are obscure; in part it may have been due to the protective effect of the A_2/Aichi/68 vaccine (available to some military personnel in mid-December) and in part to the fortuitous occurrence of Christmas leave shortly after the beginning of the influenza outbreak in CONUS.

The low incidence of influenza in CONUS military personnel emphasizes that the antigenic differences in new A influenza variants is not the sole determinant of their potential morbidity in man, and that predictions of the magnitude of influenza outbreaks on the basis of changes in antigenic composition of the virus alone are likely to be inaccurate.

IV. A Universal Method for Removing Non-Specific Inhibitors of Virus Hemagglutination from Serum.

The hemagglutination-inhibition (HI) test is one of the most useful serological methods for diagnosing virus infections. Many viruses pathogenic for man have the ability to agglutinate the red cells of one or more species of animals. Among the agents with this capacity are the myxoviruses, most arboviruses and adenoviruses, some enteroviruses and the organisms responsible for measles and rubella. The HI test has several inherent advantages over complement-fixation and neutralization assays for virus antibodies. It is simple, rapid, and particularly well suited to screening large numbers of sera. Since many HI antigens are now available from commercial sources, laboratories that lack the techniques and equipment necessary for virus propagation can do HI titrations.

Serum from man and other mammals usually possesses inhibitors for the virus hemagglutination reaction that are not associated with specific

antibody. These inhibitors are associated with several components of serum, some lipids and others protein. Since they are often present in high titers, they must be eliminated from serum before HI antibody content can be accurately measured. In many cases the removal of these non-specific inhibitors has proved difficult. A variety of techniques for removing them have been developed but these have not been entirely satisfactory since they are often time consuming, may remove specific antibody along with non-specific factors, and in many systems have failed to completely remove non-specific inhibitors.

A different approach to removing non-specific inhibitors is described herein. Instead of trying to eliminate only non-specific inhibitors, serum was treated in such a manner as to remove all serum proteins except for the major class of immunoglobulins. When test sera were mixed with the anion exchanger DEAE-Sephadex all proteins except the IgG globulins were bound, leaving only the latter protein in the supernatant fluid. The technique is simple, relatively inexpensive and rapid to perform. These studies show that DEAE extraction removes non-specific inhibitors for influenza, western equine encephalitis, dengue 2 and rubella viruses; theoretically such treatment should eliminate these factors for all types of viruses.

A. Materials and Methods:

1. Preparation of DEAE: DEAE-Sephadex A50 (Pharmacia Fine Chemicals, Piscataway, N.J.) was processed and equilibrated with low ionic strength buffer as described by Baumstartk, et al. Specifically 50 gms of DEAE was slowly added to 6 liters of water in a 16 x 45 cm cylindrical glass jar and allowed to swell for at least one hour. Fines were next removed by suspending the beads of DEAE in this solution, allowing them to settle for 10 minutes and aspirating the supernatant liquid down to the barely discernable line separating the rapidly sedimenting beads from those that settled more slowly. Water was added back to 6 liters, and the procedure was repeated 6 to 8 times or until essentially all DEAE settled rapidly and completely within 10 minutes. The DEAE was then added to a Buchner funnel and successively washed with 1 liter of .5N NaOH, 3 liters of water, 1 liter of .5N HCl, 3 liters of distilled water, and 3 liters of .01M pH 6.5 phosphate buffer. The DEAE was then resuspended in approximately 3 liters of phosphate buffer and washed twice with the .01M pH 6.5 buffer in the same manner used to remove fines. The suspension was standardized by pouring it into a 1 liter graduated cylinder, and allowing the beads to settle for one hour. At this time buffer was either removed or added so that the settled DEAE occupied 67% of the total volume of solution in the cylinder.

2. Extraction with DEAE: For each sera to be extracted 8 ml of evenly suspended 67% DEAE was added to a 40 ml round bottom centrifuge tube. Excess phosphate buffer was then aspirated through a 1.5 cm diameter glass tube that had a .1 cm thick disk of high porosity sintered glass

fused into its lower opening (available from Warren R. Foster Laboratory Glassware, 15808 Ancient Oak Dr., Gaithersburg, Md.). This was placed into the centrifuge tube and suction was applied. As buffer was drawn into the tube, the sintered glass filter was lowered onto the surface of the DEAE beads. (As soon as bubbles of air were aspirated, the suction apparatus was removed and transferred to the next centrifuge tube.)

.5 ml of each serum to be extracted was then added to a tube of aspirated DEAE. The resulting slurry was vigorously stirred on a Vortex-Genie (Scientific Industries, Inc., Queens Village, New York) initially and every 15 minutes for one hour. At this time a pasteur pipette was applied to the bottom center of each centrifuge tube and the supernatant fluid containing the IgG globulins was aspirated by a rubber bulb. The small amount of DEAE that remained in these supernatant fluids was finally removed by centrifugation at 2000 rpm for 15 minutes.

Immunoelectrophoresis was performed by the method of Scheidger. The antiserum used to develop these slides was prepared by immunizing a rabbit with six monthly injections of 1-2 ml of whole human serum in Freund's complete (first immunization) and incomplete adjuvant (subsequent five injections).

Concentrations of IgG globulins were measured by radial diffusion in commerically produced agar plates containing monospecific anti-human IgG (Hyland Laboratories, Los Angeles, Calif). Human IgG purified by DEAE cellulose column chromatography and standardized on a Beckman DU-2 spectrophotometer assuming $E^{1\%}_{1cm} = 14.3$ was the reference standard for this assay.

3. Influenza Antibody: Serological responses to influenza infections were measured in eight airmen who became ill during a recent Hong Kong influenza epidemic at a United States Air Force base in Thailand during August 1968. The acute sera were taken during the height of this illness, and convalescent bleedings were made either 13 or 16 days later. Complement-fixing (CF), HI and neutralizing antibody titers were performed by the methods outlined in the section on A_2/HK/68 influenza, this Report. The CF response was measured by microtiter techniques in "U" bottom plates (Cook Engineering, Alexandria, Va.) utilizing .025 ml dilutions of inactivated serum, .025 ml containing two units of A_2/DC/327/63 influenza antigen (Microbiological Associates, Bethesda, Md.) and two units of guinea pig complement. HI responses were measured in "V" bottom microplates using four units of A_2/HK/1/68 influenza antigen. Non-specific inhibitors were removed by DEAE or periodate treatment of serum. Neutralizing antibody was measured against 10 TCD_{50} units of A_2/Thai/303/68 virus.

4. Rubella Antibody: Rubella antibody was measured by titering plaque reduction and HI activities in single sera taken from 11 women during their first 20 weeks of pregnancy. The HI assay followed the

techniques previously described (Annual Report, this Department, 1968). Non-specific inhibitors were absorbed using Kaolin or DEAE. The plaque reduction test used is described in the rubella section of this report.

5. Arbovirus Antibody: Nineteen sera taken from 11 patients suspected of having arbovirus disease were selected from the Walter Reed serum files for assay of HI antibodies to western equine encephalitis (WEE) and dengue 2 arboviruses. Prior serology had indicated 11 of these sera had dengue 2 titers of 1:10 or less while the remaining eight contained higher levels of this antibody. HI titrations were performed according to the methods described in previous Annual Reports. Non-specific inhibitors were removed by treating serum with either DEAE or acetone.

B. Results:

1. Development of the DEAE Extraction Techniques: The DEAE extraction technique was developed with three primary objectives in mind. First, we wanted to completely separate the IgG globulins from other serum components to dispose of all possible types of non-specific inhibitors. Second, the extraction was to yield both a constant dilution and as little dilution as possible of the IgG globulins in the sera applied to it. Third, the method was to be simple, rapid to perform and inexpensive.

Pure IgG globulins are most commonly isolated from whole serum by chromatography on columns of DEAE-cellulose or DEAE-Sephadex. Serum equilibrated with dilute buffers at a pH near neutrality is applied to a column of DEAE equilibrated with the same buffer. Under these conditions, the IgG globulins, unlike other serum proteins, have a neglegible charge. They therefore fail to bind to the positively charged DEAE and are eluted from the column in pure form.

Thus DEAE could be used to satisfy the first objective. However, column chromatography requires specialized equipment and is quite time consuming. To satisfy the third requirement, a "batch" technique was developed for the extraction. Serum is mixed with DEAE equilibrated with a dilute neutral buffer and the resulting supernatant fluid then contains only IgG globulins since these are the only proteins that fail to bind to the DEAE.

We first attempted to remove the non-specific inhibitors from serum with DEAE-cellulose using a "batch" extraction technique similar to that described for DEAE-Sephadex in "methods". Although such treatment did yield pure IgG, these fractions were unsuitable for HI titrations since DEAE-cellulose contributed a factor to the supernatant buffer during extraction that agglutinated red cells. Therefore, DEAE-Sephadex was used in place of the DEAE-cellulose and provided that care was taken to remove fines, the Sephadex did not release hemagglutinins during the extraction.

The proper proportions of DEAE-Sephadex and serum and timing of the extraction procedure to yield pure IgG with the least dilution were next defined. Extraction of .5 ml serum with 8 ml of the DEAE-Sephadex suspension for one hour yielded pure IgG without impurities as shown by immunoelectrophoresis. Such treatment was found to yield consistently immunoelectrophoretically pure IgG globulins.

Table 18 summarizes the dilution of IgG globulins associated with this extraction procedure. The concentration of purified IgG in the supernatant extraction buffer was approximately 20% of its value in the corresponding whole serum and a consistent dilution of this immunoglobulin was found in the many sera extracted. Of the 16 influenza sera so treated, the IgG of 15 was diluted between 1:4.7 and 1:6. Therefore the final dilution produced by this extraction was assumed to be 1:5. A similar analysis of the IgG dilutions for the arbovirus and other sera confirmed that this 1:5 value was constant. The volume of the supernatant extraction fluid was approximately the same as the .5 ml volume of serum that had been applied. Therefore the yield of the extraction was approximately 20%.

2. Serological Diagnosis of Influenza Infections: Table 19 compares the CF, neutralizing, and HI influenza titers in acute and convalescent sera from eight patients with "flu"-like illnesses. It is evident that the HI titers of untreated and periodate treated sera were of limited value for identifying influenza responses in convalescent serum drawn two weeks after infection; only one of eight untreated serum pairs and two of eight treated with periodate had 4-fold antibody rises.

The HI titers were relatively high in both untreated and periodate treated acute sera. Although this activity might have been due to either non-specific inhibitors or pre-existing specific antibody, the neutralization data of Table 18 suggest non-specific factors were responsible. Only two acute sera had neutralizing activity and in both cases the titers were only 1:5. Furthermore, six of eight patients had 4-fold or greater neutralizing antibody responses.

The HI titers of IgG fractions produced by DEAE extraction of acute and convalescent sera agreed very closely with the neutralizing data. In both instances acute sera had little or no activity and both tests identified the same six patients as having significant serological responses to influenza. CF titers were hardly better than the periodate HI results since only three patients had 4-fold CF responses. Therefore, HI titrations of DEAE extracted sera were superior to similar titrations of periodate treated sera for the diagnosis of influenza infections, presumably because non-specific inhibitors were removed by the former treatment that were not eliminated by periodate.

3. Removal of Non-Specific Inhibitors of Arbovirus Hemagglutination: The non-specific serum inhibitors of arbovirus hemagglutination are associated with lipids and can be effectively removed by extracting

Table 18 Dilution of IgG globulins Following Treatment of Whole Serum by DEAE-Sephadex

mg IgG/100 ml

Patient	Acute Sera			Convalescent Sera		
	Whole Serum	DEAE Supernate	Dilution Factor(1)	Whole Serum	DEAE Supernate	Dilution Factor
KT 2	900	185	4.9	775	160	4.8
KT 6	1250	250	5.0	1300	240	5.4
KT 7	700	150	4.7	750	140	5.4
KT 10	800	160	5.0	750	150	5.0
KT 11	1450	280	5.2	1500	250	6.0
KT 15	1450	290	5.0	1500	300	5.0
KT 16	1350	185	7.3	1075	215	5.0
KT 17	1400	255	5.5	1300	215	6.0

(1) Whole serum IgG ÷ IgG in the DEAE supernatant fluid

Table 19 Serological Diagnosis of Influenza Infections by Various Titration Procedures

Patient	Complement Fixation	Hemagglutination-Inhibition			
		Untreated	Periodate Treated	RDE Treated	Neuraminidase
67 2	10/1[1]	ab/ab	20/20	<1/<1	1/<1
67 6	1/20	ab/80	80/80	<1/10	<1/20
67 7	20/ab	ab/80	ab/ab	<1/10	<1/20
67 10	1/<1	ab/ab	20/20	<1/<1	<1/<1
67 11	10/1	ab/80	20/80	1/ab	1/160
67 13	10/80	ab/320	ab/160	1/80	<1/160
67 16	1/ab	ab/ab	ab/80	<1/20	<1/ab
67 17	10/20	80/80	80/ab	<1/20	<1/20
No. sera showing presumptive evidence	3	1	3	0	0

[1] Non-specific results due to anti-complementary serum

serum with acetone. But since acetone extraction is time consuming, the following experiments were made to see if the inhibitors could also be removed by DEAE beads.

Table IV compares the abilities of DEAE and acetone to remove non-specific inhibitors of CEF and Sendai 2 hemagglutination reactions. All 10 untreated sera possessed high levels of inhibitors for the group A reovirus versus encephalitis virus. This activity was apparently due to non-specific factors since acetone treatment reduced the group A HI titers appreciably. DEAE extraction was at least equally effective to this capacity since titers of sera treated with it were as low or lower than those that followed acetone treatment. Both extractions also removed much activity to the group B Georgia 2 virus so that it was previously demonstrated to have low HI titers to this agent. Titers were clearly negative at a 1:10 dilution of the purified IgG preparations while traces of activity usually persisted in the acetone extracted sera.

The group C titers of the eight sera were previously found to have specific Georgia 2 HI antibody when accurately equal with one exception after both types of extraction. In this instance there was only a 4 fold difference existing in antibody when DEAE removed significant specific antibody. These preliminary results suggest that DEAE removes non-specific activities of inhibitors at least as well as acetone.

c. Removal of Non-Specific Inhibitors of Rubella Hemagglutination

Table V compares titers of rubella antibody measured by HI with those measured by plaque reduction in sera from 11 pregnant women.

Ten of the 11 sera possessed significant HI activity before extraction of non-specific inhibitors. Both acetone extraction with ether removed this activity from nine sera. DEAE removed it from six sera, while no specific antibody was demonstrated in five sera by plaque reduction. Among the plaque reduction titers on the standard for comparison, the final twelve untreated sera/six agreed with plaque data upon whether antibody was present or not in seven of 11 cases, and DEAE extracted HI titers agreed in eight of 11 cases. In all instances where the methods failed to agree titers were on the basis of detection in the test indicating antibody was present.

d. Discussion

At this preliminary stage in its development, a technique for removing non-specific inhibitors in fetal hemagglutination with DEAE-Sephadex appears promising. The extraction procedure is relatively simple to perform, consistently removes all serum components except IgG and yields a concentrated 1:2 IgG concentration for use in HI tests. Preliminary results suggest that it removes non-specific inhibitors in serum to influenza A rubella and reference hemagglutinations with a similar or greater efficiency than standard procedures. More extensive comparisons

Table 20 Removal of Serum Non-Specific Inhibitors of Arbovirus Hemagglutination by Acetone and DEAE Treatment

Patient	Serum No.	Reciprocal HI titer to group A (WEE) Untreated[1]	Acetone[2]	DEAE[2]	Reciprocal HI titer to group B (Dengue 2) Acetone[2]	DEAE[2]
Sera with low or absent group B titers						
1	DF 73	1024	<10	<10	<10 (±10)	<10
	DF 74	1024	<10	<10	<10	<10
	DF 75	256	<10 (±20)	<10	10 (±20)	<10
2	DF 43	256	<10	<10	<10	<10
3	DF 203	256	<10	<10	<10	<10
4	DE 333	1024	<10 (±20)	<10	10 (±20)	<10
5	DE 294	2048	<10 (±10)	<10 (±10)	<10 (±10)	<10
	DE 295	1024	<10 (±10)	<10	<10 (±10)	<10
6	DE 288	2048	<10 (±10)	<10	10 (±20)	<10
7	DF 314	256	<10 (±20)	<10 (±20)	<10	<10
	DF 315	1024	10 (±20)	<10	<10 (±10)	<10
Sera possessing group B antibody						
1	DF 51	256	<10	<10	320	320
	DF 52	256	<10	<10	640	640
	DF 53	256	<10	<10	640	640

2	DE	94	256	<10	(±10)	10	(±20)	80	40
	DF	48	256	10	(±20)	10	(±20)	80	20
3	DF	27	1024	<10		<10		320	320
	DF	28	2048	<10	(±10)	<10		320	320
4	DF	42	512	<10		<10		320	640

(1) Highest dilution of untreated serum that prevented complete (+ or ++) agglutination by WEE virus.

(2) Highest dilution of serum that totally prevented virus hemagglutination (O reaction). ± titers, when present, are shown in parenthesis.

Table 21 Reciprocal Titers to Rubella Virus Measured by Various Serologic Procedures

Patient	HI Unabsorbed	Simultaneous HI Kaolin treated	HI 2ME treated	Plaque reduction titer
B 204	80	10	40	9.2
D 36	320	40	160	36
D 182	80	<1	<1	1.0
E 129	<1	<1	10	1.0
E 285	40	<1	<1	1.0
F 199	[illegible]	<1	<1	0
F 226	40	<1	10	1.1
F 228	80	<1	<1	0
F 239	80	<1	<1	0
F 400	20	<1	<1	0
H 98	160	<1	1	0
Negative Control	40	<1	<1	
Positive Control	320	40	80	

of this extraction procedure with standard procedures on 88 sera with these antigens and other hemagglutinins is needed to establish the value of this theoretically-promising technique.

V. Rubella Virus.

A. Rubella Immunity Study

During the 11-month period from September 1967 to December 1968 approximately 6,000 sera were examined for rubella HAI antibody. These sera were obtained from all women entering the OB/GYN and Newborn Hospital prenatal obstetrical clinics during this period. A simple questionnaire was used to query every patient for previous history of German measles. Standard methods for assay of HAI antibody are described in the 1968 Annual Report.

Table 22 illustrates that bacterial sensitivity to an antibody does not predict whether the cell line detectable antibody. Both populations showed similar results. Approximately 10% in each bacterial sensitivity category are seronegative.

Table 23 gives the distribution of HAI titers for the two groups. The range of titers and the geometric mean titers are essentially the same for both groups. 222 of 1 PH of sera screened in the 1:8 - 1:8 range

Table 2: Correlation of History of Rubella with Rubella HAI Antibody Titer

WRCH

History	Total	# HI Positive[1]	# HI Negative[2]	% Seronegative
Yes	[illegible]	[illegible]	50	8.4
No	[illegible]	[illegible]	98	9.5
[illegible]	[illegible]	[illegible]	17	11.8
No response	[illegible]	[illegible]	13	10.8
Total	[illegible]	[illegible]	178	9.45

DEWITT

History	Total	# HI Positive	# HI Negative	% Seronegative
Yes	1034	951	73	7.1
No	764	676	88	11.5
[illegible]	333	291	34	10.4
No response	190	181	19	10.5
Total	2393	2079	214	9.4

[1] HI titer ≥1:8

[2] HI titer <1:8

Table 23 Distribution of Rubella HAI Antibody Titers

Titer[1]	Dewitt	WRGH	Both
<8	213	178	391
8	9	3	12
16	116	94	210
32	359	264	623
64	557	464	1021
128	626	496	1122
256	285	252	537
512	113	122	235
1024	15	13	28
2048	0	0	0
Totals	2293	1886	4179
GMT[2]	1:94	1:100	1:96

[1] Reciprocal of antibody titer

[2] Geometric mean titer for sera with ≥1:8 titer

B. Rubella Plaque Assay:

The development of a rubella plaque system was attempted. The hemadsorption-negative plaque assay adapted by Rawls was studied in both BHK-21 and LLC-MK_2 cell lines. Countable plaques could be obtained but results were inconsistent and not reproducible. Direct attempts under agar in BHK-21 cells were never successful. Cell sheet survival appeared to be the limiting factor; controls degenerated in 3-4 days under concentrations of agar from 0.7-1.0% and under previously described starch gel or carboxymethyl cellulose techniques. Direct attempts under similar conditions in LLC-MK_2 cells initially gave irregular results.

Eventually a satisfactory system was developed with the SEATO line of LLC-MK_2 cells, using 1% Difro purified agar, 10% heat inactivated fetal calf serum, 0.3% $NaHCO_3$ (4 ml of 7.5% $NaHCO_3$ per 100 ml) in M199. After incubation at 37°C for 7 days a second overlay was added containing 5 ml of Gibco 1:300 Neutral Red per 100 ml, 0.15% $NaHCO_3$ and 1% agar. After overnight incubation at 37°C flasks were inverted and left at room temperature. Two-three millimeter diameter discrete plaques were readily visualized and consisted of intact, unstained cells. Fluid staining tended to give a more uneven stain and less consistent plaque definition.

For the neutralization test, sera were heat inactivated at 56°C for 30 minutes and diluted in PBS containing 0.4% BPA. The antigen was strain M33, GMK22 BHK8 with a titer of c. 1.0×10^7 pfu/0.2 ml. Equal volumes of serum and virus diluted to contain 60-80 pfu/flask were incubated for one hour at 37°C. 0.2 ml of serum-virus mixture per flask (Falcon 75 mm^2) were adsorbed for one hour at 37° and overlaid with agar. Each test included a virus titration and positive and negative serum controls. Percentage virus survival at various dilutions of serum was plotted on log probit paper, and the antibody titer was taken as the dilution corresponding to 50% survival.

Serum plaque reduction (PR) titers are compared with the standard Echo-11 challenge indirect neutralization test and HAI titers in 31 sera in Table 24. Thirteen sera showed agreement with positive titers for both types of neutralization tests. Of these 13 sera six had low, but detectable, HAI titers; four were HAI negative while three were equivocal with both negative and positive titers recorded. Eight sera lacked antibody by both neutralization assay, but clearly had detectable HAI antibody. Ten sera failed to show agreement between the two neutralization assays. Eight sera lacked E-11 Neut antibody, but had demonstrable PR and HAI antibody. Two sera with minimal E-11 neut antibody, lacked both PR and HAI antibody.

The measurement of neutralizing antibody by a plaque reduction system is generally accepted as a sensitive quantitative assay since it provides for immobilization of residual uncombined virus. This latter phenomenon is an important factor in the lack of sensitivity with low titered rubella sera in the indirect neutralization test using E-11 challenge. Thus if

[illegible]

the 10 sera lacking neutralizing antibody by the E-11 challenge assay, one-half had low titer but demonstrable PR neutralizing antibody. These same 10 sera all contained low titer HAI antibody. One-half lacked PR neutralizing antibody and should be considered false HAI positives.

C. Neutralizing Antibody Assays

A total of 337 sera from the rubella immunity sera, selected for the most part because of low HAI antibody titers, were assayed by the standard E-11 challenge neutralizing antibody assay. 239 sera lacked detectable antibody by both tests (HAI <8, HAI >8). Thus 89.5% of the sera demonstrated close and agreement between both tests. The remaining 99 sera failed to agree in terms of detectable antibody in various combinations. Twenty five of these sera were also assayed for neutralizing antibody by the plaque reduction test (Table 3) lists the results of these 99 sera. Eight of these sera had detectable HAI and PR neutralizing antibody but lacked E-11 challenge neutralizing antibody. Eight sera with HAI titers >10 had PR neutralizing antibody titers [illegible]. Thus, 8/99 or 8.1% of these sera with HAI titers >10 were false positive with respect to the HAI test. The most likely explanation is incomplete removal of non-specific inhibitors of rubella hemagglutination by kaolin treatment despite careful attention to this step. Thirteen additional sera in this category were PR negative [illegible] would have fail to have PR antibody the false positive percentage may well be higher. Six sera with HAI antibody titers 8 had PR neutralizing titers >7, one additional sera equals confirmation [illegible] 2.6%) were HAI false negatives. These findings [illegible] PR neutralizing data suggest that between [illegible] seronegative sera will be demonstrable by the challenge test.

D. Background and Purpose [illegible] the Antigens.

[illegible] antigen [illegible] HAI [illegible] density gradient [illegible] of [illegible] [illegible] [illegible]

Table 25 Comparison of Rubella PR, E-11 Neut and HAI Titers in Individual Serum

Serum #	Reciprocal of Antibody Titers PR	E-11 Neut	HAI
D182	3.8	32	8
E226	6	<2, 4, >4	16
E285	3.8	2	16
E410	4.5	8	8, 16
F199	82	>16	16, 32
F226	5	>16	16, 32
B 60	7	2	<8x2, 8
D 30	19	>8	<8
D 34	43	>8	<8
F208	30	>16	<8
G 15	38	4, 8	<8, 16
G 24	14	4	<8, 32
G365	56	>16	<8, 16
334	<4	<2x3	32
F193	<4	<2	32
F228	<4	<2x2	8, 32
F239	<4	<2	16, 32x2
F400	<4	<2	16, 32
G218	<4	<2	16, 2
G238	<4	<2	16
G382	<4	<2	8, 32
138	20	<2x3	32
A 67	11	<2	16x2
A 90	8.5	<2	16x2
B173	9	<2	16
B204	10	<2	16
G294	6	<2	8, 32
G314	3	<2	8, 128
G344	2.7	<2	8, 16
A421	<4	2	<8
A444	<4	2	<8

antigen could not be sedimented under similar conditions as RNA and SVA it was designated a soluble complement-fixing (SCF) antigen. Electron micrographs of this material revealed spheres measuring 7 nm in diameter they were similar to repeating structural units seen on the surface of intact virus particles.

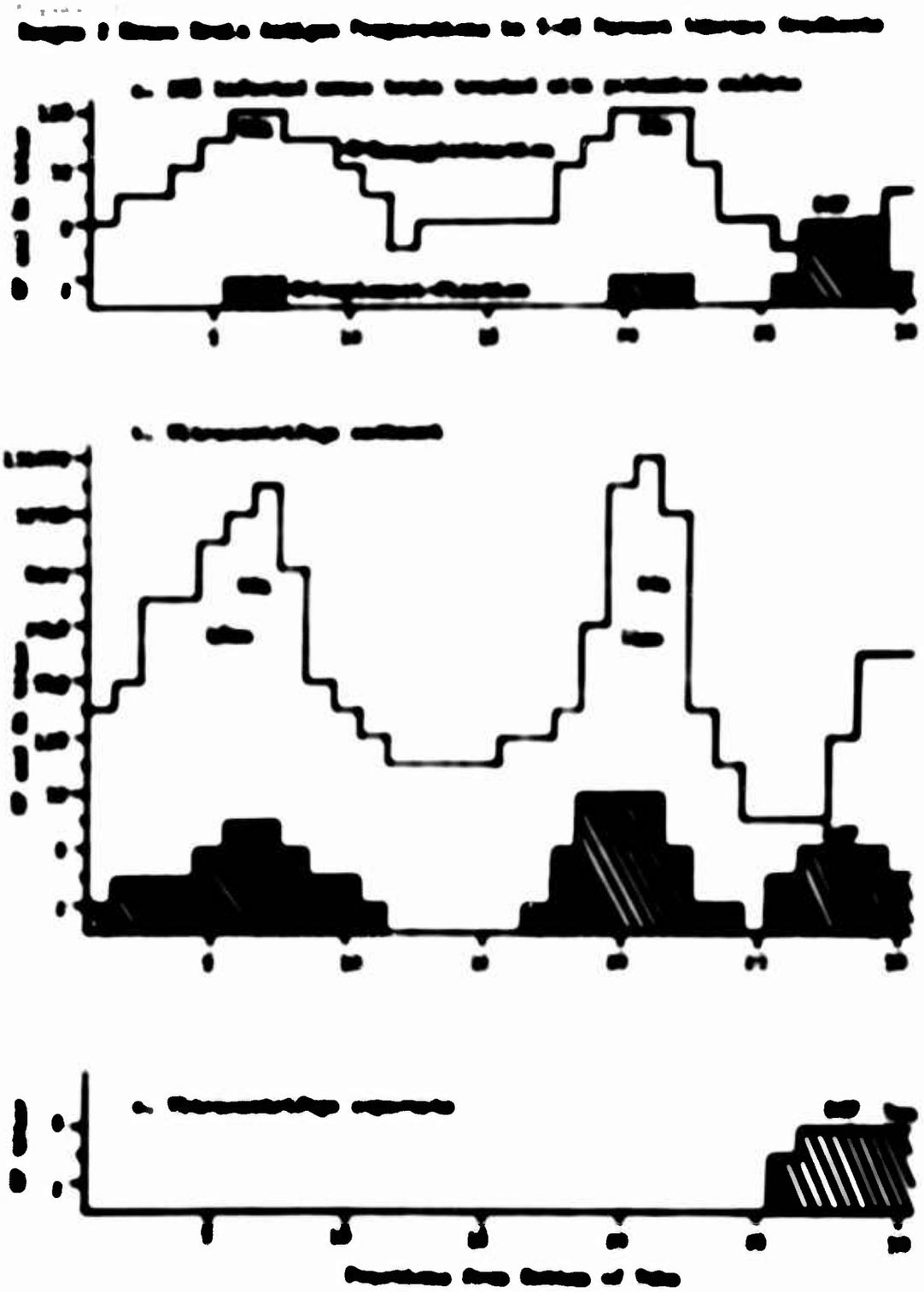
Laboratory studies during the past year have been devoted to biological and physical studies of these antigens in order to define their roles in dengue antigen populations as well as structural relationships with the intact virus.

A. Preparation and Definition of Dengue Antigens

Dengue-2 infected mouse brain was extracted from either freshly or moribund suckling mice that had been killed with chloroform and washed in 0.5 per cent benzalkonium chloride. Brain material was homogenized to a 20 per cent suspension in 0.02M Tris buffer, pH 8.2 i.e. 0.5 with 0.001M EDTA by means of an enclosed top-drive mixing blender (Waring Commander) at 0°C. Suspensions were partially clarified at 12,000 x g for 30 minutes at 4°C before addition of 2 mg of proteinase extract from a 50 mg/ml stock solution. Proteinase precipitation of pancreatic non-viral protein was allowed to continue for 60 minutes at 4°C with occasional shaking. protein precipitates were removed by centrifugation at 12,000 x g for 45 minutes at 4°C. The resultant brown opalescent clear supernatant fluid was designated proteinase purified mouse brain suspension

Physical definition of antigens contained in this and other preparations was accomplished by rate zonal sedimentation in sucrose gradients. Gradients of 5-25 per cent sucrose in 0.1 molar phosphate buffer pH 8.2 with 0.5 x 10⁻³ Britton and Roberts buffer were prepared using Beckman SW 50 L tubes and delivering a total gradient volume of 12 ml in a 1 x 3 inch cellulose nitrate tube. Samples (2-3 ml) were layered on top of the gradients and centrifuged for 3 hours at 50,000 x g at 4°C. Approximately 20 fractions were collected through a 2 inch 19 gauge stainless steel needle inserted in the bottom of the centrifuge tube. UV and CF determinations on all gradient fractions were carried out in the automated system as previously described.

Distribution of HA and CF antigens in 20 per cent mouse brain preparations analyzed in this manner can be visualized in figure one. Rapidly sedimenting hemagglutinating antigen (HAR as described above) was found about fraction 7 while the slowly sedimenting antigen (HAS) peaked about fraction 11. The majority of CF antigen in 20 per cent brain suspensions was found at the top of the gradient between fractions 25 and 30. It was designated soluble CF antigen (SCF). Little if any activity was seen associated with the intact virus particles (HAR) or the doughnut forms (HAD).



Concentration of dengue antigens from 20 per cent mouse brain preparations was accomplished by sedimentation in the ultracentrifuge at [illegible] x g for 2 hours. Sedimented material was resuspended in a volume effecting approximately a 50 to 60 fold concentration. Resuspension was facilitated by two minutes sonication at 10KC. Sucrose density gradient analysis of sedimented virus components is illustrated in Figure 10b. Concentration of BHA and SHA as determined by both HA and CF activity was achieved. SCF may or may not be found in sedimented material. Residual mouse brain protein may act as a carrier for SCF, but the amount of residual protein cannot be controlled with crude purification procedures employing protamine sulfate. In any case, SCF (soluble CF antigen) was not concentrated under these conditions. Sucrose gradient analysis of ultracentrifuge supernatant fluid (Figure 10c) shows that SCF remains primarily in suspension.

Dengue antigens utilized in studies to follow will be referred to in terms of their sedimentation characteristics in sucrose gradients as just described.

8. <u>Chronological Appearance of Dengue Antigens in Blood and Brain of Suckling Mice</u>

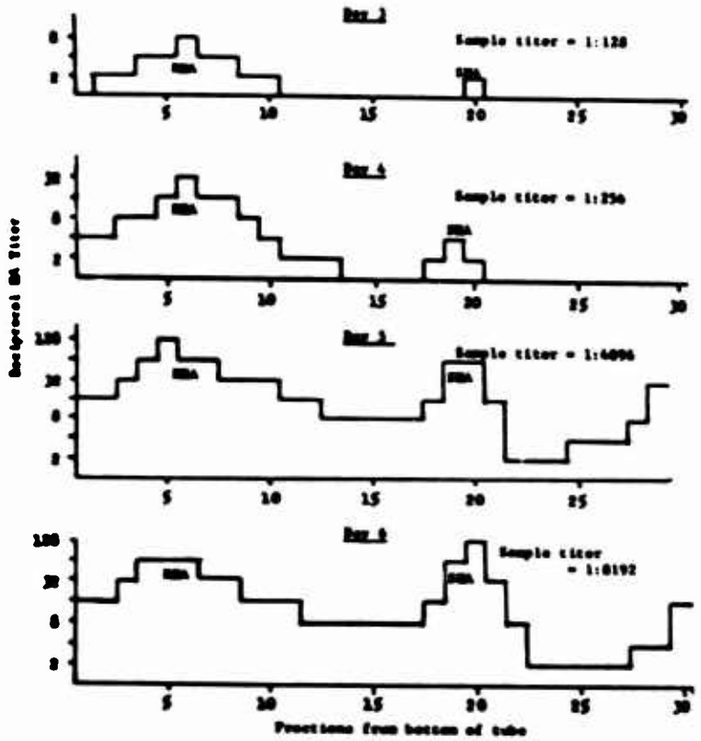
Initial studies on relationships of BHA, SHA, and SCF were carried out by determining time of appearance of each antigen in brains from dengue infected mice. Blood was also obtained in an attempt to find circulating antigens other than infectious virus. Suckling mice that were 1-3 days of age were inoculated intracerebrally (IC) with 30,000 MLDsg of dengue-2 virus in 0.01 ml. Blood was obtained by cutting jugular veins of 50 to 100 mice under anesthesia daily for 6 days at which time mortality was approximately 80 per cent.

Serum was separated from clotted blood by centrifugation at 800 x g for 15 minutes at 4°C and stored in several aliquots at -70°C.

Mice were stored frozen at -70°C after blood was obtained, and thawed at a later date when the brains were harvested by aspiration with a 18 gauge needle and syringe. Infected brain material was thoroughly ground in a mortar before gradual addition of 4 volumes of TRIS buffer. Following clarification as described above, small aliquots were stored for infectivity and SCF antigen determinations. HA antigens were obtained from the remaining infected mouse brain by treatment with protamine sulfate, sedimentation in the ultracentrifuge, and resuspension in TRIS buffer effecting a 17-fold rather than 100-fold concentration as described above.

1. <u>Appearance of Sedimentable HA Antigens in Mouse Brain</u>. HA antigen was first detected in brain material 3 days after infection, but activity (1:8) was too low for successful analysis of BHA and SHA content on sucrose gradients. Figure 11 represents sucrose gradient analysis of

Figure 11. Appearance of dengue HA antigens in suckling mouse brain harvested daily as analyzed by sucrose density gradient ultracentrifugation.

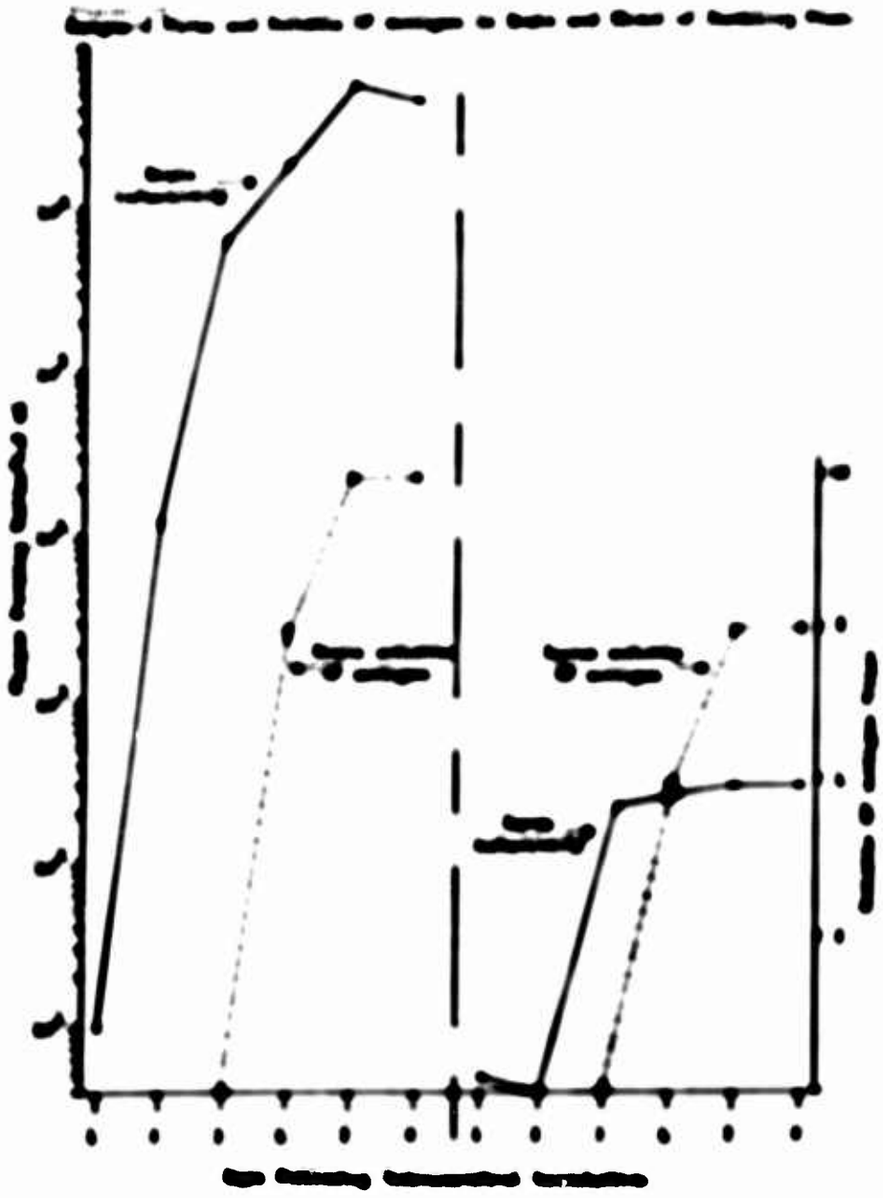


HA activity in brain material harvested on days 3 to 6, total HA titers ranging from 1:128 to 1:8192. RHA is clearly the major HA antigen component on days 3 and 4, SHA being barely detectable on day 3. (On this basis, day 2 HA antigen is probably RHA.) By day 5, SHA is present in major proportions and by day 6, total quantities of RHA and SHA appear similar. It cannot be determined whether large amounts of SHA first found on day 5 represent breakdown products during stationary phase replication, whether synthesis of SHA grossly exceeds that required for viral maturation by the fifth day after infection, or if SHA represents production of separate particles not associated with RHA. The last alternative is unlikely since experiments reported below indicate that SHA can be obtained from RHA by carefully controlled chemical extraction, as well as by induced physical stress by combined effects of ultracentrifugation and elution from inorganic precipitates. In addition, SHA is found not only in mouse brain derived virus but has been found in dengue-2 virus (SEA 30 #21868) propagated only in cell cultures.

It can also be seen in Figure 11 that some day 5 and day 6 HA antigen did not sediment (fraction 20 - top of gradient). Dialysis of this material and recentrifugation through another sucrose gradient showed that it was RHA. The initial inability of some day 5 and day 6 RHA to pass the sample-sucrose interface may be a reflection of concentration and/or an association with large quantities of soluble lipid resulting from advanced cellular injury; symptoms of CNS involvement were grossly apparent on day 5 when there was also 10 to 20 per cent mortality.

2. Appearance of HA Antigens in Mouse Serum Titers of HA total antigen in serum samples could not be determined directly due to non-specific inhibitors of hemagglutination. Standard acetone extraction procedures that remove non-specific inhibitors did not reveal any HA antigen in serum until day 5. The low titer (1:10) made further analysis of acetone extracted material impractical. Since non-specific inhibitors in serum remain near the top of sucrose gradients (previous Annual Report) and the efficiency of antigen recovery following acetone extraction is in question, untreated serum samples were applied directly to sucrose gradients. Again, no HA activity was found until day 5 at which time low level HA (1:2) was found only in the RHA region of the gradient.

3. Comparative Chronological Assay of Infectivity and Soluble CF Antigen in Serum and Brain of Suckling Mice Isolation of RHA as well as infectious virus from blood made it possible to compare time of appearance of these antigens with those in brain. Figure 12 represents daily infectivity titers measured by plaque assay in continuous monkey kidney cells (LLC-MK$_2$) and peak titers of soluble CF antigen as analyzed on sucrose gradients. Titers of infectious virus in the brain rose to 10^8 plaque forming units (pfu) on the second day after infection and reached a peak titer of almost 10^9 pfu on the fifth day, at which time no further increase was found. Soluble CF antigen in brain material could not be demonstrated until day 4, and attained a titer of 1:16 on days 5



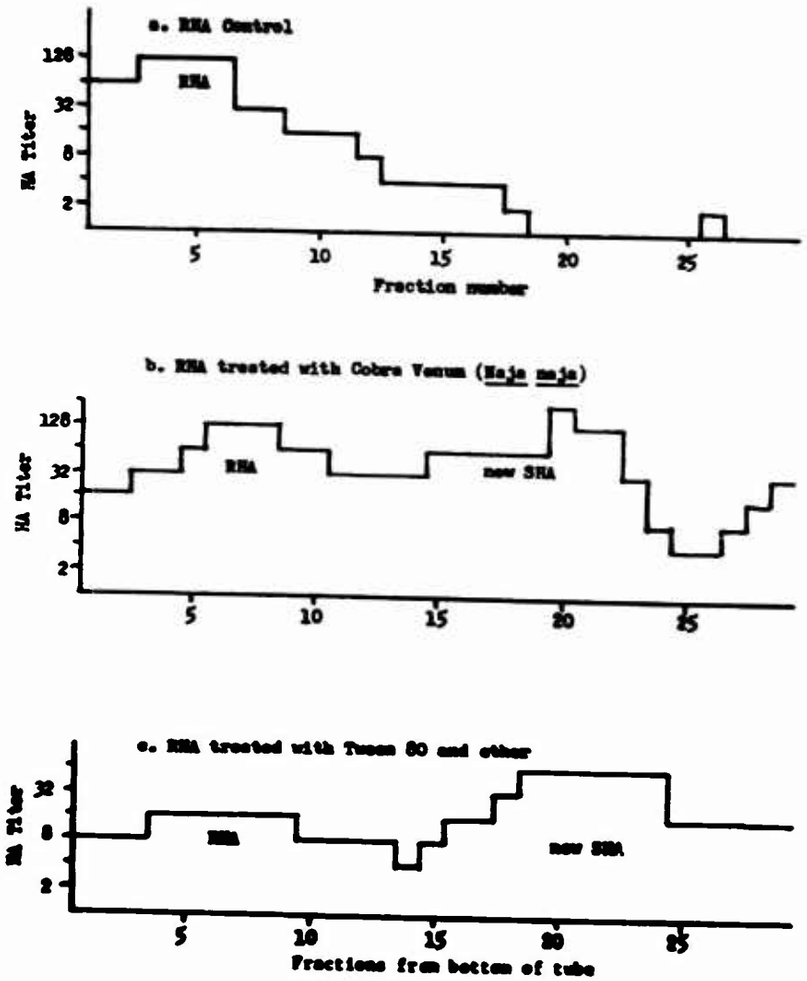
and [illegible] different proportions of infectivity and soluble CF antigen were seen in serum (right side of figure). $10^{4.5}$ pfu were present in serum in the [illegible] difference from brain infectivity titers, whereas the amount of soluble CF antigen in serum was similar to that in brain. The explanation for these observations is not readily apparent; synthesis of infectious virus under these conditions occurs predominantly in neural tissues, thus, virus and antigen found in serum probably originated in the brain. SCF, because of its small size (comparable to egg albumin as investigations as shown later), may be more readily released from infected cells than whole virus. Clearance of virus and soluble antigen from the circulation may proceed at different rates, the larger virus particle being more rapidly cleared. The lack of significant HA antigen in the serum, as shown above, indicates there is little or no accumulation of virus in blood, thus, a clearing mechanism may be active. Whatever the true explanation, the role of soluble CF antigen in infectious processes and its possible relationship to disease due to dengue virus appears worthy of further investigation.

c. Enzymatic and Chemical Extraction of SHA from RHA:

1. Analysis of the Effects of Cobra Venom and Tween-80-ether on Isolated Dengue Virus HA (RHA). RHA was obtained from a sucrose gradient of concentrated virus similar to figure 10b by pooling peak fractions and dialyzing the pool in PBS for 2 hours. The sucrose concentration was sufficiently reduced in this time interval to enable reapplication of a portion (1.3 mls) to a second sucrose gradient. Another portion of 2 mls was mixed with 8 mg of lyophilized cobra venom (Naja naja) obtained from Ross Allen Reptile Institute, Silver Springs, Florida and incubated for 30 minutes at 37°C prior to application of 1.5 mls on a second sucrose gradient. A third portion of 2.0 mls was shaken for 3 minutes with 0.2 ml of a 30 mg/ml stock solution of Tween-80, followed by another 3 minute shaking with 0.2 ml anesthesia grade ether. 1.5 ml was applied to a third sucrose gradient. Sedimentation characteristics of these three samples using hemagglutination as a marker are shown in Figure 13. It can be seen that both cobra venom and Tween-80-ether treatment, while not appreciably reducing RHA titers, produced a second distinct peak designated new SHA, with sedimentation characteristics similar to SHA previously described. Cobra venom did not appear to grossly affect overall size of the RHA peak when compared to control RHA, yet the treatment released enough HA antigen to form a new SHA peak of similar HA potency. Concomitantly, Tween-80-ether treatment caused an overall 4-fold reduction of RHA resulting in the appearance of a larger, new SHA peak. Tween-80-ether treatment constitutes a detergent-delipidating effect which has been shown to break up other RNA viruses as influenza. Cobra venom contains a variety of enzymes not completely characterized but some of which are known to be lecithinases. Isolated Phospholipase A has been shown to inactivate other lipid containing viruses.

Attempts to completely destroy RHA while maintaining SHA activity were unsuccessful. Treatment with Tween-80 for 5 minutes followed by

Figure 13. Sucrose Gradient Analysis of RNA (Intact Virus) treated with Snake Venom and Tween 80-ether.



5-minute shaking with an equal volume of ether (rather than 3 minutes) reduced activity of both RHA and SHA; 15 minute ether treatment destroyed all HA activity. Treatment of RHA with concentrations of Tween-80 and ether other than tenth volumes of sample did not result in appearance of SHA.

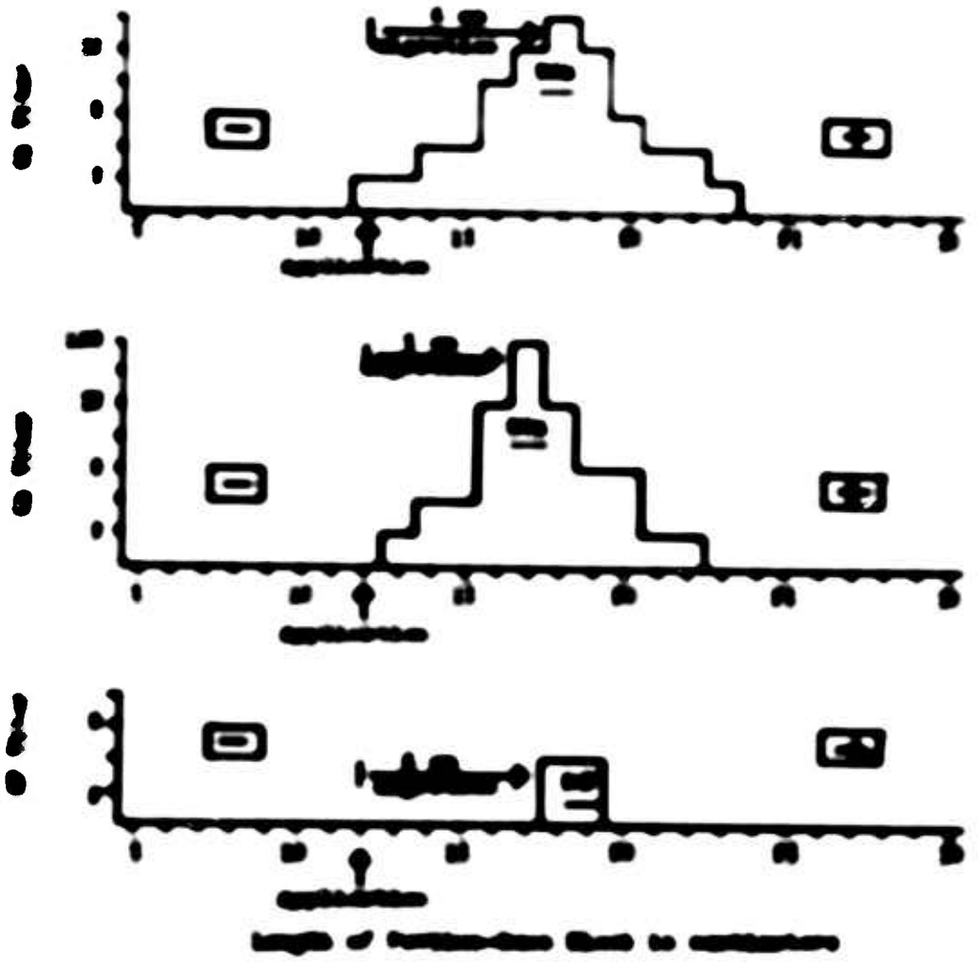
The new SHA peaks were pooled, dialyzed and recycled through succeeding sucrose gradients to insure homogeniety and sedimentation characteristics similar to previously described SHA. Electron micrographs of SHA obtained by chemical treatment of RHA are planned to ensure that SHA is indeed a structural component of RHA, and not a separate antigen particle synthesized by infected cells.

D. Electrophoretic Analysis, Adsorption and Elution Characteristics of Dengue Antigens:

The following experiments suggest that dengue antigens, RHA, SHA, and SCF, all have a similar net negative charge. Elution from inorganic precipitates facilitated by stepwise increases in ionic strength separated SCF antigen (early elution) from HA antigens and mouse hemoglobin (late elution), indicating that SCF has a lower total negative charge.

1. Electrophoresis: Preparative zone electrophoresis was carried out in an inert supporting medium of equal mixtures by weight of polyvinyl chloride (Geon) and polyvinyl chloride, polyvinyl acetate copolymer (Pevikon), in 0.06M barbital buffer x pH 8.6. Samples of 2 ml were applied 12 cm from the cathode in Pevikon-Geon blocks measuring 1 x 6 x 36 cm. Constant current of 45 milliamps and 300 volts was applied for 18 hours at 4°C. Following electrophoresis, 36 1 cm sections of each block were suspended in 2 ml of 0.02M TRIS buffer pH 8.2 by agitation on a mixer (Vortex, Jr.). Antigens eluted in this manner were separated from the Pevikon-Geon by centrifugation at 800 x g for 10 minutes. Under these conditions the electrophoretic mobility of HA and CF antigens may be visualized in Figure 14. RHA and SHA obtained from sucrose gradients, and SCF as found in ultracentrifuge supernates of infected mouse brain, all migrated 5 to 6 cm toward the anode, their mobility coinciding with the mobility of a hemoglobin marker. Thus, all are negatively charged and have similar electrophoretic mobilities. These results suggest that the 3 physically distinct dengue antigens described above possess similar net charge, i.e. same charge per unit mass.

2. Adsorption and Elution of Dengue Antigens from Inorganic Precipitates: Total charge differences among dengue antigens were analyzed by ionic strength requirements for elution from inorganic precipitates. Basically, dengue antigens were first adsorbed to inorganic precipitates in low molarity (0.005M) phosphate buffer (PB). As PB molarities were increased, particles with a lower total negative charge eluted first. Under these conditions, SCF (7 mu in diameter) was shown to have a lower total negative charge than HA antigens. RHA and SHA could not be clearly separated, but SHA (14 mu in diameter) appeared to elute before RHA (45 mu).



[illegible] and elution were carried out by both techniques utilizing [illegible] magnesium trisilicate and supernatant fluids. Precipitates were [illegible] of preimmune treated dengue-2 [illegible] and dengue types were dissolved against 0.005M PB, pH 7.0. Mixtures were centrifuged at 8000 x g for 5 minutes and precipitates washed with 1.5 volumes of 0.005M PB. Duplicate successive washings of precipitates were carried out with PB of increasing molarity from 0.01 to as high as 1.5M.

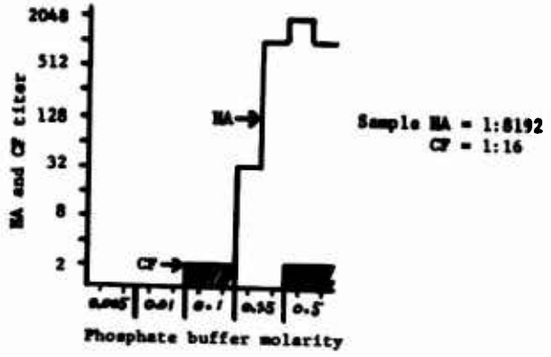
- Elution of dengue antigens from hexahitz. Hexahitz [illegible] was prepared according to Tiselius (1956) from 0.5M calcium chloride and 0.5M disodium phosphate. Precipitates were washed several times with water and stored as 0.005M PB. Figure 15a represents elution of dengue antigens from hexahitz effected with stepwise increases in molarity of PB. CF antigen that did not fix elutes first in 0.1M PB, HA antigen begins to elute at 1:320 but comes off the precipitate in high titer at 1:80 (1:640 [illegible]) enabling it to fix complement as well. The experiment was repeated with buffer molarities higher than 0.5 in an attempt to remove all CF antigen from hexahitz. However, HA antigen continued to elute at 1:80 (1:512) and 1:20 (1:128) suggesting that HA antigen is strongly bound to hexahitz in addition to its heterogeneity. Thus, total CF activity absorbed completely to hexahitz (no activity in 0.005M eluate) has also never completely eluted. Buffer molarities of 1.5 or higher precipitated hexahitz which impeded attempts to recover all of the bound CF activity.

CF antigen did not elute in buffer molarities higher than 0.5. However gradient analysis of 1:10 and 1:20 eluates indicated that CF activity of the 1:10 eluate was SCF. It was found near the gradient surface in the same position described above for SCF. In addition, soluble CF antigen extracted from ultracentrifuge supernatant fluid was absorbed to hexahitz and eluted as 1:10 PB as shown in Figure 15b. These results suggest that co-ly eluting CF antigen in Figure 15a is SCF. Thus, SCF was separated from CF antigens by elution from hexahitz with properly chosen increments of PB molarity, but the yield was poor. SCF comprises most of the CF antigen in 2% yeast extract mouse brain suspensions, yet, 0.1M (SCF) and 1:10 (whose titers in controls CHA & CHA) had similar titers. These results suggest incomplete elution of SCF and warranted investigation of other preparations.

CF activity in 1:100 and 0.5M eluates seen in Figure 15a was analyzed on sucrose gradients as illustrated in Figure 16. Only SHA was found in the 1:100 eluate while both CHA and SHA were found in the 0.5 eluate. In most experiments CHA could not be identified at all in any CF antigen eluted from hexahitz, only SHA was found. SHA either remained bound to [illegible] exchanged on precipitates with high buffer molarities dissociated. To test for recovery of CHA, it was first obtained from a sucrose gradient and then adsorbed alone onto hexahitz. Eluted antigen was analyzed again on sucrose gradients. Only CHA was recovered, providing another method of detecting CHA from SHA in addition to chemical means described above.

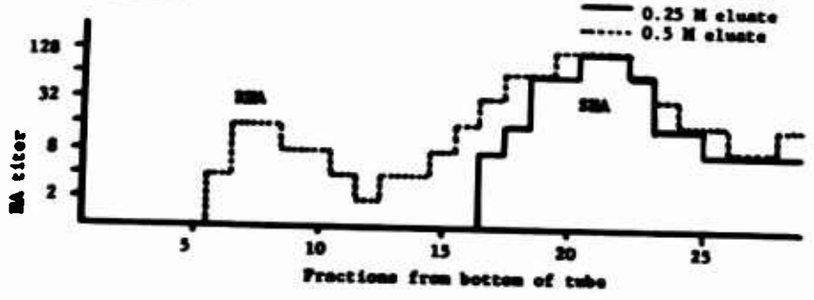
Figure 15. Elution of dengue antigens from brushite.

a. Elution of antigens in protamine purified mouse brain



b. Elution of soluble CF antigen in ultracentrifuge supernate

Figure 16. Sucrose density gradient analysis of brushite HA eluates.



[illegible] negative charge, while decreasing [illegible] the greater the total charge the stronger it binds [illegible] particles [illegible] eluted first at [illegible] followed [illegible] particles eluted next at [illegible] and SBA eluted last at [illegible] this system, indicating that [illegible] SBA.

[illegible] $Ca_3(PO_4)_2$ was prepared according to [illegible] of $CaCl_2$ and Na_2HPO_4. Precipitates were washed [illegible] 0.001N PB.

Adsorption and elution of antigens from [illegible] identical with the soluble experiments. [illegible] antigen beginning at [illegible] was found [illegible] was poor. Apparently, either hydroxyapatite [illegible] to purify SBA as described above [illegible] elution).

[illegible] $Al(OH)_3$ was prepared according to [illegible] 25 per cent aluminum sulfate with a pH [illegible] washed repeatedly with water then a pH [illegible] mutant fluids became gelatinous. They were [illegible] in water. Elution [illegible] Figure 17. [illegible] 0.01 rather than 0.1N PB. It also [illegible] starting sample, suggesting that [illegible] eluted from $Al(OH)_3$ by purification procedures. [illegible] covered from aluminum hydroxide or [illegible] apparently most BA antigen remained [illegible] linked adjuvant properties [illegible] binding properties [illegible] of laboratory animals. [illegible] animals where antigen must [illegible]

[illegible] Cells - D was washed with water and [illegible] described in previous reports. Figure 8 shows that highest [illegible] (1:64) were found in sera of [illegible] did not have the binding capacities [illegible] cipitates described above. BA titers [illegible] Significant amounts [illegible]

CF antigen was recovered and the starting sample HA titer was 1:2048. CF antigen was not recovered in an earlier experiment from DEAE cellulose column chromatography utilizing up to 0.5M NaCl as limiting eluting buffer. Experiments will be carried out to determine requirements for SCF elution.

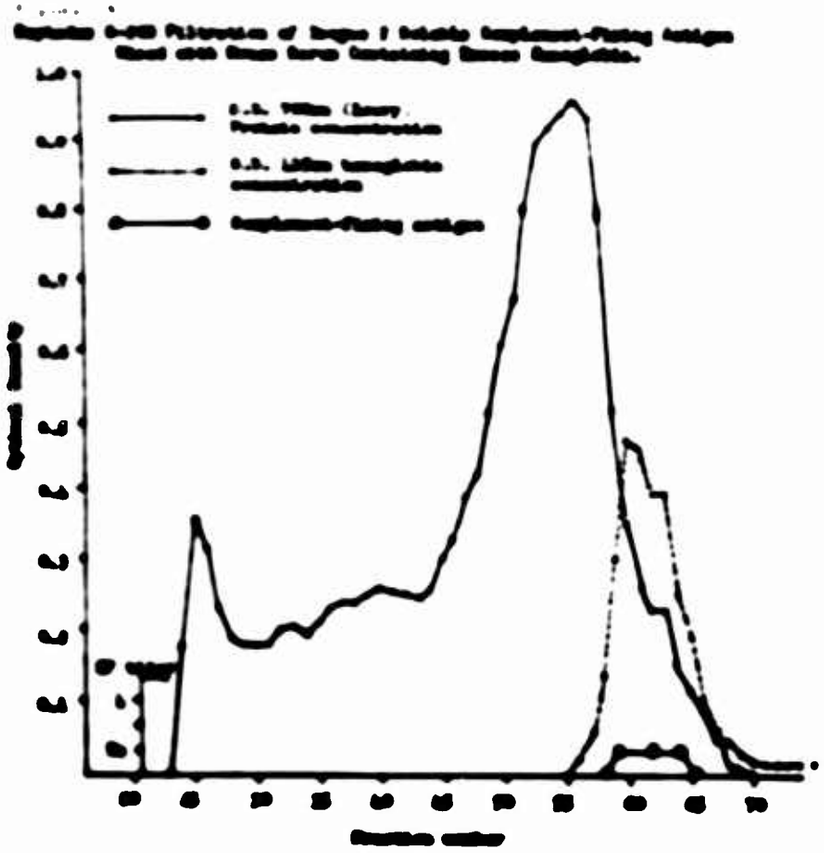
B. Purification and Characteristics of Dengue Virus Soluble Complement-fixing Antigens

SCF antigen as previously defined and characterized by ultracentrifugation was low titered (1:16), stably contaminated with hemoglobin, and apparently contaminated with residual mouse brain substances not removed by povidone treatment.

In these experiments, precipitation and concentration of antigens with ammonium sulfate left contaminating hemoglobin in solution. Sephadex gel filtration of antigen concentrates separated HA antigens as well as protein measured by absorption at 280 mu. from SCF which could only be located by serological methods. Elution of SCF from sephadex was compared to protein markers of known molecular size. Molecular diameters of 5 to 8 mu suggested measurements of 7 mu obtained in electron micrographs of negatively stained preparations previously reported and indirectly supported the concept that SCF consists of surface subunits of complete virions. Concentration of sephadex peak material of SCF by pressure dialysis resulted in an antigen product titering 100 to 150 times greater than starting material. SCF purified and concentrated in this manner retained non-sedimenting characteristics by ultracentrifugation. It was shown to be denser in CsCl (1.31 g/cm^3) than BHA or SHA (1.22 and 1.23). It reacted to a lesser degree with heterologous antibody than BHA and SHA in CF tests. Finally, it produced a single, discrete precipitin line with antibody prepared against crude virus suspensions by double diffusion in agar. Preliminary double diffusion results with SCF derived from dengue types 1 and 2 suggest that common and type-specific dengue antigens are physically linked on the same molecule (multi-determinant antigen).

