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AUTHORITY

USAMRDC ltr, 19 Jul 1976

AD

RCS MEDDH - 288 (RI)

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

Including

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

ANNUAL PROGRESS REPORT

1 July 1969 – 30 June 1970

VOLUME I

WALTER REED ARMY INSTITUTE OF RESEARCH

WALTER REED ARMY MEDICAL CENTER

WASHINGTON, D.C. 20012

760

AD

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 1969 - 30 June 1970

Volume I

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

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 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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TABLE OF CONTENTS

VOLUME I

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 0A 6477	70 06 30	DD-DR&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 07 01	K. Comp	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
63 05	Cont	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
	CURRENT 70	2	95

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

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

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

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Chromium; (U) Metals; (U) Diabetes; (U) Insulin; (U) Glucose; (U) Arteriosclerosis; (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 vitro tests are performed for biological activity of specially prepared complexes of chromium with biological materials to determine the influence of chemical structure of biological activity.

25. (U) 69 07 - 70 06 Effort was concentrated on the development of simple batch procedures as the first step of purification of natural chromium complex in yeast. Prolonged autolysis of the yeast cells releases a considerable portion of the active chromium complex (glucose tolerance factor) which can be further purified by a simple charcoal treatment followed by acidic phenol extraction. Resulting fractions were routinely tested by biological assay for increased action of insulin on glucose oxidation. The effects of the hormone were stimulated at least fivefold. Fractions extracted from Chromium-51 labeled yeast were administered to pregnant rats. Radioactivity was concentrated by the fetal liver three times greater than the content of the maternal liver. Chromium-51 yeast extract labeled the specific, regulatory chromium pool o[illegible] which responds by changing plasma chromium levels after glucose or insulin inje[illegible]. These two observations emphasize the importance of chemical binding of chromium, as it occurs in glucose tolerance factor. Project is to be considered as complete as principal investigator has resigned and the work will not be resumed by his replacement. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

* Available to contractors upon indication of approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

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: Dr. Walter Mertz

Description.

The objective of this work unit was to define and quantify the mode of action of Chromium III in metabolism. Within the work unit efforts were expended to determine the interaction of chromium and insulin and the determination of the consequences of chromium deficiency in the mammal. The work unit is to be considered complete due to the resignation of the principal investigator.

Progress.

Experiments were conducted to study the nature of chemical binding of chromium as it occurs in glucose tolerance factor. Fetal liver accumulation of chromium was three times higher than maternal liver when Chromium-51 label yeast was administered to pregnant rats.

Summary.

The research activity within this work unit has been reduced to zero due to the resignation of the senior investigator. No further work is contemplated within this area by his successor. This is a final report covering the work unit.

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

Literature Cited.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6457	70 06 30	DD-DR&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 08 31	K. Completion	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	096
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Electron and Light Microscope Study of Intestinal Vasculature in Cholera (21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology; 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 08	CONT	DA	B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 69 11 EXPIRATION: 69 12
b. NUMBER: DADA 17-70-C-0012
c. TYPE: U.CPFF d. AMOUNT:
e. KIND OF AWARD: CON f. CUM. AMT. 8791

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 69	0	0
CURRENT FISCAL YEAR 70	0.2	9

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: Biodynamics Research Corporation
ADDRESS: Rockville, Maryland 20852

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Merrill, T. G.
TELEPHONE: 301-881-4044
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Sprinz, COL H. 3
NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Electron Microscopy; (U) Cholera; (U) Intestinal Vasculature; (U) Fluid and Electrolyte Escape; (U) Light Microscopy

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

23. (U) To demonstrate the morphological alterations of the intestinal vasculature endothelium in cholera and the route by which escaping fluids and electrolytes pass from the lamina propria into the intestinal lumen across intact epithelial lining.
24. (U) Through light and electron microscopy of guinea pig tissue after prescribed times of infection with cholera.
25. (U) 69 08 - 69 12 It was found that fluid exsorption following cholera toxin administration occurred in a manner similar to that seen in the normal. No specific vascular lesions could be identified. However, the rate of fluid exsorption in cholera was greatly accelerated. These findings, suggest that the primary effect of cholera toxin may not be on the intestinal vasculature, but possibly on the epithelial lining. Term of contract expired 69 12 31 and a final report has been submitted which needs contractural obligations. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 096, Electron & light microscope study of intestinal vasculature in cholera

Investigators.
Principal: Thomas G. Merrill
Associate: Helmuth Sprinz

Description

This contract was for the study of morphological alterations of the intestinal vasculature endothelium in cholera and the route by which escaping fluids and electrolytes pass from the lamina propria into the intestinal lumen across intact epithelial lining. It was found that fluid exsorption following cholera toxin administration occurred in a manner similar to that seen in the normal. No specific vasculature lesions could be identified. The rate of fluid exsorption in cholera was greatly accelerated, suggesting that the primary effect of cholera toxin may not be on the intestinal vasculature, but possibly on the epithelial lining. This contract expired 31 December 1969.

3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 096, Electron & light microscope study of intestinal vasculature in cholera

Literature Cited

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6458	70 07 01	DD-DR&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 11 30	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	097
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Transport of Essential Metals in Simulated Biological Environments (21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 09	Cont	DA	B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 69 09 EXPIRATION: 70 08
b. NUMBER: DADA 17-70-C-0014
c. TYPE: S.CT d. AMOUNT: 27,310
e. KIND OF AWARD: New f. CUM. AMT. 27,310

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	1	23
FISCAL YEAR CURRENT 71	0.2	5

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 Maryland
ADDRESS: College Park, Md. 20740

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Rollinson, C. L.
TELEPHONE: 301-927-3800
SOCIAL SECURITY ACCOUNT NUMBER:

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

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Mineral Transport

(U) Sequential Dialysis; (U) Chromium; (U) Iron; (U) Insulin; (U) Chelating Agents;

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

23. (U) To perform kinetic studies on the interaction of trace metals with biological species, e.g., amino acids and hormones, with the aim of defining the role of trace metals in biochemical reactions.

24. (U) Through application of procedures devised by the investigator for the use of the method of sequential dialysis.

25. (U) 69 09 - 70 06 During the reporting period a number of potential ligands have been studied for Chromium (III) and Iron (III). Notably Iron (III) ligands are more rapid in reaction rate than chromium. Phosphate ion when used as a buffer was found to be reasonable ligand material at low concentration. This contract is scheduled to be terminated as of 30 August 1970 and a final report is being prepared. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 097, Transport of essential metals in simulated biological environments

Investigators.
Principal: C. L. Rollinson, Ph.D. (University of Maryland)
Associate: COL E. C. Knoblock, MSC

Description.

The technical objective of this work unit is to quantify the interaction of trace metals with biochemical moieties such as amino acids and carbohydrate intermediates and to determine the kinetics of tne reaction employing sequential dialysis as the principle analytical model system. Activities within the contract during the reporting period include:

1. Chromium - Effect of phosphate.

2. Iron - Effect of various ligands.

3. Membrane studies.

Progress.

1. Chromium - Effect of phosphate.

Phosphate ion is used to buffer reaction mixtures for kinetic studies. It has been suggested that phosphate could behave as a ligand toward Cr (III). Experiments have been performed to substantiate this effect. The results indicate that instability of reaction mixtures increases markedly as phosphate concentration is reduced from 0.2 M to 0.002 M and when Cr (III) is 10^{-4} M and 10^{-5} M. With the most potent ligands, pyrophosphate, citrate and adenosine triphosphate, the coordinating effect of phosphate does not manifest itself except at the lower concentration of ligand.

2. Iron - Effect of various ligands.

Reaction mixtures of Fe (III) were studied with various ligands and compared with reaction mixtures of Cr (III). Fe (III) reaction rates are definitively higher than those of Cr (III). Citrate, pyrophosphate, mesoxalate, triphosphate, adenosine diphosphate, oxaloacetate and 1,6-fructose diphosphate coordinate well with both Cr (III) and Fe (III). On the other hand, malate and tartronate, while excellent

ligands for Cr (III), are poor ligands with Fe (III). Fructose, contrary to published reports, has been found to have little coordinating tendency toward Fe (III).

In test mixtures of aspartic acid, methionine, arginine, ketoglutaric acid, tartrate, glutaric acid, thiamine monophosphate and sorbital, there was little diffusion of Fe (III), leading to the conclusion that these systems either formed slowly diffusing complexes or failed to prevent olation of the Fe (III).

3. Membrane studies.

A series of membranes obtained from various sources are being evaluated for their porosity characteristics in relation to sequential dialysis. Preliminary results have demonstrated the unsuitability of the silver membrane because the pores are too large. Nojax-18, a sausage casing, shows promise as a membrane because its decreased porosity may magnify observable ligand differences. Differences in experimental response to the non-uniformity of membrane materials has been studied and a procedure developed to standardize membranes in all the dialyzers employed.

Summary.

This contract is scheduled to terminate as of 31 August 1970. During the reporting period, a number of potential ligands have been studied for Cr (III) and Fe (III). Notably Fe (III) ligands are faster in reaction rate than those formed with Cr (III). Phosphate ion when used as a buffer was found to be a reasonable ligand material at low concentration.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 097, Transport of essential metals in simulated biological environments

Literature Cited.

Rollinson, C. L. and Rosenbloom, E. W.: Coordination in Biochemistry: Reactions of Chromium (III) in Coordination Chemistry. Paper presented in honor of Professor John C. Bailar, Jr., Plenum Press, New York, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0A 6480	70 06 30	DD-DR&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	9A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	K. Comp	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	098
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede 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:* DA 49-193-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 PRECEDING 69	1	13
FISCAL YEAR CURRENT 70	0.2	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 Maryland
ADDRESS:* College Park, Md. 20740

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Rollinson, C. L.
TELEPHONE: 301-927-3800
SOCIAL SECURITY ACCOUNT NUMBER:

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

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede 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. Precede 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) 69 07 - 70 06 Term of contract expired on 31 August 1969 and a final report has been submitted. 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 hormone 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 afinity 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 report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD, FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 098, Chromium complexes of insulin and related compounds

Investigators.
Principal: C. L. Rollinson, Ph.D. (University of Maryland)
Associate: COL E. C. Knoblock, MSC

Description.

The study of the interaction of trivalent chromium with biological materials that act as carriers and with insulin for which chromium is a cofactor. This contract expired on 31 August 1969 and a final report has been submitted.

Progress.

The final accomplishment of this contract effort was the development of a process of sequential dialysis that permits the study of chromium in interaction with a variety of biological materials.

Summary.

The contract terminated on 31 August 1969 and a final report has been submitted.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 098, Chromium complexes of insulin and related compounds

Literature Cited.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OB 6464	70 07 01	DD-DR&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
	A. New	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	099
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)* (U) Further Studies on the Effect of Microwave Irradiation on Turnover Rate of Serotonin and Norepinephrine in Rodent Brain

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

012900 Physiology; 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 06	CONT	DA	B. CONTRACT

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 70 06 EXPIRATION: 71 05
b. NUMBER:* DADA 17-69-C-9144
c. TYPE: S.CT d. AMOUNT: 27810
e. KIND OF AWARD: NEW f. CUM. AMT. 27810

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	0	0
	CURRENT 71	0.5	25

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:* The Johns Hopkins University
ADDRESS:* Baltimore, Md. 21205

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Snyder, S. H.
TELEPHONE: 301-HO7-3300
SOCIAL SECURITY ACCOUNT NUMBER:

21. GENERAL USE
Foreign Intelligence Not Considered

ASSOCIATE INVESTIGATORS
NAME: Holloway, LTC H. C.
NAME:

1

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Microwave Irradiation; (U) Non-ionizing Electromagnetic Irradiation; (U) Behavioral Effects; (U) Neurochemical Systems

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

23. (U) To determine the nature and extent of microwave effects on central nervous system neurotransmitters involved in thermoregulation and other autonomic functions.

24. (U) Chemical determination of changes in CNS levels and turnover rates of serotonin and norepinephrine following exposure to microwave radiation and conventional thermal stress.

25. (U) 69 05 - 70 06. Previous studies show that serotonin turnover is depressed by chronic microwave irradiation and by chronic heat stress. Experiments are underway in this new contract to determine relative efficiency of these two forms of energy in stressing thermoregulatory system and to elucidate possible differences in mechanism of action. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70)

*Available to contractors upon originator's approval.

DD, FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 099, Further studies on the effect of microwave irradiation on the turnover rate of serotonin and norepinephrine in rodent brain

Investigators.
Principal: Solomon H. Snyder, M.D.
Associate: LTC Harry C. Holloway, MC

Previous studies show that serotonin turnover is depressed by chronic microwave irradiation and by chronic heat stress. Experiments are underway in this new contract to determine relative efficiency of these two forms of energy in stressing thermoregulatory system and to elucidate possible differences in mechanism of action. Serotonin levels and serotonin turnover rates are determined by quantitative chemical procedures in rats exposed to microwaves at various strengths and durations. Results are compared to those in animals not exposed to the microwaves and to animals heated by infrared light. There are no new data to report on this contract since it has only been in effect for a month.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 099 Further studies on the effect of microwave irradiation on the turnover rate of serotonin and norepinephrine in rodent brain

Literature Cited

1. References: None

2. Publications: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6487	70 06 30	DD-DR&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 07 01	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	105
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)
(U) Metallic Micronutrients and Intermediary Metabolism (30)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
64 07	Cont	DA	B. Contract

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: 69 07 EXPIRATION: 70 03
b. NUMBER: DA 49-193-MD-5295
c. TYPE: S.CT. d. AMOUNT: 17,000
e. KIND OF AWARD: Ext f. CUM. AMT. 98,584

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	1	17
FISCAL YEAR CURRENT 70	0.8	17

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: Dartmouth Medical School
Trace Element Laboratory
ADDRESS: Bratteboro, Vt. 05301

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Schroeder, H. A.
TELEPHONE: 802-254-9637
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Mertz, W.
NAME: 1

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Chromium; (U) Vanadium; (U) Mineral Metabolism; (U) Nickel; (U) Germanium

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 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, indium, zirconium, arsenic, antimony and tin.

24. (U) Animals were 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) 69 07 - 70 06 Completed study measuring cholesterol levels and glucose levels on two diets using refined sugar versus brown sugar in chromium-deficient rats has shown that cholesterol levels were lower with the brown sugar diet. Addition of trivalent chromium to the refined sugar diet produced similar responses in both groups of animals. Life term study of rats for contamination by zirconium, niobium, antimony, vanadium and lead in the presence of adequate chromium has shown antimony to be toxic at five parts per million with increased glycosuria with zirconium, niobium and lead. Cholesterol was increased by antimony and vanadium. Antimony and lead were greatly increased in soft tissues. Trivalent chromium supplementation showed a protective effect against lead toxicity. Term of the contract expired 70 03 31 and a final report has been submitted. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 105, Metallic micronutrients and intermediary metabolism

Investigators.
Principal: H. A. Schroeder, M.D. (Dartmouth Medical School)
Associate: COL E. C. Knoblock, MSC

Description.

The technical objective of this work unit has been to study the effect of chronic nutritional deficiencies in experimental animals. Elements have included chromium, vanadium, nickel, germanium, indium, zirconium, arsenic, antimony and tin.

Progress.

In order to evaluate biological effects of trace elements, mice and rats were exposed for their lifetimes to small doses of each of 27 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 21 elements in foods, water, vegetation, wild animals were also made, by trace element analysis, and human tissue concentrations for 6. 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. 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 may suppress spontaneous tumors in mice, that selenium, rhodium and palladium are carcinogenic and that a number of other trace elements are either inert or exert vague toxicity not associated with any demonstable 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. These three changes were prevented by the feeding of trivalent chromium.

Summary.

The work unit has been terminated effective 30 June 1970 and a final report has been submitted.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 105, Metallic micronutrients and intermediary metabolism

Literature Cited.

See final report.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6490	70 06 30	DD-DR&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 07 01	K.Completion	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	108
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Alterations of cells surviving injury. (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

016200 Stress Physiology; 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
64 11	CONT	DA	C.In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

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	1	10
CURRENT 70	0.1	10

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: Walter Reed Army Institute of Research
Division of Experimental Pathology

ADDRESS: Washington, D. C. 20012

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

NAME: Glinos, A.D., M.D.

TELEPHONE: 202-576-5284

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Werrlein, R.J.; McAtee, L.T.; DA

NAME: Brown, J.R.C.

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Cell [illegible]lture; (U) Cellular Genetics; (U) Cell Freezing; (U) Radiation Injury; (U) Heat Shock; (U) Stress Physiology

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

23(U) Cell alterations such as those responsible for the untoward effects observed in individuals exposed to ionizing radiation and thermal shock and for the difficulties encountered in preserving cells and tissues through freezing, have military implications and as such constitute the specific objectives of this study.

24(U) The situations constituting the objectives of this study are due to the development of structural and functional alterations among cells which were subjected to the influence described and survived. These alterations concern the progeny of the surviving cells as well, and therefore lend themselves well to analysis through methods such as cell culture which allow extended observations over many cell generations. Accordingly, cell culture methods were used extensively in this study.

25(U) 69 07 - 70 06 In regard to ionizing radiation it was found that cells irradiated while growing are capable of a greater number of post-irradiation divisions than cells irradiated while stationary. In regard to freezing it was found that cells frozen during the duplication of their genetic material survive considerably better and have fewer chromosomal abnormalities than cells frozen prior to the initiation of this process. In regard to heat shock besides acute cell death, inhibition of DNA synthesis and mitosis during exposure to high temperatures gave rise to a subsequent synchronization of cell division with no permanent effects on generation time or the chromosomal complement of the cells. A small portion of the cell population continued to synthesize DNA during heat shock suggesting thermotolerance.

For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul. 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 108, Alterations of cells surviving injury

Investigators.
Principal: Andre D. Glinos, M.D.
Associate: R. J. Werrlein, M.S., L.T. McAtee, Ph.D.; J.R.C. Brown, Ph.D.

Problem and Background

While the mechanisms of cell growth and regeneration responsible for the repair of tissues following injury have been under intensive investigation, not sufficient attention has been given to the genetic and metabolic alterations of cells surviving injury. Yet such alterations are of great military interest as illustrated by the fact that they are responsible for the untoward effects observed in individuals exposed to ionizing radiation and for the difficulties encountered in the use of frozen cells and tissues for grafting (c.f. the work of Van Bekkum, *et al*, in Holland supported by Army contract DAJA-68-C-0081 on the use of frozen bone marrow preparations in the therapy of radiation injury). Consequently the research objectives of this study were defined as the analysis of the short and long range effects of ionizing irradiation, freezing injury and heat shock in terms of the genetic and metabolic alterations exhibited by cell populations exposed to these injurious agents. The multidisciplinary approach required to attain these objectives has been carried out in a joint effort with the Department of Zoology, University of Maryland supported by Grant No. DA-MD-49-193-65-G148. The major portion of the results obtained has been described in detail in the annual progress reports of previous years. Accordingly, the final integration of the results on heat shock described in the present report represents the completion of this work unit.

Approach

In order to maximize experimental control, accuracy of quantitation and analytical resolution, a well-defined *in vitro* culture system was used. Stock suspension cultures of L-929 mouse fibroblasts, clone WRL 10A were grown in Eagle's Minimum Essential Medium supplemented with ten percent horse serum. Temperature of incubation was 35.5° and the gas phase 5% CO_2 in air. The stock cultures were maintained in logarithmic growth by changing the culture medium on alternate days and adjusting the cell population to 4×10^5 cells/ml. Under these conditions doubling time

was 18-22 hours and plating efficiency, determined on tryprinized single-cell suspensions to avoid any errors due to clumping, 50 to 60 percent. The apparatus used to administer a 40.5°C heat-shock treatment of 12 hours duration to the experimental cultures while maintaining the control cultures at 35.5° is shown in Fig. 1. The spinner flasks containing the cell cultures were placed on a magnetic stirrer. A closed chamber was maintained around the flasks by sealing a plastic hood over the upper surface of the magnetic stirrer and the incoming gas bubbled through water in order to maintain a humid 5% CO_2 in air gas phase. The heat-treated cultures were maintained at 40.5° by water incubated from a water bath while a second water bath was used to maintain control cultures at 35.5°. Thermoprobes were used to monitor temperatures in each culture flask, water bath, and incubator for one hour preceding and during the 12-hour heat shock and for 12 hours following the temperature shock. At the termination of the 12-hour heat shock the experimental cultures were cooled rapidly or slowly according to the experimental design. Rapid cooling was accomplished by running 35.0° water through the water jackets of the heated cultures, while slow cooling involved returning the cultures to the 35.5° incubator where they eventually reached this ambient temperature. The parameters measured were cell number, mitotic activity, plating efficiency, ^3H thymidine incorporation and chromosone distribution.

Results and Discussion

It was found that the 12-hour heat shock resulted in a marked decrease of the population density of the treated cultures because of extensive cell death. There was also a decrease of the growth rates of these cultures which was most marked during the first six hours. The decreased growth rate in these cultures was a reflection of cell death but also of a reversible inhibition of cell division. Thus, within the first hour after the initiation of the thermal shock there was a complete absence of mitotic figures. This suggests that the inhibition affected cells just ready to enter mitosis, i.e. the "antephase" cells, since inhibition at the G_1, S or G_2 stages would have resulted in a gradual rather than sudden elimination of the mitotic cells. On-going DNA synthesis of cells in the S phase was also inhibited with the initiation of the heat-shock treatment but as a whole DNA systhesis was inhibited more gradually than mitosis and therefore it is unlikely that it was the main factor responsible for inhibiting cell division. In the hours following the onset of the treatment the entry of new cells into the S period of DNA synthesis was also prevented.

Following termination of the treatment and return of the cultures to 35.5° there was a 2-hour lag period before DNA synthesis was resumed. It is probable, that during this lag period, untoward thermal effects on enzymes involved in DNA synthesis were reversed and enzyme activity resumed.

The end of the heat-shock treatment was followed by partial synchronization of the cell population manifested by mitotic peaks at 30, 48 and 63 hours. In terms of DNA synthesis this synchronization was manifested by a peak which occurred prior to the peak of mitosis at 30 hours.

While these peaks in DNA synthesis and mitosis refer to the entire population, on an individual cell basis recovery from thermal shock was detected earlier through the appearance of the first ^{3}H thymidine labelled nuclei, two hours after the cultures were returned to 33.5°, and through the progressively increasing nuclear size of the treated cells between the 16th and the 20th hour. While during the thermal shock the inhibition of cell division rendered the generation time of the population indeterminant, pulse labelling experiments with ^{3}H thymidine at 24 hours, revealed that the cultures had returned to an 18-hour generation time which was approximately the same as in the control cultures. As there was an 18-hour delay in cell division following the termination of the heat shock it is suggested that thermal shock caused reversion of the cells to the early G_1 stage of the cell cycle.

It is noteworthy that during thermal shock ^{3}H thymidine incorporation continued uninterupted in approximately 7% of the cells suggesting the possibility that a portion of the population is thermotolerant.

Finally, the frequency distribution of the chromosomes of the treated cells determined 4 generations after the heat shock showed no significant difference from the controls.

Conclusions and Recommendations

From the point of view of scientific interest as well as of potential military applications, two of the findings outlined in the previous section merit special attention.

The first refers to the possibility of thermoresistance in part of the cell population subjected to heat shock. This was reflected by the fact that approximately seven percent of the treated cells continued to incorporate ^{3}H thymidine from the second to the twelfth hour of the heat treatment. While this is a relatively small number of cells if compared with the 45% of the control cells which were in DNA synthesis, it should be considered that the actual number of thermotolerant cells might be significantly higher since such cells in G_1, G_2 and M phases would not be detected by the method of ^{3}H thymidine incorporation.

The second finding refers to the reversion of heat shocked cells to the G_1 period as indicated by the timing of the partial synchronization of the population after termination of the heat treatment. Current concepts regarding the mechanism of this cellular set-back, involve partial inhibition and inactivation of RNA messengers necessary for the synthesis of the protein initiating cell division.

The overall results obtained during the course of the work in this subtask may then be summarized as follows:

a) In regard to ionizing radiation it was found that cells irradiated while growing are capable of a greater number of post-irradiation divisions than cells irradiated while stationary.

b) In regard to freezing it was found that cells frozen during the duplication of their genetic material survive considerably better and have fewer chromosomal abnormalities than cells frozen prior to the initiation of this process.

c) In regard to heat shock, besides acute cell death, inhibition of DNA synthesis and mitosis during exposure to high temperatures give rise to a subsequent synchronization of cell division with no permanent effects on generation time or the chromosomal complement of the cells. A small portion of the cell population continued to synthesize DNA during heat shock suggesting thermotolerance.

The precise molecular mechanisms responsible for these effects are not known and this makes difficult their utilization in the relevant areas of military medicine. On this basis, it is recommended that these studies be resumed as soon as the funding situation is improved.

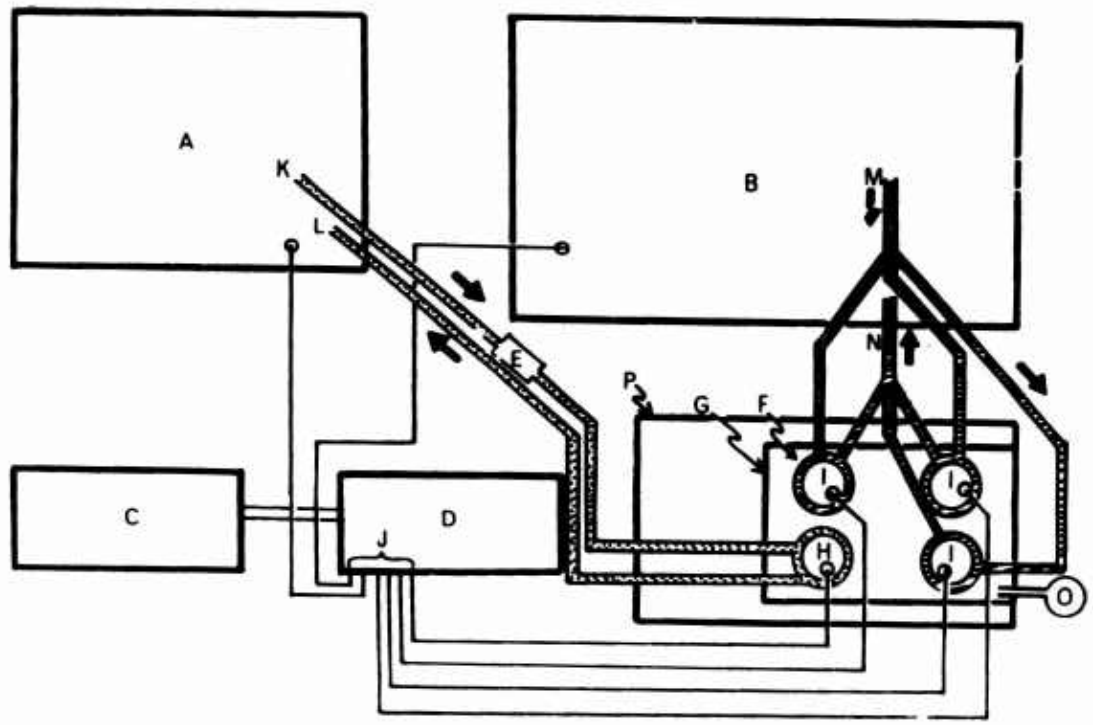

FIGURE 1

A. Water bath for maintaining 35.5°C. B. Water bath for maintaining 40.5°C. C. Recorder. D. Telethermometer. E. Water pump for transporting water to and from 35.5°C bath. F. Magnetic stirrer. G. Plastic hood. H. Control spinner flask. I. Experimental spinner flask. J. Leads for thermoprobes. K. Tubing for transporting 35.5°C water to the spinner flask. L. Tubing for returning water to the 35.5°C water bath. M. Tubing for transporting 40.5°C water to the spinner flasks. N. Tubing for returning water to the 40.5°C water bath. O. Five percent CO_2 in air tank with hose leading into the chamber. P. Plexiglass plate to increase the surface area of the magnetic stirrer providing the base to which a plastic hood was fastened. A constant atmosphere of five percent CO_2 in air was maintained under this hood.

3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 108, Alterations of cells surviving injury

Literature Cited.

1. McAtee, L.T.: Cytogenetic and kinetic effects of a sublethal heat shock on a heteroploid cell suspension culture. Thesis, University of Maryland, August 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6456	70 07 01	DD-DR&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 08 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	109
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Nuclear-Cytoplasmic Transplantation (24)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 08	CONT	DA	B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 69 08 EXPIRATION: 70 07
b. NUMBER: DADA 17-69-C-9182
c. TYPE: S.CT d. AMOUNT: 23,974
e. KIND OF AWARD NEW f. CUM. AMT. 23,974

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 70	1	18
FISCAL YEAR — CURRENT 71	0.1	5

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 Minnesota

ADDRESS: Minneapolis, Minn. 55455

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Estensen, R. D.
TELEPHONE:
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) Nuclear Transplantation; (U) Enucleated Cytoplasm; (U) Cytochalasins; (U)Sendai Virus

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

23. (U) To extend preliminary studies on nuclear transplantation of mammalian cells and to apply the technique to immunologic and other studies.

24. (U) Through a general methodology which in preliminary evaluations has been shown to be feasible; consists of separation of nucleus and cytoplasm by cytochalasins, followed by fusion of nucleus and cytoplasm with Sendai virus.

25. (U) 69 08 - 70 06 Principal effort during the period has been directed toward a characterization of low dose (1 microgram/ml) effects of cytochalasin B (CB) on N1S1-67 (Novikoff rat Hepatoma) cells and Xenopus laevis embryos. In the former system the drug prevents cytokinesis immediately without cell death or loss of viability. With Xenopus irreversible inhibition of cytokinesis takes place in a relatively well defined time period. Uptake of tritium labeld precursors of DNA, RNA, protein and membranes is also inaffected by the drug, thereby giving added support to the morphologic observation that cell growth is undisturbed. The foregoing suggests that CB specifically affects cytokinesis, however, other work indicates that other processes such as phagocytosis may also be affected. This leads to a consideration of other cell processes which involve membrane fusion. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 Jun 70

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 109, Nuclear-cytoplasmic transplantation

Investigators.
Principal: Richard D. Estensen, M.D.
Associate: COL Lyman P. Frick, MSC

Preliminary work at WRAIR confirmed observations by Carter (1967) that the drug cytochalasin B (CB) had two dramatic effects. First, at doses of 1 µg/ml in cultures of mouse fibroblasts (L-cells) the drug stopped cytoplasmic division (cytokinesis) while allowing nuclear division (karyokinesis) to proceed. Second, at doses of 10 µg/ml a significant number of cells were enucleated, leaving nuclei surrounded by plasma membrane and intact cytoplasm in a culture. The latter observation served as a basis for the suggestion that nuclear transfer or exchange might be accomplished through the use of Sendai virus cell fusion (Okada, 1962). Investigations since the beginning of the contract in August 1969 have been directed toward determining the feasibility of this methodology. Two areas have been studied: 1) the mechanism of cytokinesis through the use of low doses of CB; and 2) nuclear transfer through the use of CB in high doses.

1. Mechanism of cytokinesis -- Two experimental systems have been used to study low dose (1 µg/ml) effects of CB: 1) N1S1-67 (Novikoff rat Hepatoma) cells; and 2) Xenopus laevis embryos. Experiments on N1S1-67 cells in culture have shown that the drug prevents cytokinesis immediately without cell death or loss of viability. The inhibition can take place as late as metaphase in these cells. Work on Xenopus larvae suggests that irreversible inhibition of cytokinesis takes place in a relatively well defined time period. This suggests that such a time period may well exist in N1S1-67 or other mammalian cells. Experiments on synchronized cells will indicate if such a period of drug sensitivity also exists in mammalian cells. N1S1-67 cells exhibit an increase in numbers of nuclei and cell volume that is comparable to related parameters of cell growth in control cultures. This indicates that inhibition of general cell functions is relatively unaffected by CB. Further uptake of ^{3}H labeled precursors of DNA, RNA, protein and membranes is similarly unaffected by the drug, which gives added support to the morphologic observation that cell growth is undisturbed since these subdivisions of cell growth are not affected. Morphologic observations of growth in Xenopus embryos indicate a similar pattern. While the foregoing suggests that the process affected is specifically cytokinesis, further study has indicated that other processes that are morphologically similar--in that membrane fusion takes place--to cytokinesis, such as phagocytosis, may also be affected. These observations have

led to preliminary exploration of inhibition of phagocytosis and pinocytosis and consideration of other cell processes which involve membrane fusion.

Efforts are underway to label CB with ^{3}H and to isolate the pure form of radioactive drug. Such a compound will be useful in further description of the mode of action of the drug and its relationship to the process of cytokinesis.

A study of the ultrastructure of CB treated Xenopus embryos has been started. This has led to a new method of fixation of the embryos which makes possible studies which have not been possible before.

2. Nucleo-cytoplasmic transfer -- The concept of fusion of cytoplasm and free nuclei treated with CB needed several exploratory steps. First, a plasma membrane was necessary for fusion to take place. Ultrastructural studies have demonstrated the presence of a plasma membrane surrounding the extruded nucleus. Further, cells treated with CB could be fused with Sendai virus. It has been possible to enucleate cells and to fuse enucleated cytoplasm to chicken red cells. The resultant hybrids show incorporation of RNA precursors indicating that the cytoplasms were capable of supporting a transplanted nucleus. However, the final step remains to be accomplished, i.e., the transfer of enucleated nuclei from one cytoplasm to another. The technique can offer exciting possibilities of study of nucleo-cytoplasmic interactions as well as possible therapeutic applications, e.g., restoring loss of enzyme or other activity in patients by transplanting competent nuclei into their own cytoplasms.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 109, Nuclear-cytoplasmic transplantation

References

1. Carter, S. B.: Effects of cytochalasins on mammalian cells. Nature 213: 261-264, 1967.

2. Okada, Y.: Analysis of giant polynuclear cell formation caused by HVJ virus from Ehrlich's ascites tumor cells. I. Microscopic observation of giant polynuclear cell formation. Exper. Cell Rsch. 26: 98-107, 1962.

Literature Cited

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6495	70 07 01	DD-DR&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 07 01	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	113
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede 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
64 10	CONT	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	2	75
FISCAL YEAR 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. Academic Institution)

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3552

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Mason, Dr. J. W.

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede 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. Procede 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 07 - 70 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 still in progress. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 Jun 70.

*Available to contractors upon originator's approval

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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

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.

All hormonal and statistical analyses on this project are now complete.

Summary and Conclusions.

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

Literature Cited: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6500	70 06 30	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GROUP NUMBER	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	H. TERM.	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. MAJ CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	118
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Provide 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. DATES/EFFECTIVE: 69 06 EXPIRATION: 70 05

b. NUMBER: DA-49-193-2705

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

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
PRECEDING FISCAL YEAR 69	1	1
CURRENT FISCAL YEAR 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 (Provide SSAN if U.S. Academic participant)

NAME: Hartman, R. E.

TELEPHONE: 212-TR 6-5500

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Conrad, COL M. E. 1

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide EACH with Security Classification Code)

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

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Provide individual paragraphs identified by numbers. Provide end 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) 69 07 - 70 05 No progress was achieved during the period of this report because of the unavailability of a functioning electron microscope. Project was continued for one year without additional funds. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

Available in accordance with originator's approval

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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. A final report of the progress accomplished during the period of support has been submitted.

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 0A 6501	70 06 30	DD-DR&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 12 31	H.Termination	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 (Precede with 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. 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	2	43
	CURRENT 70	2	29

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 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 07 - 70 06 Efforts during the year have been directed toward completion of studies on the cytochemistry of plasmodial nuclei. Characterization of nucleoprotein in P. berghei, P. chabaudi, and P. vinckei infections in mice has been done. The nucleoprotein is organized in tortuous, bumpy fibrils of 420 Angstrom units diameter. There are about 100 microns of fiber in one merozoite nucleus. There are also 0.5 X 10 minus 13 g DNA in one parasite nucleus which has a total length of over 15,000 microns. DNA double helix is thus packed into chromatin fibers at a ratio of 150 microns to 1 micron of fiber. Only a triple supercoiling of DNA helix can explain the high packing ratio. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 Jun 70.

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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 C. Meszoely, MSC

The application of highly sensitive techniques for dry mass, DNA, RNA, and other cytochemically determinable substances is a specialty of the Biophysics Branch of the AFIP; some of these techniques, such as quantitative electron microscopy are world-exclusive of this group. Progress in this project is supported in the following paragraphs.

a. Dr. G. F. Bahr and U. Mikel prepared a manuscript "The Arrangement of DNA in the Nucleus of Rodent Malaria Parasites." A summary of this paper is that nucleoprotein in malarial parasites is organized in tortuous, bumpy fibrils of 420 Å diameter. There are about 100 μ of fibers in one merozoite nucleus. There are also 0.5×10^{-13} g DNA in one parasite nucleus, which have a total length of over 15,000 μ. DNA double helix is thus packed into chromatin fibers at a ratio of 150 μ : 1 μ fiber. Only a triple supercoiling of DNA helix can explain the high packing ratio.

b. A parallel investigation into the arrangement of mammalian chromatin as suggested by E. Bueding during the 1969 Panel Workshop on Malaria has been progressing rapidly in collaboration with Captain H. Golomb, MC, USA. Results from such studies are currently transferred to a computer file. This file serves as a general repository for quantitative information on chromatin throughout the animal kingdom inclusive of parasites such as malaria.

c. Ultrastructural investigations have been carried out by Captain Charles A. M. Meszoely, MC, USA. This ultrastructural study on material prepared with improved freeze etching techniques emphasizes the third dimensional aspects of the parasite, complementing and confirming earlier morphological studies on thin sections. Our study demonstrates a very intimate relationship between the conoid region and the pellicular complex suggesting that this region is highly specialized, differing markedly from the surrounding surface areas. An estimated nuclear pore count of between 30-40 is obtained for the parasite and a count of over 100 for the host erythrocyte nucleus. A surface view of the cytostome shows it to be a simple depression on the plasma membrane with an orifice of 800 Å. The surface appearance of the outer-most trophozoite membrane is smooth, while the inner surface of the inner-most membrane,

where it contacts the cytoplasma, is rough. The interphase membrane surfaces between outer and inner membranes also appears smooth. Other cell organelles are also presented in the third dimension and briefly described.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 119, Cytochemical analysis of growth of malarial parasites

Literature Cited.

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0A 6509	70 06 30	DD-DR&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 07 01	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	123
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with 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. 116,948

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 69	1	7
CURRENT FISCAL YEAR 70	1	8

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

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Rucknagel, D. L.
TELEPHONE: 313-764-5492
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) Hemoglobin E; (U) Thalassemia; (U) Genetics; (U) Population

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text 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 07 - 70 04 Term of the contract expired 70 04 30, and a final report is in preparation. Differences in gene frequencies which have been observed in the 10 provinces in Thailand which were studied suggest that genetic drift and founder effect play a large role in so-called micro-differentiation within provinces. Whereas the differences in gene frequencies in the various provinces do not correlate perfectly, the fact that so many gene frequencies vary so much from one region to another suggests that this variation is more likely to be due to gene flow or migration rather than differential selective values throughout the country. If, for example, the high frequency of thalassemia were the only characteristic of the Northern Thai, malaria selection pressure would be a reasonable explanation. If a rational relationship were apparent for several other genetic characteristics in this area, one might still invoke selection. For instance, malaria might select for genetic mutants of the Hb, G6PD and haptoglobin loci since all three of these proteins are involved with the integrity of the red blood cell. There is no rational basis for invoking entities such as the blood groups, for example, in a selection model. Migration seems a more likely common denominator. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval

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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.
Associate: COL Lyman P. Frick, MSC

The Thai population data have been analyzed using the following general approach. The phenotype frequency was determined for all of the genes after separating the Thais from hill tribesmen. From the total Thai sample we then removed first the children and then related adults. The frequency of genetic traits in the total or corrected samples did not differ significantly. These manipulations were necessary to deal with the randomness of the samples, because even truly random samples of small endogamous populations will contain relatives. Since we did not know precisely the mating structure of our populations we had no other more direct approach to the question of whether we had ascertained an inordinate number of relatives. We then calculated gene frequencies for each genetic system in each village and in each province and compared them statistically and for adherence to the Hardy-Weinberg equilibrium.

The anthropometrics and other quantitative variables such as uric acid and serum iron levels were analyzed by regressing onto age and then comparing between groups by covariance analysis. Group comparisons included comparisons between provinces in Thailand, between sexes, and for attributes such as serum iron and hemoglobin levels between the hemoglobin E, thalassemia, and normal phenotypes.

In view of the complexity of the study a table is appended to demonstrate some of the findings. First, there is a great deal of heterogeneity in gene frequency within provinces in Thailand (not shown in the table) which cannot be attributed to small sample size. Despite this variation, gene frequencies appear to vary systematically in the country as judged from the ten provinces which we have studied. In Northeastern Thailand, in addition to the high frequency of Hb E, the Thai electrophoretic variant on ceruloplasmin, transferrin D, blood group Ms, and secretor are more frequent than in other areas; Lewis (a+b-), and 6-phosphogluconate dehydrogenase type B are somewhat less frequent. Frequencies in Surin are strikingly out of line with those elsewhere in Northeastern Thailand, suggesting that Cambodians are somewhat different. For instance, the frequencies of ceruloplasmin Thai, secretor, Lewis(a-b-), and blood group B are greater; blood groups O and Ns are less frequent than elsewhere in this region.

In Northern Thailand the beta-thalassemia gene is most frequent. This does not appear to be derived from the hill tribesmen inasmuch as it was nearly absent from our small series of tribesmen. This region is characterized by elevated levels of glucose-6-phosphate dehydrogenase deficiency, Diego blood group, and Ms, and lower frequencies of 6-phosphogluconate type B, secretor, blood group B, and MS.

Southern Thailand is characterized by an elevated frequency of MS, and lower frequencies of hemoglobin E, G-6-PD deficiency, Ms, and Lewis(a-b-) than in most other areas.

The analysis of anthropometric measurements shows a large number of non-systematic differences between provinces.

Some systematic differences are apparent, however. For instance, individuals in Northern Thailand appear significantly smaller than those examined elsewhere in the country. Interpretation of these differences will be difficult, until statistical inter-racial distance measurements are completed; these are in progress.

The differences in gene frequencies outlined suggest that genetic drift and founder effect play a large role in so-called microdifferentiation within provinces. Whereas the differences in gene frequencies in the various provinces do not correlate perfectly, the fact that so many gene frequencies vary so much from one region to another suggests that this variation is more likely to be due to gene flow or migration rather than differential selective values throughout the country. If, for instance, the high frequency of thalassemia were the only characteristic of the Northern Thai, malaria selection pressure would be a reasonable explanation. If a rational relationship were apparent for several other genetic characteristics in this area, one might still invoke selection. For instance, malaria might select for genetic mutants of the hemoglobin, glucose-6-phosphate dehydrogenase, and haptoglobin loci since all three of these proteins are involved with the integrity of the red blood cell. We have no rational basis for invoking entities such as the blood groups, for instance, also in a selection model. Migration seems a more likely common denominator.

The gene frequency data have been collated, and the terminal report is being prepared on the descriptive aspects of the study. The fertility data have been analyzed in a preliminary fashion but set aside for the moment because they present special problems in analysis for which we have not as yet worked out the methodology.

	Gene Frequencies													
	HbE	β-Thal	6-PGD	Tf-D	Gc-1	Hp-1	Cp-Th	B	O	Ms	MS	Di[a]	Sec.	Le (a+b-)
Central														
Saraburi	.11	.03	.07	.01	.54	.22	.09	.19	.62	.66	.04	.0	.39	.54
Khanchanaburi	.06	.01	.07	.06	.64	.29	.07	.21	.60	.66	.07	.01	.44	.55
Northeast														
Pak Chong	.20	.004	.06	.06	.75	.28	.21	.21	.61	.69	.06	.01	.47	.49
Chum Pae	.23	.005	.05	.10	.76	.24	.18	.22	.64	.71	.04	.003	.49	.60
Udorn	.15	.010	.03	.07	.73	.24	.15	.22	.63	.74	.02	.02	.50	.52
Surin	.26	.010	.04	.07	.73	.23	.21	.35	.49	.72	.09	.01	.58	.52
North														
Chiengmai	.03	.04	.02	.02	.74	.29	.27	.17	.70	.71	.03	.04	.44	.66
Tak	.06	.01	.04	.04	.71	.20	.17	.24	.63	.60	.11	.02	.48	.63
Lom Sak	.22	.01	.04	.06	.71	.25	.18	.26	.63	.67	.06	.01	.53	.49
South														
Nakorn Srithamarat	.06	.01	.04	.04	.74	.20	.14	.21	.64	.58	.10	.002	.45	.59

Table 1

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

Literature Cited.

1. Reference

Shokeir, M. A., Rucknagel, D. L., Na-Nakorn, S., Wasi, P., and Shreffler, D. C.: Genetic polymorphism of human serum ceruloplasmin in Thailand. Amer. J. Hum. Genet., in press.

2. Publications

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	RESIST CONTROL SYMBOL
DA 0A 6510	70 06 30	DD-DR&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 07 01	H.Termination	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	124
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede 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 thou. funds)
FISCAL YEAR PRECEDING 69	1	1
FISCAL YEAR 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:

21. GENERAL USE

Foreign Intelligence Not Considered

5

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 on a plane starting from an edge rather than from the center or centers of the graph.

25 (U) 69 07 - 69 09 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. Term of the grant expried 69 09 30 and a final report is pending. For technical report see Walter Reed Army Institute of Research Progress Report, 1 Jul 69 - 30 Jun 70.

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Project 3A061101 A91C, 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. Gotlieb
Associate: Dr. A. H. Lehman

Description

This work seeks to elucidate the theoretical (mathematical) considerations in the development of a linear description of planar graphs. The standard representation of organic molecules is mathematically equivalent to a graph with colored vertices. A thorough understanding of these theoretical problems is necessary to insure that a chemical information system developed along these lines is not mathematically unsound. There are three problems which are of importance.

The first is the development of an efficient notation for the description to the theoretical limits necessary for the unequivocal representation of the structure. The representation presently under development is the "parenthesis-bracket code".

The second problem is that of graph isomorphism, i.e., the determination of whether one graph is identical with another.

The third problem is that of inclusion, i.e., given two graphs, is one of them contained within the other?

Progress

The contract with the University of Toronto is concerned mainly with the graph isomorphism aspect of the problem. The objective is to remain within the parenthesis-bracket code and develop a method for manipulation of the code to permit a canonical representation. The application of mathematical properties of planar graphs to chemical structure continues to have significant importance for computer programs involving the manipulation of chemical structure.

Summary and Conclusions

The development of sound procedures for the handling of chemical structures is of fundamental importance. During the past year significant progress has been made in developing an algorithm which will be useful for the programming connected with chemical identity.

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

1. References

Corneil, D. G. and C. C. Gotlieb, An Efficient Algorithm for Graph Isomorphisms, Extension of Ph.D. Thesis (unpublished), 1969.

Little, C. H. C., An Algorithm for Finding a Canonical Surface Imbedding for a Given Planar Connected Graph, (unpublished), 1970.

2. Publications

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6530	70 06 30	DD-DR&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 07 01	M. Termination	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

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

11. TITLE (Precede with Security Classification Code) (U) Machining the Tropical Diseases Bulletin for Use on Search Services to WRAIR and USAMRDC Personnel (R9)

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. DATES/EFFECTIVE: 69 08 EXPIRATION: 69 12
b. NUMBER: DA-49-193-MD-3039
c. TYPE: J. FFP d. AMOUNT: $5,000
e. KIND OF AWARD: SUP f. CUM. AMT: $183,064

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
PRECEDING FISCAL YEAR 69	2	71
CURRENT FISCAL YEAR 70	0.5	11

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
Washington, D. C. 20012
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RESPONSIBLE INDIVIDUAL
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TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Biological Abstracts, Inc.
Philadelphia, Pa. 19104
ADDRESS:

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Parkins, P. V.
TELEPHONE: 215-L09-1100
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Pick, CPT Robert O
NAME: 3

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Medicine in Literature; (U) Abstracting and Indexing; (U) Information Processing; (U) Information Retrieval

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

23 (U) To provide, in a readily accessible form, all data contained in Tropical Diseases Bulletin, Volumes one through sixty-three.

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

25 (U) 68 07 - 69 12 A data base in searchable format was completed for volumes forty-five through sixty-three of TDB, the subject, title and author indices for these volumes were also generated. Full text from volumes one through forty-four of TDB remains to be processed. The subject, title and author indices from volumes one through sixty-three are now stored on data cells to allow computer assisted searching. This implementation has resulted in a much greater coverage of each search question. Term of contract expired 69 12 31, and a final report is being awaited. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available in unrestricted use upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

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: CPT Robert O. Pick, MSC

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 consisting of full text, subject, title, and author indices for Volumes 1 through 63 of the Tropical Disease Bulletin has been machined.

Summary and Conclusions

This work is completed. The speed of input has been such that the building of the full text system rather than an abbreviated text system is feasible as a result of the detailed instructions generated for the typists. The availability 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 6532	70 07 01	DD-DR&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 07 01	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	170
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Biochemical Methodology and Laboratory Automation (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

008300 Inorganic Chemistry 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 10	CONT	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 70	3	55
FISCAL YEAR CURRENT 71	10	185

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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RESPONSIBLE INDIVIDUAL

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TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

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Division of Biochemistry

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NAME: Angel, LTC C. R.

TELEPHONE: 202-576-2211

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Bass, B. G.

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. 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 Department.

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 07 - 70 06 This work unit has been integrated into the broader programs of analytical chemistry methodology development and application to military clinical problems. Procedures for determining quantitatively concentrations of metallic elements in biological fluids and tissue have been developed to a highly routinized state and are being applied in support of clinical activities in the opthamology and gastroenterology clinics of the Walter Reed General Hospital and biomedical research activities of the Division of Experimental Surgery, WRAIR. The activities within this work unit are consolidated into a work unit entitled Biochemical Methodology and Laboratory Automation. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 170, Trace metal concentrations in biological matrices

Investigators.
Principal: LTC C. R. Angel, MSC
Associate: B. G. Bass, B.S.; E. A. Levri, M.S.;
R. T. Lofberg, Ph.D.; LTC D. T. Mahin, MC;
MAJ A. R. Rosenthal, MC (Division of Surgery).

Description.

The objective of this work unit has been to define and quantify the concentration of metals in a variety of biological matrices. A series of attempts to correlate the measurements of metal concentration with clinically defined states have been made. This work unit is to be terminated and combined with other analytical chemistry efforts as a part of the chemical mission of the Division of Biochemistry.

Progress.

1. Methodology.

Methodology efforts during the reporting period have been confined to refinement of neutron activation analysis and atomic absorption spectrometry for metal ions such as manganese, copper, zinc and iron. Reproducability and precision have been examined for each of the standardized procedures. Each one of the procedures for metal ions has been offered as service to the Walter Reed General Hospital, First Army Area Laboratory, Armed Forces Institute of Pathology and the Albert Einstein Medical College. Of the two principal methods employed, concentration of effort has been on atomic absorption spectrometry. This technique in most instances provides adequate information for most biochemical and physiological studies. With the shut down of the reactor facility, no further activity on neutron activation analysis techniques is contemplated. If neutron activation analysis is deemed appropriate for specific mission oriented research, neutron irradiation can be accomplished at the Diamond Ordnance Radiation Facility by coordination with that activity.

2. Biological studies.

The studies performed have been varied and limited to examination of biological matrices for sodium, copper and iron levels in a variety of specific clinical situations. Each study has been evaluated and brought to an orderly close. Further efforts will be examined in terms of missions

assigned by the Director, WRAIR. A summary of the major efforts is presented below:

a. Vitreous evaluation in globes containing intraocular foreign bodies.

As previously reported in the WRAIR Annual Report, FY 69, the analysis of intraocular foreign bodies for certain metallic elements allows the military opthalmologist to base clinical position relative to treatment on the analysis of the foreign body. During the reporting period such analyses have been performed for a number of ophthalmology services in hospitals within the Army Medical Department. The Division of Biochemistry will continue to provide service support in this area.

b. Analysis of serum and urine in patients with serum hepatitis and Wilson's disease.

As previously reported, copper and zinc levels in serum and urine has been proven to have diagnostic value in these clinical situations. Normal values for the laboratory making the measurements have been established and the analysis of numerous cases compared with established norms. As atomic absorption spectrometry is available in the clinical laboratory of most hospitals, these tests can become a routine procedure.

c. Cystic fibrosis screen by activation analysis.

The analysis of the sodium content of fingernails by neutron activation analysis as a diagnostic test for cystic fibrosis has been proposed and a number of institutions have made a number of claims relative to its reproducibility and accuracy. Studies performed in this laboratory indicate that the condition of the nails is an important consideration. It is also postulated that this technqiue is really an expensive sweat test and could be carried out successfully by more conventional means.

Summary and Conclusions.

The activities of this work unit has been confined to the application of previously developed techniques by means of applicatory studies. The work unit is to be terminated and the effort consolidated with analytical chemistry efforts within the Division of Biochemistry. Many of the procedures developed can be handled on a routine in the hospital laboratory.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 170, Trace metal concentrations in biological matrices

Literature Cited.

1. Gagnon, J. A., Mailloux, L. U., Doolittle, J. E. and Teschan, P. E.: An isotopic method for instantaneous measurements of effective renal blood flow. Am. J. Physiol. 218: No. 1, January 1970.

2. Lofberg, R. T.: A sensitive visible spectrophotometric method for copper using Bis-substituted thiocarbamoyl trisulfides. Anal. Letters 2(B): 439-448, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0A 6539	70 07 01	DD-DR&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 07 01	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	c. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 70	3	65
FISCAL YEAR CURRENT 71	3	20

19. RESPONSIBLE DOD ORGANIZATION
NAME:* Walter Reed Army Institute of Research
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20. PERFORMING ORGANIZATION
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NAME:* McClure, H. E.
TELEPHONE: NA
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign Intelligence Not Considered

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) 69 07 - 70 06 The survey of migratory birds in SEA for infections and arthropod vectors of medical and veterinary importance has continued according to plan. The bulk of the field effort has been completed, and plans for terminating the project are being drafted. According to the latest detailed report from the project (for 1968-69) over 800,000 birds belonging to 1060 species have been banded. Some 260 species of ectoparasites have been identified from 739 species of birds, and further identifications are in progress. Tissues, blood specimens, etc., have been collected from more than 700 species of birds and are currently being processed. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 172, Migratory animal pathological survey

Investigators.
Principal: H. Elliot McClure, Ph.D.

Operations of the MAPS project during 1969-70 have continued according to plan. The project consists of a coordinating group in Bangkok, Thailand, and a varying number of collaborating investigators in Korea, Japan, Taiwan, Philippines, Thailand, India, Indonesia, and Malaysia. In general, these investigators capture birds of different species, identify them, collect blood and ectoparasite specimens which are shipped to the Bangkok group, band the birds and then release them, hopefully for recapture later. By this means information is obtained on the distribution and migration of birds and their role in the dissemination of infections of medical and veterinary importance.

The latest available detailed information on the project is for the year 1968-69. Including records for that year, a total of 820,000 birds of 1060 species have been banded during the five years of the project. There are records now on 1762 recoveries from 182 species. These data have given a picture of the great movement and migration routes of birds across the land mass of Asia. A total of 259 species of ectoparasites have been identified from 739 bird species. This does not include 40 species of feather mites. Other identifications are in progress. A number of known disease vectors have been reported in the collections. To date, over 21,000 blood smears from about 700 species of birds have been studied and another 20,000 slides are awaiting processing. Up to now, 47% of the species have had infections of haematozoa, trypanosomes or microfilaria, and 16% of all blood films have been positive.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 172, Migratory animal pathological survey

Literature Cited.

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6431	70 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISS'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	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	174
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code): (U) Applications of Electroanalytical Techniques to Biochemistry and Clinical Chemistry (21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 002300 Biochemistry

13. START DATE	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 06
b. NUMBER: DADA 17-67-C-7161
c. TYPE: S.CT d. AMOUNT: 30,559
e. KIND OF AWARD: Ext f. CUM. AMT. 83,785

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 69	1	28
	CURRENT 70	1	27

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
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TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION
NAME: University of Maryland
ADDRESS: College Park, Md. 20740
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Purdy, W. C.
TELEPHONE: 301-454-2619
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Knoblock, COL E. C.
NAME:

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Clincal Chemistry; (U) Analytical Chemistry; (U) Electrochemical; (U) Biochemistry

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

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

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) 69 07 - 70 06 Coulometric titrations for the determination of amylases in serum have produced favorable comparisons with standard procedures compared with another laboratory. Constant current coulometry has been applied to titration of sodium salts of barbituric acids successfully. The same technique can be used for determination of glutathione, phosphatases, and salicylates. Polarography of 14 amino acids in dimethylsulfoxide has been studied. Equilibria have been studied by several additional electroanalytical techniques. A cadmium amalgam-cadmium chloride electrode has been used in dimethylformamide to study vanidyl sulfate analysis. Final modification by differential derivative chronopotentiograph have made transition time studies possible. This is crucial in furthering kinetic studies. Preliminary work using atomic absorption for manganese determination demonstrates feasibility from biological fluids. Vanadium has likewise been determined but difficulties are experienced in recoveries of this element from urine. Term of contract expired 70 06 30 and a final report has been submitted. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

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: W. C. Purdy, Ph.D. (University of Maryland)
Associate: COL E. C. Knoblock, MSC

Description.

The technical objective of this work unit was to extend the principles of electroanalytical chemistry to biological and clinical chemistry.

Progress.

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

Coulometric titrations have been applied to the determination of amylase in serum. Coulometrically generated bromine was used to oxidize copper (I) to copper (II). The end points were detected biamperometrically with an applied potential of 200 mV. The analysis is based upon the Sax and Trimble modification of the standard Somogyi saccharogenic procedure. A large number of Versatol samples were analyzed, employing a total sample size of 100 µl. The coulometric procedure was used to analyze a number of sera which had been analyzed at another laboratory and a comparison of results was made.

The constant current coulometric technique was applied to the titration of the sodium salts of several barbituric acid derivatives in aqueous and partially nonaqueous solutions, and to the analysis of allylic barbituric acid derivatives in blood serum samples. In the former case the titrant was electrogenerated mercury (II), and in the latter case the titrant was electrogenerated bromine. In both cases the biamperometric end point detection system was employed.

Glutathione is determined by coulometric titration with generated silver ion after precipitation of the protein in a 100-µl blood sample with metaphosphoric acid. The results of the coulometric determination compare well with those obtained by an accepted colorimetric method.

Phosphatases are determined by bromination of the p-nitrophenol released by enzymatic cleavage of p-nitrophenyl phosphate. Various

parameters associated with this reaction have been investigated including the choice of the proper substrate. Several techniques have been employed among which the "Constant excess bromine titration" method appears to give the best results.

Salicylates are titrated with bromine which is generated from a solution containing potassium bromide and sulfuric acid. The method employs a sample size of 100 µl of serum and routinely covers the range of 3 to 55 mg %. Methods are now being developed for the separation of salicylic acid from gentisic and salicyluric acids.

The polarographic characteristics of 14 amino acids in dimethylsulfoxide have been studied. Several other electrochemical techniques have been used to help elucidate the equilibria that may be present.

A $Cd(Hg)$, $CdCl_2$ reference electrode was designed for use in dimethylformamide. This electrode, when used in a 3-electrode system, gave a potential reproducible to $\pm$ 5 mV. When employed at constant temperature, the drift was less than the experimental error involved in graphically interpreting the vanadium half-wave potentials.

The polarographic technique was applied to a solution of vanadyl sulfate in dimethylformamide using 0.1 F tetraethylammonium perchlorate at the supporting electrolyte. The three resulting polarographic waves were examined and a mechanism was proposed to explain the waves. The mechanism involved an inermediate vanadium (III) dimer that is the product of a vanadium (IV) - vanadium (II) reaction. This intermediate then breaks down to the monomeric (III) state. The mechanism was studied using optical, constant-potential coulometric, and fast-sweep polarographic techniques. The final polarographic wave is attributed to a solvent bridge connecting the electrode with the vanadium (II) produced at the electrode. This bridging structure allows the solvent to be reduced at a potential more positive than when vanadium is absent.

Final modifications on the differential derivative chronopotentiograph have now 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.

Summary.

This represents the final report under this work unit as the contract is terminated effective 30 June 1970.

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

Literature Cited.

1. Purdy, W. C.: The potential of electroanalytical chemistry in clinical investigations. Enzymol. Biol. Clin. 10: 321, 1969.

2. Troy, R. J. and Purdy, W. C.: The coulometric determination of cholesterol in serum. Clin. Chim. Acta 26: 155, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6432	70 07 01	DD-DR&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 07 01	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	175
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

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

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012700 Physical Chemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 06	CONT	DA	B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 69 09 EXPIRATION: 70 08
b. NUMBER: DADA 17-67-C-7160
c. TYPE: S. CT d. AMOUNT: 37,509
e. KIND OF AWARD: EXT f. CUM. AM: 84,138

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	1.5	31
	CURRENT 71	0.5	6

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
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20. PERFORMING ORGANIZATION

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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
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TELEPHONE 202-454-2634
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Angel, LTC C. R.
NAME: 1

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede 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. Procede 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 molecules which enhance protective capacity of the chemical and to establish sufficient reference data to evaluate structure-activity relationships.

25. (U) 69 07 - 70 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 chlorquanide, daraprim, folic acid, chloroquine, quinacrine, acridine, quinine and a series of related compounds. The crystal structure of chloroquine diphosphate has been solved and refinement is in progress. With some hydrogen atoms located, the present residual, R, is 8.5%. The structure contains columns of phosphate groups, parallel to the twofold screw axis, which are closely linked by hydrogen bonding. The quinoline ring system lies in the xz plane, perpendicular to the phosphate columns. Adjacent columns are bridged by hydrogen bonds to the heterocyclic nitrogen on one side and the 4-amino group on the other. Each chloroquine side chain wraps around a phosphate column with the two aliphatic nitrogens H-bonded to neighboring phosphates. A helix-like arrangement of side chains about the phosphate columns is generated by the twofold screw axis. A set of diffractometer data has been collected for bis-(4-aminophenyl) sulphone (DDS), and is currently being processed. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*(Available to contractors upon originator's approval)

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

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: J. M. Stewart, Ph.D. (University of Maryland)
Associate: LTC C. R. Angel, MSC

Description.

The objective of this work unit is to evaluate a series of chemical compounds of particular therapeutic value by means of X-ray crystallography. The knowledge derived from such studies greatly enhances the explanation of functional structural interrelationships.

Progress.

During the reporting period, emphasis on this work unit has been directed toward the determination of the crystal structure of a series of antimalarial compounds of therapeutic interest. Included among the more important of these are diaminodiphenylsulfone (DDS), chloroquine phosphate, methylene blue, and 1-(3',4'-dichlorobenzyloxy)-2,2 dimethyl-4,6-diamino-1,2-dihydrotriazine hydrochloride. The crystal structure of each of the above compounds has been solved and information on molecular geometrics, hydrogen bonding obtained. Intensity data have been collected for quinine sulfate, 2-methyl-4-aminoquinoline.

Metal complexes of 8-aminoquinoline, chloroquine and diaminodiphenyl sulfone are well along or have been initiated. Studies on the binding of phosphate ion to chloroquine and primaquine have been initiated and are well advanced. Solution of these problems involving metal chelates can be expected to provide valuable information on the molecular location of binding sites.

Important spin off studies are being processed in a revision of computer programming for crystallographic data. The developed programs have currently led to the proving of the crystal structure of diaminodiphenyl sulfone, an acentric crystal.

Summary.

Data continues to accrue on the crystal structure of important antimalarials as well as their coordination chemistry and the ability of these compounds to form metal chelates.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

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

Literature Cited.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6434	70 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8b. DISP'N INSTR'N	8c. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 06 30	H. Termination	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	177
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Tropical Disease Bulletin Information Retrieval System (21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology; 004200 Computers

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 08	CONT	DA	B. CONTRACT

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 68 07 EXPIRATION: 69 12

b. NUMBER: DADA 17-68-C-8012

c. TYPE: U. CPFF d. AMOUNT:

e. KIND OF AWARD: CON f. CUM. AMT. $52,073

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 69	1	21
	CURRENT 70	0.5	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: Computer Applications, Inc.

ADDRESS: Silver Spring, Maryland 20910

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

NAME: Mong, W.

TELEPHONE: 301-587-3531

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Pick, CPT Robert O. 3

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Information Retrieval; (U) Malaria; (U) Information Handling; (U) Abstracts; (U) Computer Programming

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

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 TEMAC language will be used because of the ability to handle variations in spelling of words and punctuation.