1. Sephadex Gel Filtration of Dengue 2 Soluble CF Antigen

a. Recovery and characterization of SCF antigen with filtration techniques were first tested on sephadex G 200, a cross-linked dextran macromolecule matrix commonly used to fractionate globular serum proteins with molecular weights up to 1,000,000 dalton. Characteristics of serum proteins thus sorted on familiar matrices are properly functioning antigens. Accordingly, SCF antigens in povidone treated mouse brain supernatants from which CF antigens had been removed by ultracentrifugation (described above) were mixed with an equal volume of mouse serum and subjected to gel filtration on a 2.5 x 100 centimeter column. All eluates were applied to a 2 x 90 cm column and washed through with 0.025 phosphate buffered saline pH 7.2 (PBS) and 2 ml fractions collected. Figure 19 depicts a study sephadex peak (fraction 23) attributed to large molecular size mouse brain



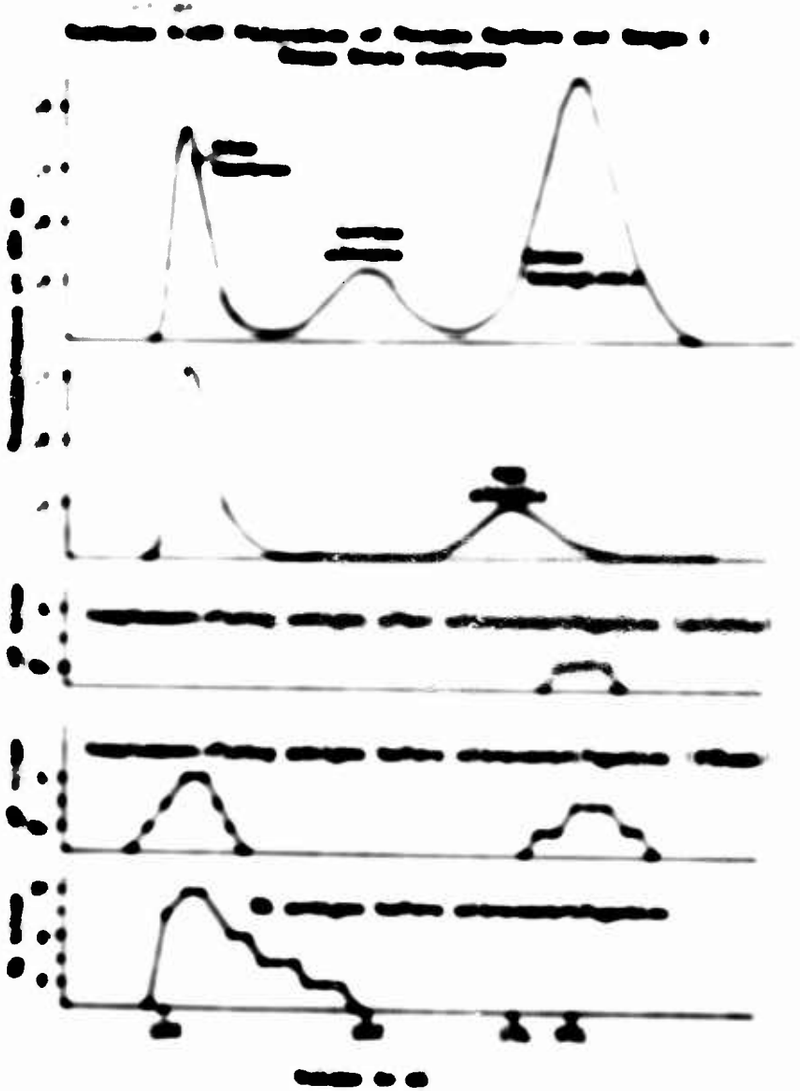
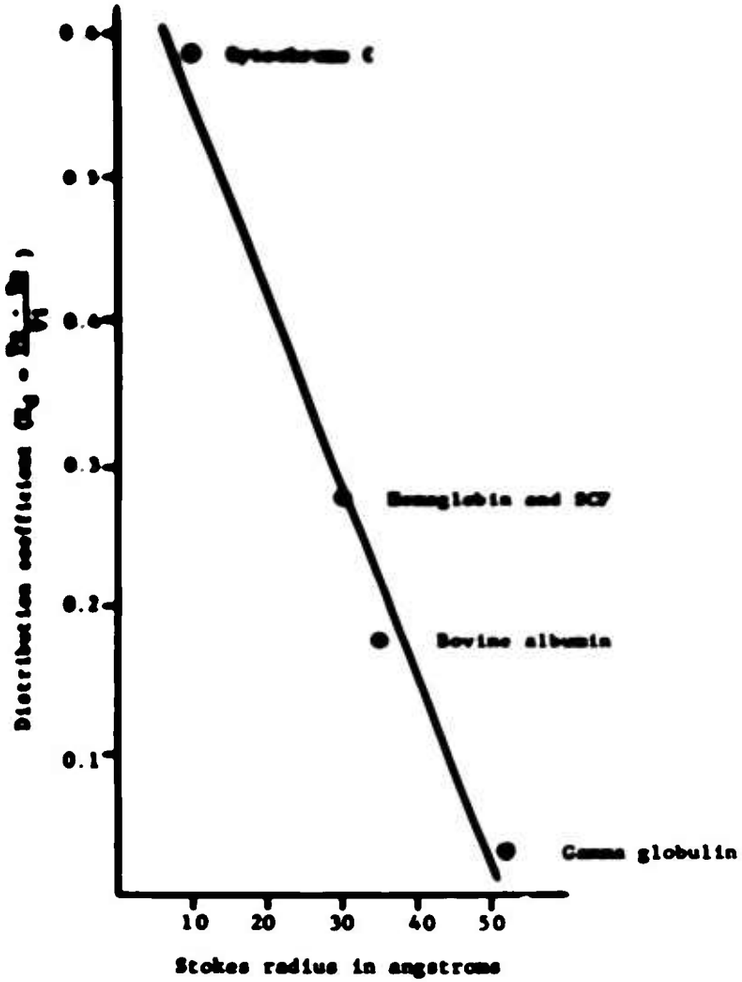


Figure 24. Elution of standard proteins and fraction I soluble GP antigen from Sephadex G-200 according to molecular size



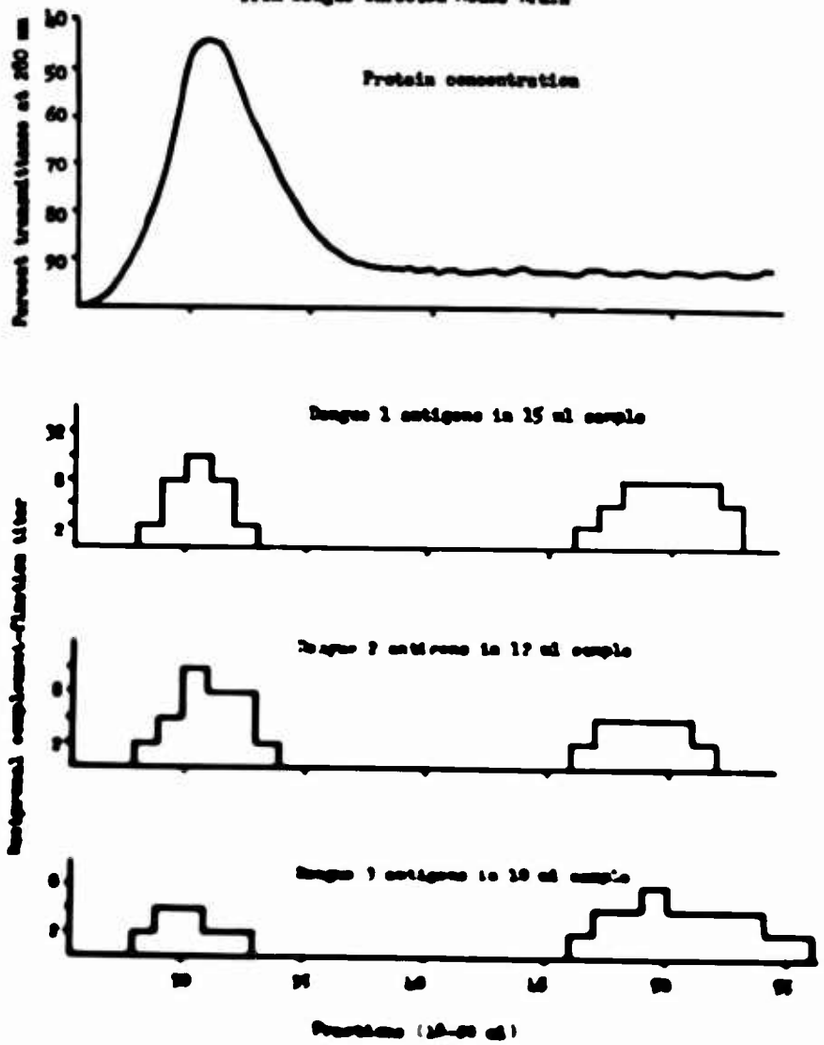
is consistent with measurements of 7 mu obtained in electron micrographs of SCF, the larger measurement possibly attributed to accumulation of negative stain around the particles.

2. Purification and Concentration of Dengue Soluble CF Antigens: Protamine purified infected mouse brain suspensions described above were optically clear but heavily colored by hemoglobin. Attempts to wash mouse brain tissue free of blood before homogenization resulted in loss of SCF while RHA and SHA were found still associated with brain tissue after washing. An alternate method of removing hemoglobin consisted of precipitation of antigens (as well as solubilized brain tissue) leaving hemoglobin in solution. Precipitation was carried out with 50 per cent saturated ammonium sulfate in protamine purified brain suspensions at 4°C for at least one hour with intermittent shaking. Precipitates were resuspended in volumes of TRIS or PBS effecting a 10-fold concentration, and sonicated for 3 1-minute periods in a Raytheon 10 KC instrument at maximum power. SCF from all 4 dengue serotypes was separated from most brain material and larger CF antigens in ammonium sulfate precipitates by Sephadex G-100 filtration as depicted in Figure 22. Samples of 10 to 15 ml were applied to 5 x 80 cm G-100 Sephadex columns and collected in 18 to 20 ml fractions. Most protein measured at 280 mu (first graph) was found in the first peak or void volume. CF activity was found in two separate peaks as described above, SCF being located in the second peak where relatively little protein was found by on-line recording spectrophotometers. Graphs 2 to 4 show excluded antigen and SCF antigen peaks of dengue types 1 to 3; distribution and titer of antigens in each peak varied among a number of separations for each dengue type, but type 3 SCF eluted from columns in a larger number of fractions implying some physical heterogeneity. Pools of SCF fractions (100 to 150 ml) were concentrated by nitrogen pressure dialysis on neutral (nonionic) membranes down to 1 to 2 ml providing an SCF antigen product titering 1:128 to 1:256. Precipitation of antigens from protamine purified mouse brain with 60 rather than 50 per cent ammonium sulfate resulted in final SCF titers of 1:512, but hemoglobin and brain proteins were also present. Recovery of SCF was not affected when RHA and SHA were removed from protamine purified mouse brain for other studies before precipitation of SCF with ammonium sulfate. Generally, final SCF antigen reagents, being relatively free of HA antigens or other virus components, were sufficiently pure for serological purposes. However, such SCF preparations were not pure in terms of molecular homogeniety; 8-10 bands were found by polyacrylamide gel electrophoresis, one of which reacted with antiserum, the remaining bands were possibly brain contaminants. Preparative electrophoresis combined with adsorption and elution on $Al(OH)_3$ will be added to the series of purification procedures in attempts to produce homogeneous preparations for chemical analysis.

3. Isopyknic Centrifugation of Dengue-2 SCF in Cesium Chloride Gradients: As previously reported (1968) mixtures of RHA and SHA banded

Figure 22

Sephadex G-100 Filtration of Ammonium Sulfate Precipitates from Dengue Infected Mouse Brain



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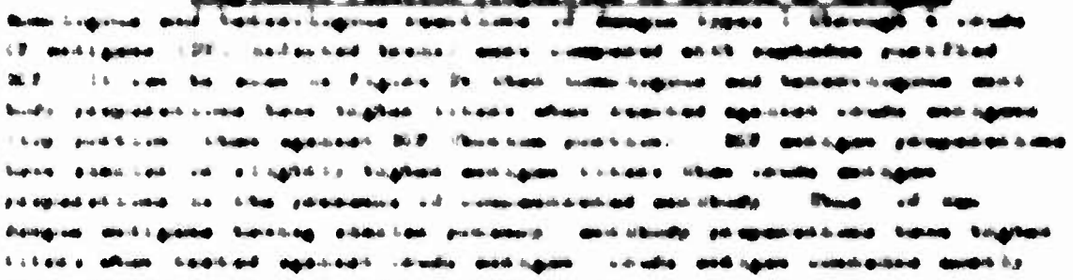

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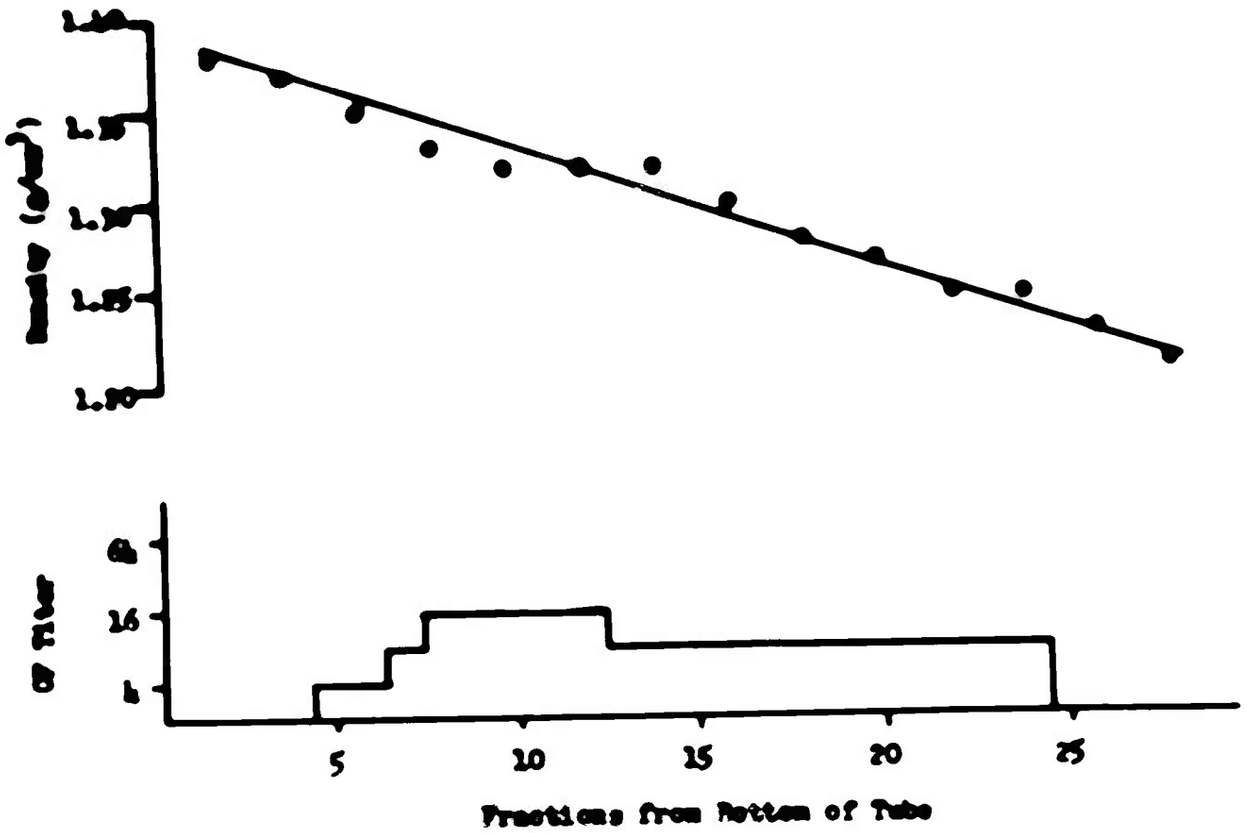
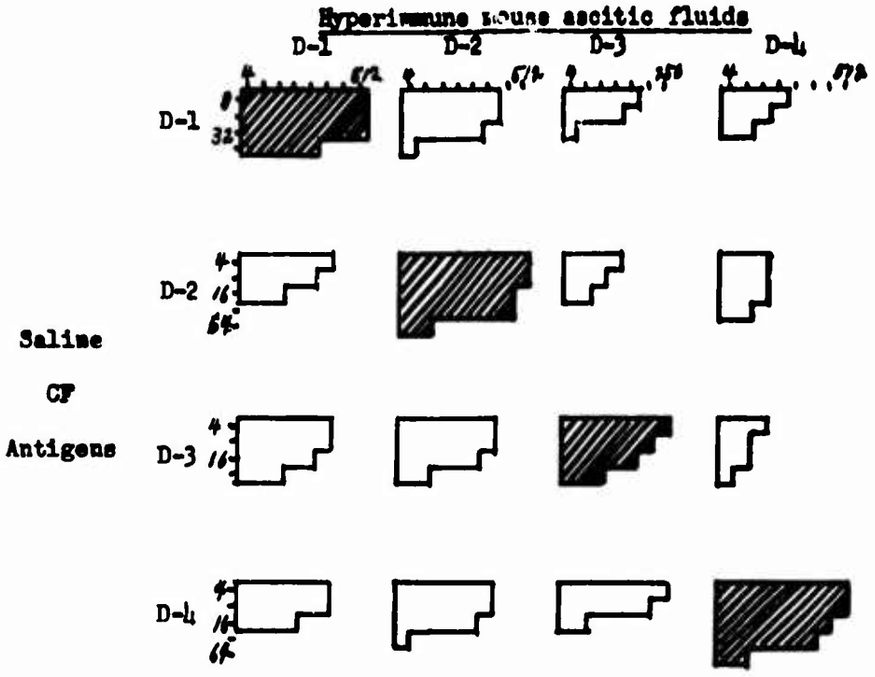
Figure 11



Soluble CF Antigen obtained from ultracentrifuge supernatant fluid by precipitation with 50 percent saturated $(NH_4)_2SO_4$

Figure 24

Homologous and Heterologous Complement-Fixation Reactions of Dengue Types 1 to 4 mouse brain saline antigens



Homologous and Heterologous Complement-Fixation Reactions of Dengue Types 1 to 4 Sephadex Purified Soluble CF Antigens

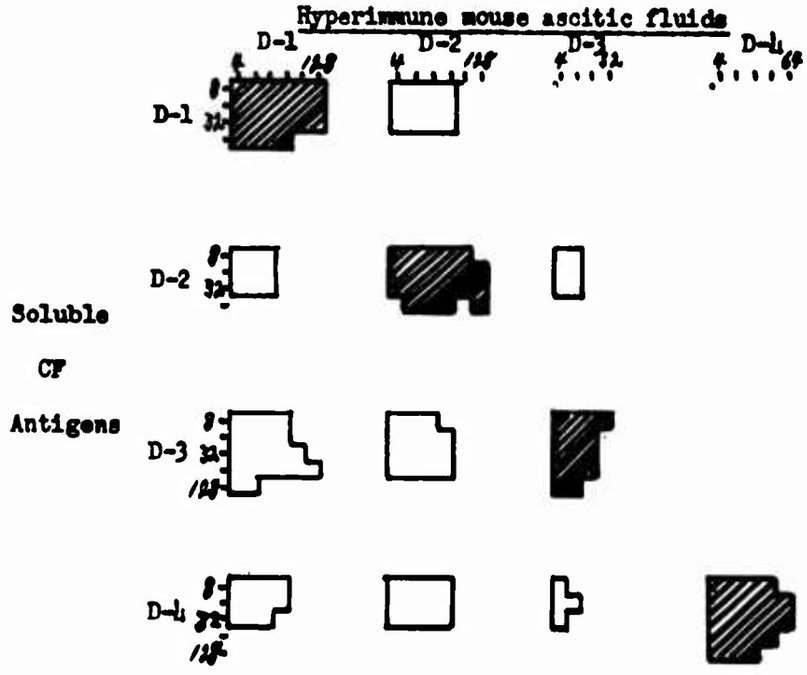
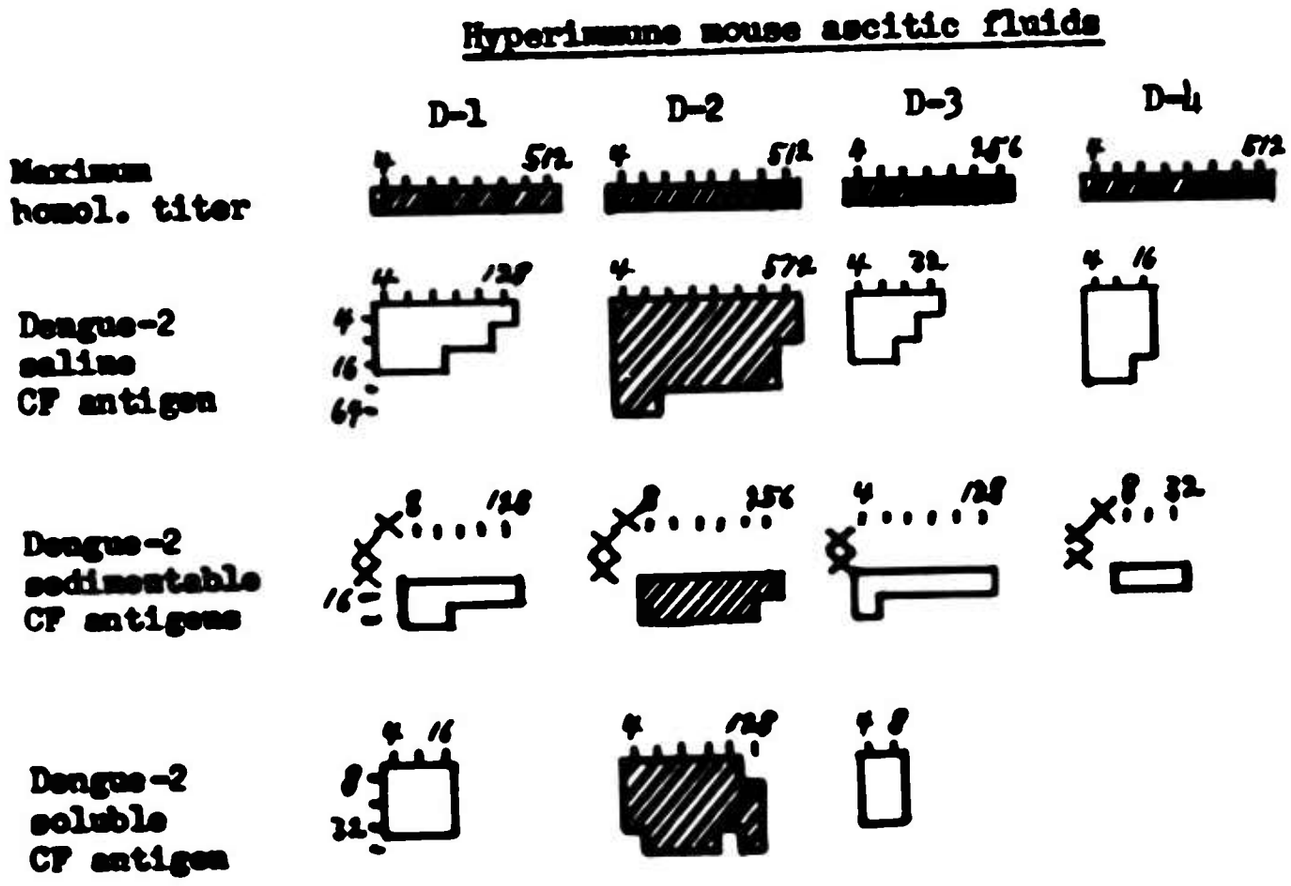


Figure 25

Homologous and Heterologous Complement-Fixation Reactions of Dengue-2 Standard Saline CF Antigen, Sedimentable CF Antigens, and Soluble CF Antigen

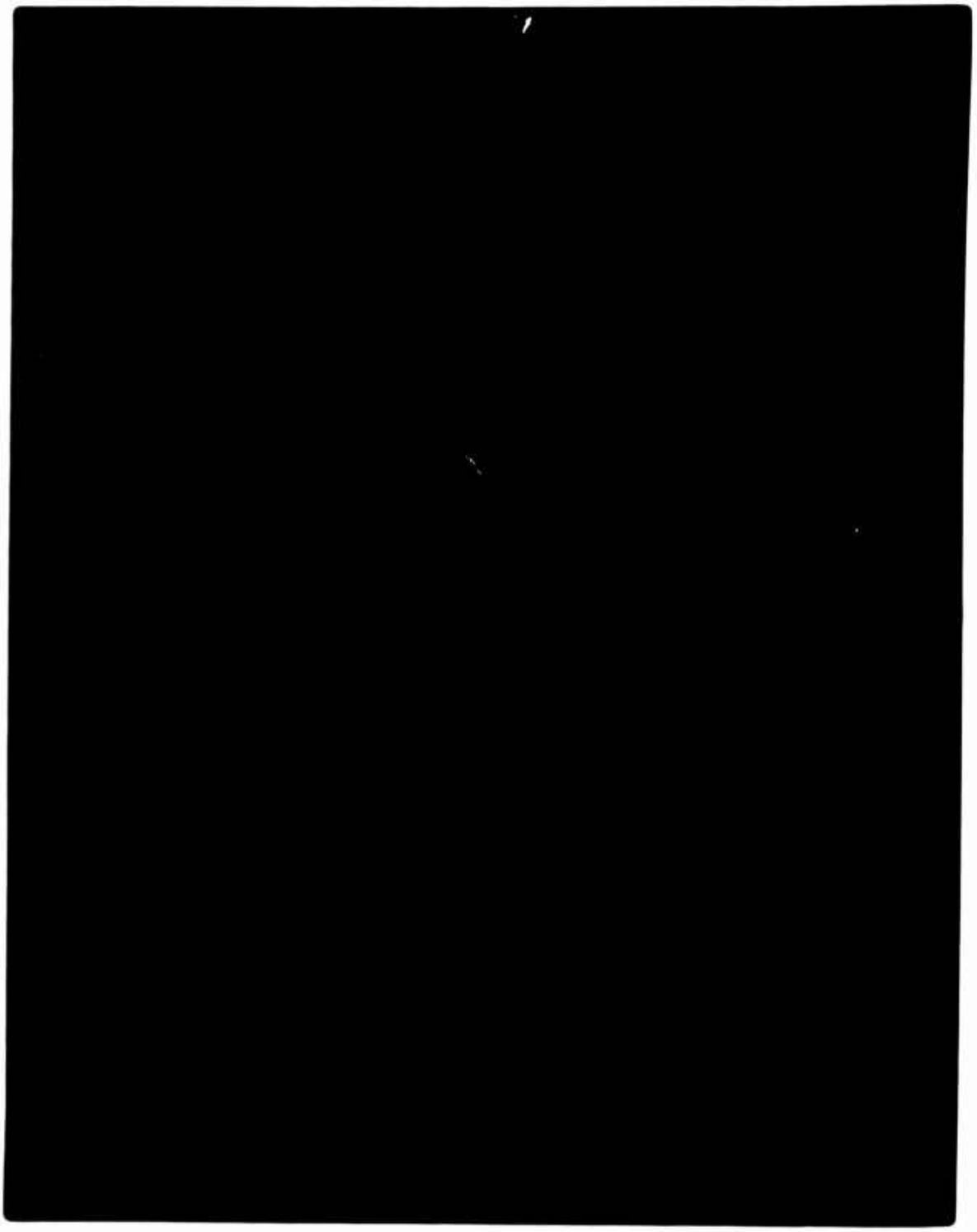


against MKF [illegible] as crude antigen derived from [illegible] MKF. Sera 8,9 and 10 appear to contribute more virus specific antigen.

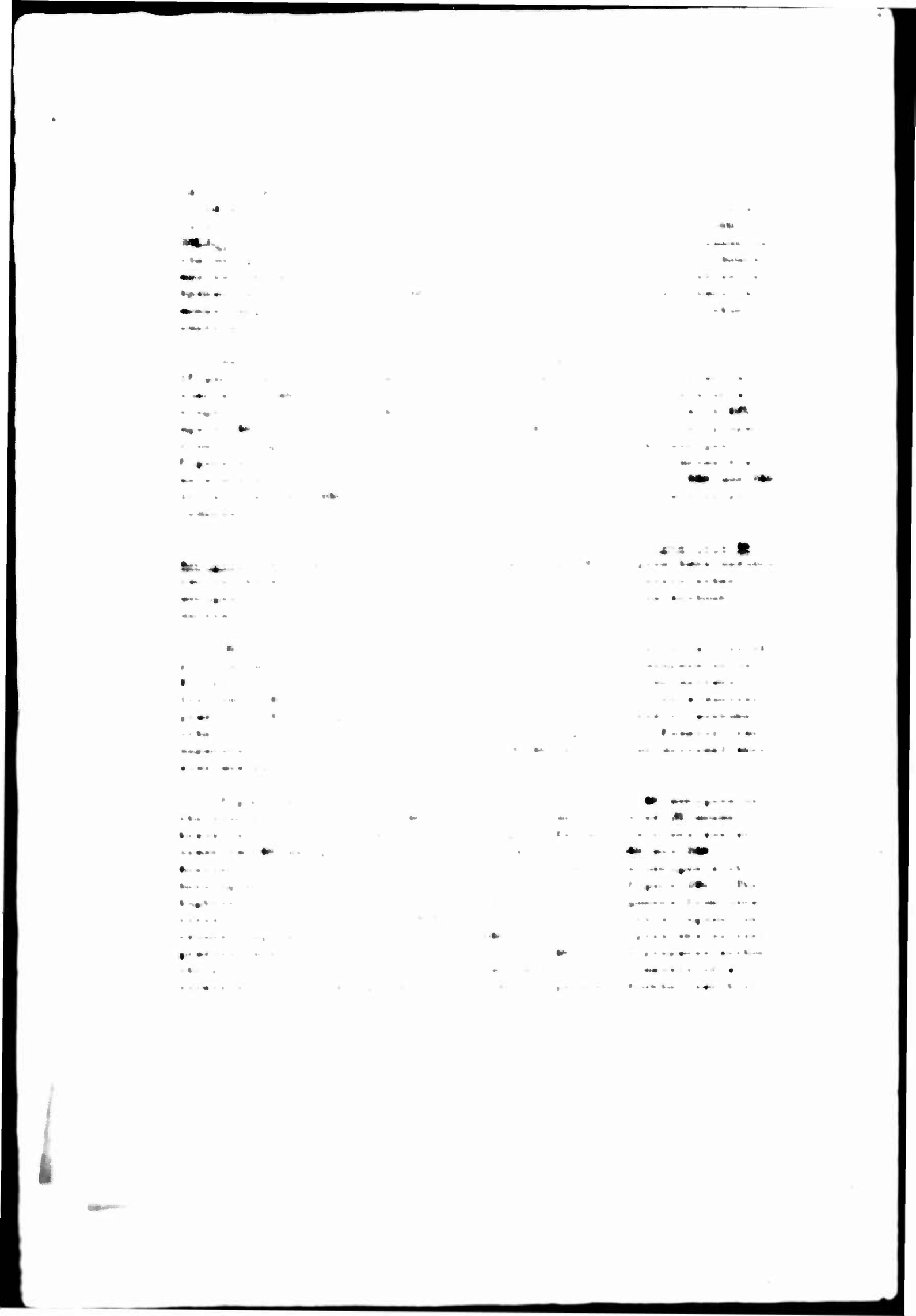
3. Immunodiffusion Studies of Reactivity of Antigens. Immunodiffusion studies were performed with soluble CF antigens (MKF) isolated from dengue types 1 through 4 infected mouse brain. With the exception of the type 3 homologous system, use of MKF in agar double diffusion studies produced single discrete precipitin lines rather than two or more generated by crude mouse brain suspensions. Utilizing antibody prepared against crude virus suspensions, relationships by discrete partial identity patterns were shown by comparing any two MKF antigen preparations against any one antibody preparation. Preliminary results below reflect studies with type 1 and type 2 systems.

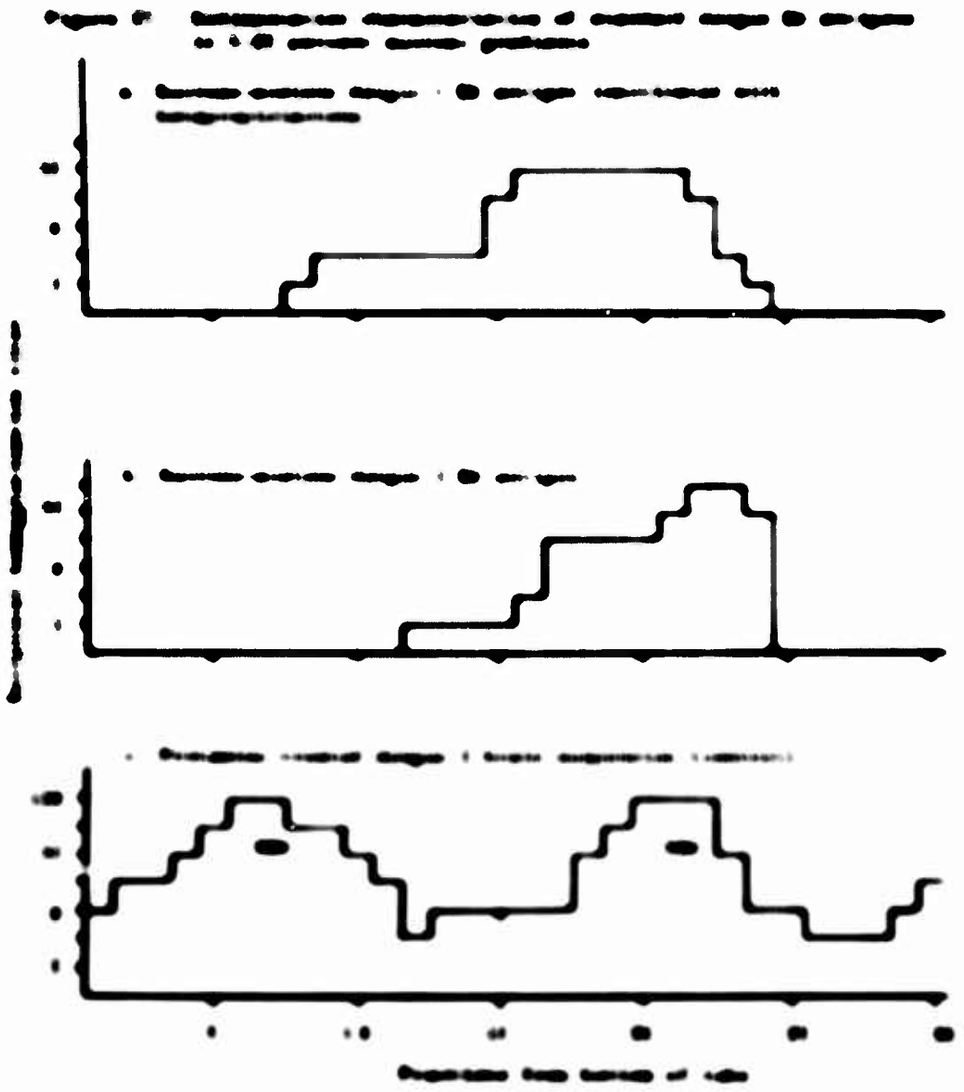
Wells measuring 3.1 mm in diameter were cut to 2.6 mm center to center in PBS with a template. Complement wells were 4 mm apart, both being 3 mm from a single well containing the appropriate component of the antigen-antibody reaction. Double diffusion systems were determined by reacting similar complement fixing units (1:2 dil) of antigen and antibody. Precipitin lines were allowed to develop for five days at which time petri dishes were washed for several days and photographed.

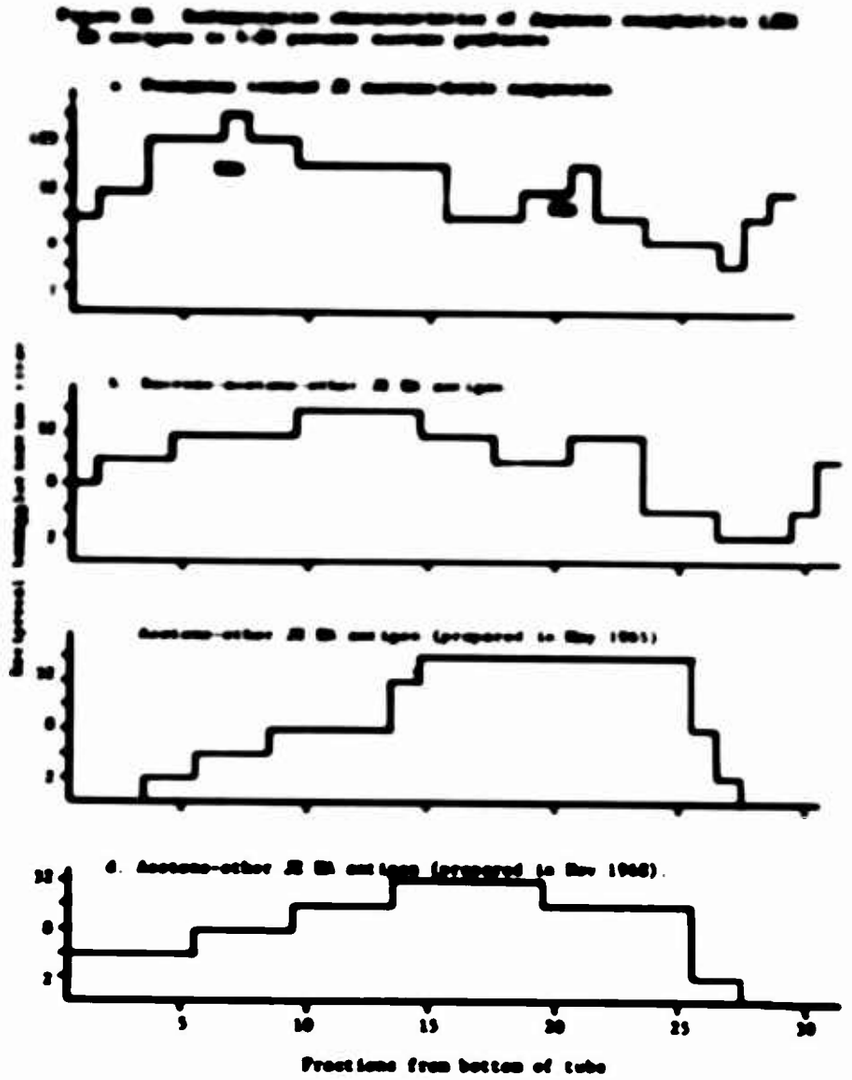
Results are diagrammed in Figure 20. Type 1 MKF diffusing toward type 1 and type 2 antibody produced a common precipitin line with both antibody preparations (identity) but did not react significantly with type 3 and 4 antibody. However, type 1 antibody diffusing toward types 1 and 2, 1 and 3, and 1 and 4 antigens produced a spur with type 1 antigen in addition to probable lines of partial identity with the other antigens. Dengue type 2 reagents produced similar results - partial identity when type 2 antibody diffused toward other dengue type antigens, but identity when type 2 antigen diffused toward type 1 and 2 antibody and insignificant reactions with type 3 (lines of bending) and type 4 antibody. When either type 1 or type 2 antigen was completely precipitated by heterologous type 1 or 2 antibody preparations forming an immunoprecipitin barrier against further diffusion and subsequent formation of spurs with homologous antibody in the adjacent well. However, a single antibody preparation, either type 1 or 2, diffusing toward type 1 and 2 antigen wells, one not completely precipitated, allowing further diffusion of antibody past the common precipitin line to form a spur with the homologous antigen. These results suggest that common and type-specific antigens of types 1 and 2 are linked on the same macromolecule (multideterminant antigen) rather than existing as physically separate antigens on the virion. Where antigens on 3 and 4 MKF reacted with 1 and 2 antibody in a typical one way cross reaction, except for a trace of bending with the type 1 antigen-type 3 antibody precipitin line, types 3 and 4 MKF do not contain significant precipitating antibody to dominant antigens on type 1 and 2 MKF. Meaningful relationships among dengue viruses may be established with MKF from all four viruses in this manner when reacted with antibody preparations prepared against crude virus, purified virus and MKF.



[redacted]






5
10
15
20
25
30

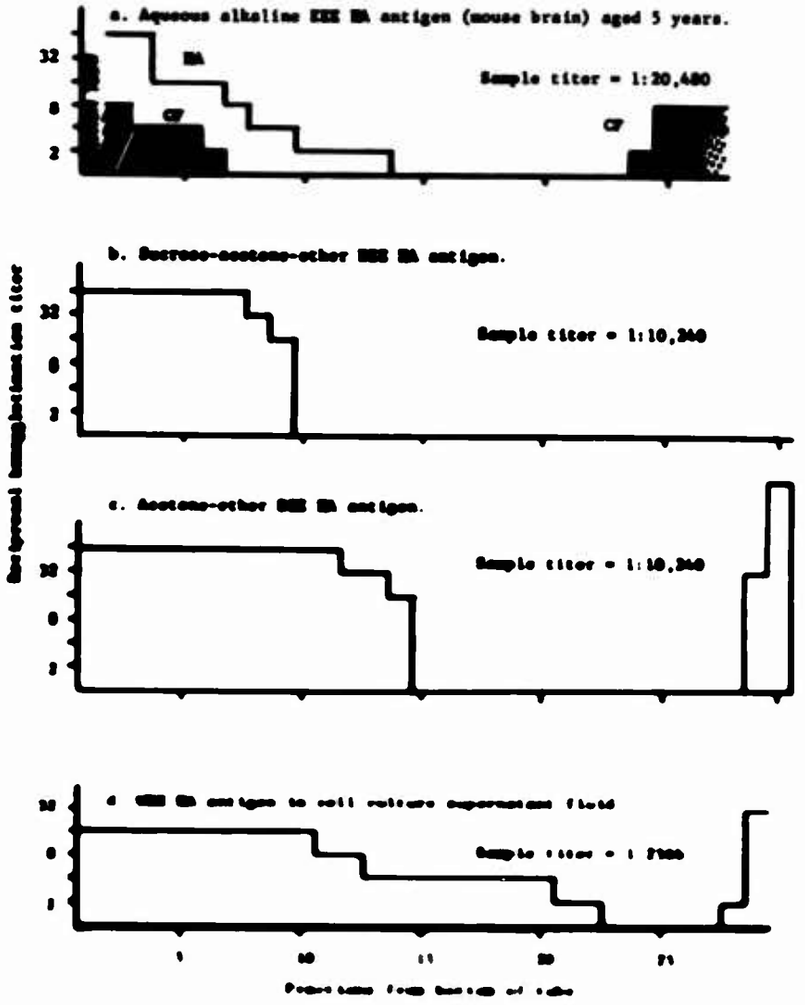
antigen prepared in the same manner appeared to peak midway in the gradient (Figure 28d), characteristic for virus fragments as described above. In any case, JE HA antigen characteristics may vary between preparations and methods of preparation; the antigen generally being physically heterogeneous.

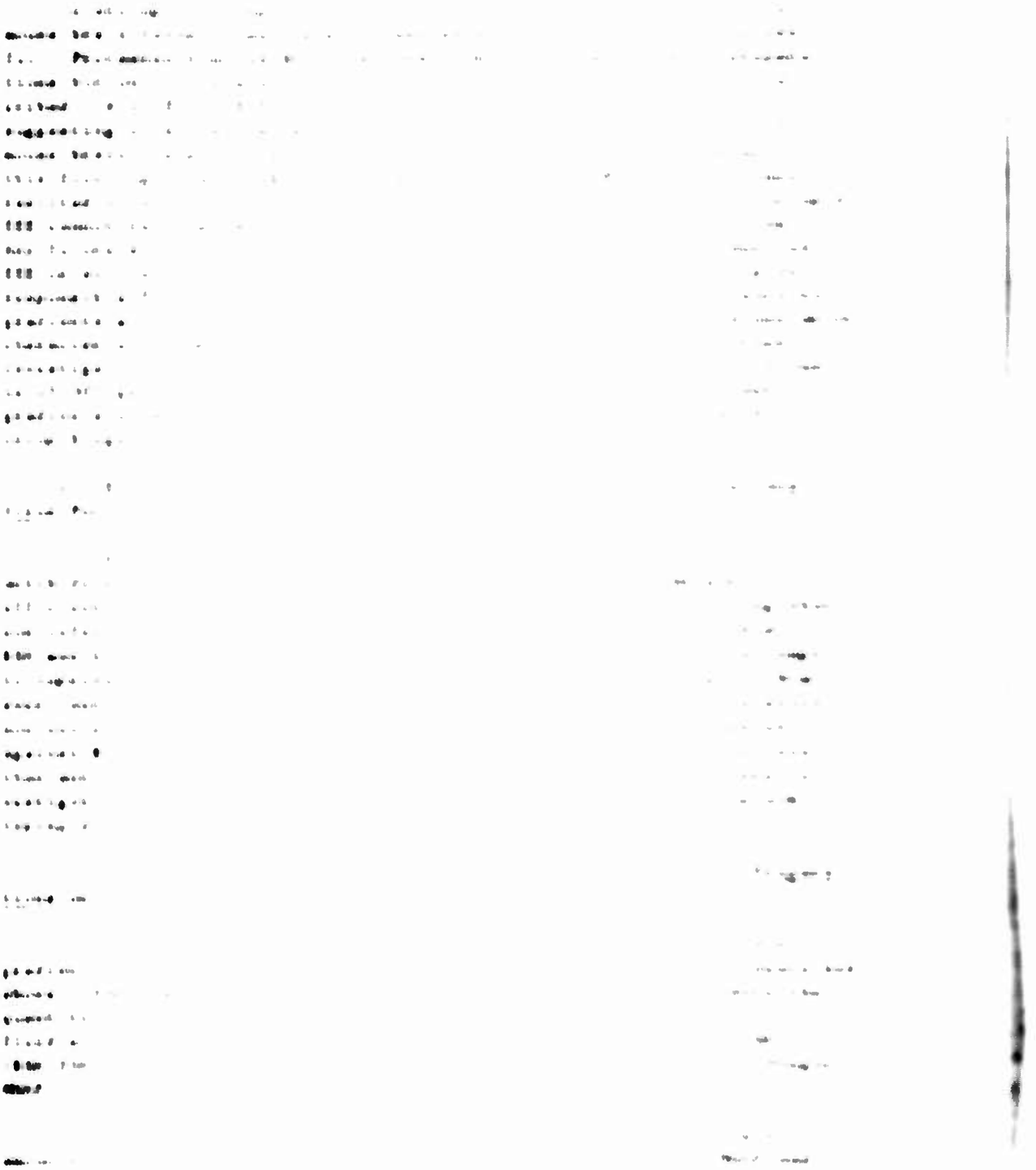
3. Sedimentation Studies of Group A Arbovirus Antigens: Eastern equine encephalitis (EEE) virus HA antigen can be readily made by homogenizing infected mouse brain in alkaline (pH 9.3) borate buffer followed by aging for several weeks, the resulting antigen being stable for several years at 4°C. Sedimentation characteristics of one such preparation (5 years old) was compared to standard acetone-ether and sucrose-acetone-ether preparations of infected mouse brain. Sedimentation of the 5-year-old alkaline aqueous antigen may be visualized in Figure 29a. Most HA antigen apparently sedimented through the entire gradient onto the bottom of the centrifuge tube; antigen that was detected (maximum titer 1:64 in fractions 1-3) accounted for only a small proportion of antigen in the sample (HA titer = 1:20,480). Sonication and dilution did not change sedimentation characteristics; however, aggregation cannot be ruled out. CF activity of this preparation has also been included (slanted parallel lines) and it can be seen that there is CF activity at the top and bottom of the gradient. Non-sedimentable CF antigen (fractions 24-27) may be similar to the soluble CF antigen described for dengue virus.

Figure 29b represents sedimentation characteristics of a sucrose-acetone-ether preparation of EEE infected mouse brain; again, most antigen is in the densest gradient region. Gradient analysis of an acetone-ether preparation (no sucrose) of EEE mouse brain is shown in Figure 29c. Trailing of antigen an additional five fractions up the gradient may indicate additional fragmentation due to use of lipid solvents without protective effects of sucrose. Antigen remaining at the gradient surface may be a reflection of insufficient solvent extraction leaving lipid with flotation properties in centrifuged fields. Fresh EEE derived from serum-free media over infected BHK cell cultures having a titer of 1:1024 could not be detected at all on the gradient, suggesting that this antigen sedimented to the very bottom of the centrifuge tube. In light of these results and the small proportion of antigen recovered in gradients compared to starting titers, EEE HA antigens detected in gradients presented in Figures 29a to 29c may represent fragmentation due to aging, lipid solvent extraction, or shearing forces during centrifugation. Source of virus (host tissue) may affect sedimentation characteristics in sucrose gradients, although this has not been true with dengue.

A western equine encephalitis cell culture HA antigen stored at 4°C for several months (Figure 29d) appears to trail from the bottom of the centrifuge tube in a manner similar to but greater than EEE HA antigens. Lack of protective protein in serum-free cell culture media may account for disrupted virions and HA fragments found on the gradient.

Figure 29. Sedimentation characteristics of group A arbovirus HA antigens in 5-25 percent sucrose gradients.





dengue 2 RHAF at room temperature. At certain time intervals during a 10-minute reaction period, 0.1 ml samples were simultaneously withdrawn from the four reaction tubes by four laboratory workers and immediately diluted 100-fold in cold growth medium (20 per cent fetal bovine serum in medium 199) to retard further neutralization. RHA and the RHA-SHA combination were also mixed with normal ascitic fluid for control purposes, being tested in a separate 10-minute time cycle 1 to 2 minutes ahead of the virus-antibody mixtures. At the end of the test, all samples were diluted further and titrated for residual virus by plaque assay in pig kidney cell culture (1968 Report).

Figure 30 represents residual virus plotted as per cent of virus found in normal ascitic fluid controls at time 0. Rates of neutralization by all four virus antibody mixtures appear similar during the first 2.5 minutes. Beginning at 3 minutes, however, neutralization of RHA-SHA mixture by dengue 2 RHAF appears terminated, whereas neutralization of RHA alone by the same antibody continued. These results suggest that SHA competed for antibody and so by being blocked neutralization of infectious virus. The obvious consideration of using RHA rather than crude virus as assay material in plaque reduction neutralization (PRNT) was tested in a later experiment. Figure 30 also shows that SHA did not block neutralization of RHA by RHAF. These results suggest that antibody to purified virus could be good kinetic reference material. A reference antibody preparation must cause the most rapid neutralization possible of homologous virus in order to minimize antigenic differences among dengue virus types. In this case RHAF was compared with an equivalent amount of antibody prepared against a mixture of two defined antigens (RHA-SHA MHAF) in kinetic analysis studies utilizing infectivity from crude cell culture assay virus. Results may be visualized in Figure 31. Similar quantities of residual virus in both antibody preparations at the end of the 10-minute test suggests they were of similar potency. However, antibody to purified virus (RHAF) neutralized crude virus infectivity at a faster rate than RHA MHAF. These results suggest that antibody preparations against more than one antigen should not be used in kinetic analysis studies. Proportions of antigens in any crude vaccine inoculum may influence neutralization kinetics of resulting antisera to the same degree that differences in antigenic composition of test viruses may influence them.

Some of these considerations were tested by comparing neutralization rates of two strains of dengue virus by antibody prepared against crude virus (standard D 1 RHAF) and antibody prepared against purified virus (RHAF) reagents were used at equivalent potencies. The experiment was also designed to determine if type 2 and type 3 dengue viruses were classified as different by kinetic analysis standards. It is evident by comparison of Figure 32 that the two viruses are identical as judged by observed neutralization rates in the presence of antibody prepared against both pure and crude virus. Both antibody preparations were of similar potency by PRNT test utilizing one hour incubation periods, but this equivalent potency was not realized in the first 10 minutes of neutralization as measured by kinetic analysis. In the previous experiment,

Figure 30
Neutralization kinetics of pure (RHA) and partially pure (RHA-SHA) dengue virus in the presence of antibody to unpurified virus (D-2 RHAF) and antibody to pure dengue virus (RHA RHAF).

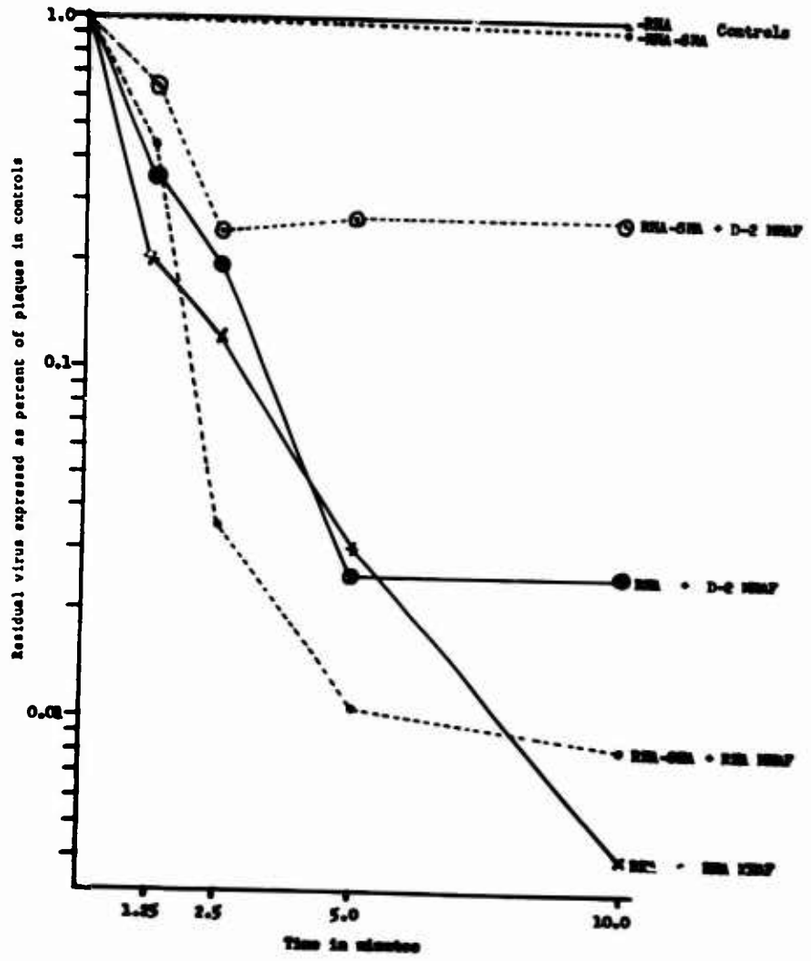


Figure 31. Neutralisation kinetics of dengue 2 cell culture virus by [redacted] and [redacted].

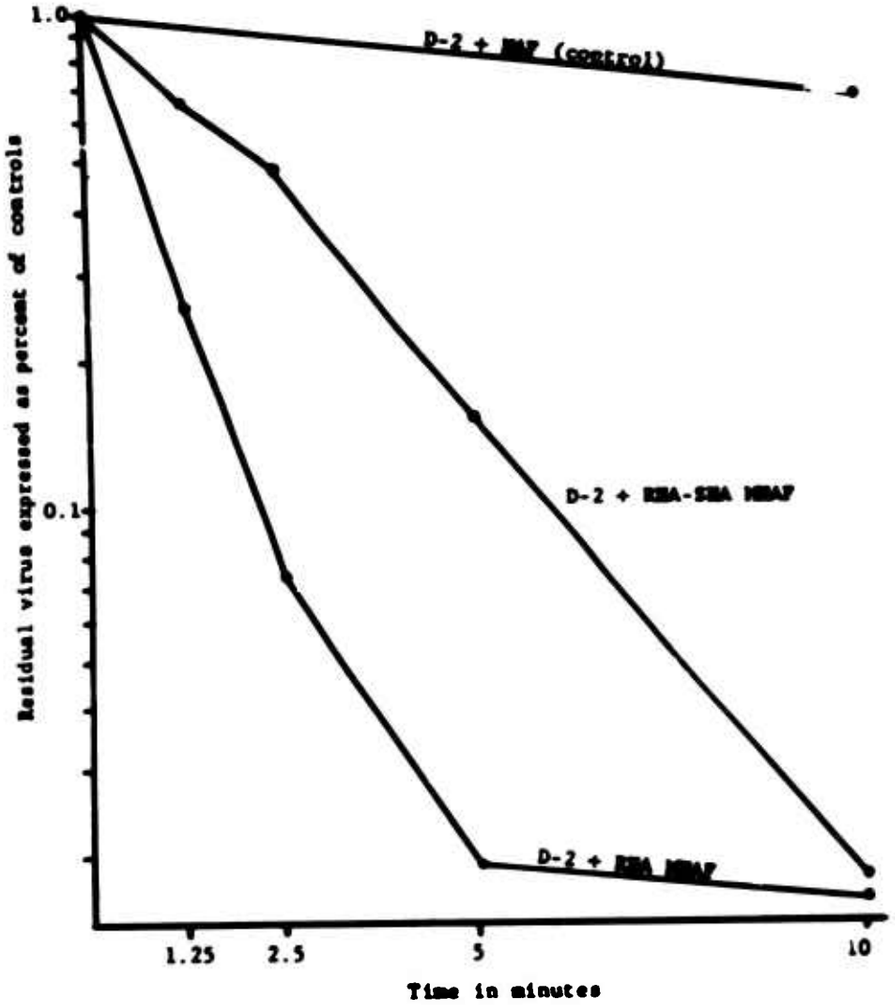
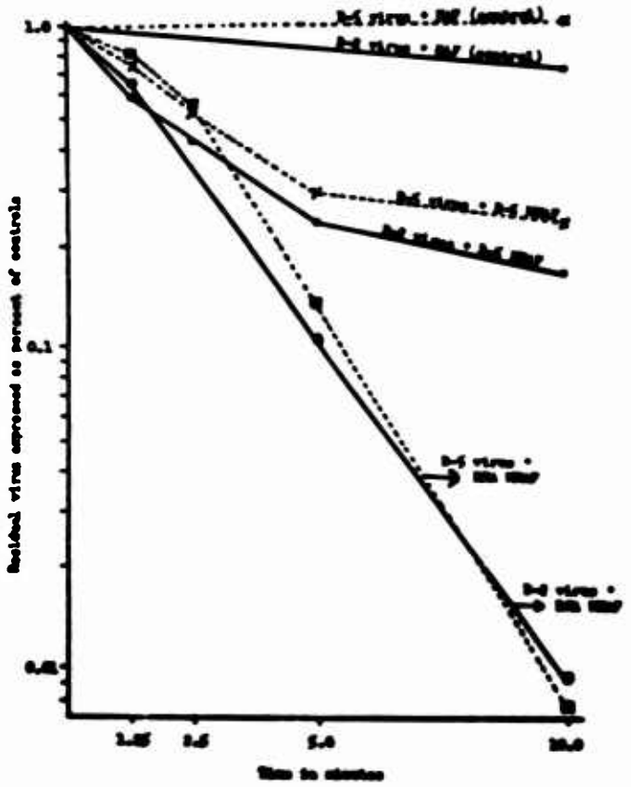


Figure 32


1.0
0.1
Residual virus expressed as percent of controls
Time in minutes

equivalent potency of both antibody preparations was realized in 10 minutes, the difference possibly being that the one antibody preparation was directed against only two purified and defined antigens (RHA and SMA) rather than three or more (RHA, SMA, SCF, fragments) unpurified antigens as in this experiment. Reduced rates of neutralization of crude viruses in the presence of antibody prepared against crude virus supports the conclusion that non-infectious antigens present in crude virus seeds compete for this type of antibody. The continuous neutralization of both crude virus preparations by RHA MHAF support the conclusion that antibody to purified virus does not react significantly with non-infectious antigens and should thus be used as reference antibody in kinetic analysis tests. It has been argued that immunization of mice with purified virus serves no purpose; probable replication of dengue in mice provides an increased antigenic mass. However, there is no evidence, nor is it likely, that non-neural replication of dengue results in the myriad or quantity of antigens found in brain tissue.

2. Effect of Virus Purification on Neutralization Endpoints of Antibody Preparations: The use of RHA as seed virus for dengue plaque reduction neutralization tests (PRNT) tests (Russell, et al, previous Reports) was analyzed by comparing PRNT antibody titers obtained with a similar plaque dose of the same virus in several physical states: a) original virus suspension in monkey kidney cell culture supernatant medium, b) in concentrated form free from soluble proteins as found in ultracentrifuge sediment, and c) purified from this sediment as found in RHA after sucrose density gradient ultracentrifugation. A dose of each virus preparation stabilized in 20 per cent FBS/199 was calculated to contain 100 plaque forming units (pfu) after mixing with 2-fold dilutions of dengue-2 MHAF. The test was incubated for 30 minutes at room temperature then held in an ice bath during inoculation of monkey kidney cell culture monolayers ($LLC\text{-}MK_2$) in 30 ml Falcon disposable flasks. Following an adsorption period of one hour, monolayers were overlaid with a half-strength nutrient agar formula developed at SEATO (1967), incubated for seven days at 37°C, stained with neutral red, and held at room temperature for two days before plaques were counted.

Numbers of plaques found in virus dose controls and at each antibody dilution are listed in Table 26. It can be seen that the virus plaque dose from each preparation was essentially the same. PFU (154) from original cell culture fluid were completely neutralized by dilutions of antibody to 1:160, the 50 per cent plaque reduction titer ($PRNT_{50}$) of dengue-2 MHAF being 1:750 as analyzed on probability paper. PFU (145) from ultracentrifuge sediment (pellet) were not completely neutralized even at low dilutions (high concentrations) of antibody; 6 to 49 pfu were scattered through antibody dilutions that neutralized essentially all pfu in the dose from original cell culture seed. The $PRNT_{50}$ was reduced to 1:300 when dengue-2 MHAF was reacted with 145 pfu of pelleted virus. $PRNT_{50}$ was reduced even further (1:80) when tested against a

Table 26 Neutralization of Dengue-2 Virus in Several Physical States Utilizing the Plaque Reduction Test

Physical state	Average plaque dose	Dilution of dengue-2 hyperimmune mouse ascitic fluid							
		20	40	80	160	320	640	1280	2560
Original	154	0*	0	0	2	21	76	113	161
CC fluid		0	0	0	3	22	60	105	182
Virus pellet	145	10	11	36	36	83	148		
		6	17	20	49	95	112		
Sucrose purified virus (RHA)	169	23	44	85	120	150			
		25	40	91	116	167			

* Number of plaques remaining after incubation of the test dose with dilutions of antibody in a plaque reduction test.

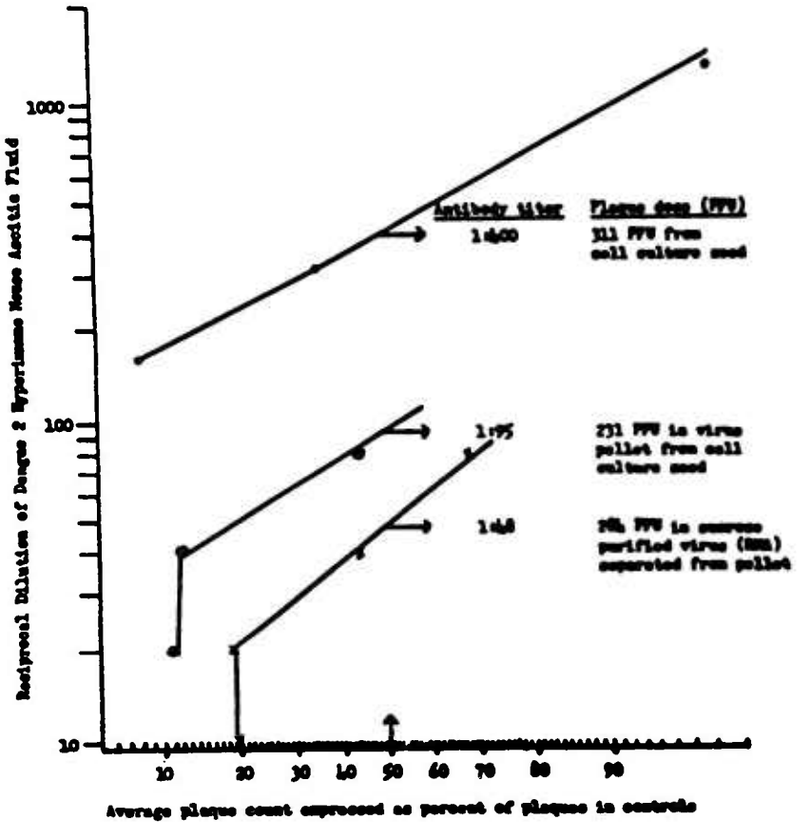
50 per cent endpoints determined on probability paper.

constant dose (169 pfu) of RHA. Thus, antibody titer decreased markedly when tested against purified virus. These results were repeated in another test graphed on probability paper in Figure 33. Plaque responses are generally dose related in this type of test as indicated by a straight line with the 311 pfu dose from original cell culture seed material, 50 per cent endpoints being determined in this manner. However, plaque responses of virus from pellet (231 pfu) and from RHA (284 pfu) were not dose related in lower dilutions of antibody. Sharp departures from linearity suggest a second mechanism of action, possibly contributing to lower $PRNT_{50}$ titers. This departure from linearity was confirmed with a plaque dose as low as 132 pfu. Purification steps may have created aggregation and fragmentation problems; aggregates release infectious particles during dilution in assay procedures and/or fragments compete or bind with antibody. The solution requires a diluent that will maintain monodisperse suspensions of intact virus particles through freezing, storage and thawing. Until such a diluent is formulated, elimination of non-infectious antigen from seed stocks is not practical. The current practical approach to improved meaningful neutralization analysis of dengue viruses may rest in production of antibody to purified virus.