25 (U) 69 07 - 69 12 Programs for merger, edits, and character combination frequency counts were written to aid in the building of the Tropical Disease Bulletin data base. Production of the data base proceeded according to schedule. Term of the contract expired 69 12 31 and a final report is pending. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available [illegible] upon originator's approval

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

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: William Mong

Description

The research effort formerly under this work unit was transferred to work unit 190 due to the transfer of the principal investigator, Mr. Harry Voccola. Mr. Mong's effort has been to support the investigations conducted under work unit 190.

Progress

Programs for merger, edits, and character combination frequency counts were written to aid in the building of the Tropical Disease Bulletin data base. These programs have been completed and delivered. Production of the data base proceeded according to schedule. Production details are enumerated in work unit 190.

Summary and Conclusions

This work unit has been terminated and the research is now being carried on under work unit 190.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 177, Tropical Diseases Bulletin information retrieval system

Publications

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6438	70 06 30	DD-DR&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 07 01	H. TERM	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	179
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede 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)
PRECEDING FISCAL YEAR 69	1	24
CURRENT FISCAL YEAR 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 (Furnish SSAN if U.S. Academic Institution)
NAME:
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Barbaro, J.F.
NAME: 3

21. GENERAL USE
Foreign Intelligence Not Considered

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

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

23 (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.

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

25 (U) 69 07 - 70 03. The alternative approach to synthesis of ethyl p-nitrophenyl w-aminoalkylphosphonates, based on the Tjioe's procedure involved the formation and subsequent hydrazinolysis of ethyl p-nitrophenyl w-phthalidimidoalkanephosphonate. The formation of the requisite precursors was achieved, however, all attempts to selectively remove the phthaloyl blocking group via hydrazinolysis were unsuccessful since the competing esterolysis reaction complicated the selective imidolysis reaction of ethyl p-nitrophenyl w-phthalimidio alkanephosphonates. Term of the contract expired 70 03 31 and a final report is pending. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

Term of the contract expired 70 03 31 and a final report is pending. For tech report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 179, Novel synthesis of organophosphonates

Investigators.

Principal: Stanley J. Brois, Ph.D.
Associate: Elmer L. Becker, Ph.D., M.D.
John F. Barbaro, Ph.D.

Description.

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

Progress.

1. The Arbusov reaction of 3-phthalimidopropyl bromide with the diethyl p-nitrophenyl phosphite to form the p-nitrophenyl ethyl 3- phthalimidopropyl phosphonate as a route to the desired p-nitrophenyl ethyl w-aminoalkylphosphonates was abandoned since nuclear magnetic resonance studies revealed that the mixed ester failed to react with the nitrogen protected amino alkyl bromide.

2. Attention was directed toward synthesizing the desired compounds utilizing the Tjioe procedure. In this approach, the phthaloyl group serves as the amine covering function. The phthaloyl group is difficult to remove and loss of the p-nitrophenol becomes competitive with its removal in the hydrazinolysis step. We were able to achieve the synthesis and characterization of the requisite precursors, specifically, the p-nitrophenyl w-phthalimidoalkyl phosphonates. The analytical and spectral data were completely consistent with the proposed structures. Our efforts to effect selective removal of the phthaloyl covering function via hydrazinolysis under various conditions resulted in the concurrent loss of p-nitrophenol due to the incursion of the esterolysis process and in no case was the desired products isolated.

Summary and Conclusions.

1. The synthetic approach based on the Tjioe's procedure involving the formation and subsequent hydrazinolysis of ethyl p-nitrophenyl w-phthalidimidoalkanephosphonate was attempted.

2. The synthesis of the requisite precursors was achieved; however, all attempts to selectively remove the phthaloyl blocking group via hydrozinolysis was unsuccessful.

3. Infrared and nuclear magnetic resonance studies indicate that the competing esterolysis reaction severely complicates the selective imidolysis reaction. Accordingly, the hydrazinolysis of the phthalimide does not appear to be a suitable route to synthesis of ethyl p-nitrophenyl w-aminoalkyl phosphonates.

Project 3A061101A91C IN HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 179, Novel synthesis of organophosphonates.

Literature Cited.

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6439	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. SUM'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 12 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. DATES/EFFECTIVE: 69 08 EXPIRATION: 70 07
b. NUMBER: DADA 17-68-C-8119
c. TYPE: S.CT d. AMOUNT: 29,524
e. KIND OF AWARD: Ext f. CUM. AMT. 59,875

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 70	1	27
FISCAL YEAR — CURRENT 71	0.1	2

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 Institution)
NAME: Doisy, R. J.
TELEPHONE: 315-473-5128
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Mertz, W.
NAME: 1

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Diabetes Mellitus; (U) Glucose Tolerance; (U) Insulin Antibodies; (U) Absorption; (U) Chromium

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 Chromium-51 will be administered and plasma and urinary Chromium-51 concentrations will be determined at intervals for 3 days. The same measurements will be made following intravenous injection of Chromium-57. 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) 69 07 - 70 06 Serum levels of Chromium III following a glucose load have been measured using radioactive chromium. Diabetic patients failed to show an increase in serum chromium levels. Normal subjects showed an increased chromium beginning at 20 minutes with a maximum of 60 minutes and a decline at 90 minutes. Protein electrophoresis showed increased chromium in both albumin and globulin, however, 80% of the chromium was not precipitated by ammonium sulfate. This low molecular weight binding suggests that transferring may not be important in binding chromium. Turnover studies following a single intravenous dose of Chromium-51 showed diabetic subjects to eliminate only one-fourth as much chromium as normal subjects. Studies are continuing to determine the differential binding of chromium between the two groups. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors unless originator's comment.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

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: R. J. Doisey, Ph.D. (State University of New York)
Associate: W. Mertz

Description.

The work unit is designed to study chromium metabolism in normal and diabetic elderly human subjects and characterize the chromium complex in circulating blood after glucose ingestion.

Progress.

Glucose loading of normal and diabetic subjects using Chromium-51 as a tracer has shown that normal subjects have elevated serum chromium levels beginning 20 minutes after ingestion, peaking at 60 minutes and falling toward normal after 90 minutes. Diabetic subjects failed to show any increase in serum chromium levels. Electrophoresis of serum proteins showed chromium in both the albumin and globulin fractions. Precipitation of proteins with ammonium sulfate failed to locate 80% of the chromium. Diabetics eliminated only one-fourth as much chromium as normal subjects.

Summary.

This contract has been terminated effective 31 July 1970 and a final report is being prepared.

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

Literature Cited.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6440	70 07 01	DD-R&E (AR) 636

3. DATE PREV SUMRY	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
69 07 01	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	181
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Development of A Meningococcal Immunizing Agent (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 09	CONT	DA	C. In-House

17. CONTRACT/GRANT N/A

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)
PRECEDING FISCAL YEAR 70	3	65
CURRENT FISCAL YEAR 71	3	60

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS:

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL, W.H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Div of CD and I

ADDRESS: Washington, DC 20012

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

NAME: Artenstein, M.S. MD

TELEPHONE: 202-576-3758

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: DA

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) N. meningitidis; (U) Meningitis; (U) Polysaccharides; (U) Vaccines; (U) Human Volunteers

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

23 (U) - To isolate, purify and characterize antigens from meningococci. To determine the protective capacity of immunogenic fractions.

24 (U) - To survey military and selected civilian populations to determine the prevalence of those serogroups of N. meningitidis causing disease. Polysaccharides will be purified and characterized by chemical and physicochemical methods. The response of animals and human volunteers will be measured by hemagglutination and bactericidal antibody tests.

25 (U) - 69 07 - 70 06 Group C polysaccharide vaccines have been administered to over 30,000 recruit volunteers without evidence of toxicity. Protection against group C disease is 90% or greater, but protection is group specific. Group A polysaccharides have been given to over 250 volunteers with no toxic effects and with antibody response in over 95%. Shelf life of lyophilized vaccine is at least 14 months. Vaccine can be stored frozen for nine months with no loss of potency. Antibodies induced by vaccine persist for 18 months or more. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*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 181, Development of meningococcal immunizing agent

Investigators.
Principal: Malcolm S. Artenstein, M.D.
Associate: MAJ Ronald Gold, MC; MAJ Edmund E. Tramont, MC; MAJ Frederic A. Wyle, MC; MAJ James G. Zimmerly, MC; CPT Richard L. Cohen, MSC: CPT Dennis L. Kasper, MC; Sanford L. Berman, Ph.D.; William C. Branche, Jr., Ph.D.; Brenda L. Brandt; Joseph P. Lowenthal, Sc.D.; Patricia L. Altieri; Hazel D. Fleet; Albert Groffinger; Charles Harkins; SP5 Howard W. Daubman; SP5 Myron V. Piziak; PFC Mitchell I. Diamond.

Description.

Field studies of efficacy of group C polysaccharide vaccine were extended and results confirmed those of the previous year in showing a 90 percent reduction in group C disease in 28,000 vaccinated recruits. Group C vaccines have a shelf life of 14 months or more. Group A polysaccharides have been administered to 250 volunteers with no apparent toxic effects and significant antibody rise in over 95 percent of individuals given 50 micrograms or more. Antibodies induced by A and C vaccines persist for at least 18 months.

Progress.

1. Prevention of disease with group C polysaccharide vaccine.

A large scale field trial during 1969 showed the efficacy of the group C vaccine in preventing disease in more than 13,000 recruit volunteers. Studies were continued during the fall of 1969 and winter of 1970 to determine the incidence of meningococcal disease among vaccinated and non-vaccinated recruits in three basic training centers.

Description of the study:

Approximately 20 percent of newly inducted recruits received the vaccine during their first week at the training center. This was accomplished by obtaining voluntary consent from recruits as they arrived at the dispensary for their mandatory vaccinations (influenza, tetanus, poliovirus). Pertinent data on numbers of vaccinees at each post are given in Table 1.

Table 1. Distribution of vaccine by Post.

Army Post	Vaccine Lot No.	Inclusive Dates of Study	No. of Men Vaccinated
Ft. Ord	C-8	11 June 69-22 Apr 70	7,924
Ft. Lewis	C-6, C-7	21 Oct 69-5 May 70	3,783
Ft. L. Wood	C-8	11 Feb 70-15 May 70	2,244
			13,951

Results:

No adverse reactions were caused by the vaccine.

Data on illness are incomplete at the time of this summary since some men have not completed their basic combat training. Preliminary data are given in Table 2.

Table 2. Number of meningococcal illnesses among study group.

	No. diagnosed illnesses		Dx not confirmed	Other
	Group C	Group B		
Ft. Ord				
Vaccine	1	0	0	
Control	6	0	1	3 AIT
Ft. Lewis				
Vaccine	0	0		
Control	19	0		3 in non-vaccinated companies
Ft. L. Wood				
Vaccine	0	0	0	
Control	5	0	1	

It is of some interest that no group B meningococcal infections were documented in either group this year. The previous study had shown more group B disease among these men who had received group C vaccine than among the controls, suggesting that administration of the group C vaccine increased the risk of acquiring group B disease. Our entire experience with group C vaccine is presented below in Table 3.

Table 3. Effectiveness of group C vaccine.

Study year	Number of group C meningococcal illnesses: vaccine	control
1968	0	4
1969	1	38
1969-1970	1	30
Totals	2	72
Population (approx.)	28,000	112,000

Conclusions:

The group C polysaccharide vaccine has been 90 percent effective in preventing group C meningococcal disease in Army recruits. This material has been free of toxicity in approximately 30,000 volunteers.

Meningococcal carrier surveys following group C vaccination.

Meningococcal carrier surveys were conducted at Ft. Lewis and Ft. Ord by the Sixth Army Medical Laboratory and at Ft. Leonard Wood by the Fifth Army Lab in order to observe the effect of group C vaccination on transmission. Recruits were surveyed during both Basic Combat Training (BCT) and Advanced Individual Training (AIT). The methods used were described in previous annual reports.

Between October 1969 and April 1970, there was relatively little transmission of group C meningococci at Ft. Ord so that no statistically significant difference in group C carrier rates was noted in eight companies studied during their 6th week of basic training (Table 4). Group C transmission was low at Ft. Leonard Wood also. However, in one of the three BCT companies at Ft. Lewis, sufficient group C transmission occurred and the vaccinated recruits were found to have significantly lower group C carrier rates.

Five companies were surveyed at Ft. Ord during the 6th week of AIT between October and December 1969 (Table 5). In two of the five companies there was significant group C transmission. The vaccinees in these two companies had significantly lower carrier rates than the control recruits who had had their BCT at Ft. Ord, suggesting that the local pharyngeal protective effect of the vaccine persists for at least 14-16 weeks.

Table 4. Prevalence of meningococcal carriers at 6th week of BCT.

Post	Date of Survey	Vaccine group #	Vaccine group % +	Vaccine group C	Vaccine group B	Vaccine group Other	Control #	Control % +	Control C	Control B	Control Other
Wood	8 Apr 70	23	30	3	2	2	75	28	11	7	3
	13 May 70	20	25	2	2	1	80	26	8	10	3
	Total	43		5	4	3	155		19	17	6
	% (Mean)		28.0	11.6	9.3	7.0		27.1	12.3	10.9	3.9
Lewis	1 Dec 69	23	35	0	2	6	93	33	2	3	25
	12 Jan 70	16	56	0	1	8	78	67	10	5	37
	2 Feb 70	20	40	4	0	4	80	65	38	2	12
	Total	59		4	3	18	251		50	10	74
	% (Mean)		42.4	6.8	5.1	30.5		53.4	19.9	4.0	29.5
Ord	27 Oct 69	19	5	0	0	1	81	14	0	5	6
	24 Nov 69	23	17	0	3	1	84	13	2	1	8
	1 Dec 69	19	22	0	3	1	88	18	2	7	7
	2 Jan 70	20	35	0	4	3	77	30	3	8	12
	24 Feb 70	32	22	2	3	2	121	35	3	7	18
	24 Feb 70	24	17	0	2	2	121	24	0	7	18
	6 Apr 70	21	28	0	2	4	71	24	0	5	14
	6 Apr 70	17	47	1	1	6	83	58	5	17	24
	Total	145		3	18	20	726		15	57	107
	% (Mean)		28.3	2.1	12.4	13.8		24.7	2.1	7.9	14.7

Table 5. Prevalence of meningococcal carriers at 6th week of AIT.

Date	Vaccine group					Control-BCT at Fort Ord					Control-BCT not at Fort Ord				
	#	%+	C	B	Other	#	%+	C	B	Other	#	%+	C	B	Other
13 Oct 69	25	40	1	1	7	79	28	3	5	14	68	34	1	4	18
20 Oct 69	19	68	0	5	8	69	46	6	3	23	95	45	20	6	17
3 Nov 69	29	59	1	5	11	91	65	23	10	26	59	56	9	5	19
20 Nov 69	25	52	1	5	7	81	41	3	15	19	85	48	5	25	11
8 Dec 69	21	57	1	6	5	86	77	14	20	32	83	59	11	12	26
Totals	119		4	22	38	406		49	53	114	390		46	52	91
% (Mean)		53.8	3.4	18.5	31.9		53.2	12.1	13.1	28.1		48.5	11.8	13.3	23.3

2. Stability of polysaccharide vaccines.

Determination of storage conditions is one of the practical problems related to vaccines and other biological preparations. A number of experiments have been undertaken to provide such information regarding meningococcal polysaccharide vaccines. Stability of the lyophilized product and various conditions of storage after hydration was measured by assessing immunogenicity in man.

Antibody assays were carried out by hemagglutination technique.

Lyophilized group C vaccine has been stored at refrigerator temperature (4-5°C) with no apparent loss of potency. Lot C-6 was tested for immunogenicity after as long as 13 months of storage; Lot C-7, after 14 months and Lot C-8 after 9 months. Results of antibody tests in recruit volunteers are shown in Table 6. The minor fluctuations in antibody titers reflect variations in sensitivity of the hemagglutination tests which were not performed on the same day. These data provide evidence that the shelf life of the lyophilized product exceeds one year.

Table 6. Immunogenicity of lyophilized vaccine after storage at 4°C.

Lot No.	Duration of storage	No. men	Mean antibody rise*
C-6	3 mo.	34	4.6
	11 mo.	22	5.1
	13 mo.	25	4.1
C-7	1 mo.	29	5.3
	3 mo.	26	5.1
	14 mo.	49	6.9
C-8	3 mo.	29	6.0
	9 mo.	35	5.3

*No. of 2-fold dilutions, hemagglutination test.
Sera obtained before and 2 weeks following immunization.

In order to determine storage conditions of the hydrated group C polysaccharide vaccines, recruit volunteers at Fort Lewis and Fort Ord were immunized with vaccine which was diluted and stored under different conditions. For controls, freshly hydrated vaccine was used in a comparable group of men. Serum and nasopharyngeal cultures were

obtained prior to vaccination and two weeks later. As before, group C carriers were deleted from the antibody data. Control and vaccinee sera were tested for hemagglutination antibody titers on the same day. Results are shown in Table 7. It can be seen that storage under refrigeration for 20 days or frozen for as long as 9 months had no effect upon the immunogenicity of the group C polysaccharide. However, because there is no preservative in the vaccine it would seem preferable to avoid refrigeration of vaccine once the cap has been punctured.

3. Studies of group A polysaccharide vaccine.

a. Lot A-6.

(1) This lot was prepared by the Department of Biologics Research, Walter Reed Army Institute of Research and amounted to approximately 7,000 doses. Initial studies consisted of immunization of five laboratory volunteers with 50 micrograms administered intradermally. Skin reactions measured 1-2 cm at 24 hours and were gone in 48 hours. Antibody response is shown in Table 8.

Table 8. Antibody response to Lot A-6.

Volunteer	Serum HA titer*	
	0	2 weeks
Bra	32	256
Rou	64	256
Alt	8	32
Gro	16	64
But	32	512

*Reciprocal of serum dilution

(2) Lot A-6 was next tested in military personnel who were observed for changes in red and white blood cell counts and urinalysis as well as for antibody development. Forty-two volunteers from the 18th Medical Brigade and the 6th Armored Cavalry Regiment, Fort George G. Meade, Maryland received the vaccine subcutaneously in a dose of 50 micrograms. Two subjects complained of gastrointestinal distress and diarrhea beginning 6 hours after the vaccination but were asymptomatic after 24 hours.

Urinalysis: Twenty-one subjects submitted urine specimens prior to and at 24 and 48 hours after vaccination. The only abnormalities noted were traces of albumin in 3 specimens (3 subjects: 1 before and 1 at

Table 7. Stability of group C meningococcal polysaccharide vaccine.

Vaccine	Conditions	Serum	Geometric mean HA titer[a]		No. men with no response[b]	
			Control[c]	Experimental	Control	Experimental
C-6	Refrigerated 20 days	Pre-	1.6	1.7		
		Post-[d]	5.7	5.9	2/25	1/23
C-8	Frozen 3 months	Pre-	1.2	1.4		
		Post-	6.5	6.4	0/35	2/35
C-6	Frozen 9 months	Pre-	2.0	2.5		
		Post-	7.1	7.0	1/22	0/21

[a] Log 2

[b] Less than four-fold increase

[c] Control = freshly hydrated

[d] Two weeks post vaccination

each other time interval).

CBC and hematocrit determinations were recorded from 22 subjects before and after immunization. Hematocrit values fluctuated somewhat but all were normal before vaccine and were not significantly changed afterwards. Fluctuations in total WBC were random and abnormal cells were not seen. Platelet estimates were based upon smears in 6 individuals and were normal. Serological studies were performed on 15 individuals from whom a 2 week serum specimen could be obtained. Antibodies were measured by hemagglutination test (Table 9).

Table 9. Antibody response to Lot A-6 vaccine (Ft. Meade).

Time	No. Men	Geometric mean titer*
Prior to vaccine	15	3.20
2 weeks after vaccine	15	5.27
		2.07 mean tube increase

*No. of 2-fold dilutions starting at 1:2

Eighty per cent of the men (12/15) showed a 2 tube (4-fold) or greater rise in titer.

(3) Meningococcal vaccine, Lot A-6, was tested in 44 volunteers from Company B, 5th Battalion, 3rd Training Brigade, U. S. Army Training Center, Infantry, Fort Dix, New Jersey on 27 August 1969.

Vaccine was administered subcutaneously in a dose of 50 micrograms. All subjects were examined on three occasions within the 48 hour period immediately following vaccination. There were no local or systemic reactions to the vaccine in the 44 subjects.

Temperatures were recorded using an oral thermometer, prior to and at 18, 25 and 35 hours following vaccination. There were no significant changes in each subject's temperature.

Carrier status - 10 of the subjects were carriers of a strain of Neisseria meningitis prior to vaccination. Two weeks post vaccination there were 9 carriers, including 7 of the original 10 carriers. Serogroups present included B, C and 29E. Vaccination with type A vaccine did not alter the carrier status or type of organism found.

Serological response to vaccine. Hemagglutination tests using group A polysaccharide-sensitized human erythrocytes as antigens were performed with the following results (Table 10).

Table 10. Antibody response to Lot A-6 vaccine (Ft. Dix).

Time	No. Men	Geometric mean titer*
Prior to vaccine	37	3.56
2 weeks after vaccine	37	6.62
		3.06 mean tube increase

*No. of 2-fold dilutions starting at 1:2

Eighty-four per cent of the men (31/37) showed a 2 tube (4-fold) or greater rise in titer.

These data, plus those from 42 volunteers at Fort Meade provide evidence of the lack of acute toxicity of Lot A-6 vaccine. Antibody tests (hemagglutination) showed that 83 percent of volunteers had a 4-fold or greater rise by two weeks after vaccination.

b. Lot A-7.

(1) This lot was prepared by Squibb Institute and consisted of 33,000 doses (50 micrograms/dose). Initial tests were conducted on 7 laboratory volunteers.

Vaccine was administered subcutaneously in a dose of 50 micrograms. All subjects were examined periodically for 48 hours. There were no local or systemic reactions to the vaccine in the 7 subjects.

CBC, Hgb, Hct, MCV, MCH and MCHC were recorded prior to and at 24 and 48 hours following vaccination. No consistent or significant changes were noted in any of the parameters tested. None of the volunteers were carriers of N. meningitidis at the time of vaccination.

Antibody tests on these individuals are shown in Table 11.

(2) Titration of Lot A-7 - One hundred and eleven recruit volunteers were vaccinated with varying amounts of group A vaccine at Fort Knox on 10 November 1969. Vaccine was administered subcutaneously in doses of 10, 50 or 100 micrograms. There were no adverse reactions to these injections.

Serologic responses as measured by hemagglutination are presented in Table 12. From these data it is apparent that the response to 50 and 100 micrograms is identical and that the antibody response to 10 micrograms is much lower. Expressed in another way (Table 13) only 1 of 68 men failed to show a 4-fold or greater titer rise following the 50 or 100 microgram dose whereas 5/33 (15%) failed to respond to the 10

Table 11. Antibody response to Lot A-7.

Subject	Reciprocal of HA titer at indicated week following vaccination		
	0	2	4
Dan	4	32	64
Sam	4	16	64
Piz	16	128	32
Mar	32	128	128
OBr	8	1024	64
Coa	4	32	32
Zim	8	128	256

Table 12. Antibody response to group A vaccine.

Dose group	Geometric mean HA titer (log 2) at indicated week			
	0	2	4	8
10 mcg	0.45	2.9	3.5	3.6
50 mcg	0.32	5.6	5.9	5.6
100 mcg	0.29	5.3	5.3	5.7

Table 13. Number of men who failed to respond to group A vaccine.

Dose group	No. failures*/No. tested at indicated week following vaccination			
	2	4	8	Total
10 mcg	14/33	9/31	5/31	5/33
50 mcg	2/36	0/35	0/35	0/37
100 mcg	3/30	1/30	1/27	1/31

*Less than 4-fold antibody titer rise

microgram injection. From this table it is also apparent that a significant proportion of men who received the lowest dose failed to show antibody rise within two weeks but developed increasing titers over the next 6 weeks. Carrier studies on this company are shown in Table 14. No group A strains were identified.

Table 14. Carrier studies on company vaccinated with Lot A-7.

Week of training	No. pos./No. sampled	% pos.	Serogroup						
			A	B	C	Bo	29E	135	Non-groupable
1	23/118	19.5	0	1	1	1	9	1	10
3	34/120	28.4	0	3	3	3	8	1	16
5	51/117	43.5	0	2	8	16	11	0	14
7	50/124	40.3	0	1	16	12	9	0	12

Conclusions:

To date our experience with group A polysaccharides has been as follows:

	Lab volunteers	Military
Lot A-5	9	53
Lot A-6	5	66
Lot A-7	7	111
	21	230

There have been no adverse reactions. Tests for toxicity of Lot A-6 showed no urine abnormalities in 21 subjects and no effects on CBC tests in 22 men who received Lot A-6 and 7 volunteers given Lot A-7; platelet estimations were normal in 6 men given Lot A-6. No temperature elevations occurred in 44 volunteers who received Lot A-6. Immunogenicity has been dose dependent in that 10 micrograms produced lower HA titers and 15 percent failure rate compared to 50 micrograms. Five to 15 percent of men given 50 mcg did not develop significant HA antibody rises within 2 weeks but almost all showed rises by 4 weeks.

These results indicate that these vaccines are suitable for large scale field tests of protective capacity should an outbreak of group A disease occur.

4. Duration of vaccine induced antibody.

Polysaccharide antigens appear to provide prolonged stimulation of antibody levels in man. Studies by Heidleberger showed that precipitin antibody titers following pneumococcal vaccination persisted at 50 percent of peak levels for over 3 years. Most of our current data on group A and C meningococcal polysaccharide induced antibodies has been obtained in Army recruits and these studies have been limited to 6-8 week time intervals. This report presents antibody titers of laboratory volunteers up to 18 months following vaccination.

In 8 volunteers who received group C vaccines HA titers increased significantly within 1 week following immunization reaching peak values (1:128-1:2048) between 2 and 8 weeks (Table 15). Occasionally titers fell several fold between 2 and 8 weeks. At 14-18 months after vaccination, 7 of the 8 showed decreases of 2-5 tubes from the peak titer. The remaining volunteer (SB) had a titer at 18 months only 1 tube less than his peak titer at 8 weeks. Despite these changes, geometric mean HA titer at 14-18 months was 1:64 compared to <1:2 prior to immunization. Antibody (IGG) determined by immunofluorescence showed rising titers which paralleled HA titers but did not rise over 1:256. Three of 5 volunteers with 14 or 18 month data showed persistence of peak FAB titers. The other 2 persons showed only 2 tube decreases from the peak. Only 1 individual (PE) was known to be a nasopharyngeal carrier at 18 months (29E serogroup). Of the three volunteers who showed FAB rise to Lot A-5 vaccine, titers remained very high throughout the 18 months of the study.

Thus, by 2 different assays, vaccine induced antibodies can be detected in high concentrations for at least 18 months. This interpretation is tempered by the fact that these laboratory volunteers were working with viable meningococci and their products or were exposed to recruit carriers on numerous occasions during the period of observation. Confirmation of these results by studies in other populations will be desirable.

5. Immunogenicity of Lot C-10.

Lot C-10 (March 1970) consists of 1.2 grams of group C polysaccharide (over 13,000 doses). This lot was prepared at WRAIR (Department of Biologics Research) using 12 liter fermentations in carboys whereas previous lots (including C-7) were produced in 1 liter volumes in flasks. Immunogenicity of C-10 was compared to that of C-7 by vaccinating recruit volunteers at Fort Lewis. Fifty micrograms of vaccine was administered by subcutaneous injection. No adverse reactions occurred. Nasopharyngeal cultures and serum specimens were obtained prior to and 2 weeks after injection. No group C carriers were detected. Results of hemagglutination tests are shown in Table 16.

Table 15. Duration of antibodies induced by group A or C polysaccharide vaccines.

Vaccine	Volunteer	Assay	0 wk	1 wk	2 wk	4 wk	8 wk	5 mo	8 mo	12 mo	14 mo	17-18 mo
A-5	BLB	FAB-A	4		64	64	256	128	128	128		64
	HS	FAB-A	16		64		64	32		64		64
	CH	FAB-A	8		64	64	32	64				64
	JS	FAB-A	64		64	32	32	32		64		32
C-7	WB	FAB-C	<2				256				256	
		HA-C	<2				128				16	
	JD	FAB-C	8		32		32				8	
		HA-C	<2		1024		128				256	
	WB	FAB-C	4				128				128	
		HA-C	2				512				64	
C-6	JL	FAB-C	4	4	64		256					-
		HA-C	<2	32	128		64					16
	SB	FAB-C	8	128	128		256					64
		HA-C	32	32	128		256					128
	PE	FAB-C	<2	-	256		128					256
		HA-C	<2	-	>2048		128					64
	DW	FAB-C	4	16	32		64					64
		HA-C	<2	256	256		256					64
C-4	FT	HA-C	<2		1024		1024					64

Table 16. Group C antibody response to two lots of polysaccharide vaccine.

Vaccine Lot No.	No. men tested	Serum date	Geom. mean HA titer	Mean antibody rise	No. men with no rise
C-7	49	0 wk	0.7		
		2 wk	7.6	6.9	3
C-10	48	0 wk	1.3		
		2 wk	7.6	6.3	0

Failure of occasional individuals to respond with antibody increase within two weeks has been noted before. The antibody titers indicate that both lots of vaccine are comparable in their immunogenicity.

Acknowledgements:

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LTC Ignacio Hernandez-Fragoso, MC, Fort Knox, Kentucky

LTC Joseph Smith, MC, Fort Dix, New Jersey

LTC Llewellan J. Legters, MC, Fort Ord, California

LTC John M. Sowell, MC, Fort Ord, California

LTC Raymond L. Coultrip, MC, Fort Ord, California

CPT Curtiss E. Weidmer, MC, Fort Lewis, Washington

LTC Gilberto E. Varela, MC, Fort Leonard Wood, Missouri

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 181, Development of meningococcal immunizing agent

Literature Cited.

1. Artenstein, M. S., Gold, R., Zimmerly, J. G., Wyle, F. A., Schneider, H. and Harkins, C. Prevention of meningococcal disease by group C polysaccharide vaccine. New England J. of Med. 282:417-240, 1970.

2. Artenstein, M. S., Gold, R., Zimmerly, J. G., Wyle, F. A., Branche, W. C., Jr. and Harkins, C. Cutaneous reactions and antibody response to meningococcal group C polysaccharides in man. J. Infectious Dis. 121:372-377, 1970.

3. Artenstein, M. S. and Buescher, E. L. Advances in meningococcal meningitis control. Army Research and Development 11:32-33, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION* DA 0B 6443	2. DATE OF SUMMARY* 70 07 01		REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUM'RY 69 11 30	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	182
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with security Classification Code)* (U) Correlation of Performance, Aggression, Stress, Combat and Group Position with Testicular and Adrenal Secretions. (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: 69 08 EXPIRATION: 70 07
b. NUMBER:* DADA 17-69-C-9014
c. TYPE: S.CT d. AMOUNT: P 9860
e. KIND OF AWARD: EXT f. CUM. AMT. P 79116

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	1	15
	CURRENT 71	0.1	1

19a. RESPONSIBLE DOD ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Rose, R. M., M.D. 1
NAME:

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Performance Under Stress; (U) Psychiatry; (U) Endocrine Response; (U) Coping Behavior; (U) Aggressive Behavior

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

23. (U) To assess the relevant social behaviors that operate to influence future performance and endocrine response to stressful tasks simulating military training and combat.

24. (U) As relatively complete control and observation of a group of unrestricted monkeys is feasible, animals with known behavioral histories will be subjected to various stresses and their behavioral and endocrine responses recorded to clarify social influence on success of task performance and aggressive behavior. The relationship between hormone secretions and performance success, breakdown, and aggressive outbursts will be determined.

25. (U) 69 07-70 06 Observation, recording and computer analysis of all behavioral interactions in a group of 34 adult male rhesus monkeys over a 3 month period is complete. Plasma testosterone was found to correlate positively with various aggresive behaviors, as well as with dominance rank order. Dominance rank is maintained primarily by threat gestures rather than by actual physical contact after the first few days of establishment of the group. Animals who most frequently threaten subordinates have the highest level of endogenously secreted testosterone. Work is in progress for the testing of high and low aggressive animals in standard psychological test paradigms. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 Jun 70.

*Available to contractors upon originator's approval.

DD, FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 65 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 182, Correlation of performance aggression, stress, combat and group position with testicular and adrenal secretion

Investigators.
Principal: Irwin S. Bernstein, Ph.D.
Associate: Robert M. Rose, M.D.