3. Isolation of Dengue Type 3 in 1960 Singapore and 1968 Jamaican and Tahitian Outbreaks: A frequently fatal hemorrhagic fever disease in pediatric populations was first recorded in 1954 in Manila and Bangkok; similar epidemics occurred again in 1956 in Manila and in 1958 in Bangkok (Hammon). Serum specimens obtained in 1956 yielded virus isolates in 1957 that were identified by 1960 as being two new serotypes of dengue virus, designated as types 3 and 4. Isolation and characterization of these new viruses (especially type 3) encompassed some 10 to 20 passages in suckling mice before incubation periods were reduced from three weeks to less than one week, and before virus titers reached sufficient levels for serological analysis and neutralization tests in mice. Lengthy procedures in dengue virus identification were virtually eliminated when Russell et al recently reported isolation procedures in cell culture and application of plaque reduction neutralization (PRNT) tests for rapid and unequivocal identification of dengue viruses. The method was used in experiments below to identify the etiologic agents of a dengue-like disease in Singapore and elsewhere in Malaysia during 1959 to 1960, as well as recent outbreaks in Jamaica and Tahiti (1968). Results show that the majority of these viruses were type 3 dengue. Its global distribution can be underscored by noting isolations in Pakistan and Puerto Rico in previous Reports.

a. Methods: Seed viruses were obtained from monkey kidney or pig kidney cell cultures incubated at 36°C for 8 to 14 days and used at the first to fourth cell culture passage. A constant dose of 25 plaque-forming units (pfu) or greater was mixed with 2-fold dilutions of dengue types 1 through 4 hyperimmune mouse ascitic fluid, incubated for

Figure 33
Neutralization of Dengue 2 Virus in Several Physical States



30 minutes at room temperature and inoculated onto monkey kidney monolayers as described above or pig kidney monolayers (1968 Report) in PRNT tests. Results in Tables 27 and 28 list the dilutions of antibody in hyperimmune mouse ascitic fluids that neutralized 50 per cent of the indicated plaque dose as determined on probability paper. The majority of the isolates are clearly type 3 dengue. It can be seen that there is considerable variation in titer of dengue MHAF. Experiments described above showed that purified virus produced even lower antibody titers and further suggested that this problem might be due to using antibody prepared against crude virus rather than using antibody prepared against purified virus; non-infectious antigen appeared to block neutralization of infectivity by the former; but not the latter. It remains to be seen whether antibody prepared against purified virus will minimize extreme variations in titer when tested in PRNT tests.

VII. The Clearance of Horse IgG-Globulins from Patients who have Allergic Reactions to Equine Anti-Rabies Prophylaxis.

At the present time, rabies represents a considerable threat to military personnel troops in the Republic of Viet Nam. Rabies virus is endemic in this region, and a significant number of military personnel become exposed to rabies during their tour in Viet Nam. Hyperimmune equine anti-rabies serum is frequently used for prophylaxis in exposed personnel, and allergic reactions to equine antiserum are not uncommon. The work described below was designed to ask the specific question; will equine anti-rabies serum confer adequate protection to the individual who develops an allergic reaction to this horse serum?

A. Background:

Upon injection of foreign serum proteins to animals or humans, three phases in the clearance of these proteins from serum can be recognized. The initial phase, the period of equilibration, lasts approximately 24 to 48 hours and represents the period of equilibration of the foreign protein between the extra-vascular and the intra-vascular spaces. If the protein has been given intramuscularly, one will find a rapid increase in its concentration in serum over this 48 hour period; while if it has been given intravenously, one will find a rapid decrease in its serum concentration over a 48 hour period. The second phase, the period of exponential decline, begins at about 48 hours and extends for a variable period of time thereafter. During this phase, the serum concentration of foreign protein decreases at an exponential rate, demonstrable as a straight line by plotting the concentration of foreign protein on a log scale versus the time following injection on an arithmetic scale. The third phase, the immune stage, is one of rapid elimination of the foreign protein, due to the production of antibodies to the foreign protein by the host. The straight line of the exponential phase abruptly declines downward at the onset of the immune phase as the protein very rapidly disappears from the serum. Humans treated with horse serum products who develop allergic reactions to these products might therefore

Table 27 Identification of 1960 Elisberg Malaysian Dengue Isolates by PRNT in Pig Kidney Cells

Isolate designation and plaque dose	Mouse hyperimmune ascitic fluid D-1	D-2	D-3	D-4
Loo Bee Lay 57 pfu	<10*	<10	80	<10
Goh Chew Wan 223 pfu	<10	<10	30	<10
Dunsford 72 pfu	<10	<10	80	<10
Yin Kwai Lan, 25 pfu	<10	10	160	<10
1000 pfu	<10	<10	23	<10
Warner 38 pfu	<10	20	320	<10
CSK, 14 pfu	10	>1280	80	10
66 pfu	20	>1280	70	12
Julleatt 126 pfu	<10	200	10	<10
Yacob bin Chenob 78 pfu	<10	10	45	<10
L/Cpl Wilson 68 pfu	<10	10	54	<10
Embahadur, 518 pfu	<10	<10	45	<10
29 pfu	16	<10	56	<10
Bertie 29 pfu	34	25	130	11
Homologous virus	300	1200	160	90

* Reciprocal of 50 per cent plaque reduction titer

Table 28 Identification of 1968-69 Jamaican and Tahitian Dengue Isolates from Human Serum

Jamaican Viruses	HMAF D-1	D-2	D-3	D-4
J-384 4/29/68[a]	30[b]	80	>160	<10
J-403 5/5/68[a]	<10	12	160	<10
J-837/68	10	<10	110	<10
J-1005/68	<10	<10	200	<10
J-1007/68	<10	<10	120	<10
J-1096/68	<10	<10	36	<10
J-1203/68	<10	600	<10	<10
J-19 28 Jan 69[c]	<10	>160	20	<10
Tahitian Viruses[d]				
Str 4, 28354	<10	<10	75	<10
Str 18, 28356	20	20	>160	10
Str 22, 28358	<10	<10	80	<10
Homologous Viruses	300	1200	160	90

[a] Serum received 12 May 69 from CDC, Atlanta, for identification.
[b] Reciprocal of 50 per cent plaque reduction titer.
[c] Received as first suckling mouse brain passage.
[d] Received as second cell culture passage from Dr. Leon Rosen.

be expected to clear the foreign proteins very rapidly from the serum through an immune reaction. There is, however, very little data in the literature pertinent to the clearance of horse serum in individuals developing allergic reactions after its administration. An opportunity to study this problem arose when LTC Bruce Melnly, stationed at U.S. Army Tripler General Hospital, obtained sequential bleedings from eight patients who developed allergic reactions after receiving equine anti-rabies serum. He sent these serum specimens to WRAIR requesting hemagglutination titrations of antibody to whole horse serum. In addition, assays of antibody to horse IgG and assays of the concentrations of horse IgG in these sera were performed.

B. Methods:

1. Measurement of Human Antibodies to Whole Horse Serum and to Horse IgG: Antibody to whole horse serum was measured in all sera by a passive hemagglutination test. Human type O red cells were washed three times with 10 volumes of pH 7.2 buffered saline and made up to a 2.5% suspension in pH 7.2 saline. 15 ml of this red cell suspension was mixed with 15 ml of a 1:20,000 tannic acid solution and incubated for 10 minutes at 37°C. The solution was then centrifuged at 1,200 rpm for 5 minutes and washed with 30 ml of pH 7.2 saline. The red cells were centrifuged and resuspended with 15 ml of plain saline. To 10 ml of this red cell suspension, 4 ml of pH 6.4 buffered saline and 10 ml of normal horse serum diluted 1:200 with pH 6.4 saline were added. After 15 minutes incubation at room temperature, the cells were centrifuged, washed once with 20 ml of 1:100 normal rabbit serum in plain saline and diluted to 10 ml with 1:100 normal rabbit serum: this suspension served as antigen for the titration reactions. Titration was performed by adding equal volumes of the tanned red cell-antigen suspensions to equal volumes of serial dilutions of the human serum to be assayed. Titrations were done both in tubes utilizing a total volume of .5 ml in each tube and in microtiter plates utilizing a total volume of .1 ml per cup.

A similar type of passive hemagglutination titration was performed on all sera with horse IgG as antigen instead of whole horse serum in order to measure the antibodies produced to the horse IgG. The technique of this titration was similar to that used with whole horse serum with the exception that tanned red cells were sensitized with 10 ml of 1.5 mg/ml horse IgG in pH 6.4 buffered saline instead of the 10 ml of 1:200 whole horse serum.

2. Purification of Horse IgG: The gamma globulins were precipitated from whole horse serum by making the serum 37% saturated with ammonium sulfate. After washing the precipitated globulins once with 50% saturated ammonium sulfate the resuspended gamma globulins were dialyzed over night against .00525 M pH 8.6 phosphate borate buffer (Biochem Journal 74:201, 1960). The gamma globulins were then added to a large column containing TEAE (tetraethylaminoethyl cellulose) that had

been equilibrated with the same phosphate borate buffer. Fractions were collected and the protein content in each was measured by absorption at 280 mu. Those fractions containing the highest concentrations of horse IgG were concentrated by vacuum dialysis and tested for purity using immunoelectrophoresis and a rabbit anti-horse whole serum to identify the components of horse serum present in the IgG-globulin fraction. Although traces of non-IgG components were present in such preparations, the concentrations appeared very small in comparison to that of the IgG-globulin.

3. Reduction of Horse IgG into Heavy and Light Chains: A 2% solution of purified horse IgG that had been dialyzed against .55M TRIS buffer brought to pH 8.2 with concentrated hydrochloric acid, was reduced according to the methods described in Arch. Biochem. Biophys. supplement 1, 174, 1962. Briefly, 2-mercaptoethanol was added to give a final concentration of .75M. After incubation for one hour at room temperature, the reduced gamma globulins were cooled in an ice bath and an equal volume of recrystalized .8M iodoacetamide was added. The pH of the solution was maintained at 8 with trimethylamine. After one hour, the reduced and alkalinated IgG was dialyzed overnight against saline and any precipitate was removed by centrifugation. It was then dialyzed against cold in propionic acid and applied to a G 100 column equilibrated with this same acid solution. The first protein peak eluted from the column represented the heavy chains of horse IgG while the second peak represented the light chains. Two rabbits received 3 mg each of the heavy chains of IgG and were bled approximately one month later. Each produced antibody that precipitated horse IgG, horse IgG heavy chains and small amounts of antibody were also found specific for the light chains of horse IgG. Antisera was absorbed with increments of light chain, and increments of whole human serum to remove any antibodies to these substances. The final preparation of rabbit anti-horse IgG-chain precipitated only the IgG-globulins from whole horse serum and from the equine anti-rabies serum as demonstrated by immunoelectrophoresis. Monospecificity of the antigen was further demonstrated by comparing the quantitative precipitin curves of it versus both whole horse serum and the purified IgG-globulin from whole horse serum. The two curves formed by these substances indicated that at equivilance the same amounts of protein were precipitated from the IgG preparation as were precipitated from the whole horse serum. This is strong evidence that components other than horse IgG were not precipitated by the anti-heavy chain.

4. Measurement of the Concentration of Horse IgG in Patient Serum: Two types of assays were used to measure the horse IgG contents in the patients who received equine anti-rabies therapy. The first technique was radial diffusion and utilized 8% ion agar that had been filtered at 50°C, 2% of the mono-specific anti-heavy chain, and 20% of the normal human serum to prepare plates. Normal human serum was added

to prevent non-specific precipitation. Buffer used in the plates was pH 7.5 phosphate buffered saline. This mixture was then added to used plastic immunoplates that had been cleaned and dried (Hyland Laboratories, Los Angeles, California). 2.9 ml was used for each plate. After the agar solidified, six holes were punched in the plates and the holes were filled with the serum to be assayed for IgG. After 24 hours at room temperature, the diameter of the precipitin rings was measured. A standard curve was obtained by using known concentrations of purified horse IgG diluted in normal human serum. The standard concentrations of IgG were measured by ascertaining the absorbency at 280 mu, and assuming that the extinction coefficient of horse IgG was 14.

Concentrations of horse IgG in the patient's sera were also measured by the more accurate quantitative precipitin test. For this test, between .05 and .2 ml of the human serum to be assayed was added to tubes containing .8 ml of mono-specific rabbit anti-horse IgG-heavy chain. After one hour at 37° and 3 days at 4°, the tubes were centrifuged and washed three times with cold phosphate buffered saline. Precipitates were then measured in 3.3 ml of folin reagent. A standard curve was obtained by using varying concentrations of purified horse IgG diluted in normal human serum.

5. Immunoelectrophoresis: All patient's sera were examined for precipitating antibody to the proteins of horse serum. This was done by a simple immunoelectrophoretic test, using a single slide for each serum. The upper hole on the slide was filled with equine anti-rabies and the lower hole was filled with whole normal horse serum. After the electrophoretic run, the patient's serum was added to the trough. In this manner, one could identify those human sera with precipitins to horse serum proteins and determine for which horse serum proteins the human antibody was specific.

C. Results:

Table 29 illustrates the clinical data of the eight patients included in the study group. The patients varied in age, 3½ years to 42 years, and received doses of equine anti-rabies serum that was consistent with their weight. It is evident that all had varied allergic reactions to the horse serum. Although these individuals also received the duck embryo rabies vaccine, it is unlikely that these allergic reactions were related to this vaccine, since but one of 18 patients who received the duck embryo vaccine alone during this same epidemic had an allergic reaction. The clearance of the horse IgG of individual patients and their antibody titers to whole horse serum and to horse IgG-globulin are illustrated in Tables 30 through 37. Patient #1 (Table 30) developed serum sickness beginning on about day 6 and was hospitalized. High titers of agglutinins to tanned human 0 cells sensitized with whole horse serum were found by day 15 following injection. In contrast, this patient showed quite low titers to 0 cells sensitized with purified horse

Table 29 Clinical Data of the Study Population

Patient No.	Name	Age	Wt. (lbs)	Dose equine anti-rabies	Allergic Manifestations: Day of Appearance	Allergic Manifestations: Type
1	Clohecy	42	156	40000	4	[illegible]
2	Brown	30	142	40000	4	[illegible]
3	Henry	12	80	20000	4	[illegible]
4	Maul	30	175	40000	5	[illegible]
5	Johnson	9	42	13000	10	[illegible]
6	Branch	35	135	40000	7	[illegible]
7	Hinkley	21	152	40000	4	[illegible]
8	Montgomery	31	94	10000	7	[illegible]

Table XX Response of [illegible] to [illegible]
of Digoxin and Ferritin

	Day	Day	Day	Day
	[illegible]	[illegible]	[illegible]	[illegible]
Titre to ferritin				
Rabbit serum	0	[illegible]	[illegible]	[illegible]
Titre to ferritin				
IgG globulins	0	[illegible]	[illegible]	[illegible]
mg ferritin IgG precipitated in				
serum				
Rabbit [illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Guinea pig [illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Precipitation to				
ferritin sodium phosphate	0	+	+	+

IgG globulins. The titre showed considerable [illegible] which [illegible] action was produced at this dilution. Precipitation of [illegible] in antisera containing [illegible] acids at pH 4.5 [illegible] It is possible that the [illegible] at 1:20 may [illegible] specific [illegible] sodium antibody to ferritin IgG was [illegible]

Cross-identification of ferritin IgG in rabbit [illegible] from [illegible] is also illustrated in Table XX. The guinea pig [illegible] found to be [illegible] guinea pig [illegible] diffusion test. The lowest concentration of IgG [illegible] diffusion test was approximately .01 mg/ml which is [illegible] of ferritin IgG could be detected by guinea pig [illegible] In any event, ferritin IgG [illegible] sodium is [illegible] both techniques agreed within the limits of the [illegible] It is evident that [illegible] from this patient still [illegible] ferritin IgG. [illegible] sodium ferritin IgG concentrations were plotted on a semi-log graph paper [illegible] obtained and no evidence of an [illegible] following immunization was found. No evidence of an immune elimination of IgG was thus apparent. Clearance of ferritin IgG from the body of this [illegible] patients was similar. None had evidence of an immune elimination of this IgG.

Patient #1 was one of three individuals who [illegible] to ferritin sodium to precipitate with rabbit [illegible] of [illegible] ferritin is an immunoelectrophoretic analysis. On all study days after the second week following immunization with [illegible]

[illegible] through the [illegible] specificity of the [illegible] [illegible] [illegible]

[illegible]

	[illegible]					
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]						
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]						
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]						
[illegible]						
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]						
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

had refused for an alpha of beta globulin. The response of patient #3 (Table 36) was similar to all other patients, horse IgG was detectable by both techniques as late as 33 days post-immunization. Patient #6 (Table 33) was the third individual who was found to have precipitins to horse serum proteins, and his serum precipitated an alpha or a beta globulin present in both whole horse serum and in the equine anti-rabies. Horse IgG was detectable 30 days after immunization in this individual but could not be found 61 days following injection. Patient #7 had detectable horse IgG as late as 67 days post-immunization (Table 36) while patient #8 (Table 37) had horse IgG as late as 83 days post-immunization.

These data show the following points: 1) All eight patients had manifestations of an allergic reaction which were almost certainly attributable to the equine anti-rabies preparation, 2) all patients produced antibody to the equine anti-rabies as detected by the agglutination of tanned human O cells sensitized with whole horse serum proteins, 3) three of the eight patients produced antibody that precipitated with one or more components of horse serum. In no case could precipitating antibody to horse IgG be demonstrated; however, the antibody appeared specific toward either alpha or beta globulins. 4) little or no antibody was produced to the horse IgG component of horse serum as detected by the agglutination of tanned human O cells sensitized with purified IgG and 5) two techniques for measuring the catabolism of horse IgG in sequential bleedings from the eight patients indicated that in no case was there evidence of an immune clearance. Horse IgG was always detectable 30 days or more after immunization and in two patients small amounts of this globulin persisted as long as approximately 80 days

[illegible]

Table 33 [illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]

Table 21 [illegible]

	[illegible]					
	[illegible]					
Titer to whole horse serum	[illegible]					
Titer to horse IgG-globulins	[illegible]					
mg Horse IgG per ml serum:						
Radial Diffusion	[illegible]					
Quantitative Precipitins	[illegible]					
Precipitins to horse serum proteins	[illegible]					

[illegible]

	[illegible]				
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]

[illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]

Table 38 Precipitation of Neutralizing Activity from Equine Anti-Rabies by Anti-IgG

Tube	Anti-Rabies Exp. Date	Anti-Rabies ml	ml Anti-IgG	ml normal rabbit serum	IgG mg/ml remaining	Neutralizing titer remaining
1	Nov 68	.005	1.0		<.01	<1:2
2	Nov 68	.005		1.0	.38	1:4.7
3	Jun 70	.005	1.0		<.01	<1:2
4	Jun 70	.005		1.0	.41	1:6.2

in preliminary experiments, be to use an animal system. The clearance of horse IgG-globulins following the administration of whole equine anti-rabies to two species of animals was the next study. Groups of eight mice were injected with five units of equine anti-rabies per pound, 30 units of equine anti-rabies per pound, 300 units of equine anti-rabies per pound and 3000 units of equine anti-rabies per pound. The injections were by the intraperitoneal route as intravenous injections of the equine anti-rabies in moderately high concentrations, proved lethal for mice. Serum was obtained from the mice by bleeding them via the tail on days 3, 10, 17, 21, 27, 39, 49 and 64 following infection. Blood was collected in capillary tubes, centrifuged to remove the red cells, and then frozen in sealed capillary tubes until the time of assay. IgG contents were measured in radial diffusion plates using the same mono-specific antisera and techniques used to measure the levels of horse IgG in the human sera. The rabbit anti-horse heavy chain did not have to be absorbed with mouse serum since this antiserum did not precipitate anything from normal mouse serum. The results of these experiments strongly suggest that mice respond to horse serum very much the same way humans do. In the mice administered 6 units per pound, horse IgG was usually detectable 10 days after the injection. In those administered 30 units per pound, IgG was present 21 to 27 days following injection and IgG was present 27 to 49 days after 300 to 3000 unit injections. Only in rare cases did the clearance of horse IgG follow an immune pattern during the time of clearance. A semi-log plot of the data (concentration of horse IgG on the log scale and the day following injection on the arithmetic scale) showed that clearance almost always was in straight line suggesting that the mice did not respond by producing antibody to the IgG. Clearance of horse IgG in nine rabbits administered equine anti-rabies was also measured in similar fashion. Three animals each received 6 units per pound, 30 units per pound and 100 units per pound equine anti-rabies given intravenously. Concentration of horse IgG was measured by radial diffusion in serum obtained from the rabbits 10 minutes after injection, and at 2, 4, 6, 9 and 13 days after injection. The data suggested that rabbits did respond to

the IgG component of horse serum and cleared this protein by immunological mechanisms. In all animals the concentration of horse IgG dropped rapidly between 10 minutes and 2 days following injection, during the equilibration phase. Concentrations leveled off at a slower rate between 2 and 4 days following infection and then disappeared very rapidly and were always undetectable at 9 days.

D. Discussion:

Data presented here shows that patients with manifestations of serum sickness following administration of equine anti-rabies serum do not necessarily clear horse IgG-globulin as a result of an immune response. It appears that serum sickness is probably due to other components in the equine anti-rabies serum, probably beta or alpha globulins. None of those persons studied were known to receive any horse serum products previously. It, therefore, remains to be determined whether individuals who are previously sensitized to horse serum have similar exponential clearance of horse IgG as the patients studied. It is possible that given enough injections of horse serum one would ultimately develop an immune response to the horse IgG and clear it quickly from the circulation. Less allergic reactions might occur if an adequate preparation of purified IgG from equine anti-rabies were administered to patients rather than the whole equine anti-rabies. Even a more promising alternative would be to administer gamma-globulin of immunized humans since one would completely eliminate the potential for allergic reactions by this treatment. Hyperimmune horse serum, however, continues to be used in other forms of therapy such as for treating snake bites, botulism and organ transplant rejection (horse anti-lymphocyte serum). Use of purified horse IgG-globulin prepared from such hyperimmune sera should result in fewer allergic reactions than currently occur after administration of whole horse serum.

VIII. The IgM Response of Children to S. typhosa Vaccine.

Abstract.

Children immunized with commercial typhoid vaccine had elevations of total IgM-globulins that averaged 55% above pre-immunization levels within ten days. Concentrations of IgM specific for the surface antigens of S. typhosa were found by measuring the decrease in total IgM levels that followed absorption of the children's sera with whole S. typhosa organisms. On the days when IgM-globulins were highest, sera contained at least 22 to 32 mg/100 ml of IgM anti-typhoid. This antibody accounted for 18 to 30% of all IgM in these sera and with one exception, most of the IgM-globulin increase that had occurred since immunization.

The amounts of IgM specific for the O somatic, H flagellar and Vi antigens of typhoid were compared in the sera of four immunized children. The assay involved measuring the concentrations of IgM that various

antigens absorbed from sera taken near the height of the response. The concentrations specific for Vi were probably quite low, the amounts directed to the O antigen were measurable in 2 of 4 children but constituted less than half of their anti-typhoid, while IgM anti-H predominated in this response. Polymerized flagellin absorbent containing H and O antigens removed 22 to 76 mg IgM antibody/100 ml from sera taken within two weeks of immunization. This accounted for as much as 50% of all serum IgM-globulins and in 7 of the 8 children essentially all of the total IgM-globulin increase that followed immunization.

Background.

In a previous report (see Annual Report, Dept of Virus Diseases, 1967-1968) serum IgM levels in Basic Combat Trainees were found to rise during the first three weeks of training and return to normal levels by the end of basic training. One hypothesis to explain this increase in IgM is that it represents a response to the several immunizations received during the first weeks of BCT. Children immunized with S. typhosa vaccine developed increased serum IgM levels within two weeks. The following experiments were performed to determine what proportion of the serum IgM increase was specific anti-typhoid antibody and to determine the components of the IgM response which are specific for the O, H, and Vi antigens of S. typhosa.

A. Methods and Materials:

1. Immunization: A commercial heat-phenol vaccine (E. Lilly and Co., Indianapolis, Illinois) containing 1×10^9 S. typhosa (9, 12, Vi:d:-) per ml was given subcutaneously to eight children 3-7 years of age. Four received 0.5 ml on day 0 and four 0.5 ml on days 0 and 7. Sera obtained immediately before immunization and on days 7, 10, 14 and 28 were stored at -20°C. No child had a history of previous typhoid immunization, although three (W.S., W.D. and M.R.) possessed low levels of flagellar (H) and somatic (O) agglutinins to S. typhosa in day 0 bleedings.

2. Total Serum Immunoglobulins: Serum IgG and IgA levels were quantitated by radial diffusion using commercially produced (Hyland Laboratories, Los Angeles, California) agar plates and standards. Serum IgM concentrations were assayed by a more accurate micro-quantitative precipitin technique; IgM was purified from euglobulins of normal human serum by block electrophoresis with polyvinyl chloride particles and Sephadex G-200 gel filtration. The protein content of the purified IgM was measured in a Beckman DU2 Spectrophotometer at 280 mu assuming an $E^{1\%}_{1cm}$ of 11.85. This preparation was judged pure by immunoelectrophoresis and the identification of a single 18S component by analytical ultracentrifugation. Rabbit anti-IgM was prepared by injecting two animals with three monthly intradermal doses of 1 to 5 mg each, the first in Freund's complete and subsequent two in incomplete adjuvant. Serum taken

three weeks after the last immunization [illegible]
by immunoelectrophoresis but [illegible]
specificities were absorbed with a fraction [illegible]
a DEAE-cellulose column with [illegible] phosphate buffer.
Immunoelectrophoretic analysis of the final absorbed antiserum against
whole human serum usually identified a single broad precipitin arc
occupying a position characteristic of IgM globulin. Occasionally,
however, the line appeared to split into two or more parallel arcs of
identical shape and mobility. Dr. M. Metzger of the National Institutes
of Health provided four purified Waldenström's macroglobulins to test
the possibility that different allotypes of chains were recognized by
the antiserum. Similar splitting of IgM arcs was observed in each case
and the phenomenon was considered an artifact.

The quantitative precipitin test involved mixing a measured volume
(usually equivalent to .01 ml) of unknown serum with .05 ml of anti-
specific anti-IgM in 12 x 75 mm acid cleaned tubes. Volumes were brought
to .25 ml with saline buffered to pH 7.3 with .02M phosphate (PBS) and
the tubes incubated for one hour at 37° and three days at 4°. The pre-
cipitates were then centrifuged, washed twice with 1.5 ml of cold PBS
and measured by the Lowry reaction. That the absorbed rabbit anti-IgM
precipitated only IgM was confirmed by comparing quantitative precipitin
curves of it versus both purified IgM and whole serum. The curves had
almost identical shapes, both antigens formed equal maximum precipitates
at equivalence, and IgM levels in the whole serum were the same when
calculated from several points on the purified IgM curve. This whole
serum, with its IgM content established in this manner, was used to pre-
pare a standard reference curve during each precipitin assay.

The precision of these methods was such that the mean difference
and 95% confidence limits of at least 100 duplicate assays in mg/100 ml
were 87 ± 12 for IgG, 13 ± 3 for IgA and 2.7 ± .5 for IgM measurements.

3. Absorption. Absorbents were prepared from a culture of
S. milwaukee (4) f, g, -) and the vaccine strain of S. typhosa (9, 12,
Vi:d:-). The organisms were grown overnight on brain-heart-infusion agar,
harvested in PBS and killed with daily .02% increments of thimerosal.
Just before use, suspensions were gently washed three times, resuspended
in PBS and standardized by measuring optical density at 450 mμ.

To absorb IgM anti-typhoid, a measured volume of serum was mixed
with an equal volume of a bacterial suspension. After incubation for
one hour at 37° and overnight at 4°, the bacteria were removed by cen-
trifugation at 4° and 2,000 G for two hours. Complement was then ab-
sorbed from supernatant fluids and IgM levels assayed by the quantitative
precipitin test.

4. Complement Removal. Complement was removed from [illegible]
sera to prevent it from adding to specific precipitates in the quantitative

precipitin assay. 2 mg of rabbit IgG anti-egg albumin precipitated at equivalence with antigen was added per 1 ml of human serum. The suspension was incubated for two hours at room temperature, three days at 4° and the precipitate then removed by centrifugation.

5. Titers of S. typhosa Agglutinins: Agglutinins were titered in V-bottom microplates (Cook Engineering, Alexandria, Virginia). Human O red cells coated with purified Vi antigen, generously provided by Dr. S. Formal were used for Vi titers, formalized S. typhosa H901 was used to measure H agglutinins, and S. typhosa 0901 killed with alcohol and phenol was the antigen for O titrations. Endpoints for the three procedures were identified by settling patterns. PBS was the diluent for O and Vi assays, but in order to read settling patterns the H antigen had to be diluted in PBS containing .5% normal rabbit serum. This latter procedure was very sensitive; H titers of individual sera were usually 16-fold higher than those measured by standard tube dilution assays; it was less selective, however, since sera containing only O antibody also agglutinated formalized antigen. This was of little consequence since H titers were much higher than O titers under the conditions of these experiments.

Agglutinins due to IgM antibody were identified by reduction with 2-mercaptoethanol. Equal volumes of serum and .1M 2-ME were incubated in pH 7.5 PBS for one hour and then diluted with PBS and titered. As a control of this technique, a serum with only macroglobulin agglutinins and a serum with only 7S agglutinins, as determined by sucrose gradient ultracentrifugation, were included as controls each time the reduction was performed.

6. IgM Assayed by Absorption with Polymerized Flagellin: Polymerized flagellin was prepared from S. typhosa H901 according to methods modified from those outlined by Ada et al. This organism was grown overnight on heart infusion broth containing .9% agar, harvested with PBS and killed by adding daily .02% increments of thimerosal. The flagella were then sheared from bacteria in a blender and purified by four cycles of differential centrifugation alternating between 5000 G for 25 minutes to remove bacterial debris and 40,000 G for 45 minutes to sediment flagella. The purified flagella were next depolymerized to their protein constituent flagellin by adding 1/20 volume of 1N HCl. This was accompanied by a marked clearing and a decreased viscosity of the solution. After insoluble materials were removed by centrifugation at 80,000 G for one hour, the supernatant fluid containing the flagellin was neutralized with NaOH, and two volumes of cold saturated ammonium sulfate were added to polymerize and precipitate the flagellin. After recovery by centrifugation at 20,000 G, the flagellin was placed through the depolymerization cycle again to insure its purity.

The protein contents of purified flagellin solutions were measured either directly by drying an aliquot to constant weight at 105° after

it had been dialyzed against distilled water, or indirectly by comparing the OD at 280 mu of an unknown depolymerized flagellin solution with the OD of a solution standardized by the direct method.

IgM anti-flagellin was measured by mixing serum from which complement had been removed with a volume of purified polymerized flagellin to yield a final serum dilution of 1:10. Flagellin was used within three weeks of preparation since aging seemed to increase its tendency to remove IgM non-specifically. After the mixtures were incubated for one hour at 37°C and overnight at 4°C, the solutions were transferred to 26 x 90 mm polycarbonate tubes and centrifuged at 4° for one hour at 60,000 G in a Type 30 Beckman rotor. When the fixed angle rotor stopped, a clear pellet of flagellin remained above the upper level of liquid (usually 1 ml in volume). At least 90% of the 280 mu absorbing material was removed from solutions of flagellin by this treatment.

B. Results:

1. Serum Immunoglobulin Levels Following Typhoid Immunization: Total levels of IgG-, IgA- and IgM-globulins were measured in sera taken immediately before and 7, 10, 14 and 28 days after injection of typhoid vaccine. The differences between levels on days 0 to 7, 0 to 10, 0 to 14 and 0 to 28 were calculated for each immunoglobulin in each child. Figure 34 illustrates the means of these differences for all eight children. IgG and IgA changed relatively little after immunization; neither was more than 15% above baseline (the day 0 level) on any study day. In contrast, IgM increased rapidly to a peak averaging 55% above baseline on day 10 before declining towards starting levels. The IgM levels of individual children are illustrated in Table 39. Their percentage changes from baseline to highest levels ranged from 21 to 127% and averaged 68%. In all but two cases (M.R. and J.B.) IgM was falling towards baseline on day 28. The IgG, IgA and IgM changes of children given a single dose were not noticeably different from the changes induced by two doses of vaccine.

2. IgM Removed by S. typhosa: Concentrations of specific IgM typhoid antibody were determined by measuring the reduction in total serum IgM-globulins that followed absorption with whole S. typhosa organisms. Six sera taken from four of the children on days when IgM-globulins were highest were absorbed as follows. Measured aliquots of serum from day 0 and the day or days of highest IgM levels were mixed with equal volumes of PBS (the unabsorbed control) and with the same volume of S. typhosa and S. milwaukee suspensions in PBS. The latter organism does not share H, O or Vi antigens with the typhoid bacillus. After incubation, bacteria were removed by centrifugation, complement was absorbed and the IgM remaining in the supernatant fluids assayed by quantitative precipitins. The dilutions of all such steps were exact so that the IgM ultimately measured by quantitative precipitins could be converted by calculation to mg/100 ml in the original undiluted serum.

Figure 34. Changes of total serum immunoglobulins following typhoid vaccination expressed as the mean of mg/100 ml differences between baseline and each study day (circles), the 95 percent confidence limits of these values (brackets) and the mean percent change (mean mg/100 ml difference divided by average baseline concentration X 100)

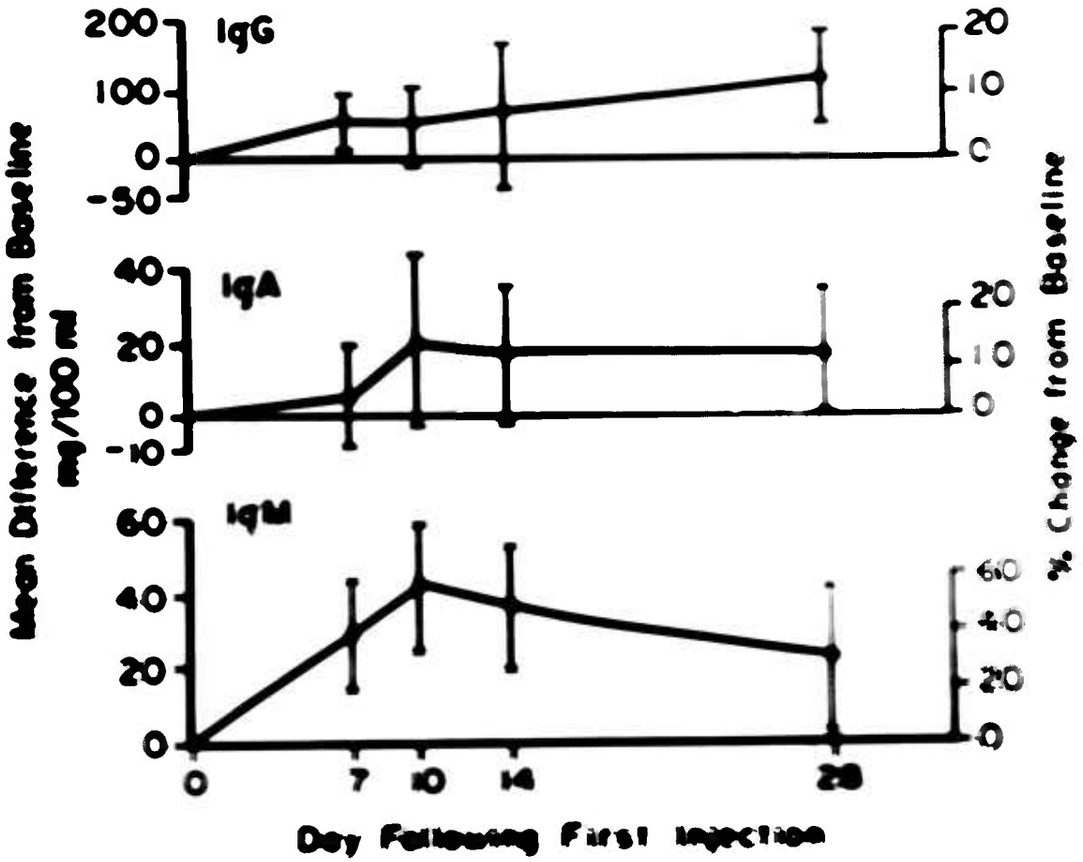


Table 39 Total Serum IgM Levels Following Typhoid Immunization

Vaccine	Subject	mg IgM/100 ml Serum					% Change Day 0 to Highest Level
		Day 0	Day 7	Day 10	Day 14	Day 28	
.5 ml on day 0	PR	41	93	77	73	49	127
	MM	105	152	182	175	120	73
	JT	37	65	63	52	41	76
	MR	72	90	101	91	107	49
.5 ml on day 0 and 7	AW	89	130	127	122	106	46
	WS	103	111	157	147	121	52
	WD	85	90	97	103	87	21
	JB	75	107	141	135	151	101

As shown in Table 40, IgM was not removed from sera that lacked antibody to its absorbent. Day 0 levels were not lowered by either organism, and S. milwaukee, having no specific typhoid antigens, did

Table 40 IgM Remaining after Serum was Absorbed with S. milwaukee and S. typhosa

Subject and Vaccine Dose	Day	mg IgM/100 ml Serum Remaining after Absorption[a]		
		Saline	S. milwaukee	S. typhosa
PR .5 ml	0	40	42	41
Day 0	7	87	89	59
	10	71	76	49
MM .5 ml	0	108	112	108
Day 0	10	179	182	147
MR .5 ml	0	70	79	72
Day 0	10	99	103	74
WS .5 ml	0	103	113	106
Day 0 and 7	10	160	169	128
	14	146	149	119

(a) Sera of PR and MM were absorbed with an equal volume of 1.5×10^{11} S. typhosa and S. milwaukee per ml while MR and WS were absorbed with 2.5×10^{11}/ml.

not absorb IgM from sera taken at the height of the response. In contrast, S. typhosa removed IgM from all post-immunization sera studied, presumably by absorbing specific antibody. The amounts taken by this organism were calculated by subtracting IgM left after absorption from the unabsorbed (saline) values. For example, in the day 7 bleeding of P.R., S. typhosa reduced IgM from 87 to 59 mg/100 ml. Presumably the 28 mg removed is specific IgM typhoid antibody. The results of similar calculations for all six sera of Table 40 are summarized in Table 41. Between 22 and 32 mg IgM anti-typhoid/100 ml serum were removed by S. typhosa at the peak of the response. Table 41 also relates these antibody concentrations to the other IgM-globulins in these sera. IgM typhoid antibody accounted for 19 to 32% of the total IgM-globulins in their respective sera and, with one exception, accounted for the majority of the increase in IgM-globulins that had occurred since day 0.

3. Typhoid Agglutinins: H, O and Vi agglutinins were titered in day 0, 7, 10, 14 and 28 bleedings of the eight children studied.

Table 41 IgM Removed by S. typhosa

Subject	Day	IgM Anti-Typhoid mg/100 ml	% of Total Serum IgM(a)	% IgM Increase From Day 0(b)
PR	7	28	32%	60%
	10	22	31%	71%
MM	10	32	18%	45%
MR	10	25	25%	86%
WS	10	32	20%	56%
	14	27	19%	63%

(a) Antibody IgM on indicated day ÷ total serum IgM on indicated day x 100.

(b) Antibody IgM on indicated day ÷ (total serum IgM on indicated day - day 0 level of same child) x 100.

Peak levels for each antigen occurred on days 7 to 14 in every case. At their highest values, O agglutinins ranged from 1:320 to 1:2560 and H microtiters ranged from 1:10,240 to 1:81,920. Vi titers were not detected in M.M.'s sera and were low in the others, not surpassing 1:80 in any child. The H, O and Vi agglutinins of sera listed in Table 41 were apparently due to macroglobulin antibodies since titers were below 1:20 after reduction with 2-ME.

H, O and Vi agglutinins were also titered in aliquots of supernatant fluids left after the absorption shown in Table 40 to monitor removal of these antibodies by S. typhosa. Table 42 summarizes representative data of one child. As expected, S. milwaukee did not lower H, O or Vi agglutinins from their unabsorbed (saline) values. In contrast, S. typhosa removed essentially all Vi and O agglutinins and reduced H titers 4-fold on day 7 and 8-fold on day 10. This pattern of essentially complete removal of anti-Vi and anti-O but only a 2- to 8-fold reduction of anti-H was found in all sera absorbed with S. typhosa. Thus, since a portion of the IgM anti-H remained in the supernatant fluids, the actual concentrations of antibody may be higher than those calculated in Table 41. For reasons not entirely clear, absorption of these sera at up to 1:5 dilutions with larger numbers of S. typhosa generally did not remove significantly more IgM or H agglutinins. Perhaps the portion of this early IgM antibody not absorbed has a very low binding strength for its antigen.

Table 42 Agglutinins Following Absorption of Sera of PR

Antigen	Day	Absorbent: Saline	Absorbent: S. milwaukee	Absorbent: S. typhosa
H	0	<10	<10	<10
	7	10,240	10,240	2,560
	10	10,240	10,240	1,280
O	0	<20	<20	<20
	7	1,280	2,560	<20
	10	1,280	1,280	<20
Vi	0	<10	<10	<10
	7	80	80	<10
	10	40	40	<10

4. Non-Specific Absorption of IgM-Globulins: All interpretations of these data hinge upon the assumption that only specific IgM was absorbed by S. typhosa. This contention is supported by the failure of this organism to remove IgM in sera taken before immunization and by the failure of S. milwaukee, lacking typhoid O, H and Vi antigens, to reduce IgM in post-immunization sera. It remained conceivable, however, that small amounts of anti-typhoid in immune sera could trap significant amounts of non-anti-typhoid IgM during absorption. The experiment summarized in Table 43 tested this possibility. Antiserum was obtained

Table 43 Failure of Rabbit Anti-S. typhosa to Non-specifically Trap Normal Human IgM

Subject	Day 0 Serum PBS S. typhosa	Day 0 Serum Rabbit Anti-Typhoid S. typhosa
WS	101	108
MM	111	113
MR	73	77

IgM assayed in supernatant fluids by quantitative precipitins is expressed in mg human IgM/100 ml of day 0 serum. .08 ml each of day 0 human serum, 5×10^{11} S. typhosa/ml and either PBS or rabbit anti-typhoid sera were incubated and centrifuged as before. Human IgM was not detected by quantitative precipitins in a control of equal parts of rabbit anti-typhoid, S. typhosa and PBS.

[illegible]

[illegible]

In the preceding sections, the quantities of antibody directed to the vaccine strain of typhoid (Ty21a) were measured in the sera taken from four children during the days when their IgG globulins were tagged. These same sera were absorbed with the O and with H suspensions. Table VI compares the IgG removed by these cells with that absorbed chemically by equal concentrations of the Ty21a organism. As one also knows for the vaccine (O+H+Vi) strain, saline of the same chromatographic fraction of IgG globulins non-specifically. But O concentrations often absorbed with O or O+H were unchanged from the values obtained when no cells were added. In contrast, Ty21a removed IgG from all pooled human sera and to reduced levels in sera from two of the four children.

The quantities of IgG specific for each chromatographic area calculated by subtracting the IgG left after absorption from the corresponding un-absorbed (saline) value. The results, shown in Table VII, indicate O antigen removed the least. Ty21a removed intermediate amounts and Ty21a

Table 44 IgM Remaining in Sera after Absorption with Different Strains of S. typhosa[a]

Subject and Vaccine Dose	Day	mg IgM/100 ml Serum Remaining After Absorption with Indicated Antigens: Unabsorbed (Saline)	0 (0901)	O+H (H901)	O+H+Vi (Vaccine)
PR	0	40	40	43	41
.5 ml	7	87	75	65	59
day 0	10	71	65	56	49
MR	0	70	72	74	72
.5 ml	10	99	98	82	74
day 0					
MM	0	108	111	107	108
.5 ml	10	179	182	156	147
day 0					
WS	0	103	104	101	106
.5 ml	10	160	147	129	128
day 0 and 7	14	146	131	122	119

(a) PR and MM were absorbed with 1.5×10^{11} of each organism/ml of serum, while MR and WS were absorbed with 2.5×10^{11}/ml of serum.

Table 45 Concentrations of IgM Removed by Various Strains of S. typhosa

Subject	Day	mg IgM Antibody/100 ml Serum: 0 (0901)	O+H (H901)	O+H+Vi (Vaccine)
PR	7	12	22	28
	10	6	15	22
MR	10	1	17	25
MM	10	-3	23	32
WS	10	13	31	32
	14	15	24	27

took the most IgM from these sera. Presumably these values represent antibody specific for the different absorbents. We expected to calculate the amounts of IgM directed to the individual typhoid antigens by a series of subtractions of these data. Anti-O should have equaled the IgM absorbed by O antigen, anti-H should have equaled anti-O+H minus anti-O, while anti-Vi should have equaled anti-O+H+Vi minus anti-O+H.

It was only possible, however, to discern IgM specific for the O antigen with this information. O, H and Vi agglutinins were titered in aliquots of all unabsorbed (saline treated) and absorbed sera to monitor the removal of the corresponding antibodies. Table 46 illustrates representative data from a single child. The three strains of typhoid

Table 46 Titers of Agglutinins Remaining in Sera After Absorption with Different Strains of S. typhosa[a]

Agglutinin Titered	Day of Bleeding	Absorbent: None (Saline)	0 (0901)	O+H (H901)	O+H+Vi (Vaccine)
O	7	1,280	<20	<20	<20
	10	1,280	<20	<20	<20
H	7	10,240	10,240	5,120	2,560
	10	10,240	10,240	2,560	1,280
Vi	7	80	80	20	<10
	10	40	40	<10	<10

[a] Sera of PR.

removed essentially all O agglutinins from the children's sera, and the organism possessing only O did so without reducing H or Vi titers. This suggests the latter organism removed only that IgM specific for its O antigen. But the titers also indicated absorption with these organisms could not be used to differentiate IgM specific for H and Vi. First, although neither O+H nor O+H+Vi removed all H agglutinins, the latter organism consistently reduced these titers slightly more than the former. Thus, the difference between IgM absorbed by O+H+Vi and O+H is not necessarily all anti-Vi; some may be anti-H. Further, O+H although allegedly devoid of Vi, consistently reduced Vi agglutinins by one or two 2-fold dilutions. Therefore, the difference between IgM removed by O and O+H is not necessarily all anti-H; some may be anti-Vi.

As shown in Table 45, sera of two children lost small amounts of IgM to the organism containing only O antigen. To substantiate that

this represented anti-O, the O and O+H+Vi strains were held at 100°C for 2.5 hours and washed three times with PBS. Although this treatment does not destroy the heat-stable O antigen, it eliminates the heat-labile H antigen and releases Vi into solution where it can be washed away. Single sera of the children with measurable anti-O were absorbed with these new preparations of somatic antigen. Only O agglutinins were removed by the heated organisms and as shown in Table 47, both strains removed 10 mg of IgM/100 ml from the day 7 bleeding of PR and 15 to 19 mg/100 ml from the day 10 serum of WS. These values are similar to the 12 and 13 mg removed respectively by the unheated O strain and suggest the latter organism removed only IgM specific for the heat-stable O antigen.

Table 47 IgM Remaining in Sera after Absorption with Heated S. typhosa 0901 and Vaccine[a]

		mg IgM/100 ml Serum Remaining After Absorption with Indicated Antigens		
Subject	Day	None (Saline)	O (Boiled 0901)	O (Boiled Vaccine)
PR	0	38	39	38
	7	88	78	78
WS	0	99	97	99
	10	159	140	144

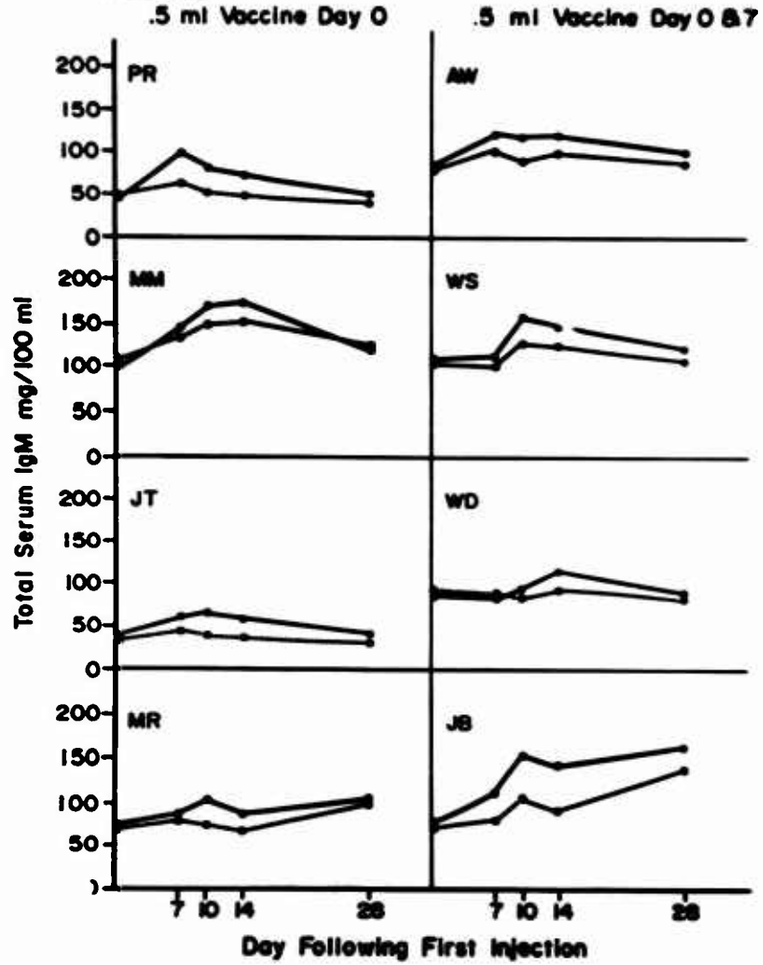
(a) Sera were absorbed with equal volumes of 2.5×10^{11}/ml of both organisms.

6. IgM Removed by Polymerized Flagellin: Since H titers were at least 1:10,000 at the height of the IgM response while the greatest Vi titer was only 1:80, it seemed likely that most of the anti-typhoid was anti-H. We therefore measured the IgM absorbed from sera by purified H antigen in the form of polymerized flagellin.

The general method of absorption was similar in principle to that used for whole bacteria. Absorption was carried out with 1.5 mg flagellin/ml to avoid non-specific removal of IgM encountered when more than 5 mg/ml of flagellin was used. Sera taken near the time when antibody levels were maximal were also absorbed with 3 mg/ml to see if additional IgM could be removed.

Figure 35 compares total IgM levels (absorbed only with saline) with the IgM left after absorption with flagellin for all bleedings. In every

Figure 35. Removal of IgM by polymerized flagellin at different times during the response to typhoid vaccine. The area between the unabsorbed IgM curve (•————•) and curve of IgM remaining after absorption (□————□) represents specific antibody removed by 1.5 mg flagellin/ml of human serum.



child the two curves originate at a common point indicating IgM was not removed from day 0 sera. They then diverge showing relatively large amounts were absorbed at the peak of the response and finally begin to converge again by day 28. The area between the curves presumably represents specific antibody, and its rapid increase and decline are consistent with the view of the IgM response gained by titration data.

The quantities of specific antibody removed near the height of the response by 3 mg flagellin/ml serum are summarized in Table 48. Flagellin at this concentration usually absorbed more IgM than it had at 1.5 mg/ml, although again IgM was not taken from pre-immunization sera. Measured in this way, the children produced 22 to 76 mg of IgM antibody/100 ml serum within two weeks of immunization. With the exception of MM, anti-typhoid accounted for 20 to 50% of their total IgM levels and at least 76% of the increase in IgM-globulins induced by vaccine. The response of MM differed from that of the other children, since both flagellin and whole bacteria (vaccine) removed less of his post-immunization of IgM than either removed from any of the others.

7. Specificity of IgM Removed by Flagellin: Agglutinins titered before and after absorption indicated flagellin absorbed anti-H more effectively than any of the whole bacteria. H titers were reduced 16- to 64-fold by flagellin as compared with only a 2- to 8-fold drop following treatment with O+H or O+H+Vi organisms. As expected, Vi agglutinins were not changed by flagellin so there was no indication that IgM of this specificity was removed. But since O titers were reduced 2- to 8-fold, absorption with flagellin did not distinguish between IgM anti-O and anti-H. To be certain IgM anti-O represented a minor component in all eight children, the sera listed in Table 48 were absorbed with the O bacterial antigen. As shown in Table 48, the same two individuals previously found to have measurable anti-O (Table 45) were the only children who lost definite quantities of IgM to boiled S. typhosa 0901. Therefore, the majority of IgM antibody elicited by typhoid vaccine appears to be specific for the H antigen.

8. Failure of a Flagellin - Anti-Flagellin Complex to Trap Normal Human IgM: Equal volumes of normal human sera and the ten-day rabbit anti-S. typhosa serum were mixed with polymerized flagellin so that the human sera were exposed to 3 mg flagellin per ml and diluted 1:10. After the usual incubation and centrifugation at 60,000 G, quantitative precipitins indicated all human IgM remained in the supernatant fluids. Normal IgM was not trapped by polymerized flagellin combined with its specific antibody.

C. Discussion:

Children immunized with S. typhosa vaccine have elevations in total serum IgM-globulins averaging 60% above baseline within ten days.

Table 48 IgM Absorbed by Flagellin From Sera Taken Near the Height of the Response to Typhoid Vaccination

Vaccine	Subject	Day	mg/100 ml Absorbed by: Flagellin[a]	mg/100 ml Absorbed by: O[b]	% All[c] IgM Absorbed by Flagellin	% Total[d] IgM Increase Absorbed by Flagellin
.5 ml day	PR	7	41	14	42%	77%
0	MM	14	22	1	13%	30%
	JT	10	27	7	40%	93%
	MR	10	40	1	38%	121%
.5 ml day	AW	10	34	-1	29%	103%
0	WS	10	49	18	31%	100%
and 7	WD	14	22	-2	19%	76%
	JB	10	76	-3	50%	104%

(a) Sera were absorbed with 3 mg flagellin per ml of serum.

(b) Sera were absorbed with 2.5×10^{11} S. typhosa 0901 that had been held at 100°C for 2.5 hours and washed three times with PBS.

(c) IgM anti-flagellin on indicated day ÷ total IgM on indicated day x 100.

(d) IgM anti-flagellin on indicated day ÷ (total IgM on indicated day - day 0 level of same child) x 100.

This finding confirmed and extended that antibody responses and antibody levels of this immunoglobulin and that the vaccines stimulated found in army recruits could have been due to the booster of immunogenicity they received during basic combat training.

Most of the IgM globulin increase was specific and typhoid the cause of the magnitude of the IgM rise. Because antibody response increase in bactericidal antibodies that are specific for endotoxin related to the immunogen, and because added endotoxin would have been required to elicit non-antibody immunoglobulins, we anticipated that most of the increase would probably not be specific for S typhosa. The children's response to typhoid vaccine, however, was accompanied by elevations of from 22 to 32 mg IgM/100 ml serum was specific typhoid antibody and this accounted for 19 to 27% of total IgM globulins and to fifty of the more of the increase in IgM from pre-immunization levels. All of these estimations may be lower than actual values since only a portion of the agglutinins were measured by absorption. Lipopolysaccharide prepared from the typhoid bacillus removed most of this and, and showed that the IgM rise appeared specific for the immunizing endotoxin.

The technique of measuring antibody by studying the decrease in serum immunoglobulins that follows absorption with homologous endotoxin as applied here has limited potential as an analytical method. The greatest disadvantage is insensitivity since antibody must accumulate for a significant proportion of the total serum immunoglobulin class to be detected. At least 5 to 10 mg of IgM antityphoid per 100 ml serum was necessary for measurement against the background of normal serum IgM. Total serum IgA and IgG levels are higher than IgM levels, so even more antibody would be required for a similar quantitation of these antibodies.

The technique does, however, have a few basic advantages over other quantitative methods. It depends only on the most basic characteristic of antibody - the ability to combine with antigen. Precipitation is not necessary, nor are the washing steps of quantitative agglutination which might elute antibody of low avidity. Also, antibody of a single class can be quantified without prior separation steps. Finally, the total response to infectious agents with multiple surface antigens is measurable.

The assay of IgM anti-O was more definitive than that for anti-H or anti-Vi because absorption with S. typhosa O-901 deprived the supernatant and exclusive removal of antibody directed to the somatic antigen. Sera so treated lost essentially all O agglutinins while H and Vi titers remained unchanged. Further, after the O-901 and reactive antibody had been held at 100°C for 2.5 hours and washed, they both removed approximately the same amounts of IgM as untreated O-901. Since heat destroys H antigens and elutes Vi to be washed away, all of the IgM absorbed by untreated O-901 appears specific for the heat-stable O somatic antigen. And this antibody accounted for a minor portion of the total anti-typhoid IgM.

The specificity of that anti-typhoid not directed to the O antigen was less certain, but the data suggest practically all of it was anti-H rather than anti-Vi. Absorption of sera with purified H antigen as polymerized flagellin removed even more IgM-globulins and more H agglutinins than any of the bacterial absorbents; however, purified flagellin removed more than just anti-H. Although Vi agglutinins were not affected, some O agglutinins were absorbed along with H antibody. Apparently, small amounts of the somatic antigen had been carried with flagellin during the purification procedure. The absorption of anti-O with the anti-H was of little consequence in these measurements since the concentrations of IgM absorbed by O were low or not measurable. It therefore appears that practically all of the anti-typhoid at the height of the IgM response is specific for the H determinant on flagellin.

IgM antibody has several properties that suggest this class of immunoglobulin is suited for combating bacterial infections in the systemic circulation. First, IgM is essentially limited to blood; only about 10% of total body IgM is in extravascular tissues. Second, IgM has very potent anti-bacterial properties. IgM anti-S. typhimurium O promotes opsonization and bactericidal effects 100 to 1000 times as well as the IgG counterpart. Also horse IgM anti-pneumococcal polysaccharide was shown to protect mice from the corresponding organism as well as 100 and 100,000 times as much IgA and IgG antibodies of the same specificity. Third, systemic bacterial infections are life threatening. So any protective mechanism would have to be brought into play rapidly to be of value. The IgM response complies with this; macroglobulins generally have the first and dominant antibody activities during the early period following exposure to new antigens. The present study adds yet another property that would be of obvious advantage to the host with a serious infection. IgM antibody is not only made rapidly but also in quite large amounts. Within 7 to 14 days, these children produced 5 to 30 mg of specific IgM per 100 ml of serum. By assuming a plasma volume of 5%, one can calculate that a 25 kg child is capable of producing ½ to 1 gram of IgM antibody within this period. In all probability, however, most of the IgM induced by vaccine in these experiments is of little value in prophylaxis. Of the specificities induced by S. typhosa, antibodies directed to O and Vi are generally considered most important while anti-H, predominating in these children, seems to have little value. It would be interesting to compare concentrations of IgM elicited by the three typhoid antigens following infection. Perhaps in this situation, greater quantities would be specific for the more important O and Vi antigens.

18 Ecological Studies of Western and Eastern Equine Encephalitis in Maryland.

Epizootics of eastern equine encephalitis have occurred at three to five year intervals in Eastern Maryland, Delaware, and New Jersey over

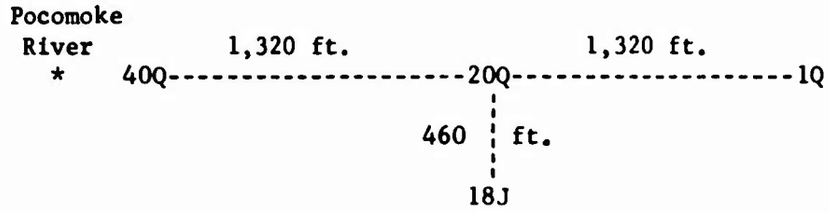
the past few decades. One such episode occurred on the Chesapeake Bay's Eastern Shore in 1956; another in 1960. After the 1960 outbreak, ecological investigations were undertaken by WRAIR on the Eastern Shore to study this phenomenon, and eastern equine encephalitis (EEE) and western equine encephalitis (WEE) viruses were isolated in 1964 from mosquitoes collected in the Pocomoke River Swamp, located a few miles south of Pocomoke City, Maryland. In 1965, a third epizootic of EEE occurred at a time when WRAIR was continuing ecological studies in the swamp. Studies have continued to the present time, and in 1968, data were collected in the course of a fourth epizootic of EEE on the Eastern Shore. Thus, for the first time, the cycle from one epizootic to a second epizootic has been documented with data collected over many years in the same study area using essentially the same methods. Following is a description of the 1968 swamp study and the 1968 epizootic. Trends in the ecology of the principal viruses from the 1965 to the 1968 epizootics are discussed.

A. Study Approach:

Ecological studies completed prior to 1968 satisfactorily demonstrated that WEE and EEE viruses were maintained each year within the Pocomoke River Swamp, at least from June through October, via mosquito-bird-mosquito cycles. It was shown that wild birds function as reservoir hosts, and that the principal and possibly only ecologically significant vector is the mosquito Culiseta melanura. In Maryland, this mosquito occurs predominantly in river swamps. In the Pocomoke Swamp, C. melanura larvae apparently live in subterranean cavities within the swamp floor. Such cavities can result when a tree falls and roots are displaced. Adult mosquitoes disperse to some distance outside of the swamp, but their greatest densities are maintained within it. Data collected in 1967 indicated that mosquito densities were greater in some parts of the swamp than in others. Since vector densities could conceivably correlate with the amount of virus transmission, the 1967 data suggested that some areas of the swamp might be more favorable than others for maintenance of viruses. The 1968 study was designed to evaluate this hypothesis.

Most of the 1968 effort was directed towards a more complete description of the swamp ecosystem than what had been achieved in past years. Much of the emphasis in previous years had been on upland sites or on the edge of the swamp. However, in 1968 over 75% of the data collected came from within the swamp. Only a single upland site was included in the 1968 study, and it was very close (20-60 ft) to the swamp. The upland site, named 18J, was used to obtain some comparative data between swamp and upland. Work within the swamp was done along a line extending from the Pocomoke River to 2,640 ft. into the swamp. Three sites on the line, called the "Q-line", were singled out for special emphasis. The sites had been studied in 1966 and 1967, when they were called 40Q, 20Q and 1Q. The 40Q site was 50-100 ft. into the swamp from the Pocomoke River. The 20Q site was another 1,320 ft. into the swamp, and 1Q was an

equal distance from 20Q (2,640 ft. from 40Q). The upland site (18J) was perpendicular to the line connecting the other sites and was located approximately 460 ft. from 20Q.



The three swamp sites and the upland site were compared to determine if virus activity in some locations was greater than that in others. Antibody appearance rates in sentinel animal populations and virus infection rates in mosquito vector populations were used as indicators of virus activity.

B. Methods:

1. Sentinel Studies: Work completed in 1966 through 1967 showed that Bobwhite Quail (Colinus virginianus) were good sentinel animals. They survived in the swamp environment, were infected with EEE and WEE viruses, and produced detectable neutralizing antibody after infection. Thus, quail were used again in 1968. Four to six week old birds were purchased from a farm in Savannah, Georgia. Upon arrival, the quail were maintained indoors under close surveillance until 2-2½ months of age. Prior to using birds as sentinels, blood samples were taken to make certain that WEE or EEE neutralizing antibodies were not present. When birds were moved to the swamp, two cages of ten birds each were established at sites 18J, 40Q, 20Q and 1Q. The sentinel quail studies started on 15 March 1968. Replacement birds were added to cages to compensate for mortality. Alternate cages of quail were bled on consecutive weeks such that each bird was sampled at approximately 14 day intervals. Blood samples were taken by jugular venipuncture with syringes wetted with a dilute heparin solution.

Plasma samples were used in neutralization tests for detecting WEE and EEE antibodies. The procedure used was similar to that described in the 1967 Annual Report. In brief, sera were diluted 1:5 and challenged with 10-100 $TCID_{50}$ of infectious virus propagated in cell culture. Viruses used were WEE m-3249/65 and EEF m-2449/64; both strains were initially obtained as mosquito isolates from the Pocomoke River Swamp and identified as either EEE or WEE virus by comparison with prototype strains. WEE m-3249/65 had been passed twice in hamster kidney cell culture; EEE m-2449/64 had been passed twice in mice and once in hamster kidney cell culture. After incubation at 37°C for 30 minutes, the challenged sera were inoculated onto confluent monolayers of primary

hamster kidney cells. Culture tubes were examined periodically for cytopathic effect and compared to control tubes. If cytopathic effect did not occur, a serum was considered to contain antibody to the challenge virus. If equivocal results were obtained, the sample was retested.

2. Mosquito Population Studies: As in past years, mosquitoes were captured by CDC miniature light traps. Two traps were operated side by side at 1Q, 20Q, 40Q and 18J. Double traps were employed to reduce the risk of losing a site sample on any given night due to light trap failure, a malady for which the traps are well known. Early in the study, traps were operated one night a week. After 1 July, mosquitoes were collected on two consecutive nights each week. Mosquitoes were killed at -65°C and sorted on ice. Pools of one to 25 mosquitoes were prepared according to species, sex, location and date of capture and stored at -65°C until virus isolation attempts were made. Pools were ground in Ten Broeck grinders to which 1.5 cc of diluent had been added. The diluent consisted of Eagle's basal medium containing 20% heat inactivated fetal bovine serum, bicarbonate and antibiotics. The ground suspensions were centrifuged for 15 minutes at 3000 rpm to remove particulate debris, and 0.1 ml of the supernatant was inoculated into two or three primary hamster kidney cell culture tubes, which subsequently were examined for cytopathic effect over a period of five days. If an isolate was recovered from a mosquito pool, it was passed in cell culture and identified using WEE and EEE antisera prepared in domestic rabbits. Reisolation was attempted from the original mosquito pool, and if accomplished, the reisolated agent was identified by neutralization test with the appropriate antiserum. Where reisolation could not be achieved, the identity of the initial isolate was confirmed in a second neutralization test with the appropriate antiserum. All isolation work was performed in a room quarantined against materials known to contain group A arboviruses.

Mosquito larval populations were studied at 40Q, 20Q, 1Q and at several additional sites on the "Q-line". Sites, 10Q and 15Q were located between 1Q and 20Q; sites 25Q and 30Q were located between 20Q and 40Q. One other site, 10L, located near 10Q was studied. Three holes were dug at each of these sites on 5 March 1968. The holes were roughly square, with 2-2½ ft. on a side. Earlier (2 Nov 67) a smaller (approx 1 ft sq) hole was dug at each location. The smaller holes were sealed closed with 3/4"x2'x2' plywood boards and covered with leaf litter. The three open holes at each site were sampled regularly by dipping for larvae. The holes sealed with boards were opened and samples only three times during 1968.

Larvae were collected from two other habitats. Several old holes, which were dug early in 1967, were sampled. These were essentially round and averaged 15" in diameter. Three large standing pools of water were sampled which measured 6'x18', 14'x43', and 9'x25'.

from August to mid November 1966 an experiment was conducted to determine how larval population would change in a variety of tidal environments to sites totally open to the atmosphere. Four 1m square, were dug near the water's edge. These were offset 2 m apart with the tubes "A" and "C" were randomly paired & positioned that a pond was at the base. Thus, mosquitoes were prevented access to the tubes. Tubes "B" and "D" remained uncovered. The four tubes were prepared by taking the sand removed from each hole and screening it to remove any mosquito larvae dipped. Then, each was returned placing a measured volume of larval population determined by using a standard dipper sites.

Collected larvae were preserved in 95% alcohol. All larvae were measured, and identified to species. The number of specimens of each species were recorded in a data sheet for each hole and sites.

Chemical analyses were performed on water samples taken from open holes at 10, 20, and 30 cm depth. Water samples were collected in glass bottles and refrigerated until the analyses could be performed. Chemical analyses were done using a Beckman Model G pH meter (Hellige Aqua Indicator) for pH and chlorides. The determinations were performed by the mercuric nitrate titration method. Other parameters measured were temperature, salinity, and dissolved oxygen. Water samples were collected under suction through No. 40 Whatman paper and transported to the testing.

). Mosquito Survey. The mosquito survey was conducted by MEAIR, consisted of the study of the mosquito fauna, University of Maryland. During the 1966 season, 7 stations of aquatic and flying mosquitoes were collected from the study area using a New Jersey light trap. Larvae were collected from flying mosquitoes, and pupae were reared to adulthood (Adult) and identified to species. The specimens were in Mi tests following the procedure given in the mosquito (1966-67). The identified to species.

The host preferences of mosquitoes, a wild birds, was studied during the season in 1966. The determination was done in the survey and in the laboratory with the bird meal. These data will be used to determine the mosquito tions are considered as a possible in the study. However, bird and mammal inclusion in this report

C. Results and Discussion:

1. The 1968 EEE Epizootic at Willard's, Maryland: In Maryland, birds began dying on or about 16 July at the Ronald Davis Farm (11,500 pheasants and 2,500 partridge), Willards. Deaths increased steadily between 16-26 July to approximately 200 pheasants per day and 10 partridge per day. On 26 July, the flock morbidity exceeded 10% for pheasants and 5% for partridge; brain and blood samples were collected from moribund birds. EEE virus was isolated from pheasant brains. Pheasant sera (13/13) were positive for EEE-HI antibody. Of 12 sera tested for neutralizing antibody (EEE-NT), seven were positive, one was equivocal, and four were negative.

Illness in equines was first reported on 19 July. A pony at Whaleysville, Md., was sick on 18 July and died the following day. Three ponies at Willards became sick on 24 July; two died and one recovered. The three ponies that died were EEE-HI equivocal and EEE-NT positive. The pony that recovered was EEE-HI positive, as well as EEE-NT positive, seven days after onset of disease. Other equine deaths occurred in the Willards vicinity, but specimens were not obtained from them.

The Communicable Disease Center (CDC), Atlanta, Georgia, sent a team to the Willards area to collect mosquitoes. A large number of EEE virus isolations were made by CDC from Culiseta melanura collected between 31 July and 6 August. However, no WEE isolations were made from that species. Three WEE isolations were obtained by CDC from other mosquito species. Considering these findings, WEE probably did not play a significant role in the Willards epizootic.

The last confirmed equine case of EEE in the epizootic occurred on 12 August at Berlin, Maryland, a town approximately eight miles southeast of Willards. EEE virus was isolated from the brain of this equine at WRAIR.

Overall, the Willards epizootic lasted from mid-July to mid-August. No human cases of EEE were reported in the area; potentially dangerous locations were subjected to mosquito abatement by State authorities. However, the Willards epizootic was only one of several outbreaks of EEE which occurred in the summer of 1968. In Delaware, 1,500 pheasants died between mid-July and September; EEE virus was confirmed in two of four suspect flocks. Twenty suspect equine cases occurred in Delaware in the same period. An epizootic in New Jersey resulted in nine cases of human encephalitis between mid-July and mid-September (Morbidity & Mortality Weekly Report 17:371., 5 Oct 68), and 115 suspect EEE equine deaths were reported. Virus evidently continued to circulate in the environment in New Jersey after mid-September, since two additional human cases occurred between mid-September and early October.

2. Virus Activity in the Pocomoke River Swamp in 1968: EEE virus was first isolated in the swamp from a pool of C. melanura collected on 6 August (Table 49). Virus transmission, however, must have started

Table 49 Virus Isolations Made from Pools of Female Culiseta melanura Collected in 1968

Site	Date	Identification No.	Number in pool	Virus Isolated	Reisolation
1Q	6 Aug	m-300	25	WEE	yes
	13 Aug	m-321	21	WEE	no
	19 Aug	m-348	25	EEE	yes
	26 Aug	m-384	25	EEE	yes
		m-386	25	EEE	no
	28 Aug	m-415	25	EEE	yes
	2 Sep	m-469	25	EEE	yes
		m-471	25	EEE	yes
	9 Sep	m-523	25	EEE	yes
		m-527	25	EEE	yes
	8 Oct	m-642	25	EEE	yes
	23 Oct	m-697	25	WEE	yes
20Q	29 Jul	m-279	25	WEE	yes
	6 Aug	m-308	25	EEE	yes
	13 Aug	m-336	25	EEE	yes
		m-338	25	EEE	yes
		m-340	25	WEE	yes
	19 Aug	m-357	25	WEE	yes
	28 Aug	m-411	25	WEE	no
	2 Sep	m-426	25	EEE	yes
		m-427	25	EEE	yes
		m-444	25	EEE	yes
		m-453	25	WEE	yes
	1 Oct	m-624	25	EEE	yes
	8 Oct	m-635	25	EEE	yes
40Q	19 Aug	m-377	25	EEE	yes
	9 Sep	m-530	25	WEE	yes
18J	29 Jul	m-283	16	WEE	yes
	14 Oct	m-649	21	WEE	yes

prior to that time, since two sentinel quail at 1Q were found to have EEE-NT antibody in blood taken on 6 August, and work by this laboratory has shown that quail do not develop neutralizing antibody until at least

seven days post-infection with virus. The average rate of infection in the C. melanura population from 26 August to 23 October was at least one infected for each 852 individuals tested (1:852), and the peak of EEE infection in C. melanura occurred from 26 August to 3 September. The last EEE virus isolation came from mosquitoes collected on 8 October. However, very few mosquitoes were collected thereafter, and virus still may have been circulating in the C. melanura population.

WEE virus appeared before EEE. WEE was isolated from sentinel blood collected on 22 July. Isolations were made from C. melanura collected on and after 29 July (Table 49). Virus infection rates in the C. melanura population remained relatively constant until 28 August, after which a slight decrease occurred (Table 50). WEE was found in

Table 50 WEE and EEE Virus Infection Rates for Female Culiseta melanura Mosquitoes Collected During 1968 in the Pocomoke Swamp, Maryland.

Period	Number Tested	Infection Rate WEE	Infection Rate EEE
1 May - 9 Jul	2,723	0	0
14-15 Jul	2,718	0	0
21-22 Jul	407	0	0
29 Jul	302	1:151	0
5-6 Aug	730	1:730	1:730
12-13 Aug	611	1:306	1:306
19 Aug	923	1:923	1:462
26-28 Aug	881	1:881	1:294
2-3 Sep	1,929	1:1,929	1:386
9-10 Sep	1,372	1:1,372	1:686
16 Sep - 23 Oct	2,538	1:1,269	1:846
29 Oct - 18 Nov	209	0	0
Year Totals	15,333	1:1,394	1:852

C. melanura collected up to 23 October. Over the year, the WEE infection rate (1:1,394) was about half the EEE rate.

Sentinel conversion (antibody appearance) rates provided insight into virus transmission trends (Table 51). A sentinel conversion in serum collected on a given date indicated that virus was transmitted to that quail 7-14 days previous (not earlier, since birds were bled every two weeks). Sentinel WEE-NT conversions started at a relatively high level

Table 51 The Appearance of Neutralizing Antibody in the Sera of Sentinel Quail

	Jun 5	Jun 12	Jun 27	Jul 1	Jul 8	Jul 15	Jul 22	Jul 29	Aug 6	Aug 14	Aug 20	Aug 28	Sep 4	Sep 10	Sep 17	Sep 25	Oct 1	Oct 9	Oct 15	Oct 24	Oct 29
WEE-NT																					
1Q	-	-	-	-	-	-	-	2*	3	5	6	1	1	1	-	-	-	-	-	-	-
18J	-	-	-	-	-	-	-	4	-	2	4	1	4	-	-	1	1	-	-	-	-
20Q	-	-	-	-	-	-	-	1	4	3	3	2	1	1	-	-	-	1	-	-	-
40Q	-	-	-	-	-	-	-	1	2	1	4	6	2	0	2	-	-	-	1	-	-
all areas	-	-	-	-	-	-	-	8	9	11	17	10	8	2	2	1	1	1	1	-	-
EEE-NT																					
1Q	-	-	-	-	-	-	-	-	2	9	5	-	2	1	-	-	-	-	-	-	-
18J	-	-	-	-	-	-	-	-	-	6	2	4	5	-	-	-	-	-	-	-	-
20Q	-	-	-	-	-	-	-	-	-	4	4	3	3	1	-	-	-	1	1	-	1
40Q	-	-	-	-	-	-	-	-	-	-	1	4	3	3	-	1	1	0	3	1	-
all areas	-	-	-	-	-	-	-	-	2	19	12	11	13	5	-	1	1	1	4	1	1

* No. of birds exhibiting neutralizing antibody for the first time.

on 29 July and reached a slight peak by 20 August. A decrease occurred from 20 August to 10 September, after which the number of WEE susceptible sentinel quail in the swamp was not adequate to measure WEE transmission trends. However, conversions occurred sporadically among the few susceptibles that were left. Of a total of 77 quail sentinels resident in or near the swamp between 22 July and 29 October, only four failed to produce WEE-NT antibody. In contrast, EEE-NT conversions first appeared on 6 August and reached a very high, early peak one week later (14 August). Conversions were slightly lower during the three weeks which followed. A dramatic decline in EEE-NT conversions occurred thereafter, except for a second small peak observed on 15 October. Only five quail failed to produce neutralizing antibody to EEE between 6 August and 29 October.

The initial high rate of WEE-NT conversions observed on 29 July and the initial low rate of EEE-NT conversions observed on 6 August may be effects of mosquito density (Table 52). The WEE-NT conversions resulted via virus transmission in the period 15-22 July. On 15 July, the C. melanura population was at the summer maximum, although numbers declined between 15-22 July. The EEE-NT conversions resulted via virus transmission in the period 22-29 July, when the C. melanura population was very low. Thus, in the early phases of virus transmission, the potential for WEE transmission was greater than the potential for EEE virus transmission.

The incidence of EEE-NT conversions differed with study site, whereas the incidence of WEE-NT conversions did not (Table 51). WEE-NT conversions started on the same date at the four study sites, and about the same number of birds produced WEE-NT antibodies between 29 July and 10 September at each location. To determine if there were significant differences in the number of WEE-NT conversions at different sites or on different dates, 29 July through 10 September data (Table 51) were subjected to analysis of variance. Differences were not statistically significant. EEE-NT conversions first appeared deep in the swamp at 1Q. The following week, EEE-NT antibody appeared in birds at 18J and at 20Q. Conversions at the river, at 40Q, did not begin until later. The majority of sentinels at 1Q converted in the two week period 6-20 August. Birds at sites 18J and 20Q required a four week period (6 August-10 September). Conversions at 40Q were drawn out over a much longer period of time. Thus, compared to EEE virus transmission to sentinels deep in the swamp at 1Q, transmission was about half as fast in the 18J-20Q region and relatively slow near the river. Since WEE virus transmission was the same everywhere, it appears that the initial distribution or the mechanism of transmission for EEE must in some way differ from that for WEE. However, the same mosquito species, Culiseta melanura, transmits both viruses. Perhaps another vector, mosquito or otherwise, is being overlooked for WEE. Furthermore, since WEE was initially more dispersed than EEE, WEE may be continually present in the swamp, whereas EEE may be introduced each year from outside.

Table 52 Female Mosquitc Densities as Determined by Light Trap Capture

Date**	Total	Culiseta melanura	Other
April 23	4	0	4
May 1*	3	2	1
8*	5	1	4
15*	157	128	29
21*	42	27	15
28	1099	607	492
June	152	95	57
[illegible]	426	196	230
[illegible]	305	212	93
[illegible]	652	453	199
July	400	308	92
	223	201	22
	703	493	210
	1429	1365	64
	1441	1353	88
	218	198	20
	239	209	30
	345	302	43
Aug 5*	269	248	21
[illegible]	489	482	7
12*	95	95	0
13*	523	516	7
19	1009	923	86
26*	272	265	7
27*	115	113	2
28	508	503	5
Sep 2	1380	1367	13
3	565	562	3
9	639	630	9
10	751	744	7
16	141	140	1
17	72	70	2
24	241	231	10
25	581	557	24
30	13	13	0

Table 52 (continued)

Date	Total	Culicoides spp.	Others
Oct 1	129	125	4
7	36	32	4
8	239	235	4
14	392	387	5
15	293	293	0
22	325	313	12
23	165	162	3
29	4	4	0
Nov 4	70	69	1
5	117	115	2
11	5	5	0
18	25	16	9

* All sites not represented on these dates due to trap failure.

** Light trapping began approximately 1900 hours on given dates.

). Tested Species and Populations: After 15 July, Culiseta melanura appeared to be the predominant mosquito species in the swamp (Table 51). Small numbers of other species were collected (Table 53) and tested for virus, but no isolations were made. Likewise, no virus was recovered from any mosquitoes present before 15 July. Thus, as in the past, C. melanura must be considered the primary vector of WEE and EEE in the Freemans River Swamp. However, the potential of other species to act as supplementary vectors cannot be ruled out since some species may have been attracted only slightly to light traps. Thus, adequate samples of these species have not been obtained for the measurement of virus infection rates. One such species is Culex salinarius. In past years, two isolations of EEE virus have been made from C. salinarius pools collected from the swamp. In 1968, only small numbers of this species were collected. Furthermore, other species of mosquitoes, whose probable presence in the swamp is indicated by large numbers of bites, were not captured in light traps in 1968. The vector potential of these species remains unmeasured.

Study sites differed in the numbers of infected Culiseta melanura present (Table 49) and in the total number of C. melanura present (Table 50). These two categories of data were somewhat correlated. The greatest densities, as indicated by light traps, occurred at 1Q. Captures of C. melanura at 20Q were usually lower. To determine if there was an overall significant difference between these sites, data given in Table 50 were subjected to a χ^2 Sign Test (Steel & Torrie, 1960. Principles and Procedures of Statistics. McGraw-Hill, N.Y.). Only values were used where all sites were represented on the given collection date and if a total of 100 or more mosquitoes was collected on a given date. Over the year, captures at 1Q were significantly greater than captures at 20Q (χ^2 = 6.3, $p < .025$). However, approximately equal numbers of WEE and EEE virus isolates were obtained from the two sites. Light trap captures were higher at 20Q than at 40Q (χ^2 = 6.3, $p < .025$) and C. melanura densities at 40Q were consistently higher than at the upland site 18J (χ^2 = 8.0, $p < .005$). Few virus isolates were obtained at sites of low mosquito density (40Q and 18J).

From the preceding data, a possible mode of transmission of EEE virus through the swamp can be inferred. EEE virus may have been introduced into the swamp at or near 1Q where it proliferated rapidly due to the abundance of mosquitoes in that area. Sentinels at 1Q were infected rapidly in the process (Table 51). Soon virus was spread to 20Q, where a considerable mosquito population also was present. The upland site 18J was close to 20Q, and EEE virus may have arrived there via mosquitoes dispersing from 20Q. Strangely enough, sentinels at 18J converted as or more rapidly than those at 20Q even though 18J had a very small population of C. melanura. A different arthropod vector may have caused sentinel infection in the upland. Finally, EEE-NT conversions at 40Q appeared after 18J and 20Q. The virus may have reached 40Q from 20Q in

Table 53 Female Mosquitoes Collected by Light Trap (Other Than *Culiseta melanura*) in 1968

Species	#	Capture period
Aedes cantator	10	1-21 May
	756	28 May - 9 Jul
	46	14 Jul - 14 Oct
	812	
Aedes canadensis	40	23 Apr - 21 May
	466	28 May - 1 Jul
	150	8 Jul - 19 Aug
	7	26 Aug - 10 Sep
	663	
Culex salinarius	1	15-21 May
	74	28 May - 9 Jul
	83	14-15 Jul
	70	21 Jul - 19 Aug
	8	26 Aug - 18 Nov
	236	
Anopheles bradleyi-crucians	11	28 May - 19 Jun
	32	26 Jun - 15 Jul
	12	22 Jul - 17 Sep
	53	24 Sep - 23 Oct
	3	29 Oct - 5 Nov
	111	
Aedes vexans	47	12 Jun - 9 Jul
	17	14 Jul - 22 Oct
	64	
Aedes atlanticus	21	28 May - 26 Aug
Anopheles punctipennis	21	15 May - 23 Oct
Aedes sollicitans	17	12 Jun - 25 Sep
Uranotaenia sapphirina	13	19 Aug - 23 Oct
Mansonia perturbans	9	15 Jul - 3 Sep
Aedes taeniorhynchus	8	12 Jun - 25 Sep

Table 53 (continued)

Species	#	Capture period
Culiseta inornata	8	18 Nov
Culex territans	5	21 May - 15 Jul
Aedes triseriatus	5	15 Jul - 2 Sep
Anopheles quadrimaculatus	4	14 Jul - 24 Sep
Psorophora ferox	3	12 Jun - 1 Jul
Psorophora confinnis	1	22 Jul
Toxorhynchites rutilis	1	21 Jul

Table 54 Numbers of Female Culiseta melanura Trapped at Different Sites

Date**	Site 1Q	20Q	40Q	18J
1 May	ns*	1	0	1
8 May	0	1	ns	ns
15 May	87	ns	36	5
21 May	19	ns	6	2
28 May	249	44	286	28
3 Jun	17	63	6	9
12 Jun	127	19	24	26
19 Jun	32	63	104	13
26 Jun	202	114	93	44
1 Jul	80	131	42	55
8 Jul	97	71	12	21
9 Jul	341	49	73	30
14 Jul	607	351	195	212
15 Jul	859	250	125	119
21 Jul	100	90	4	4
22 Jul	25	151	18	15
29 Jul	75	150	55	22
5 Aug	33	116	99	ns
6 Aug	250	116	92	24
12 Aug	50	ns	ns	45
13 Aug	96	368	ns	52
19 Aug	304	485	96	38
26 Aug	183	69	ns	13
27 Aug	98	12	ns	3
28 Aug	329	132	39	3
2 Sep	677	561	101	28
3 Sep	406	70	73	13
9 Sep	401	104	95	30
10 Sep	550	100	44	50
16 Sep	110	21	7	2
17 Sep	36	25	7	2
24 Sep	142	66	15	8
25 Sep	329	181	41	6
30 Sep	0	0	5	8
1 Oct	69	43	11	2
7 Oct	26	0	4	2
8 Oct	121	61	43	10
14 Oct	239	55	72	21
15 Oct	154	16	105	18
22 Oct	84	129	73	27
23 Oct	90	31	9	12
29 Oct	0	3	0	1

[illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]

[illegible]

the same fashion as from 20Q to 18J. Thus, 1Q and 20Q may have functioned as reservoirs of EEE infected mosquitoes, which dispersed to other places such as 18J and 40Q.

4. Distribution of Mosquito Larvae in the Pocomoke River Swamp: The species composition of mosquito larval populations differed with size of habitat, location in the swamp and time of year (Table 55). In natural pools of water, Culex territans was essentially the only species present. This species also was common in the open holes dug early in 1968. Culex territans was abundant in these holes from mid-July to late October and 1Q to 40Q. C. territans was found less frequently in the smaller (15" dia.) old holes dug in 1967.

Culiseta melanura was abundant after mid-June in the old holes located near 20Q. Larvae were often found in the 1968 holes between 20Q and 1Q. C. melanura larvae were seldom collected from the natural pools or in the 1968 holes between 25Q and 40Q. However, the species may be present everywhere in the swamp within subterranean cavities. The plywood sealed holes dug from 1Q to 40Q are thought to resemble such cavities. When opened on 19 August, no larvae were observed in any of them. These holes could not be resealed tightly, and adult mosquitoes probably had access to them via small openings at the corners. When subsequent collections were made on 28 August and 23 October, C. melanura larvae were found in all enclosed holes which contained water, including 40Q. In fact, it was the only species present in the holes, which suggests that C. melanura is the only larval species which characteristically occurs in that type of habitat. The open vs. covered hole experiment conducted at 15Q also indicated that C. melanura occurs in greater abundance within covered holes (Table 56).

Covered holes probably represented a habitat intermediate between open holes and holes sealed with plywood boards. Each of the three Culex species were found in about equal numbers in open and covered holes. Thus, these species are capable of surviving in a semi-enclosed environment. Culiseta melanura larvae made up the greatest percentage of the total larval population in covered holes. The abundance of C. melanura in covered holes may be due to a preference among adult females for egg deposition in enclosed places.

Aedes canadensis larvae were abundant early in the season at all open sites except 40Q, where it was never found (Table 55). After April, A. canadensis was most commonly found in the old holes dug in 1967. The species virtually disappeared from most sites by mid-July.

Culex salinarius was never collected deep in the swamp at 1Q, 10Q, or 10L. It was rarely found at 15Q and 20Q. The species was occasionally abundant closer to the river between 25Q and 40Q.

Table 55 The Percentage of the Total Population of Larvae 3 mm or More in Length Made up by Various Species of Mosquitoes

Site	Time period	Samples in period	Per cent (%)				
			Aedes canadensis	Culiseta melanura	Culex salinarius	Culex restuans	Culex territans
Natural Pools	24-29 Apr	1	100	0	0	0	0
	16 Jul	1	0	11	0	0	89
	6-30 Sep	4	0	0	0-10	0	90-100
Old Holes	24-29 Apr	2	99-100	0	0	0-1	0
	8-30 May	4	78-100	0-12	0	0	0
	5-12 Jun	2	94-100	0-6	0	0	0
	20-27 Jun	2	35-46	54-65	0	0	0
	1-29 Jul	5	16-67	20-68	0	0-12	0-13
	8-21 Aug	3	0-13	63-87	0-28	0	0-32
	6-30 Sep	4	0-18	82-100	0-2	0	0-2
1Q	24-29 Apr	2	92-100	0-8	0	0	0
	16 Jul	1	0	70	0	0	0
	6-30 Sep	4	0	0-8	0	0	92-100
	10-29 Oct	4	0	5-19	0	0-86	6-95
	11 Nov	1	0	26	0	51	23
10L	24-29 Apr	1	100	0	0	0	0
	6-30 Sep	5	0-9	0	0	0	91-100
	10-29 Oct	3	0	3-73	0	0	27-97
	11 Nov	1	0	83	0	17	0

Table 55 (continued)

Site	Time period	Samples in period	Per cent (%)				
			Aedes canadensis	Culiseta melanura	Culex salinarius	Culex restuans	Culex territans
10Q	24-29 Apr	1	100	0	0	0	0
	6-30 Sep	5	0-5	0-10	0	0	85-100
	10-29 Oct	4	0	0-8	0	0	92-100
	11 Nov	1	0	81	0	0	19
15Q	24-29 Apr	1	100	0	0	0	0
	16 Jul	1	8	8	0	0	84
	6-30 Sep	5	0-5	0-4	0-4	0	92-100
	10-29 Oct	3	0	0-9	0-3	0	91-97
20Q	24-29 Apr	2	100	0	0	0	0
	16 Jul	1	15	0	0	33	52
	6-30 Sep	5	0	1-40	0-3	0-7	53-96
	10-16 Oct	2	0	8-19	0	0	81-92
	22-29 Oct	2	0	7-8	0	85-91	1-8
	11 Nov	1	0	20	0	79	1
25Q	24-29 Apr	1	100	0	0	0	0
	16 Jul	1	6	0	0	53	41
	6-30 Sep	5	0-2	0	4-42	0-22	37-96
	10-16 Oct	2	0	0-3	0-20	0-6	74-97
	22-29 Oct	2	0	0	0	90-95	5-10
	11 Nov	1	0	3	3	91	3

Table 55 (continued)

Site	Time period	Samples in period	Per cent (%)				
			Aedes canadensis	Culiseta melanura	Culex salinarius	Culex restuans	Culex territans
30Q	6-30 Sep	5	0-26	0	0	0-5	62-100
	10-16 Oct	2	0	0	0-33	0-36	64-100
	11 Nov	1	0	0	0	0	0
40Q	24-29 Apr	2	0	0	1-6	94-98	0-1
	16 Jul	1	0	0	80	17	3
	6-30 Sep	5	0	0	24-75	0-69	0-43
	10-29 Oct	4	0	0	0	0-100	0-100
	11 Nov	1	0	0	0	100	0

Table 56 Mosquito Larvae Collected in the Open Versus Covered Hole Experiment Conducted 15 August - 14 November 1968

	Species of Larvae			
	Culex salinarius	Culex territans	Culex restuans	Culiseta melanura
Covered sites:				
hole "A"	21	31	0	131
hole "C"	16	44	23	101
Open sites:				
hole "B"	3	5	0	21
hole "D"	28	70	4	1
Sums:				
Covered sites	37	75	23	232
Open sites	31	75	4	22
Species composition (%)				
Covered sites:	10.1	20.4	6.3	63.2
Open sites:	23.5	56.8	3.0	16.7

Like C. salinarius, C. restuans was frequently found near the river. It often comprised the major part of the larval populations in the region from 40Q to 20Q. C. restuans was infrequently abundant deeper into the swamp. It was never collected in the natural pools and only a few larvae were ever found in the old holes dug in 1967.

In some cases, larval abundance correlated with light trap captures of adults. A. canadensis larvae were abundant in old holes until late June (Table 55), and most adults of this species were captured before July (Table 53). Culiseta melanura larvae were found more frequently after mid-June. Overall, adults of C. melanura were not captured in large numbers until mid-June (Table 52). By contrast, Culex territans and C. restuans were seldom captured in light traps, although the abundance of larvae of these species suggests that adults should have been abundant also.

Additional insight into larval distribution, and therefore factors which may determine adult distribution, was provided by a consideration of larval habitats.

5. Differences in Larval Habitats: The larval data clearly show that some species were more characteristic of breeding sites near the river (Culex salinarius and C. restuans) while others were not (Aedes canadensis and Culiseta melanura). In an effort to explain larval distribution, habitats were examined to determine their similarities and differences. Environmental parameters which were similar at all sites could be eliminated as influences on larval distribution in 1968. Parameters which did vary from site to site might suggest mechanisms by which larval populations were limited.

All larval habitats were similar in a few respects. The pH of swamp waters was about the same everywhere and equaled pH 4.6 or lower (Table 57). The acidity was in part due to the acidity of Pocomoke River water, which was pH 6.4 at Pocomoke City when measured by Hulme on 23 July 1963 (State Md. Dept. Geology, Mines & Water Res. Bull. 16, 1955. Baltimore). However, organic acids and free CO_2 produced via decomposition most certainly contributed to the acidity of waters within the swamp. It is unlikely that pH was a factor in determining the distribution of larvae.

Hydrogen sulfide was common throughout the swamp and indicated that anaerobic decomposition occurred at all larval sites. Thus, waters of larval habitats probably were low in dissolved oxygen content. The deoxygenation of swamp waters probably resulted via processes of decomposition. In addition, oxygen may have been removed from waters by bubbling of CO_2, methane, H_2S, etc. Since iron was plentiful (Table 57), oxidation of soluble iron to insoluble ferric hydroxide possibly contributed to deoxygenation.

Table 57 Chemical Analysis of Waters in the Pocomoke Swamp for 1968

	15 Aug 68	19 Sep 68	24 Oct 68	26 Nov 68
Turbidity (ppm):				
1Q	84	73	84	44
2Q	48	26	24	11
4Q	74	28	24	14
Silica (ppm SiO_2):				
1Q	3.9	3.2	1.0	3.0
2Q	7.7	9.3	1.0	9.0
4Q	>12.0	>12.0	2.1	9.2
pH:				
1Q	4.3	4.1	4.4	4.1
2Q	3.8	3.6	4.0	3.9
4Q	4.2	4.0	4.6	4.5
Dissolved Iron (ppm Fe)				
1Q	0.5	0.1	0.1	0.2
2Q	0.9	0.5	0.5	1.6
4Q	0.9	1.4	0.5	2.2
Total Iron (ppm Fe)				
1Q	1.2	0.5	2.0	0.2
2Q	1.0	1.1	1.1	1.9
4Q	0.9	1.9	0.9	2.2

Table 10 (continued)

	[illegible]	[illegible]	[illegible]	[illegible]
Sulfate (ppm SO_4)				
10	1.0	1.0	1.0	1.0
200	1.1	11.0	11.0	24.0
400	34.0	>130.0	>130.0	>130.0
Sulfite (ppm SO_3)				
10	12.0	11.0	..	11.7
200	3.0	3.6	..	3.6
400	3.0	3.3	..	3.6
Total Phosphates (ppm PO_4)				
10	1.0	0.6	2.2	0.3
200	0.7	0.4	0.3	0.2
400	1.4	0.7	0.3	0.1
Orthophosphates (ppm PO_4)				
10	0.4	0.1	1.6	0.2
200	0.3	0.2	0.3	0.2
400	0.6	0.7	0.4	0.1
Nitrates (ppm NO_3)				
10	0.5	3.5	5.5	0.5
200	1.5	0.5	3.5	1.5
400	2.5	0.5	4.0	0.5

Larvae resident deep in the swamp probably inhabited a more anaerobic aquatic environment than those near the river. The distribution of sulfates, sulfites and irons suggests such an interpretation. Sulfates were more abundant at 40Q than at 1Q, although 40Q concentrations were also much larger than river concentrations (ppm SO_4 = 15; Hulme, op. cit.). The increased concentrations of sulfates at 40Q may have arisen via oxidation of anaerobically produced sulfites. Although the concentration of total irons was not different in the swamp from the quantity measured for the Pocomoke River by Hulme in 1963 (ppm Fe = 1.4, op. cit.), dissolved irons were more abundant at 40Q than at 1Q, and suspended irons were frequently more abundant at 1Q. Probably soluble irons were oxidized to form insoluble suspended forms and these accumulated at 1Q. Flushing by the river may have reduced the concentration of suspended irons at 40Q. If in fact oxygen concentrations were higher at the river, and dissolved oxygen was not measured directly, it probably resulted from the penetration of river water through the swamp floor or by river flushing of larval holes. Thus, larval distribution may have been influenced by the effect of the river on the oxygen content of the water. However, physical disturbance caused by river flushing may have limited the survival of some species near the river.

Conditions at 1Q, and to a lesser extent at 20Q, were similar to those found in some bog situations. Whereas water at 40Q was light yellow, water from 1Q and 20Q was tea color; 1Q had the deeper color. Also, turbidity was greater deep in the swamp but could not be attributed to dissolved solids, since silica and dissolved iron were more abundant at 40Q. The tea colored water and high turbidity in the swamp indicated the presence of humic acids, tannins, lignins or other decomposition products in the form of organic colloids. Suspended but filterable (through Whatman No. 40) irons may have contributed to turbidity also.

The preceding data indicate that some substances were in greater abundance at some sites than at others. Areas near the river probably had the greatest concentrations of dissolved oxygen, sulfates, dissolved irons and silicates. Areas deeper in the swamp had increased concentrations of free CO_2, sulfites and organic compounds. It is not unlikely that bacterial and plankton populations would differ at sites that are so dissimilar. If such populations did not differ in species content, the relative abundance of each species may have differed. However, biological productivity in the holes dug in 1968 was probably about the same everywhere in the swamp since phosphate and nitrate concentrations, which are good indicators of productivity, were similar at the three sampling sites (Table 57). These observations suggest that mosquito-larval food may differ with site in kind but not in quanti . The mosquito-larval distributions for Culiseta melanura, Aedes canadensis, Culex restuans and C. salinarius may have depended to some degree on food quality rather than food availability.

6. Factors Which Limit the Distribution of Mosquito Species:

Summary: It is clear from foregoing results, that no species of larva is found with the same frequency at all places or in all habitats within the swamp. Culex territans was the most cosmopolitan species, and it may have either wider chemical tolerances or a greater ability to survive varied physical conditions than do the other species. However, even Culex territans was limited. It was not present in the plywood sealed holes, and it was not abundant in the small holes dug in 1967. Aedes canadensis never was found at 40Q in 1968; perhaps this species could not tolerate the frequent flushing by river water. On the other hand, Culex restuans and Culex salinarius were found most frequently nearer the river, between 20Q and 40Q. Culiseta melanura larvae were found more frequently in open water between 1Q and 20Q than in the open waters closer to the river.

Various mechanisms may be at work which limit the distribution of mosquito larvae in the swamp. Some species may be influenced by the amount of dissolved oxygen in the water. Food quality could limit survival. There is also the possibility of competition. The presence of C. melanura near the river in open holes may be precluded by the abundance of Culex in the region. Competition with other species may be less intense or absent within enclosed habitats. Indeed, competitive forces may have selected for a Culiseta melanura population in which females actively seek enclosed places to lay eggs. Or like A. canadensis, C. melanura larvae may prefer the more stable environment in the deep swamp, which probably more closely reflects the anaerobic conditions which prevail within cavities in the root mat.

The distribution of adult mosquitoes is probably not as limited as the distribution of their larvae, since adults can disperse, or be dispersed, to considerable distances after emergence. But at least in some species, adult mosquito populations reach their highest densities at the sites where larvae are most frequently found and where larvae are most abundant. The C. melanura data show that larvae were most frequent around 1Q, where the greatest adult captures were made. Preliminary analyses of adult capture data for several other species collected in 1968 also show site preferences. Therefore in some species, the key to adult distribution is larval distribution, and larval distribution depends on habitat conditions. Eggs which are deposited in habitats which are not suitable simply will not develop through the larval instars. In periods of virus transmission, the greatest number of EEE and WEE infected C. melanura will be present in areas where there are suitable breeding sites. EEE virus transmission probably will be more rapid at such locations, although WEE virus transmission may not be.

In order to understand mosquito distribution in the swamp, much more systematic work will be required on interactions between larval species and with the parameters, biotic and abiotic, of their environments. Experiments must be performed in the laboratory and in the field to determine what limiting factors are at work. Such knowledge is of

particular interest for vectors of disease. When the factors which regulate the distribution and survival of C. melanura are known, the capacity to predict the times and locations of adult densities could be at hand. It may even be possible to enhance the effects of natural regulating mechanisms to reduce C. melanura larval populations. Thus, it might be possible to prevent a large emergence of adults, such as that which occurred on 14-15 July in 1968. As adult populations diminish so does the potential for virus transmission. In effect, epizootics similar to the Williards episode of 1968 might be prevented. If emergence could not be controlled, then at least there would be a forewarning of a potentially dangerous situation, and mosquito abatement could be initiated. However, prediction and control of vector population densities will only be possible after further studies of larval populations and the variables which regulate them.

7. Mosquito Infection Rates: 1964-1968: Epizootics of EEE have occurred with a periodicity of three to five years on the Eastern Shore of Maryland. Mosquito data collected from 1964 through 1968 in the Pocomoke River Swamp demonstrate the trends in WEE and EEE virus infection in one such cycle (Table 58). The year 1964 was characterized by low infection rates for both viruses in the C. melanura population. In 1965, however, EEE virus reached epizootic proportions, and WEE virus infection was increased also. The overall infection rate (WEE & EEE) was 1:495 individual mosquitoes. EEE infection decreased in 1966. However, WEE virus infection continued to climb. WEE was as common in 1966 as EEE had been in 1965, and the overall infection rate for 1966 (1:484) was the same as that observed in 1965. In 1967, rates for both viruses dropped. No EEE virus isolates were made from C. melanura, but a single isolation was obtained from Culex salinarius. Virus infection again increased in the Culiseta melanura population in 1968. The increases in WEE and EEE observed from 1967 to 1968 were similar to increases observed from 1964 to 1965. Of course, the 1968 EEE increase was to epizootic proportions.

If the trends indicated by Table 58 continue to hold true, then the year 1969 should evidence a substantial decrease in EEE infection in the C. melanura population. Also, 1969 should be a year when WEE virus reaches epizootic proportions.

The number of female Culex salinarius captured in 1968 was approximately an order of magnitude lower than what would have been predicted on the basis of collection data for the years 1964 through 1967 (Table 58). C. salinarius has twice provided EEE virus isolates. Therefore, population fluctuations, as the reduction in 1968, are of interest. No specific reason for the 1968 decrease can be given at this time, but the data suggest that 1968 was a year of low density for mosquito species other than Culiseta melanura.

Table 10 [illegible] and [illegible] Incidence from Three Cohort Studies [illegible] in the Framingham Heart Study [illegible]

	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Number of					
Incidence					
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Number of					
[illegible] [illegible]					
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Number of					
[illegible] [illegible]					
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Person-Years Data					
Sex					
[illegible] [illegible]					
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

* [illegible] [illegible] [illegible] [illegible]

Summary.

1. Acute Respiratory Disease in the 12 Basic Combat Training (BCT) posts from July 1968 to May 1969 was caused principally by Adenovirus Types 4 and 7 (ADV-4 and ADV-7). Live Adenovirus Type 4 oral vaccine (L-AV-4) was given to incoming recruits at seven posts in early winter 1969 and resulted in the suppression of ADV-4 associated ARD by a time when 75% of the BCT strength was immunized. In most posts, ADV-7 became the predominant pathogen after L-AV-4 immunization and in the Northern most posts was associated with high ARD rates during spring 1969. A_2/HK/68 influenza occurred on most BCT posts in December and January but accounted for less than 20% of ARD hospitalization in those months. Type B influenza caused significant morbidity at Fort Bliss and mi morbidity at Forts Bragg and Wood during February 1969.

2. Three separate studies of a live, enteric-coated, Type 7 Adenovirus oral vaccine (L-AV-7) were undertaken. Administration of the vaccine to volunteers resulted in no adverse effects. L-AV-7 containing $10^{5.4}$ TCD_{50} virus caused gastrointestinal infection without illness and produced type-specific serum N antibodies in all susceptible volunteers immunized. Simultaneous administration of both L-AV-4 and L-AV-7 vaccines was safe and no significant decrease in immunogenicity of either vaccine was demonstrated. The administration of L-AV-4 and L-AV-7 vaccines to trainees at Fort Dix, N.J. produced serum ADV-7 N antibodies in 85% of susceptibles immunized, and resulted in a 47% suppression of febrile ARD and a 96% suppression of ADV-7 associated ARD as compared to a group immunized with L-AV-4 and a placebo capsule.

3. An epidemic of A_2 influenza was first recognized in Hong Kong in July 1968. A_2/HK/68 strains were readily isolated in embryonated eggs or monkey kidney tissue culture. The serologic response of individuals infected with this strain resembled a primary antibody response. Significant differences in the antigenic composition of the A_2/HK/68 strains to previous A_2 strains was evident from HI and neutralizing antibody tests; hyperimmune rooster antisera to a 1957 A_2 strain did not neutralize A_2/HK/68 strains, nor did hyperimmune rooster antisera to the A_2/HK/68 strains neutralize the 1957 strain. The 1968 military polyvalent influenza vaccine did not induce significant titers of N antibody to A_2/HK/68 strains in immunized recruits.

4. Treatment of human sera with a slurry of DEAE-Sephadex removed all serum components except IgG and removed all non-specific inhibitors of hemagglutination to influenza A_2, arbovirus and rubella virus hemagglutinins. The extraction procedure is simple and reproducible and may represent a universal method for removal of non-specific serum inhibitors to a variety of viral hemagglutinins.

5. Rubella HI titers were measured on over 4,200 sera obtained from all women entering WRAIR and Dewitt Army Hospital prenatal clinics. Approximately 10% of the women were seronegative to rubella, historical recall of previous rubella or lack of rubella infection by individuals did not predict their susceptibility to rubella. A plaque reduction neutralization test for rubella was developed with LLC-MK2 cells, this serologic test was found more sensitive than existing neutralization tests for rubella.

6. Serial serum specimens from patients developing allergic reactions after administration of equine anti-rabies serum were tested for antibodies to whole horse serum and horse IgG and for clearance of horse IgG-globulins. While all eight patients developed high or moderate antibody titers to whole horse serum, none developed significant antibody titers to equine IgG and the clearance of equine IgG from their serum was exponential. Precipitins, measurable in three patients, were directed against equine alpha or beta globulins and not equine gamma globulins. Despite their allergic reactions to horse serum, these patients did not develop detectable antibodies to equine IgG (containing the [illegible] antibodies) and did not clear equine IgG by an immune mechanism.

7. Children immunized with typhoid vaccine responded with an increase in serum levels of IgM. The major part of this IgM increase was specific anti-typhoid antibodies, and antibodies to the flagellar antigen (H) constituted most of the specific anti-typhoid IgM response.

8. Isolates obtained from patients with dengue-like disease in 1968 epidemics in Jamaica and Tahiti were clearly identified as dengue type 1, although a few isolates from Jamaica were dengue type 2. Type 3 strains were also isolated retrospectively from specimens obtained from an epidemic in Malaysia in 1960.

9. Three physically defined dengue antigens were previously isolated from infected mouse brain by sucrose gradient fractionation, 1) rapidly sedimenting HA antigen (RHA) containing infectious virus particles measuring 45 mu, 2) slowly sedimenting HA antigen (SHA) containing non-infectious 14 mu particles, and 3) a soluble non-sedimenting, non-hemagglutinating CF antigen (SCF) containing 7 mu particles similar to surface projections on RHA. RHA was first detected in mouse brain two days after infection, SHA on day 3, and SCF on day 4. Large quantities of SHA and SCF were present at 5-6 days immediately prior to death. SCF was found circulating in blood at similar levels found in brain, while RHA infectivity in blood was 10^5-fold less than that found in brain. SHA could be derived from RHA by extraction with Tween-80 or treatment with cobra venom.

10. Kinetic analysis methods suggested that SHA blocked neutralization of RHA by antibody prepared against crude virus but not by antibody raised against purified virus. Antibody raised to purified virus neutralized infectivity in crude virus suspensions to a greater rate than antibody raised to crude virus suggesting use of the former as a reference research reagent for kinetic analysis techniques. Use of purified virus (RHA) in standard plaque reduction neutralization tests was unsuccessful; antibody titers were drastically reduced from titers obtained using crude virus suspensions due to aggregation of the purified virus releasing infectious virus on dilution.

11. Migration of RHA, SHA, and SCF in an electric field indicated that these antigens have a similar net negative charge. Their elution from inorganic precipitates suggested that the total charge of each antigen increased with its size. The elution characteristics of SCF in Sephadex gel filtration suggests a stoken radius of 25 angstroms or a diameter of 5 mu, consistent with measurements of 7 mu obtained from electron micrographs.

12. Purified SCF reacted incompletely with antibody prepared against crude virus, but exhibited the same degree of cross reactions as crude antigens except that type 1 SCF did not react with types 3 and 4 antibody and types 2 and 3 SCF did not react with type 4 antibody. RHA and SHA cross-reacted to a greater degree than SCF of crude antigens which contain mostly SCF and but small amounts of RHA and SHA. Dengue SCF antigens with the exception of type 3 produced single, discrete precipitin lines in agar double diffusion tests with homologous and heterologous antisera. Results suggested that common and type-specific antigens were linked on the same molecule rather than as physically separate antigens on the virion.

13. An epizootic of Eastern Equine Encephalitis occurred around Millard's, Maryland from mid-July to mid-August 1968. Eastern equine encephalitis (EEE) virus was first isolated in the Pocomoke Cypress Swamp, Maryland in early August and peak activity of this virus occurred in mid-August, it was last isolated in early October. Western Equine Encephalitis (WEE) virus was first isolated in late July and activity of this virus was fairly constant to September, WEE was last isolated in late October. Culiseta melanura was the predominant mosquito species in the swamp as determined by light trapping and larval surveys. EEE and WEE were isolated from mosquitoes of this species alone, overall infection rates for melanura were 1:856 for EEE and 1:194 for WEE. The incidence of EEE antibody conversions in sentinel quail differed with site within the swamp, while the incidence of WEE antibody conversions did not. Differences in the larval habitats of mosquito species in the swamp were found and compared with the distribution of adult mosquitoes in swamp study sites.

Project 3A061102B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 166, Viral Infections of Man

Publications.

None.

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(U) Rickettsial Infections (09)

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PII Redacted

(1) Rickettsia, (2) Epidemic, (3) Murine, (4) Tsutsu

(U) Investigate the incidence and distribution of rickettsial diseases.

1) (U) Study typhus rickettsiae by immunological methods. 2) (U) Study of epidemic typhus rickettsiae in the U.S. 3) (U) Maintenance of rickettsial diseases for investigational purposes.

24 (U) Determine the host specificity of murine typhus rickettsiae in wild rodents. 2) Rickettsial immunochemistry. 3) Study host-parasite relationships of rickettsial infection and immunity. 4) Investigation of biochemical & physical properties of R. prowazekii. 5) Investigate feasibility of using fecal antigen in "Weil-Felix" test. 6) Study factors which influence rickettsial virulence.

25 (U) Study [illegible] rickettsiae by immunological methods. 2) Determine host specificity of murine typhus rickettsiae in wild rodents. 3) Study host-parasite relationships of murine typhus rickettsiae in reservoir hosts. 4) Investigate the biochemical & physical properties of R. typhi. 5) Investigate the feasibility of using fecal antigen in "Weil-Felix" test. 6) Study factors which influence rickettsial virulence.

26 (U) Study [illegible] rickettsiae by immunological methods. 2) Determine host specificity of murine typhus rickettsiae in wild rodents. 3) Study host-parasite relationships of murine typhus rickettsiae in reservoir hosts. 4) Investigate the biochemical & physical properties of R. typhi. 5) Investigate the feasibility of using fecal antigen in "Weil-Felix" test. 6) Study factors which influence rickettsial virulence.

Project 3A061102B710 COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 167 Rickettsial Infections

Investigators.

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Description.

During the current reporting period, research efforts have been concerned with: [1] the scrub typhus vaccine development program including (a) continued adaptation of newly recognized prototype strains in embryonated hens' eggs and (b) determining the applicability of certain physico chemical methods to the concentration and purification of scrub typhus rickettsiae from lightly infected yolk sacs; [2] collection of serologic evidence that (a) R. canada, a newly recognized member of the Typhus Group, causes acute febrile illness in man, and (b) an enzootic focus of infection with a member of the Typhus Group exists in the flying squirrel population near Montpelier, Virginia; [3] attempts employing indirect immunofluorescent technics to differentiate primary and secondary antibody responses in Typhus Group infections in man and to identify the class of immunoglobulins present in sera collected in the early phase of disease, during and after convalescence; [4] investigations of the anti rickettsial activity of a number of the newer antibiotics, and [5] studies to definitively identify 7 strains of Chlamydiae recently from the blands of 7 patients in Thailand.

Progress

1. Scrub Typhus Vaccine Development. Over the past several years the Department of Rickettsial Diseases has been evaluating the feasibility of developing a killed polyvalent vaccine which would protect man against the antigenically diverse strains of Rickettsia tsutsugamushi that exist in nature. An antigenic analysis of 70 strains of scrub typhus isolated in Thailand from patients, wild mammals and trombiculid chiggers was carried out. Classification of the scrub typhus strains was based upon the complement fixing reactivity of immune sera from guinea pigs infected with the wild strains. The antigens for the complement fixation test were comprised of partially purified suspensions of 7 different strains established by Japanese workers as prototype strains to represent all of the scrub typhus rickettsiae recovered in Japan, as well as other

distinctive that strains as they were recognized. Previous annual Reports have presented the evidence which demonstrated the presence of at least 8 antigenically diverse strains in the Thai collection. At the present time, in addition to the 3 Japanese prototypes, 21 additional Thai strains which are candidates for prototype status and for inclusion in the polyvalent vaccine include TA76, TA716, TA720, TA804 and TH101. The difficulties encountered in obtaining the concentrations of organisms in yolk sacs infected with the Thai strains needed for the preparation of antigens and experimental vaccines have been described previously. This problem must be resolved before studies establishing the purity of the candidate strain can be completed. In addition, characterization of the major and minor antigenic components of these agents must be carried out as this is a prerequisite to determining the fewest number of strains to be incorporated into a polyvalent vaccine to provide the broadest degree of protection against scrub typhus strains in nature. Consequently, efforts during the past year have been concentrated with (a) improving the growth of scrub typhus rickettsiae in the embryonated hens' eggs and (b) development of means of purification which would permit the preparation of large suspensions of scrub typhus rickettsiae from lightly infected yolk sacs.

a. Propagation of Candidate Prototype Thai Strains of Scrub Typhus. Serial passages of 3 to 7 lines of Thai strains TA76, TA716, TA720, TA804 and TH101 were made at approximately 10-day intervals in order to better adapt these agents to cultivation in embryonated eggs. The passages were carried out with freshly harvested yolk sacs. When bacterial contamination, or unidentified abnormalities in the agents from the eggs were encountered, stock tissue material from an earlier passage was used. Clarified stock suspensions of yolk sacs were examined microscopically to assess the concentration of organisms. In the succeeding 10 to 12 passages there has been little consistent improvement in the growth of any of the Thai strains. Other workers have reported that injection of embryonated eggs with substances pooled to concentrate with S. rickettsii increases infectivity titers of harvested yolk sacs. When the same doses of substances were administered to eggs yolk to infection with strains of S. tsutsugamushi, there was no improvement in growth of the rickettsiae. Attempts to promote the propagation of scrub typhus rickettsiae by injecting the eggs with 1 mg of antibacterials or antifungal agents failed.

b. Applicability of physicochemical methods for concentration to the purification of scrub typhus rickettsiae. An important prerequisite to the production of a potent experimental vaccine against scrub typhus and complement-fixing antigens is the preparation of concentrated and purified suspensions of S. tsutsugamushi organisms. However, a number of biological characteristics of this organism have made it a formidable and, to date, almost an insurmountable task

fixation completely detached from the slides during rinsing stages. Therefore, these substances had to be removed from the antigen suspension before the material could be examined satisfactorily. Covering the smears after fixation with a fine coat of acrylic plastic from a spray can did not interfere with the staining process but markedly reduced the loss of material from the slide.

Rickettsiae are known to maintain cellular integrity, infectivity and pathogenicity when suspended in solutions containing sodium chloride, potassium chloride, glutamate, sodium and potassium phosphate buffer systems and supplemented with various concentrations of sucrose and albumin (Bovarnick, M.R., et al., J. Bact. 59:509-522, 1950). Therefore, a solution with some of these constituents which closely mimics the composition of intracellular fluid, the natural habitat of rickettsiae, was devised for this series of studies. The final solution developed resembled K-7.5 (op. cit.), but differed by containing more electrolytes and less sucrose, and was called "Modified K-7.5." (See Table 1.) The pH of this solution was near 7.5, specific gravity = 1.010 and osmolarity was approximately 300 milliosmoles.

TABLE 1. MODIFIED K-7.5 SOLUTION

	Gm/L.	Millimoles/L.
[illegible]	9.000	125.00
[illegible]	.100	1.34
[illegible]	.160	1.19
[illegible]	1.300	10.9
[illegible]	7.5	21.9

[illegible] Density Gradient Centrifugation. Preliminary tests had shown that density gradient centrifugation was more effective if maximum amounts of pH and material were obtained from the suspension containing a [illegible] while removing the organisms. The following procedure, all performed at 4°C, were used to prepare infected yolk sacs for density gradient centrifugation. Sucrose and Sucrose 6:1 solution to produce a pH suspension (w/v) was added to density fractured infected yolk sac, homogenized by vibrating for three 30-second intervals separated by 30-second periods during which the suspension was not agitated

The homogenate was then centrifuged for 5-7 minutes at 150 g and 4 C. The midzone, that is, the volume between the sedimented cellular debris and floating fatty material, was harvested. This combination of low speed centrifugation and midzone harvest was performed 3 times. The midzone volume harvested from the third low speed centrifugation was then centrifuged at 20,000 g and 4 C for 30 minutes. After the supernatant containing soluble yolk material was discarded, the sediment composed of rickettsiae and yolk sac material was resuspended in an appropriate volume of Modified K-7.5 solution, carefully layered on a gradient. Ficoll, a synthetic inert non-ionized molecule of polymerized sucrose, was utilized for density gradients because of the following advantageous physical and chemical properties. The high molecular weight (about 400,000) and the extreme solubility of the compound allow for the formulation of solutions with both a high Ficoll content and specific gravity with comparatively low viscosity and negligible osmotic pressure. The low osmotic pressure was considered important in the preservation of the fragile R. tsutsugamushi. Ficoll gradients containing R. tsutsugamushi can be broken rapidly and easily by dilution without osmotic disruption of the organisms. A 50% (w/v) solution of Ficoll in Modified K-7.5 was used as the stock solution and was diluted with additional Modified K-7.5 to obtain the concentrations needed in the various studies.

Linear gradients ranging from 10 to 50% (specific gravity = 1.045-1.185) were prepared and the specimen applied. After 90 minutes centrifugation at 16,100 g the particles attained isopycnic position. All operations were carried out at 4 C. The scrub typhus rickettsiae did not accumulate within a narrow range of specific gravities as do many other cells and subcellular particles, but were distributed with the host tissue components throughout most of the gradient without significant concentration at any particular level. Although the cause for the distribution of the rickettsiae throughout this great range of specific gravities is not known, the following explanations may apply. The buoyant density of R. tsutsugamushi may vary widely, and therefore, any randomly selected population might contain organisms spanning a wide range of specific gravities. However, if the buoyant density of these organisms actually is within a narrow range, the great variation in specific gravity demonstrated could be attributed to an intimate association of the organisms with varying amounts of light or heavy density host products or other host tissue components.

The sensitivity of egg yolk lipoproteins to aqueous solutions is affected by sodium concentrations of magnesium sulfate (Hanahan, C and M *, Cook, Biochim Biophys Acta, 44-64, 1960). This phenomenon was utilized in an attempt to change the physicochemical relationship between R. tsutsugamushi organisms and the sodium yolk sac components, and thereby obtain a partial or positive purification separation of these organisms with the exception that 0.1 M magnesium sulfate with

solubilizes all but high density lipoproteins was substituted for Modified K-7.5 diluent, the processing of infected yolk sacs prior to density gradient centrifugation was similar to that previously described for the pretreatment of yolk sacs; i.e., 3 low speed centrifugations at 150 g from which the midzones were recovered, followed by one high speed centrifugation at 20,000 g. Compared with the K-7.5 treatment, the sediment resulting from the high speed centrifugation contained most of the rickettsiae and far less egg material. The majority of the yolk proteins was either in solution or floating near the surface and did not contribute to the sediment.

Although the processing of infected yolk sac suspensions with $MgSO_4$ solution was effective in partially purifying R. tsutsugamushi, it was evident that the residual host tissue components had to be removed before the rickettsiae could be concentrated. The high speed sediment was resuspended in 0.45 M magnesium sulfate and diluted slowly overnight with distilled water to 0.15 M in an attempt to remove lipoprotein, phosvitin and the livetins from the sediment. However, this treatment did not produce the desired results for the concentration of contaminants was not appreciably reduced.

When suspensions of rickettsiae partially purified by the magnesium sulfate treatment were subjected to Ficoll density gradient centrifugation, the organisms with the egg material again were distributed throughout the gradient. No distinct and definite concentration of a particular specific gravity was found. However, when infected yolk sac suspensions treated in a similar manner were placed on a Ficoll sucrose density gradient having a range of specific gravities comparable to the Ficoll gradients, bands containing both rickettsiae and egg material were detected at sucrose concentrations of approximately 33% and 37%. Attempts were made to purify each of the 2 bands by concentration on appropriate density sucrose gradients. There was no detectable change in the position of distribution of the lighter band, and the heavier band contained a 35-43% sucrose gradient. The approximate similarity in specific gravity of material in the heavier band may have been caused by a dissociation of particles containing egg-associated antigens and rickettsiae. It was concluded that preparations of yolk sac suspensions treated with magnesium sulfate and Ficoll density gradient centrifugation, although satisfactory, could not provide the high degree of purification required.

A [illegible] fraction of a number of attempts were made to concentrate rickettsiae from yolk sac suspensions by precipitation with ammonium sulfate. Fractions separated that contained the greatest portion of the rickettsiae often ammonium sulfate and other egg components were found with either the lipoproteins, the livetins or phosvitin. Attempts were made to purify rickettsiae and chromatograph egg-associated yolk antigens and antigenic [illegible] was found to

of biological properties. Although little is known of the factors determining the behavior of cells and particles in these aqueous polymer phase systems, the area as well as the chemical and physical properties of the surface are apparently significant. The usefulness of this less severe, highly efficient method of phase partition for the separation of scrub typhus rickettsiae from suspensions of infected yolk sacs was evaluated.

The constituents of all stock solutions and phase systems were measured on a weight basis because of the high viscosity of the polymer solutions.

Stock solutions of the various reagents were prepared in the following manner. A potassium phosphate buffer (KPB), consisting of equimolar proportions of KH_2PO_4 and K_2HPO_4, was used generally as the diluent in the phase systems and stock solutions. In certain specific instances a sodium phosphate buffer (NaPB), consisting of equimolar proportions of NaH_2PO_4 and Na_2HPO_4, or distilled water (D/W) was used. When diluents other than KPB were used, the stock solutions consisted of the polymer in the designated solution.

Dextran, marketed under the trade name "Dextran 500," was obtained from Pharmacia Fine Chemicals, New Jersey. The material used had a limiting viscosity number of 53 ml/g, weight average molecular weight of 490,000 and number average molecular weight of 185,000 and is referred to as D^{53}. A 20% (w/w) of D^{53} was utilized as the stock solution. Polyethylene glycol was obtained as "Carbowax 6000" from the Union Carbide Company, New York, New York. This product has a number average molecular weight of 6,000 to 7,500 and is referred to as PEG^{6000}. A 30% (w/w) solution of PEG^{6000} was used as the stock solution. Methylcellulose (MC), U.S.P., was obtained from Fisher Scientific Company, Fairlawn, New Jersey. Three different products, referred to as MC^{4000}, MC^{400} and MC^{15} were 1%, 3% and 5% (w/w), respectively.

In the potassium phosphate-polyethylene glycol-water phase system a 30% (w/w) stock solution of potassium phosphate (KPHOS) in distilled water was used. The potassium phosphate used was a mixture with the following ratio: 306.9 g K_2HPO_4 to 168.6 g KH_2PO_4. A 15% (w/w) stock solution of reagent grade $MgSO_4 \cdot 7H_2O$ in distilled water was used in the $MgSO_4$-PEG^{6000}-H_2O phase system. The concentration of the stock solution represents the actual amount of $MgSO_4$ and not the water of hydration.