DESCRIPTION

Considerable interest has developed in the relationship of testosterone to aggressive behavior. There is good evidence documenting the necessity of testosterone to be present during crucial periods of brain development for the appearance in adulthood of male sexual and aggressive behavior. However, there has been no data available on the relationship between the normal aggressive behavior associated with living in social groups and the animal's level of endogenously secreted testosterone. This relationship is of interest from two standpoints. First, regulation of testosterone secretion is susceptible to environmental influences via the hypothalamic-pituitary-gonadal system. Aggressive behavior may also be effected by changes in brain concentration of testosterone secondary to increased or decreased secretion.

Previous work has suggested that an animal's performance during stress is related to his aggressive behavior as well as testosterone level. The relationship between these three variables of aggressiveness, testosterone and performance is being systematically studied in this joint project involving the Yerkes Regional Primate Center and Department of Psychiatry, WRAIR.

PROGRESS

An all male group of 34 adult rhesus monkeys was formed and placed in a large outdoor compound measuring 125' X 125'. Animals had access to an enclosed, temperature-regulated building for shelter. These conditions permitted careful observation and recording of all interactions between animals as well as providing in excess of 1/3 of an acre excluded so that the possible effect of differential sexual activity would not confound any relationship observed between testosterone and aggressive behavior.

Behavioral data were collected in formal sessions consisting of 1,000 seconds per animal repeated 10 times over a 3 month period. During these times behavior was recorded using a standard inventory describing 73 different activities, including all social interaction as

well as individual (self-directed) activities. Data were transferred to cards and computer analysis performed to list doer and recipient of all dyadic interactions as well as total frequencies of every response for every animal. In addition, analysis was made to reveal the status hierarchy in the group based on classical "peck order" description of dominance. Wins and losses in agonistic encounters for each dyad in the group were listed and a dominance matrix was ordered from these observations. And essentially linear hierarchy was produced.

The group was established in September 1969. Two months elapsed to permit the dominance hierarchy to be firmly established. After the behavioral interactions were observed for three months all animals had blood removed for plasma testosterone determinations.

Aggressive behavior, mostly non-contact in the form of threat and chase, correlated positively and significantly with testosterone ($r = .515$). Being submitted to also correlated significantly with plasma testosterone. Aggression and being submitted to were related ($r = .534$), but do not measure the same thing. The converse relationship of high submissiveness and low testosterone does not appear to be very strong. Animals who showed frequent submissive responses did not show significantly lower testosterone, although the findings are in this direction. These relationships suggest that any animal below the alpha male who shows a high frequency of aggressive behavior to his subordinates will tend to have higher testosterone levels regardless of how frequently he shows submissive responses to the animals above him in the dominance hierarchy. Testosterone therefore appears to be more related to the frequency of aggressive behavior than to the opposite and reciprocal behavior of submissive responses.

This is the first study done revealing significant relationship between social status and the level of endogenously secreted testosterone. Studies are in progress to determine whether the higher plasma testosterone that is frequently seen in more aggressive animals precedes or is the result of their dominant and aggressive status. In addition, the behavioral and endocrine responses of individual dominant and submissive animals to shock avoidance will be studied this coming year in collaboration with the Department of Experimental Psychology, WRAIR.

SUMMARY AND CONCLUSIONS

Aggressive and dominance behavior was closely studied in a group of 34 adult male rhesus monkeys living in a large outdoor compound. These behaviors correlated positively with plasma testosterone, which may be regulated by the position the animal achieves in the social group, and alter his performance under stress.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 182 Correlation of performance aggression, stress, combat and group position with testicular and adrenal secretion

Literature Cited

1. References: None.

2. Publications: None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6444	70 07 01	DD-DR&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 07 01	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	6 11 01 A	3A061101A91C	00	183
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Diseases of Military Animals in Southeast Asia (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 005900 Environmental Bio

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
68 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 70	3	60
FISCAL YEAR CURRENT 71	6	120

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Hildebrandt, MAJ P. K. DA

21. GENERAL USE

Foreign intelligence not considered.

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Tropical Canine Pancytopenia; (U) Ehrlichia canis; (U) Babesia gibsoni; (U) Acute glossitis; (U) Chloroquine; (U) Styrid-Caricide

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, study, diagnose & control known & potential infectious diseases of military dogs in Southeast Asia. The major effort is directed toward the cause, pathogenesis, treatment, and control of Tropical Canine Pancytopenia (TCP), which has jeopardized the operational efficacy of military dogs in SE Asia. Studies are also being conducted on the epidemiology, treatment and control of Babesia and other parasitic infections which are known to occur in military dogs in SE Asia.

24. (U) Conventional epidemiologic and microbiologic techniques are employed and new procedures will be developed as needed.

25. (U) 69 07 70 06 Tropical Canine Pancytopenia (TCP), a highly fatal hemorrhagic disease, has been responsible for the death of more than 180 U. S. military dogs in Southeast Asia since the outbreak began in 1968. This includes approximately 80 deaths since 1 July 1969. The disease has also occurred in military and privately owned dogs in the Caribbean. Ehrlichia canis has been consistently recovered from affected dogs in Southeast Asia, Puerto Rico, the Virgin Islands, Florida and Panama. Experimentally infected Beagles and mongrels develop clinical signs consistent with the natural disease; however, clinical signs of hemorrhage including epistaxis have been experimentally induced only in the German Shepherd. Laboratory studies have provided evidence that tetracycline may be effective during the early stages of disease. Toxicity of chloroquine in dogs has been determined and a solvent extraction and thin-layer chromatographic analysis of blood and urine for chloroquine have been developed. Studies in progress to define clinical B. gibsoni infection in laboratory Beagles indicate the disease is characterized by a progressive, macrocytic anemia. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 -- 30 Jun 70.

*Available to contractors upon originator's approval.

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Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 183, Diseases of Military Animals in Southeast Asia

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Description.

To define, study, diagnose and control known and potential infectious diseases of military dogs in Southeast Asia. The major effort is directed toward elucidation of the cause, pathogenesis, treatment and control of a highly fatal hemorrhagic disease, Tropical Canine Pancytopenia. This disease has been responsible for the death of numerous military dogs in Southeast Asia where it has jeopardized the operational efficacy of combat units dependent on military dogs. Studies are also being conducted on the epidemiology, treatment, and control of Babesia and other diseases which are known to occur in military dogs in Southeast Asia.

Progress.

I. Tropical Canine Pancytopenia.

1. Introduction

Tropical canine pancytopenia (TCP) is a newly recognized, highly fatal, infectious disease of dogs in diverse tropical and subtropical areas of the world. In Southeast Asia this disease, which has also been called "idiopathic hemorrhagic syndrome", "tracker dog disease" and "canine hemorrhagic fever", has been responsible for the death of numerous military dogs. The history, as well as clinical and pathologic studies of the natural disease, has been reported in detail (Walter Reed Army Institute of Research Annual Progress Report, 1 July 1968 - 30 June 1969). During the past year studies have continued on the etiology, pathogenicity and control of TCP with special emphasis on the experimental disease.

In Southeast Asia TCP was first recognized in 1963 in British military dogs in Singapore (Wilkins, J. H. et al., Vet Rec. 81: 57, 1967). Between 1963 and 1967 this disease, which is characterized by pancytopenia, hemorrhage and severe emaciation, was responsible for the death of numerous military and privately owned dogs in Singapore (Wilkins, J. H. et al., Vet Rec. 81: 57, 1967: Spence, J. B. et al., Vet Rec. 81: 328, 1967). Studies in Singapore (Wilkins, J. H. et al., Vet Rec. 81: 57, 1967) and Malaysia (Mac Vean, D. W., Malaysian Kennel Review, 66, 1968) provided evidence that TCP was infectious; however, an etiologic agent was not recovered.

TCP was first recognized in the Republic of Vietnam in 1967 in several Labrador Retrievers which had previously been trained as tracker dogs in Malaysia (Walker, J. S. et al., J.A.V.M.A. in press). In 1968 an epizootic of this disease occurred in Vietnam among German Shepherds which had originated in the United States. To date approximately 180 U. S. military dogs have died of the disease in Southeast Asia. The disease has been reported recently in military and privately owned dogs in Puerto Rico and the Virgin Islands (Huxsoll, D. L. et al., Vet Rec. 85: 587, 1969). TCP has also occurred in the Mideast (Seamer, J. and Snape, T., Vet Rec. 86: 375, 1970) and in Florida (Ridout, S., Eau Gallie, Florida, Personal Communication). TCP has been reported most frequently in the German Shepherd; however, dogs of other breeds have also been affected. The disease is reported more often in purebred dogs than in mixed breeds (Mac Vean, D. W., Malaysian Kennel Review, 66, 1968).

A sudden onset of epistaxis, the most dramatic sign of the disease, is often the first indication that a dog is affected with TCP. In some instances epistaxis is the only apparent clinical sign; however, it is often accompanied by one or more of the following signs; anemia; edema of limbs and scrotum; loss of weight; ecchymotic hemorrhages on the abdomen; petechial hemorrhages in the mucosa of the penis, buccal cavity and conjunctiva; anorexia; dyspnea; fever; corneal opacity; lethargy; lymphadenopathy; posterior weakness; melena and hyphema (Wilkins, J. H. et al. Vet Rec. 81: 57, 1967; Mac Vean, D. W., Malaysian Kennel Review, 66, 1968; Huxsoll, D. L. et al., Vet Rec. 85: 587, 1969; Walker, J. S. et al., J.A.V.M.A. in press). Death usually occurs within a few days following onset of epistaxis; however, if hemorrhage is profuse, death may occur within several hours. Some dogs survive the initial hemorrhagic episode; however, epistaxis often reoccurs, and the dog eventually succumbs to the disease. Some dogs become chronic bleeders and have intermittent episodes of epistaxis over a period of several months prior to death. Many of the chronic bleeders become severely emaciated. Bleeding is not limited to epistaxis. In some instances severe intestinal hemorrhage occurs as evidenced by large quantities of blood in the stool. Coagulation time and prothrombin time are normal; however, bleeding time is prolonged. Severe anemia, leukopenia and thrombocytopenia occur. The erythrocyte sedimentation

rate is often elevated. Electrophoretic examination of sera from affected dogs reveals an increase in the gamma globulin fraction and a depression of the albumin factor.

A large number of dogs with TCP do not develop epistaxis or other clinical signs of hemorrhage; however, the hematologic signs in these dogs are similar to those observed in dogs with epistaxis in that severe pancytopenia occurs (Huxsoll, D. L. et al., Vet Rec. 85: 587, 1969; Walker, J. S. et al., J.A.V.M.A. in press, 1970). These dogs, which have been referred to as pancytopenics as opposed to bleeders, usually become severely debilitated prior to death.

A review of clinical records of several hundred dogs affected with TCP has provided evidence that the clinical course of the disease is prolonged (Walker, J. S. et al., J.A.V.M.A. in press, 1970). Dogs with TCP usually have a history of a febrile episode occurring 2 or more months prior to the onset of epistaxis. The duration of the febrile episode may vary from a few days to several weeks. The fever may be accompanied by anorexia, severe weight loss, decreased stamina, and edema of the limbs and scrotum. In a few instances mild epistaxis may be evident during the febrile period. In all dogs red and white blood cell counts are lowered.

The febrile episode is usually followed by a period of apparent recovery which has been referred to as the subclinical phase (Walker, J. S. et al., J.A.V.M.A. in press, 1970). Dogs will usually regain a normal physical appearance; however, the anemia and leukopenia often persist. Therefore, prior to the onset of epistaxis, the only sign of disease in many dogs is an altered hemogram. In those dogs that die without clinical signs of hemorrhage, death may be attributable to extensive internal hemorrhage or secondary infections which are associated with severe anemia and leukopenia.

Pathological findings have been consistent in all forms of the disease. Macroscopic findings include a generalized lymphadenopathy, petechial and ecchymotic hemorrhages on serosal and mucosal surfaces of most organs and in subcutaneous tissue, brownish mottling of the lungs, and a moderate enlargement of the spleen in some dogs. The most striking histologic finding is a perivascular plasma cell infiltrate in numerous organs, especially the lungs, meninges, kidney and spleen. The bone marrow is hypoplastic.

The tick is the apparent vector of the disease. Outbreaks of TCP have often been associated with severe tick infestations, and the incidence of the disease has decreased or disappeared in some kennels after rigid tick control measures were enforced (Mac Vean, D. W., Malaysian Kennel Review, 66, 1968).

2. Transmission Studies in Laboratory Dogs.

The successful transmission of TCP to laboratory Beagles by intravenous inoculations of fresh whole blood from affected dogs in Southeast Asia has been reported (Walter Reed Army Institute of Research Annual Progress Report, 1 July 1968 - 30 June 1969). During the past year it became apparent that military and privately owned dogs in the Caribbean were affected with TCP. Blood specimens from affected dogs in Puerto Rico, the Virgin Islands, Florida, and Panama have been used in attempts to transmit the disease to laboratory dogs (Beagles, mongrels and German Shepherds). Transmissible disease compatible with TCP has been produced in laboratory dogs inoculated with specimens from affected dogs in the Caribbean as well as Southeast Asia.

Beagles and mongrels develop signs of disease within 10-15 days following intravenous or intraperitoneal inoculation of fresh whole blood from an affected dog. Early signs of disease are fever, serous nasal and ocular discharge, anorexia, depression, lowered thrombocyte count, elevation of the erythrocyte sedimentation rate and lowered red and white blood cell counts. The duration and severity of these signs are extremely variable; however, in many experimentally infected Beagles signs of disease persist for several weeks. Most dogs develop anemia, leukopenia and thrombocytopenia. Intracytoplasmic inclusions of Ehrlichia canis can be demonstrated in monocytes in capillary blood smears prepared during the early stages of the disease (Huxsoll, D. L. et al., Vet Rec. 85: 587. 1969). These inclusions are more readily found in mononuclear cells in impression smears prepared from lung tissue. In many instances inclusions of Ehrlichia canis can also be found in impression smears of spleen, liver and kidney tissue. The inclusions can be demonstrated with any Romanovsky type stain. They occur as single or multiple morula-like bodies in the cytoplasm and appear to be aggregates or colonies of elementary bodies. These inclusions have been demonstrated in capillary blood smears or tissue impressions of all experimentally infected dogs.

Most experimentally infected Beagles and mongrels recover from the disease but remain infected. In a few instances relapses characterized by a reappearance of earlier signs, have occurred. Epistaxis has never been observed in experimentally infected Beagles or mongrels.

German Shepherds inoculated with fresh whole blood from an affected dog develop disease indistinguishable from the natural disease. Onset of disease in the German Shepherd is similar to that in the Beagle although the disease is generally more severe. A few experimentally infected German Shepherds have not recovered from the early stages of the disease. Most, however, survive the early stages and may regain a normal physical appearance. Abnormal hematologic signs may partially disappear; however, relapses frequently occur and, as in the Beagle,

are characterized by a reappearance of earlier signs. Epistaxis and other forms of hemorrhage have occurred in experimentally infected German Shepherds as early as 10 days and up to 120 days post inoculation. During the early stages of the disease a blood tinged, seropurulent nasal discharge is evident in most experimentally infected German Shepherds. This sign, however, is distinct from the profuse epistaxis which usually occurs later in the course of the disease. Hemorrhage is associated with severe thrombocytopenia (Fig. 1). Other signs observed in experimentally infected German Shepherds during the course of the disease include ulceration of the nasal mucosa, corneal opacity, hyphema, petechial and ecchymotic hemorrhages in the mucosa of the penis and buccal cavity, posterior weakness, dyspnea and edema of the limbs.

Ehrlichia canis has been recovered from 12 dogs with signs of tropical canine pancytopenia in Southeast Asia, Puerto Rico, the Virgin Islands, Florida, and Panama (Table 1). Five of the 12 dogs had concurrent infections with Babesia canis. However, the production of experimental TCP in laboratory dogs is not dependent on dual infections. Experimental disease, indistinguishable from the natural disease, is produced with Ehrlichia alone. Two isolates have each been serially passed 11 times in laboratory dogs with no apparent alteration in the character of the disease.

Impression smears were prepared from frozen tissue collected from dogs with TCP in Southeast Asia. The impression smears were stained with Wright's stain and examined for inclusions of Ehrlichia canis. Inclusions were found in tissue from 11 of 14 dogs examined.

Dogs that recover from the disease remain infected. Following inoculation, dogs have remained infected during an observation period of more than one year (Table 2). However, the incubation period in subinoculated Beagles has progressively increased during the course of the observation period. Although infectivity titrations have not been done on blood from the donor dog, the evidence serves to suggest that infectivity has decreased. It is apparent that healthy dogs may be carriers and may serve as a source of infection.

The conclusion that Ehrlichia canis is the etiological agent of tropical canine pancytopenia is based on the consistent recovery of Ehrlichia from dogs affected with TCP from diverse geographical locations and the production of disease, indistinguishable from the natural disease, in laboratory dogs experimentally infected with Ehrlichia. The identification of Ehrlichia canis is based on its morphological characteristics in infected cells. The agent of TCP is indistinguishable from Ehrlichia canis, originally described by Donatien and Lestoquard in 1935 (Bull. Soc. Path. Exot. 28: 418, 1935). Tropical canine pancytopenia may be a previously unrecognized manifestation of infection

Fig. 1 Fluctuations in packed cell volume, leukocyte count, and thrombocyte count of 9 month old German Shepherd following intravenous inoculation with agent of TCP.

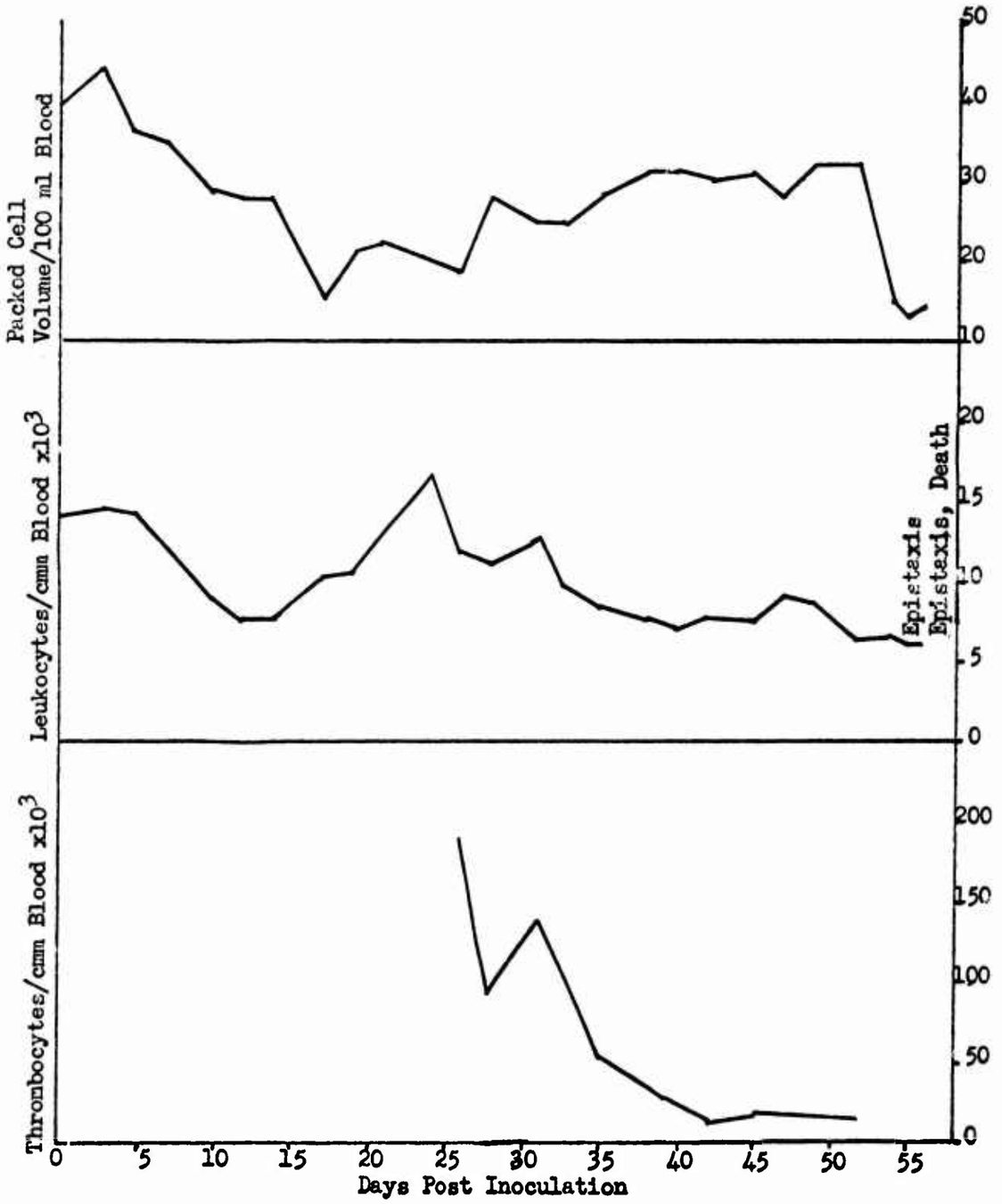

Table 1. Summary of Isolates of Ehrlichia canis from Dogs with TCP

Location	Number of Isolates	Breed of dog Providing Isolate	Epistaxis in Dog Providing Isolate	Concurrent Infection with Babesia canis
Southeast Asia	2	German Shepherd*	+	-
		German Shepherd*	-	+
Virgin Islands	3	German Shepherd	+	+
		Old English Sheepdog	+	-
		Mongrel	-	-
Puerto Rico	4	German Shepherd*	-	-
		German Shepherd*	-	-
		German Shepherd	+	-
		Labrador Retriever	-	-
Florida	2	German Shepherd	+	+
		German Shepherd	+	+
Panama	1	German Shepherd	+	+

* Military dog

Table 2. Persistence of E. canis Infection in Experimentally Infected Beagles

Day Post Infection of Donor Dog	Inoculum[1]	Day Clinical Signs Appeared in Subinoculated Beagles			
		Fever	Decrease in RBC[2]	Decrease in WBC[3]	Elevated ESR[4]
19	20 ml whole blood	9	8	11	11
90	25 ml whole blood	11	7	10	12
221	20 ml whole blood	17	17	17	19
381	20 ml whole blood	19	20	20	23

(1) Blood was collected in sodium citrate and inoculated immediately into recipient by intravenous route.

(2) RBC: Red Blood Cells

(3) WBC: White Blood Cells

(4) ESR: Erythrocyte Sedimentation Rate

with Ehrlichia canis. The precise relationship of the agent of TCP with previously described strains of Ehrlichia canis has not been resolved.

3. Pathological Findings in Beagles Experimentally Infected with the Agent of TCP.

Sixteen to eighteen-week-old Beagles were inoculated intravenously with whole blood from dogs that were acutely ill with TCP. These were followed clinically and sacrificed at intervals from 10-180 days post inoculation.

Gross lesions included generalized lymphadenopathy. The lymph nodes were enlarged and the medullas reddened. The intestines had prominent Peyer's patches. Small focal red areas were disseminated over the liver. The lungs were often mildly mottled and did not collapse completely.

Histologically there was an early non-suppurative pneumonitis, non-follicular reticuloendothelial hyperplasia of lymph nodes and hyperplasia of the bone marrow.

Endothelial cells of pulmonary vessels were often swollen and contained cytoplasmic inclusions (morulae) of Ehrlichia canis. Early in the disease reticuloendothelial cells were very numerous in the lumena of pulmonary vessels and many contained inclusion bodies. Impression smears prepared from lungs yielded numerous mononuclear cells with cytoplasmic inclusions.

A non-suppurative meningitis occurred in all dogs consisting primarily of immature R.E. cells, lymphocytes and plasma cells.

As the disease progressed the non-suppurative pneumonitis subsided with plasma cells remaining about many of the small and medium sized blood vessels. There were often focal subendothelial accumulations of mononuclear cells in the pulmonary vessels. Plasma cells became more prominent in the lymphoreticular tissues and meninges particularly surrounding the veins.

There were small focal disseminated areas of necrosis in the liver with accumulations of mononuclear cells. The bone marrow became hypoplastic and was accompanied by minimal extramedullary hematopoiesis occurring even in dogs anemic for several months. The gross and histologic changes seen in young Beagles chronically infected with Ehrlichia are similar to changes seen in natural terminal cases of TCP in German Shepherds.

4. Chemotherapeutic Studies.

Since the operation of military dog units in Southeast Asia is dependent on control of TCP, studies were initiated to establish a prophylactic and therapeutic treatment for this disease. The treatment of terminal and chronic cases of TCP in Southeast Asia has met with little success. Rickettsia are sensitive to broad spectrum antibiotics, and tetracycline has been reported to be effective in the treatment of canine Ehrlichiosis (Ewing, S. A. Adv. Vet. Sci. and Comp. Med. 13: 331, 1969). Chemotherapy studies have been initiated to test therapeutic and high and low level prophylactic doses of tetracycline using the standard Medical Supply drug.

A. Preliminary Prophylactic Studies. A preliminary study was directed toward prevention of disease with high levels of tetracycline. Four laboratory Beagles were utilized in this study. Two dogs were each given 750 mg of tetracycline daily; the other 2 dogs served as untreated controls. Two days later, all four dogs were inoculated intravenously with 25 cc each of whole citrated blood from a dog acutely ill with TCP. The treated dogs received the tetracycline regimen for 14 days after inoculation.

The two untreated dogs developed signs of TCP while the treated dogs did not. After two months, the treated dogs were challenged with infective blood and developed typical TCP.

B. Therapeutic Studies. In this study tetracycline therapy was commenced after early signs of disease (fever and an altered hemogram with increased ESR and slight leukopenia) were allowed to develop. This was intended to simulate typical TCP suspects presented to military veterinarians in Southeast Asia.

Two weeks of baseline data were collected on each dog prior to inoculation. Each dog was inoculated intravenously with 5 cc of whole blood collected in sterile sodium citrate from a common donor dog ill with TCP. Thirteen adult laboratory Beagles were used, ten were treated and three served as untreated control dogs. The treated dogs received 500 mg of tetracycline orally b.i.d. for 14 days. Treatment was initiated after all of the thirteen dogs had developed a fever and most had altered hemograms. The dogs for each group were selected randomly.

Each dog was examined and its temperature recorded daily. Blood was collected twice weekly in EDTA for determination of ESR, hematocrit, RBC, WBC, platelet counts, MCV, SGPT and BUN. Serum was taken once a week for determinations of the A/G ration and tetracycline levels using a biological assay system in which Bacillus cereus var mycoides is used as the test organism. One month after the last day of treatment, blood from five of the treated dogs will be inoculated into

susceptible dogs to determine if the treated dogs have become carriers. Blood from the remaining five treated dogs will be inoculated three months after the last day of treatment to allow maximum time for the organisms to multiply or for relapses to occur.

Within 48 hours after treatment commenced none of the treated dogs had a fever. In general, blood values of the treated dogs remained stable or shifted toward normal while those of the untreated dogs continued to decline. Erythrocyte sedimentation rates were elevated for a few weeks in spite of treatment but in most cases eventually returned to normal.

C. Therapy of a Chronic Carrier Dog. A German Shepherd which had been infected with TCP for six months and represented a chronic case, was placed on 2 gms of tetracycline per day for 14 days. Prior to treatment the dog was found to be infected by passage of blood into a susceptible dog. One month after treatment the dog had gained ten pounds, leucopenia had disappeared, and the PCV had risen from 37 to 42 percent. At this time 20 ml of blood was inoculated into a susceptible dog and did not cause disease.

5. Tick Transmission of Ehrlichia canis.

Outbreaks of TCP have often been associated with severe tick infestations, and the incidence of the disease has decreased or disappeared in some kennels after rigid tick control measures were enforced (Mac Vean, D. W., Malaysian Kennel Review, 66, 1968). The common dog tick, Rhipicephalus sanguineus, has been incriminated as the vector of E. canis, the etiologic agent of TCP. Donatien and Lestoquard (Arch. Inst. Past.Algerie, 1937, 15: 142-187 have reported that R. sanguineus is capable of both transovarial and transtadial (stage-to-stage) transmission of E. canis. However, Ewing and Philip (Am. J. Vet. Res., 1966, 27: 67-69) were unsuccessful in transmitting E. canis with this species. In both studies the transmission experiments were done in enzootic areas where prior infection with or immunity to E. canis could not be excluded.

In this laboratory over 100 Beagles have been inoculated with E. canis, and all have been susceptible. Furthermore, none of the Beagles maintained as controls in the Ehrlichia studies have become infected with E. canis. Using the laboratory's supply of susceptible, uninfected Beagles, studies to define the role of the tick as a vector of E. canis were initiated.

A. Establishment of a Laboratory R. sanguineus Colony.

Engorged R. sanguineus females, collected in the TCP enzootic areas of Puerto Rico and the Republic of Vietnam, were sent to this laboratory for study. During the year 28 females were received from Puerto

Rico and 60 from Vietnam. From these 88 ticks, three were selected to establish a continuous laboratory colony. The laboratory strains all originated from ticks removed from scout dogs in Vietnam and were designated VN 6, VN 48, and VN 52.

All ticks are housed in an incubator at 26-27°C and approximately 90% relative humidity. A period of 12 to 14 hours of light per day is furnished the ticks to prevent diapause. Individual ticks or groups of ticks are maintained in small, plastic vials with stainless steel wire tops and snap-cap bottoms.

Ticks have three distinct stages in their life cycle, larvae, nymphs, and adults. Each stage must feed on an animal before proceeding to the next stage or, in the case of adults, laying ova. The feeding time on dogs has varied from 3 to 10 days depending on the stage. Because of the difficulty in restraining dogs for this long feeding time, great difficulty was encountered in feeding and recovering large numbers of ticks using reported methods. With the cooperation of the U. S. Army Medical Biomechanical Research Laboratory, a highly successful method for accomplishing this was devised (Fig. 2).

Ticks are fed on the dorsal and lateral areas of the dog's neck. The feeding site is prepared by closely clipping the hair and washing the neck with tap water. No soaps or solvents are used. Next, one to three, small, rigid, vial holders are sutured to the dog's neck over the feeding sites. The holders are designed to accept the plastic vials in which the ticks are maintained once the vials' snap-cap bottoms have been removed. The vials fit snugly into the rigid holders and give a tight seal. To insure that no ticks escape from beneath the rigid holders, a flexible polyethylene collar is adhered with DOW Corning Medical Adhesive B around the holder and the dog's neck for four to five inches beyond the holder. All dogs infested with ticks are kept in a tick-proof, plastic cage developed by Instrumentation Division, Walter Reed Army Institute of Research.

Using the above method, over 20 tick feedings on dogs have been carried out. Usually 90-100% of the original population placed on the dog is recovered as live, engorged ticks. The small larval and nymphal stages have a higher mortality rate and are more apt to be trapped in the edges of the rigid holder than are the adults. These losses are not excessive, however, and seldom exceed 20% of the unengorged ticks placed on the dog. Even though no restraint measures are used on the dogs, dislocation of the tick vials has occurred in only one instance. Minor skin irritation at the site of attachment of the holder has been occasionally noted, especially when feeding periods have been prolonged.

All three laboratory strains of R. sanguineus have been reared through two complete life cycles involving 24 separate feedings on dogs. Life-cycle data on the three strains are summarized in Table 3.

Fig. 2 Diagram of tick feeding apparatus.