The infected yolk sacs in the appropriate buffers were homogenized and centrifuged at low speed as previously described to partially remove cellular debris and extraneous fat. The midzone fluid recovered after the third low speed centrifugation was added to the phase systems.

In a number of instances, PEG^{6000} was added to the yolk sac-diluent mixture prior to blending in order to inhibit protein and lipoprotein denaturation. In most instances separation and partitions of the phases occurred after the mixture was allowed to stand at room temperature for varying periods of time (1 g/time). Because of the viscosity of certain phase systems, it was necessary to centrifuge the mixtures at low speeds to form the phases. The organisms were detected as previously described by indirect immunofluorescent staining after the hydrophilic polymers were removed by dilution with K-7.5 diluent and sedimentation by centrifugation. The amount of contaminating yolk was estimated after visual inspection of the harvested fractions.

The details and results of the experiments carried out with the methylcellulose (MC)-dextran (D) phase system are summarized in Table 2. After the low speed centrifugation, which was necessary to effect partition, all of the material in the infected yolk sac suspension was present in the bottom phase. Irrespective of the formulation of the partition system, rickettsiae and yolk sac components were distributed between a sediment and the remaining supernatant fluid of the dextran phase. There was no evidence of selective separation or concentration of the rickettsiae.

The results of attempts to purify Kato-infected yolk sac suspensions with polyethylene glycol (PEG)-dextran (D) phase systems prepared with either potassium phosphate buffer (KPB), sodium phosphate buffer (NaPB) and sucrose, or with $MgSO_4$ are presented in Table 3. Scrub typhus rickettsiae were not separated from the yolk sac material in the PEG^{6000}-D^{53}-KPB phase system. Somewhat different distributions were obtained when the partition was established after standing (1 g/22 hours) and after low speed centrifugation (225 g/40 min.). Nevertheless, the finding of rickettsiae in a particular phase was always accompanied with the presence of considerable amounts of host tissue components.

Unlike the methycellulose-dextran phase system, the distribution of molecules and particles in the polyethylene glycol-dextran system is affected by both the type of electrolytes in the media and the ionic strength. Therefore, in an attempt to alter the distribution of organisms and yolk material in the polyethylene glycol-dextran system, sodium phosphate buffer (NaPB) or $MgSO_4$ was incorporated into the system. Sucrose, which is distributed equally throughout both phases, was added when needed to prevent a hypo-osmolar state.

In comparison with the results obtained with the PEG^{6000}-D^{53}-KPB partition, separation of the phases of the PEG^{6000}-D^{53}-NaPB system after standing or centrifugation, was associated with a slight shift in the distribution of both rickettsiae and yolk from the interface into the

TABLE 2. PHASE PARTITION OF YOLK SAC SUSPENSION OF KATO STRAIN R. TSUTSUGAMUSHI IN METHYLCELLULOSE-DEXTRAN SYSTEMS

PRETREATMENT OF SPECIMEN	PHASE SYSTEM PREPARATION CONSTITUENTS	g FORCES AND TIME	DISTRIBUTION OF COMPONENTS OF SPECIMENS IN PHASES TOP (MC)	INTER-FACE	BOTTOM* FLUID	(D) SEDIMENT
1) 10% ys** (Kato) in 0.45 M $MgSO_4$ blended 3X	2% MC^{15}					
	2% D^{53}	165 g/10 min.	—	—	2 R	3-4 R
2) Centrifuged 3X @ 365 g/10 min.	10% ys				2 Y	3-4 Y
3) Midzone #3 centrifuged @ 20,000 g/30 min.	0.1 M KPB					
4) Sediment resuspended in 0.45 M $MgSO_4$	1% MC^{400}					
	2% D^{53}	165 g/10 min.	—	—	3 R	3 R
5) Diluted $\bar{c}$ D/W to 0.15 M $MgSO_4$	10% ys				3 Y	3 Y
6) Centrifuged @ 20,000 g/20 min.	0.1 M KPB					
7) Sediment resuspended in 0.5 M KPB	0.5% MC^{4000}					
	1.0% D^{53}	165 g/10 min.	—	—	3 R	3 R
8) Diluted $\bar{c}$ D/W to 0.10 M KPB	10% ys				3 Y	3 Y
9) Added to phase	0.1 M KPB					

* Concentration of R (Rickettsiae) and Y (Yolk sac components) graded on a scale from 0 to 6.

** Yolk sac.

upper phase. There was, however, no selective separation evident. (See Table 3.) The addition of $MgSO_4$ to the polyethylene glycol-dextran system containing infected yolk sac suspensions partially purified by $MgSO_4$ pretreatment resulted in a shift of the rickettsiae along with egg material from the top phase into the interface and bottom phase. (See Table 3.)

When partially purified infected yolk sacs were incorporated into polyethylene glycol (PEG)-potassium phosphate (KPHOS) phase systems, partition resulted in the fairly homogeneous distribution of organisms and egg material throughout all phases (Table 4). Changes of the concentrations of PEG^{6000}, potassium phosphate and yolk sac as well as the substitution of $MgSO_4$ for potassium phosphate resulted in some shifting of the rickettsiae from one phase to another along with the host tissue components.

Purification by a single phase partition depended upon the sequestration of the desired particles, the rickettsiae, either into one of the phases or the interface, with the exclusion elsewhere in the system of all the unwanted cell debris, lipids, proteins and other components. Alternatively, distribution of most of the components, either the rickettsiae or the host tissue material, into one of the phases, with a fairly uniform dispersion of the other component throughout all phases, could be utilized for effective purification provided phase partitions were carried out repeatedly.

The treatment of infected yolk sac suspensions with selected polymer phase systems; viz., methylcellulose-dextran, polyethylene glycol-dextran and polyethylene glycol-salt, did not result in either of the favorable distributions. R. tsutsugamushi was not separated from the egg material. Although there was a different distribution of the components when one type of phase partition was compared to another, in no instance were the rickettsiae distributed in a pattern different from that of the egg material.

2. Rickettsia canada: A New Member of the Typhus Group. The isolation of Rickettsia canada from Haemaphysalis leporispalustris ticks removed from indicator rabbits near Richmond, Ontario, Canada, was reported by McKiel et al. in 1967 (Canad. J. Microbiol. 13:503, 1967). On the basis of antigenic relationship with Rickettsia prowazekii and Rickettsia mooseri the microorganism was considered to be a new member of the Typhus Group of Rickettsieae. Additional information on the geographic distribution, the arthropod vectors and vertebrate hosts involved in the infection cycle in nature, and the pathogenicity of the rickettsia for man has not been published.

TABLE 3. PHASE PARTITION OF YOLK SAC SUSPENSIONS OF KATO STRAIN R. TSUTSUGAMUSHI IN POLYETHYLENE GLYCOL-DEXTRAN PHASE SYSTEMS

PRETREATMENT OF SPECIMEN	PHASE SYSTEM PREPARATION: CONSTITUENTS	PHASE SYSTEM PREPARATION: g FORCES AND TIME	DISTRIBUTION OF COMPONENTS OF SPECIMENS IN PHASES: TOP (PEG)	INTER-FACE	BOTTOM* (D)
1) 12% ys,** PEG^{6000} & 0.1 M KPB blended 3X	4.0% PEG^{6000}	225 g/ 40 min.	1 R	3 R	—
	5.0% D^{53}		2 Y	3 Y	—
2) Centrifuged 3X @ 225 g/10 min.	9.0% ys	1 g/ 22 hrs.	1 R	2 R	1 R
3) Midzone #3 into phase	0.1 M KPB		1 Y	2 Y	1 Y
1) 17% ys, 1.2% PEG^{6000}, 0.2 M Sucrose & 0.01 M NaPB blended 3X	3.6% PEG^{6000}	225 g/ 30 min.	2 R	1-2 R	—
	4.5% D^{53}		2 Y	2 Y	—
2) Centrifuged 3X @ 225 g/10 min.	0.2 M Sucrose	1 g/ 40 min.	2 R	2 R	—
3) Midzone #3 into phase	10% ys 0.01 M NaPB		2 Y	2 Y	—
1) 10% ys in 0.15 M $MgSO_4$ blended 3X	4.0% PEG^{6000}				
2) Centrifuged 3X @ 365 g/30 min.	5.0% D^{53}			2 R	1 R
3) Midzone #3 centrifuged @ 20,000 g/ 30 min.	4.0% $MgSO_4$	1 g/120 min.	—		
4) Resuspended in 0.15 M $MgSO_4$ & into phase	13.0% ys			3 Y	1 Y
1) 10% ys in 0.45 M $MgSO_4$ blended 3X					
2) Centrifuged 3X @ 365 g/30 min.	4.0% PEG^{6000}				2 R
3) Midzone #3 centrifuged @ 20,000 g/ 30 min.	5.0% D^{53}	1 g/ 30 min.	—	—	
4) Resuspended in 0.45 M $MgSO_4$	4.0% $MgSO_4$				
5) Diluted c D/W over 18 hours to 0.15 M $MgSO_4$	13.0% ys				3 Y
6) Added to phase					

* Concentration of R (Rickettsiae) and Y (Yolk sac components) graded on a scale from 0 to 6.

** Yolk sac.

TABLE 4. PHASE PARTITION OF YOLK SAC SUSPENSIONS OF KATO STRAIN *R. TSUTSUGAMUSHI* IN POLYETHYLENE GLYCOL-SALT SOLUTION SYSTEMS

PRETREATMENT OF SPECIMEN	PHASE SYSTEM PREPARATION CONSTITUENTS	& FORCES AND TIME	DISTRIBUTION OF COMPONENTS OF SPECIMENS IN PHASES TOP (PEG)	INTER-FACE	BOTTOM* (SALT)
1) 10% ys** (Kato), 4% PEG^{6000}, 0.1 M KPB & blended 3X	10.0% PEG^{6000} 10.0% K Phos. 4.0% ys	1 g/180 min.	2 R 2 Y	2 R 2 Y	1 R 2 Y
	7.9% PEG^{6000} 12.3% K Phos. 5.0% ys	1 g/ 45 min. 365 g/ 20 min.	4 R 4 Y 3 R 3 Y	1 R 1 Y 1 R 1 Y	1 R 1 Y 1 R 1 Y
2) Centrifuged 3X @ 365 g/10 min.	8.0% PEG^{6000} 14.0% K Phos. 4.5% ys	1 g/ 30 min.	2 R 2 Y	1 R 1 Y	± R ± Y
3) Midzone #3 into phase	7.0% PEG^{6000} 14.8% K Phos. 2.0% ys	1 g/ 60 min.	1-2 R 1-2 Y	1-2 R 1-2 Y	± R ± Y
1-3) as above 4) Midzone #3 centrifuged @ 20,000 g/30 min.	7.5% PEG^{6000} 15.0% K Phos. 10.0% ys	1 g/ 30 min.	2 R 2 Y	± R ± Y	± R ± Y
5) Resuspended in 0.1 M KPB & 3.0% PEG^{6000}	9.0% PEG^{6000} 6.0% $MgSO_4$ 9.0% ys	1 g/ 30 min.	— —	1 R 1 Y	1 R 1 Y

* Concentration of R (Rickettsiae) and Y (Yolk sac components) graded on a scale from 0 to 6.

** Yolk sac.

a. Serologic Evidence of Rickettsia canada Infection of Man. In the fall of 1968, paired serum specimens from a patient with a clinical diagnosis of Rocky Mountain spotted fever (RMSF) were submitted to the Department of Rickettsial Diseases, Walter Reed Army Institute of Research, for confirmatory serologic diagnosis. Complement fixation (CF) tests with a variety of soluble antigens prepared from members of the Spotted Fever Group failed to demonstrate antibodies, but tests with group-reactive and specific antigens prepared with members of the Typhus Group established a causal relationship with R. canada. This finding prompted the retesting of serum specimens submitted during previous years that had been obtained from: (1) cases diagnosed as RMSF which could not be substantiated by serologic tests, and (2) cases of RMSF confirmed by CF tests. Serologic evidence found thus far indicated that 4 patients had experienced a severe febrile disease caused by R. canada.

Group-reactive soluble antigens used in the study were prepared from yolk sacs infected with 2 different strains of R. rickettsi (Bitterroot and Sheila Smith) and R. akari (Hartford). In addition, 2 antigens were prepared from different passage levels of strain TT-118. The latter strain is a Spotted Fever Group rickettsia recovered from a mixed pool of Rhipicephalus and Ixodes larval ticks collected in Huai Mae Sanom, Thailand, in November 1962.

Each of the spotted fever soluble antigens exhibited a high degree of intragroup cross-reactivity. The difference in the complement-fixing antibody titer of each pool of antisera obtained from guinea pigs infected with the Spotted Fever Group strains detected in tests with 4 to 8 units of the homologous and heterologous antigens was 2-fold or less. None of the spotted fever soluble antigens fixed complement in the presence of a 1:10 dilution of sera obtained from guinea pigs infected with R. mooseri, R. prowazekii or R. canada.

The typhus group antigen was made by mixing equal volumes of soluble R. mooseri and soluble R. prowazekii antigens having comparable titers. CF tests showed that 4 to 8 units of the composite typhus group antigen detected the same level of antibody in murine and epidemic typhus immune guinea pig sera as was found when the same sera were tested with the homologous antigen only. The titer of R. canada immune guinea pig sera with the typhus group antigen was 2-fold lower than with the homologous antigen. The typhus group antigen did not fix complement in the presence of a 1:10 dilution of sera from guinea pigs infected with the 4 different Spotted Fever Group rickettsiae.

The specific epidemic and murine typhus antigens were corpuscular antigens prepared from infected yolk sac suspensions by repeatedly washing the organisms in 0.5 M KCl, resuspending the rickettsiae in

normal saline, followed by 2 ether extractions and concentration by centrifugation. When 2 to 4 units of the specific antigens were tested with the murine and epidemic typhus immune guinea pig sera, the heterologous complement-fixing antibody titer was 8 to 32-fold less than the homologous titer. These antigens did not fix complement in the presence of a 1:10 dilution of sera from guinea pigs infected with R. canada or the Spotted Fever Group strains.

The R. canada antigen was prepared in the same manner as the spotted fever soluble antigens. However, the R. canada antigen was highly strain specific. The only heterologous activity found was with the epidemic typhus immune guinea pig sera and the titer was 8-fold less than levels demonstrated in tests with the typhus group-reactive and specific R. prowazekii antigens.

Serum specimens from patients were tested with 4 to 8 units of each of the soluble antigens and 2 to 4 units of the corpuscular antigens. To insure performance of the rickettsial antigens, appropriate positive and negative control sera, as well as an antigen prepared from yolk sacs of uninfected embryonated eggs were included in each test.

Table 5 presents the results of CF and Weil-Felix tests on the sera of the 4 patients which exhibited R. canada antibodies. The first 2 cases listed, W.G. and L.G., developed complement-fixing antibodies that were detected only with the R. canada antigen. Tests with the 5 different soluble antigens prepared from strains of Spotted Fever Group rickettsiae were negative. Although R. canada is a member of the Typhus Group, fixation of complement with the group-reactive soluble antigen and specific epidemic typhus and murine typhus antigens was not observed. Although the titer of Proteus OX_{19} was 4-fold greater than OX_2 agglutinins in the early specimen of case W.G., the 2-week serum contained high levels of both agglutinins. The Weil-Felix reaction with sera from case L.G. exhibited a significant increase in Proteus OX_{19} antibodies only.

A similar Weil-Felix reaction was observed with the third case, C.M. Complement-fixing antibodies detected on the 9th day after onset reacted only with the R. canada antigen but the 24th day serum fixed complement in the presence of all the antigens except the specific murine typhus and epidemic typhus antigens.

Both Proteus OX_{19} and OX_2 agglutinins developed in the last case, J.S. Complement-fixing antibodies detected by the R. canada antigen were present in the 25 day serum. However, tests on later convalescent sera revealed the presence of spotted fever antibodies when tested with a R. rickettsi antigen prepared from the Bitterroot strain. The sera were negative with the other spotted fever group-reactive antigens.

TABLE 5. SEROLOCIC EVIDENCE OF R. CANADA INFECTION OF MAN

PATIENT & LOCATION	SERUM DATE	DD*	COMPLEMENT-FIXING ANTIGENS: SPOTTED FEVER GROUP: R. RICKETTSI: BITTER-ROOT	R. RICKETTSI: SHEILA SMITH	R. AKARI: HART-FORD	R. AKARI: TT-118**	TYPHUS GROUP	SPECIFIC: R. PROWAZEKII	SPECIFIC: R. MOOSERI	SPECIFIC: R. CANADA	WEIL-FELIX REACTION†: OX_{19}	WEIL-FELIX REACTION†: OX_2
W.G.	9/12/63	6	-††	-	-	-	-	-	-	40 §	640	160
♂ 23yrs. TEXAS	9/20/63	14	-	-	-	-	-	-	-	320	≥ 1280	≥ 1280
L.G.	8/2/67	9	-	-	-	-	-	-	-	-	20	40
♀ 33yrs	8/7/67	14	-	-	-	-	-	-	-	-	20	20
NORTH	8/22/67	29	-	-	-	-	-	-	-	20	640	20
CAROLINA	10/3/67	62	-	-	-	-	-	-	-	10	≥ 1280	20
C.M.	8/2/67	3	-	-	-	-	-	-	-	-	< 20	< 20
♀ 30yrs	8/7/67	9	-	-	-	-	-	-	-	20	640	< 20
NORTH CAROLINA	8/22/67	24	80	80	40	160	5	-	-	40	320	< 20
J.S.	8/13/68	10	-	-	-	-	-	-	-	-	160	80
♂ 15yrs	8/28/68	25	-	-	-	-	-	-	-	80	2560	2560
NORTH	9/30/68	58	20	-	-	-	-	-	-	20	160	160
CAROLINA	3/27/69	209	10	-	-	-	-	-	-	-	< 20	< 20

* Day of Disease.
** Results obtained with 2 different TT-118 antigens.
† Proteus OXK agglutinins were < 1:20.
†† Complement was not fixed at 1:5 dilution.
§ Reciprocal of serum dilution.

The etiology of the illnesses in these patients cannot be established definitely on the basis of the serologic data only, in the absence of the causative agent. At the present time, the only explanation that can account for the presence of R. canada complement-fixing antibodies in the convalescent sera of the first 2 patients (W.G. and L.G.) is either an infection with the newly recognized rickettsial species or a closely related strain. Interpretation of the results obtained in the last 2 cases depends upon the cross-reactivity that may be expected when sera from patients with infections with members of the Spotted Fever Group and Typhus Group are tested with the respective group-reactive soluble and specific antigens representative of the agents in both groups. There is little information in the literature which deals with this aspect of the serologic response in human disease. It is not common practice for diagnostic laboratories to routinely test specimens with antigens representative of all of the different groups of rickettsiae when the clinical picture and circumstances of infection suggest a single disease. Published reports by other workers demonstrating antigenic relationships between the 2 groups have been based principally on the development of rickettsial agglutinins, toxin-neutralizing antibodies, and immunity to heterologous challenge following experimental infection of a variety of laboratory animals. Sera from many other cases of RMSF and murine typhus from different areas of the United States and from cases of murine typhus and tick typhus occurring in other parts of the world have been tested with the same antigens used in this study. These data will be reported later, but a brief review of the results obtained thus far is pertinent to the present study. The majority of the patients with RMSF developed antibodies which fixed complement only in the presence of all of the soluble spotted fever group antigens. When reactivity with antigens prepared from a member of the Typhus Group was demonstrated it was present in specimens from military personnel or their dependents who most likely had been immunized with epidemic typhus vaccine. In these instances, significantly lower levels of antibodies were detected with the typhus group-reactive antigen as well as with the specific R. prowazekii, R. mooseri and R. canada antigens. Generally, the titers with R. prowazekii antigen were greater or equal to the R. mooseri titers while the levels of R. canada antibody, when present, were considerably lower.

The last 3 cases in Table 5 had never been immunized with epidemic typhus and there was no history of prior disease caused by a member of the Typhus or Spotted Fever Groups of rickettsiae. Information about these factors in the first case (W.G.) was not available. The development of antibodies detected by the spotted fever group and R. canada antigens in case C.M. may have resulted from a dual infection with R. rickettsi and R. canada. Since both agents are probably present in the same geographic regions and most likely employ the same tick vectors and vertebrate hosts, it is not unreasonable to expect to encounter

single ticks infected with both rickettsial species. Similarly, in case J.S., a dual infection may have caused the sequential appearance of first R. canada antibody and then the spotted fever antibody detected by the Bitterroot antigen. The appearance of low titers of spotted fever antibody 2 or 3 months after onset of disease has been observed occasionally in other cases clinically diagnosed as RMSF. It is possible that the Spotted Fever Group organism causing the disease may be a strain that, antigenically, differs markedly from the strains used for the preparation of antigens.

The clinical features of the illnesses of each of the patients were indistinguishable from those usually associated with RMSF. Patient W.G. became ill in Laredo, Texas in September 1963. The only available clinical information indicated that the patient had headache, fever, chills, marked anorexia and constipation. He developed a macular rash on the hands, palms, legs and abdomen which persisted for 3 or 4 days. There was no known exposure to insects. The other 3 cases which were documented more extensively occurred in the Fort Bragg-Fayetteville area of North Carolina where RMSF is known to be endemic. The most recent case occurred in early August 1968 while the other 2 patients became ill in late July 1967. The history prior to onset of disease and the clinical course were similar in all 3 cases. Each lived or had spent time in wooded areas and had removed an attached tick from his or her scalp 4 to 9 days before onset of fever, chills, malaise and headache. All had severe illnesses, the most prominent features of which were fever of 104 to 106 F, severe frontal headache, vomiting and appearance of a maculo-papular rash initially on arms and legs, including palms and soles, and spreading to the abdomen and chest. In addition, one patient had a stiff neck, muscle and joint pains, dizziness and blurred vision. Following the initiation of therapy with tetracycline in one case and chloramphenicol in the other 2 cases, there was a drop in temperature and marked clinical improvement within 24 to 36 hours.

The species identity of the ticks removed from these patients is unknown. The original isolation of R. canada was made from H. leporispalustris ticks which are not known to attack man but are found primarily on rabbits, small mammals and birds. The ticks found in the eastern United States that will feed on man are Dermacentor variabilis, the American dog tick, which is considered the most important vector of R. rickettsi in the east; Amblyomma americanum, the Lone Star tick,which is particularly prevalent in the Fayetteville area and in the south central U. S.; and Ixodes scapularis, the black-legged tick. Like H. leporispalustris, these species are found on rabbits and small mammals; in addition, A. americanum and I. scapularis also infest birds. The simultaneous feeding of several species of tick on animals and birds provides a link for the exchange of infection among the ticks. In studying the parasite-vector-host relationship of R. canada, Burgdorfer

reported that intracoelomic injection of infectious yolk sac suspensions into adult H. leporispalustris and D. andersoni (the Rocky Mountain wood tick) produced a generalized infection of all tissues and the rickettsia was transmitted during feeding on susceptible host animals. Furthermore, D. andersoni larvae became infected by feeding on rickettsemic meadow mice and the infection was maintained transstadially without adverse effects on the ticks. Transovarial transmission of R. canada in a tick species has not been reported. A collaborative study has been initiated with members of the staff of Womack Army Hospital and the Preventive Medicine Activity, Fort Bragg, North Carolina, to attempt recovery of the rickettsial agents causing disease in the Fayetteville area and to obtain information about the infection cycle in nature.

b. Enzootic Focus of a Member of the Typhus Group of the Tribe Rickettsieae. In May 1963, the Department of Rickettsial Diseases, the Department of Biostatistics, Walter Reed Army Institute of Research, the Department of Biology, Old Dominion College, Norfolk, Virginia, and the Virginia State Department of Health initiated a collaborative ecologic study of Rocky Mountain spotted fever. (See Annual Reports, 1 Jul - 30 Jun, 1963-1964, -1965, -1966, -1967.) Field and laboratory studies were designed to provide coincident qualitative information on the interaction of the different components involved in the infection cycle; viz., adult and subadult vector ticks, their vertebrate hosts and Rickettsia rickettsi. For the field investigations a 60 acre study area, known to be an enzootic focus of Rocky Mountain spotted fever, was selected near Montpelier, Virginia. From 1963 through 1966, 962 small and medium-sized mammals representing 14 different species were live-trapped, tagged, bled for serum and released. Each serum was tested for the presence of complement-fixing antibodies against Rocky Mountain spotted fever, murine typhus and Q fever. The results of the serologic studies on the incidence of spotted fever antibodies in the animals were presented in prior Annual Reports.

Not previously reported was the presence of typhus group antibodies in the sera of some of the animals. Of 31 flying squirrels (Glaucomys volans) taken during 1963-1966, 13 (42%) had antibodies which fixed complement in the presence of a soluble typhus group-reactive antigen. The only other animals in the study area that had typhus group antibodies were 1 Pitymys (pine vole) of 14 collected and 2 of 419 Peromyscus (white-footed mouse). Serologic evidence of spotted fever infection was found in only 2 of the flying squirrels and the serum from these animals was negative in tests with the typhus group antigen.

The typhus group antigen employed in these tests was a soluble-type antigen prepared by ether extraction of an aqueous suspension of yolk sacs infected with R. mooseri. The 4 units of the antigen used in the tests detected maximum antibody in both murine typhus and epidemic typhus immune guinea pig sera.

The pertinent data on the flying squirrels that had typhus antibodies are presented in Table 6. It is of interest to note that infection apparently was occurring during the fall and winter months. Each year animals with antibody were not trapped after June or July and serologic positives were not encountered until the following December or January. In 2 of the animals, No. 30 and No. 37, serologic conversions were documented. Each of these squirrels, when first trapped in November or December, did not have demonstrable typhus group antibodies. The following month when each was trapped again, titers of 1:32 and 1:16, respectively, were demonstrated. There was no correlation between the presence of typhus group antibodies and infestation of the animals with ticks or fleas. Lice were observed only on 2 animals which did not have typhus antibodies.

Murine typhus is world-wide in distribution and is probably enzootic throughout a large portion of the United States. The principal reservoirs of the disease are rats and mice and infection is transmitted sporadically to man by the rat flea, Xenopsylla cheopis. It was presumed that the finding of a high incidence of typhus group antibodies in the sera of flying squirrels was evidence of spread of murine typhus from commensal rodents to a wild animal reservoir. Murine typhus in the past, has always been considered to be maintained by a rat-rat flea cycle. In recent years, however, evidence has been accumulating from studies in Ethiopia and Egypt that ticks and domestic animals may serve as reservoirs of R. prowazekii. These reports and the isolation of a new member of the typhus group, R. canada, from Haemaphysalis leporis-palustris ticks in 1961, prompted a reexamination of the ecologic significance of typhus group antibodies in the sera of flying squirrels.

Live-trapping was begun again on the study area near Shenandoah, Virginia, and 18 flying squirrels were captured between March and June, 1962. They were tied for search and released at the same location where they were trapped. The sera were tested by complement fixation tests with a spotted fever group antigen, a typhus group antigen, a specific reacting R. canada antigen and specific complement fixing antigens for murine typhus and Brazil typhus. (See Proceedings for Serologic Evidence of R. canada Infection of Man for description of preparation and complement fixing sensitivity of the antigens.) The sera from 4 of the squirrels were negative with all of the antigens. Sera from 14 squirrels reacted with the murine typhus complement fixing antigen. Of these 9 sera were positive for typhus group antibodies. The complement fixing antibody titers on the sera from the various 9 animals are presented in Table 7.

In the case of 1 of the animals (Nos. 1 and 2) the sera had the same or equivalent titers with the spotted fever group antigen, R. prowazekii and R. canada antigens and these sera were also

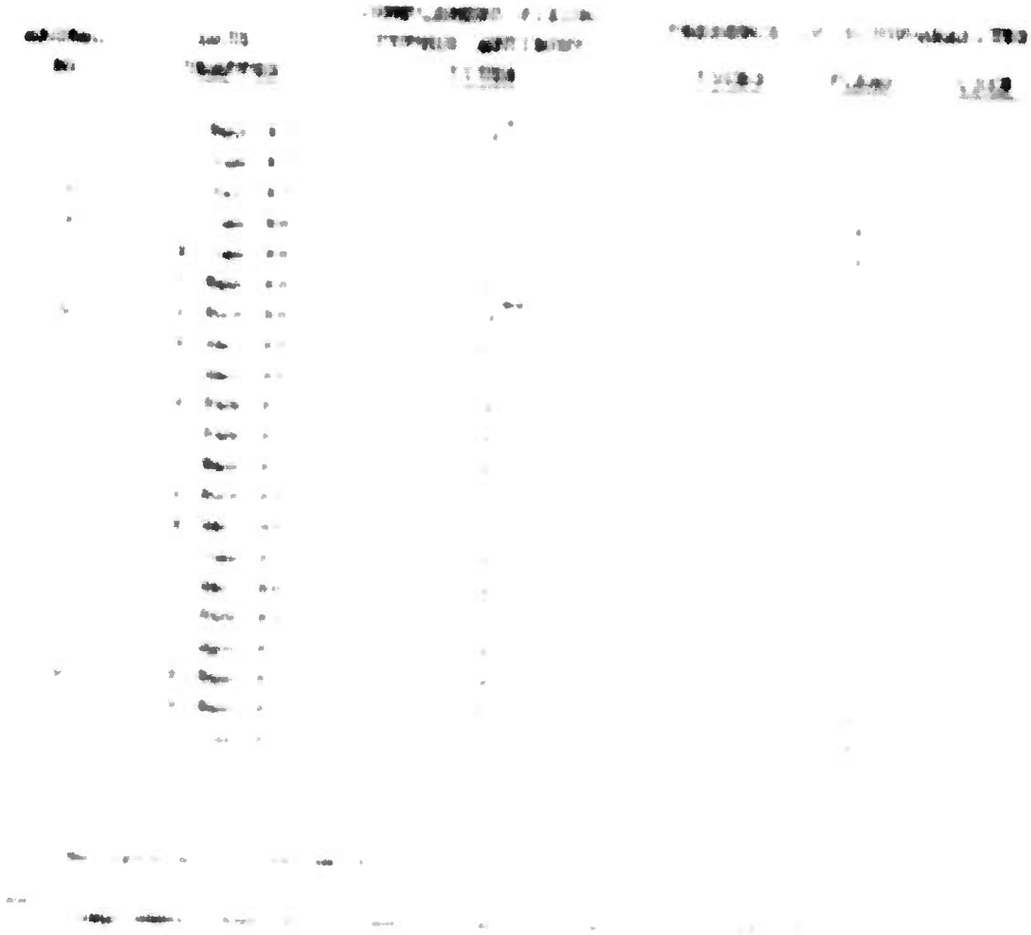

TABLE 1. COMPLEMENT-FIXING ANTIBODY TITERS OF SERA FROM FLYING [illegible]

([illegible]) TRAPPED IN VIRGINIA IN 1944

		ANTIGENS				
		GROUP-REACTIVE		SPECIFIC		
ANIMAL NO.	DATE TRAPPED	SPOTTED FEVER	TYPHUS	B. [illegible]	B. [illegible]	[illegible]
3	6 [illegible] 44	0	1:20	1:4	0	0
6	20 [illegible] 44	0	1:2	1:2	0	0
	6 [illegible] 44	0	1:4	0	0	0
7	20 [illegible] 44	0	0	0	0	0
	11 [illegible] 44	0	0	0	0	0
	3 [illegible] 44	0	1:4	1:4	0	0
	24 [illegible] 44	0	1:2	1:2	0	0
	13 [illegible] 44	0	1:4	1:2	0	0
17	28 [illegible] 44	0	0	1:4	0	0
18	17 [illegible] 44	0	1:4	0	0	1:2
	0 [illegible] 44	0	1:4	0	0	1:2
20	13 [illegible] 44	0	1:4	1:2	1:8	1:8
22	24 [illegible] 44	0	1:8	1:4	0	1:2
24	27 [illegible] 44	0	0	0	0	0

* [illegible] was not tested on the animals [illegible] to 1:40 dilutions. The same [illegible] was usually not sufficient to begin at a 1:10 dilution.

** [illegible] of serum dilutions.

R. mooseri antigen. One animal, No. 26, had essentially the same titer, 1:16 or 1:32, with all 3 antigens. Squirrels No. 7 and No. 17 exhibited a 4-fold greater titer with the R. prowazekii antigen than with the R. canada antigen, while in animal No. 21 the reverse was the case. R. mooseri antibodies were of low titer or negligible in these animals. Spotted fever antibodies were not detected in any of the sera. Interpretation of the typhus group antibodies demonstrated in the sera of flying squirrels is not possible at the present time. It appears, however, that R. mooseri is not the organism responsible for infection in the area. The usual criterion for establishing a causal relationship between the serologic response and the etiologic agent is that antibody levels in complement fixation tests with the homologous specific antigen are at least 4-fold higher than the titer with specific antigens prepared from the other members of the group of rickettsiae. On this basis, one could conclude that R. prowazekii and R. canada, or other closely related organisms, are enzootic in the flying squirrel population in Montpelier, Virginia.

In late July 1968, an attempt was made to recover the etiologic organism(s) from the tissues of a flying squirrel trapped at the study area. Serum collected 2 days before the squirrel was sacrificed had a complement-fixing antibody titer of 1:32 with the epidemic typhus specific antigen and 1:4 and 1:8 with the R. mooseri and R. canada antigens, respectively. After exsanguination the brain, liver and spleen were removed aseptically. A suspension of the tissues was inoculated into the yolk sacs of ten 7-day old embryonated eggs and intraperitoneally into 3 adult male guinea pigs.

The eggs were candled daily and the yolk sac of an embryo found dead on the 9th day after inoculation was harvested and passed to another group of eggs. No rickettsia-like organisms were observed in Macchiavello-stained smears of the yolk sac. No additional deaths of embryos originally inoculated occurred. All second passage embryos survived until the 12th day at which time 3 yolk sacs of the latter passage were harvested, smeared and passaged to a third set of eggs. None of the third passage embryos died and smears made of 4 yolk sacs harvested on the 12th day were negative. The guinea pigs receiving the tissue suspension did not develop fever or scrotal reaction and serum collected 28 days post inoculation did not fix complement in the presence of the typhus or spotted fever group-reactive antigens.

Final interpretation of the serologic results is dependent upon the recovery of the responsible organism from a naturally infected flying squirrel. Future studies include making additional isolation attempts from animals trapped in the wild, as well as following the course of infection and serologic response in flying squirrels experimentally inoculated with each of the 3 Typhus Group rickettsial strains and with R. rickettsi.

3. Differentiation of Primary and Secondary Antibody Responses in Typhus Group Rickettsial Infections. The results of investigations to characterize the type of immunoglobulins responsible for the serologic reactivity found in serial serum specimens from a patient with murine typhus (RJH) were presented in prior Annual Reports. Complement fixation and agglutination tests with a variety of antigens were performed with the whole specimens and with fractions collected after gel filtration with Sephadex G-200. The presence of IgM, IgG and IgA in the fractions was identified by agar double diffusion technics. Complement-fixing (CF) antibodies detected with group-reactive soluble antigen and specific washed rickettsial suspensions were both IgM and IgG as early as 13 days after onset of illness. IgM CF antibodies persisted for more than 3 months but after 7 3/4 months the CF antibodies were IgG. Agglutinating antibodies reacting with suspensions of Proteus bacillus OX_{19} and Rickettsia mooseri, as well as with erythrocytes sensitized with rickettsial antigens (ESS) were only IgM. Proteus OX_{19} and ESS agglutinins disappeared by 90 days, but R. mooseri agglutinins were still present in the 236th day specimen. Absorption of fractions containing antibody with specific anti-immunoglobulin sera did not reveal any serologic reactivity attributable to IgA.

Other workers have been able to differentiate, by relatively simple serologic means, between primary epidemic typhus and Brill-Zinsser disease which is a recrudescence of a R. prowazekii infection contracted years earlier. Employing complement fixation, the titer of serum specimens before and after treatment with 2-mercaptoethanol or ethanethiol was determined in tests with soluble epidemic typhus antigen. In primary epidemic typhus there was a significant reduction in antibody titer caused by the sulfhydryl reducing agents which was attributed to dissociation of IgM antibodies. The antibodies, developing in Brill-Zinsser disease, were only IgG and the complement-fixing reactivity was unaffected by the reducing agents. Thus, primary infection was associated with the appearance of antibodies of both the IgM and IgG classes, but recrudescence manifested a secondary or booster-type response in which only IgG antibodies were produced.

In the past, difficulties have been encountered in establishing the etiology of Typhus Group rickettsial diseases in certain cases by serologic means. When the patient had been previously immunized with epidemic typhus vaccine, complement fixation tests with specific antigens very often would not differentiate between murine typhus and epidemic typhus. It was postulated that R. mooseri infection in an individual previously immunized with epidemic typhus vaccine would be associated with a primary antibody response characterized by the appearance of both IgM and IgG antibodies to the specific antigenic components of R. mooseri shared with R. prowazekii. Following an R. prowazekii infection in a patient who had been previously immunized with epidemic typhus vaccine

only a secondary or booster response would be expected and only IgG antibodies would be produced. The study to be reported here was undertaken to determine if indirect immunofluorescence with specific fluorescein isothiocyanate conjugated (FITC) anti-human immunoglobulins could be used to differentiate primary and secondary antibody responses in Typhus Group rickettsial diseases when the patient has had prior immunologic experience by infection or immunization with the same or a related member of the group.

The method for performing the indirect fluorescent (IF) antibody test was the same as has been described in previous reports. The acetone-fixed antigens employed were smears of suspensions of yolk sac infected with either R. mooseri, R. prowazekii, or R. canada, a newly recognized member of the Typhus Group. The results presented were obtained with these antigens. Attempts to increase the specificity of the antibody activity in the IF test with the use of highly purified washed rickettsial suspensions which displayed specific CF and agglutinating reactivity were not successful. Indeed, more specific reactivity was not obtained and difficulty was encountered in determining if fluorescence in terminal dilutions was due to antibody or autofluorescence of the rickettsiae. Similar difficulties have been encountered when other formalin-inactivated microorganisms were employed as antigens. Other efforts to increase the specificity of each antigen by pretreatment with a pool of immune sera from guinea pigs infected with the other 2 members of the group were also unsuccessful.

The IF titers of IgM and IgG antibodies in the patients' sera reacting with each of the rickettsial antigens were determined respectively with FITC-labeled goat anti-human IgM and anti-human IgG. The specificity of the anti-immunoglobulins was established by completely blocking or markedly reducing the fluorescence with a homologous unconjugated anti-immunoglobulin but not with heterologous unconjugated anti-immunoglobulin. In addition the titers of antibodies were also determined with FITC-labeled horse anti-human whole serum (ws). The serum specimens studied were obtained from personnel accidently infected in this and other laboratories. The etiology of each of the illnesses was based principally upon the occurrence of disease within 2 weeks after known exposure to the infective agent.

The results of IF tests on serial serum specimens obtained from the patient (RJH) with murine typhus described in the introduction who had not been previously immunized with epidemic typhus vaccine are presented in Table 8. Previously, rickettsial agglutination tests and complement fixation with specific washed corpuscular antigens showed the serum titers to be significantly higher with murine typhus antigens than with epidemic typhus antigens. With the exception that the IF

TABLE 8. SUSPECTED MURINE TYPHUS INFECTION IN A PATIENT NOT PREVIOUSLY IMMUNIZED WITH EPIDEMIC TYPHUS VACCINE

INDIRECT FLUORESCENT ANTIBODY TITERS

PATIENT AND SPECIMEN	ANTIGENS								
	R. MOOSERI ANTI-GLOBULIN CONJUGATE*			R. PROWAZEKII ANTI-GLOBULIN CONJUGATE			R. CANADA ANTI-GLOBULIN CONJUGATE		
	IgM	IgG	ws	IgM	IgG	ws	IgM	IgG	ws
(RJH) pre	--†	--	--	--	--	--	--	--	--
11 dd	160	160	640	160	160	640	160	160	160
13 dd	2560	2560	10240	2560	2560	2560	640	640	2560
18 dd	2560	2560	10240	2560	2560	2560	640	2560	2560
30 dd	640	2560	10240	640	640	2560	640	640	640
58 dd	640	640	2560	--	640	640	--	640	160
90 dd	160	640	640	--	640	640	--	160	160
236 dd	40	160	640	--	160	160	--	160	160
1710 dd	40	160	640	--	160	160	--	40	160

* IgM = FITC-labeled goat anti-human IgM
IgG = FITC-labeled goat anti-human IgG
ws = FITC-labeled horse anti-human whole serum

† Negative with an initial 1:10 dilution

titers with the R. canada antigen were somewhat lower than with other antigens, there were no significant differences among the levels of antibody reacting with the 3 different antigens and the corresponding anti-globulin conjugates. Significant differences were not encountered until 58 days after the onset of illness when only R. mooseri IgM antibodies were demonstrable. It was noteworthy that this specific IgM reactivity was still demonstrable more than 4 years later.

The IF antibody responses after murine typhus infection in patients having been inoculated with epidemic typhus vaccine within the preceding 1 1/2 years are shown in Table 9. The success of immunization, determined by the development of antibodies post vaccination, was not known in any of the cases. However, in no instance was IF antibody to any member of the Typhus Group detected in sera collected prior to onset of disease. In the first case, (RFP) the titers of R. mooseri IgM antibodies were consistently higher than the corresponding antibodies reacting with R. prowazekii and R. canada. There was no apparent decline in the level of IgM rickettsial antibodies in the third month as was seen with the previous case (RJH). An intercurrent spotted fever infection which occurred 46 days after the onset of the murine typhus infection probably complicated the antibody response. The second case illustrates a different pattern of antibody reactivity. The levels of R. mooseri IgM and R. prowazekii antibodies were the same and only low titers of R. canada IgM antibody were found. IgM antibodies again declined after the third month and in the 220th day specimen the R. mooseri IgM antibodies were only 4-fold greater than the other corresponding antibodies. The consistently higher R. prowazekii IgG antibody may be indicative of a booster response caused by the current infection.

When murine typhus developed in patients who had demonstrable antibodies resulting from prior successful immunization with epidemic typhus vaccine, IgM antibodies were not produced. (See Table 10.) Higher levels of IgG antibodies, which did develop, were detected with the R. prowazekii antigen than with the other 2 antigens in the first case (RNM). In the second case (CLW) the levels of R. mooseri and R. prowazekii IgG antibody were the same. Information about the relationship of the serum specimens to the day of disease was not available for this case. Both patients manifested only low levels of antibodies reacting with R. canada.

IgM antibodies were not produced in a patient who developed epidemic typhus in spite of successful prior immunization. (See Table 11.) The general predominance of the levels of the R. prowazekii IgG antibody over the R. mooseri antibody in this case (RP) is similar to the response observed in the case (RNM) which also had demonstrable antibodies following epidemic typhus immunization but contracted murine typhus. However, in case RP, significant activity was detected with the R. canada antigen.

TABLE 9. SUSPECTED MURINE TYPHUS INFECTION IN PATIENTS PREVIOUSLY IMMUNIZED WITH EPIDEMIC TYPHUS VACCINE

INDIRECT FLUORESCENT ANTIBODY TITERS

PATIENT AND SPECIMENS	ANTIGENS								
	R. MOOSERI ANTI-GLOBULIN CONJUGATE*			R. PROWAZEKII ANTI-GLOBULIN CONJUGATE			R. CANADA ANTI-GLOBULIN CONJUGATE		
	IgM	IgG	ws	IgM	IgG	ws	IgM	IgG	ws
(RFP) pre	--†	--	--	--	--	--	--	--	--
4 dd	--	--	--	--	--	--	--	--	--
10 dd	160	160	160	160	160	160	40	40	160
17 dd	640	2560	2560	160	2560	2560	160	160	640
24 dd	640	640	640	160	640	640	40	160	160
31 dd	640	640	640	160	160	640	40	160	160
34 dd	640	640	2560	640	640	640	40	160	160
59 dd††	640	2560	2560	160	2560	2560	40	640	640
76 dd	640	2560	640	160	2560	640	160	640	2560
213 dd	640	2560	640	160	640	640	40	160	160
(WJB) pre	--	--	--	--	--	--	--	--	--
2 dd	10	10	10	--	160	160	--	--	--
10 dd	160	160	160	40	640	640	10	40	40
14 dd	160	160	160	160	640	640	10	40	40
21 dd	160	160	640	160	640	640	10	10	40
31 dd	160	160	160	160	640	640	10	10	40
38 dd	160	160	160	160	640	640	--	10	40
52 dd	160	640	640	160	640	640	40	40	40
220 dd	40	160	160	10	640	160	10	40	40
(TFB) pre	--	--	--	--	--	--	--	--	--
1 dd	--	--	--	--	--	--	--	--	--
8 dd	10	10	40	10	10	40	10	--	40
11 dd	160	40	160	160	40	160	160	40	160
15 dd	160	160	640	640	160	640	160	40	160
24 dd	160	160	160	160	160	160	160	40	160

* IgM = FITC-labeled goat anti-human IgM
IgG = FITC-labeled goat anti-human IgG
ws = FITC-labeled horse anti-human whole serum

† Negative with an initial 1:10 dilution

†† Corresponds to 13th day od disease caused by Sported Fever Group infection

TABLE 10. SUSPECTED MURINE TYPHUS INFECTION IN PATIENTS PREVIOUSLY IMMUNIZED WITH EPIDEMIC TYPHUS VACCINE

INDIRECT FLUORESCENT ANTIBODY TITERS

ANTIGENS

PATIENT AND SPECIMENS	R. MOOSERI ANTI-GLOBULIN CONJUGATE*			R. PROWAZEKII ANTI-GLOBULIN CONJUGATE			R. CANADA ANTI-GLOBULIN CONJUGATE		
	IgM	IgG	ws	IgM	IgG	ws	IgM	IgG	ws
(RNM) pre	--†	10	10	--	640	640	--	10	10
3 dd	--	40	40	--	160	160	--	10	10
10 dd	--	640	640	--	2560	2560	--	10	10
14 dd	--	640	640	--	2560	2560	--	10	10
21 dd	--	640	640	--	2560	2560	--	10	10
31 dd	--	10240	640	--	10240	10240	--	40	10
35 dd	--	2560	640	--	2560	2560	--	40	40
49 dd	--	2560	640	--	10240	2560	--	40	40
66 dd	--	640	640	--	10240	2560	--	40	40
91 dd	--	640	640	--	2560	2560	--	40	40
(CLW) pre	--	40	10	--	40	40	--	--	--
21 Jun 50	--	160	40	--	160	160	--	--	--
26 Jun 50	--	640	160	--	640	640	--	10	10
5 Jul 50	--	640	640	--	640	640	--	40	10
22 Sep 50	--	640	160	--	640	640	--	10	10
13 Nov 50	--	160	160	--	640	640	--	10	10

* IgM = FITC-labeled goat anti-human IgM
IgG = FITC-labeled goat anti-human IgG
ws = FITC-labeled horse anti-human whole serum

† Neagative with an initial 1:10 dilution

TABLE 11. SUSPECTED EPIDEMIC TYPHUS INFECTION IN A PATIENT PREVIOUSLY IMMUNIZED WITH EPIDEMIC TYPHUS VACCINE

INDIRECT FLUORESCENT ANTIBODY TITERS

PATIENT AND SPECIMENS	ANTIGENS								
	R. MOOSERI ANTI-GLOBULIN CONJUGATE*			*R. PROWAZEKII* ANTI-GLOBULIN CONJUGATE			*R. CANADA* ANTI-GLOBULIN CONJUGATE		
	IgM	IgG	ws	IgM	IgG	ws	IgM	IgG	ws
(RP) pre	--†	10	40	--	40	40	--	--	--
1 dd	--	2560	2560	--	2560	2560	--	40	40
3 dd	--	10240	2560	--	10240	10240	--	160	160
4 dd	--	10240	10240	--	40960	10240	--	640	640
9 dd	--	10240	10240	--	40960	40960	--	640	640
15 dd	--	2560	2560	--	40960	40960	--	640	640
668 dd	--	40	40	--	160	160	--	40	40

* IgM = FITC-labeled goat anti-human IgM
IgG = FITC-labeled goat anti-human IgG
ws = FITC-labeled horse anti-human whole serum

† Negative with an initial 1:10 dilution

It was evident from these data that the close relationships between the surface antigens of R. mooseri and R. prowazekii, and the sensitivity of the indirect immunofluorescent technic, made it impossible in almost all instances, to identify the etiologic agent on the basis of the IF antibody response. The antigenic composition of R. canada was sufficiently different that it could have been excluded as the cause of disease in all cases. Indeed, when the human host had been successfully immunized with epidemic typhus vaccine, and then was infected with murine typhus, antigenic differences were not recognized. The antibody response resembled a secondary or booster reaction with the production of only IgG antibodies.

4. Effects of Newer Antimicrobials on Rickettsiae. In the WRAIR Annual Report (1 Jul 67 - 30 Jun 68) a preliminary report was made of investigations to determine if any of the more recently developed antibiotics had a rickettsiocidal effect. There has been little effort on the part of the pharmaceutical industry to improve upon chloramphenicol and the tetracyclines for the treatment of rickettsial diseases. Antimicrobials are needed that could prevent the high relapse rate that occurs in certain rickettsial diseases when treatment is initiated early in the course of disease and terminated after the patient has been afebrile for 48 to 72 hours. Rickettsiocidal drugs might also prevent the development of latent infections in epidemic typhus and thus eliminate the presumed major interepidemic reservoir of this disease. The primary purpose of the study to be reported was to identify antibiotics which had a direct effect on rickettsiae and secondly, to evaluate the anti-rickettsial activity of classes of antibiotics not previously tested for this effect. Since scrub typhus is the most important military rickettsial disease problem at this time, strains of Rickettsia tsutsugamushi were employed.

The details of the in vitro and in vivo test procedures used in the study were described last year. Briefly, in the in vitro method yolk sac suspensions of the Karp strain of R. tsutsugamushi diluted in Snyder I diluent to a concentration of 1:100 were incubated with 10, 50 or 250 micrograms/ml of the antibiotic for 2 hours at 37 C. Under these conditions the rickettsiae are known to continue certain enzyme activities, although the full state of metabolic function is not known. After incubation, serial 10-fold dilutions of the suspensions with antibiotic as well as the control suspensions without antibiotic incubated under the same conditions, were prepared; and 0.2 ml aliquots of each dilution were inoculated into six 14 to 18 gram white mice. The mouse LD_{50} titers were calculated after 28 days. The control suspensions usually contained between $10^{7.5}$ and $10^{8.6}$ mouse LD_{50}. Antibiotics having a direct antirickettsial effect caused a significant reduction in the LD_{50} titer of the Karp suspension.

The in vivo tests were identical to those employed previously by Smadel et al. (J. Immunol. 57:273,1947). Sets of eleven 6-day old embryonated hens' eggs were injected with 0.2 ml of each of the desired concentrations of the antibiotics under study. Doses of antibiotics tested ranged from 3.9 to 500 micrograms per egg in 2-fold increments. Within 20 minutes after inoculation of the antibiotic all test eggs were injected with 0.2 ml of a yolk sac suspension of the Gilliam strain of R. tsutsugamushi diluted to 1:100 with Snyder I diluent. Appropriate infectivity and drug toxicity controls were included in each trial. All eggs were incubated at 35 C and candled daily. Any egg dying within the first 48 hours was discarded and the mean day of death of the remaining embryos was calculated. The usual time of death of the infected control eggs without antibiotic was 7.4 days.

In the in vitro test a drug was considered to have an anti-rickettsial effect if it effected a 100-fold or more reduction in the mouse LD_{50} titer of the Karp seed material. In the in vivo system, the efficacy of an antimicrobial on the growth of Gilliam strain rickettsiae was considered significant if the mean day of death of the drug-treated eggs was extended 2 or more days beyond the infected control eggs without antibiotic.

A list of the antibiotics that have been tested and the minimal dose that reduced the mouse infectivity titer of the Karp seed suspension or significantly prolonged the life of embryos infected with the Gilliam strain are presented in Table 12. The anti-rickettsial activity of tetracycline, oxytetracycline and chlortetracycline on experimental infections in laboratory hosts and the value of these antibiotics in the treatment of rickettsial diseases of man has been well documented. As expected, the newer tetracycline analogs also exhibited anti-rickettsial activity. On a weight basis, doxycycline was slightly more potent in the in vivo assay system, while chlortetracycline and methacycline were more effective in the in vitro system.

Neither penicillin G nor the 2 "broad spectrum" synthetic penicillins displayed significant anti-rickettsial properties at the concentrations tested.

Chloramphenicol is of value as a prophylactic drug in animals experimentally infected with R. tsutsugamushi and in man infected with scrub typhus by exposure in the field under controlled conditions. It has been well established that it is a rickettsiostatic drug rather than rickettsiocidal. Cetophenicol, an analog of chloramphenicol, displayed similar activity; i.e., it was relatively efficacious in the living system but had no activity in the in vitro mouse assay test.

TABLE 12. MINIMALLY ACTIVE DOSES (MICROGRAMS) OF CERTAIN ANTIMICROBIALS

CLASSES	ANTIBIOTICS	IN VITRO*	IN VIVO**
TETRACYCLINES	Tetracycline	50	—***
	Oxytetracycline	50	15.6
	Chlortetracycline	10	15.6
	Doxycycline	50	7.8
	Rolitetracycline	50	15.6
	Methacycline	10	—
PENICILLINS	Penicillin G	NSA (250)****	—
	Ampicillin (Polycillin)	NSA (250)	NSA (500)
	Hetacillin	NSA (250)	NSA (500)
CHLORAMPHENICOLS	Chloramphenicol	250	—
	Cetophenicol	NSA (250)	62.5
MACROLIDES	Erythromycin	250	62.5
	Amphomycin	NSA (250)	—
	Novobiocin	250	—
NEOMYCIN-KANAMYCIN GROUP	Neomycin	NSA (250)	—
	Paromomycin	NSA (250)	—
	Kanamycin	NSA (250)	—
CEPHALOSPORINS	Cephaloridine	NSA (250)	—
POLYMYXIN POLYPEPTIDES	Polymyxin	10	—
ANTI-TUBERCULOUS AGENTS	Isoniazid	NSA (250)	—
	Viomycin	50	—
	Ethionamide	NSA (250)	—
ANTI-FUNGAL AGENTS	Griseofulvin	NSA (250)	—
ANTI-VIRAL AGENTS	Amantadine	NSA (250)	NSA (500)
	Idoxuridine	NSA (250)	NSA (500)
	Methisazone	NSA (250)	NSA (500)

* Smallest dose of antibiotic (mcg/ml) tested which reduced infectivity of scrub typhus suspension 100-fold or more.

** Smallest dose of antibiotic (mcg/egg) tested which prolonged mean survival time of infected eggs 2 or more days.

*** Not tested.

**** No significant activity (highest dose tested).

Among the macrolides, erythromycin and novobiocin had in vitro anti-rickettsial activity at the highest dose tested (250 mcg) and the former, at 62.5 mcg/egg, significantly prolonged the survival time of Gilliam-infected eggs.

Except for polymyxin and the anti-tuberculous drug, viomycin, which exhibited some in vitro rickettsiocidal activity, none of the other antimicrobials; viz., neomycin, kanamycin, cephaloridine, griseofulvin, the antiviral and other anti-tuberculous agents had an anti-rickettsial effect over the range tested.

Results of current tests to compare the efficacy of a number of the newer antibiotics on the in vitro and in vivo effect on scrub typhus rickettsiae indicated that none had anti-rickettsial activity comparable to or greater than the tetracyclines and chloramphenicol.

5. Recovery of Chlamydial Agents from Thailand. In the course of studies being conducted by the Thai Component, SEATO Medical Laboratory, Bangkok, to investigate the presence and extent of rickettsial diseases in Thailand, an agent which was highly lethal for mice was recovered from the blood of a hill tribe boy who had an influenza-like illness accompanied with a rash. The agent, designated TH4043, was identified as a chlamydia by complement fixation tests.

Currently, the genus Chlamydia is divided into Group A and Group B. The former group includes the etiologic agents of trachoma, inclusion conjunctivitis, lymphogranuloma venereum and mouse pneumonitis. These agents form compact round or oval intracytoplasmic inclusions or microcolonies which produce glycogen and are sensitive to sulfadiazine. Within Group B are the agents of psittacosis, meningopneumonitis, feline and human pneumonitis, bovine and guinea pig conjunctivitis and avian-derived strains. These latter organisms produce diffuse intracytoplasmic inclusions, do not produce glycogen, and are sulfadiazine insensitive.

Preliminary studies on the agent recovered in Thailand indicated that it belonged to the B group of Chlamydia. (See Annual Report, 1 Jul 67 - 30 Jun 68.)

Table 13 summarizes the results of the tests done for final group identification of TH4043. Included in the studies were strains of lymphogranuloma venereum (LGV) and ornithosis to serve as Group A and Group B controls, respectively.

Cover slip cultures of 14pf cells were infected with about 10^5 egg LD_{50} of infected yolk sac seed material of each agent. At 24, 40, 48 and 72 hours after infection, duplicate cover slips containing infected

TABLE 13. GROUP IDENTIFICATION OF A CHLAMYDIAL AGENT CAUSING HUMAN DISEASE IN THAILAND

GROUP	AGENT	GROWTH IN CELL CULTURES*		SENSITIVITY TO SULFADIAZINE EGG LOG ID_{50} TITER	
		TYPE OF INCLUSION	GLYCOGEN PRODUCED	WITH SULFADIAZINE (1 mg/egg)	NO DRUG (CONTROL)
A	Lymphogranuloma venereum (JM strain)	Compact	Yes	2.7	7.7
B	Ornithosis (P4 strain)	Diffuse	No	7.7	7.7
	TW4043	Diffuse	No	8.9	9.1

* Hep I line of normal rat fibroblasts

cells as well as cover slips of uninfected cells were removed. One set was stained with Macchiavello's stain and the other set was fixed in methanol and then immersed in a mixture of iodine and potassium iodide which stains glycogen. Examination of the Macchiavello-stained cells revealed compact round or oval intracytoplasmic microcolonies of elementary bodies and larger reticular inclusions in the LGV-infected cells, while the ornithosis and TH4043 inclusions were much more diffuse.

The formation of glycogen by chlamydiae is a transient phenomenon that occurs from the first through the third day of growth. Intracytoplasmic clusters of chlamydiae, containing glycogen, appear brown against a light tan background, while non-glycogen-forming microcolonies are indistinguishable from other cellular material.

In observing the above infected and uninfected cultures, no differences were noted between the cells infected with ornithosis, TH4043 and the uninfected control cultures. Cells infected with LGV, however, contained large dark brown glycogen-stained inclusions.

For purposes of taxonomic differentiation, susceptibility to sulfadiazine is defined as a 100-fold or more reduction in the egg LD_{50} titer of a suspension of chlamydiae containing 10^6 LD_{50} or more. The results of the comparative titrations in embryonated eggs of the LGV, ornithosis and TH4043 seed suspensions in the presence of 1 mg/egg of sulfadiazine and without drug are shown in Table 13. There was essentially no difference in titer of the ornithosis and TH4043 seed material in the sulfadiazine-treated eggs as compared to the titers obtained in the untreated eggs, indicating that these agents are insensitive to sulfadiazine. On the other hand, LGV was markedly inhibited by the drug; i.e., there was $\geq$100,000-fold decrease in titer in the presence of sulfadiazine.

The findings of insensitivity of the TH4043 agent to sulfadiazine and failure to produce glycogen in diffuse intracytoplasmic microcolonies indicate that the organism belongs to the B group of psittacosis-type agents.

In June 1968 another agent (TH5000) which could not be identified as a rickettsia and failed to grow on bacteriologic media was recovered from a patient from Prachin province in eastern Thailand. The Thai male patient, who was about 60 years old, went to the hospital with the chief complaint of having had fever for 8 days. An eschar was observed on the right buttock and there was enlargement of the regional lymph glands. A rash or other abnormal findings were not noted. The patient responded satisfactorily upon chloramphenicol therapy. Complement fixation tests with acute and convalescent phase sera with typhus and spotted fever

group antigens and Q fever antigen as well as the indirect fluorescent antibody test for scrub typhus were negative. Mice inoculated with the patient's blood became ill on the 9th day. On autopsy, peritoneal fluid was sticky and stained smears contained numerous intracytoplasmic chlamydia-like organisms.

In late August 1968, spleens from the 5th mouse passage were sent to the Department of Rickettsial Diseases for identification. A suspension of the mouse spleens received at WRAIR was inoculated into yolk sacs of embryonated eggs and intraperitoneally into mice and guinea pigs. The yolk sacs of 2 embryos dead on the 6th day were passaged to additional eggs. Smears of the yolk sac membranes, stained with Macchiavello stain, contained numerous red elementary bodies. The mice were sacrificed on the 5th day when they were lethargic and had ruffled fur. Examination of Giemsa-stained smears of the spleens revealed elementary bodies as well as larger intracytoplasmic inclusions. None of the guinea pigs developed fever ($\geq$ 104 F), but they did exhibit a scrotal reaction which persisted from the 3rd to the 8th day. They were exsanguinated 28 days after inoculation to obtain convalescent serum.

A complement-fixing antigen prepared from a suspension of infected yolk sacs by repeated centrifugation and resuspension of the elementary bodies in 0.5 M KCl and then ether extraction titered 1:128 with 2-4 units of a psittacosis convalescent human serum pool, a lymphogranuloma venereum control serum and a TH4043 guinea pig antiserum. Similarly, TH5000 and TH4043 immune mouse and guinea pig sera fixed complement in the presence of TH5000 and TH4043 antigens.

Preliminary studies on the group identification of TH5000 indicate that it, like TH4043, is a psittacosis-like agent belonging to the B group of Chlamydia. Cover slip cultures of 14pf cells infected with TH5000 and stained with iodine failed to show the presence of dark brown glycogen producing plaques.

Summary and Conclusions.

1. Scrub Typhus Vaccine Development.

a. Studies have continued on 5 strains of R. tsutsugamushi recovered in Thailand which are antigenically distinctive from the established prototype Gilliam, Karp and Kato strains. Two or 3 different lines of each strain have been carried through 8 to 10 additional passages in order to better adapt these agents to cultivation in embryonated eggs, but there has been very little consistent improvement in the growth of any of the Thai strains. Inoculation of the eggs with cortisone or

sulfadiazine, which have been reported to increase infectivity titers of other groups of Rickettsieae, did not improve the yield of scrub typhus rickettsiae.

b. A number of physico-chemical procedures which have been successful in other types of molecular separations were evaluated in an attempt to eliminate host material and recover a significant amount of scrub typhus antigen. The Kato strain was selected for these studies because it has been the least satisfactory of the 3 original prototype strains for preparing complement-fixing antigens. The technics employed included density gradient centrifugation and phase partition with aqueous-organic and polymer systems. Although much information has been gained on the intrinsic properties of the Kato strain, none of the procedures employed was wholly successful in producing highly concentrated suspensions of rickettsiae free of contaminating host material.

2. Rickettsia canada: A New Member of the Typhus Group.

a. Serologic evidence has been obtained that the etiology of severe febrile illnesses in 4 patients was caused by R. canada, a new species belonging to the Typhus Group of Rickettsieae. Complement fixation tests with soluble spotted fever antigens and group-reactive and specific antigens prepared with members of the Typhus Group established the causal relationship. Three of the patients had sustained a tick bite and all of them had clinical histories indistinguishable from that observed in Rocky Mountain spotted fever. The finding of R. canada antibodies in the convalescent sera of these patients can be considered only presumptive evidence that this new species of rickettsia has caused severe illness in man. However, the serologic evidence is sufficiently unusual to justify alerting physicians and laboratory personnel who are concerned with providing serologic diagnoses to the possible existence of a previously unrecognized rickettsial disease in the United States.

b. Thirteen of 31 flying squirrels trapped at the Rocky Mountain spotted fever Study Area near Montpelier, Virginia during 1963-1966 had antibodies which fixed complement in the presence of a soluble group-reactive typhus antigen. The isolation of R. canada and the recently published reports that ticks and domestic animals in Ethiopia and Egypt may be reservoirs of R. prowazekii prompted a reevaluation of the ecologic significance of typhus group antibodies in the sera of flying squirrels. Between March and July 1968, 18 flying squirrels were live-trapped, bled and released. On the basis of the results of CF tests on sera employing a group-reactive typhus antigen, and specific antigens for R. prowazekii, R. mooseri and R. canada, it appears that R. prowazekii and R. canada, or other closely related organisms are enzootic in the flying squirrel population.