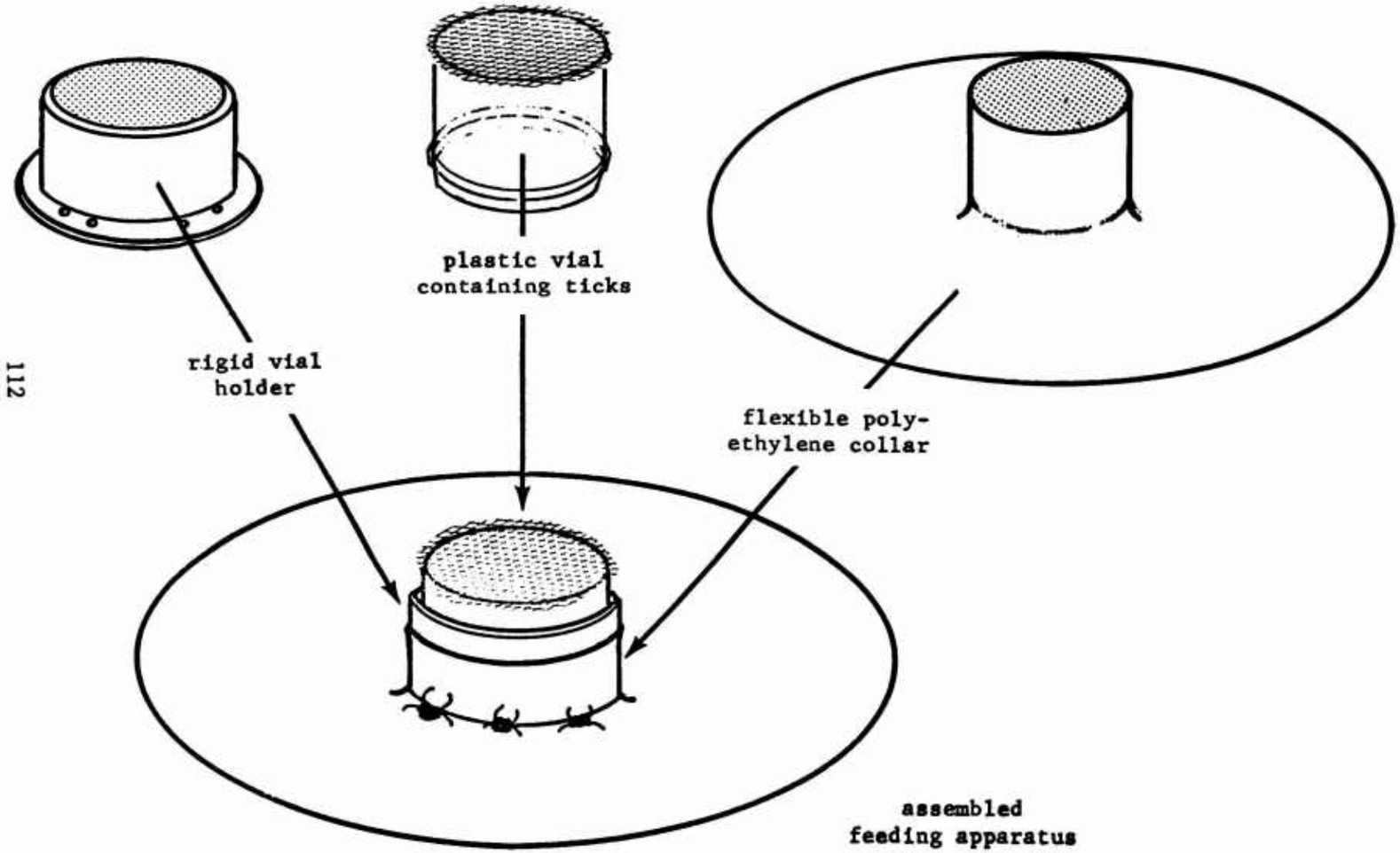

Table 3. Life Cycle Summary of Laboratory R. sanguineus Strains

Stage	Feeding Time	Molting Time	Pre-oviposition	Oviposition	Incubation
Ova	--	--	--	--	23-31 days
Larvae	3-5 days	10-17 days	--	--	--
Nymphs	4-7 days	15-20 days	--	--	--
Adults	5-10 days	--	3-5 days	8-13 days	--

B. Transmission Studies. All laboratory Beagles used in the tick transmission studies were monitored with daily temperatures and twice weekly SGPT, ESR, WBC, and PCV determinations. Previous studies have shown these tests to be the best indicators of E. canis infection. Giemsa-stained blood smears were examined twice weekly and whenever fever or abnormal laboratory results were noted.

In an effort to prove transovarial transmission of E. canis with R. sanguineus, larval offspring of seven female ticks from Vietnam and one female tick from Puerto Rico were fed on four susceptible Beagles. All ticks, with the exception of the single tick from Puerto Rico, came from either dogs known to be infected with E. canis or suspected of having TCP. Three of the Vietnam ticks were from a dog which had both E. canis and Babesia canis present in blood smears. Neither E. canis nor B. canis was detected in any of the four Beagles.

The inability to achieve transmission with larvae derived from the eight ticks does not eliminate the possibility of transovarial transmission. Many factors could account for the failure of the female ticks to acquire and transmit E. canis. Dogs in Southeast Asia suspected of having TCP were frequently placed on high daily doses of tetracycline, an effective antibiotic in preventing E. canis infection. Also, clinically recognized TCP occurs weeks to months after the original infection with E. canis takes place and at a time when antibody levels may be high. Thirdly, some of the dogs from which ticks were taken were classified as "suspects" on the basis of blood studies, and a diagnosis of E. canis infection was not confirmed.

Ehrlichia canis transmission studies using experimentally infected Beagles and two of the three laboratory strains of R. sanguineus, VN 6 and VN 48, were done. Each strain was fed through one complete life cycle (larvae-nymph-adults) on normal laboratory Beagles to determine if either the VN 6 and VN 48 strain naturally transmitted any disease which could confuse the studies. All dogs were monitored clinically and with laboratory tests as detailed above. None of the dogs showed signs of disease. The third tick strain, VN 52, has been maintained on uninfected dogs throughout, and none of these dogs have shown signs of disease.

Each stage of both tick strains used in the experimental transmission studies was divided into two approximately equal populations before feeding. One-half of the population was fed on an experimentally infected Beagle and one-half on a normal Beagle. Subsequent feedings were on normal dogs for both populations. Control ticks fed on uninfected Beagles paralleled all transmission experiments. None of the control ticks transmitted E. canis or any other recognized disease to the normal dogs.

Larvae offspring of VN 6 were fed on a Beagle with acute TCP. Numerous

E. canis inclusions were seen in the monocytes of this dog on the day prior to placing the larvae on it. These ticks failed to transmit the disease to normal laboratory Beagles when fed as nymphs and as adults.

A second E. canis infected Beagle was used to feed VN 6 nymphs and larvae offspring of VN 48. Transtadial transmission was achieved with both these strains. The VN 48 ticks transmitted E. canis both as nymphs and as adults, and the VN 6 ticks transmitted disease as adults. The incubation period from the day the ticks were placed on the dogs until a body temperature greater than 103.5°F and E. canis inclusions were found in the dogs' monocytes was 20 days for the VN 48 nymph transmission and 14 days for the two adult transmissions.

Transovarial transmission attempts with ticks fed on experimentally infected dogs are in progress. The females from the two groups of ticks which transmitted E. canis as adults have laid ova. Larvae from those ova will be fed on normal Beagles.

6. Preservation of E. canis. Following the isolation of E. canis from clinical cases of TCP in Southeast Asia, detailed studies on the pathogenicity and chemotherapy of this disease were initiated. However, the only certain way of preserving E. canis was in the living dog. The disease could be transmitted to laboratory dogs by whole blood collected from affected dogs and maintained for short periods of time on wet ice. Prolonged storage of infectious material was uncertain. Due to the increasing numbers of isolates from Southeast Asia, Puerto Rico, the Virgin Islands, Florida and Panama, maintenance of the organisms in dogs became impractical. Therefore, a study on preservation of infectious material from dogs with TCP was initiated.

A. Preservation of Frozen Blood. Eighteen ml of whole blood were collected in sterile sodium citrate from a dog affected with TCP. Also, 15 ml of whole blood were collected in EDTA from the same dog. Both samples were quick frozen in a dry ice-alcohol bath and stored for two and one half months at -60°C. The two samples were thawed and each inoculated intravenously into adult laboratory Beagles. Both dogs developed TCP.

B. Preservation of Frozen Lung Tissue. Since the lung is the site of the highest demonstrated concentration of Ehrlichia canis organisms, attempts were made to preserve lung tissue and transmit the infection by means of lung suspensions. Two adult laboratory Beagles were inoculated with lung suspensions prepared from two different dogs with TCP. The first was inoculated intraperitoneally with 30 cc of a 20% lung suspensions prepared from a laboratory Beagle with TCP. The second received 18 cc of a 20% lung suspension from a German Shepherd which died of TCP in Vietnam.

The lung from the laboratory Beagle was quick frozen in a dry ice-alcohol bath and stored at -100°C for one month. After thawing, it was minced and placed in a blender with veal infusion broth and 0.5% bovine serum albumin to make a 20% suspension. The suspension was refrigerated for thirty minutes and decanted through gauze. The resulting suspension was then used as the inoculum.

The lung from the German Shepherd was prepared in the same manner except that the early handling in Vietnam was not known. It had been stored for approximately nine months at -100°C.

The dog receiving the lung suspension from the laboratory Beagle developed TCP, while the dog which received the lung suspension from the German Shepherd remained normal.

7. Transmission Studies in Cell Cultures and Embryonating Eggs.

Attempts to grow the agent of TCP in cell cultures and embryonating eggs have continued. Following the recovery of a blood-borne agent and subsequent demonstration of E. canis in mononuclear cells, modifications were made in the approach to culture the agent. Specifically, in latter experiments, antibiotics were omitted from culture media wherever possible and an attempt was made to culture cells in which the agent was present.

Initial unsuccessful attempts to recover the TCP agent from frozen tissues of field cases in cell cultures were described (Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1968 -- 30 June 1969). In a later study, the bone marrow from a field case (Smokey 434X) was inoculated into the continuous baby hamster kidney cell line (BHK-21), and continuous African green monkey kidney cell line (Vero). A transmissible cytopathic agent tentatively designated, "Smokey B. mar.", was recovered in BHK-21 cells. Subsequent studies described below, indicated the agent was unrelated to TCP. After 4 passages in BHK-21 cells, the agent was readily propagated in primary dog kidney (PDK) cells. The Smokey B. mar. agent caused localized cytopathic effects (CPE) which were evident in 48 to 72 hours. Preparations of the agent contained 10^7 to 10^8 tissue culture infectious dose 50 ($TCID_{50}$ ml). Smokey B. mar. was completely inactivated by chloroform and pH 3.0 treatment. The agent passed through 450 and 220 nM millipore filters with a 3.6 log $_{10}$ loss of infectivity in the latter filter and did not pass through a 100 nM filter. The development of CPE was completely suppressed by 100 mcg of aureomycin. Small intracytoplasmic coccus-like bodies were evident in Giemsa and Wright stained infected cells. Clusters of morulae of the coccus bodies were not evident, rather the cocci were seen in or on protoplasmic strands of infected cells. Attempts to culture the agent in bacteriologic and mycoplasma media were unsuccessful. Two Beagles were given approximately 10^8 $TCID_{50}$ of Smokey B. mar. by the intraperitoneal route.

Neither dog developed signs of disease in the 38 days postinoculation. Both dogs developed typical signs of TCP when challenged with the Schatzie line of TCP. Antibody to Smokey B. mar. agent was not detected in canine serum from field cases or laboratory infections. Attempts to reisolate the agent from the original tissue were unsuccessful. The experimental data suggests that an aureomycin-sensitive agent approximately 150-300 nM in size was recovered from cell culture or media used to recover the agent and is not related to TCP. Further studies will be required to identify the agent.

Two series of experiments were done to recover the TCP agent in embryonating eggs. In the first experiments, infectious blood from dogs given the Schatzie line of TCP were inoculated into the yolk sac of 6-day-old embryos. At 14 day intervals, 2 passages of yolk sac suspensions were done without antibiotics. The embryos were examined for viability and for the presence of organisms in Macchiavello stained yolk sacs. There were no deaths attributed to the TCP agent and the presence of rickettsia-like organisms were not evident in stained smears of yolk sac. In the second series of experiments, leukocytes from acutely ill dogs were used. The leukocytes were separated from the erythrocytes, lysed in water and resuspended in Hank's balanced salt solution (HBSS). The suspension was inoculated into embryonated eggs by the chorioallantoic, amniotic and intravenous routes. Appropriate materials were collected at the death of the embryos for ten days following inoculation and given one blind passage by the original route. No significant differences in embryonic deaths were noted between cells from normal and diseased dogs.

Further experiments were done to recover the TCP agent in canine cell cultures inoculated with infectious blood from a dog given the Schatzie line of TCP. The cell cultures were washed to remove all antibiotics and observed for 21 days postinoculation. Cytopathic effects were not evident in PDK, Madin-Darby canine kidney cell line (MDCK), and the Walter Reed canine cell line (WRCC). The cells were trypsinized and subcultured for 14 days. There were no CPE or intracellular organisms in Wright and Giemsa stained cell cultures.

Testis, thymus, lung, kidney, lymph node and bone marrow cell cultures were prepared from dogs infected with the Schatzie line of TCP and uninfected dogs. The cell cultures were prepared without antibiotics. No CPE was observed in 21 days cultivation, and there was no interference of vesicular stomatitis virus (VSV) when the cell cultures were challenged. Intracellular organisms were not evident in Giemsa, hematoxylin and eosin, and Wright stained cell cultures. Since the TCP agent has been consistently demonstrated in lung macrophages and in mononuclear blood cells, these cells were cultured from an infected dog. Alveolar macrophage cell cultures were prepared using Appel's technique (PSEB&M 126: 157, 1967). Attempts to culture alveolar macrophages without antibiotic were unsuccessful and penicillin (100

units), streptomycin (100 mcg/ml) and fungizone (2.5 mcg/ml) were used. Alveolar macrophage cultures were prepared from infected dogs and maintained for 2-to-3 weeks without evidence of inclusion body development. Similarly, lymphocyte cultures were prepared from infected and normal dogs. Cultures were examined daily for the development of Ehrlichia-like inclusions for 8-10 days. Three attempts were made utilizing fetal bovine serum in the culture medium without success. One attempt made using homologous pre-infection plasma added to the culture medium showed promising results in that an increasing number of Ehrlichia-like inclusions were noted in the cultures for 72 hours but were not apparent beyond this time. In addition, cultures of blood monocytes were prepared from infected dogs. After centrifugation of the blood and careful removal of the buffy coat, the procedure of Bennett and Cohen was used (J. Exp. Med. 123: 145, 1966). The monocytes were resuspended in autogenous serum in antibiotic free media. Intracellular organisms did not develop in cultured infected mononuclear cells. Further isolation studies in cell cultures are planned with irradiated cell cultures (Gordon et al. J. Inf. Dis. 120: 451, 1969.

8. Electron Microscopic Studies.

The cause of Tropical Canine Pancytopenia has been attributed to a tick borne rickettsia-like agent, Ehrlichia canis. There are no serological, cultural, histochemical, or biochemical procedures which can be used to define the infectious agent. Its identity is based solely on the morphologic characteristics in infected cells. The cytoplasmic inclusion, characteristically found in mononuclear cells. appears as a morula which is an aggregate of individual elementary bodies. It was decided that an electron microscopic description of the infectious agent's ultrastructure would be of great aid to substantiate its classification and elucidate cultural conditions necessary for its propagation.

Capillary blood smears were prepared from laboratory Beagles experimentally infected with Ehrlichia canis. When a large number of monocytes with morulae were found, 5 ml samples of venous blood were collected in EDTA coated tubes. The monocytes were then separated from the whole blood by the selective floatation method of Marikovsky and Danon (J. Ultrastructure Res. 18: 176-180, 1967). A series of phthalate ester mixtures of different specific gravities were prepared. The percentage of monocytes isolated compared to other white blood cells was correlated with the specific gravity of the phthalate mixtures. For example:

specific gravity	% monocytes
1.062	85
1.066	93

specific gravity	% monocytes
1.070	94
1.074	92
1.078	81

However, it was later found that when there was a high incidence of morulae in capillary smears, they were rarely detectable in the blood taken from the jugular vein. This method has now been abandoned.

A method was needed which would make relatively rare cells available for electron microscopy. The method of Gaynor, Bouvier and Cintron (Proc. Soc. Expt. Biol. & Med. 133: 520-523, 1970) seemed ideal. The procedure permitted light microscope scanning of cells embedded in an epoxy resin. The location of the cell is then marked with a stylus and the cell is sectioned for electron microscopy. The cells were obtained at necropsy by lavaging the lungs of a diseased animal with tissue culture medium 199 containing Hank's balanced salt solution and sodium bicarbonate. The cells were fixed, stained, dehydrated and imbedded according to Gaynor, et al. The preparations are now being sectioned for electron microscopy.

II. Canine Babesiosis.

Canine babesiosis due to B. gibsoni is enzootic in India (Kapur, H. R. Indian Vet. J., 1944, 19: 199-205), Ceylon (Seneviratna, P. Ceylon Vet. J., 1965, 13: 1-6), and Malaysia (Groves, M. G. and Yap, L. F., J.A.V.M.A., 1968, 158: 689-694). Recently, the parasite has been found in Korea (LTC J. L. Fowler, 406th Medical Laboratory, Japan; Personal communication). The principal vector of B. gibsoni in enzootic areas is the tick Haemaphysalis bispinosa. Because this tick is widely distributed throughout Southeast Asia, B. gibsoni represents a potentially important disease of military dogs.

Most published work on B. gibsoni has originated from countries where the disease is enzootic. These reports usually deal with clinical signs and treatment of the naturally occurring disease. Detailed blood studies and, with one exception, pathological examinations of infected dogs have not been reported.

1. Infections in Laboratory Beagles: The source of our laboratory strain of B. gibsoni was infective blood from a Bull Terrier, which contracted B. gibsoni infection in Malaysia. It was brought to the United States in May 1967, shortly after clinical recovery. This latent infection has been followed by inoculation of blood into splenectomized dogs. The dog was last demonstrated to be positive in August 1969. Since reinfection can be excluded, this confirms a persistent B. gibsoni infection of over 27 months.

The strain of B. gibsoni obtained from this Bull Terrier has been maintained in two intact Beagles. These two dogs initially suffered an acute anemia following the inoculation of infective blood, followed by a spontaneous remission of disease. Both are now inapparent carriers of B. gibsoni. Infections in three splenectomized dogs have been fatal; two were splenectomized prior to infection and one after it had become an inapparent carrier of the disease.

Studies are being conducted to define clinical B. gibsoni infection in intact, laboratory Beagles. Nine dogs were simultaneously infected with blood from a splenectomized dog with an acute B. gibsoni infection. The infected dogs are being examined and temperatures recorded daily. During acute infection, hematocrits, parasite counts, and reticulocyte counts are also done daily. Hemoglobin, RBC, WBC, differential WBC, SGPT, BUN, total bilirubin, urine occult blood, urobilinogen, and urine protein values are determined two to three times a week. Four uninfected, intact Beagles act as controls.

Although this study has not been completed, certain characteristics of the disease are evident. A progressive anemia has been the only consistent sign of disease. The blood picture is typically one of a macrocytic anemia resulting from an increased destruction or removal of RBC's. There is a very active hemopoietic response; polychromasia, anisocytosis, and reticulocytosis are characteristic. Numerous nucleated RBC's are evident late in the infection. Although total white blood cell counts fluctuate within the normal range, there is a slight lymphocytosis during the acute phase of disease.

Body temperature is extremely variable during infection and no correlation between temperature fluctuations and changes in parasite numbers has been observed. Splenomegaly is a common finding; however, this organ is frequently difficult to palpate. Elevated urinary bilirubin has been the only consistently abnormal urinalysis finding. In marked contrast to acute B. canis infections, icterus and hemoglobinuria are extremely rare, even in severe B. gibsoni infection. Total bilirubin, SGPT, and BUN determinations have been within normal ranges.

The objective of the B. gibsoni studies is to define experimental clinical disease in Beagles, after which chemotherapy studies will be initiated.

2. Preservation of B. gibsoni.

Blood from a dog with a high B. gibsoni parasitemia was preserved by the addition of glycerol and freezing. Blood in EDTA was filtered through gauze to remove any fibrin. Glycerol was then added to a concentration of 12%, and the blood-glycerol mixture stirred continuously for two hours at 5°C. Vials containing 25 ml. aliquots of the mixture were transferred to a -100°C freezer and allowed to freeze at their own rate. The preserved blood was infective for an intact

Beagle 17 days post freezing. This experiment is continuing.

III. Acute Canine Glossitis, "Red Tongue Syndrome".

Since July 1969, approximately 25 cases of non-fatal, acute, non-suppurative glossitis have occurred in U. S. military dogs in the Republic of Vietnam. In most cases, the signs regress and the dogs return to normal within 3-7 days. However, some chronic cases recur after an apparent recovery. Other clinical signs exhibited include a marked gingivitis and edema of the gums, excessive salivation, and at times a serous conjunctivitis. The tongues are sensitive to palpation and the dogs eat with difficulty. A significant consistent clinical finding is a normal total WBC count with an eosinophilia. To date bacterial and fungal cultures have been inconclusive and histopathologic findings are insignificant. There is no consistent correlation between this condition and TCP. Transmission studies were done to determine if an infectious agent could be demonstrated.

1. Transmission Studies.

Frozen biopsies of two affected tongues were received from RVN. Serial ten-fold dilutions of affected tissue suspensions were inoculated into the tongues of normal dogs. A suspension of unaffected tissue was also inoculated for control purposes. During a six-week period of observation, none of the inoculated dogs had any gross changes in the tongue or gingiva, increased rectal temperature, or alteration in total or differential white blood cell counts. The dogs continued to eat and drink without difficulty.

Tissue suspensions were also inoculated into primary and continuous canine cell cultures. Cytopathic effects or hemabsorption were not observed in these cultures. Bacteriologic cultures were negative.

2. Pathological Studies.

Several lingual biopsy specimens have been submitted in 10% formalin for examination. There is a mild accumulation of monocytes and plasma cells in the subepithelial connective tissue, particularly about blood vessels. There has been, in two cases, a polymorphonuclear infiltrate in the epithelium in addition to the subepithelial change. It was not possible to determine if the epithelial change was primary or secondary. Fungal stains were negative. In all cases there was mild hyperactivity of epithelial germinal cells, and in several clusters of such cells, nucleoli were quite large and somewhat suggestive of viral inclusion bodies. Enlarged nucleoli are common findings in activated epithelium and can often resemble viral inclusion bodies. The histologic changes noted are nonspecific and the etiology cannot be established.

IV. Chloroquine Toxicity in Dogs.

Chloroquine- primaquine tablets are widely used thoughout Southeast Asia for malaria prophylaxis. Although there is no therapeutic requirement for administering such tablets to military dogs, a few reports have been received of severe illness in military dogs following the alleged administration of chloroquine-primaquine tablets. In such instances veterinarians responsible for the care of these dogs requested appropriate analyses on blood and tissue from affected dogs. It is often difficult to collect urine from dogs under field conditions; therefore analytical procedures were limited to blood analysis. Current methods of analysis did not meet the needs for a rapid, simple method which could be used in almost any laboratory.

The first step in the study was to define chloroquine-primaquine toxicity in dogs. Since the available methods for chloroquine analysis of dog blood were cumbersome, studies were also initiated to develop a simple, rapid test that could be used on whole blood. It was also felt that a simple, rapid quantitative test for chloroquine would be useful in laboratories where chloroquine analysis on human specimens are frequently required.

The discussion of chloroquine determination by Undenfriend in "Fluorescence Analysis in Biology and Medicine" was used as a starting point in methods development.

1. Chloroquine-Primaquine Toxicity in Laboratory Dogs.

Since the usual malaria prevention medication in use is a combined tablet of 500 mg Chloroquine phosphate with 79 mg primaquine phosphate, this combination was tried first in toxicity trials. It had been reported that one tablet was sufficient to produce illness in German Shepherds. Therefore, three levels of one-tablet, one-half tablet and one-fourth tablet were given to laboratory Beagles weighing approximately 10 Kg each with the following results:

Dog. No. 1: One tablet (500 mg Chl-P04, 79 mg Prim-P04). The dog showed signs in less than four hours after administering the drug. Primarily CNS signs were observed with loss of motor control and equilibrium. The dog died approximately two hours after onset of signs. Chl-P04 level in blood was approximately 8 mg/L.

Dog. No. 2. One-half tablet (250 mg Chl-P04, 39.5 Prim-P04). Signs developed suddenly four hours after drug was administered. The signs were essentially the same as for dog No. 1, but suddenly disappeared after about two hours. No residual effect was noted. Blood taken at onset of signs contained about 5 mg/L of Chl-P04.

Dog No. 3: One-fourth tablet (125 mg Chl-P04 and 19.8 mg Prim-P04). Dog exhibited no abnormal behavior or signs. A blood specimen taken four hours after drug was administered contained 1.5 mg/L Chl-P04.

When the drugs were administered separately, Chloroquine 125 and 250 mg as phosphate and primaquine phosphate at 26.3 and 52.6 mg doses, the previous experience was confirmed with Chloroquine alone, but at the levels administered, primaquine was apparently not toxic.

Dog No. 4: (125 mg Chl-P04) Dog did not develop signs of toxicity. The post inoculation blood levels as Chl-P04 were: 1.5 mg/L at 4 hrs, 0.4 mg/L at 24 hrs and a trace at 52 hrs.

Dog No. 5: (250 mg Chl-P04) Dog developed signs four hours after the drug was given and persisted for approximately two hours. The signs were flaccid paralysis and loss of equilibrium. The blood levels as Chl-P04 were: 3 mg/L at 4 hrs, 1.5 mg/L at 21 hrs, 0.15 mg/L at 52 hrs and a trace at 72 hrs.

Dog No. 6 receiving 26.3 mg Prim-P04 and Dog No. 7 receiving 52.6 mg Prim-P04 developed no signs of toxicity.

It was concluded that the toxic dose of Chloroquine phosphate falls between 25 and 50 mg per Kg. The no-effect level of primaquine phosphate equals or exceeds 5.3 mg/Kg, which is considerably higher than the primaquine content of the Chloroquine-primaquine tablet which produced signs in experimental dogs. The toxicity appears to be due to Chloroquine.

Three of the dogs that had been used 3 months previously for the acute toxicity studies were used for more chronic studies. One dog that became ill following the initial 250 mg dose of Chloroquine phosphate was given 250 mg Chloroquine phosphate three times a week for a total of eight doses. Two that had received 125 mg Chloroquine doses were each given 125 mg Chl-P04 three times a week for 8 doses. One dog was maintained as a negative control. At no time during dosage regimen or afterward did signs of drug toxicity appear even in the dog that three months previously had developed severe signs from one dose of 250 mg Chl-P04. Although the blood from the original dose contained only Chloroquine, small amounts of an apparent metabolite appeared in a blood sample four hours after the first dose in the multiple dose studies. On succeeding days other fluorescent metabolites appeared rapidly until four were detected and appeared regularly, attached to the red cells. In one human subject who had on two occasions taken chloroquine tablets weekly for a year and whose blood and urine were analyzed repeatedly over an extended period before and after administration of a single dose of chloroquine, only chloroquine and no metabolites could be detected.

Table 4 summarizes the analysis of blood collected from dogs during repeated administration of chloroquine. Except where shorter intervals are shown the blood samples was drawn 48 to 72 hours after the previous dose and just prior to an additional dose of chloroquine. Levels reported are in fluorescent equivalents of chloroquine. The column headed "Metabolite 3" was usually the most intense and since it remained at or almost on the origin it was difficult to estimate accurately. A light-absorbing (not fluorescent) blue spot was present in extracts of blood from all dogs including the controls but was not present in extracts of washed cells. This spot was near or partly on "Metabolite 4" and could have decreased its fluorescent intensity. Metabolite 1, which appeared following the first dose in this series, migrated well above chloroquine on alumina thin layer chromatography (TLC) while all other metabolites appearing later migrated much less than chloroquine.

Four chromatographically different fluorescing metabolites were found in blood of dogs that were given chloroquine repeatedly. These are attached to the red blood cell since washed cells showed the same levels as did whole blood while separated plasma showed no detectable chloroquine or metabolites.

2. Methods Development for Analysis of Chloroquine and Metabolites in Blood and Urine.

To be useful in field laboratories, a method for chloroquine analysis in dogs should require minimal equipment. However, due to materials in blood and urine that have absorbent, fluorescent or fluorescence quenching properties, the chloroquine must be adequately separated to be determinable. Spectrophotometry had inadequate sensitivity and specificity in detecting low levels of chloroquine in small size samples. Other fluorescing and quenching materials in blood and urine interfered in spectrofluorometry. Thin layer chromatography provided a means of concentrating a large sample into a small area to permit quantitation based on fluorescence. Also chloroquine could be separated from interfering materials in the samples.

Chloroquine phosphate is water soluble; chloroquine base is soluble in organic solvents such as petroleum ether and chloroform. By extracting the organic solvent solution with acid, the chloroquine passes into the aqueous phase. Undenfriend reported that his aqueous solution could be used for spectrophotometric measurements. However, trials in this laboratory indicated that, with blood or urine, too much interference was present for the weak chloroquine absorbance to be measured.

Table 4: Chloroquine and its Metabolites in Blood of Dogs, as Chloroquine Equivalent Flurorescence, mg/L

Dog CP-1 125 mg doses

Date	Hours since last dose	Chloroquine	Metabolite 1 high	Metabolite 2 low	Metabolite 3 origin	Metabolite 4
1-15	pre-treatment	0	0	0	0	0
1-15	4	0.6	0.1	0	0	0
1-16	28	0.3	0.2	0	*	
1-17	48	0.3	0.2	0.5	1.5	
1-20	72	0.3	0.4	*	*	*
1-22	48	0.3	tr	*	1	*
1-24	48	0.3	0.2	*	1	*
1-27	72	0.4	0.6	0.1	1	0.2
1-29	48	0.3	0.4	0.4	2	0.1
1-31	48	0.3	0.5	0.2	2	0.2
2-3	72	0.4	0.8	0.4	1	0.1
2-5	48	0.4	0.5	0.4	2	0.2

* not determined

Table 4. Continued.

Dog CP 2 125 mg doses

Date	Hours since last dose	Chloroquine	Metabolite 1 high	Metabolite 2 low	Metabolite 3 origin	Metabolite 4
1-15	pre-treatment	0	0	0	0	0
1-15	4	0.6	0.5	0	0	0
1-16	28	0.3	0.3	0.2	1	
1-17	48	0.2	0.4	0.1	1.5	
1-20	72	0.3	0.4	*	*	*
1-22	48		0.1	0.1	1	
1-24	48	0.1	0.2	*	1	*
1-27	72	0.3	0.6	0.2	1	0
1-29	48	0.1	0.4	0.8	3	0.1
1-31	48	0.2	0.5	0.2	2	0.2
2-3	72	0.2	0.4	0.4	3	0.1
2-5	48	0.4	0.6	0.8	2	0.4

* not determined

Table 4. Continued.

Dog CP 3 250 mg doses

Date	Hours since last dose	Chloroquine	Metabolite 1 high	Metabolite 2 low	Metabolite 3 origin	Metabolite 4
1-15	pre-treatment	0	0	0	0	0
1-15	4	1.1	0.4	0	0	0
1-16	28	0.6	0.5		1	
1-17	48	0.5	0.5	*	*	*
1-20	72	0.1	0.5	*	*	*
1-22	6	5	1.5	*	1	*
1-24	48	0.8	0.3	*	2	*
1-27	72	0.6	0.4	1.0	2	0
1-29	48	0.4	1.0	1.0	3	0.2
1-31	48	0.6	0.7	0.4	3	0.2
2-3	72	0.6	1.0	0.5	3	0.1
2-5	48	0.6	0.8	0.5	2	0.2

* not determined

Therefore, extraction and cleanup procedures were begun with the aim of using TLC for quantitation. Preliminary trials showed that pure chloroquine phosphate made basic with sodium hydroxide and extracted into organic solvent would migrate on alumina TLC sheets. Use of ultraviolet exposure during viewing showed that as little as 0.1 microgram chloroquine phosphate was detectable. Aqueous solutions were unsatisfactory for TLC.

Single extractions of blood or urine, made alkaline with sodium hydroxide, with petroleum ether or hexane gave very erratic recoveries of added chloroquine. Single extractions with chloroform or methylene chloride contained too many interferences. However, by first extracting the alkalinized blood or urine with chloroform or methylene chloride followed by extraction of the organic phase with dilute sulfuric acid and then after adding excess sodium hydroxide and re-extracting into chloroform or methylene chloride, an extract was obtained giving consistent high recovery suitable for thin layer chromatography. It was found that usual ACS grades of organic solvents had excessive fluorescent and fluorescence quenching interferences. Use of special grades designed for pesticide analysis, namely, Burdick and Jackson "Distilled in Glass" or Mallinckrodt "Nanograde", eliminated this problem.

Other interferences can come from plastics (polyethylene or vinylite screw cap liners) or rubber. Glass apparatus or Teflon-lined caps or tin foil lined caps did not interfere. Tin foil liners tend to corrode slowly and may be limited to about ten uses. Glass stoppered apparatus often leaked.

Three methods of producing purified chloroquine extracts of blood and one method for preparing extracts of urine were developed to serve varying needs. One method was developed for detecting extremely low levels in blood down to 10 mcg/L as chloroquine phosphate or 6 mcg/L as chloroquine base in blood. This method requires 20 ml of blood which is made alkaline with sodium hydroxide and extracted with chloroform or methylene chloride. An aliquot of the extract is extracted with 0.1 $\underline{N}H_2SO_4$ and an aliquot of this is made alkaline and back-extracted into methylene chloride. The final extract representing 14 ml of blood is evaporated and made up to 0.070 ml. Up to 0.050 ml of this (representing 10 ml of blood) is analyzed for chloroquine by alumina TLC.

A second method was essentially the same but designed for levels which would be found in "poisoned" dogs or human therapeutic doses. It uses 5 ml of blood and smaller glassware and small volumes of reagents. The third blood method, designed to eliminate interferences in determining the metabolites produced in chronically treated dogs, is very similar except the blood cells were washed virtually free of serum by repeated washes with normal saline before proceeding with the chloroquine extraction.

Although the first two blood methods are applicable to urine a much simpler extraction procedure proved satisfactory. When 5 to 10 ml of urine is made alkaline, extracted into 5% methylene chloride in petroleum ether and extract washed once with alkaline water, an entirely acceptable extract for determining chloroquine by TLC was obtained. Actually this gives a cleaner extract from urine than does the multi-step blood methods.

An apparent simple test for detecting chloroquine in urine is to spot urine on filter paper and examine under short wave ultraviolet light. However for our purposes this test had very limited usefulness due to other fluorescent materials normally present in urine. Attempts to develop paper chromatograms to separate chloroquine from interferences were unsuccessful due to persistent streaking or failure of materials to move on paper. However, since thin layer chromatography permits a much greater choice of chromatographic materials all further work concentrated on thin layer chromatography. Commercially prepared alumina and silica coated sheets were tried. Chloroquine will not migrate from origin on silica sheets (Eastman Chromagram, Machery-Nagel Polygram, Baker Flex). However, various solvent systems produced movement of chloroquine on alumina sheets (Eastman Chromagram, with and without fluorescent indicator, Machery-Nagel Polygram, Baker Flex). With sheets containing fluorescent indicator the background fluorescence raised the detection limit and made quantitation difficult. All sheets except Eastman had 250 micron layers. On these the detection limits were higher (larger amount required for detection) than on the 100 micron Eastman layers. However, in some cases interferences could be separated more readily on the thicker layers.

The usual method of TLC calls for use of 20 x 20 cm plates and a migration distance of at least 10 cm. Trials were made using sheets cut to 10 cm height and migrating the solvent only 5 cm above the spotting line. This proved very adequate for most blood and urine samples, although migration for 8 cm is possible giving even better separation of spots.

Samples and standards may be spotted as close as 8 mm apart. A 10 x 10 cm sheet will take 9-10 spots. Spotting is done 1.5--2 cm from the bottom edge. Spotting points are marked by laying a ruler on the sheet and touching with a pencil at the desired points. In all cases at least one standard is spotted since Rf values are influenced by temperature, humidity and saturation of development jar.

A blotter lined jar is used for development and developing solvent poured directly in the jar a few minutes before the sheet is placed for development. A glass plate or aluminum foil is used to close the development jar. For large numbers of samples the Eastman Chromagram developer is used with 10 x 20 cm sheet. Since it is developed between two glass plates no pre-saturation or blotter liner is needed.

Thin-layer sheets, or plates, tend to migrate faster along the edges than in the middle. This can be avoided by stripping the coating for 1 millimeter along the edge. If not done the spots from different points migrate at different rates and move out of line.

Many solvent systems were tried and several can be used. For human blood not having the metabolities found in dogs chloroform-hexane mixtures (1 + 3, 1 + 1, or 3 + 1) adequately separate chloroquine from interferences. For dog blood development with acetone or 1 + 1 mixture of acetone with petroleum ether or hexane is required for adequate separation of all spots. This is also satisfactory for human blood.

For urine best separations from interferences were obtained with acetone alone, but in some cases 1 + 1 acetone-petroleum ether or 3 + 1 acetone-chloroform are useful. Development time for 5 cm is usually about 10 minutes, whereas 8 cm development may require 20 minutes.

3. Recovery Trials of Chloroquine Added to Dog Blood and Human Urine.

To determine the accuracy of the test, known quantities of chloroquine phosphate were added to human urine and dog blood and analyzed as unknowns. The results are summarized in tables 5 and 6. Generally there was excellent agreement between the actual concentration and test results.

In a human trial, the chloroquine levels in blood and urine following administration of 500 mg Chl-P04, are given in Table 7. The levels are affected both by the time the urine sample is collected and the intake of fluids.

4. Discussion.

Toxicity trials in mature Beagle dogs disclosed that chloroquine has a LD/50 between 25 and 50 mg chloroquine phosphate per Kg body weight. Continued sublethal administration of the drug may induce the production of several metabolites. Further work is contemplated to determine the nature of the metabolites and their presence in other species. However, only chloroquine was found in the blood or urine of the human volunteer.

The methods of analysis developed for blood have proved their ability to recover very low levels of added chloroquine. The ability to separate chloroquine from at least four metabolites should simplify the characterization of the metabolites.

The methods are simple and only a limited amount of equipment, reagents and technicians' time are required for screening a large number of specimens.

Table 5. Recoveries of Chloroquine Phosphate in Urine

	Actual Concentration	Test Results
1.	Blank	Negative
2.	0.1 mg/L	0.1 mg/L
3.	0.5 mg/L	0.5 mg/L
4.	1.0 mg/L	1.0 mg/L
5.	0.5 mg/L	0.5 mg/L
6.	1.0 mg/L	1.0 mg/L
7.	0.1 mg/L	0.1 mg/L
8.	Blank	Negative
9.	0.1 mg/L	0.05 mg/L
10.	1.0 mg/L	1.0 mg/L
11.	0.05 mg/L	0.05 mg/L
12.	0.5 mg/L	0.5 mg/L
13.	Blank	Negative
14.	0.5 mg/L	0.75 mg/L
15.	0.05 mg/L	Trace

Table 6. Recovery of Added Chloroquine Phosphate in Dog Blood

Actual Concentration	Test Results
1. 70 mcg/L	60 mcg/L
2. 50 mcg/L	45 mcg/L
3. Blank	Negative
4. 20 mcg/L	20 mcg/L
5. 70 mcg/L	60+ mcg/L
6. 50 mcg/L	40+ mcg/L
7. 100 mcg/L	100 mcg/L
8. 200 mcg/L	200 mcg/L

Table 7. Chloroquine, As Phosphate, in Human Blood and Urine from One 500 mg Tablet

Blood:	4 hours	600 mcg/L
	48 hours	100 mcg/L
	30 days	30 mcg/L
Urine:	Residual from previous administration - 1.0 mg/L	
	2 hours	5 mg/L
	6 hours	18 mg/L
	20 hours	30 mg/L (overnight collection)
	26 hours	8 mg/L
	52 hours	6 mg/L
	4 days	1.5 mg/L
	30 days	2.5 mg/L

The method for chloroquine in urine should be of value in screening humans to determine if weekly preventive dose of chloroquine is actually being taken. Since chloroquine appears in higher concentrations in urine than in blood, simpler methodology is possible. A simple extraction without further cleanup will detect levels of chloroquine that should be of interest.

Thin layer chromatography is rapid, simple and inexpensive and best suited of any procedure for multiple analyses. Although theoretically not as accurate as some more sophisticated measurement methods it proved more than adequate for a clinical procedure.

V. Evaluation of Chemoprophylactics for Control of Heartworm and and Hookworm in Military Dogs.

Hookworm and heartworm infections are serious problems in military dogs. Treating dogs for these diseases poses several problems. The dogs are often debilitated and susceptible to secondary bacterial or viral infections. Damage caused by heartworms to the heart and pulmonary vessels may be irreparable, and treatment itself may require four to six weeks.

Under laboratory conditions, the drugs styrylpyridinium (Styrid, Americal Cyanamid Company) and diethylcarbamazine citrate (Caricide, American Cyanamid Company) have proven effective in preventing hookworm and heartworm infections (G. S. Tullock, et al., Am. J. Vet. Res. 31: 437-448, 1970; I. B. Wood, et al., J. Parasitol. 51: 34-37, 1965). With the cooperation of personnel at Fort Benning and in Vietnam, field studies to evaluate these two drugs were conducted.

1. Fort Benning Study.

After successful completion of initial trials, which were previously reported (Walter Reed Army Institute of Research Annual Progress Report, 1 July 1968 -- 30 June 1969), all dogs at Fort Benning were placed on the drugs. Results of this larger field study were comparable to the initial trials.

The studies at Fort Benning suggest that styrylpyridinium and diethylcarbamazine provide effective control of intestinal parasites, cause no side effects, and do not impair performance of the dogs.

2. Vietnam Studies.

One Infantry Scout dog Platoon, placed on Styrid-Caricide during training at Fort Benning, was continued on the drugs after shipment to the Republic of Vietnam in January 1969. In this unit the drugs have been administered as 200 mg Styrid and 400 mg Caricide tablets.

One Caricide tablet per dog is administered daily. The Styrid is not given on a continuous basis. Instead, the dogs are given styryl-pyridinium daily for a period of five days then taken off the drug for seven days. At the end of seven days the dogs are placed back on the drug, and the cycle is repeated. All dogs in this unit are followed with bimonthly Knott's tests for microfilaria and monthly fecal floatations for hookworm ova. Microfilaria have not been found in any of the study dogs during this year. Occasional hookworm ova have been seen, but the incidence has been low.

Two additional units in RVN were placed on the Styrid-Caricide evaluation program in December 1969. Approximately half the dogs in these two units are on a Styrid and Caricide regimen identical to the one outlined above; the remaining dogs serve as untreated controls. One unit has approximately 20 dogs; the other, approximately 36 dogs. Data from these two units have been difficult to evaluate because of inability to maintain the integrity of treated and control groups.

VI. Summary and Conclusions.

1. Tropical canine pancytopenia (TCP) is a newly recognized infectious disease of dogs in diverse tropical and subtropical areas. The disease is characterized by hemorrhage, pancytopenia, severe emaciation and persistent infection. Dogs with TCP are often presented with epistaxis, which is the most dramatic sign of the disease; however, a large number of affected dogs develop severe pancytopenia and die without manifesting clinical signs of hemorrhage. The disease has been reported most frequently in the German Shepherd. Pathological findings consist of petechial and ecchymotic hemorrhages on serosal and mucosal surfaces of numerous organs. The most prominent histological finding is a perivascular plasma cell infiltrate in most organs. The disease has been successfully transmitted to laboratory dogs. Experimentally infected Beagles and mongrels develop clinical signs consistent with the natural disease; however, clinical signs or hemorrhage including epistaxis have been experimentally induced only in the German Shepherd. Ehrlichia canis has been identified as the etiologic agent of TCP and has been recovered from dogs affected with TCP in Southeast Asia, Puerto Rico, the Virgin Islands, Florida and Panama. Attempts to transmit the disease to other laboratory animals and to propagate the agent in cell cultures and embryonating eggs have been unsuccessful. The organism has been preserved for 10 weeks in blood frozen at 60°C. Transtadial transmission of Ehrlichia canis by laboratory reared ticks, Rhipicephalus sanguineus, has been demonstrated. Preliminary studies have provided evidence that tetracycline may be effective in treating the early stages of the disease.

2. Canine babesiosis due to B. gibsoni is a disease of potential importance to military dogs in Southeast Asia. The disease in experimentally infected laboratory Beagles is characterized by a macrocytic, hypochromic anemia and elevated urinary bilirubin.

3. Acute canine glossitis (red tongue syndrome) has occurred in approximately 25 dogs in the Republic of Vietnam. This is a non-fatal condition, however, the dog is incapacitated and cannot be utilized in military operations for 3 to 7 days. Transmission studies using frozen tongue biopsy specimens from affected dogs in the Republic of Vietnam were negative. Cell cultures were also inoculated with negative results. Histologic changes in formalin fixed specimens were non-specific and at this time the etiology cannot be established.

4. Chloroquine-primaquine toxicity studies in dogs provided evidence that early, acute signs of toxicity are attributable to chloroquine. The toxic dose of chloroquine phosphate falls between 25 and 50 mg per Kg. Methods of chloroquine analysis using solvent partitioning and alumina TLC were devised for varying levels of chloroquine in blood and urine. In the dog at least four fluorescing metabolites of chloroquine appear in the erythrocytes. Both blood and urine methods are adaptable to mass screening.

5. The evaluation of chemoprophylactics for control of parasites in military dogs has continued. All dogs at Fort Benning, Georgia and several units in Vietnam have been placed on styrylpyridinium and diethylcarbamazine, a combination of drugs which provide effective control of heartworms, hookworms and roundworms. These field studies have provided evidence that the drugs are effective in control of parasites, cause no side effects and do not impair performance of the dogs.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 183, Diseases of Military Animals in Southeast Asia

Literature Cited

1. Hildebrandt, P. K., Huxsoll, D. L., and Nims, R. M.: Experimental Ehrlichiosis in Young Beagle Dogs. Fed. Proc. 29: 754, 1970.

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

23. (U) To determine and characterize causative agents of infectious diseases occurring in military dogs during their induction and training; to determine how the diseases are spread; and to develop effective control measures. Studies on agents associated with acute respiratory disease outbreaks are specifically emphasized. The potential military importance of infectious agents newly found in dogs are evaluated.

24. (U) Conventional epidemiologic and microbiologic technics are employed and new procedures will be developed as needed.

25. (U) 69 07 - 70 06 Outbreaks of respiratory disease occurring at induction and training centers from 1966 to 1968 affected approximately 25% of the dogs. These disease episodes seriously disrupted the shipment of dogs to operational areas. Outbreaks are primarily related to infection with parainfluenza virus SV-5. This virus was found to be highly communicable in field and experimental studies. It has been further established that over 90% of dogs at time of military induction are susceptible to SV-5 infection. In 1969 and 1970, SV-5 infections continued to occur at the induction and training centers. Antibody studies of infected dogs provided little or no evidence of infection with other canine viruses. Three new viruses with properties of rhinoviruses and a fourth virus with properties of a coronavirus were recovered from dogs. These agents are being further characterized and studied for ability to produce disease. The incidence of Brucella canis infections and disease in the WRAIR beagle colony was reduced by a microbiological surveillance program. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 184, Diseases of recruit military animals

Investigators.

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Description.

To determine the etiology and epidemiology of respiratory disease in military dogs and to develop methods of control and prevention. To identify and characterize viruses of military dogs and laboratory animals which may be potential zoonotic agents, or interfere with the utilization of the animals in the research programs or diagnostic services at WRAIR.

Progress.

1. Respiratory Disease in Military Dogs.

Investigation of the first epizootic (May-June 1966) of upper respiratory disease at Fort Benning led to the recovery of a highly communicable parainfluenza SV-5 (Am. J. Vet. Res. 29: 1809, Sept. 1968). At Lackland Air Force Base (LAFB), Crandell and his co-workers also recovered SV-5 from military dogs (Am. J. Vet. Res. 29: 2141, Dec. 1968). In 1967 and 1968, respiratory disease and SV-5 infections continued to occur at the LAFB induction center and at Fort Benning, Ga. Nearly 25% of the dogs developed mild respiratory disease and almost all the dogs had serologic evidence of SV-5 infections. In many instances, the SV-5 infections could be associated with the disease. The respiratory disease was essentially a recruit disease occurring predominantly within 2 to 3 months of entry into service (Annual Report 1 July 1968 - 30 June 1969).

In June 1969, in conjunction with Major Olson, Chief, Military Dog Veterinary Service, LAFB, and Colonel Ritter, Post Veterinarian, Fort Benning, the epidemiology and etiology of respiratory disease in military dogs was studied further. Dogs were followed serologically from time of induction at LAFB and at time of arrival and departure from Fort Benning. In contrast to previous experience, few cases of respiratory disease have occurred in military dogs during the past year.

On arrival at the LAFB procurement center, 90.2% or 618 of 685 dogs had no SV-5 antibody. These findings indicated that new dogs are highly susceptible to SV-5 infection and were consistent with earlier observations (Annual Report 1 Jul 1968 - 30 June 1969). The 67 serotest positive dogs originated from 24 states, representing all geographic areas of the continental United States. A relatively high prevalence of SV-5 antibody was found in dogs from California (15 of 95 or 15.8%) and Ohio (7 of 38 or 18.4%).

Serum specimens from all of 22 dogs which were received at Fort Benning in August 1969 were serotest negative for SV-5 antibody. Similarly of 22 dogs arriving in September and October 1969, only 4 or 18% had SV-5 antibody. Two of the 4 serologically positive dogs were negative at LAFB. In November, the incidence of SV-5 antibody in dogs arriving at Fort Benning markedly increased. Twenty-two of the 23 dogs had antibody; a rise in titer occurred in 16 of 17 dogs between the period of procurement at LAFB and shipment to Fort Benning. It appeared that little or no SV-5 infections occurred in the summer and early fall at LAFB but infections suddenly increased in Oct-Nov 1969.

After completion of scout dog training at Fort Benning, SV-5 antibody was present in 83 of 109 dogs (76%) tested from November 1969 to January 1970. Only 3 of 14 serotest negative dogs completing training Nov-Dec 1969 converted to positive SV-5 status. However, rises in titer occurred in 15 of 27 susceptible dogs completing training in January 1970. The findings at Fort Benning were similar to LAFB, in that after a period of little virus activity, there was an increased rate of infection. Further observations on the epidemiology of SV-5 infections are in progress.

During the investigation of respiratory disease outbreaks at Fort Benning, not all cases could be associated with SV-5 infection. In the investigations of outbreaks in 1967-1968, SV-5 infections could only be demonstrated in approximately 40% of the cases. In view of this and also of disclosures of new canine viral agents, additional serological studies were done on sera obtained during the 1967-1968 Fort Benning study. Findings are summarized in Table 1. With the exception of canine herpes, antibody was detected in varying degrees to each virus in the acute phase serum. More than 90% of the initial sera had antibodies to canine distemper and canine adenoviruses and probably reflected previous immunizations. The high prevalence of canine parvovirus antibody (96%) was consistent with observations in other studies. The reactivity found in the acute phase serums against the canine rhinovirus and coronavirus isolates were often at the lowest levels tested and should be interpreted cautiously. Ten of 30 dogs tested had an increased antibody titer to SV-5. Increases in antibody titer were demonstrated in 8 dogs for canine parvovirus (4 of 26 dogs), canine coronavirus (3 of 31 dogs), and the canine rhinovirus L198T (1 of 31 dogs). Five of the 8 dogs also had increased SV-5 titers. Although viral infections by agents other than SV-5

Table 1. Serological Tests with Established and New Canine Viruses and Serums from Military Dogs with Respiratory Disease at Fort Benning 1967-1968.

Virus	Serological test	No. dogs with antibody in initial specimen Total tested (%)	No. dogs with increased antibody Total tested (%)
Parainfluenza SV-5	Neutralization	20/30 (66.6)	10/30 (33.3)
Canine distemper	"	11/12*(91.5)	0/12 (0)
Canine adenoviruses:			
Infectious canine hepatitis	"	32/32 (100)	0/32 (0)
Toronto A26/61	"	32/32 (100)	0/32 (0)
Canine herpes	"	0/31 (0)	0/31 (0)
Reovirus type I	Hemag. - inhibition	5/28 (17.8)	0/28 (0)
Canine parvovirus	"	25/26 (96.3)	4/26 (15.4)
Canine coronavirus**	Neutralization	4/31 (12.9)	3/31 (9.7)
Canine rhinovirus**			
3/68	"	14/21 (66.6)	0/21 (0)
L198T	"	20/31 (64.7)	1/31 (3.2)
A128T	"	8/29 (27.6)	0/29 (0)
A128Thr.	"	16/31 (51.7)	0/31 (0)

* Immunized with canine distemper-hepatitis vaccine.
** Provisional classification of WRAIR isolates.

were demonstrated in military dogs, they do not appear at this time to be important causes of respiratory disease.

2. Virus Studies of Newly Procured Laboratory Dogs.

Initial findings on viral studies of respiratory disease in newly procured "conditioned" mongrel dogs were previously presented (Annual Report 1 July 1968 - 30 June 1969). During quarantine 13 of 35 dogs developed respiratory disease and 5 died. Eight transmissible agents were recovered from 4 of 10 dogs with respiratory disease (Table 2). Specimens from 3 fatal cases were not available for culture. Both parainfluenza SV-5 and minute virus of canines (MVC) were recovered from each of 2 fatal cases which were cultured; in addition, 2 new viruses were isolated from 1 of these dogs (L198). Five of the 8 isolates were recovered from different cell cultures or from more than 1 specimen. Convalescent sera were available from the 2 surviving dogs from which SV-5 and canine herpes were isolated. A rise in SV-5 titer occurred in the first dog, but a rise in titer to canine herpes was not demonstrated in the other dog. Rises in titer to each of the 5 different isolated viruses were found among the 30 dogs surviving the quarantine period. The multiple isolations and the rises in antibody titer to each virus support the conclusion that each agent was present.

Each of the 2 new viruses, designated L198T and L198R, produced a rapid, distinctive, cytopathic effect (CPE). The L198T isolate produced an "enterovirus-like" CPE in the Walter Reed canine cell line (WRCC). High titer virus preparations produced CPE within 15 hours and end-points of titration were reached in 72-96 hours. Inclusion bodies were not seen in microscopic examinations of infected cells stained with hematoxylin and eosin (H&E). The L198R produced similar CPE in primary dog kidney and thymus cells. In the first 2 passages, focal clumping of cells occurred, and in the third and later passages, giant cells were seen. After H&E staining of infected cells, clumping of the chromatin and karyorrhexis were evident in the giant cells. Following recovery and establishment of each virus, the agents were purified by 3 terminal dilutions and virus preparations were made for characterization studies and immunization of rabbits. Antiserums against other viruses recovered from dogs, i.e., canine distemper (CD), canine infectious hepatitis (ICH), Toronto A26/61, canine herpes, parainfluenza SV-5, reovirus type 1, MVC, and rabies did not neutralize the 2 viruses.

The chemical and physical properties of L198T and L198R are summarized (Table 3). L198T was resistant to chloroform, ether, and 5-iododeoxyuridine (IUDR) but was labile at pH 3.0. The virus readily passed through a 50 nM filter which retained ICH virus. These properties are similar to the rhinoviruses in the picornavirus group. This conclusion was supported by electron microscope examination of partially purified negatively stained L198T virus. The virions were

Table 2. Viruses Recovered from Throat and Rectum Swab Specimens from 10 Laboratory Dogs with Respiratory Disease

Dog No.	Specimen	Agent recovered	Viral recovery in cell culture*	Serum neutralization test titer	
				Before onset	After onset
L133	Throat	Canine herpes	DK; DT	0	0
L198**	Throat	SV-5	DK	< 4	not available
		L198T	WRCC***	4	" "
	Rectum	L198R	DT, DK	< 4	" "
		MVC	WRCC	< 20	" "
L205	Throat	SV-5	DK; DT; WRCC	< 4	64
L204**	Throat	SV-5	DK; DT; WRCC	< 4	not available
	Rectum	MVC	WRCC	< 20	" "

* DK = primary dog kidney cell culture; DT = primary dog thymus cell culture; WRCC = Walter Reed canine cell line.

** Fatal respiratory disease.

*** Agent recovered on days 1, 2, and 6.

Table 3. Chemical and Physical Properties of Canine Virus Isolates L198T and L198R

Treatment	Loss virus titer (log 10) after treatment	
	L198T	L198R
Chloroform*	0.0	>3.0
Ether*	0.1	>3.0
pH 3.0	>2.8	0.8
50°C - 1 hr.	not done	3.0
5-Iododeoxyuridine**	+0.2	+1.1
Filtration		
100 nM (Millipore-VC)***	+0.3	1.2
50 nM (" -VM)***	+0.8	>2.7

* Reference resistant and susceptible viruses used as controls.
** Reference RNA and DNA viruses used as controls.
*** Reference viruses of known size was mixed with test virus for control purposes.

approximately 27 nM in diameter, had cubic symmetry and a few empty or hollow particles were evident. An attempt was made to relate L198T with other picornaviruses. Antiserums to the human picornaviruses, poliovirus types I to III, Coxsackie A-9 and B-1 to B-6, Echo 1 to 32, and 6", human rhinoviruses (20 types) and encephalomyocarditis virus did not neutralize the L198T isolate.

L198R virus was susceptible to chloroform and ether treatment, and resistant to pH 3.0 and IUDR. The virus passed through a 100 nM filter which retained SV-5, but not through a 50 nM filter. These findings indicate that L198R is a middle sized enveloped RNA virus. To more definitely classify the virus, ultrathin sections of infected cell cultures were examined by electron microscopy. [Electron microscope examinations were done by Dr. A. Strano and Mr. W. Engler of the Armed Forces Institute of Pathology.] Virus particles were found in cytoplasmic vesicles. The virions had envelopes and measured 60-100 nM with a central core of 50-60 nM. These findings suggest that L198R virus may be a member of the coronavirus group. The L198R isolate was serologically compared with other similar viruses. It was not neutralized by infectious bronchitis virus (Mass. and Conn. strains) polyvalent mouse hepatitis virus, rat coronavirus, lymphocytic choriomeningitis virus, rubella virus, respiratory syncytial virus, hog cholera virus, and bovine virus diarrhea antiserums.

Attempts were made to propagate the 2 viruses in a large variety of primary and continuous cell cultures (Table 4). The L198T isolate could be propagated only in canine continuous cell cultures, i.e., the WRCC and Madin-Darby canine kidney and not in primary or diploid canine

Table 4. Susceptibility of Primary and Continuous Cell Cultures to Canine Viruses L198T and L198R

Cell culture	Development of cytopathic effects w/Th virus	
	L198T	L198R
Primary:		
Dog kidney	0	+
Dog thymus	0	+
Human embryonic kidney	0	0
Rhesus monkey kidney	0	0
Afr. green monkey kidney	0	0
Rabbit kidney	0	0
Porcine kidney	0	0
Feline kidney	0	0
Hamster kidney	0	0
Bovine embryonic kidney	0	0
Chick embryo	0	0
Diploid:		
German shepherd embryo	0	+
Beagle embryo	0	+
Human Wl-38*	0	0
Equine testicle (MA188)	0	0
Continuous:		
Walter Reed continuous canine (WRCC)	+	0
Madin-Darby canine kidney	+	0
Human HEp #2	0	0
Baby hamster-kidney 21	0	0
Madin Darby bovine kidney	0	0

* The Wl-38 cells were susceptible to human rhinovirus type 4 strain 1660.

cell cultures tested. In contrast, the L198R isolate produced CPE in primary and diploid canine cell cultures but not in the continuous cell cultures. L198T and L198R viruses were not pathogenic for suckling mice, ferrets and rabbits. The L198R virus was also not pathogenic for weanling mice, guinea pigs and suckling hamsters. The hamsters did not develop tumors 7 months after subcutaneous inoculation. The restrictive growth of these 2 viruses in canine cell cultures and the failure of antiserums to human picornaviruses to neutralize L198T and antiserums to the coronaviruses and other RNA enveloped viruses to neutralize L198R suggest these are new canine viruses. Further studies of these 2 viruses are in progress.

Serological tests were done to measure the spread of canine viruses during the quarantine period. Upon arrival at the conditioning facility, approximately 80% of the dogs had antibody to CD and ICH viruses, reflecting previous immunization. Antibody to MVC was present in 58.8%, to parainlfuenza SV-5 in 28.6%, and lower percentages (8.6% to 25.7%) of the dogs had antibody to canine herpesvirus, reovirus type I, and the provisional canine rhinoviruses and canine coronavirus (Table 5). After quarantine, increased antibody titers were found in each of the recovered viruses and CD, ICH, and A26/61 viruses. The rises to CD and ICH viruses may reflect recent vaccination. Para-influenza SV-5 was the most communicable, infecting 63.4% of the dogs and MVC was second, infecting 46.2%. Only 10% of the dogs had evidence of canine herpes infection. The 2 new canine viruses L198T and L198R did not appear to be highly communicable, as only 6.7% (2/30) and 13.3% (4/30) had rises to L198T and L198R, respectively. The 3 other canine rhinoviruses (described below) and reovirus type I did not appear to be active in this population. Many of the dogs had more than 1 viral infection.

The findings in the present study are consistent with previous studies of respiratory disease in "non-conditioned laboratory dogs." In the earlier investigation, the presence of multiple agents was observed with a high communicability of SV-5 and low of canine herpes, and multiple virus infections occurring in many dogs (Am. J. Vet. Res. 31: 697, Apr. 1970). Another striking parallel with previous observation was the high incidence of fatal respiratory disease in dogs without CD virus antibody, and the occurrence of non-fatal disease in dogs with CD virus antibody. In the present study, 4 of 7 dogs susceptible to CD infections died, while only 1 of 27 of the resistant group died.

3. Recovery of a Canine Rhino-like Virus from a Bio-Sensor Dog at the University of Maryland.

In February 1968, investigators under contract to the Army at the University of Maryland, requested professional veterinary assistance for a breeding problem in a group of dogs. During physical examination of a former scout dog, specimens for virus isolation were

Table 5. Serological Tests with Established and New Canine Viruses and Serums from Conditioned Laboratory Dogs with Respiratory Disease

Virus (strain)	Test	No. dogs with antibody in initial specimen Total tested (%)	No. dogs with increased titer/ Total tested			
			Sick	Contact	Total	%
Parainfluenza SV-5 (D008)	Neut.	10/35 (28.6)	4/8	15/22	19/30	(63.4)
Canine distemper (Onderstepoort)*	"	27/34 (79.5)	1/7	1/1	2/8	(25.0)
Canine adenoviruses						
Infectious hepatitis (Cornell)*	"	28/35 (80.0)	1/8	1/21	2/29	(6.9)
Toronto (A26/61)	"	28/35 (80.0)	1/8	1/22	2/30	(6.7)
Canine herpes	"	3/35 (8.6)	1/8	2/22	3/30	(10)
Reovirus Type 1	Hemag. Inhib.	7/35 (20.0)	0/8	0/22	0/30	(0)
Canine parvovirus	Neut.	20/34 (58.8)	5/7	7/19	12/26	(46.2)
Canine coronavirus**	"	3/35 (8.6)	0/8	4/22	4/30	(13.3)
Canine rhinoviruses**						
(3-68)	"	9/35 (25.7)	0/8	0/22	0/30	(0)
(L198T)	"	8/35 (22.8)	0/8	2/22	2/30	(6.7)
(A128T)	"	6/35 (17.1)	0/8	0/22	0/30	(0)
(A128Thr.)	"	3/35 (8.6)	0/8	0/22	0/30	(0)

* Immunized with canine distemper-hepatitis vaccine.

** Provisional classification of WRAIR isolates.

obtained from the penis by swabbing the proximal end and a blood specimen for serological studies was obtained. A transmissible cytopathic agent was obtained in the WRCC line. The agent was reisolated and also recovered from a second similar specimen obtained a month later. A rise in neutralizing antibody titer for the isolates was demonstrated. The agent, designated 3-68, produced an "enterovirus-like" CPE similar to L198T virus. Inclusion bodies were not evident in H&E stained infected cells. The virus produced CPE in only the WRCC and Madin-Darby continuous canine kidney cells and not in any of the other cell cultures listed in Table 4. The virus was not pathogenic for suckling or adult mice, ferrets, or rabbits.

The chemical and physical properties of 3-68 virus was similar to those of L198T virus. The virus was resistant to chloroform and ether, significantly inactivated at pH 3.0, and passed through a 100 nM and 50 nM millipore filter without loss of titer. The growth of 3-68 virus was not inhibited by IUDR. Cross-neutralization between the 3-68 and L198T viruses with reference rabbit serums did not occur.

4. Recovery of Two Viruses from Laboratory Foxhounds.

The introduction of a new source of dogs into the WRAIR quarantine facility provided an opportunity to conduct prospective studies of respiratory disease and to enlarge our limited knowledge of dog viruses. In December 1968, the first shipment of foxhounds from Florida arrived at WRAIR. Throat and rectal specimens and a blood specimen were obtained from each dog on the day of arrival. A second blood specimen was obtained 25 days later. Respiratory disease did not occur in this group of dogs. The throat and rectal specimens were inoculated into primary dog kidney and the WRCC line. From the throat specimen of foxhound A128, two transmissible agents were recovered. One virus, A128Thr., produced CPE in primary dog kidney and the other, A128T, in the WRCC line. Each agent was reisolated and a rise in antibody titer to the agent recovered in WRCC line was demonstrated, but the same titer, 1:4, occurred with A128Thr. in the arrival and 25 day serums. Detailed characterization studies are in progress. However, initial studies indidate both viruses are small (<50nM), naked, and labile at pH 3.0. Cross-neutralization tests of A128T, L198T and 3-68 viruses resulted in the demonstration of partial cross-neutralization by A128T serum of L198T virus. The homologous titer was 16-times higher than the heterologous titer.