3. Fluorescein isothiocyanate conjugated anti-human IgM and IgG goat sera and antigens prepared from suspensions of yolk sacs infected with R. mooseri, R. prowazekii and R. canada were used to study the IF antibody responses of patients infected with members of the Typhus Group. Primary antibody responses characterized by the development of both IgM and IgG antibodies were demonstrated when murine typhus was contracted by individuals who had been inoculated with epidemic typhus vaccine but did not have detectable levels of antibody prior to infection. Some of these responses were not appreciably different from that seen in a patient with murine typhus who had never been vaccinated. When murine typhus or epidemic typhus occurred in patients who had levels of circulating antibody from previous epidemic typhus vaccination, a secondary or booster antibody response was observed. Only IgG antibodies were produced. Because of the close relationship between the surface antigens of R. mooseri and R. prowazekii, and the sensitivity of the immunofluorescent technic, it was not possible in most of the cases to identify the etiologic agent causing the disease.

4. Results of current tests to compare the efficacy of a number of the newer antibiotics on the in vitro and in vivo effect on scrub typhus rickettsiae indicated that none had anti-rickettsial activity comparable to or greater than the tetracyclines and chloramphenicol.

5. In 1967, an agent which was highly lethal for mice and embryonated eggs was recovered in Thailand from the blood of a patient whose illness was associated with fever and rash. Serologic and biologic studies by the Department of Rickettsial Diseases, WRAIR, identified it as a chlamydia belonging to the B group of psittacosis-like agents. In June 1968, another chlamydial agent was recovered from the blood of another patient in Thailand with a febrile illness. Preliminary studies indicate that it is also a Group B chlamydia.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 167 Rickettsial infections

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26. OBJECTIVE, 27. APPROACH, 28. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) - Studies on the etiology, ecology, epidemiology, pathogenesis, physiological, immunological and diagnostic aspects of diseases of microbial origin which are current or potential problems to military forces. Current emphasis on meningococcal infections and mycoplasma infections in military forces.

24 (U) - Development of bacteriological techniques - holding media, genetic homology, serological bacteriophage and bacteriocine typing systems, antibiotic sensitivity tests, and cultural methods - for recovery and study of bacteria, bacterial L forms and mycoplasma, collection of sera, case histories, clinical materials, etc. from cases of above disease entities. Field studies on prophylactic regimens, spread and persistence of meningococci in recruit populations - air sampling of transmission of acute respiratory disease agents.

25 (U) - 69 01 - 69 06 Group C sulfadiazine resistant meningococci have spread throughout the Army training centers and now account for 85 per cent of meningitis cases. Carrier rates for group C meningococci have varied in the different posts, but the carrier rate in group C vaccinated recruits was reduced. The polysaccharide from serogroup Bo (Y) has been isolated and purified and is distinct from classical groups A, B and C. All types and representative strains of mycoplasmas and reference strains of L-phase variants in the American Type Culture Collection have been fully characterized in terms of their diagnostically significant biochemical reactions and DNA base compositions. A new serologic test for influenza virus infection is being developed. Studies of adenovirus immunity have shown that serum and nasal secretory antibodies develop in all recruits who are naturally infected. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68-30 Jun 69.

(FOR ARMY USE)

Project 3A061102B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 168, Bacterial diseases

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Description.

Studies of meningococcal meningitis have covered a variety of epidemiologic observations, transmission experiments, extraction of antigens and measurement of antibodies. Diagnostic methods are being evaluated as are attempts to develop new chemoprophylactic agents.

Investigations of mycoplasma and bacterial variants were mainly at the level of isolation, identification and classification; techniques which are necessary before it will be possible to ascertain the etiological role of the organisms in human disease.

Bacteriologic support has been provided to a malaria project involving growth of sporozoites in mosquitos. Diagnosis of viral infections by serologic means and studies of local respiratory tract antibodies in viral diseases are other microbiological problems under investigation.

Progress.

1. Studies of meningococcal meningitis.

a. Prevalence of the various serogroups among case strains of Neisseria meningitidis submitted to WRAIR.

During the reporting year 9 May 1968 to 15 May 1969 a total of 396 strains of N. meningitidis isolated from systemic infections were submitted by other laboratories for confirmatory and sulfadiazine resistance studies. Only a few, sixteen, were submitted from laboratories outside of CONUS. The majority of these strains were isolated from cases of clinical meningitis in military personnel, primarily recruits, and a much smaller number were isolated from dependents. A summary of these strains indicating their source by Army area, serogroup and sulfadiazine resistance is presented in Table 1. Of the 396 strains studied, 349 (88 percent) were serogroup C and 35 (9 percent) were serogroup B. Eleven of the remaining 12 (3 percent) were either of the WRAIR serotypes Boshard ("Slaterus Y") or 135-III. The one remaining strain, isolated from the CSF of an enlisted man recently returned to CONUS from South Korea, was identified as belonging to serogroup A. This is only the second such clinical isolation from military personnel in CONUS since WRAIR initiated intensive meningococcal studies in 1964.

Table 2 summarizes the meningococcal experience of the U. S. Army from 1964 through 15 May 1969. It should be noted that the data in Table 2 is constructed on the basis of a calendar year rather than reporting year. It can be seen here that in 1968 and 1969 the replacement of serogroup B by serogroup C (88 percent of all isolations) as the prime cause of meningococcal meningitis in the U. S. Army is essentially complete. Table 3 demonstrates a similar picture with regard to sensitivity of these group C strains to sulfadiazine. As the incidence of group C meningococcal isolates increased from 1965 to 1967 within these group C organisms, a great increase, from 4 percent to 51 percent, was noted in the strains resistant to one mcg or more sulfadiazine per ml. Nationwide the percentage of sulfadiazine resistant group strains appears to have reached a plateau at 96 percent in 1968, a level which completely destroys the utility of sulfadiazine as a therapeutic or chemoprophylactic agent.

In the past annual reports the data concerning the characterization of case strains was presented on a nationwide basis which provided information on gross epidemiologic trends. Reexamination of this same data on the basis of yearly trends within specific Army areas has cast additional light on the events that led up to present epidemiologic picture. Table 4 describes the yearly changes in prevailing meningococcal serogroups within each Army area. As early as 1965 a slight shift could be detected in the percentage of group C strains isolated from

Table 1. Source, serotypes and sulfadiazine resistance of case strains of *N. meningitidis* received by WRAIR from 9 May 1968 to 9 May 1969.

	Serogroup*										
	A		B		C		Bo(Y)		135-III		
Army Area	S	R	S	R	S	R	S	R	S	R	Area Total
I	0	0	4	0	2	129	1	0	0	0	136
II	0	0	0	0	2	22	0	1	1	0	26
III	1	0	7	7	1	65	1	0	1	0	83
IV	0	0	1	6	1	24	1	0	0	0	33
V	0	0	0	0	0	17	3	0	0	0	20
VI	0	0	1	3	0	78	0	0	0	0	82
Europe	0	0	7	0	3	5	0	0	0	0	15
Other**	0	0	0	0	1	0	0	0	0	0	1
Total	1	0	20	16	10	340	6	1	2	0	396
% of serogroup sulfa resistant		0%		44%		97%		14%		0%	

* WRAIR serogroup 29E not encountered; sensitive (S) or resistant (R) to 1 mcg/ml sulfadiazine.

** Puerto Rico; one strain.

Table 2. Changes in prevalence of serogroups B and C N. meningitidis among case strains submitted to WRAIR from 1964 to 1969*.

	1964		1965		1966		1967		1968		1969*	
Serogroup	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
B	295	85.8	157	84.0	231	80.5	54	35.3	35	17.1	26	10.3
C	33	9.6	24	12.8	41	14.3	86	56.2	268	84.5	223	88.1
Other**	16	4.6	6	3.2	15	5.2	13	8.5	14	4.4	4	1.6
Total	344	100.0	187	100.0	287	100.0	153	100.0	317	100.0	253	100.0

*1969 through 15 May.

**Includes serogroup A and WRAIR serogroups Boshard, 29E and 135-III.

Table 3. Changes in occurrence of sulfadiazine resistance among case strains of N. meningitidis submitted to WRAIR from 1964 to 1969.

		1964	1965	1966	1967	1968	1969*
	No. received	297	157	231	54	35	26
Group B	No. Sul R	194	89	162	35	16	14
	% Sul R	65%	57%	70%	65%	46%	54%
	No. received	33	24	41	86	268	223
Group C	No. Sul R	3	1	21	75	258	213
	% Sul R	9%	4%	51%	87%	96%	96%

* Only through 15 May 1969.

Table 4. Increase in the prevalence of serogroup C N. meningitidis among strains received from cases of meningococcal disease in CONUS from 1964 to 1969.

Year	U. S. Army Area I	II	III	IV	V	VI	Total
1964	4% (1/28)*	4% (1/24)	11% (5/47)	6% (3/46)	19% (9/47)	8% (7/91)	9% (26/283)
1965	23% (5/22)	4% (1/23)	7% (2/27)	12% (4/32)	7% (2/29)	14% (1/7)	11% (15/140)
1966	40% (23/58)	0% (0/23)	10% (5/52)	4% (3/80)	11% (5/44)	10% (1/10)	14% (37/267)
1967	82% (49/60)	53% (9/17)	33% (11/33)	50% (5/10)	50% (9/18)	25% (2/8)	58% (85/146)
1968	95% (142/149)	83% (19/23)	73% (61/83)	59% (10/17)	73% (8/11)	83% (19/23)	85% (259/306)
1969**	99% (79/80)	95% (19/20)	78% (45/58)	71% (15/21)	83% (10/12)	96% (68/71)	90% (236/262)

* (Group C N. meningitidis/Total N. meningitidis)

** (1969 - 1 Jan through 15 May)

cases of meningitis in the 1st Army Area. By 1967, 82 percent of the cases were the result of group C infections and by 1968 group B infections are as rare as group C infections were in 1964. During the period 1965 to 1966 group C infections occurred at a low rate in the other five Army areas. The increased occurrence of group C strains was not apparent in the 2nd, 3rd, 4th and 5th Army areas until 1967, and in the 6th Army Area until 1968. These data suggest a fan-like geographical spread of group C strains out from the Northeast U. S. starting in 1966 and reaching the West coast in 1968.

A very similar picture is seen when data concerning the occurrence of sulfadiazine resistant group C meningococci is classified by year and Army area (Table 5). An almost linear increase in the percentage of resistant strains took place in the 1st Army Area from 1965 to 1967. Similar increases in the remaining Army areas also lagged a year behind the 1st Army Area. During these same periods when group B strains were declining the percentage of sulfadiazine resistant group B strains among all the group B isolates remained relatively stable, fluctuating about the 60 percent level.

We are presented with two probably related phenomena; first an increase in the incidence of group C meningococcal meningitis over group B which appeared initially in the Northeast U. S. followed by a rapid spread south and west to the Pacific coast, and secondly, within the group C meningococci a rapid increase in the incidence of sulfadiazine resistant organisms showing the same geographic movement to the west. One may postulate (1) the introduction of sulfadiazine resistant group C strains into the Northeast U. S. in 1965 or 1966, (2) that these strains were better able to colonize the nasopharynx than the then prevailing strains, (3) that they were also more able to produce systemic infections and (4) that sporadic sulfadiazine chemoprophylaxis exerted sufficient selective pressure to enable these strains to become fully established and spread throughout the U. S. However, lacking information on (1) virulence, (2) the possible relationship of sulfadiazine resistant to virulence and (3) the nature and effect of chemoprophylaxis, all of the foregoing are at best conjecture.

b. Meningococcal carrier surveys.

Meningococcal carrier surveys were performed in basic training companies to determine the prevalence of carriers and of the various serogroups and to observe the effect of group C vaccination upon transmission.

The methods used were the same as previously described, namely, streaking high pharyngeal swabs on to chocolate agar media containing lincocin and polymixin. Isolates were confirmed by carbohydrate fermentations and serogrouping.

Table 5. Increasing occurrence of sulfadiazine resistant* group C N. meningitidis by Army area from 1964 to 1969**.

Year	I	II	III	IV	V	VI	Total
1964	0% (0/1)***	100% (1/1)	0% (0/5)	0% (0/3)	22% (2/9)	0% (0/7)	12% (3/26)
1965	20% (1/5)	0% (0/1)	0% (0/2)	0% (0/4)	50% (1/2)	0% (0/1)	7% (1/15)
1966	65% (15/23)	0% (0/0)	20% (1/5)	33% (1/3)	80% (4/5)	100% (1/1)	50% (18/36)
1967	94% (46/49)	89% (8/9)	54% (6/11)	60% (3/5)	100% (9/9)	100% (2/2)	87% (74/85)
1968	99% (140/142)	95% (18/19)	95% (61/64)	90% (9/10)	88% (7/8)	95% (18/19)	96% (253/262)
1969	99% (78/79)	95% (18/19)	98% (44/45)	100% (15/15)	100% (10/10)	97% (66/68)	98% (231/236)

* Resistant to 1.0 mcg sulfadiazine/ml medium.

** Through 15 May 1969.

*** (Sulfadiazine resistant group C/no. group C received from Army area)

Results in Table 6 are from eight BCT companies at Fort Dix, N. J. Vaccine was not being used during this period.

In general, carrier rates were lower than those observed in the previous year. The low rate of 24 percent was seen in early fall; the highest rate was 63 percent in E-3-3 in December. The data on these small samples must be interpreted cautiously because of the variation in percentage of carriers in different cohort companies; i.e., C-3-3 and E-3-3. Also, different patterns of prevalence of serogroups occurred in different training companies: C-3-3 showed group C strains to be most common; E-3-3 had Bo organisms predominating: A-1-3 had NT strains in larger numbers than C or Bo; B-1-3 showed C strains primarily.

In two companies, A-4-3 and C-4-3, in which approximately 20 percent of the men had received group C polysaccharide vaccine, carrier surveys performed at two week intervals revealed significant reductions in group C meningococcal acquisitions (Table 7). Total carrier rates and total acquisitions were not different between vaccinated and nonvaccinated men. These results are in agreement with those observed a year ago and are indicative of the ability of the vaccine to provide a local nasopharyngeal immunity to the meningococcus.

Two other companies were surveyed at two week intervals during BCT, one other was tested during the seventh week only and one company at Fort Bragg was surveyed during the sixth week. These data are shown in Table 8. In each instance total meningococcal acquisitions were comparable in the vaccine and control groups. In companies B-4-2 and B-3-2, although the differences in C carrier rates were not significant at the 5 percent level, there was a decided trend for fewer C carriers amongst the vaccinated groups. Companies D-4-2 (Fort Dix) and D-6-2 (Fort Bragg), which had the lowest total transmission showed, no differences in the group C carrier rates between vaccinated and control groups.

In these companies there was no apparent trend for any particular serogroup to fill the niche created by reduced group C carriers amongst vaccinated recruits. Serogroups B and 29E were increased in vaccinated men in one company each; group Bo spread in vaccinees in two companies. Further observations along these lines are needed.

c. Dissemination of meningococci by carriers.

Most cases of meningitis are acquired from contact with asymptomatic carriers rather than contact with a diseased individual. Thus, for several years we have attempted to study those factors responsible for person-person transmission. Epidemiologic analysis of pharyngeal acquisitions among roommates and air sampling of rooms and

Table 6. Meningococcal carrier surveys - Fort Dix, nonvaccinated units.

Company	Date	Week of training	No. pos/ No. tested	% Pos.	Serogroup						
					B	C	Bo	29E	135	Non-groupable	Other
D-3-3	10 Sept 68	7	30/100	30	5	4	13	0	0	8	
E-6-3	30 Sept 68	7	24/100	24	9	1	5	1	2	6	
C-3-3	26 Nov 68	4	29/97	30	2	10	5	0	0	11	1
	9 Dec 68	6	27/84	32	4	14	3	0	0	6	0
E-3-3	26 Nov 68	4	35/97	36	2	5	17	0	0	10	1
	9 Dec 68	6	50/79	63	4	11	30	1	0	4	
B-5-3	15 Jan 69	3	19/100	19	5	2	6	0	0	6	
A-5-3	15 Jan 69	3	22/100	22	1	2	6	4	0	9	
A-1-3	26 Jan 69	7	38/80	48	2	11	8	0	0	17	
B-1-3	28 Jan 69	7	48/84	57	5	26	3	1	0	13	

Table 7. Effect of group C vaccine on meningococcal acquisitions, Fort Dix, 1969.

Company and % vaccinated	Carrier status	Week of training								Total acquisitions	
		1		3		5		7			
		V	C	V	C	V	C	V	C	V	C
A-4-3 V=20%	Total Mgc	27*	36	29	29	34	55	77	81	76	83
	group C	5	3	5	6	3	19	13	32	12**	42
C-4-3 V=20.9%	Total Mgc	31	22	23	14	29	35	76	69	77	76
	group C	2	0	3	1	0	11	17	36	21***	38

V - Vaccinated group

C - Nonvaccinated controls

* - Percentage of those tested

** - P = <0.01

*** - P = <0.02

Table 8. Percentage of vaccinated and control recruits acquiring meningococci in the upper respiratory tract, Fort Dix.

Unit and vaccine	status	No. of men	Serogroup acquired (% of men)						
			B	C	Bo	29E	135	NT or Ro	Total pos[1]
B-4-2	V	33	6	15	6	15	0	18	58
	C	109	12	31	9	5	1	10	66
D-4-2	V	34	6	12	6	3	0	15	42
	C	101	6	13	1	9	0	13	33
B-3-2[2]	V	19	11	11	16	5	0	26	69
	C	116	12	28	5	6	0	20	72
A-4-3	V	43	24	12	22	12	0	14	76
	C	173	14	42	22	9	2	22	83
C-4-3	V	45	5	21	21	10	0	44	77
	C	174	8	38	7	7	0	31	76
D-6-2[3]	V	29	3	3	14	3	3	6	34
Ft. Bragg	C	134	7	6	13	8	7	12	52

[1] Total positive = % of men who became carriers. Men who acquired more than 1 serogroup are counted once.

[2] Single 7th week survey - only 65% of men sampled.

[3] Single 6th week survey - 88% of men sampled.

coughs from individual carriers have shown that transmission is primarily direct via large droplets but many acquisitions occur outside sleeping quarters. Carriers varied in the numbers of organisms dispersed by coughing but these variations did not correlate with presence or absence of respiratory symptoms or presence of a concurrent viral infection.

Continuation of studies of dispersal by single coughs from carriers utilized the Large Volume Air Sampler (LVS) with attached pre-impactor and collection methods described previously. Special emphasis was placed on chronic carriers as compared to recruits studied last year. Subjects were selected from laboratory personnel known to be chronic carriers of meningococci.

Five carriers were tested from two to seven times over a period of one to two months. With three exceptions less than 25 organisms were dispersed with each cough. One individual, H. Sch., expelled 68 organisms on one occasion five weeks after acquisition of a group C meningococcus. Prior to and following this one cough other tests showed minimal or negligible dispersal. J.S. excreted 32 organisms; identified as group B on one occasion. Three days earlier and one month later no aerosolized meningococci were found; at these times he was carrying a NT meningococcus. F.T., a Bo carrier, coughed 155 organisms on one occasion; ten days prior and one month later less than 25 were expelled.

These data were summarized together with chronic laboratory carriers tested last year and with results of tests on recruits last year (new carriers). Results are shown in Table 9. The chronic carriers (defined as duration more than eight weeks) are, in general, minimal dispersers; only three of 12 had a maximum cough dispersal of more than 25 organisms. Eight of 16 acute carriers (less than eight weeks) were expelling 25 or more organisms per cough and six of these recruits had been nasopharyngeal carriers less than two weeks.

Previous data comparing relation of dispersal to serogroup of organism and sulfadiazine resistance among recruit carriers was revised and is shown in Tables 10 and 11. The conclusions have not changed; i.e., neither serogroup nor sulfa resistance correlated with dispersal.

Summary of the various factors related to dispersal of meningococci by coughing is shown in Table 12.

d. Meningococcal antigens.

(1) Endotoxins. Studies of meningococcal endotoxins have been undertaken to establish the role of these antigens in determining strain specificity within the serogroups. Chemical, serologic and biologic assays are being developed for this purpose.

Table 9. Duration of carrier state compared with magnitude of dispersal.

Duration	No. of organisms				
	0-12	13-25	26-50	51-100	101-200
Less than 2 weeks	4*	0	1	2	3
2-3 weeks	3	0	0	0	0
Less than 8 weeks	1	0	0	1	1
More than 8 weeks (recruits)	1	0	0	1	0
More than 8 weeks (lab personnel)	5	3	1	0	1

* No. of individuals; maximum sample used.

Table 10. Comparison of meningococcal serogroup carried and number of organisms dispersed in new carriers.

Serogroup	No. or organisms				
	0-12	13-25	26-50	51-100	101-200
NT	4*	0	0	1	1
B	3	0	2	0	0
C	5	1	2	2	1
Bo	2	0	0	0	0
29E	0	0	0	1	0

* No. of men based on initial cough sample.

Table 11. Relation of sulfa resistance to dispersal.

	No. of organisms				
Sulfonamide	0-12	13-25	25-50	51-100	101-200
Resistant	6*	0	3	2	1
Sensitive	8	1	1	2	1

Table 12. Factors related to magnitude of dispersal.

Factor	Conclusion
1. Magnitude of nasopharyngeal swab culture	Not related
2. Simultaneous viral infection	Not related
3. Presence of respiratory symptoms	Not related
4. Serogroup of organism	Not related
5. Sulfadiazine resistance	Not related
6. Variation in dispersal from day to day	Minimal
7. Duration of carrier state	Suggestive
8. Ambient temperature	Not tested
9. Ambient relative humidity	Not tested
10. Viscosity of secretions	Not tested
11. Presence of local antibodies	Not tested

(a) Preparation of endotoxin:

Organisms: Well defined N. meningitidis serogroup C strains from the departmental collection were used. All were clinical isolates from blood or CSF. Inoculum seed cultures were taken from lyophilized vials, at same level of passage, as near to original isolate as possible (within three-six passes), plated on Barile's agar and incubated at 37°C in CO_2 incubator overnight. This plate culture was checked for purity by Gram stain and a heavy loopful inoculated into 1000 cc of Mueller-Hinton Broth (Difco) in a two-liter flask which was incubated at 37°C on rotary shaker for six hours and then checked again for contamination by Gram stain. The inoculum flask was then aseptically added to mass culture media. Initially this was a 26 liter carboy containing 14 liters of Mueller-Hinton Broth; later this was done in a 14 liter jar of Microferm Fermentor (New Brunswick Scientific Co.). The mass culture was grown with aeration through a sparger, with stirring, at 37°C for 18 hours. In the morning, mass culture was checked for contamination by Gram stain and plate culture. Organisms were removed from media by continuous flow centrifugation in RC2 Centrifuge and KSB Continuous Flow accessory (Sorvall) at 15,000 rpm. The cell pack was suspended in 0.5 percent formalin saline solution for 30 minutes and then washed twice with normal saline; cells were then suspended in small amount of distilled water to give a thick slurry, shell frozen and lyophilized. Next day, dry cells were weighed and stored under vacuum over P_2O_5 at 4°C until used for extraction.

Endotoxin extraction: The hot phenol-water method of Westphal was used to prepare crude endotoxin which was extensively dialyzed against frequent changes of distilled water for four days and then concentrated in vacuo to one-fifth its original volume. The concentrated endotoxin was then ultracentrifuged 105,000 x g for three hours to separate the endotoxin (gel layer) from the nucleic acid (supernatant). Ultracentrifugation was repeated until endotoxin was free of U.V. absorbing material at 270 mμ. Gel layers containing purified endotoxin were lyophilized. Yields average 1-2 percent of dry weight of cells.

Chemical analysis: Earlier chemical analysis on a serogroup C meningococcal endotoxin (F. Wyle, unpublished) show it to contain:

total hexose (as glucose	14%
total lipid	20%
fatty acid	24%
protein	1%
sialic acid	2%
KDO	2%
heptose	4%
phosphate	6%

The present lots of purified endotoxin have a total hexose content (as glucose) of 8-13 percent.

While investigating the sialic acid content using the Resorcinol test, it was found that the percentage of sialic acid varied from 7.5 percent to 2.5 percent depending on the number of ultracentrifugation steps and the degree of purification. This is interpreted as further purification removing contamination group specific polysaccharide which itself contains approximately 80 percent sialic acid.

The Thiobarbituric Acid (TBS) assay also detects sialic acid but unlike the Resorcinol test measures presence of all 2-Keto, 3-Deoxy sugars. The TBA test on unhydrolyzed endotoxin showed a content of 2.75 percent; this is unusual in that KDO or sialic acid is usually found in bound form and the TBA test only measures the free forms. After acid hydrolysis there was found 16.6 percent TBA reactive material. This probably indicates that there is about 14 percent KDO present in the endotoxin.

It was hoped that neuraminidase (sialidase) could be used to remove any contaminating C polysaccharide. However, using V. cholera neuraminidase, it was found that not only did the enzyme not remove sialic acid from the endotoxin but it failed to digest the C polysaccharide (approximately 80 percent sialic acid) and also would not digest Boshard polysaccharide (approximately 48 percent sialic acid).

Lipid was extracted from endotoxin, methylated and assayed by gas-liquid chromatography. (GLC performed by CPT Standefer, Department of Biochemistry, WRAIR.) The patterns, with large peaks of C-16 and C-18 fatty acids, were identical to previously published GKC examinations of Neisseria (Yamakawa and Ueta, Japan J. Exp. Med. 34:361, 1964).

Preliminary paper chromatography of endotoxin carbohydrates have shown the presence of glucose, galactose, ribose and, as yet, two unidentified spots.

Further efforts in purification using sepharose gel filtration and quantitative and qualitative analysis are in progress.

(b) Serology:

The antigenic characteristics of meningococcal endotoxin are being explored to determine what serologic specificities are carried by this structure. With analogy to Salmonella, where "O" antigen (strain) specificity resides in its endotoxin, it is hypothesized that meningococcal strain specificity is in its endotoxin.

By slide immunodiffusion the endotoxins of serogroup C organisms reacted with serogroup C antisera to give a single line. This precipitin line cross-reacted with a line for C polysaccharide. There was also a cross reaction with some serogroup B antisera. There were no precipitin lines formed with serogroup A, Bo, X, or Z antisera.

Most of the recent endotoxin serology has involved the hemagglutination test and the hemagglutination inhibition test. Sheep red blood cells were fixed with pyruvic aldehyde according to the method of Ling (Brit. J. Hemat. 7:299, 1961). Grids were run for each endotoxin to determine the optimal concentration of antigen for sensitization. While it was found that endotoxins with high sialic acid content could directly sensitize the fixed RBC's, those with low sialic acid content had to be treated with dilute NaOH (0.1 N, 56°C for 6 min.) and then neutralized to pH 7.5 (0.1 N NaOH) to allow sensitization. Endotoxin sensitized RBC's gave a sensitive and reproducible system for detection of anti-meningococcal antibody in both human and rabbit sera. The specificity of this test was for serogroups; as with meningococcal polysaccharide it differentiated between different serogroups with only minimal cross reaction. It proved to be somewhat more sensitive than C polysaccharide, averaging two to three tubes higher titer when the two were run in parallel. However, no significant differences between serogroup C sera made against different strains were detected. One finding, which is as yet unexplained, is that individuals immunized with C polysaccharide vaccine have antibody that reacts with endotoxin sensitized RBC's, and do so usually to a higher titer than with C polysaccharide sensitized RBC's. There are two fragmentary pieces of information that seem to indicate that this phenomenon is not due to C polysaccharide contamination of endotoxin. First, there are some vaccine/human sera that do not have a significant titer against endotoxin sensitized RBC's but do have a high titer against C polysaccharide sensitized RBC's; secondly, in the hemagglutination inhibition test, C polysaccharide did not inhibit the hemagglutination of endotoxin sensitized RBC's while completely inhibiting C polysaccharide sensitized RBC's. Attempts to clarify these findings are in progress.

Preliminary indications are that the meningococcal bactericidal test (described in another section of this report) may be the technique that can best demonstrate strain specificity.

(c) Biologic activity:

In conjunction with the chemical and serologic investigations of meningococcal endotoxins, the biologic activity of endotoxin preparations is being tested. Endotoxins are lethal to 21 day old Swiss-Webster mice (LD_{50}-750 µgm/mouse), produce both the localized and the generalized Shwartzman reactions, and produce febrile responses in rabbits. One assay of interest is chorioallantoic membrane inoculation of 10 day old chick embryos. The toxicity (LD_{50}) of our preparations

in this assay varies from 0.5 μgm and 2 μgm per egg. We have also been exploring the technique of *in vitro* neutralization of chick embryo toxicity as another serologic assay. Here, too, further work is in progress.

(2) Polysaccharide of serogroup Boshard. Polysaccharide antigens of group A and C meningococci have been shown by Gotschlich, et al. to be immunogenic in man. Group B polysaccharide has been isolated but has not been analyzed for chemical or immunologic properties. Serogroup Boshard (Bo), although a rare cause of disease, has been frequently found in carrier surveys. Previous investigations have shown that Bo strains and Y strains of Slaterus appear to be identical based upon agglutination reactions. In order to better define these relationships isolation of the polysaccharide antigen was undertaken.

Initial attempts to extract Bo polysaccharide by using the cetavlon method of Gotschlich did not yield a satisfactory Bo antigen as it does with groups A and C meningococci. A new method was devised which incorporated procedures of Sherp and Rake and those of Gotschlich. This report summarizes the method of extraction and some of the properties of the purified polysaccharide.

The following procedure has given satisfactory preparations. The original strain, 31-M, was grown in Frantz medium, a chemically defined medium consisting of casamino acids, dextrose, cystine and inorganic salts, for 18 hours with vigorous shaking. After checking each flask by Gram stain and macroscopic agglutination, toluene was added to each flask for increased lysis. Twenty liters of culture were concentrated *in vacuo* by flash evaporation to 600 ml. After dialysis against distilled water, the insoluble debris was removed by centrifugation and discarded. To the supernatant fluid was added absolute ethanol and $CaCl_2$ to a final concentration of 30 percent and one percent respectively resulting in the immediate precipitation of deoxyribonucleic acid and the slower precipitation of ribonucleic acid. The nucleic acids were then removed by centrifugation and the supernatant fluid was brought up to a concentration of 75 percent ethanol resulting in the immediate precipitation of the polysaccharide. The polysaccharide was washed in absolute ethanol to remove excess calcium salts, washed in acetone, then in ether and dried *in vacuo*.

The precipitate was pulverized and made soluble by grinding with a glass rod in M sodium acetate, clarified by filtration through a Millipore prefilter pad and reprecipitated with absolute ethanol. The dried polysaccharide was dissolved in distilled H_2O and removal of proteins in the form of a cake was accomplished by repeatedly shaking the clear supernatant fluid with chloroform and butanol according to the method of Sevag. Addition of 2/3 volume of saturated cupric acetate

to 1/3 volume of the polysaccharide was added to remove any residual contaminating proteins. After centrifugation, the polysaccharide was removed from solution by the addition of ethanol, dissolved in saturated sodium acetate at pH 7 and reprecipitated in ethanol. The process was repeated twice to remove the blue color of the cupric acetate and to assure that the polysaccharide was recovered as the sodium salt. The maximum polysaccharide yield from a 20 liter culture was 200 mg.

Studies of the chemical composition of Boshard polysaccharide were undertaken. Resorcinol-HCl tests on the purified Boshard antigen were performed to determine the presence of sialic acid which is a major component of the polysaccharides from groups B and C. The Boshard antigen was found to be composed of 48 percent sialic acid. Further analyses to determine the remaining constituents of the antigen were performed. The anthrone test using varying glucose standards revealed the presence of a hexose which constitute 36 percent of the polysaccharide antigen. Chromatographic data indicated that the neutral hexose was glucose. To exclude the possibility that glucose was present only as a contaminant and not as a constitutive portion of the Boshard antigen, the "glucostat" test, which is an enzymatic test specific for glucose, was employed. The presence of glucose as a major antigenic component was confirmed.

The Boshard polysaccharide was also analyzed for the presence of protein and nucleic acid contamination. By the method of Lowry, et al. a protein content of 0.3 percent was found. Nucleic acid determinations were made by ultraviolet spectroscopy at 260 mμ. Only 0.1 percent nucleic acid contamination was detected.

The molecular weight of Boshard polysaccharide was determined by agarose columns. Three Biolgel A columns were prepared - 0.5 M, 100-300 mesh with a range of 10,000-500,000; 1.5 M, 50-100 mesh with a range of 10,000-1,500,000 and 15 M, 100-200 mesh with a range of 40,000-15,000,000. In each column one ml containing one mg of Boshard polysaccharide was compared to blue dextran which has a molecular weight of two million. In the first two columns, the Boshard polysaccharide, as well as the blue dextran, were found in the void volume. Although both blue dextran and Boshard polysaccharide were retained by the 15 M column, the blue dextran was eluted before Boshard. Therefore, the molecular weight of Boshard polysaccharide is larger than 1.5 million and less than 2 million.

Dry weight analysis of the purified Boshard polysaccharide was performed by Dr. W. C. Alford at the National Institutes of Health. The results are shown in Table 13.

Table 13. Dry weight analysis of Boshard polysaccharide

Element	Percent calculated*	Percent present
C	40	42.90
H	-	5.60
N	2.5	2.87
P	-	0

* As 48 percent sialic acid.

By immunoelectrophoresis the Boshard polysaccharide is composed of at least two fractions. One has a net neutral charge and does not migrate; the other component, probably sialic acid polymer, is negatively charged and migrates to the anode.

The Bo polysaccharide was used in a passive hemagglutination test. A 10 percent suspension of glutaraldehyde fixed erthrocytes was washed 3X in .15 M phosphate buffered saline (PBS) at pH 7.3. The concentration was adjusted to two percent and the purified antigen was added and incubated at 37 C for 30 min. It was determined that 20 μg/ml of polysaccharide was the optimal antigen concentration required for sensitization. The sensitized cells were washed 3X in PBS and readjusted to 0.5 percent concentration. The microtiter system was employed using 0.05 amounts of the constituents in round bottom plates. To perform the test, 0.05 ml of a heat-inactivated serum was added to the first well and serially diluted with 0.05 loop in 0.05 ml PBS. The sensitized cells were added and the plates incubated at 4 C overnight. Titers of the antisera were expressed as the last well showing 3+ (75 percent) agglutination. The serologic specificity of the antigen is shown in Table 14. In this table only serogroup Y has high cross reactions with Boshard. Moreover, sera to both Boshard and Y absorbed with Boshard or Y bacteria showed complete identity in the Bo HA test (Table 15).

Specificity of the polysaccharide and high titered cross reactions with Y were further demonstrated by hemagglutination-inhibition (HI) test. In this test both crude and purified antigens, as well as whole cell bacterial antigens, could be tested. The latter were prepared by making a heavy suspension of an agar grown log phase culture in one ml of PBS followed by high speed centrifugation. Performance of the test was accomplished by diluting the antigen serially followed by the addition of 0.025 ml containing two units of Boshard antiserum. After incubation at room temperature for at least

Table 14. Specificity of the passive hemagglutination test with Boshard polysaccharide sensitized preserved cells against homologous and heterologous meningococcal antisera.

Serum Designation	Strain used for immunization	Reciprocal of titers
592	A	0
000	A	320
718	A	160
95	A	220
572	A	0
Zuesher	B	160
881	B	0
880	B	0
887	B	0
40	B	0
1628	C	0
N.I.H.-SB	C	0
9 mis	C	0
601	C	0
JE5	C	10
96	X	320
97	X	160
98	X	80
100	Y	2560
99	Y	160
646	Y	5120
653	Z	20
650	Z	20
602	Bo	2560
642	Bo	640
636	Bo	2560
644	Bo	1280
707	Bo_6	2560
717	Bo_{13}	5120
719	Bo_{13}	320

Table 15. Titers of the passive hemagglutination test with Boshard polysaccharide sensitized preserved cells against Boshard and Y absorbed antisera.

Serum	Strain used for immunization	Absorbing antigen	Reciprocal titer
602	Bo	-	2560
602	Bo	Bo	0
602	Bo	Y	0
602	Bo	X	2560
602	Bo	Z	2560
646	Y	-	5120
646	Y	Bo	0
646	Y	Y	0
646	Y	X	5120
646	Y	Z	5120

15 min., 0.05 ml of sensitized RBC's were added to each well and the plates were reincubated at 4 C overnight. Boshard and Y supernate fluids inhibited the test to the same titer, once again showing identity. In addition, the variation in specific soluble substance produced by Boshard strains was revealed by this sensitive test. Thus, despite the antigenic distinctness of the Boshard serogroup to the other established serogroups, it has consistently shown identity or near identity to the Y serogroup by HA, HI, agglutination, agar gel diffusion and immunoelectrophoresis.

The halo precipitation test was also employed to test antigenic relatedness. In this method 0.5 ml of antiserum was incorporated in 10 ml of Eugon agar in petri dishes and log phase cultures were stamped on the plates with a modified Lidwell apparatus. After incubation at 37 C for 24 hrs. followed by incubation for an additional 24 hrs. the precipitates which are formed are a direct reaction of the specific soluble polysaccharides produced by a colony with the specific antiserum in the agar. In Table 16 Boshard and Y are not serologically related to the other serogroups but are identical to each other. Again it is demonstrated by this test that among 23 Boshard strains there were wide variations in the amount of specific polysaccharide produced or in the ability of the soluble antigen to diffuse.

The fluorescent antibody test was used as final test of the relationship of Boshard and Y serogroups. It was felt that because log phase bacterial cells are employed, the normal steric relationships of the surface and subsurface antigens were preserved. An agar grown log phase culture was suspended to approximate 1×10^9 organisms/ml which was diluted with PBS to a final yield of 2×10^8 organisms/ml. One drop of the suspension was placed on microscope slides and incubated at 37 C until dried and fixed. A drop of each dilution of immune serum was placed on a single spot of dried bacteria and placed in a moist chamber for 20 min. After washing 3X in PBS and removing excess fluid around the spots, a drop of fluorocein tagged goat-antirabbit serum at a 1:20 dilution was added to each slide. After further incubation in a moist chamber for 20 min. the slides were again washed 3X in PBS, dried around the spot and mounted with a coverslip and buffered glycerin. The results of the fluorescent antibody test are shown in Table 17.

Although each antigen exhibited high titers in its homologous antiserum, there were only low titer cross reactions to the heterologous antiserum. The apparent contradiction of this test to the other serological tests which confirmed the serological identity of the two serogroups has at least one possible explanation. The phenomenon called steric hindrance describes a situation where one organism may react in two antisera whereas the other may react in its homologous serum and very little or not at all in the heterologous serum. Although both organisms may possess a common antigenic component, it may be located on the surface layer of one and may be situated more deeply or

Table 15. Halo precipitation of N. meningitidis strains in the presence of Boshard and Y antisera.

Strain	Sero-group	Antisera 602 Bo(31M)	Antisera 646 Y	Antisera 719 Bo(13)	Antisera 138 Y
3-Lm	Bo	2+	-	2+	-
VII	Bo	2+	2+	-	-
2-I	Bo	2+	2+	-	NG
27-I	Bo	2+	+	-	-
33-I	Bo	2+	-	2+	-
56-I	Bo	±	+	-	-
76-I	Bo	-	3+	-	-
77-I	Bo	2+	3+	-	-
38-II	Bo	-	2+	-	NG
50A-II	Bo	-	2+	-	-
82-II	Bo	2+	3+	-	PG
101-II	Bo	-	+	-	-
104-III	Bo	+	+	2+	-
37-IV	Bo	+	+	2+	-
90-IV	Bo	2+	2+	-	PG
142-IV	Bo	-	±	-	-
117-III	Bo	3+	+	-	-
213-III	Bo	+	-	3+	-
139-III	Bo	-	2+	-	PG
147-III	Bo	2+	-	3+	-
173-III	Bo	+	3+	-	-
178-III	Bo	2+	2+	3+	+
191-III	Bo	+	+	3+	-
120 EUR	A	-	-	-	-
19 EUR	A	-	-	-	-
45 EUR	A	-	-	-	-
141-IV	B	-	-	-	-
130 EUR	B	-	-	-	-
153-I	B	-	-	-	-
166-IV	B	-	-	-	-
107-VI	C	-	-	-	-
97-V	C	-	-	-	-
51-V	C	-	-	-	-
68 Misc	X	-	-	-	-
75 Misc	X	-	-	-	-
76 Misc	X	-	-	-	-
18 Misc	Y	+	2+	+	+
69 Misc	Z	-	-	-	-
70 Misc	Z	-	-	-	-
71 Misc	Z	-	-	-	-

Table 16. (Continued)

* = Halo diameters recorded at 48 hrs.

\- = No halo

± = Questionable

\+ = <1 mm

2+ = 1-2 mm

3+ = 2-3 mm

NG = No growth

PG = Poor growth

[illegible] reactivity by titers of Bushard and Y antisera to Bushard and Y antigens.

Rabbit antisera	Bushard antigen: Reciprocal of titer	Y antigen: Reciprocal of titer
[illegible] - Bushard prebleed	[illegible]	[illegible]
[illegible] - Bushard postbleed	512	[illegible]
99 - Y prebleed	[illegible]	[illegible]
99 - Y postbleed	8	512

masked by other surface antigens in the latter. This phenomenon could prevent the group antigen site from reacting with a molecule of antibody.

It is concluded that serogroups Bosbard and Y possess identical group specific polysaccharides. There appear to be variations in the amount of polysaccharide produced by different strains and the structural relationships of the polysaccharide to the intact organism may also vary.

e. Meningococcal antibodies.

(1) Strain specific bactericidal antibodies.

(a) Background and objectives: Currently available serologic techniques permit the classification of meningococci into broad groups on the basis of the agglutination of whole organisms by specific rabbit antiserum. The existence of strains within these broad serogroups (A, B, C, Bo, 29E, 135, X, Z) has long been suspected, but no practical method of detecting antigenic differences between strains has been developed.

Serum bactericidal activity is a very sensitive indicator of antibody against meningococci. Previous work at WRAIR and by Roberts (Roberts, R. J. Exp. Med. 126:795-818, 1967) has suggested that the bactericidal reaction could be used to detect antibody differences between strains of the same serogroup.

The work to be described has several goals:

1. To develop a reproducible bactericidal assay using rabbit antiserum against case strains.

2. To use the test to distinguish antigenic differences between strains within a serogroup.

3. To determine the antigenic components against which the bactericidal antibody is directed.

The ability to identify strains within the broad serogroups would greatly enhance the study of the dynamics of transmission of meningococci among recruits. Equally important, serologic identification of strains will benefit the study of drug resistance, genetic transformation, and other basic aspects of the microbiology of meningococci.

b. Methods.

Culture methods - Lyophilized group C meningococci from the USAIR collection were suspended in 5.0 ml of phosphate buffered saline (pH 7.0, 0.1M) and plated on Barile's agar. After overnight growth at 37 C in a CO_2 incubator, a loopful of organisms were inoculated into 25 ml of Mueller-Hinton broth (Difco) in a 250 ml Nephelometer flask and incubated aerobically at 37 C in a rotary shaker water bath at 125 rpm.

The optical density at 650 mμ was measured every 30 min. in a Coleman Jr. Spectrophotometer. An optical density of 0.1 was found by colony count to correspond to a concentration of approximately 3×10^8 organisms/ml. This O.D. value was attained 30-90 min. after inoculation of the flask and fell on the early log phase of the growth curve.

Preparation of antiserum - Antisera to 14 group C strains were prepared in New Zealand rabbits (1-2 Kg) by the administration of a single intravenous injection of 5×10^8 live, early log phase organisms. The rabbits were bled weekly from the marginal ear vein. Serum was sterilized by filtration through a 0.45 μ Millipore filter and stored in screw top vials at -70 C. Before being used in the bactericidal test, all antisera were inactivated at 56 C for 30 min.

Bactericidal assay - The method employed is a modification of the procedure described by Roberts (Roberts, R. J. Exp. Med. 126:795-818, 1967).

1. Diluent (Gel-Gey Solution consisted of 0.1 percent galatin (Difco) in Gey's Balanced Salt Solution (Microbiological Associates). Fresh Gel-Gey solution was prepared before each test by adding 5 ml of sterile 10 percent gelatin to 500 ml of sterile Gey's Balanced Salt Solution.

2. Complement: The reaction mixture always contained 10 percent normal rabbit serum as a complement source. Rabbits were selected for complement sources only after their sera was shown to lack bactericidal activity against meningococci. The normal rabbit serum was stored at -70 C and thawed immediately before use. Serum was discarded after four weeks of storage.

3. Meningococci: The strain to be tested was grown as described above. The inoculum consisted of 0.1 ml of a broth culture with an optical density of 0.1, i.e. approximately 3×10^7 organisms in the early log phase of growth.

-- Reaction Mixtures were set up in 16 x 75 mm screw top tubes.

Complement control

Gel-Gey Solution	0.8 ml
Normal Rabbit Serum	0.1 ml
Meningococci	0.1 ml

Test mixture

Gel-Gey Solution	0.7 ml
Normal rabbit serum	0.1 ml
Test antiserum	0.1 ml
Meningococci	0.1 ml

The meningococci were always added last.

The reaction tubes were incubated aerobically at 37 C with shaking. Colony counts were determined at 0 and 120 min. by the plate method. 0.05 ml of each reaction mixture was diluted with 0.9 ml of Gel-Gey Solution from which serial 10-fold dilutions were made. 0.1 ml of each dilution was transferred to a Mueller-Hinton agar plate (dried at room temperature for two-three days to ensure rapid absorption of the fluid) and spread with a bent glass rod while rotating the plate on the Spray-Fisher Dish Turntable (Fisher). The plates were incubated overnight at 37 C in a candle jar or in a CO_2 incubator and the number of colonies were counted.

The relative percentage of killing was calculated by comparing the number of organisms present in the test mixture at two hours to the number in the complement control at two hours. Significant bactericidal activity was taken to be greater than a 99 percent or 2-log reduction in the viable colony count at two hours.

Absorption of antiserum with whole organisms - A loop-ful of meningococci from an overnight growth on Barile's agar was added to 0.5-1.0 ml of antiserum which had previously been heated at 56 C for 30 min. After incubation for one hour at 4 C, the bacteria were removed by centrifugation and the absorption step was repeated with fresh organisms for one hour at 4 C. After centrifugation, the doubly absorbed antiserum was filtered through a 0.45 μ Millipore filter and stored at 4 C.

Control antiserum was treated in like fashion, omitting the addition of organism.

Absorption of antiserum with C-polysaccharide - After heating at 56 C for 30 min. antiserum was mixed with an equal volume of

a 50 percent suspension of pyruvic aldehyde fixed human O Rh negative red cells sensitized with C-polysaccharide (Lot C_4). The mixture was incubated for one hour at 37 C with frequent shaking and then for 18 hrs. at 4 C. The red cells were removed by centrifugation and the antiserum sterilized by filtration.

Control antiserum was prepared by absorption with unsensitized, pyruvic-aldehyde fixed red cells.

(c) Results.

Reproducibility of the bactericidal test - The coefficient of variation of replicate plate counts averaged about 10 percent. Nevertheless, the reproducibility of the test from day to day was high. With the prototype system of 60 Eur (C_{11}) strain versus anti-60 Eur rabbit antiserum, kills of greater than 99 percent were obtained in 11 tests. Similar reproducibility was achieved with several other strains.

The results of a typical test of strain 60 Eur vs. anti-60 Eur are shown in Table 18. After incubation for two hours there is growth in the complement control tube and a 2.5 log fall in the number of organisms in the test mixture. The relative kill at two hrs. is 99.98 percent or 3.5 logs.

Table 18. Results of reaction between 60 Eur strains and anti-60 Eur antiserum.

	Organisms/ml	
Reaction mixture	0	2 hr
Complement control	3.2×10^7	5.1×10^8
Test mixture	3.2×10^7	8.7×10^4

Effectiveness of immunization method - A single intravenous injection of 10^8 live meningococci resulted in the production of bactericidal activity in sera of all 20 rabbits tests five-six weeks after immunization. All antisera gave at least 99 percent kill when tested at a 1:10 dilution against homologous strains. Antisera against 60 Eur and against 59 Eur were titrated and found to give a 50 percent kill (calculated by the Reed-Muench method) at dilutions of 1:320 and 1:160 respectively. The immunization procedure did not result in the development of detectable agglutinating or precipitating antibody as measured by the standard slide agglutination method or by agar gel

diffusion. Low titers (<1:32) of hemagglutinating antibody were detected by one week in all rabbits but the titers declined to less than two by six weeks after immunization.

Antibody nature of the bactericidal activity - Evidence supporting the involvement of specific antibody in the bactericidal test is three-fold.

1. As indicated in Table 19, there was no bactericidal activity detected in sera of rabbits prior to immunization. Such activity regularly appeared following immunization.

Table 19. Bactericidal activity in pre- and post-immunization rabbit antisera tested against homologous strains.

	Relative % kill at 2 hours	
Strain	Pre-immune sera	6-week post-immune sera
60 Eur	0	99.9
158-III	0	99.9
118-V	0	99.0

2. Absorption of specific antiserum with the homologous strain used in immunization results in removal of almost all bactericidal activity, as seen in Table 20.

Table 20. Bactericidal activity before and after absorption of antiserum with homologous strain.

	Relative % kill at 2 hours	
Strain	Unabsorbed antiserum	Absorbed* antiserum
60 Eur	99.9	12
138-I	99.7	0
142-Eur	99.6	10

* Absorbed antiserum was doubly absorbed with whole organisms of the homologous strain.

3. Bactericidal activity is complement dependent. Heating of both the antiserum and the normal rabbit serum for 30 min. at [illegible] resulted in complete loss of activity.

Differentiation of group C strains with the bactericidal test -

1. Reaction of strains against unabsorbed heterologous antisera - Three group C strains (60 Eur, 138-I, and 142 Eur) were compared for reactivity towards homologous and heterologous antisera and the results are shown in Table 21.

Table 21. Bactericidal activity of homologous and heterologous antisera against strains 60 Eur, 138-I and 142 Eur.

	Relative % kill by indicated antisera		
Strain	Anti 60 Eur	Anti 138-I	Anti 142 Eur
60 Eur	99.9	63.2	91.4
138-I	95.4	99.7	99.7
142-Eur	87.9	99.9	99.6

Each strain was killed by its homologous antiserum and strains 138-I and 142 Eur were killed equally well by the antisera to the other. However, the reduced activity of anti 138-I antiserum against 60 Eur and of anti-60 Eur against 138-I suggested that these two strains may be antigenically distinct. Since the use of unabsorbed antiserum did not make the differences clear-cut, experiments were carried out to eliminate antibody directed against the C-polysaccharide common to all eight strains.

2. Bactericidal activity in antisera absorbed with C polysaccharide - The results of reacting the three strains against antisera absorbed with C polysaccharide are incubated in Table 22.

Table 22. Bactericidal activity of homologous and heterologous antisera after absorption with C-polysaccharide.

	Relative % kill with C-polysaccharide absorbed antisera		
Strain	Anti 60 Eur	Anti 138-I	Anti 142 Eur
60 Eur	99.8	0	92.9
138-I	0	99.3	99.5
142 Eur	0	98.3	99.9

A comparison of the results in Tables 21 and 22 reveals that the antigenic differences suggested by the reactions against unabsorbed heterologous antisera become much more distinct when strains are tested against antisera absorbed with C polysaccharide.

Thus 60 Eur is demonstrated to be antigenically different from 138 I, since there is no cross reactivity with the corresponding absorbed antisera. The failure of absorption of anti-142 with C-polysaccharide to remove all bactericidal activity against 60 Eur may be due either to incomplete absorption of anti-142 Eur or to the sharing of minor antigens between 60 Eur and 142 Eur. The data do not permit the resolution of these alternatives.

The marked reduction in bactericidal activity of several of the absorbed antisera against heterologous strains indicates that much or all of the activity of the unabsorbed antisera against heterologous strains was due to the presence of antibody against the C-polysaccharide.

The effect of absorption with C polysaccharide on the bactericidal activity against the homologous strain is shown in Table 23.

Table 23. Bactericidal activity of unabsorbed and C-polysaccharide absorbed antisera against homologous strains.

	Relative % kill with indicated antisera	
	Unabsorbed homologous	Absorbed homologous
Strain	Antisera	Antisera
60 Eur	99.9	99.8
138-I	99.6	99.3
142 Eur	99.7	99.9

The lack of reduction in bactericidal activity of absorbed antiserum against homologous strains emphasizes the importance of bactericidal antibodies to antigens other than the C-polysaccharide.

3. Bactericidal activity in antisera absorbed with whole organisms - To define further the antigenic differences among these three strains, each strain was reacted against antiserum absorbed with whole organisms. As indicated in Table 20, double absorption of the antiserum with the homologous organism removes almost all activity against that strain. The results of absorption with heterologous strains are shown in Tables 24-26.

Table 24. Bactericidal activity of antisera against strains 60 Eur and 138 I after cross absorption.

	Relative % kill in indicated antisera			
	Anti 60 Eur		Anti 138 I	
Strain	Unabsorbed	Absorbed with strain 138 I	Unabsorbed	Absorbed with strain 60 Eur
60 Eur	99.9	99.7	63.2	22.3
138 I	95.4	0	99.7	98.4

Table 25. Bactericidal activity of antisera against strains 60 Eur and 142 Eur after cross absorption.

	Relative % kill in indicated antisera			
	Anti 60 Eur		Anti 142 Eur	
Strain	Unabsorbed	Absorbed with strain 142 Eur	Unabsorbed	Absorbed with strain 60 Eur
60 Eur	99.9	99.5	91.4	25.0
142 Eur	87.9	0	99.6	99.9

Table 26. Bactericidal activity of antisera against strains 138 I and 142 Eur after cross absorption.

	Relative % kill in indicated antisera			
	Anti 142 Eur		Anti 138 I	
Strain	Unabsorbed	Absorbed with strain 138 I	Unabsorbed	Absorbed with strain 142 Eur
142 Eur	99.6	51.0	99.9	22
138 I	99.7	0	99.6	Not Done

Marked reduction in bactericidal activity of antisera against heterologous strains without any significant change in activity against homologous strains occurred with the pairs 60 Eur-138 I (Table 24) and 60 Eur-142 Eur (Table 25), confirming that 60 Eur is antigenically different from 138 I and 142 Eur. On the other hand the similarity between 138 I and 142 Eur was seen by the effect of cross absorption on reducing activity in the cross reactions (Table 26).

The fact that the absorbed antisera often retained activity against heterologous strains suggests that either the method utilized did not result in complete absorption of antibody against C-polysaccharide or that other common antigens exist.

In summary, the determination of bactericidal activity against homologous and heterologous strains in rabbit antiserum absorbed with C-polysaccharide or with whole organisms permits the identification of antigenic differences between strains of group C meningococci.

(2) Studies of the passive hemagglutination test.

Previous studies in this laboratory have demonstrated that polysaccharide sensitized erythrocytes agglutinate (HA) in the presence of antibody and that the test is group specific. In 200 individuals vaccinated with A or C vaccines, bactericidal and HA antibody responses showed perfect correlation. Further studies have been undertaken to improve the HA procedure.

C polysaccharide microtiter passive hemagglutination tests - Tests are performed in the microtiter system. For the test human O, Rh negative red cells are tanned by the pyruvic aldehyde method. (N. R. Ling, 1961, Brit. J. Haemat., 7:299-302.) The cells are suspended at a 10 percent concentration and stored at 4 C. Prior to sensitization, the cells are washed three times with phosphate buffered saline pH 6.9 (PBS) and suspended to a concentration of two percent. The amount of antigen solution needed for optimal sensitization was determined to be 20 µg/ml by grid titration. The concentration of C polysaccharide needed for sensitization has remained constant for eight lots. After incubation with the antigen for 30 min. in a 37 C water bath, the cells are washed three times in PBS and diluted further to a concentration of 0.5 percent. For stabilization of the cells, bovine serum albumin (BSA) is added to a final concentration of 0.5 percent w/v. It was found that the use of round bottom plates and 0.05 ml amounts of reagents gave patterns which were most easily read and consistent. To perform the test, two-fold dilutions of 0.05 ml of heat inactivated sera are made in 0.05 ml PBS with 0.05 ml diluters. After 0.05 ml of sensitized cells is added to each well, the plates are rotated gently and incubated at room temperature for two hours. Titers of sera are

expressed in terms of the last wells showing 3+ (75 percent) agglutination. Controls consist of a specific rabbit antiserum, two sera from a vaccinee having titers of 1:256 or greater and two human sera having no titer. The 12th well in each titration serves as the cell control.

The test is considered valid when the negative sera remain negative and the positive sera vary no more than one dilution higher or lower than their mean titers.

One group of sera from vaccinated individuals required absorption with nonsensitized RBC's to remove agglutinins which interfered with the polysaccharide HA test.

Studies to determine the length of time that sensitized cells remain usable were undertaken. Results showed that although the cells remain sensitized for 10 days to the same degree as the initial day, bacterial contamination, which is very common, causes significant decrease in titers. Sensitized cells, therefore, are kept and used for three days but for no more than seven days. Sensitized RBC's stored over three weeks agglutinate nonspecifically.

Attempts to preserve sensitized cells by two methods have been unsuccessful. In the first method cells were sensitized in the usual manner, diluted to a concentration of 10 percent and quickly frozen and kept at -20 C. When the cells were thawed for use beginning with the first day after freezing little or no settling of cells was observable, probably due to cell rupture.

A second method used to preserve sensitized cells was lyophilization. Cells were sensitized by the usual method, washed three times in PBS, diluted to a cell concentration of two percent, quickly shell frozen in alcohol and dry ice and lyophilized. Each bottle was to be reconstituted with five ml of PBS with BSA added which would provide sufficient cells for one microtiter plate. However, these cells failed to settle at room temperature within 18 hrs.

In an attempt to resolve this problem cells were lyophilized in the same manner as before but the BSA was added before lyophilization. Table 27 shows the titers of control sera with freshly sensitized cells as compared to lyophilized cells.

These data indicate that lyophilization appears to be a promising tool and further tests will explore its use. BSA is an essential ingredient in the initial freezing step.

Table 27. Comparison of hemagglutination titers obtained with fresh and lyophilized erythrocytes.

	Reciprocal of titer (8 determinations)	
	Fresh cells	Lyophilized
Antisera	mean	mean
A rabbit	0	2
B (881) rabbit	0	8
C14 (1:20) rabbit	64	64
C19 (1:20) rabbit	32	64
075 human	256-512	256
690338 human	256	256
064 human	256	256
PBS control	0	0

F. Transport media for isolation of Neisseriae.

Background: Various transport media have been tried for the preservation of gonococcal specimens. The concentrations of the inorganic salts are critical for preventing rapid autolysis and the toxic effects of other organisms present. A satisfactory medium must maintain the optimal osmotic pressure. Calcium chloride is required for good growth of the gonococcus. In the presence of sodium chloride, cells will be very mucoid. If .01-.02 percent calcium chloride is added, non-mucoid colonies are obtained. At the same time it must be remembered that calcium ions in high concentration are toxic for some enzymes. Stuart's medium as a solid agar has increased the recovery of gonococci compared to the original formul with 0.3 percent agar.

C&B and Amies (1 percent charcoal) media have been compared using six stock strains of Neisseria gonorrhoeae. Four vials were inoculated with each strain. The media was stored at 25 C and subcultured daily for four days. A charcoal swab was used in C&B medium and a cotton swab in Amies medium. From overnight growth on chocolate agar containing lincomycin and polymixin (chocolate LP) suspensions were made in the basic salt solutions of each medium to a density of #1 MacFarland standard. Each swab was immersed in one ml of the cell suspension and then placed in the holding medium. All strains were viable for 48 hrs. in each medium but two vials of the Amies medium were contaminated with a mold. No strains were recovered after this time.

Six fresh isolates of N. gonorrhoeae were used for comparison of two variations in the basic salt mixture:

A.			B.		
	0.25%	NaCl		0.5%	NaCl
	0.1%	Na_2HPO_4		0.1%	Na_2HPO_4
	0.02%	KCl		0.005%	$CaCl_2$
	0.005%	$CaCl_2$			

Each was made up with one and three percent agar. Sodium thioglycollate was added and the final pH of each was 7.4. Charcoal treated swabs were used for inoculation. One set was stored at 25 C and one at 3 C. After three days at 25 C, only two were still viable in medium B with three percent agar. Four were still viable in both A and B with three percent agar after three days at 3 C. After four days under refrigeration, two were viable in both media with three percent agar. Using one percent agar, no strains survived three days at 25 C and only two of six (B salts) were recovered at 3 C storage.

Cotton swabs are toxic for the pathogenic Neisseria. Gluun and Kennett found that charcoal could be used as a detoxifying agent. Previous trials have shown that when one petri dish is inoculated with a swab from transport medium stored three-four days and another inoculated with some of the surrounding medium in the vial, growth is obtained in some cases only on the second plate. Glutamine is used in several supplements for N. gonorrhoeae. It is also a detoxifying agent. Since sodium glutamate can be substituted for glutamine in the supplements, alkaline treated swabs with sodium glutamate were used with fresh isolates of N. gonorrhoeae in transport medium but no increase in viability was observed.

For transport of N. meningitidis the original C&B medium was prepared with one modification only: 0.25% NaCl was used. The final pH was 7.5. The medium was used by CPT Schrot of the First Army Medical Laboratory, Fort George G. Meade, in a carrier survey at Fort Knox. Sixty-five pharyngeal specimens were obtained in duplicate using charcoal treated swabs. One swab was streaked immediately on a chocolate L.P. plate and the other placed in holding medium. All vials were refrigerated at 8 C for 90 hrs. before subculture. There were 42 original positives and after 90 hrs. storage 22 were recovered. One possible explanation for the low recovery may be the observation of Flexner that at low temperature more N. meningitidis survive in concentrated suspensions while at high temperatures more survive in more dilute suspensions.

Table 28 shows the results of a number of transport medium studies for meningococcal recoveries using storage at room temperature. It is apparent that about 70 percent of carriers can be detected using C&B media if the swabs are alkaline or charcoal treated. Further studies are indicated, especially to test whether storage at colder temperatures will increase the yield.

Rapid diagnostic methods: In 1965, White and Kellogg published a fermentation medium for N. gonorrhoeae consisting of GC medium base and one percent defined supplement. The supplement indredients are:

glutamine	1 gm	ferric nitrate	50 mgm
.2% cocarboxylase	1 ml	distilled water	100 ml

The medium was prepared in screw cap tubes as slants and inoculated with a 2 mm bacteriological loop from overnight chocolate agar plates. After five hrs incubation the reactions could be read.

Table 28. Summary of studies of transport medium for meningococcal carrier surveys.