Further studies on the growth of the viruses in different cell cultures, animal pathogenicity, relationships to other viruses, and occurrence in dogs are in progress.

5. Canine Brucellosis.

Brucella canis infections had been found to be enzootic in WRAIR breeding colony of 300 female and 14 male beagle dogs at Cumberland, Va. The infections were responsible for a high incidence of abortion and sterility. In October 1969, a concerted effort was made to eradicate the disease from the WRAIR colony. On the basis of the known association of B. canis bacteremia and B. canis agglutinins in infected dogs (Moore, J. A. and Gupta, B. N., J.A.V.M.A. 156: 1737, 1970), a program of bacteriological and serological testing was initiated to identify and eliminate carrier dogs. All dogs were tested at approximately monthly intervals for the presence of B. canis agglutinins. Animals seropositive at titers of 1:200 or greater were quarantined. Quarantine animals were eliminated from the colony only after B. canis was cultured from the blood. Animals that were culturally-negative on repeat examinations were readmitted to the colony providing agglutinin titers had receded to titers below 1:200.

The scheme for culturing and identifying B. canis is as follows: Approximately 5 ml of blood is drawn directly from the dog's jugular vein into a blood culture bottle containing 50 ml of Tryptic Soy Broth (Difco Laboratories). Blood cultures are incubated aerobically at 37°C. Subcultures onto Brucella Agar (Albimi Laboratories) or Brain Heart Infusion Agar (Difco Laboratories) containing 0.05% sodium thiosulfate are made every 3-4 days for 2 weeks. A 10 unit bacitracin and a 300 unit polymyxin B sensitivity disc (Difco Laboratories) are placed on the area of heaviest inoculation. Above the same area a lead acetate strip is suspended from the lid of the culture plate. Subcultures are examined after 2-3 days, aerobic incubation at 37°C. Brucella canis grows slowly and typical colonies are circular, entire, convex, smooth, and translucent. The organism is resistant to polymyxin B and bacitracin and produces H_2S. Suspect colonies are further verified by the absence of growth on MacConkey's or EMB agar and rapid (5 to 10 minutes) hydrolysis of urea broth when incubated at 52°C.

Since the eradication program was initiated, the incidence of significant serological reactions (e.g., titers of 1:200) has been reduced from 4.1% to 1.3% and was consistent with decreased incidence of inapparent infection and abortion.

Summary and Conclusions.

1. Respiratory Disease in Military Dogs.

Studies were continued on the etiology and epidemiology of respiratory disease in military dogs. Parainfluenza SV-5 infections continued to occur at the Lackland Air Force Base induction center and at the Fort Benning training center in 1969 and 1970. More than 90% of the newly procured dogs are susceptible to SV-5 infection at time of induction.

Antibody studies were carried out in dogs with respiratory disease to other known and new canine viruses. A lower rate of infection to 3 new canine viruses was found. However, the incidence of infection suggests that they do not play a large role.

2. Virus Studies of Newly Procured Laboratory Dogs.

Virus studies were carried out on a group of "pre-conditioned" laboratory dogs which developed respiratory disease during conditioning. In addition to SV-5 and the minute virus of canines (MVC), 2 new canine viruses L198T and L198R were isolated. The new viruses have the properties of a rhinovirus and a coronavirus. Serological tests indicated the presence of at least 5 viruses during the conditioning period. SV-5 and MVC were the most communicable. Increased antibody titers to the 2 new canine virus isolates were also found.

3. Recovery of a Canine Rhino-like Virus from a Bio-Sensor Dog at the University of Maryland.

A rhino-like virus (3-68) was recovered from the penis of a bio-sensor dog at the University of Maryland. The dog developed a rise in titer to the isolated virus. The isolate is not antigenically related to other canine viruses.

4. Recovery of Two Viruses from Laboratory Foxhounds.

Two small acid labile viruses, A128T and A128Thr., were recovered from the throat of an apparently normal laboratory foxhound. Both agents were reisolated and a rise in titer occurred in the serum one month later to one of the isolates. Antiserum to the A128T isolate neutralized the L198T virus at 1/16 homologous titer. Further studies are in progress.

5. Canine Brucellosis.

The incidence of inapparent and frank B. canis infections (manifested by abortions and infectivity) in the WRAIR beagle dog colony has been reduced by a program of serological and bacteriological testing to identify and eliminate carrier animals.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 184, Diseases of Recruit military animals

Literature Cited.

1. Swango, L. J., Eddy, G. A., and Binn, L. N. Serologic comparisons of infectious canine hepatitis and Toronto A26/61 canine adenovirus. Am. J. Vet. Res. 30(8): 1381-1387, 1969.

2. Binn, L. N., Lazar, E. C., Helms, J., and Cross, R. Viral antibody patterns in laboratory dogs with respiratory disease. Am. J. Vet. Res. 31(4): 697-702, 1970.

3. Binn, L. N., Lazar, E. C., Eddy, G. A., and Kajima, M. Recovery and characterization of a minute virus of canines. Infect. & Immun. 1(5): 503-508, 1970.

4. Gillespie, J. H., Carmichael, L. E., Gourlay, J. A., Dinsmore, J. R., Abbott, G. W., Binn, L. N., Cabasso, V., Fox, F. H., Gorham, J. R., Ott, R. L., Peacock, G. V., and Sharpless, G. R. Report of the panel on symposium on immunity to selected canine infectious diseases. JAVMA 156(12): 175-182, 1970.

5. Binn, L. N. A review of viruses recovered from dogs. JAVMA 156(12): 1672-1677, 1970.

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7. Huxsoll, David L., and Hemelt, Irene E. Clinical observations of canine herpesvirus. JAVMA 156(12): 1706-1713, 1970.

8. Binn, L. N., and Lazar, E. C. Comments on epizootiology of parainfluenza. JAVMA 156(12): 1774-1777, 1970.

9. Swango, Larry J., Wooding, W. L. Jr., and Binn, L. N. A comparison of the pathogenicity and antigenicity of infectious canine hepatitis and the A26/61 virus strain (Toronto). JAVMA 156(12): 1687-1696, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OB 6446	70 06 30	DD-DR&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 07 01	K. Completion	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	185
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)*

(U) Veterinary Combat Surgery (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

012900 Physiology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
68 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	1	25
FISCAL YEAR CURRENT 70	1	25

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:* 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.

TELEPHONE: 202-576-5379

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

21. GENERAL USE

Foreign Intelligence Not Considered

ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Cyanoacrylates; (U) Veterinary surgical equipment

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

23. (U) To adapt newly developed veterinary surgical procedures to combat situations 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 07 - 70 06 The principal investigator studied veterinary combat surgical problems during a recent TDY tour in Viet Nam (Oct-Nov 69). The cyanoacrylate spray and a prototype canine field surgical table were taken to Viet Nam for field testing. Non-fatal combat wounds to military dogs were found to represent a small percentage of deaths. None were presented for treatment during above TDY. The prototype table and hemostatic spray were left with units for field evaluation. There is minimal aequirement for this type study, and the work unit is therefore terminated. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

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 to Termination:

1. A prototype canine surgical table for field use has been fabricated by the Division of Instrumentation, WRAIR. (Fig 1 through 5) 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. (Fig 4) The surface consists of two identical halves, hinged with a flexible material which also serves as a drainage trough. (Fig 4 & 5) 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. (Fig 2) The table surface is also capable of being tilted along its long axis (Fig 3) The surface of the table is slotted to improve drainage (Fig 5) Being removable from its base, it is potentially useful as a stretcher or litter. (Fig 5)

The table base of tubular stainless steel, a relatively light weight metal, will collapse for crating and movement. (Fig 1 & 2)

Dimensions and configuration of the table permits the surgeon closer proximity to the surgical field. The trough configuration of the surface minimizes the need for restraining straps and sandbags which are necessary on conventional flat surfaced surgery tables. (Fig 2 & 4)

The prototype table has been tested both in the laboratory and in the field. The following evaluation report has been forwarded from the 459th Veterinary Detachment in Bien Hoa, Viet Nam where the table is currently being utilized.

"1. Advantages

a. Detachable stretcher type top simplifies post surgical movement.
b. V-trough enhances dog immobilization after anesthesia has been administered.
c. V-trough adjustment further helps immobilization.

2. Disadvantages

1. Table is too heavy and bulky for easy transportation.
2. A prolonged time for constructing is necessary.
3. Height adjustment is inadequate, as longer legs had to be installed before use.
4. The table is much too unsteady. It has been found to be nearly impossible to keep a large Scout Dog on the table to administer anesthesia with the constant movement.
5. The table top is too short for the large Scout Dogs.
6. A stop should be placed on the V-trough, as dogs are squeezed when carried-sometimes being squeezed out of the stretcher.
7. The dips, cracks, and crevices are present in the top make it difficult to properly clean.
8. Snaps for carrying the legs together are not of sufficient strength."

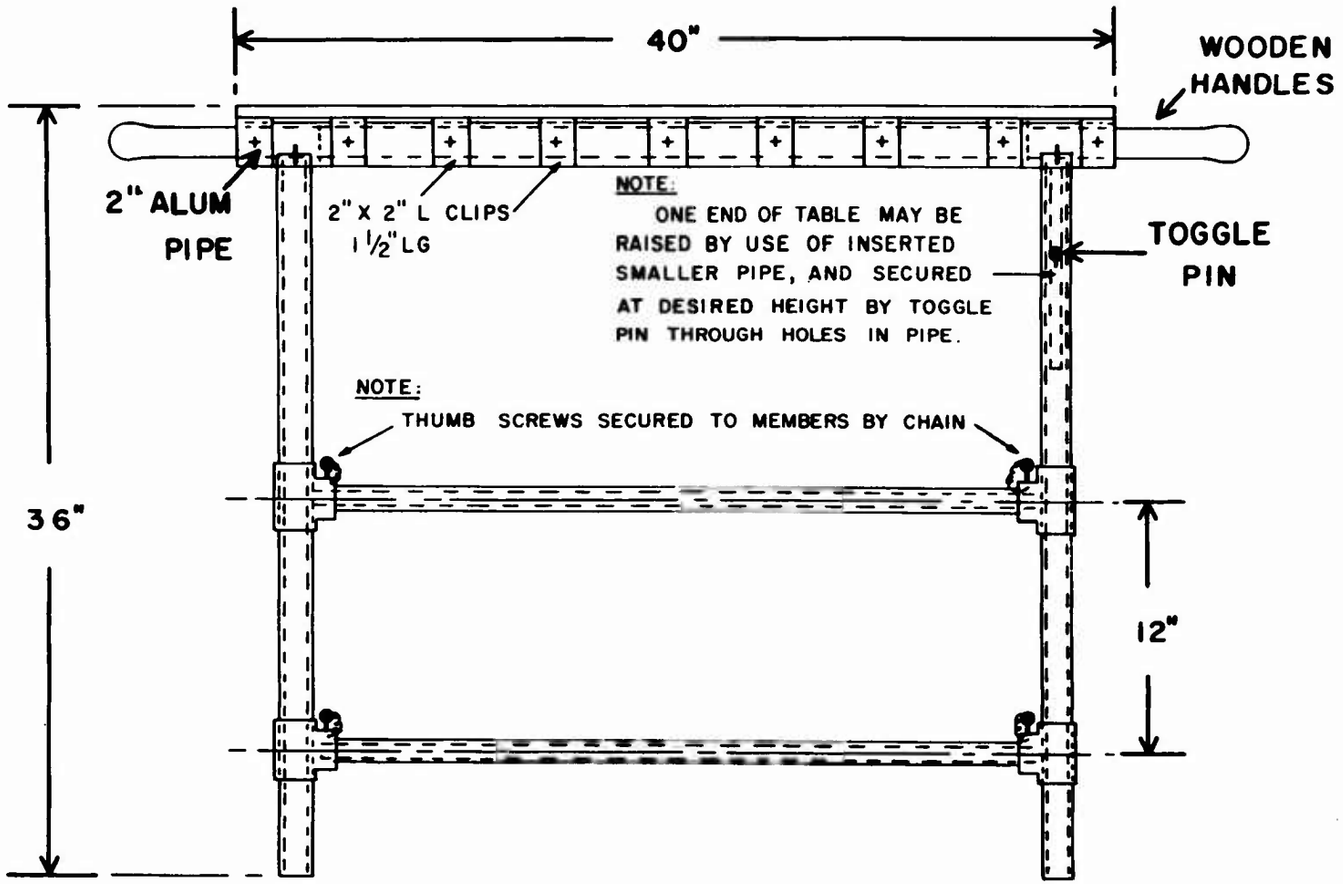
2. The cyanoacrylic hemostatic sprays developed at the Division of Experimental Surgery, WRAIR, were evaluated using the canine as the experimental model. These sprays have proven useful for hemostasis of solid organ and soft tissue trauma in humans. Their adaptability for field surgical procedures has been studied in humans and dogs and found to be very useful. The sprays have been extensively evaluated in the laboratory and found to be highly effective hemostatic agents in the canine, and have potential life-saving value for combat wounded canines.

During a recent six week TDY trip to Viet Nam (Oct-Nov 69), veterinary combat surgical problems were investigated. Two general categories of combat wounds occur to military dogs. The first is the fatal injury from missiles, mines, and booby traps. The second is injuries from shrapnel, fragments, and mortars. Wounds in the latter category are usually very superficial in nature. Medical evacuation procedures for wounded military dogs in RVN is efficient and effective. Most injured canines reach a veterinary hospital within 1-2 hours after injury. No combat wounded military dogs were presented to the 936th Veterinary Hospital during the above TDY; thus the cyanoacrylic sprays were not evaluated in actual field cases. A small supply of the cyanoacrylic hemostatic sprays was furnished to the major veterinary hospitals in Viet Nam for experimental use, after demonstration of their proper use. Cases in which the spray is used will be evaluated, and all data, including necropsy results, will be forwarded to WRAIR.

3. Pneumosplint:

A commercially available inflatable splint (Readisplint; 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 & punctures. A desirable feature of the device is the hemostatic effect of the uniformly applied pressure.

There is a very low incidence of combat wounded military dogs in RVN whose wounds are amenable to surgical treatment. Further studies under this work unit are unwarranted at this time. This constitutes a final report.


40"
WOODEN HANDLES
2" ALUM PIPE
2" X 2" L CLIPS 1½" LG
NOTE:
ONE END OF TABLE MAY BE RAISED BY USE OF INSERTED SMALLER PIPE, AND SECURED AT DESIRED HEIGHT BY TOGGLE PIN THROUGH HOLES IN PIPE.
TOGGLE PIN
NOTE:
THUMB SCREWS SECURED TO MEMBERS BY CHAIN
36"
12"

FRONT

Fig 1

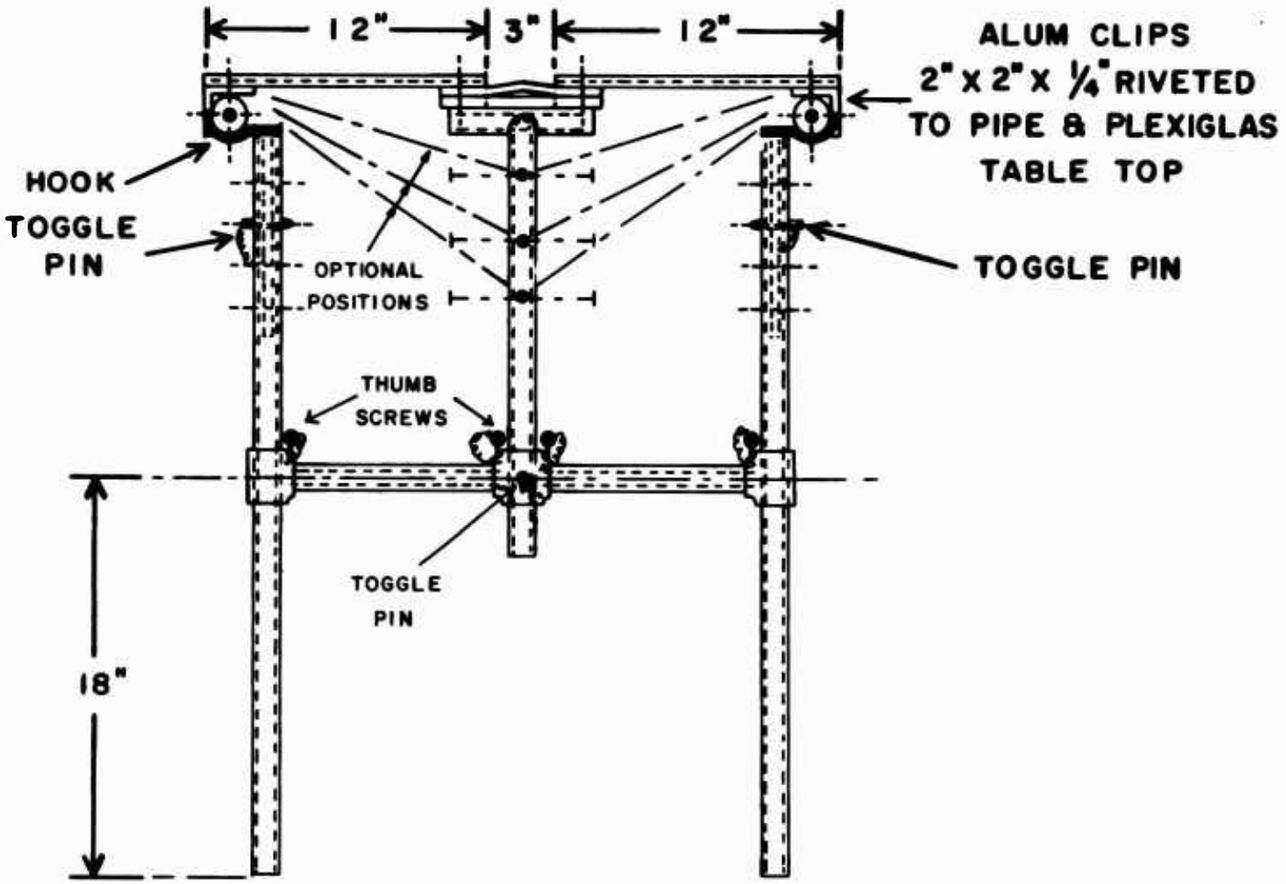
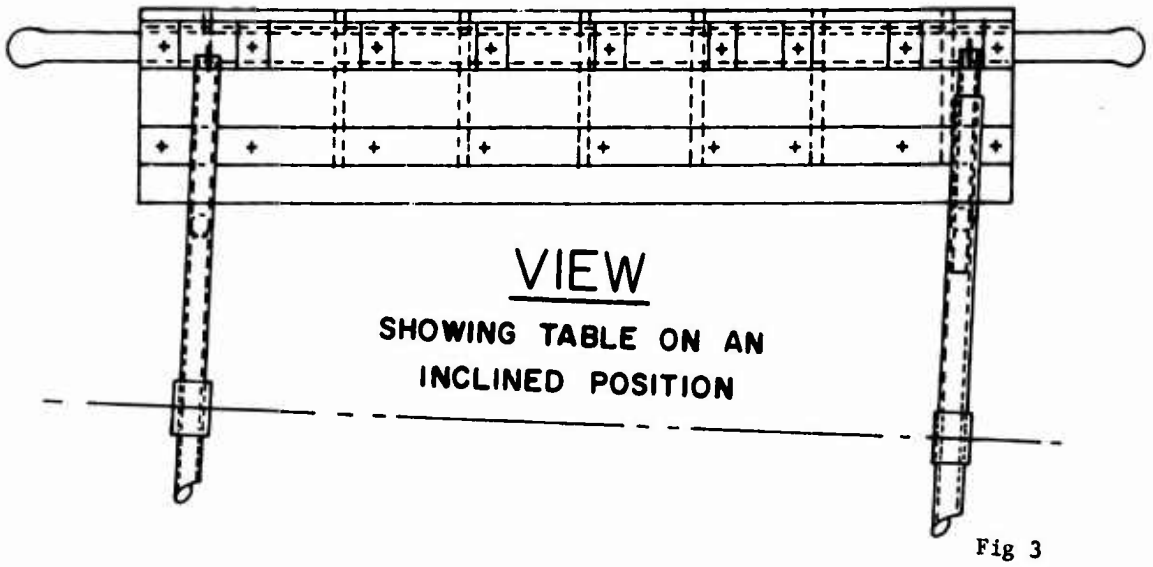

12"
3"
12"
ALUM CLIPS
2" X 2" X ¼" RIVETED
TO PIPE & PLEXIGLAS
TABLE TOP
HOOK
TOGGLE
PIN
OPTIONAL
POSITIONS
TOGGLE PIN
THUMB
SCREWS
TOGGLE
PIN
18"

Fig 2

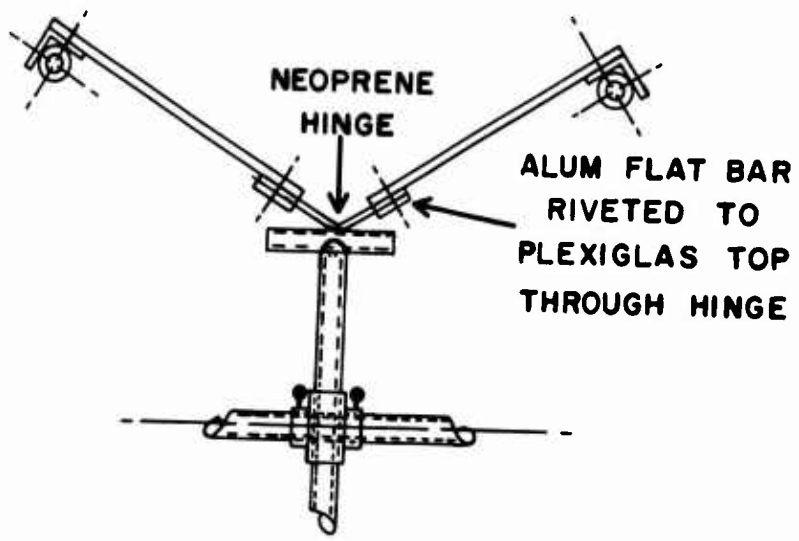
END



VIEW

SHOWING TABLE ON AN INCLINED POSITION

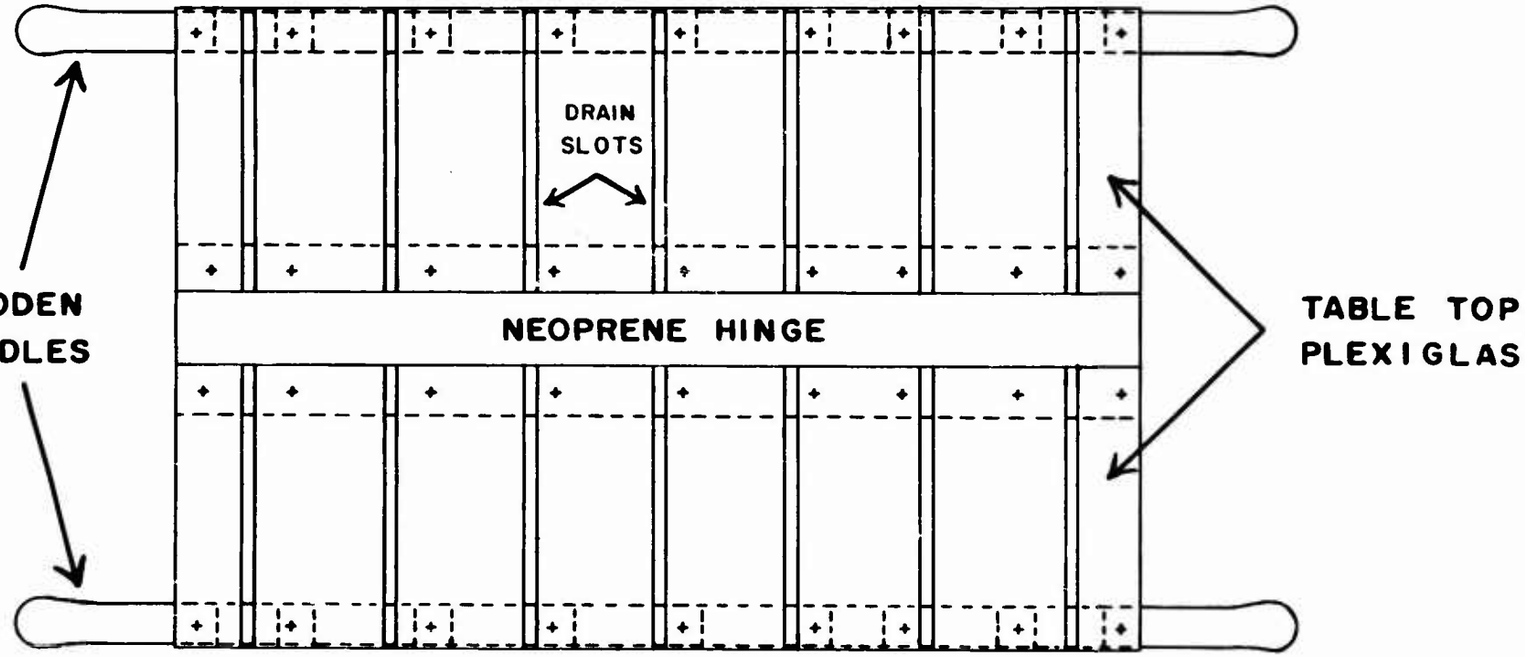
Fig 3



END VIEW

SHOWING TABLE IN DEPRESSED POSITION

Fig 4


DRAIN
SLOTS
WOODEN
HANDLES
NEOPRENE HINGE
TABLE TOP
PLEXIGLAS

TOP

Fig 5

PROJECT 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 185 Veterinary combat surgery

Literature Cited

1. References: None

2. Publications: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA OB 6447	70 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 07 01	H. Termination	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	186
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede 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. PERFORMANCE METHOD
68 10	CONT	DA			B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 69 10 EXPIRATION: 70 06
b. NUMBER: DADA 17-69-C9029
c. TYPE: U. CPFF d. AMOUNT: None
e. KIND OF AWARD: CON f. CUM. AMT. $164,955

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 69	2	122
	CURRENT 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 participation)
NAME: Berk, B.
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Artenstein, M.S.
NAME: 3

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code) (U) Meningococcal Polysaccharide A; (U) Meningococcal Polysaccharide C; (U) Neisseria meningitidis

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) - 69 07 - 70 01 Approximately 20 grams (350,000 doses) of group C polysaccharide were produced in 100 liter fermentation cultures and have satisfied all safety and sterility tests. An equal quantity of group A polysaccharide is undergoing final sterility testing. Term of the contract expired 70 01 31 and a final report is pending. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 -30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 67 1498-1 (FOR ARMY USE)

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 186, Production of meningococcal polysaccharides, types A & C

Investigators.
Principal: Bernard Berk, Ph.D., Squibb Institute for Medical Research
Associate: Malcolm S. Artenstein, M.D.

Description.

Approximately 40 grams of meningococcal polysaccharide vaccine group C and group A have been prepared for immunization of humans.

Progress.

Using a 100 liter capacity fermentation process, 18 grams (approximately 350,000 doses) of group C vaccine have been prepared and bottled as 10 dose and 50 dose lyophilized vials. This material has satisfied all specifications and requirements for human use. An equal amount of group A polysaccharide vaccine has been prepared and is undergoing final assays. Experimentation with lactose or mannitol instead of sodium chloride as a carrier material showed no differences in the molecular size of the final lyophilized product; thus, sodium chloride was used.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 186 Production of meningococcal polysaccharides, types A & C

Literature Cited: None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6449	70 06 30	DD-DR&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 07 01	H.Termination	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	187
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Computer Aided Printing of Chemical Structures (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012100 Organic Chemistry; 019600 Display Devices and Equipment

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 01	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	0	5
FISCAL YEAR CURRENT 70	0.1	5

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: 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: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Typesetting; (U) Computers; (U) Publishing

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

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 or any Army program involving chemicals and chemical structures, 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 07 - 69 12 Debugging of the preliminary program written to test the logic to be used in converting typewriter to typesetter font has been done. The next step is to produce the font and test on Government Printing Office machines. Work unit was terminated 69 12 31 due to the transfer of the principal investigator and lack of competent replacement. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

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: Alfred P. Feldman, M.S.

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.

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). The test programs have been debugged. The font must still be produced and tested. This work unit was terminated 69 12 31 due to the transfer of the principal investigator.

1. D. P. Jacobus, D. E. Davidson, A. P. Feldman and J. A. Shafer: "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).

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 187, Computer aided printing of chemical structures

Publications

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6451	70 06 30	DD-DR&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 07 01	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	188
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Behavioral Baselines for the Experimental Study of Uremia (21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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. DATES/EFFECTIVE: 70 02 EXPIRATION: 70 06
b. NUMBER: DADA 17-69-C-9094
c. TYPE: U.CPFF d. AMOUNT: $21,532
e. KIND OF AWARD: EXT f. CUM. AMT. $65,161

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 69	0.5	18
FISCAL YEAR — CURRENT 70	1	43

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 (Furnish SSAN if U.S. Academic Institution)
NAME: Taub, E.
TELEPHONE: 301-585-3915
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Cirksena, LTC W. J.
NAME: Carter, MAJ C. B. 2

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Uremia; (U) Behavioral Baselines; (U) Kidney; (U) Body Fluids; (U) Hemodialysis

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number, 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 07 - 70 06 - In the past it has been demonstrated that repetitive counting tasks can be taught to rhesus monkeys and that the experimental induction of uremia will interfere with the accuracy of counting. In addition, peritoneal dialysis has been shown to correct the defects induced by uremia. During this year, attempts were made to mimic uremia, as measured by disturbances in counting behavior, by infusions of urea into animals with bilateral ureteral obstruction. In this manner elevation of urea concentration is possible without corresponding elevation of other uremic metabolites. In the two animals studied this year, elevation of BUN levels to 400 mg/100 ml have lead to disturbances in counting behavior and EEG disturbances similar to uremic monkeys. This level of BUN is the same as those noted to be associated with counting disturbances in the natural course of uremia. Urea may be involved in the pathogenesis of uremia. However, the data available are insufficient to implicate urea as the cause of the uremic syndrome. Term of the contract expired 70 06 30 and a final report is pending. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 June 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 188, Behavioral baselines for the experimental study of uremia

Investigators: MAJ Charles B. Carter, MC and Edward Taub, Ph.D.

Description: This project consists of the study of neurobehavioral manifestations of uremia in rhesus monkeys by operant conditioning behavior techniques. Utilizing these techniques, an attempt is made to evaluate the effects of specific compounds in the pathogenesis of uremia.

Progress: In the past, it has been demonstrated that repetitive counting tasks can be taught to rhesus monkeys and that the experimental induction of uremia will interfere with the accuracy of counting. In addition, peritoneal dialysis has been shown to correct the defects induced by uremia.

During this year, attempts were made to mimic uremia, as measured by disturbances in counting behavior, by infusions of urea into animals with bilateral ureteral obstruction. In this manner, elevation of urea concentration is possible without corresponding elevation of other uremic metabolites.

Results: In the two animals studied this year, elevation of BUN levels to 400 ml/100 ml have lead to disturbances in counting behavior and EEG disturbances similar to uremic monkeys. This level of BUN is the same as those noted to be associated with counting disturbances in the natural course of uremia. Urea may be involved in the pathogenesis of uremia. However, the data available are insufficient to implicate urea as the cause of the uremic syndrome.

Conclusions and Recommendations: It has been possible to study uremia in the rhesus monkey by operant conditioning behavior techniques. This project has been terminated.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 188, Behavioral baselines for the experimental study of uremia (contract with Behavioral Rsch-Med)

Publications:

1. Teschan, P. E., Carter, C. B., and Taub, E.: Direct assay of toxic factors in uremia. (Abstract) The American Society of Nephrology Third Annual Meeting, 68, 1969.

2. Teschan, P. E., Carter, C. B., and Taub, E.: Experimental studies of toxic factors in uremic encephalopathy. NIAMD Conference on Uremic Toxins. Monterey, California, March 18-20, 1970 (to be published).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6450	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISS'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	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	189
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Preparation of Tubercular Antigens (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 03	CONT	DA	B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 70 03 EXPIRATION: 71 02
b. NUMBER: DADA 17-69-C-9108
c. TYPE: S. CT d. AMOUNT: P$10,336
e. KIND OF AWARD: EXT f. CUM. AMT. P$17,310

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	0.5	8
FISCAL YEAR CURRENT 71	0.3	7

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
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TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: George Washington University
ADDRESS: Washington, D. C. 20006

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Reich, Dr. M.
TELEPHONE: 202-331-6533
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Fife, E. H , Jr.
NAME:

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; (U) Immunofluorescence

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

23. (U) Preliminary studies indicate the SAFA test using purified tubercular antigen fractions superior to conventional tuberculin tests for early detection of active tuberculosis in monkeys. In addition, the SAFA test has shown potential for appraising the efficacy of tuberculosis therapy in humans and could play an important role in effective treatment and management of the disease. Present contract to supply specified quantities of A-protein, C-protein and polysaccharide tubercular fractions, for comprehensive evaluation of the SAFA procedure and for monitoring tuberculosis in the WRAIR non-human primate colony.