	Ft. Ord (1963) C&B Medium[1] pH 7.9 alkaline swab		Ft. Dix (5/31/67) C&B Medium[2] pH 7.5 alkaline swab	Ft. Dix (4/23/68) C&B Medium[3] pH 7.4 alkaline swab	Ft. Dix (5/27/68) C&B Medium[3] pH 7.5 polyester swab (Falcon)	Ft. Dix (9/30/68) C&B Medium[4] pH 7.5 alkaline-charcoal swab	Ft. Dix (11/13/68) Leibovitz CVTM medium cotton swab
144 specimens			50 specimens	100 specimens	60 specimens	50 specimens	130 specimens
Orig. Isol.	34%		68%	32%	73%	24%	35%
% groupable	100%		52%	43.2%	93%	75%	-
3 days Room Temp.				of 15 positives			
% Isol.	30%[5]		66%	12	75%	69%	Not Done
% groupable	94%		34%	7	7[illegible]%	88%	Not Done
4 days Room Temp.				of 9 positives	of 15 positives	of [illegible] positives	
% Isol.	0		63%	5	13	1	23%
% groupable			4.6%	2	5	3	-

1 Orig. formula with 5.0 gm NaCl, 5.0 gm Difco agar.
2 Orig. formula with lincocin (.006 mgm/ml) and polymyxin (45 units/ml).
3. Orig. formula modified: .02% starch, 2.5 gm. NaCl/liter, 5 gm. purified Difco agar and drugs.
4. Orig. formula.
5. % of original isolates.

The following media were compared for rapid fermentation:

White & Kellogg's medium
MH Medium with White's supplement
HI medium with White's supplement
MH medium with 1% rabbit serum

Freshly isolated strains of N. gonorrhoeae and several strains of N. meningitidis were used. When all were inoculated with a 2 mm loop, MH medium with White's supplement had slightly more growth and was easier to read in five hrs. One set of MH medium with no carbohydrate was inoculated as a control on the utilization of starch. For stamping plates, suspensions were made in saline with one percent Trypticase Soy Broth. Density of N. gonorrhoeae was equivalent to #/ MacFarland standard and N. meningitidis equivalent to #/ MacFarland. No more than 12 strains were inoculated on each plate. After five hrs. incubation with carbon dioxide, dextrose was positive for all strains. The colonies were yellow as was the surrounding medium. Maltose was utilized by the N. meningitidis strains. All negatives were quite pink. MH medium with White's upplement had more growth than the same base with one percent rabbit serum.

c. Chemoprophylaxis - Studies with folic acid antagonists.

A procedure to test antifolate compounds for activity against N. meningitidis and for ability to potentiate the activity of sulfadiazine was described last year. The method, i.e. testing a large variety of N. meningitidis strains against serial plate dilutions of the compounds in question and of combinations of the compounds and sulfadiazine, proved extremely burdensome and time consuming. Therefore, attempts were made to simplify the procedures and still derive data which would be accurate and indicative of potential therapeutic usefulness.

A new set of 12 case strains was formulated which encompassed serogroups A, B, C and Bochard. Where possible, within each serogroup strains were chosen which were sensitive to sulfadiazine (MIC = 0.1-0.2 mcg/ml), resistant (MIC = 1-2 mcg/ml) and highly resistant (MIC = $\geq$10 mcg/ml). These strains were recharacterized bacteriologically and their sulfadiazine patterns rechecked. To eliminate the preparation of large numbers of plates containing serial dilutions of sulfadiazine and anti-folate compounds, the gradient concentration plate method of Szybalski was used. Gradient drug concentrations prepared in this fashion permitted the simultaneous testing of six strains against a wide range of drug concentrations on single plates.

This concentration gradient plate method was used to test ten compounds with known binding activity for the enzyme dihydrofolate reductase involved in the synthesis of dihydrofolic acid from

dihydropteroic acid. The ten compounds provided by Burroughs-Wellcome Co. were tested in two concentration gradients, one with a high concentration of 10.0 mcg/ml and the other with a high of 1.0 mcg/ml, against six strains of meningococci which had sulfadiazine MIC's of 12.5 or more mcg/ml. Seven of the ten compounds showed no activity at 10 mcg/ml and were eliminated. The specific activities (MIC) of the remaining three compounds were then precisely defined in a short series of plate dilutions. Plates were then set up using constant 0.1 MIC of test compound in sulfadiazine gradients starting with 25 mcg and 6.25 mcg/ml and the reverse, gradients starting with 10 or 1.0 mcg/ml of test compound with constant 1.56 mcg/ml sulfadiazine present. This latter concentration of sulfadiazine was 8 to 30 fold less than the MIC values of the six test strains.

Results from the above experiments showed that of the three Burroughs-Wellcome compounds which possessed activity against the meningococci, two, 49-171 and 49-272, when present at 0.1 MIC were able to reduce the sulfadiazine inhibitory concentration by factors of four to eight. The results from the third compound, 48-142, were equivocal. Conversely the inhibitory concentrations of compounds 49-171 and 49-272 were reduced by a factor of two to four in the presence of a low concentration, 1.56 mcg/ml of sulfadiazine. This data supports the conclusion that the antifolate compounds which bind the enzyme dihydrofolate reductase, and sulfadiazine, which acts at another site, act synergistically in the disruption of folic acid metabolism of sulfadiazine resistant strains of N. meningitidis.

The procedure described above has greatly simplified the testing of antifolate compounds. It has permitted the rapid and graphic illustration of synergistic activity against six meningococcal strains of two classes of compounds on four gradient plates, two of which are controls.

Selected compounds are being examined for structure-activity relationships.

2. Investigations on mycoplasmas and L-phase variants of bacteria.

Studies on the isolation, characterization, identification, and classification of Mycoplasma species, including the T-strain mycoplasmas, and of L-phase variants of bacteria have been pursued systematically and most of the methodology is now directly applicable to problems in clinical medicine.

a. Modified bacteriologic techniques for the characterization of Mycoplasma species.

Biochemical characterization of mycoplasma strains is a

simple and useful adjunct to other systems of identification of these organisms. As more and more species of Mycoplasma are recognized, precise characterization of each species in terms of its biochemical and physiological activities is highly desirable. In addition, simple, standardized methods for such characterization are essential for preliminary narrowing of the range of possible species to which a new isolate might belong. As early as the 1950's Edward and Freundt developed the basis for most of the biochemical procedures currently in use, but no attempt has since been made to characterize under standardized conditions both the old and the new species and strains now available.

The objectives of this project were to modify and standardize existing bacteriologic procedures and to develop new tests to extend the biochemical basis for mycoplasma identification. All of these tests were then applied to 53 strains comprising more than 22 species of mycoplasmas to determine their efficacy in differentiating and characterizing species.

The mycoplasmas used were obtained from the American Type Culture Collection. The materials and methods employed were described in detail elsewhere (B. B. Aluotto. 1969. Ph.D. Thesis, Univ. of Md., College Park, Md.).

Of prime importance for the initial sorting of mycoplasmas into groups of biochemically similar species were the tests for breakdown of urea, glucose and arginine. This initial study did not include the T-strain mycoplasmas, which hydrolyze urea; all the included strains were confirmed to be negative for hydrolysis of urea. Thus the mycoplasmas in this study displayed one of the following patterns:

(1) Glucose positive, arginine negative.

(2) Glucose positive, arginine positive.

(3) Glucose negative, arginine positive.

(4) Glucose negative, arginine negative.

Further subdivisions within each of these major groups could be effected by applying the following tests: oxidation or fermentation of glucose, reduction aerobically and anaerobically of tetrazolium, tellurite, and methylene blue, phosphatase activity, production of film and spots, hydrolysis of gelatin, digestion of casein and coagulated serum, sensitivity to optochin, and hemolysis of sheep red blood cells.

Although a number of other tests were investigated, they were found to be unsuitable for purposes of characterization or differentiation. Tests for hydrolysis of tributyrin, starch, hippurate, DNA,

and DNA were unsatisfactory because the modified methods employed yielded false reactions. Modified tests for catalase, oxidase, deamination of phenylalanine, hydrolysis of gluconate, and the benzidine test all gave negative results for all of the strains. The catalase and the benzidine tests were apparently not sensitive enough to detect positive reactions reported by other investigators. Tests for production of indole, hydrogen sulfide, and acetylmethylcarbinol, for nitrate reduction, for litmus milk reactions, and the methyl red test also yielded negative results on all of the organisms tested, thus offering no help in differentiation of strains.

The reactions, recorded in detail elsewhere (J. V. Aluotto. 1969. Ph.D. Thesis, Univ. of Md., College Park, Md.), provide the characterization data for all of the type or representative strains of mycoplasma that were included in the American Type Culture Collection at the time this study was in progress. At least 12 of the species investigated produced distinctive test result patterns. These species were M. arthritidis, M. gallinarum, M. gateae, M. hyorhinis, M. maculosum, M. pneumoniae, M. equigenitalium, M. bovirhinis, M. felis, M. fermentans, M. sp. (sheep), and M. bovigenitalium. Many new strains have been added recently to the Collection, and characterization of the new strains is now in progress. The results to date on the latter strains are shown in Table 29.

By use of the 13 tests found to be suitable for determining biochemical activity, together with knowledge of the gaseous environment preferred, and the ability to grow in the absence of serum and at room temperature, an unknown mycoplasma isolate could tentatively be assigned to three or four species at most. Application of the growth inhibition test (preferably along with other types of serologic tests) using antiserum to the three or four most probable species should in most cases identify the organism, or else indicate that it is a species or subspecies unlike any of the reference strains.

It is a generally accepted requirement that new bacterial isolates and strains be characterized as fully as possible biochemically and biologically when studies on such organisms are published. Thus, it would seem to be equally desirable and important that newly isolated or unusual mycoplasma strains be similarly characterized using standardized methods that could readily be duplicated by other laboratories. The methods and results described in this investigation could serve as one framework of reference when characterization of new strains is undertaken.

Furthermore, the above methods are now being used routinely in this laboratory on clinical isolates and are allowing specific identification of such isolates to be made much more rapidly than in the past.

Table 29. Differential physiologic reactions displayed by recently acquired mycoplasmas.

Mycoplasma species	ATCC No.	Strain	Glucose breakdown	O-F test	Arginine hydrolysis	Tetrazolium reduction	Tellurite reduction	Methylene blue reduction[a]
						ae/an	ae/an	ae/an
M. arginini	23838	G230	-		+	-/-	+/+	-/-
M. orale, Type 1	23714	CH19299	-		+	-/-	-/-	-/-
M. orale, Type 2	23636	CH20247	-		+	-/+	+[c]/+[c]	-/-
M. pharyngis	19524	LGM	-		+	-/+	+[c]/+[c]	-/-
M. salivarium	23557	Manire	-		+	-/-	-[c]/+[c]	-/-
M. sp.	-	S	+	F	-	-/+	+/+	-/-
M. sp.	-	14	+	F	+	+/+	+/+	+/+

Table 29. (Continued)

Mycoplasma species	ATCC No.	Strain	Phosphatase	Film and spots	Gelatin hydrolysis	Casein digestion	Serum digestion	Sensitivity to optochin	Hemolysis[b] (sheep cells)	Preferred gaseous environment
M. arginini	23838	G230	-	-	-	ND	ND	+	α'r	Ae
M. orale, Type 1	23714	CH19299	-	-	-	ND	ND	+	-	An
M. orale, Type 2	23636	CH20247	+	-	-	-	ND	+	α'	An
M. pharyngis	19524	LGM	-	-	-	-	ND	+	-	An
M. salivarium	23557	Manire	-	+	-	ND	ND	+	-	An
M. sp.	-	S	-	-	-	ND	ND	-	α	Ae
M. sp.	-	14	±	-	-	ND	ND	-	α'r	Ae

Key: +, positive reaction; ±, weak positive reaction; -, negative reaction; F, fermentative reaction; α, alpha hemolysis; α', alpha prime hemolysis; r, green ring at outer zone of hemolysis; ND, not determined; Ae, aerobic; An, anaerobic.

a. Reading at 24 hr.

b. Reading at 4 days.

c. Growth inhibited by test substrate.

A somewhat similar scheme for characterization is also being developed for the urea-hydrolyzing T-strain mycoplasmas, which to date have been distinguished from each other solely on the basis of immunologic differences.

b. Molecular genetic studies on mycoplasmas and L-phase variants of bacteria.

The WRAIR Annual Report for 1968 presented the preliminary Tm values for 23 Mycoplasma strains, 19 of which were type or representative strains. The study has been expanded to include a total of 25 Mycoplasma strains and six L-phase variant strains. Tm values were determined for five control organisms, a standard curve derived, and final base ratio (% guanine + cytosine) values calculated. The results are given in Table 30.

The base composition values obtained for most of the type or representative strains of Mycoplasma correlated well with those of other strains of the respective species examined in other investigations. Likewise % G+C values for five of the six L-phase variants fell within the respective G+C range of the classical bacterial form of the species.

Streptobacillus moniliformis L-phase variant, L1 Rat 30, was found to have a base ratio of 23.9% G+C, the lowest value recorded to date for a member of the Schizomycetes, and as low as values previously considered unique to some members of the class Mollicutes. Interpretation of this finding awaits base ratio data on the bacillary phase of S. moniliformis strains.

These results contribute data pertinent to the characterization of the strains investigated and provide reference criteria for identification and classification of new strains.

An experiment to evaluate the applicability of base ratio determination by hyperchromic spectra analysis (Hirschman & Felsenfeld, J. Mol. Biol., 16:347, 1966) were initiated simultaneously with the Tm studies. Aliquots of DNA were taken from a single DNA extraction of each strain and used for base composition derivation by both methods. Thirty strains, including 21 Mycoplasma, two L-phase variants each with its parent form, two other L-phase variants, and three bacterial strains were examined by the two methods. The resulting base ratio values and differences in values by the two methods are presented in Table 31.

Correlation of % G+C values obtained by the two methods was good. Twenty-six of the 30 samples showed differences by the two methods of less than the 3% G+C maximum error expected. The four remaining samples differed by 3.1 to 5.9 % G+C but were still less than the 6% error found between the Tm method and still another method.

Table 30. DNA base composition values of mycoplasmas and L-phase variants.

Organism	ATCC No.	Strain	Tm(°C)[a]	% G+C[b]
Mycoplasmas:				
M. arthritidis	19611[c]	PG6 (Preston)	82.97	31.3 ± 0.2
M. bovigenitalium	19852[c]	PG11 (B2)	82.58	30.4 ± 0.2
M. bovirhinis	19884[c]	PG43 (5M331)	80.38	25.4 ± 0.3
M. felis	23391[c]	CO	80.26	25.2 ± 0.2
M. fermentans	19989[c]	PG18 (G)	81.81	28.7 ± 0.4
M. gallinarum	19708[c]	PG16 (Fowl)	81.06	27.0 ± 0.7[d]
M. gallisepticum	19610[c]	PG31 (X95)	83.17	31.8 ± 0.2
M. gateae	23392[c]	CS	81.71	28.5 ± 0.1
M. histotropicum	23115	Sabin Type C	82.02	29.2 ± 0.6
M. hominis, Type 1	23114[c]	PG21 (H50)	82.03	29.2 ± 0.1
M. iners	19705[c]	PG30 (M)	82.01	29.1 ± 0.2
M. laidlawii	23206[c]	PG8 (Sewage A)	83.69	33.0 ± 0.1
M. laidlawii	23217	PG9 (Sewage B)	83.42	32.4 ± 0.2
M. neurolyticum	19988[c]	Sabin Type A	80.72	26.2 ± 0.1
M. orale, Type 1	23714[c]	CH19299	81.44	27.8 ± 0.4
M. orale, Type 2	23636[c]	CH20247	80.69	26.1 ± 0.2
M. pharyngis	15544	Patt	79.70	23.9 ± 0.2
M. pneumoniae	15531[c]	FH	86.74	39.9 ± 0.4
M. pulmonis	19612[c]	PG34 (Ash)	81.48	27.9 ± 0.3
M. salivarium	23064[c]	PG20 (H110)	82.99	31.4 ± 0.1
M. spumans	19526[c]	PG13 (C48)	81.79	28.6 ± 0.2
M. sp. (dog)	23462	PG24 (C21)	82.04	29.2 ± 0.1
M. sp. (goat)	15718	C30 KS-1	81.90	28.9 ± 0.2
M. sp. (human)	15497	Navel	80.97	26.8 ± 0.4
M. sp. (sheep)	23243	67-166	81.17	27.2 ± 0.2
L-phase variants:				
Corynebacterium sp.	23831	D-Campo B-L	97.17	63.7 ± 0.2
Staphylococcus aureus	19640	Smith L	83.46	32.4 ± 0.5
Streptobacillus moniliformis	14075	L1 Rat 30	79.72	23.9 ± 0.1
Streptococcus faecalis	23242	G-K L (T53)	85.97	38.2 ± 0.2[e]
Streptococcus pyogenes	19563	Richards L	85.55	37.2 ± 0.3
Streptococcus sp.	19617	Bruno L		37.2 ± 0.1[f]

Table 30. (Continued)

a - Tm expressed as the mean of three or more thermal denaturation curves.

b - DNA base composition values expressed as the molar ratio of guanine + cytosine X 100/adenine + thymine + guanine + cytosine with standard deviation of mean. The standard regression curve by which % G+C values were derived was obtained using the chemically determined base ratio values given by McGee and the following Tm values: Diplococcus pneumoniae (86.31), Escherichia coli (91.05), M. gallisepticum (83.74) Neisseria meningitidis (91.48) and Proteus mirabilis (86.41).

c - Type or representative strain.

d - A difference of greater than 2.8% G+C in the base composition values of two organisms is significant using the largest standard deviation encountered (0.7% G+C) and 95% confidence intervals to determine significance.

e - Values for parent streptococcus G-K (B14) are Tm:85.46°C and G+C:37.0 ± 0.2% (data of this laboratory).

f - Unpublished data of Zell A. McGee.

Table 31. Comparison of DNA base composition values derived from hyperchromic spectra with values derived from Tm's.

Organism	ATCC No.	Strain	% G+C[a] derived from hyperchromic spectra[b]	% G+C derived from Tm's[c]	Difference in the two % G+C values
Mycoplasmas:					
M. bovirhinis	19884	PG43 (SM331)	24.9 ± 2.1	25.4 ± 0.3	0.5
M. felis	23391	CO	28.2 ± 1.5	25.2 ± 0.2	3.0
M. fermentans	19989	PG18 (G)	27.8 ± 0.2	28.7 ± 0.4	0.9
M. gallinarum	19708	PG16 (Fowl)	30.2 ± 0.8	27.0 ± 0.7	3.2
M. gallisepticum	15302	S-6	27.2 ± 1.5	33.1 ± 0.3	5.9
M. gatesae	23392	CS	27.1 ± 1.1	28.5 ± 0.1	1.4
M. histotropicum	23115	Sabin Type C	28.2 ± 0.6	29.2 ± 0.6	1.0
M. hominis, Type 1	23114	PG21 (H50)	26.8 ± 0.4	29.2 ± 0.1	2.4
M. laidlawii	23206	PG8 (Sewage A)	31.7 ± 0.3	33.0 ± 0.1	1.3
M. laidlawii	23217	PG9 (Sewage B)	31.0 ± 0.6	32.4 ± 0.2	1.4
M. neurolyticum	19988	Sabin Type A	24.2 ± 1.7	26.2 ± 0.1	2.0
M. orale, Type 1	23714	CH19299	27.2 ± 0.8	27.8 ± 0.4	0.6
M. orale, Type 2	23636	CH20247	23.3 ± 1.2	26.1 ± 0.2	2.8
M. pneumoniae	15531	FH	40.1 ± 0.5	39.9 ± 0.4	0.2
M. pulmonis	19612	PG34 (Ash)	26.1 ± 3.7	27.9 ± 0.3	1.8
M. salivarium	23064	PG20 (H110)	28.3 ± 3.1	31.4 ± 0.1	3.1
M. spumans	19526	PG13 (C48)	28.6 ± 1.2	28.6 ± 0.2	0.0
M. sp. (dog)	23462	PG24 (C21)	29.8 ± 1.4	29.2 ± 0.1	0.6
M. sp. (goat)	15718	C30 KS-1	28.0 ± 1.8	28.9 ± 0.2	0.9
M. sp. (human)	15497	Navel	24.2 ± 0.3	26.8 ± 0.4	2.6
M. sp. (sheep)	23243	67-166	27.8 ± 3.4	27.2 ± 0.2	0.6

Table 31. (Continued)

Organism	ATCC No.	Strain	% G+C[a] derived from hyperchromic spectra[b]	% G+C derived from Tm's	Difference in the two % G+C values
L-phase variants and parent forms:					
Staphylococcus aureus	19640	Smith L	33.6 ± 3.5	32.4 ± 0.5	1.2
Streptococcus faecalis	23242	G-KL (T53)	39.8 ± 3.6	38.2 ± 0.2	1.6
Strep. faecalis	23241	G-KP (B14)	37.7 ± 2.6	37.0 ± 0.2	0.7
Strep. faecalis	19635	F24L	35.2 ± 1.0	37.9 ± 0.3	2.7
Strep. faecalis	19634	F24P	34.9 ± 1.5	38.1 ± 0.3	3.2
Streptococcus pyogenes	19563	Richards L	36.1 ± 1.6	37.2 ± 0.3	1.1
Bacterial strains:					
Escherichia coli	-	K12 (Y10F⁻)	49.9 ± 0.6	49.8 ± 0.6	0.1
Neisseria meningitidis	23253	Nel5	50.2 ± 0.1	50.8 ± 1.0	0.6
Proteus mirabilis	14273	9	36.9 ± 1.4	39.2 ± 0.5	2.3

a - DNA base composition values expressed as the molar ratio of guanine + cytosine X 100/adenine + thymine + guanine + cytosine.

b - Derived from three hyperchromic spectra determinations with standard deviation of the mean.

c - Derived from three or more thermal denaturation curves with standard deviation of the mean.

i.e. buoyant density (Marmur and Doty, J. Mol. Biol., 5:109, 1962). Variation between the values was random, nonlinear, and independent of any particular G+C range. (Marmur and Doty found their greatest deviation between Tm and Buoyant density values when the guanine-cytosine content was below 33%.)

Reproducibility is not as good by the hyperchromic method as it is by the Tm method. Where the highest standard deviation by hyperchromicity is 3.7% G+C, the greatest deviation by Tm is only 1% G+C. Furthermore, the degree of deviation by the hyperchromic method shows no correlation with the degree of difference between G+C values by the two methods, i.e. they are independent of one another leaving no explanation for the variation within the one system as well as between the two systems. Therefore, where a quick estimate of base composition is desired, the hyperchromic spectra analysis method of determination can be useful. However, where more accurate and more reliable results are required for characterization of Mycoplasma and L-phase variant strains the Tm method is preferred.

c. Type culture collection of mycoplasmas and L-phase variants of bacteria.

The collection of mycoplasmas and L-phase variants developed by the American Type Culture Collection and Walter Reed Army Institute of Research has continued to increase in size and diversity. It contains 26 named species and many still unnamed species, including a prototype T-strain mycoplasma. In all 69 strains of mycoplasmas are available from the collection, and more than 20 other strains are stored at WRAIR while awaiting processing and submission to the collection. In addition, the collection now includes 21 strains of L-phase variants, 18 strains of their parent bacteria, and 9 strains of their revertant bacteria.

A new policy for the acquisition of strains by the ATCC has been established. The earliest pure culture passage from the initial isolate of each strain is obtained directly from the original investigator. Formerly, pure cultures of later passages carried by secondary recipients of the strain were accepted by the ATCC.

Several significant improvements in preparation, lyophilization, and storage of mycoplasmas and L-phase variants have increased the yield of viable organisms after freeze-drying and greatly reduced the number of seed-stock replenishments required. As reported last year, the use of the one-step manifold method of lyophilization allows 90 vials of a given strain to be freeze-dried at one time. These vials are stored at -70°C instead of +4°C, so that viability is extended for many years. During the current year only three strains of mycoplasma and one L-phase variant strain required seed-stock replenishment.

A preliminary study has been conducted on the viability of mycoplasmas lyophilized in the presence and absence of sucrose. Broth-grown cultures were harvested by centrifugation and resuspended at 15-times concentration in broth medium without sucrose and in broth medium containing 12% (w/v) final concentration of sucrose. Samples of each suspension were withdrawn before and after lyophilization, serially diluted, plated on appropriate agar, and the colony forming units counted.

Table 32 presents a summary of the colony counts performed on nine different strains of mycoplasmas. The colony counts of strains lyophilized in the presence of sucrose, when compared with those lyophilized without sucrose, were higher in eight of the nine strains studied. Only one strain (Mycoplasma bovimastitidis) showed a slightly lower count when freeze-dried in the presence of sucrose.

Two L-phase variants (Corynebacterium sp. D-5-L and Corynebacterium sp. D Campo-B-L), which were suspended and lyophilized in a suspension of 12% (w/v) sucrose + 1% (v/v) horse serum (no base medium included), yielded after freeze-drying counts of 3.5×10^7 and 4.4×10^7 organisms per ml. respectively. Comparative studies were not performed, but heretofore many unstable L-phase variants prepared by this laboratory had not been found to survive freeze-drying. Lyophilization in the presence and absence of sucrose will be continued for all strains submitted to the ATCC until sufficient comparative data is available to verify these preliminary findings.

Pertinent data on the current status of the culture collection is summarized below:

Number of colony counts and purity tests performed	236
Number of vials of mycoplasmas supplied by ATCC to investigators throughout the world	179
Number of vials of L-phase variants supplied by ATCC to investigators throughout the world	16
Number of vials of mycoplasmas and L-phase variants supplied to investigators at WRAIR or other military installations	86

The total number of cultures supplied by ATCC to military installations free of charge represented a significant contribution to the military.

Table 32. Comparison of colony counts of freeze dried mycoplasmas employing two different suspending media.

Mycoplasma species	Medium without sucrose		Medium with 12% (w/v) sucrose	
	Before freeze-drying	After freeze-drying	Before freeze-drying	After freeze-drying
Mycoplasma arginini (ATCC #23838) G230	2.6×10^9	1.5×10^8	2.8×10^9	4.8×10^8
Mycoplasma bovimastitidis 01 (ATCC #25025)	2.6×10^9	2.7×10^7	2.4×10^9	1.4×10^7
Mycoplasma felis (ATCC #23391) CO	1.4×10^7	0	4.8×10^7	3.6×10^5
Mycoplasma hominis (ATCC #23114) PG21 H50	1.7×10^9	1.2×10^6	3.5×10^9	6.7×10^8
Mycoplasma hyorhinis (ATCC #25021) PG29 (Leach)	1.4×10^{10}	3.6×10^8	1.7×10^{10}	5.1×10^9
Mycoplasma hyorhinis (ATCC #25026) PG29 (Edward)	1.5×10^{10}	1.3×10^9	1.3×10^{10}	9.8×10^9
Mycoplasma hyorhinis (ATCC #25077) PG29 (Freundt)	1.1×10^{10}	2.0×10^8	1.0×10^{10}	5.6×10^9
Mycoplasma orale, Type 1 CH19299 (ATCC #23714)	2.9×10^9	5.0×10^7	3.4×10^9	2.6×10^8
Mycoplasma pneumoniae (ATCC #15531) FH	4.2×10^7	3.8×10^5	Not done	5.4×10^7

It is worth noting that the ATCC mycoplasma collection is the only one known in which nearly every strain is fully characterized in terms of its G+C ratio and biochemical activities. Work is now in progress to obtain significant immunologic information on each of these strains.

3. Bacterial flora of the mid-gut of Anopheles stephensi (India).

Chao and Wistrich examined the mid-gut sections of Culex quinquefasciatus and fourth-instar larvae for bacterial flora. A Bacillus sp. and a yeast Saccharomyces sp. were the only organisms isolated from the larvae. Isolates from adults included Flavobacterium sp., Klebsiella sp., Micrococcus caseolyticus (Evans), and a Pseudomonas. Jadlin isolated a Pseudomonas (photosynthetic purple pigmented species) from the mid-guts of Anopheles quadrimaculatus and A. durenti. This organism contained a number of amino acids required for the development of the sporozoites of Plasmodium berghei. To date, very little has been done on the role of the bacterial flora in the development of sporozoites.

A pilot study was carried out with Captain Peach of the Dept. of Entomology on the isolation and identification of the predominating organisms in the mid-gut of Anopheles stephensi (India), host for simian and human malaria, particularly Plasmodium falciparum.

Six females were used for each group studied. The following schedule was used:

A. No meal (newly emerged)
B. Sugar meal, 10% sucrose, 3 day old adults.
C. Blood meal (specimens taken immediately after feeding)
D. 24 hrs. after blood meal.
E. 48 hrs. after blood meal.
F. 72 hrs. after blood meal.
G. 96 hrs. after blood meal.

Seventy percent ethanol was used for surface sterilization. Slides and instruments were autoclaved. Each mid-gut was dropped into 1 ml of sterile Heart Infusion Broth (Difco), crushed, and the following media inoculated:

Casman's Blood Agar
Chocolate Brain Heart Infusion (BBL) Agar
Thioglycollate Broth

Incubation procedure:

Casman's Blood Agar - 37 C aerobically, anaerobically
30 C aerobically, anaerobically
23 C aerobically, anaerobically

Chocolate Agar - 37 C with carbon dioxide

Thioglycollate Broth - 37 C

All subcultures with no growth were incubated for a maximum of five days before discarded. Before the initial blood meal 10 ml of sterile heart blood was obtained from the rabbit and subcultured as a control.

Bacteria isolated:

1. Newly emerged mosquitoes.

WRAIR No.	11 -	Specimen #2	Corynebacterium sp.
	12	" #3	No growth after 7 days incubation
	13	" #4	" " " " " "
	14	" #5	" " " " " "
	15	" #6	" " " " " "
	16	" #7	Klebsiella pneumoniae
			Enterobacter liquefaciens

2. Specimens submitted after a sugar meal.

WRAIR No.	17 -	Specimen #1	Enterobacter liquefaciens
	18 -	" #2	Enterobacter liquefaciens
	19 -	" #3	Enterobacter liquefaciens
	20 -	" #4	Pseudomonas aeruginosa
	21 -	" #5	Enterobacter liquefaciens
	22 -	" #6	Enterobacter liquefaciens
			Pseudomonas aeruginosa

3. Specimens submitted after first blood meal.

WRAIR No.	23 -	Rabbit blood -	no growth after 5 days incubation
	24 -	" " -	" " " " " "
	25 -	Specimen #1	Enterobacter liquefaciens
	26 -	" #2	Enterobacter liquefaciens
	27 -	" #3	Pseudomonas aeruginosa
	28 -	" #4	Enterobacter liquefaciens
	29 -	" #5	Enterobacter liquefaciens
	30 -	" #6	Pseudomonas aeruginosa
			Enterobacter liquefaciens

4. Specimens submitted 24 hrs. after blood meal.

WRAIR No. 39	Specimen	#1	Pseudomonas aeruginosa
			Enterobacter cloacae
40	"	#2	Serratia marcescens
41	"	#4	Serratia marcescens
			Enterobacter cloacae
42	"	#5	Enterobacter cloacae
			Serratia marcescens
43	"	#6	Serratia marcescens
44	"	#7	Serratia marcescens
			Enterobacter cloacae

5. Specimens submitted 48 hrs. after blood meal.

WRAIR No. 46	Specimen	#1	Serratia marcescens
47	"	#2	Serratia marcescens
			Enterobacter aerogenes
48	"	#3	Serratia marcescens
49	"	#4	Serratia marcescens
			Pseudomonas sp.
50	"	#5	Enterobacter cloacae
			Pseudomonas sp.
51	"	#6	Enterobacter cloacae
			Serratia marcescens

6. Specimens submitted 72 hrs. after blood meal.

WRAIR No. 52	Specimen	#1	Serratia marcescens
53	"	#2	Serratia marcescens
			Enterobacter cloacae
54	"	#3	Serratia marcescens
55	"	#4	Serratia marcescens
56	"	#5	Enterobacter aerogenes
			Enterobacter liquefaciens
			Serratia marcescens
57	"	#6	Serratia marcescens

7. Specimens submitted 96 hrs. after blood meal.

WRAIR No. 58	Specimen	#1	Serratia marcescens
59	"	#2	Serratia marcescens
60	"	#3	Serratia marcescens
61	"	#4	Serratia marcescens
62	"	#5	Pseudomonas sp.
63	"	#6	Serratia marcescens

A few Micrococci, Corynebacterium, and a Bacillus sp. were seen in some of the original smears but none were recovered in subcultures. Experiments designed to measure the role of the predominating bacterial species in relation to the sporozite population are being considered.

4. Immunology of viral infections.

a. Serologic diagnosis of influenza by SAFA test.

Clinical diagnosis of viral diseases is, with rare exceptions, merely an educated guess. Precise etiologic diagnosis requires laboratory assistance and is usually not available in general hospitals. Attempts to develop simple serologic tests which could be performed in routine laboratories have been under study. The SAFA (soluble antigen fluorescent antibody) technique has been investigated as a serologic test for influenza infection.

(1) Methods.

(a) Whole virus antigen: Two strains of Asian Influenza, A2/Jap/305/57 and A2/HK/19/68 were used in this study. These viruses were grown in the allantoic sac of nine day old chick embryos. Each egg was inoculated with 0.1 ml of a 10^{-4} dilution of stock virus. The eggs were incubated at 37 C. for 48 hrs. then harvested. In preliminary studies crude allantoic fluid virus was used as the SAFA antigen. In later experiments the virus harvest was adsorbed twice onto Human "O" red blood cells (0.1 ml packed cells/10 ml allantoic fluid) at 4 C. for 1½ hours. The agglutinated cells were removed by low speed centrifugation at 4 C. and the cells washed once with cold pH 7.2 PBS. The virus was eluted into small volumes of pH 7.2 PBS over a period of 5-6 hrs. at 37 C. The virus was further purified by differential centrifugation as follows: (1) a 3,000 rpm centrifugation for 30 min. at room temperature immediately after the rbc's were removed; (2) a 10,000 rpm (12,100 G) centrifugation for 30 min. at 4 C.; and (3) finally, centrifugation at 25,000 rpm (56,573 G) for 3 hrs. at 4 C. to pellet the virus. The virus pellet was resuspended in pH 7.2 PBS.

(b) Type and group specific influenza antigen: Several methods for releasing the HA antigen from the intact viron have been described in the literature. (Laver, Virology 20:251, 1963; Lief and Henle, Virology 2:753, 1956; Davenport et al, J. Expt. Med. 112:765, 1960; Mizutani et al, Virology 17:210, 1960; Hobson, J. Expt. Path. XLII:257, 1966). The method of Laver using sodium deoxycholate was tried on several occasions with no success. Influenza viral antigen was prepared by a combination of the methods of Lief and Henle and Mizutani et al. The method is as follows: One volume of purified influenza virus was treated with three volumes of anesthetic ether for 2 hrs. at 4 C. Agitation was accomplished by use of a magnetic stirrer,

and the phases were separated using a separatory funnel. Excess ether was removed by drawing air over the aqueous phase overnight.

The hemagglutination (HA) antigen was removed from the soluble antigen by adsorbing the former onto human "O" cells (0.1 ml of packed rbc's was added to 10 ml of ether treated virus for 1 hour at 4 c). The cells were removed by low speed centrifugation, washed once with cold pH 7.2 PBS, then the antigen was eluted into a small volume of PBS over a period of 2 hrs.

(c) SAFA test: The improved SAFA test described by Toussaint (Expt. Parasit. 19:71, 1966) was used with one modification in the preparation of the antigen disc. The method of Toussaint recommends that the ¼ in. in diameter cellulose acetate disc, used as a matrix for the antigen, be immersed in the antigen solution for 30 seconds then allowed to dry overnight. However, when crude allantoic fluid influenza was applied in this manner only a small amount of the antigen adsorbs to the disc and the antigen did not adsorb uniformly over the entire surface of the disc. The first modification tried was to filter the crude virus through a 0.45 μ Millipore membrane. An HA test was performed on the material before and after filtration to determine how much, if any, of the antigen was adsorbed to the disc. The HA results showed that only 2 of the original 16 units present were adsorbed onto the membrane. This result was not unexpected because Elford (J. Path. Bacteriol. 34:505, 1931) recommended that viruses should be suspended in proteinaceous dilluents to prevent non-specific adsorption of the virus to the collodion membranes used for sizing viruses, allantoic fluid influenza virus is nothing more than a protein suspension of virus.

Wallis and Melnick (Bull. Wld. Hlth. Org. 36:219, 1967; J. Virol. 1:472, 1967) found that organic material in polio virus harvested from cell culture and the organic material found in raw sewage contained a membrane coating compound (MCC) which prevents polio virus from being adsorbed to Millipore membranes. They later found, however, that when low concentrations of NaCl or $MgCl_2$ were added to the above types of samples that polio virus was effectively adsorbed. It seemed probable that allantoic fluid also contained MCC, so allantoic fluid virus was diluted in 0.15M NaCl, then filtered through a 0.45 μ Millipore membrane. The results of this experiment are shown in Table 33.

Thus, discs for the following experiments were prepared by filtering a saline solution of intact influenza virus through a 0.45 μ Millipore membrane. The membrane, after filtration, was removed from its holder, placed on a piece of filter paper and allowed to dry overnight. The ¼ in. discs were punched out just prior to doing the test.

Table 33. The effect of sodium chloride on the adsorption of influenza virus in allantoic fluid to a Millipore membrane.

Dilution of virus in 0.15M NaCl	Units of HA activity before filtering	Units of HA activity adsorbed
Undilute	128	1
1:5	64	2
1:10	32	4
1:20	16	16
1:40	8	8
Ultracentrifugation pellet	128	128

(d) Hemagglutination Inhibition Test: The micro-hemagglutination inhibition test was performed basically as described by Sever (J. Immunol. 88:320, 1962). Group "O" human red blood cells were used in the test and the tests were read after standing at room temperature for 60 min. (Hilleman and Werner, J. Immunol. 71:110, 1953). The sera were treated with receptor destroying enzyme (Mulder et al., Ant. von Leeunwenhoek 32:145, 1952) prior to testing to remove non-specific inhibitors of hemagglutination.

(2) Results.

Acute and convalescent serum pairs from patients showing a rise in antibody by HI or CF to A2/Jap, A2/HK or B influenza were used to evaluate the SAFA test.

The results in Table 34 show that in the homologous A2/Jap system the fluorometer dial reading (FDR) of the convalescent serum of each pair was significantly higher than the acute sera. This increase in FDR was in agreement with the rise in antibody shown by the HI test. It can also be noted that even though the above patients have no HI antibody to the A2/HK virus the convalescent sera of all three pairs showed an increased FDR over the acute when the SAFA test was run using the A2/HK virus as the antigen. The increase in FDR seen with sera from patients with A2/Jap infections was always higher in the homologous system even though heterologous cross reactivity was seen.

In the A2/HK homologous system the convalescent serum of each pair also showed an increase in FDR over the acute, which was in agreement with the HI results. Cross reaction was seen when these sera were reacted with the A2/Jap antigen. Both the acute and convalescent sera of all three patients tested showed strong reactions with the A2/Jap antigen, indicating a previous exposure to the A2/Jap antigen. Two of the three patients tested showed an HI antibody rise to the A2/Jap antigen; these also showed a change in FDR in the SAFA test. The third patient showed no increase in HI or change in FDR to the A2/Jap antigen. It is of interest that the two patients with the HI rise to the A2/Jap antigen showed a greater change in FDR to this, the heterologous antigen, than to the homologous antigen. The higher FDR in the heterologous system may, however, be due to antigen concentration since the test contained 1024 units of A2/Jap virus and only 128 units of A2/HK virus.

Table 34 shows that when the SAFA test was performed on two different days the FDR reading was quite different. This difference was possibly due to the antigen preparation. The concentration of antigen in both tests was approximately the same, however, the antigen used in the first test was a cleaner preparation. The second antigen still contained traces of hemoglobin from the

purification procedure. This could possibly have blocked part of the antigen-antibody-fluorescein antihuman globulin reaction.

The paired sera from patients who showed only B influenza antibody rise by either the HI or the CF test showed only a very small change in FDR to either of the A2 antigens. Both the acute and convalescent sera of these patients reacted to about the same extent with both of the A2 antigens indicating the possibility of a previous exposure to an A2 influenza strain; in two of the three individuals an unchanged CF or HI titer was shown with the A2 antigen.

Because of cross reactions the SAFA test using whole virus antigens can not be used to show a strain specific A2 influenza antibody rise. In order to try to make the test strain specific, the HA and the soluble antigens were released from the intact viron using ether. The antigens were separated by the adsorption-elution of the HA antigen onto human "O" rbc. Neither the HA nor the soluble antigens showed any reactivity in the SAFA test. The inactivity of these antigens may be due to insufficient concentration or purity; the HA antigens were heavily contaminated with hemoglobin from the rbc adsorption.

(3) Conclusions.

The above results indicate that even though the SAFA test in its present form cannot be used to differentiate between A2 influenza strains it can probably be used to differentiate between an A2 and a B influenza infection.

b. Serum and local nasal antibodies following natural adenovirus infections.

The serum antibody response of 23 individuals with adenovirus infection (types 4, 1, 6 and 7) was studied. Neutralizing antibody tests correlated well with radioimmunodiffusion; the latter technique, however, offered the advantages of simplicity and distinct separation of specific immunoglobulin antibodies. The antigen used in radioimmunodiffusion measured group rather than type specific adenovirus antibody.

Only four patients demonstrated a serum response consisting of early macroglobulin antibody development followed by and then replaced by IgG. The majority of individuals showed a prolonged IgM response accompanied by high titered IgG antibody. IgA serum antibody developed in almost all the patients. Following type 4 adenovirus infection nasal secretory antibody, both IgA and IgG, but not IgM, was detected at the same time or within a week of the detection of serum antibody and was universally found in the 35 individuals studied.

Table 34. Comparison of influenza HI, CF, and SAFA test.

Serum		HI			CF		SAFA[2]							
							A2/Jap/57		ΔFDR		A2/HK/68		ΔFDR	
Pt.	Date	A2/Jap/57	A2/HK/68	B/Lee	A	B	1.	2.	1.	2.	1.	2.	1.	2.
D.A.	10/18/57	<5 (20)[1]	<5	(320)			135	65	1431	682	189	29	1132	187
	10/23/57	20 (160)	<5	(320)			1566	747			1321	216		
J.M.	10/11/57	<5 (10)	<5	(160)			148	87	1241	435	311	69	903	117
	10/23/57	20 (160)	<5	(320)			1389	540			1414	186		
S.G.	9/17/57	<5 (20)	5	(320)			148	50	1228	436	216	41	705	100
	9/27/57	40 (320)	10	(320)			1376	486			921	141		
D.C.	1/29/65				(5)	(<5)	635		-217		715		-404	
	2/26/65				(5)	(40)	418				311			
R.G.	CI-287	(10)		(20)			230		67		230		189	
	CI-274	(10)		(320)			297				419			
R.T.	2/11/66				(<5)	(<5)	527		30		271		51	
	2/24/66				(<5)	(10)	557				322			
J.Z.	12/2/68	40	<5					423		287		159		255
	12/31/68	160	160					710				414		
S.R.	12/16/68	20	<5					279		216		69		192
	1/13/69	80	80					495				261		
B.B.	12/2/68	20	<5					396		-27		76		185
	12/31/68	40	160					396				261		

Table 34. (Continued)

[1] Results of test in the year serum was received.

[2] When FDR readings were over 100 the slit size of the UV orifice was decreased to reduce the intensity of the fluorescence and the FDR was multiplied by an appropriate factor (3, 9, 27).

1. = Test performed 11/25/68.

2. = Test performed 5/26/69.

Summary and conclusions:

The meningococcal meningitis investigations have shown increasing prevalence of group C, sulfadiazine resistant strains as the cause of disease in the Army; approximately 85 percent of recent cases. Carrier surveys have shown variations in rates in Army recruits in different training companies and at different posts. The reduced group C carrier rates among vaccinated recruits observed last year were confirmed, although the effect is seen only when controls have high group C carrier rates, over 35 percent. It was found that dispersal of meningococci by new carriers is greater than that of chronic carriers. Studies of meningococcal endotoxins are in preliminary stages in which techniques for extraction and analysis are being developed. The polysaccharide of the Boshard serogroup has been isolated and purified and has been shown to be distinct from the classical serogroups A, B and C but to be identical to that of serogroup Y. Bactericidal tests have shown that antigenic similarities and differences among strains of serogroup C can be identified. Isolation techniques and in vitro antibiotic tests are under development.

Studies on classical mycoplasmas, T-strain mycoplasmas, and L-phase variants of bacteria have dealt with development and improvement of methods for characterization and identification of strains, and with resolution of various problems in classification by use of molecular genetic techniques. All type and representative strains of mycoplasmas and reference strains of L-phase variants in the American Type Culture Collection have been fully characterized in terms of their diagnostically significant biochemical reactions and DNA base compositions. Current emphasis is now directed to improved and standardized methods for isolation of the above groups of organisms from clinical specimens and their rapid identification by biochemical and serological methods.

The bacterial flora of the mid gut of mosquitos (A. stephensi) has been characterized, thus paving the way for studies of the relationship of this flora to experimental malarial sporozoite development. A new serologic test (SAFA) for influenza can differentiate type A from type B infections. Serum and nasal antibodies develop in all recruits naturally infected with type 4 adenovirus.

Project 3A061102B71Q, COMMUNICABLE DISEASE AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 168, Bacterial diseases

Publications.

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	1412A(2)						

(U) Zoonotic Diseases (09)

010100 Microbiology

06 62	NA	DA		C. In-House
NA				
		FISCAL YEAR 69	12	250
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DA

(U) Zoonoses; (U) Epidemiology; (U) Leptospirosis; (U) Melioidosis; (U) Leptospiras; (U) Animal Viruses

23. (U) To study diseases of animals that have or may have potential military significance. To determine basic biological characteristics of infectious agents, epidemiological features of disease, the mode and course of infection, to define factors in infection and resistance to disease, factors in drug susceptibility and resistance, to develop improved methods for diagnosis, treatment and control.

24. (U) Conventional epidemiological and microbiological technics are used, Cryogenic technics used to develop methods for preserving organisms. Immunochemical methods used to define antibody components in antisera from patients and immunized animals.

25. (U) 69 01 - 69 06 Methods for definitive identification of leptospiral strains were simplified by use of modified agglutinin-adsorption technic. The use of suckling rabbits to determine strain virulence was evaluated with hamster-virulent and hamster-avirulent (stock culture) pathogenic serotypes, a biflexa type and a new serotype isolated from a turtle with biological characteristics of both pathogenic and saprophytic leptospiras. The suckling rabbit afforded no advantage over hamsters in determining virulence. The turtle strain was non-infectious for both experimental hosts. Isolates from South African swine associated with abortion episodes were identified to be canicola serotypes. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 68 - 30 Jun 69.

DD [illegible] 1498-1

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 170, Zoonotic diseases

Investigators.
Principal: A. D. Alexander, Ph.D.
Associate: CPT Stanley S. Green, VC; L. B. Evans, B.S.;
V. M. Shepler, M.S.

Progress.

1. Leptospirosis.

a. Preservation of Leptospiras by Liquid Nitrogen Freezing. Observations on the viability of leptospiras after storage under liquid nitrogen vapor (-130 C) were continued. On the basis of previous findings (Annual Report 1 Jul 1967 - 30 Jun 1968) it was deemed practicable to use liquid nitrogen for maintenance of stock cultures.

Ninety stock strains were grown in Stuart's media. Glycerol was added to final concentration of 10% and subcultures were distributed in multiple ampoules, rapidly frozen and stored. After 11 months of storage, 16 of the strains were tested for viability. All but two, serotypes sejroe and ricardi, were recovered in culture. The exceptional strains, however, were viable by microscopic examination immediately after thawing. Additional check tests are planned. Preliminary tests were done on the use of dimethylsulfoxide as a cryoprotective agent for liquid N_2 storage. When added to a mature culture of a pathogenic leptospira (L. bataviae, 1415) in concentrations greater than 5%, no viable leptospiras were seen 24 hours after treated cultures were held at room temperature.

b. Serological Studies. Two of 35 isolates obtained at a focus of infection for troops in Panama were studied further and identified as serotype shermani. Results of culture typing tests on 10 isolates submitted from various institutions in the United States and overseas are shown in Table 1.

The new findings for the United States were icterohemorrhagiae in swine, ballum and grippotyphosa in muskrats. The occurrence of pyrogenes in swine has not heretofore been reported. Swine infections with canicola in South Africa has not been reported previously.

Table 1. Results of Culture Typing Tests on Leptospiral Isolates Submitted During Fiscal Year 1968.

Source Country	Institute	Host	Serogroup identification
U.S.	U. of Illinois	swine	icterohemorrhagiae
"	"	cattle	"
"	"	muskrat	ballum
"	"	"	grippotyphosa
"	Wisconsin Animal Health Laboratory	swine	pomona
"	"	cattle	"
Philippine Rep.	U. of Philippine	swine	pyrogenes
Haiti	Cambridge City Hosp.	human	icterohemorrhagiae
South Africa	Vet.-Invest.-Center	porcine	canicola

Serological tests for leptospiral antibodies were done on 58 bovine sera submitted by the Veterinary College, Kerala, India. Significant antibody titers at 1:100 were found in 4 samples (2 hebdomadis serogroup, 1 each hyos and autumnalis serogroups). Partial reactions were seen in 16 other sera.

Agglutinin adsorption technics for identification of leptospiras were simplified by use of standardized (by nephelometry) concentrations of absorbing antigen which were added directly to specified amounts of serum. The modified technic reduces the need for multiple adsorptions, and the incubation time for adsorbing mixtures.

c. Comparison of Suckling Rabbits and Hamsters for Determining Strain Virulence. The suckling rabbit is reported to be highly susceptible to frank infection with pathogenic leptospiras. Its potential usefulness to demonstrate infectiousness of biflexa strains and newly disclosed hamster avirulent types with intermediary characteristics of pathogenic and saprophytic strains were examined. Infection could not be induced with a stock strain of biflexa nor with a recently isolated strain from turtles (intermediate type). In comparative tests done with pathogenic leptospiras, the suckling rabbit did not prove to be more susceptible to infection or frank disease than hamsters.

2. Melioidosis.

Immunoglobulin studies on sera from melioidosis patients were initiated to clarify the course of the serological response; its relationship to persistence of infection and to determine the reactivity of immunoglobulin fractions in hemagglutination (HA) and complement-fixation (CF) tests.

Serum globulin fractions were separated by the use of sucrose density gradients centrifugation and gel filtration methods. In the first procedure continuous gradients were formed at 4 C from 10 and 40% solutions of sucrose in phosphate buffered salt solution (pH 7.2) by the use of a mixing chamber. Sera were heated at 56 C for 30 minutes and diluted 1:2 with phosphate buffered saline (pH 7.2) and layered on this gradient in 0.5 ml amounts with an equal amount of 10% sucrose solution. Gradients were prepared in triplicate and centrifuged in a Spinco Model L centrifuge at 36,000 rpm for 18 hours. Ten serial fractions, approximately 0.5 ml each, were collected from a perforated hole in the bottom of a centrifuge tube. Protein concentrations of the fractions were determined by the Lowry method. The fractions were dialyzed against phosphate buffered saline pH 7.2. The gel filtration column, 70 cm long and 2.5 cm in diameter, was prepared with Sephadex G-200. The Sephadex was washed several times with distilled water and the "fines" were removed by decantation. The gel was equilibrated with phosphate-buffered saline (pH 7.2). A 2.0 ml serum sample previously tested at 56 C for 30 minutes and dialyzed against the buffered diluent was applied to the column. As soon as the serum was diffused into the column the buffered diluent was added. The flow rate was 15 ml/hr. Eluates were collected in 5 ml amounts. The protein content of fractions was determined by measuring the optical density at 280 mμ in a Beckman DU spectrophotometer. The IgM and IgG content of the immunoglobulin fractions from both procedures were determined by immuno-diffusion tests with anti-human IgM and IgG. Fractions were then tested for the presence of antibodies reactive in HA and CF tests.

IgM and IgG immunoglobulin fractions of 12 sera from nine patients were obtained by the use of sucrose gradients and tested for HA and CF antibody. The samples comprised specimens taken at different times of disease and specimens with little or no HA or CF reactions. Results of tests are summarized in Table 2. IgM antibody was found in sera obtained during the first few weeks of disease, but was not evident in 4 of 5 sera obtained 3 months or more after disease onset. IgM antibodies did not appear to figure in CF reactions but were reactive in the HA test. The CF activity of sera could be related to IgG antibody. IgG antibody of serum obtained early in the course of disease appeared to have relatively poor hemagglutinating antibody.

Gel filtration techniques were used to separate immunoglobulin components of serum obtained 218 days after disease onset from patient W. W. The whole serum had an HA titer of 1:2560 and CF titer of 1:512. HA and CF antibody was only found in IgG components. Serum from R. W. (obtained on 132nd day of disease) with 1:64 CF titer and negative HA reaction was also fractionated by use of gel filtration techniques. The IgM fraction was inactive. The IgG fraction reacted only in CF test. These findings were consistent with that obtained on fractions of same serum separated by the use of sucrose gradients.

Table 2. Complement Fixation and Hemagglutination Test Reactions of IgG and IgM Fractions of Sera from Melioidosis Patients

Patient	Day of Disease	Fraction	Reciprocal of Titer HA	CF
F. M.	1 (?)	Whole serum	160	512
		IgM	4	AC
		IgG	16	32
A. N.	5	Whole serum	20	Not tested
		IgM	8	-
		IgG	-	16
K. M.	14	Whole serum	5120	16
		IgM	128	AC
		IgG	32	16
D. R.	23	Whole serum	1280	512
		IgM	128	AC
		IgG	-	32
W. J.	17	Whole serum	1280	128
		IgM	32	AC
		IgG	-	16
	164	Whole serum	640	128
		IgM	16	AC
		IgG	2	16
W. R.	31	Whole serum	40	16
		IgM	2	-
		IgG	2	AC
	59	Whole serum	40	8
		IgM	4	-
		IgG	4	4
	120	Whole serum	-	8
		IgM	-	-
		IgG	2	AC
R. W.	132	Whole serum	40	64
		IgM	-	-
		IgG	-	16
	221	Whole serum	-	64
		IgM	-	-
		IgG	-	16
W. W.	218	Whole serum	2560	512
		IgM	-	-
		IgG	512	128

In view of the small amount of serum or serum fractions availabe for serological testing, it was deemed advisable to adapt the CF test to microtiter techniques.

Sixteen diagnostic melioidosis serums were selected for study which had both high and low CF antibody titers as determined by the tube test. The serums were diluted two-fold in standard U microtiter plates using 0.025 ml of serum and equal amounts of TBS saline. The plates were refrigerated for several hours and 0.025 ml of complement (the same concentration as used in the tube test) was added followed by 0.025 ml of the same antigen dilution as employed in the tube test. The plates were placed at 4 C for overnight incubation and the next morning they were placed in a 37 C incubator for 10 minutes. Sensitized RBC's (0.05 ml) were added to each well. Plates were covered with a plastic film and floated in a 37 C water bath for 45 minutes and shaken at 15 minute intervals. After incubation the plates were centrifuged at 1500 rpm for 5 minutes in a PR-2 International centrifuge. The titers in the microtiter test were 2 or 3 dilutions lower than those in the tube test.

In an attempt to increase the sensitivity of the microtiter CF test, melioidosis CF antigen was titrated in microtiter plates. The optimum antigen dilution was found to be the same as that used in the tube test. Next, the microtiter CF test was performed using varying amounts of complement. Six dilutions of complement starting with 5.0 units (the amount used in the tube test) and ending with 2.6 units of complement were tested in microtiter technique. The optimum amount of complement was found to be 4 units. When the amount of complement used in the microtiter test was more than 4 units, less anti-complementary activity was observed but the sensitivity of the test was decreased three-fold. Conversely, with less complement the sensitivity of the test was increased but there was marked anti-complementary activity. By adapting the CF test to the microtiter technique, it is possible to assay all fractions of whole serum for both HA and CF antibody content.

It was not possible to determine CF antibody titer in most of the fractions containing IgM due to anti-complementary activity. The same phenomenon has been reported for macroglobulins of other immune sera.

Summary and Conclusions.

1. Leptospirosis.

The feasibility of preserving leptospiras by storage in liquid nitrogen refrigeration was demonstrated further in tests conducted on stock cultures after an 11 month storage period. Toxic properties

of dimethylsulfoxide served to limit its usefulness as a cryoprotective agent for preservation of leptospiras by liquid nitrogen freezing. Culture typing tests on leptospiras received from various institutions in the United States and overseas have provided new information on the distribution of various serotypes in select mammals. Additional evidence was obtained on enzootic occurrence of leptospirosis in India. Methods for definitive identification of leptospiral strains were simplified by use of modified agglutinin-adsorption test. The use of suckling rabbits to determine strain virulence was affirmed; however, this host afforded no advantage over hamsters in determining virulence.

2. Melioidosis.

Studies on immunoglobulin serum fractions from melioidosis patients indicated that both immunoglobulin fractions IgM and IgG have hemagglutinating activity, but that CF antibodies appear to be IgG fraction only.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 170 Zoonotic diseases

Publications

D. K. Haapala, B.S.; M. Rogul, Ph.D.; L. B. Evans, B.S.; and A. D. Alexander, Ph.D. Deoxyribonucleic and Acid Base Composition and Homology Studies of Leptospira. J. Bact. 98:421-428, May 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6448	69 07 01	DD-R&E (AR) 613

3. DATE OF SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. ENTER ESTAB	9. SPECIFIC DATA CONTRACTOR ACCESS	10. LEVEL OF SUM
31 01 69	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. THIS UNIT

11. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A061102B71Q	00	171
b. CONTRIBUTING				
c. [redacted]	1412A(2)			

12. TITLE (Precede with Security Classification Code)

(U) Development of Biological Products (09)

13. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

14. START DATE	15. ESTIMATED COMPLETION DATE	16. FUNDING AGENCY	17. PERFORMANCE METHOD
05 58	NA	DA	C. In-House

18. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

19. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR 69 (PRECEDING)	6	190
FISCAL YEAR 70 (CURRENT)	6	170

20. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Considered

22. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Berman, S. L.

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24. KEYWORDS (Precede each with Security Classification Code)

(U) Biological Products; (U) Eastern Equine Encephalomyelitis; (U) Freeze-Drying; (U) Meningococcus Polysaccharides; (U) Plague; (U) Q Fever; (U) Shigella; (U) Vaccines

25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. This work unit is concerned with the development of manufacturing methods for the production of new effective vaccines and for the modification existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.

24. (U) Increased effectiveness and reduced reactivity are pursued by use of new physical and chemical methods for processing. Improvement in stability and reduction of logistic requirements are achieved by application of modern freeze-drying and packaging techniques.

25. (U) 69 01 - 69 06 Investigations have continued on the development of new and improved biological products. - 1. Studies on the improvement of methods for pilot scale production of purified Meningococcal Polysaccharides have been carried out. Significantly greater yields may be obtained as the result of a modification of the growth medium and the application of simpler and more efficient processing procedures. 2. Zonal centrifugation of Genetron-extracted rickettsial suspensions yielded purified Q fever vaccine with significantly reduced protein and lipid content. 3. Long term surveillance of the stability of freeze-dried live Plague vaccines revealed no significant loss in viability after 3 years storage at -20°C. 4. Improved yields of Eastern equine encephalomyelitis virus were obtained in roller culture bottles seeded with 5×10^6 chick embryo cells per ml. 5. An experimental lot of freeze-dried live shigella vaccine was prepared with a Streptomycin-dependent strain of S. flexneri, 2a. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 68 - 30 June 69.

Available to contractors upon request approval.

DD FORM 1498-1 (FOR ARMY USE)

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 171, Development of biological products

Investigators.
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Associate: Sanford Berman, PhD; Patricia L. Altieri, BS; Arthur White, MS; Calvin Powell, MS; Doria Dubois, BS; Albert Groffinger

Description.

This work unit is concerned with the development of manufacturing methods for the production of new effective vaccines and for the modification of existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.

Progress.

1. Meningococcus Polysaccharides.

During this period studies have been carried out on the development of pilot scale methods for the preparation of purified high molecular weight specific polysaccharides derived from Neisseria meningitidis, for use in the immunization of man against meningococcal meningitis.

a. Small scale lots of purified polysaccharides derived from group A and group C meningococcal strains were prepared in the spring of 1968 by the method developed by CPT Emil C. Gotschlich of the Department of Bacteriology, WRAIR (Annual Report, 1968). Initial attempts in this laboratory to scale up the volumes of production of groups A and C polysaccharides from one-liter per container to twelve-liters per container were unsuccessful. It appeared that the additional time required to adequately sterilize by autoclaving twelve-liter volumes of Franz's medium (employed by Dr. Gotschlich in one-liter volumes) adversely affected the medium so that satisfactory growth of the meningococci could not be obtained. On the other hand, a medium containing a 1:5 dilution of Medium 199, in lieu of the casamino acids in the growth medium, supported the growth of the meningococci in the twelve-liter volumes desired and yielded purified polysaccharides which were indistinguishable from those

prepared in Franz's medium. Consequently the 199 medium was used for the production of 17,500 doses of group C polysaccharide and 7,500 doses of group A polysaccharide. These preparations are currently being used in clinical studies to evaluate the efficacy of the polysaccharides in the protection of man against meningococcal infection.

b. Subsequent laboratory investigations have been conducted in an attempt to simplify the processing procedures and to increase the yield of purified group C polysaccharide. It was found that Franz's medium could be successfully utilized in twelve-liter volumes if the heat-sensitive portion (casamino acids and dextrose) was prepared as a concentrate and sterilized by filtration before dilution to final concentration in the autoclaved, salt-containing portion of the medium. Consistently good growth of meningococci was obtained in a medium prepared in this fashion, provided the cultures were subjected to a combination of agitation and aeration during incubation.

Investigation of the processing procedures revealed that a lengthy centrifugation step and all but 5 successive Sevag extraction steps (to remove contaminating protein) could be eliminated from the method outlined by Gotschlich (Annual Report, 1968) without affecting yield, purity and character of the polysaccharide.

The preparation of pilot scale lots applying the information obtained in these studies has resulted in yields of purified polysaccharides at least 5 times greater than those obtained in the pilot scale production described in paragraph a. above.

c. Currently, the procedures described above are being applied to the production of group B meningococcal polysaccharide, in an attempt to prepare a sufficient quantity of this material for clinical use.

2. <u>Q Fever</u>.

During the past year an investigation on the use of the continuous flow zonal centrifuge for the preparation of purified Q fever vaccine was initiated.

Initially, an attempt was made to introduce into the Spinco B XVI continuous flow zonal rotor an inactivated Henzerling phase 2 rickettsial preparation consisting of a 10% yolk sac membrane suspension which had been previously centrifuged at low speed to remove cellular debris, etc. However, this was not successful, as the particulate matter remaining in the suspension caused

blockage in the small channels in the rotor and in the rotating seal. In addition, fatty material collected on the core of the rotor, causing an abnormal rise in pressure within the rotor.

Since Genetron-113 (trichlorotrifluoroethane) had previously been successfully used in this laboratory to extract fat from yolk sac membrane suspensions (Annual Report, 1966), the crude rickettsial preparation was treated with this fluorocarbon before introduction into the rotor. Ten parts of the yolk sac membrane suspension was blended with one part of Genetron for one minute, and the resulting mixture was centrifuged at 800 x g for 30 minutes. The upper aqueous layer, containing the rickettsiae, was collected and was fed into a sucrose-gradient in the zonal centrifuge. This Genetron-treated material passed through the rotor at the recommended pressure, without blockage or accumulation of fatty material on the core of the rotor.

A series of experiments was carried out to determine the effect of pH on the efficiency of the Genetron extraction. The most satisfactory results were obtained at pH 6.6. At a lower pH clarification was improved, but this was accompanied by a reduction in the number of rickettsiae recovered in the extract.

A series of 50 ml fractions was collected from the rotor and the density of each fraction was determined by means of a refractometer. To each fraction 35 ml of 0.85% saline was then added and the fractions were centrifuged at 22,000 x g for one hour. The pellets were resuspended with 5 ml of 0.85% saline, yielding a 10X concentration for each fraction. The amount of rickettsial antigen in each fraction was then determined by complement fixation. The results were as follows:

TABLE I

Fractionation of Genetron-Extracted Rickettsial Suspension by Density-Gradient Zonal Centrifugation

Fraction	% Sucrose	Density	CF Titer
1	8.5	1.032	8
2	13.5	1.053	8
3	17.0	1.069	3
4	22.0	1.090	2
5	28.0	1.119	0
6	35.0	1.151	0
7	42.0	1.190	4

TABLE I
(cont'd)

Fraction	% Sucrose	Density	CF Titer
8	50.0	1.230	256
9	55.0	1.258	512
10	61.0	1.292	64
11	63.2	1.306	8

As can be seen from the CF titers, the bulk of the rickettsiae banded over a range of densities from 1.23 to 1.29 g/cc. Fractions 8, 9 and 10 were therefore pooled and 35 ml of 0.85% saline was added to this pool, yielding 50 ml of Genetron-extracted, zonal centrifuged material, designated vaccine G-Z.

This vaccine G-Z was compared with vaccines prepared from the same crude rickettsial suspension 1) treated with Genetron, but without zonal centrifugation (vaccine G), and 2) extracted with sulfuric ether by the standard procedure described by Berman et al. (J. Bacteriol., 1961, 81: 794-799) (vaccine E). Vaccines G and E, following extraction with Genetron or ether, were subjected to centrifugation at 22,000 x g for one hour, the resulting pellets resuspended with 0.85% saline to a volume of 50 ml. Thus each of the 3 vaccines was prepared from one liter of the crude 10% yolk sac membrane suspension, and each was in a final volume of 50 ml. A standard Q fever vaccine (Lot DP-7) was included as a reference.

TABLE II

Comparative Assays of Vaccines G-Z, G, E and DP-7

Vaccine	Nitrogen (mgN/ml)	Protein (mg/ml)	Fat (mg/ml)	CF Titer
G-Z	0.125	0.71	2.87	96
G	0.250	3.26	18.1	120
E	0.195	1.36	8.32	56
DP-7	0.016	0.22	0.7	6

Since the reference vaccine (DP-7) had a CF titer of 6, vaccine G-Z was diluted 1:16, vaccine G 1:20, and vaccine E 1:10, to bring them all to the same antigen level. On the basis of these dilutions, the volume of vaccine G-Z was 800 ml, vaccine G 1000 ml, and vaccine E 500 ml. The results of the nitrogen, protein and fat determinations on the diluted preparations are given in Table III.

TABLE III

Comparative Assays on Vaccines with Equivalent Antigen Content (CF Titers = 6)

Vaccine	Nitrogen (mgN/ml)	Protein (mg/ml)	Fat (mg/ml)
G-Z	0.0078	0.044	0.17
G	0.012	0.163	0.9
E	0.0195	0.13	0.83
DP-7	0.016	0.22	0.7

Vaccine G-Z appears to be significantly lower in nitrogen, protein and fat content than the other 3 preparations. The toxicity and immunogenicity for guinea pigs of these vaccines is currently under investigation.

3. Plague.

During the past several years the Department of Biologics Research has been engaged in laboratory investigations on the development of a stable living plague vaccine, prepared with attenuated strains of Pasteurella pestis. As the result of these studies, reproducible methods have been worked out for the preparation of freeze-dried living plague vaccine suitable for human use (Annual Report, 1966). Employing these methods, a supply of freeze-dried seed material and one or more batches of freeze-dried vaccine for human use were prepared with each of 4 attenuated P. pestis strains: 1) EV 76, Saigon (obtained from Institut Pasteur, Saigon, Vietnam), 2) EV 76, Devignat (obtained from Ft. Detrick, Md.), 3) EV 76, 51f (obtained from Dr. K. F. Meyer, University of California Medical School, San Francisco, Calif.) and 4) NPM-23V (obtained from Ft. Detrick, Md.).

The freeze-dried living vaccine prepared with the EV 76, Saigon strain produced excellent antibody and protective responses in guinea pigs. However, when tested in limited human trials, carried out by the USAMU, Ft. Detrick and by the University of California School of Medicine, this vaccine failed to elicit a satisfactory F-1 antibody response in the volunteers.

The freeze-dried living vaccines prepared with the other 3 attenuated plague strains are currently undergoing extensive testing in animals prior to use in man. In the meantime, the stability of these 4 plague vaccines during prolonged storage at -20°C, +4°C, 22°C

and 37°C was evaluated.

a. Long term surveillance of the viability of the freeze-dried EV 76, Saigon strain, prepared in Nov. 1965, was continued during this period. There was no apparent loss in viability of those samples stored at -20°C for a period of three and a half years (172 weeks). At 4°C, maximum viability was maintained for 41 weeks, while at higher storage temperatures significant loss in viability occurred within two weeks.

b. Stability studies were also carried out on freeze-dried vaccines prepared in early 1968 with two other attenuated P. pestis strains, EV 76, Devignat and EV 76, 51f, and a vaccine prepared in Dec. 1968 with a fourth strain, NPM-23V. A summary of the results of periodic titrations of the number of viable organisms in samples of these preparations which were stored at -20°C and +4°C is recorded in Table IV.

TABLE IV

Stability of Viable Count, Freeze-Dried Plague Vaccines

Storage (weeks)	EV 76 Devignat Strain		EV 76 51f Strain		NPM-23V Strain	
	-20°C	+4°C	-20°C	+4°C	-20°C	+4°C
2	7.2×10^9		8.1×10^9		5.6×10^9	4.8×10^9
4	4.6×10^9	6.5×10^9	8.2×10^9	5.2×10^9	7.0×10^9	4.9×10^9
8	5.7×10^9	4.5×10^9	9.1×10^9	5.4×10^9	3.8×10^9	2.0×10^9
12	6.5×10^9	5.8×10^9	5.0×10^9	2.9×10^9	5.8×10^9	2.0×10^9
24	6.1×10^9	1.8×10^9	4.9×10^9			
52	5.6×10^9	1.9×10^9	5.3×10^9			

These results indicate that, for maximum stability during long-term storage, these preparations should be kept at -20°C. At +4°C storage maximum viability is maintained for at least 12 weeks. Although not shown in the Table, at higher storage temperatures (+22°C and +37°C) significant loss in viability occurs within two weeks.

4. Eastern Equine Encephalomyelitis.

Employing EEE virus in chick embryo fibroblast cell cultures as a model system, laboratory studies were continued during this period to determine the possible practical value of modifications of usual tissue culture procedures for the production of viral antigens and vaccines.

Additional studies on the use of a tissue culture system employing continuous monolayers around the entire inner surface of cylindrical bottles rotating on a commercially manufactured roller apparatus confirmed the results obtained earlier with a crude home-made roller (Annual Report, 1967). This system provides a practical method for producing large amounts of virus for antigen or vaccine production, utilizing a minimum number of containers.

A series of experiments designed to determine the optimum conditions for obtaining maximum virus yields in the roller cultures was initiated. It was found that consistently greater yields of virus were obtained when the roller bottles were seeded with 250 ml of growth medium containing $5x10^6$ cells per ml, than with the same volume of medium containing $1x10^6$ cells per ml. Results of a typical titration are given in Table V.

TABLE V

Comparison of EEE Virus Yields in Roller Cultures and Stationary Cultures of Chick Embryo Fibroblasts

Harvest Time	Roller Cultures: Seeded with 250 ml containing $1x10^6$ cells/ml	Roller Cultures: Seeded with 250 ml containing $5x10^6$ cells/ml	Stationary Cultures: Seeded with 50 ml containing $1x10^6$ cells/ml	Stationary Cultures: Seeded with 50 ml containing $5x10^6$ cells/ml
16 hrs.	6.66	7.66	6.83	7.83
18 hrs.	6.83	7.99	7.16	8.16
24 hrs.	7.5	9.16	N.D.	8.16

The effect of other factors, such as temperature of incubation, speed of rotation, size of inoculum, medium composition, etc., on virus yield are currently under investigation.

5. Shigella.

During this period the Department of Biologics Research has continued to provide freeze-drying support to Dr. S. B. Formal of the Department of Applied Immunology, WRAIR, in his work on the development and evaluation of a live, attenuated oral Shigella vaccine. A lot of freeze-dried vaccine, suitable for human use, was prepared in Aug. 1968 from another candidate strain, a streptomycin-dependent mutant of Shigella flexneri 2a. This vaccine has been provided to Dr. Formal for evaluation in animals and man.

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4	4.6×10^9	6.5×10^9	8.2×10^9	5.2×10^9	7.0×10^9	4.9×10^9
8	5.7×10^9	4.5×10^9	9.1×10^9	5.4×10^9	3.8×10^9	2.0×10^9
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	Roller Cultures		Stationary Cultures	
Harvest Time	Seeded with 250 ml containing $1x10^6$ cells/ml	$5x10^6$ cells/ml	Seeded with 50 ml containing $1x10^6$ cells/ml	$5x10^6$ cells/ml
16 hrs.	6.66	7.66	6.83	7.83
18 hrs.	6.83	7.99	7.16	8.16
24 hrs.	7.5	9.16	N.D.	8.16

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Summary and Conclusions.

1. Studies on the development of pilot scale methods for the preparation of meningococcal polysaccharides have continued. Using a growth medium developed in this laboratory, a total of 17,500 doses of purified group C polysaccharide and 7,500 doses of group A polysaccharide were prepared for human field studies. Additional investigations have resulted in simplication of the processing procedure and have provided greater yields of purified A and C polysaccharides. The methods developed are currently being applied to the production of group B specific polysaccharide.

2. Studies on the use of the zonal centrifuge for the preparation of purified Q fever vaccine were initiated. Preliminary extraction of the crude yolk sac membrane suspension with Genetron-113 was necessary to remove the bulk of the lipid and tissue debris prior to centrifugation. Passage through the continuous flow density gradient centrifuge resulted in a product with a significantly reduced nitrogen, protein and fat content. The toxicity and immunogenicity of the zonal centrifuged vaccine is currently being evaluated.

3. Laboratory investigations on the freeze-dried attenuated plague vaccine have continued. Surveillance of the stability of the vaccines prepared from four different attenuated strains of the plague bacillus indicate that a storage temperature of -20°C will provide maximum stability of the freeze-dried product. These vaccines are currently being evaluated in animals prior to consideration for use in man.

4. Studies of the effect of modifications of tissue culture procedures on the production of viral vaccines and antigens were continued, employing EEE virus in chick embryo cell cultures as a model system. Consistently greater yields of virus were obtained when roller bottles were seeded with 250 ml of growth medium containing 5×10^6 chick embryo cells per ml than with the same volume of medium containing 1×10^6 cells per ml.

5. An experimental freeze-dried live attenuated Shigella vaccine was prepared with a streptomycin-dependent strain of Shigella flexneri 2a.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 171, Development of biological products

Publications.

Sorrentino, J. V., Berman, S., Lowenthal, J. P. and Cutchins, E. The Immunologic Response of the Guinea Pig to Eastern Equine Encephalomyelitis Vaccines. Am. J. Trop. Med. and Hyg., 1968, 17: 619-624.

Berman, S., Altieri, P. L., Groffinger, A., Lowenthal, J. P. and Formal, S. B. Freeze-drying Various Strains of Shigella. Applied Microbiol. 1968, 16: 1779-1781.

Project 3A061102B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 172, Sero-recognition of microbial infections

Investigators.
Principal: Earl H. Fife, Jr., M.S.
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Description.

This task is concerned with the mechanisms and patterns of immune responses. In vitro and in vivo methods are used to study host response to antigens. In vitro studies involve the development, improvement, and evaluation of procedures for detection of host antibodies. The studies also entail isolation, purification and identification of antigens by chemical and serological methods. In vivo studies include: (1) investigations on the ability of antigens to stimulate serologically detectable antibodies, (2) cellular level immune response to microbial infection, and (3) production of specific antisera by infection and/or experimental antigens or antigen fractions. Antigens which show a high level of serological sensitivity and specificity are evaluated for immunogenicity.

Progress.

1. Soluble antigen fluorescent antibody (SAFA) tests for serodiagnosis of infections diseases. Details of the development and progressive technical improvements of the SAFA test have been presented in previous reports on this Work Unit (WRAIR Research & Development Reports, 1966 et seq.). The procedure continues to show considerable promise for the serodiagnosis of wide variety of infectious diseases. In addition to the applications of the SAFA test described in this report, other investigators in WRAIR are investigating the potential of the procedure for the serodiagnosis of schistosomiasis, amebiasis, filariasis, malaria, trichinosis, meningococcosis, and a variety of viral diseases. Experience has shown that the method required for fixing a given antigen to the test disc may vary from antigen to antigen. However, once this is achieved, the basic SAFA technic usually can be employed without modification, regardless of the antigen-antibody system involved.

During the course of screening sub-human primates for tuberculosis, it was recognized that the original SAFA technic was somewhat cumbersome and difficult to employ in a mass-testing program. Studies therefore were initiated to determine whether certain technical modifications would make the procedure more suitable for mass-screening, without sacrificing the inherent specificity and sensitivity of the original method. This was achieved by introducing the following

innovation in methodology. After completion of the primary reaction (reaction of the antigen-sensitized disc with test serum), the discs were washed with tris-buffered saline (TrBS) and then immediately mounted on the adhesive side of black plastic electrical tape. The mounted discs then were allowed to dry for ca. 20 minutes at room temperature to insure adherence of the discs to the tape. The secondary reaction was accomplished by first moistening each disc with TrBS, then adding one drop of optimally diluted fluoresceine-conjugated anti-globulin antiserum and incubating in a moist chamber at room temperature for 30 minutes. It was observed that addition of the conjugated antiserum to moist rather than dry discs significantly reduced inherent nonspecific fluorescence. At the completion of the secondary reaction, the tape containing the test discs was submerged in TrBS and washed three times to remove unbound conjugate. The washed discs were allowed to dry for 1 1/2 - 2 hours and then read on the fluorometer in the manner prescribed for the original technic. This modification of the SAFA procedure eliminates many of the manipulations involved in processing each individual disc, significantly reduces the time required to perform the tests, and minimizes the risk of losing the identity of a specimen. This modification of the SAFA was used exclusively in the studies reported herein.

a. Tuberculosis. The need for a reliable immunodiagnostic test for early detection of tuberculosis in sub-human primates and the deficiencies of the currently recommended palpebral intradermal tests were discussed in detail in a previous report on this Work Unit (WRAIR Research & Development Report, 1968). Preliminary results on sera from human and simian subjects suggested that the SAFA test, using purified antigens from cultures of M. tuberculosis, possibly could be of value in this respect. In view of the encouraging findings summarized in the previous report (op. cit.), a comprehensive collaborative study was initiated with investigators at the US Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Md.

These studies were designed to investigate various aspects of the natural transmission of tuberculosis in sub-human primates and to critically evaluate the serological and immunological response in this disease. A group of 30 Rhesus monkeys was selected for these investigations. Each animal was carefully examined for evidence of tuberculosis, and all gave negative tuberculin and SAFA tests. In addition, X-rays showed no evidence of tuberculosis or other pulmonary disease. Six of the monkeys then were exposed to an aerosol of M. tuberculosis (human strain) administered in such a manner that each animal inhaled ca. 5000 organisms. The exposed animals were scrubbed with a disinfectant to remove any tubercle bacilli deposited on the external body surfaces and then were placed in specially designed holding cages. Eighteen of the unexposed animals were employed either as "cage mates" of the exposed monkeys, or were placed in adjacent cages, or in remote cages receiving air circulated through the cage of an exposed animal. The remaining 6 animals were placed on therapeutic doses of Isoniazid and housed in cages adjacent to those of the aerosol-exposed monkeys. Each animal was bled, skin tested and X-rayed at three week intervals during

the first four months of the study, and at monthly intervals thereafter. All sera from a given animal were stored at -70°C until the monkey expired or was sacrificed, and then all were tested in the SAFA test on the same day. Three antigens were used for serologic evaluation of the sera. These included "A" and "C" protein fractions derived from culture filtrates of M. tuberculosis cultures, and a polysaccharide antigen isolated from the cell walls of the tubercle bacilli. All were supplied by collaborators at the George Washington University Medical Center.

Sixteen of the 24 untreated monkeys acquired tuberculosis either from the aerosol or by exposure to an infected animal. All infections were confirmed by isolation of the organism in cultures. Results of the SAFA and tuberculin tests on this group are summarized in Table 1. Thirteen of the 16 infected monkeys gave reactions in the SAFA test and all 8 noninfected animals were nonreactive. Using the criteria recommended by Remin and Wilkerson (J. Chron. Dis., 13 : 6, 1961) for evaluating the sensitivity and specificity of diagnostic tests, the SAFA test showed a sensitivity of 81% and 100% specificity. These findings are in marked contrast to those obtained with the tuberculin test. In the latter, only 7 of the 16 infected animals were reactive. Moreover, 2 of the 8 noninfected monkeys also exhibited reactions with the tuberculin antigen. The sensitivity of the tuberculin test therefore was 44% and the specificity 75%. Review of the data obtained from the three infected animals that failed to react in the SAFA test revealed information of note. One of these monkeys developed a fulminating infection and expired at about the time detectable antibodies would have been expected to appear. The other two animals had very mild infections and showed no clinical evidence of active tuberculosis. They appeared to be healthy, exhibited normal weight gain throughout the observation period, and showed no pathology other than granulomata of the hylar lymph nodes. Cultures of the lymph nodes, however, did reveal the presence of M. tuberculosis. Analysis of the findings obtained from animals that were SAFA-tuberculin-necropsy positive revealed that the SAFA always became positive (14-64 days) before the tuberculin test. Although the X-ray findings on the individual animals have not been received from the radiologist at this time, it is his impression that the X-rays became positive before reactions were observed in the SAFA tests. The animals that received Isoniazid throughout their period of exposure were monitored for 6 months following withdrawal of the drug. None of this group became infected. The Isoniazid therefore appeared to have excellent prophylactic properties. Nevertheless, it remains to be determined whether the drug can effect a radical cure in monkeys after the infection has become established. It should be noted that ancillary studies have shown that repeated tuberculin tests, even when performed at bi-weekly intervals, do not stimulate production of antibodies that react in the SAFA test. Thus, it is unlikely that the periodic tuberculin tests conducted in the present studies influenced the serological findings.

The practice of performing SAFA tests for tuberculosis on all sub-human primates during their quarantine period has been continued. A total of 1271 monkeys were screened in this manner during the present reporting period. Of this group, 29 reacted in tests with one or more

Table 1

Relative Sensitivity and Specificity of SAFA and Taberculin Tests for Detection of Tuberculosis in Monkeys

SAFA Tests

SAFA Test Results*	True Disease Classification	
	Diseased	Not Diseased
R	13 (A)**	0 (B)**
NR	3 (C)**	8 (D)**
TOTAL	16	8

$$\text{Sensitivity} = \frac{A}{A+C} \times 100 = \frac{13}{16} \times 100 = 81.25\%$$

$$\text{Specificity} = \frac{D}{B+D} \times 100 = \frac{8}{8} \times 100 = 100\%$$

****

Tuberculin Tests

Tuberculin Test Results*	True Disease Classification	
	Diseased	Not Diseased
R	7 (A)**	2 (B)**
NR	9 (C)**	6 (D)**
TOTAL	16	8

$$\text{Sensitivity} = \frac{A}{A+C} \times 100 = \frac{7}{16} \times 100 = 43.75\%$$

$$\text{Specificity} = \frac{B}{B+D} \times 100 = \frac{6}{8} \times 100 = 75.0\%$$

*R = Reactive; NR = Nonreactive

** A = Number of True-Positives
B = Number of False-Positives
C = Number of False-Negatives
D = Number of True-Negatives

of the employed M. tuberculosis antigens. Since facilities for the isolation of individual animals were not available locally, it was an established practice to sacrifice all animals that reacted in the SAFA test for tuberculosis or were tuberculin positive. Gross autopsies were performed on each of these animals. However, limitations of personnel, etc. made it impractical to perform detailed histopathological examinations of tissue sections, or attempt to isolate the organism by culture methods. Unfortunately, under these conditions, definitive information concerning the true disease status of many of the animals was not obtained. These problems, however, are being resolved. Arrangements recently were made to transfer all "suspect" monkeys to USAMRIID, Ft. Detrick, hold them in isolation for an indefinite period, and observe whether they develop active tuberculosis. To date, 4 SAFA-positive monkeys have been monitored in this manner and thus far 2 developed fulminating tuberculosis and expired.

Further collaborative studies on the various aspects of simian tuberculosis are being planned with investigators at the USAMRIID. In addition, the feasibility of field testing the SAFA test for tuberculosis in troops at Ft. Ord is being investigated.

b. Histoplasmosis. Details of the methodology developed for use of the SAFA test for the serodiagnosis of histoplasmosis were presented in the previous report on this Work Unit (WRAIR Research & Development Report, 1968). The SAFA test has been performed in parallel with the standard complement fixation and precipitin tests on all sera submitted for the serodiagnosis of mycotic diseases. In general, the SAFA test results have shown little correlation with the results obtained in the standard tests. The majority of the sera that were reactive in the complement fixation and precipitin tests were non-reactive in the SAFA test. On the other hand, many sera that gave strong reactions in the SAFA test were negative in the standard tests. These findings suggest that the antibodies detected in the SAFA test may be different from those that react in the standard tests. Efforts now are being made to correlate the serological findings with clinical data to determine whether the various reactivity patterns have pathognomonic value.

c. Echinococcosis. Dr. Alfonso Trejos, Chief of Laboratories at the Centro Panamericano de Zoonosis, Ramos Majia, Argentina, recently called our attention to the prevalance of hydatid disease in domestic animals and humans residing in certain areas of Argentina. He also discussed the problems he had encountered in attempting to use complement fixation and agglutination tests to identify infected individuals. These problems were particularly serious in tests on dog and sheep sera. It was suggested that use of the SAFA procedure might overcome these difficulties and collaborative effort along these lines was initiated. It was observed that the antigen (hydatid fluid from infected sheep), after simple air-drying (no chemical fixative required), adhered well to the test discs, and that the standard SAFA technic could be employed. The results of preliminary tests on sera from humans, dogs and sheep presumed to be infected with Echinococcus

granulosis are summarized in Table 2. It was apparent that the SAFA test worked equally well with sera from humans, dogs and sheep. This was in contrast to earlier studies in which the complement fixation test was employed. Although the reason for the phenomenon is not known, it has been well established that the majority of dog sera become anticomplementary during the heat inactivation required to destroy the native complement. This essentially precludes the use of complement fixation tests on dog sera unless special technics are employed. A discussion of this problem is presented in another section of this report. In view of the encouraging results obtained thus far with the SAFA test for echinococcosis, collaborative studies are being continued with investigators at the Centro Panamericano de Zoonosis. Plans are being made to experimentally infect a number of dogs and sheep with E. granulosis, and follow the patterns of serologic response throughout the course of the infection. From these studies, it should be possible to obtain information concerning the time of appearance of antibody and determine whether the antibody titers are influenced by the length and severity of the infection. The results of these investigations will be given in a subsequent report on this Work Unit.

2. Serodiagnosis of American trypanosomiasis (Chagas' disease). The importance of Chagas' disease as a major public health problem in Central and South America was noted in the previous report on this Work Unit (WRAIR Research & Development Report, 1968). Recent conversations with Dr. Joffre M. Rezende of the Universidade Fedral de Goias Hospital das Clinicas, Goiania, Brasil, indicate that the magnitude of the problem is even greater than earlier epidemiological information suggested. Field studies regularly are revealing new hyper-endemic areas and it is currently estimated that there are 5-6 million cases of Chagas' disease in Brasil, with at least 600,000 cases in the State of Goias alone. Moreover, Dr. Rezende said that in addition to the usual cardiopathy of chronic Chagas' disease, one of six cases of the disease acquired in the Goias area either has or will develop megaesophagus and/or megacolon. During the present reporting period, this Laboratory has continued to participate in the activities of the Chagas' Disease Study Group organized by the PAHO. An investigator from this Department has collaborated with Dr. Jose Almeida, Universidade de Sao Paulo, Ribeirao Preto, Brasil, in the development of the quantitatively standardized complement fixation procedure that is being used to critically evaluate currently employed antigens, and is being employed as the standard procedure for routine serodiagnosis throughout Central and South America. At the present time, the relative specificity and sensitivity of nine candidate antigens are being evaluated in collaborative studies in seven laboratories in North, Central and South America. These findings will be used to select a standard reference antigen to be used for evaluating future antigens and technics, and for routine serodiagnosis in endemic areas.

Development of a hemagglutination test for Chagas' disease. The technical intricacies and the requirement for overnight incubation seriously limit the suitability of the complement fixation (CF) procedure for use in small field laboratories and blood banks. In addition, a

Table 2

Soluble Antigen Fluorescent Antibody (SAFA)
Tests for Echinococcosis on Sera From Humans,
Dogs and Sheep Presumed to Have Hydatid Disease

Serum Source	No Tested	Number giving indicated results: Reactive	Weakly Reactive	Nonreactive
Human	28	21	2	5
Dog	12	10	1	1
Sheep	12	8	2	2

question has been raised concerning the ability of the CF test to detect antibody in individuals with asymptomatic chronic Chagas' disease. In view of the inherent sensitivity and technical simplicity of indirect hemagglutination (IHA) procedures, studies were initiated to investigate the feasibility of employing IHA tests for the serodiagnosis of Chagas' disease. Results of preliminary studies were presented in the previous report on this Work Unit (WRAIR Research & Development Reprt, 1968). The IHA test using the purified protein antigen described in earlier reports (WRAIR Research & Development Reports, 1960, et seq) proved to be considerably more sensitive than the standard CF procedure, and no serious problems of specificity were encountered. However, the suitability of the IHA test for routine blood bank screening is somewhat compromised by the requirement to sensitize fresh sheep cells each day tests are performed. Previous experience with other IHA systems has shown that treatment with pyruvic aldehyde renders erythrocytes essentially impervious to mechanical damage; red cells treated in this manner can be stored for extended periods at 3°C or even lyophilized without apparent change in physical characteristics. Investigations are in progress to determine whether the T. cruzi protein or exoantigen can be adsorbed on tanned or untanned pyruvic aldehyde treated sheep cells, and whether the sensitized cells can be lyophilized without alteration of their serological properties. Results of these studies will be given in a subsequent report on this Work Unit.

3. Experimental mouse syphilis: Immunological and physiological responses in mice infected with Treponema pallidum. Studies presented in the previous report on this Work Unit (WRAIR Research & Development Report, 1968) were continued for a portion of this reporting period. The kinetic studies of the antibody response to T. pallidum outlined in the previous report have been completed. None of the mice in these studies developed antibodies that reacted with the lipoidal (cardiolipin) antigens even though they were periodically tested for a period of 339 days after inoculation with virulent T. pallidum. This was in contrast to the development of treponemal antibodies. TPI and FTA tests became positive within 4 weeks after infection and this reactivity persisted for the life of the animal.

Mice receiving an inoculum of treponemes emulsified in Freund's complete adjuvant exhibited a peculiar, unexpected immunological response. Serum pools obtained 45, 82 and 155 days after injection all gave strong reactions in FTA tests in which the Reiter treponeme sonicate (sorbent) was omitted. However, these sera showed no reactivity whatsoever in the standard FTA-ABS tests employing the Reiter sonicate. These results suggested that the subcellular material liberated from the disintegrated treponemes incorporated in Freund's adjuvant elicited a strong antibody response to Treponema group antigens but lacked the capacity to stimulate production of the T. pallidum specific antibodies which appeared after inoculation with motile, virulent treponemes.

Antibody response to various doses of intact heat-inactivated and motile treponemes also was investigated. Accordingly, a series of 6 groups

consisting of [illegible] mice each were inoculated intraperitoneally with [illegible] ml of Nelson's medium containing 10-fold dilutions of motile treponemes ranging from 10^7 through 10^2 organisms. A second series of [illegible] groups received similar inocula except that the treponeme suspensions were heated in a 56°C water bath for 40 minutes before injection. The control group received 0.1 ml of Nelson's medium alone. Serum was obtained from each animal 42 and 90 days after inoculation and stored at -70°C until examined in serologic tests. Ancillary studies have shown that treatment of a serum with a sonicate of avirulent Reiter treponemes, as is the practice in the FTA-ABS test, blocks the generic Treponema antibodies and leaves only the T. pallidum species specific antibodies available to react in the test. On the other hand, both the generic and species specific antibodies may contribute to the reactions observed in FTA tests on untreated sera. In the present studies, all sera were tested in both immunofluorescence (FTA and FTA-ABS) procedures to determine the time of appearance and quality of antibodies induced by the various inocula. The results are summarized in Table 3. Sera collected 42 days after inoculation with heat-inactivated treponemes showed some evidence of immune response, but the antibodies appeared to be directed almost exclusively against the generic antigens of the organisms; only occasional sera reacted in the FTA-ABS test. Moreover, the frequency of detectable antibody response appeared to be independent from the size of the inoculum. Examination of the data obtained from the 90-day sera revealed that species-specific antibodies also ultimately were developed in many of the animals inoculated with the inactivated treponemes. This was evident from the near identity of FTA and FTA-ABS test results on sera from mice receiving 10^3 or more heat-inactivated organisms. These findings are in contrast to those obtained from mice inoculated with more than 10^4 motile treponemes. High levels of species-specific antibodies were present in the majority of the 42-day sera from animals receiving 10^5 - 10^7 living organisms. On the other hand, animals receiving smaller inocula showed an immunological response similar to that exhibited by the mice inoculated with the heat-inactivated treponemes; the generic rather than species-specific antibodies predominated. However, the immune mechanisms in mice receiving motile treponemes continued to function at an effective level and essentially all animals, regardless of the size of the inoculum showed high levels of species-specific antibodies in the 90 day serum specimens.

These findings suggest that motile treponemes injected into mice remain viable and probably multiply for an extended period in the tissues, thus providing a continuing antigenic stimulus. The long duration of infective treponemes in the mouse model was established in studies on a group of 30 mice selected for subsequent experiments. In these studies, each animal was infected with 10^6 motile treponemes and then sacrificed 196 days later. The thymus, spleen, and axillary lymph nodes of each animal were excised, placed in cold Hank's balanced salt solution (BSS), and homogenized in a Duall tissue grinder. The lymphoid cell suspension then was centrifuged at 800 rcf for 5 minutes and washed three times with 30 volumes of cold BSS. The washed cells suspension was counted in a hemocytometer and diluted in BSS to give a suspension containing ca. 32×10^7 lymphoid cells/ml. Trypan blue exclusion tests showed that greater than 90% of the lymphoid cells were viable. A 0.5 ml volume of

Table 3

Antibody Response in Mice Inoculated with Graded Numbers of Heat-Inactivated or Motile T. pallidum (Nichols strain)*.

Number of treponemes in inoculum	Heat Inactivated Treponemes				Motile Treponemes			
	Days after inoculation							
	42		90		42		90	
	FTA	FTA-ABS	FTA	FTA-ABS	FTA	FTA-ABS	FTA	FTA-ABS
10^7	2/5	1/5	2/5	4/5	4/5	4/5	5/5	5/5
10^6	2/4	0/4	2/4	2/4	4/5	3/5	5/5	5/5
10^5	2/5	0/5	3/5	3/5	5/5	4/5	5/5	5/5
10^4	2/5	1/5	3/5	3/5	4/5	1/5	5/5	5/5
10^3	2/5	0/5	4/5	3/5	1/5	0/5	5/5	5/5
10^2	2/5	0/5	0/5	0/5	2/5	0/5	4/5	4/5

*FTA (without Reiter sorbent) and FTA-ABS (with sorbent) test results expressed as the number of reactions/number of sera tested.

the cells suspension was injected into each testis of a mature rabbit to determine whether virulent treponemes could be demonstrated. A preinjection serum sample obtained from the recipient rabbit was non-reactive in the TPI and FTA-ABS tests. Seven weeks after infection, the rabbit developed a palpable orchitis, and serum collected at this time was reactive in the TPI and FTA-ABS tests. One week later (8 weeks post injection), the rabbit was sacrificed, a bi-lateral orchiectomy performed, and the testes diced and shaken for 10 minutes in Nelson's medium in the manner used for preparation of TPI test antigen. Dark-field examination of the testicular extract revealed ca. 25×10^6 motile T. pallidum/ml. It is believed that the persistence of infective treponemes in the lymphoid tissues accounts for the observed vigorous, prolonged immune response of the mouse to this organism.

The findings obtained in these studies illustrate certain unique features of the mouse model for studying the mechanisms of the pathogenesis of T. pallidum infection. The mouse harbors a primary infection for life, and although the organisms become widely disseminated throughout the tissues and organs, no clinical or pathological evidence of disease can be observed. On the basis of these observations, it appears that the severe pathology and symptomology associated with human and rabbit syphilis are due to factors other than the direct action of endo- or exotoxins produced by actively mitotic treponemes. A further curious feature of T. pallidum infection in the mouse is the uniform failure to develop the anti-lipid antibodies ("reagins") that are characteristic of human and rabbit syphilis. Some investigators have postulated that the reaginic antibodies are not elicited by an antigen component of the treponeme per se, but rather, are produced by an immune response to host tissues that have been altered by the infection. The findings obtained in the mouse syphilis model are consistent with this postulate since no lesions are produced and reagins are absent even though the mouse shows a persistent, vigorous response to antigens of the treponemes.

The principal investigator in these studies completed his obligated tour of service during this reporting period and currently is preparing a manuscript to be submitted for publication in a scientific journal. No further studies along these lines are being planned at the present time.

4. Immunological and serological studies on individuals with schistosome dermatitis. These studies were conducted in collaboration with members of the U.S. Army Medical Research Team (WRAIR) Vietnam. Initial surveys for schistosomiasis conducted on natives residing in several locations in the Mekong River delta revealed that a considerable number of these individuals were hypersensitive to Schistosoma antigens. Positive schistosomiasis skin tests were observed in 6.29 per cent of the more than 2000 individuals tested. However, multiple stool and rectal biopsy examinations on skin test positive individuals were uniformly negative for schistosome eggs, and only rare infections with other trematodes were observed. These findings suggested that exposure to zoophilic (non-human) schistosomes might account for the dermal hypersensitivity observed among the native residents. In an effort to confirm this hypothesis, surveillance for episodes with features of

schistosome dermatitis was maintained in one US infantry battalion deployed at Dong Tam in the Mekong River delta. On 14 July, 196[illegible], such an episode occurred among members of one platoon during combat operations west of Dong Tam. The patrol had been exposed to canal water by wading. Three weeks later, a second episode occurred among members of a second platoon after it crossed the same canal as that used by the first platoon. This report summarizes the immunological and serological findings on individuals involved in these episodes.

Immediately after return from the patrol in which an episode of dermatitis occurred, each member of the platoon was given a complete physical examination and questioned concerning his dermatitis experience. In addition, blood was collected at this time for serologic tests and skin tests for schistosomiasis were performed. Multiple stool specimens also were obtained from each individual, concentrated by the formalin-ether technic, and examined for parasite ova. Rectal biopsies were performed on all persons who exhibited cutaneous lesions. Although the military tactical situation prevented 100 per cent participation in follow-up studies, efforts were made to collect additional blood specimens and to repeat the skin tests at monthly intervals wherever possible.

Of the 67 men that forded the canal at the point designated earlier, 29 (43.3%) experienced severe itching and 9 (13.4%) developed a rash. It is noteworthy that two members of the first platoon were reexposed three weeks later while crossing the canal with the second platoon. Both experienced more severe cutaneous reactions following the second exposure.

According to accounts given on return from patrol, the itching sensation had its onset 20 to 60 minutes after the men forded the canal; one man experienced such severe cutaneous irritation that he ran about screaming for a brief period and had to be restrained to prevent exposure to enemy fire. Some described the pruritis as a "biting-digging" sensation, while others noticed the itch but were not particularly uncomfortable. The itching sensation did not occur above mid-thigh (the approximate depth of the canal), and lasted for 1 to 24 hours.

The rash was maculopapular. Lesions were 1 to 3 mm in diameter and were surrounded by a 1-mm rim of erythema. In each case there were 15 to 30 lesions, and these occurred predominately on the calves. Although the papules disappeared in ca. five days, areas of hyperpigmentation were still present 2 to 3 weeks later.

Results of initial and repeated skin tests on members of the exposed platoons are summarized in Table 4. Although a number of individuals in this group eventually became skin test positive, these "conversions" did not usually occur until 2 to 3 months after the episode of dermatitis. Nevertheless, there did appear to be a correlation between the skin test reactions and the severity of cutaneous manifestations. The more severe the cutaneous reaction, the greater the probability of skin test conversion. The 3 individuals showing positive skin test reactions on return from patrol warrant

Table 4

Serial Schistosomias Skin Test Results on Members of Exposed Platoons

	Months after exposure			
	0	1	2	3
Individuals tested	67	50	40	37
Conversions	0	1	5	4
% Conversions	0	2.0	10.4	10.8
Cumulative totals of reactors	3*	4	9	13
Per cent total reactors	4.5	8.0	18.8	35.2

*Three individuals were skin test positive on return from patrol in which exposure occurred.

special comment. All experienced particularly severe cutaneous reactions less than 30 minutes after fording the canal. The severity and rapidity of onset of these reactions strongly suggest that these individuals had previously been sensitized with schistosome cercariae and the possibility of frank schistosomiasis was considered. However, repeated stool examinations and rectal biopsies on these and the other members of the exposed group uniformly failed to reveal the presence of schistosome ova. On these bases, it was concluded that the observed hypersensitivity was due to exposure to non-human schistosomes that penetrated the skin but were not capable of migrating through the tissues to sites suitable for maturation and deposition of ova.

Results obtained with the various serologic tests for schistosomiasis are summarized in Table 5. The most striking feature of these findings was the high incidence of reactions in the Fluorescent Antibody (FA) test. Forty-seven per cent of the group reacted in FA tests on sera collected shortly after the episode of dermatitis, and the incidence of reactions persisted at a high level (greater than 50%) for the duration of the observation period. Some persistent reactivity also was observed with the Plasma Card (PC) test. However, the frequency of reactions was much lower than that exhibited by the FA test. These findings are in marked contrast to those obtained with the Slide Flocculation (SF) and Complement Fixation (CF) tests. In the latter, only an occasional reaction or weak reaction was observed, and these were transitory in nature, seldom persisting for more than one month after exposure.

These studies have shown that the skin test and FA test for schistosomiasis are of no value for diagnosis or epidemiological surveys unless the possibility of exposure to non-human schistosomes can be excluded. On the other hand, sensitization with zoophilic schistosomes has relatively little effect on the specificity of the SF and CF tests. Thus the latter obviously are the methods of choice for the diagnosis and epidemiological study of human schistosomiasis.

5. Mass cultivation of yeast-phase Histoplasma capsulatum for production of antigen. For years, the standard procedure for mass cultivation of yeast-phase H. capsulatum used for the preparation of serodiagnostic antigens has been to culture the organisms in Kolle flasks containing Francis' Cystine Agar. However, this procedure has certain inherent limitations. These include: 1) growth is only modest and numerous flasks are required to provide the volume of organisms necessary for antigen production; 2) growth rate is relatively slow, requiring at least 7 days to achieve maximum yields; and 3) difficulty of removing agar particles from the harvest. The present report describes studies undertaken to determine whether these limitations could be overcome by employing a liquid rather than solid medium for mass cultivation.

Four media regularly used for cultivation of fungi were evaluated in these studies. These included: 1) Difco Brain Heart Infusion Medium; 2) a Malt Extract Medium (containing malt extract, peptone and dextrose);

Table 5

Results of Serologic Tests for Schistosomiasis
on Sera from Members of Exposed Platoons

Serologic test*	Months after exposure: 0		1		2		3	
	No. tested	No. reactors	No. tested	No. reactors	No. tested	No. reactors	No. tested	No. reactors
FA	66	31 (47.0%)	53	33 (62.4%)	38	19 (50.0%)	17	9 (53.0%)
PC	67	5 (7.5%)	50	3 (6.0%)	48	11 (23.0%)	37	4 (10.5%)
SF	64	4 (6.2%)	54	4 (7.4%)	42	1 (2.4%)	19	0 (0%)
CF	62	3 (1.6%)	52	2 (3.8%)	40	0 (0%)	19	0 (0%)

*FA = Fluorescent antibody test
PC = Plasma card test
SF = Slide Flocculation test
CF = Complement fixation test

3) a Glucose Medium (containing glucose and peptone); and 4) Emmons' modification of Sabouraud Medium (containing dextrose and neopeptone). Each was modified by omitting the prescribed agar and adding 0.1 gm of L-cystine per liter of medium. Other investigators have reported that L-cystine promotes cell division and enhances the growth of yeast phase H. capsulatum. The media were adjusted to pH 6.8-7.0 before transferring 100 ml aliquots to side-arm flasks and autoclaving for 15 minutes at 15 pounds pressure.

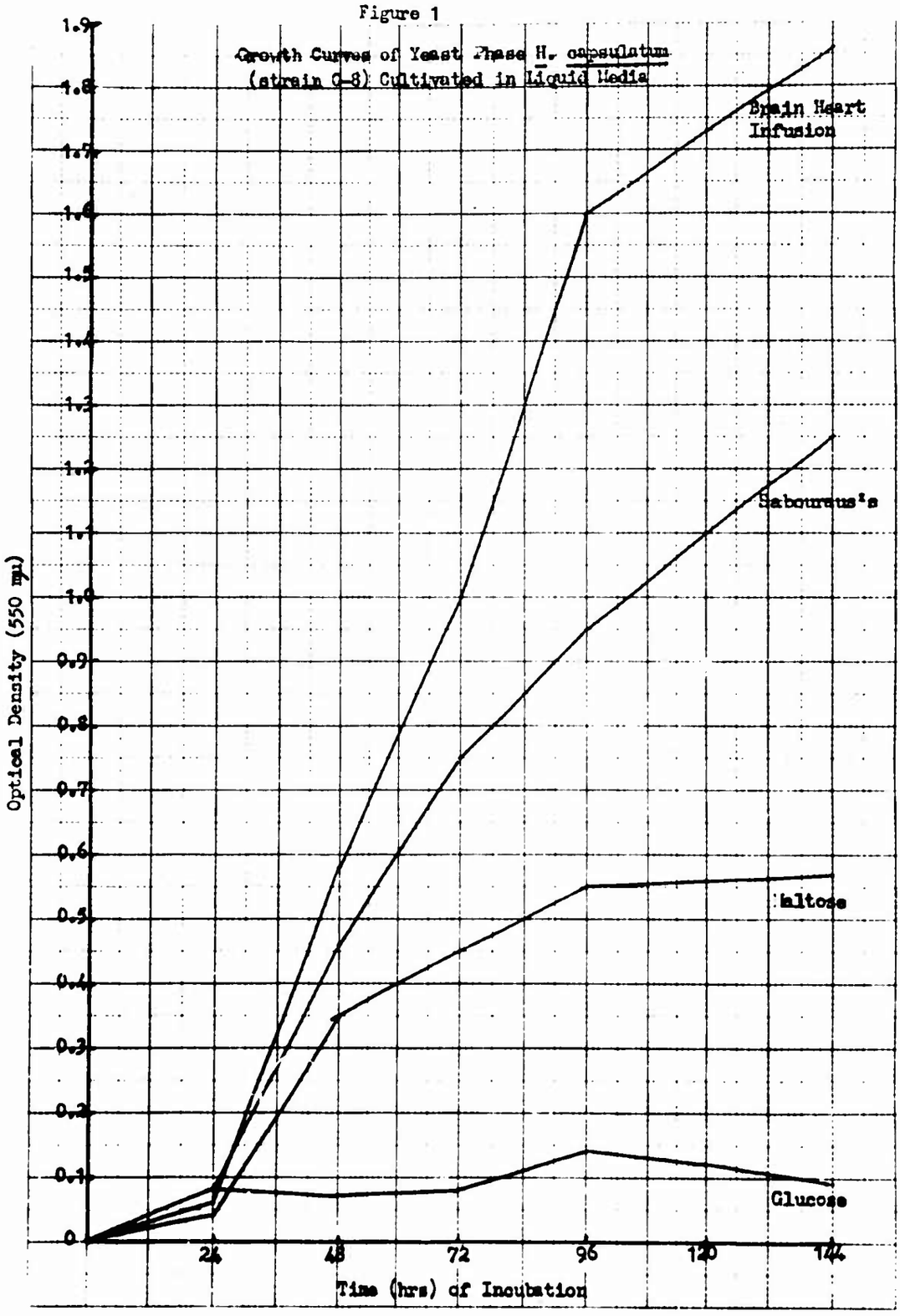
The three strains of H. capsulatum (G-8, G-92, and G-95) normally employed in preparation of standard complement fixing antigens were used to evaluate the experimental media. A loop of organisms from a stock culture grown on Francis' Cystine Agar slant was used as inoculum for each flask. The flasks then were placed on a mechanical shaker and incubated at 37°C for 6 days. The 37°C temperature was employed to assure yeast phase rather than mycelial growth. The relative growth of each culture was monitored by optical density measurements at 24 hour intervals. The growth curves obtained with strain G-8 are presented in Figure 1 and are similar to those obtained with the other strains.

It is readily apparent that the Liquid Brain Heart Infusion Medium (LBHI) is superior to the other media employed. This was a consistent finding with all strains of H. capsulatum used in the evaluation. Certain features of the growth curve with the LBHI medium are of particular interest. With each strain of organism used in these studies, a characteristic lag phase of growth was noted during the first 24 hours of incubation. The logarithmic growth phase occurred during the 24-96 hour incubation period. Growth then continued through hour 144, but at a reduced rate. Studies are in progress to determine whether the lag phase period can be decreased and the log phase extended by using log phase organisms as inoculum.

Studies were conducted to compare the yield of organisms obtained with the new LBHI medium with that obtained with the standard Francis' Cystine Agar cultures in Kolle flasks. Thus, 15 flasks containing 200 ml of LBHI medium, each were inoculated with one loop of organisms from the stock culture, placed on a mechanical shaker, and incubated at 37°C for 96 hours. The cultures then were pooled and the organisms washed in saline and packed by centrifugation for 5 minutes at 1000 rcf. Fifteen Kolle flasks containing 150 ml of Francis' Cystine Agar also were inoculated with one loop of the stock culture and incubated at 37°C for 7 days. The organisms were washed from the surface of the agar with saline, the washings pooled, and the cells packed by centrifugation for 5 minutes at 1000 rcf.

Harvests obtained from the standard solid medium yielded a packed cell volume of 7.3 ml, whereas harvests from the new liquid medium (LBHI) yielded 18.5 ml of packed organisms. Thus the harvest from the LBHI medium cultures was 2.5-times greater than that obtained from a comparable number of standard medium cultures, and moreover, was completely free from solid medium components. Studies are in progress to determine whether antigens prepared from organisms cultivated on the two media

Figure 1


Growth Curves of Yeast Phase H. capsulatum (strain G-8) Cultivated in Liquid Media
Brain Heart Infusion
Sabouraud's
Maltose
Glucose
Optical Density (550 mμ)
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
24
48
72
96
120
144
Time (hrs) of Incubation

differ serologically.

6. Stabilization of sheep erythrocytes for use in serologic tests. When Bukantz, et al (J. Lab. & Clin. Med., 31 : 394, 1946) first recommended the use of modified Alsever's solution for the preservation of sheep blood, it was reported that blood collected in the prescribed manner was satisfactory for use in serologic tests after storage for as long as 12 weeks. More recent studies, however, have failed to corroborate these findings. Investigations in this laboratory (Fogel, et al, Proc. Soc. Exper. Biol. & Med., 121 : 186, 1966), using osmotic fragility, spontaneous lysis, and susceptibility to immune lysis as parameters to critically evaluate the stability of sheep red cells preserved in Alsever's solution, revealed that erythrocytes stored for periods up to 4 weeks exhibited a significant, progressive increase in fragility. Moreover, these investigators noted that cells stored longer than 3 weeks often were unduly fragile and hypersensitive to immune lysis, thus rendering them unsuitable for serologic tests. Part of this problem may be related to the fact that the present donor sheep must be treated periodically to keep the worm burdens of persistent Haemonchus infections at acceptably low levels. Since all of the antihelminthics used for this purpose contain phenathiazine, a drug known to cause severe blood dyscrasias, treated sheep routinely are rested for 60 days before blood is collected for use in serologic tests. Nevertheless, some increased erythrocyte fragility may persist beyond this period.

DeVenuto (Proc. Soc. Exper. Biol. & Med., 128 : 997, 1968) recently reported that the addition of progesterone (0.05 μM/100 ml suspension) to a suspension of human erythrocytes preserved in ACD solution significantly reduced the rate at which the osmotic fragility increased and spontaneous hemolysis developed. In view of these findings, consideration was given to the possibility that addition of progesterone likewise would increase the storage life of sheep cells collected for use in serologic tests. Accordingly, 50 ml of sheep blood collected in Alsever's solution was aseptically transferred to each of four sterile vaccine bottles. Three of the bottles received 0.025, 0.05 and 0.075 μM respectively of aqueous progesterone. The fourth bottle served as the control (no progestrone). The blood was stored at 3°C and at bi-weekly intervals a complement titration, osmotic fragility test and spontaneous lysis determination were performed on each specimen.

Although the osmotic fragility of the cells gradually increased during storage, the rate of increase of the progesterone-treated cells was significantly less than that of the control. After 6 weeks of storage, the osmotic fragility of the treated cells was the same as that shown by the control after storage for 2 weeks. Significant changes in the fragility of the progesterone-treated cells did not appear until after 8 weeks of storage. The control, however, rapidly deteriorated and the cells were extremely fragile after storage for 4 weeks.

The complement titers obtained with the progesterone-treated cells

did not change during the first six weeks of storage. However, a slight reduction of titer was noted after storage for eight weeks.

All three levels of progesterone used in these studies effectively stabilized the cells. However, the cells treated with 0.024 μM progesterone/50 ml of cells suspension showed the least change in osmotic fragility, and this concentration was selected for use in subsequent investigations.

These studies have shown that the addition progesterone to preserved sheep cells increases the storage life of the cells at least two-fold. Moreover, progesterone treatment does not render the cells unsatisfactory for subsequent use in complement fixation tests. Studies are in progress to determine whether progesterone-treated cells can be used in indirect hemagglutination tests.

7. A modified complement fixation procedure for tests on dog sera. It has long been known that dog sera frequently become anticomplementary following heat inactivation (56°C for 30 min) to destroy the native complement. The reason for this phenomenon is unknown. Nevertheless, it constitutes a serious problem when attempts are made to use such sera in diagnostic complement fixation tests. Although investigators generally circumvent this problem by employing other serodiagnostic procedures (e.g. indirect hemagglutination or flocculation), this is not always feasible since certain antigens cannot be adsorbed on erythrocytes or inert carriers.

Some investigators have attempted to minimize these difficulties by assuming that the specific reactivity can be expressed as the difference between the per cent hemolysis obtained in the test with antigen and that observed in the serum control. This assumption is not valid and often will lead to erroneous interpretations. However, the problem can be approached on a theoretically sound, practical basis. It is well established that there is a linear relationship between the amount of immune complex formed and the units (C'H50) of complement required for 50 per cent hemolysis. The specific reactivity therefore can be expressed as the ratio between the units of C' required for 50 per cent hemolysis in the test with serum + antigen ($K'_{S,A}$) and the units of C' required for 50 per cent hemolysis in the serum control (K'_S), i.e. the serum titer, $T_S = \frac{K'_{S,A}}{K'_S}$.

In practice, a single dilution of serum + optimal antigen is tested in parallel with 5, 7.5 and 10 units of complement. The serum control (no antigen) is tested with 1, 2.5 and 5 units of C' The $K'_{S,A}$ and K'_S then can be estimated graphically on log-probit paper by selecting the tubes showing partial hemolysis and plotting the logarithms of the units of C' used vs the probits of the observed percentages of hemolysis, and fitting a straight line to the experimental points. The intercept of the line with the coordinate of 50 per cent hemolysis indicates the units of C' required for 50 per cent hemolysis in the test with antigen and the serum control.

This approach is illustrated by the example presented in Table 6 and Figure 2, which shown the results of a histoplasmosis complement fixation test on serum from an infected dog. In the tests with serum + antigen, it was observed that 40% hemolysis was obtained with 7.5-unit complement and 60% hemolysis with 10-unit C'. Similarly, the serum controls showed 25% hemolysis with 2.5-unit C' and 80% hemolysis with 5-unit C'. Figure 2 shows a plot of these data on log-probit graph paper. The $K'_{S,A}$ (units of C' required for 50% hemolysis) for tests on the serum + antigen proved to be 8.7 units whereas the K'_S for tests on the serum alone was 3.4 units. The serum titer $(\frac{K'_{S,A}}{K'_S})$ therefore was 2.3. In histoplasmosis CF tests conducted in this manner, sera showing titers of 2.0 or greater are considered to be reactive. The possibility that this method may provide a capability for performing leptospirosis and echinococcosis CF tests on dog sera is being investigated.

Summary and Conclusions.

1. The soluble antigen fluorescent antibody (SAFA) procedure has continued to show excellent diagnostic potential and the technic is being employed for the serodiagnosis of a variety of parasitic, bacterial, mycotic and viral diseases. Further technical innovations have been introduced to simplify performance of the test and facilitate use of the procedure in mass screening.

a. Studies on the use of the SAFA test for the serodiagnosis of tuberculosis have been continued. In carefully controlled investigations on experimentally infected monkeys, the sensitivity and specificity of the SAFA test was far superior to that obtained with the tuberculin intradermal test. In addition, the SAFA test always became positive (14-64 days) before the tuberculin test. All monkeys received at WRAIR are being screened with the SAFA test for tuberculosis. Animals showing reactions in the SAFA test are immediately isolated and observed for a period to determine whether they develop clinical disease.

b. The SAFA test for histoplasmosis has been performed in parallel with the standard complement fixation and precipitation tests on all sera submitted for the serodiagnosis of mycotic disease. Little correlation of results was obtained, indicating that the SAFA and standard tests may detect different antibodies. Serologic results and clinical data are being evaluated to determine whether the reactivity patterns obtained in the serologic tests have pathognomonic value.

c. The suitability of the SAFA test for serodiagnosis of echinococcosis has been investigated. Preliminary studies on sera from humans, dogs and sheep with naturally acquired hydatid disease indicate that the SAFA test may be superior to the conventional complement fixation and flocculation tests for serodiagnosis of this infection. The problems usually encountered in performing complement fixation and flocculation tests on dog and sheep sera were not encountered in the SAFA system. Investigations are being continued on experimentally infected animals.

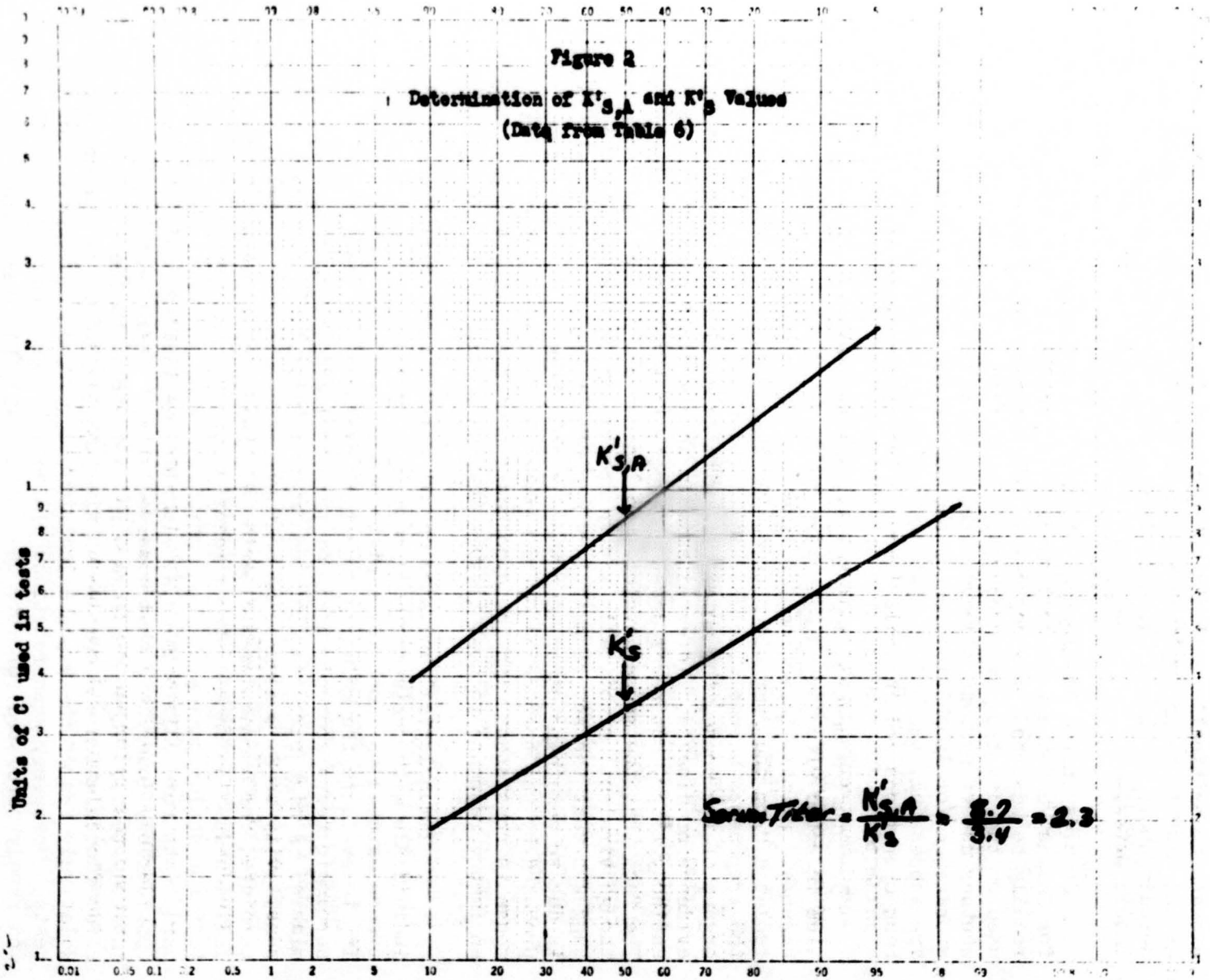
Table 6

Results of a Histoplasmosis CF Test on Dog Serum

Units of C' used	Per cent hemolysis				
	1U	2.5U	5U	7.5U	10U
Serum (1:8) + antigen			0	40	60
Serum Control	0	25	80		

Figure 2

Determination of $K'_{S,A}$ and K'_S Values
(Data from Table 6)



$$\text{Serum Titer} = \frac{K'_{S,A}}{K'_S} = \frac{8.2}{3.4} = 2.3$$

2. Efforts to further improve serodiagnostic tests for Chagas' disease have been continued. A new, quantitatively standardized complement fixation procedure has been developed. This procedure currently is being used by members of the PAHO Study Group on Chagas' Disease to critically evaluate antigens now in general use. The method also is being employed as the standard procedure for routine serodiagnosis of Chagas' disease in Central and South American laboratories.

Studies on the development of an indirect hemagglutination test for Chagas' disease continued. The purified protein antigen can readily be adsorbed to sheep cells, but the sensitized cells must be used on the day they are prepared. Studies are in progress to determine whether use of pyruvic aldehyde-treated cells will permit prolonged storage and/or lyophilization of the sensitized cells. If these efforts are successful, the IHA test would be ideally suited for use in blood bank operations and in small laboratories with minimum equipment.

3. Studies on the physiological and immunological responses in mice infected with T. pallidum were continued. The mouse apparently harbors a primary infection for life; virulent treponemes have been isolated from the lymphoid tissues 196 days post infection. Although the treponemes become widely disseminated throughout the tissues and organs of the mouse, the animal exhibits no clinical or pathological evidence of disease. Infected animals show a rapid rise of treponemal antibody titer, but never develop detectible levels of reaginic antibody. The fact that the mouse developes no lesions or clinical symptoms, even though virulent, mitotically active treponemes are present in the tissues, suggests that the severe pathology manifested in human and rabbit syphilis is due to something other than the direct action of exo- or endotoxins produced by the treponemes. The absence of reaginic antibodies in the mouse is consistent with the hypothesis that the latter are the result of an immune response to host tissues that have been altered by the infection.

4. Immunological and serological studies were conducted on individuals with schistosome dermatitis. Episodes of dermatitis in troops exposed to zoophilic schistosome cercariae provided an opportunity to evaluate the relative specificity of various immunologic and serologic tests commonly used for the serodiagnosis of human schistosomiasis. A high percentage of the exposed individuals developed a hypersensitivity to S. mansoni skin test antigen. Moreover, these reactions persisted for a considerable period of time. The majority of these individuals also showed strong reactions in the fluorescent antibody test for schistosomiasis. Some persistence of reactivity was noted in card tests for schistosomiasis, although the incidence of reactions was considerably lower than that observed with the skin tests or fluorescent antibody tests. These findings were in contrast to those obtained with slide flocculation and complement fixation tests for schistosomiasis. Relatively few of the exposed individuals reacted at all in the latter tests. Moreover, when reactions did occur, they were transitory in nature and rarely persisted for more than one month after exposure. The studies show that the skin test and fluorescent antibody test are of no value for

individual diagnosis or for epidemiological studies on human schistosomiasis unless exposure to zoophilic schistosomes can be excluded. On the other hand, reliable serological information can be obtained with the flocculation and complement fixation tests even in areas endemic for non-human schistosomes.

5. Efforts have been made to improve the methods for mass cultivation of yeast-phase H. capsulatum required to prepare antigens for serologic tests. A liquid brain-heart infusion medium supplemented with cystine was superior to the other media that were investigated, and yielded harvests that were 2.5 times greater than those obtained with the standard Francis' cystine agar medium. The serologic properties of organisms cultivated in the new liquid medium are under investigation.

6. Studies were undertaken to determine whether the storage life of sheep cells preserved in Alsever's solution could be extended by incorporating progesterone in the suspension. Results showed that the addition of 0.025 μM of progesterone/50 ml of cells suspension had an effective stabilizing effect on the cells. Although the osmotic fragility of the progesterone-treated cells gradually increased during storage, the rate of increase was considerably lower than that of the cells without progesterone. The progesterone-treated cells were suitable for use in complement fixation tests after storage for 6 weeks. In contrast, the untreated cells became hyper-fragile when stored more than 3 weeks. Complement titrations performed after various periods of storage showed that progesterone treatment of the cells had no adverse effect on the hemolytic activity of the complement. The possible effect of progesterone treatment on cells to be used in indirect hemagglutination tests is being investigated before a decision is made concerning its routine use in cells collected for serologic tests.

7. Since dog sera often become anticomplementary when heat-inactivated to destroy native complement, it is difficult to use such specimens in diagnostic complement fixation tests. With some antigen-antibody systems, it may be possible to circumvent this problem by employing an alternative serologic technic such as a hemagglutination or flocculation procedure. However, this may not be feasible for certain antigens, and a modified complement fixation procedure has been devised for use under these conditions. Since there is a linear relationship between the amount of immune complex formed and the units of complement required for 50 per cent hemolysis, the specific reactivity can be expressed as the ratio between the units of complement required for 50% hemolysis in the test with serum + antigen, and the units of complement required for 50% hemolysis in the serum control. Details of the procedure for determining these values are presented in the text. The procedure has been used successfully for the serodiagnosis of histoplasmosis in dogs, and studies are in progress to determine its suitability for the serodiagnosis of canine leptospirosis and echinococcosis.

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Publications.

None.