24. (U) By procedures described by the investigator in the scientific literature.

25. (U) 69 07 - 70 06. Regular deliveries of the A-protein, C-protein and polysaccharide antigen fractions have been made according to the agreement in the contract. In addition, experimental antigens from atypical Mycobacterium species (photochromogens) are being prepared for evaluation in the SAFA test. Problems relating to deterioration of the C-protein antigen during storage have been encountered. Investigations to devise methods for overcoming this difficulty are in progress. Findings thus far indicate that the polysaccharide antigen is superior to the others for detection of simian tuberculosis, and that the C-protein antigen is best for detection of human tuberculosis. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon ord/holder's approval.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 189, Preparation of tubercular antigens

Investigators.
Principal: Melvin Reich, 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 and his associates are used to isolate A-protein, C-protein and polysaccharide antigens from the culture filtrates and cell walls of M. tuberculosis. These antigens are used by investigators in the Department of Serology in studies on the serodiagnosis of simian and human tuberculosis.

Progress.

Regular deliveries of the A-protein, C-protein and polysaccharide tuberculin antigen fractions have been made to the Department of Serology, WRAIR, in accordance with the terms of the contract. 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 tubercular antigens

Literature Cited: None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6454	70 07 01	DD-DR&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 08 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	190
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with security Classification Code)

(U) Tropical Disease Bulletin Information Retrieval System (21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology; 004200 Computers

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 07	CONT	DA	B. CONTRACT

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 70 06 EXPIRATION: 71 05

b. NUMBER: DADA 17-69-C-9171

c. TYPE: U. CPFF

d. AMOUNT: $35,483

e. KIND OF AWARD: EXT

f. CUM. AMT. $63,018

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	1	30
FISCAL YEAR CURRENT 71	1	33

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: CompuMath, Inc.

ADDRESS: Silver Spring, Maryland 20910

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

[redacted]occola, H. W.

TELEPHONE: 301-587-3531

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Schafer, June A.

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

3

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Information Retrieval; (U) Information Handling; (U) Abstracts; (U) Computer Programming; (U) Text Processing

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 a computer system to create, maintain and index abstracts from the Tropical Disease Bulletin, and to develop manipulative capabilities such as the updating of the master file and sorting in certain fields within the file.

24 (U) The problem-oriented TEMAC language will be used because of its ability to handle variations in spelling and punctuation.

25 (U) 69 07 - 70 06 Production work to incorporate information from volumes one through sixty-five of the Tropical Disease Bulletin (TDB) into a machine searchable data base has been completed. Both a file containing all special characters from the original flexowriter input tapes and a second file with these special characters eliminated are available for searching. The Title and Author Index for all volumes has been completely processed. Preliminary experimentation on a screening technique for rapid searching has begun. Time tests have been made to compare screen searching to character by character searching. Further analysis and experimentation is needed to develop an optimum set of screens for rapid search. The Subject Index File will be completed and used as an interim search tool and as a control on screen search output. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 190, Tropical Disease Bulletin information retrieval system

Investigators:
Principal: Harry W. Voccola
Associates: June A. Schafer; CPT Robert O. Pick, MSC

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 the programs written in TEMAC and MAP have been used to build and correct the file needed by the Tropical Disease Bulletin project. In addition, subject, title, and author indices have been created for use by the Tropical Disease Bulletin project and the Project EXPERT system. In addition preliminary experiments have been made with two different search techniques. The first type is a character by character comparison to identify words or parts of words. Studies have been done on the comparative speed and accuracy of the two techniques. The future work includes conversion of the programs to the CDC 3300 so that future production and experimentation can be done at the WRAIR computer installation.

Summary and Conclusions:

This work has made excellent progress in that the entire data base has been successfully compiled into a machine searchable master file with appropriate indices. The preliminary experiments give good indication that the screen bit approach to full text searching will be fruitful. The capability of handling relatively unpurified text should be of broad interest to the Army Medical Service. With increased computer capabilities at WRAIR we look forward to bringing this project in-house. Conversion of the programs to the WRAIR configuration is the first step toward that goal.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 190, Tropical Disease Bulletin information retrieval system

Publications

None.

PROJECT 3A061102B71Q
COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00
Communicable Diseases and Immunology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6440	70 07 01	DD-DR&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 07 01	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	61102A	3A061102B71Q	00	165
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Parasitic Diseases (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	CONT	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	70	3	75
FISCAL YEAR — CURRENT	71	3	75

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: MERONEY, COL W. H.

TELEPHONE: 202-576-3308

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of CD&I

ADDRESS: Washington, D. C. 20012

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

NAME: SADUN, E. H., Sc.D.

TELEPHONE: 202-576-3308

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

21. GENERAL USE

Foreign intelligence not considered

ASSOCIATE INVESTIGATORS

NAME: MOON, A. P. DA

NAME:

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Parasite; (U) Serology; (U) Immunology; (U) Antibody; (U) Allergy
(U) Hypersensitivity; (U) Schistosomiasis; (U) Echinococcosis

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

23(U) The purpose of this research is to study various physiological, immunological and ecological aspects of parasitic diseases of military importance toward the goal of gaining a better understanding of natural susceptibility, acquired resistance and the effectiveness of therapeutic agents for the prevention, suppression and treatment of these infections.

24(U) Through careful perusal of pertinent literature and discussions with other scientists both classical and new methods are used to set up controlled experiments.

25(U) 69 07 - 70 06 The immunological response of human beings, chimpanzees, monkeys, rabbits, guinea pigs and mice to infections with Schistosoma mansoni or S. haematobium was studied by comparing antibodies detected by fluorescent, flocculating and anaphylactic reactions. Detailed parasitologic, serologic, clinical and pathologic studies in chimpanzees infected with Schistosoma haematobium were completed. There was a correlation between the findings. Chimpanzees are well suited for studies on the evolution of schistosomiasis haematobia. Two antigens were compared in a soluble antigen fluorescent antibody test for echinococcosis. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

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: E. Bueding, M.D.; A. W. Cheever, M.D.; R. E. Duxbury; LTC D. G. Erickson, MSC; MSG R. W. Gore; MAJ A. Hernandez, MSC; W. H. Hildreth; CPT R. Hoff, MSC; CPT A. J. Johnson, VC; SP4 C. E. Jones; H. R. Langbehn; F. von Lichtenberg, M.D.; A. P. Moon; CPT R. H. Perry, MSC; SP5 D. Rose; M. J. Schoenbechler; CPT T. J. Sullivan, MC; SP5 R. B. Tomlinson; J. S. Williams

1. Homocytotropic reagin-like antibodies in man and experimental animals with schistosomal infections.

Many serological tests are available for the laboratory diagnosis of schistosomiasis. These tests detect antibodies which appear to bear little relationship to the course of the infection, its clinical features or to the host's response. Therefore, none of these tests can be used to assess adequately the intensity of infection, prognosis or cure.

Helminthic infections frequently produce allergic clinical manifestations and immediate hypersensitivy reactions which can be detected by skin tests. Reagin-like antibodies in schistosomiasis have been reported recently. Some of these observations prompted a series of investigations to detect a possible relationship between homocytotropic antibodies and the clinical features of schistosomiasis or to the immunity which results from repeated infections. Ogilvie et al. detected reagin-like antibodies in rats and monkeys infected with Schistosoma mansoni, but attempts to induce reaginic antibodies artificially by vaccinating rats with freshly homogenized adult worms with or without Freund's adjuvant failed. Since the reaginic antibody appeared to be stimulated only by a living infection, they suggested that these antibodies might be responsible for immunity. Hsu and Hsu found that rhesus monkeys infected repeatedly with X-irradiated cercariae of S. japonicum may develop reaginic antibodies. However, they were unable to correlate the presence of this kind of antibody with resistance to a challenge infection. Furthermore, the transfer of sera containing passive cutaneous anaphylactic (PCA) antibodies did not show any protective effect against a challenging exposure. They concluded that reagins are not involved in acquired resistance to S. japonicum.

A series of investigations was conducted in this laboratory to gather evidence which might indicate relationships between the development of reagin-like antibodies and host resistance to schistosomal infections. Anaphylactic antibodies in man and experimental animals

were compared with conventional IgG and IgM antibodies detected by other serological tests. Preliminary accounts of some of these investigations have been presented elsewhere.

Irradiation was performed with a "Gamma-cell 200" cobalt 60 irradiator with strich adherence to the technic described previously.

Human Infections

A total of 118 serum specimens from infected individuals living in hyperendemic areas was studied. All diagnoses were confirmed by the presence of eggs in the stools or urine. In addition, 14 serum specimens from healthy individuals from nonendemic areas were used as controls.

Experimental Animals

Eighteen West African chimpanzees (Pan satyrus), presumably free of schistosome infections as determined by repeated stool examinations, were exposed to single and multiple infections with varying numbers of S. mansoni or S. haematobium cercariae. In addition, seven chimpanzees were used as uninfected controls. All of these animals were bled at monthly intervals beginning with the day they were exposed to infection until the time of necropsy.

Four rhesus monkeys (Macaca mulatta) were exposed to single doses of 1,[illegible] cercariae each of S. mansoni. They were bled every two weeks from the time of exposure until the end of the experiment.

Nine cercopithecus monkeys (Cercopithecus sabaeus) were exposed to 100 S. mansoni cercariae each and were bled 11 and 19 months after exposure. One additional animal served as an uninfected control.

Twelve albino rabbits were exposed to either 1,000, 5,000 or 25,000 S. mansoni cercariae each and were bled at weekly intervals. Thirty-six weeks after the initial exposure, the 11 surviving animals were re-exposed to 5,000 cercariae each. Three additional rabbits were used as uninfected controls.

Five guinea pigs were exposed to 100 S. mansoni cercariae each and were bled at regular intervals up to 10 weeks after exposure. Two guinea pigs served as uninfected controls.

Over 300 ICR, Bagg and Hairless male mice were exposed to varying numbers of S. mansoni cercariae. Some received a single exposure, others were re-exposed once or twice as indicated in the experimental results. Some mice were given 100 Trichinella spiralis larvae each 4 weeks after exposure to S. mansoni. Other mice were exposed to 10,000 cercariae which had been irradiated with approximately 3,000 roentgens. Twenty mice were used as uninfected controls.

Testing of Sera

Passive cutaneous anaphylaxis (PCA) reactions were employed to detect homocytotropic antibodies. S. mansoni cercarial antigen was used throughout these studies. Serum specimens from human and subhuman primates were tested in the skin of rhesus monkeys weighing 2 to 3 kilograms each. The presence of homocytotropic antibodies in sera from other animlas was tested in the skin of recipients of the same species and strain as those of the donors. The PCA test was carried out with strict adherence to the published method. Cross reactions were regularly observed between S. mansoni antigen and S. haematobium infections.

Soluble antigen fluorescent antibody (SAFA), cercarial fluorescent antibody (FA) and slide flocculation (SF) tests were also conducted according to the methods previously described.

In a positive PCA test a blue spot develops at the intradermal injection site within 30 minutes after challenge. The resulting lesions were arbitrarily assigned ratings of 1+, 2+, 3+ and 4+ depending on their size and coloration. The size and the intensity of the bluing are roughly proportional to the amount of homocytotropic anaphylactic antibody present. Sera were treated with mercaptoethanol or heat and fractionation by gel filtration (Sephadex G-200) or by DEAE Sephadex chromatography as described previously.

Serological Reactions with Human Sera

Schistosome eggs had been found by stool or urine examinations in 118 patients from hyperendemic areas. Of these, 82 excreted S. mansoni eggs, 18 S. haematobium and 18 both S. mansoni and S. haematobium eggs. Homocytotropic (reaginic) antibodies were detected in rhesus monkeys from 83 of the specimens from infected individuals (70%), while none of the sera from healthy controls gave a positive PCA test (Table 1). The percentage of serum specimens reacting in the SAFA and SF tests among infected individuals from hyperendemic areas was higher than in the PCA test. There was no obvious correlation between the results of these three tests and age of the host, schistosome species, presence or absence of other helminthic infections and presence or absence of hepatosplenic disease in those cases in which such information was available. A direct correlation was observed between the antibody titer detected by the SAFA test and the proportion of specimens reacting in the PCA test (Table 2).

Serological Reactions from Experimentally Infected Animals

Serum specimens from 3 of the 4 chimpanzees exposed once to S. mansoni cercariae gave positive PCA reactions. No PCA antibodies were detected in any of the chimpanzees before the 5th month after exposure. In one animal PCA was no longer demonstrable 10 months after exposure; in another animal positive reactions persisted throughout the experiment 36 months after exposure (Fig. 1). S. mansoni worms were recovered at

necropsy from all the animals including the one in which no PCA antibodies were detected. Fluorescent antibodies were observed in all of the animals 1 to 2 months after infection, reached a peak in 6 to 10 months, and then declined gradually but remained elevated throughout the study (Fig. 1).

Table 1

Antibodies Detected in Human Sera with *Schistosoma mansoni* Antigen

Infection	No. sera tested	PCA Reactors		SAFA Reactors		SF Reactors	
		No.	%	No.	%	No.	%
S. mansoni	82	52	63	76	93	10	100
S. haematobium	18	13	72	17	94	18	100
S. mansoni and *S. haematobium*	18	18	100	18	100	18	100
Total	118	83	70	111	94	46	100
Normal controls	14	0	-	0	-	0	-

Table 2

Relationship Between Titers in the SAFA Test and Percent PCA Reactors in Human Sera

SAFA Reactors		Results of PCA Test	
Titer (reciprocal)	Number specimens	Number reacting	Percent
<8	7	1	14
8	25	15	60
32	33	23	70
128	27	21	77
512	26	22	85
Total	118	82	70

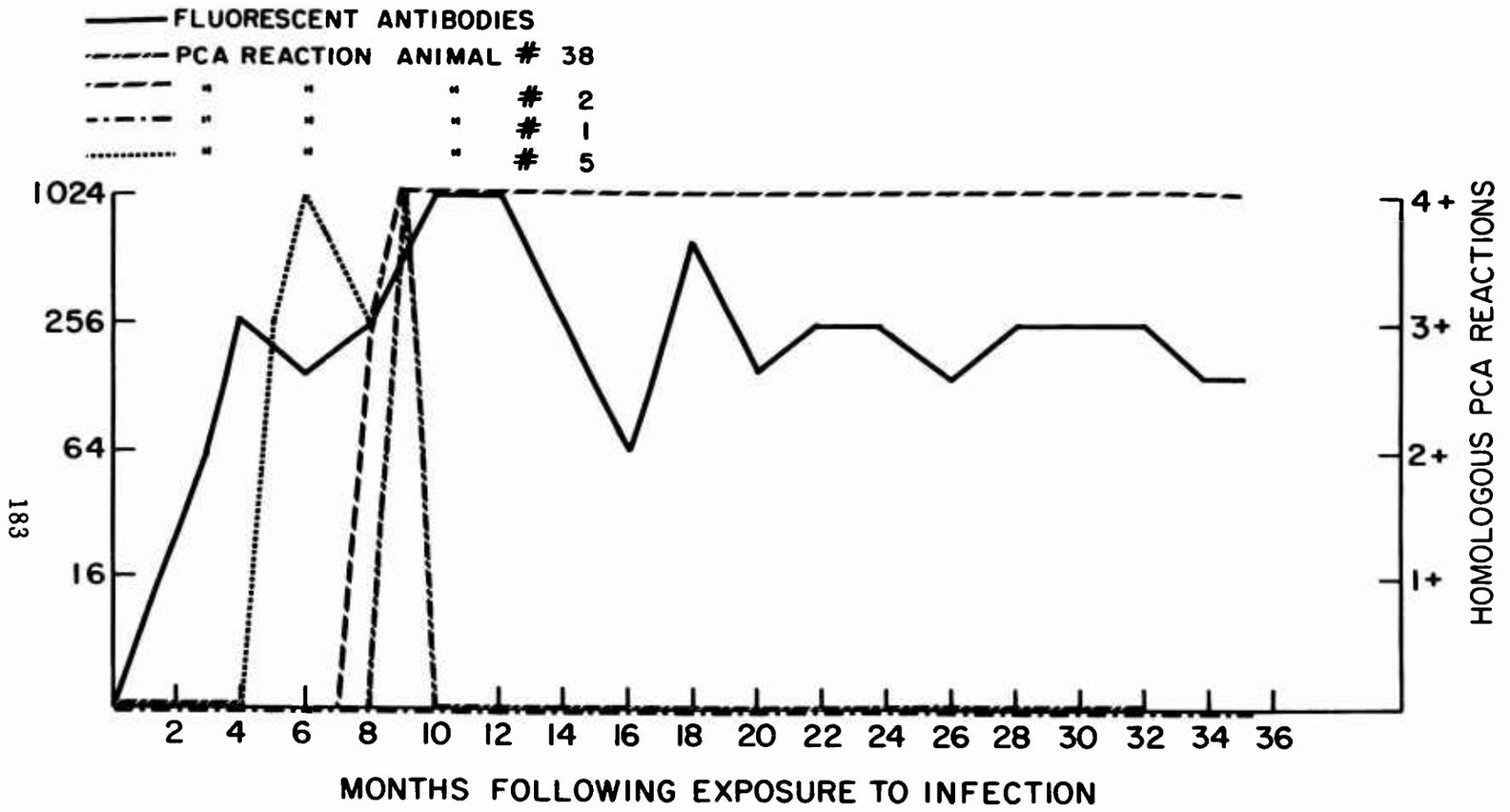

Fig. 1. Patterns of development of fluorescent and homologous PCA antibodies in chimpanzees exposed to a single dose of 1,000 S. mansoni cercariae.

The time-course development of reaginic antibodies varied in 4 chimpanzees exposed repeatedly to S. mansoni cercariae (Fig. 2). PCA antibodies were detected in the sera of 2 of the animals in the 6th and 10th month after infection. However, all 4 animals were infected since worms were found by perfusion at necropsy. Conversely, the course of antibody production as measured by the FA test was quite similar for all the chimpanzees in this group. Fluorescent antibodies were detected in the sera of all the animals. Serum titers increased rapidly and remained relatively constant in all animals for the duration of the experiment (Fig. 2).

In the chimpanzees exposed either once or repeatedly to S. haematobium cercariae the time-course development of PCA antibodies was irregular and transient as observed in S. mansoni infections (Fig. 3). However, the antibodies appeared much earlier in 3 of the 4 animals. The time-course development of fluorescent antibodies was essentially the same as that observed with S. mansoni infections in this species (Fig. 3), except that titers tended to decrease 9 months after exposure.

The anaphylactic antibody response varied in rhesus monkeys infected with S. mansoni also. PCA antibodies were detected in 2 of 4 animals as early as 2 months after exposure. In one of them PCA reactivity was no longer demonstrable 6 months after exposure to infection. However, with reinfection there was a prompt reappearance of PCA antibodies in the animal which had lost its reactivity as well as in the other two animals that did not react following a primary infection (Fig. 4). Fluorescent antibodies were demonstrable in all of the infected animals 2 months after infection; they reached a peak almost immediately, and then they remained elevated throughout the study. There was little change in titers following reinfection (Fig. 4).

The antibody reactions obtained with serum specimens from 10 cercopithecus monkeys are shown in Table 3. Flocculating antibodies at relatively low titers were demonstrated in 7 of 9 infected monkeys, but PCA antibodies were detected in only 2 of them. All of the animals in which no PCA antibodies were detected were found to be infected at necropsy.

The individual PCA reaction patterns for rabbits infected with 1,000, 5,000 and 25,000 S. mansoni cercariae are shown in Figures 5, 6 and 7 respectively. Anaphylactic antibody activity was seldom present in the sera obtained before the 6th week after infection and they did not persist beyond 20 weeks after initial exposure. Nine of the 11 sera from infected rabbits demonstrated anaphylactic antibodies after primary infection, although all were infected, as demonstrated at necropsy. After reinfection, 7 of the 9 animals which had anaphylactic activity after the initial infection showed a secondary response. The 2 animals which did not show a primary response did not develop anaphylactic antibodies after reinfection. Fluorescent antibodies were demonstrated 3 to 4 weeks after infection, they reached a peak in 8 to 10 weeks and remained elevated throughout this study. There was little

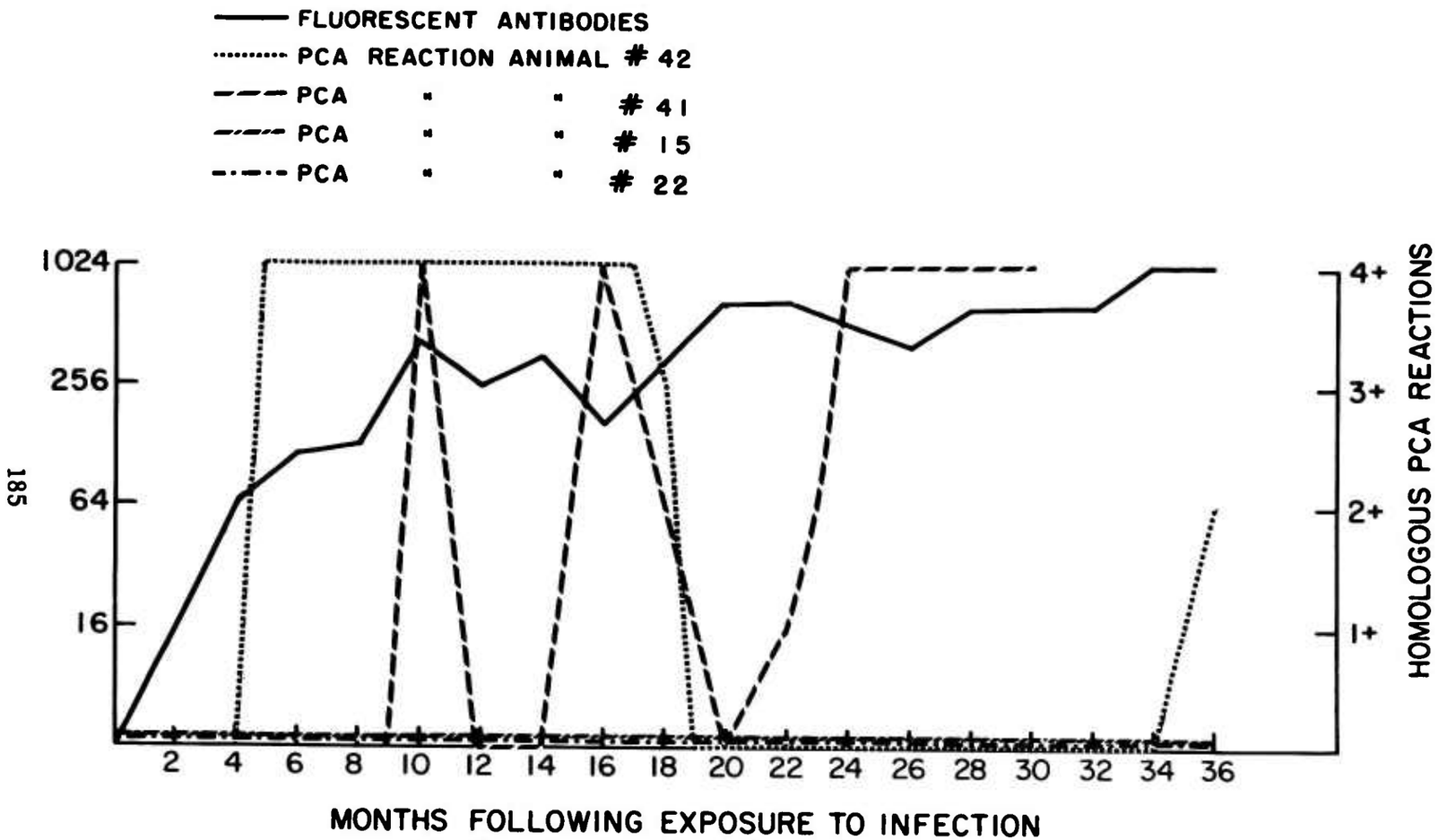

Fig. 2. Patterns of development of fluorescent and homologous PCA antibodies in chimpanzees exposed to monthly doses of S. mansoni cercariae.

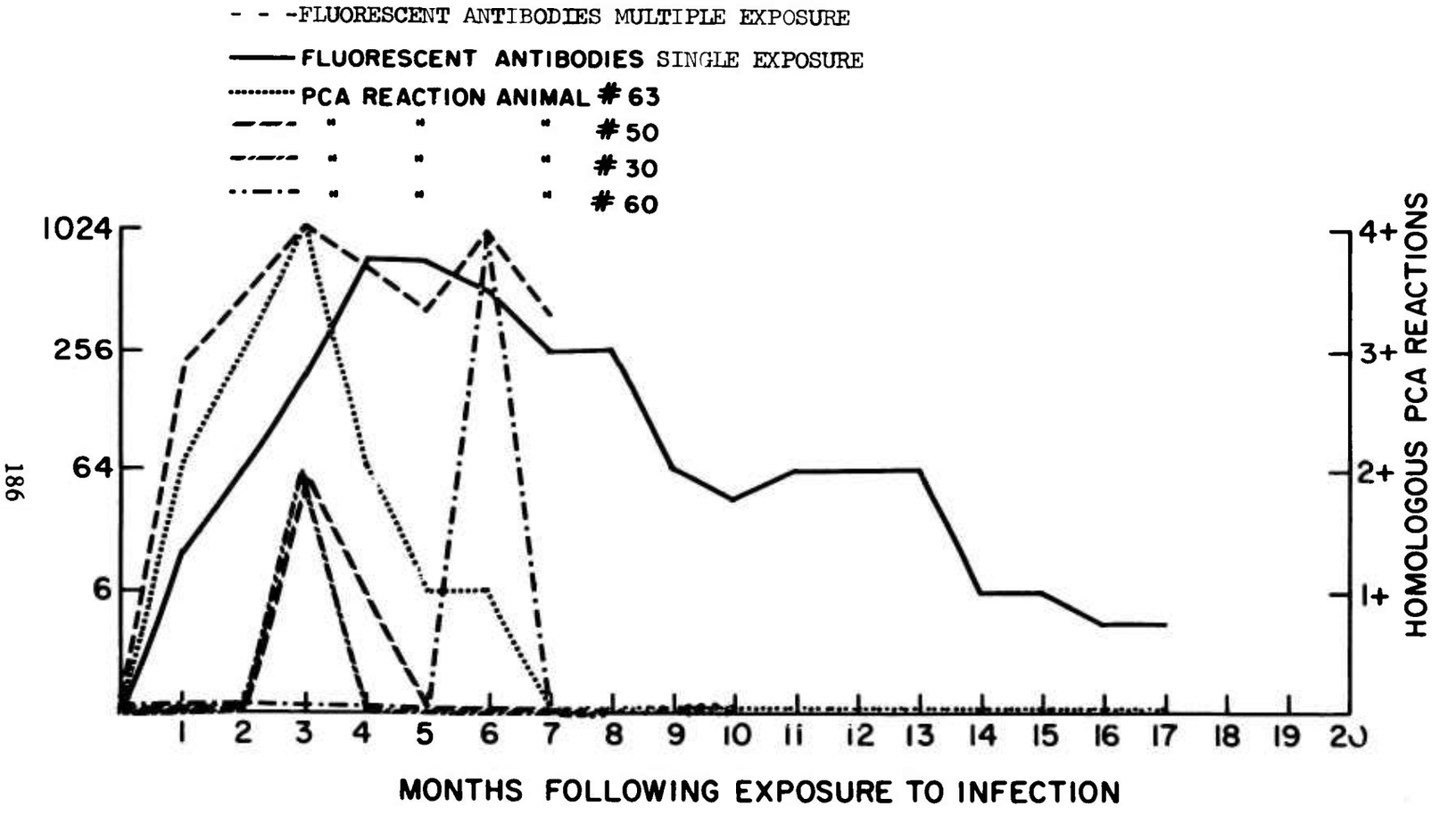

Fig. 3. Patterns of development of fluorescent and homologous PCA antibodies in chimpanzees exposed to varying single or multiple doses of S. haematobium cercariae.

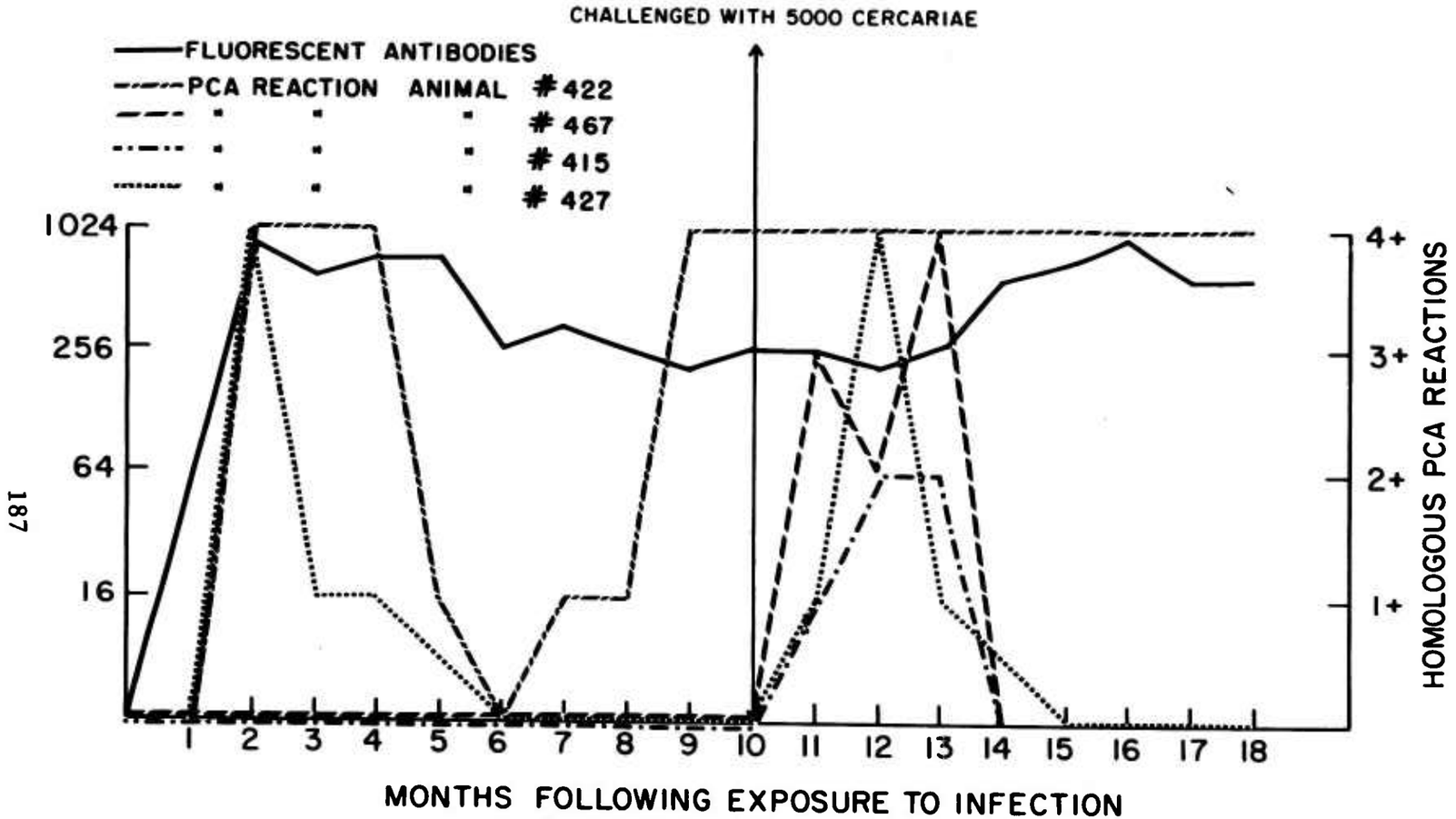

Fig. 4. Patterns of development of fluorescent and homologous PCA antibodies in rhesus monkeys exposed to 800 S. mansoni cercariae and subsequently challenged with 5,000 cercariae each.

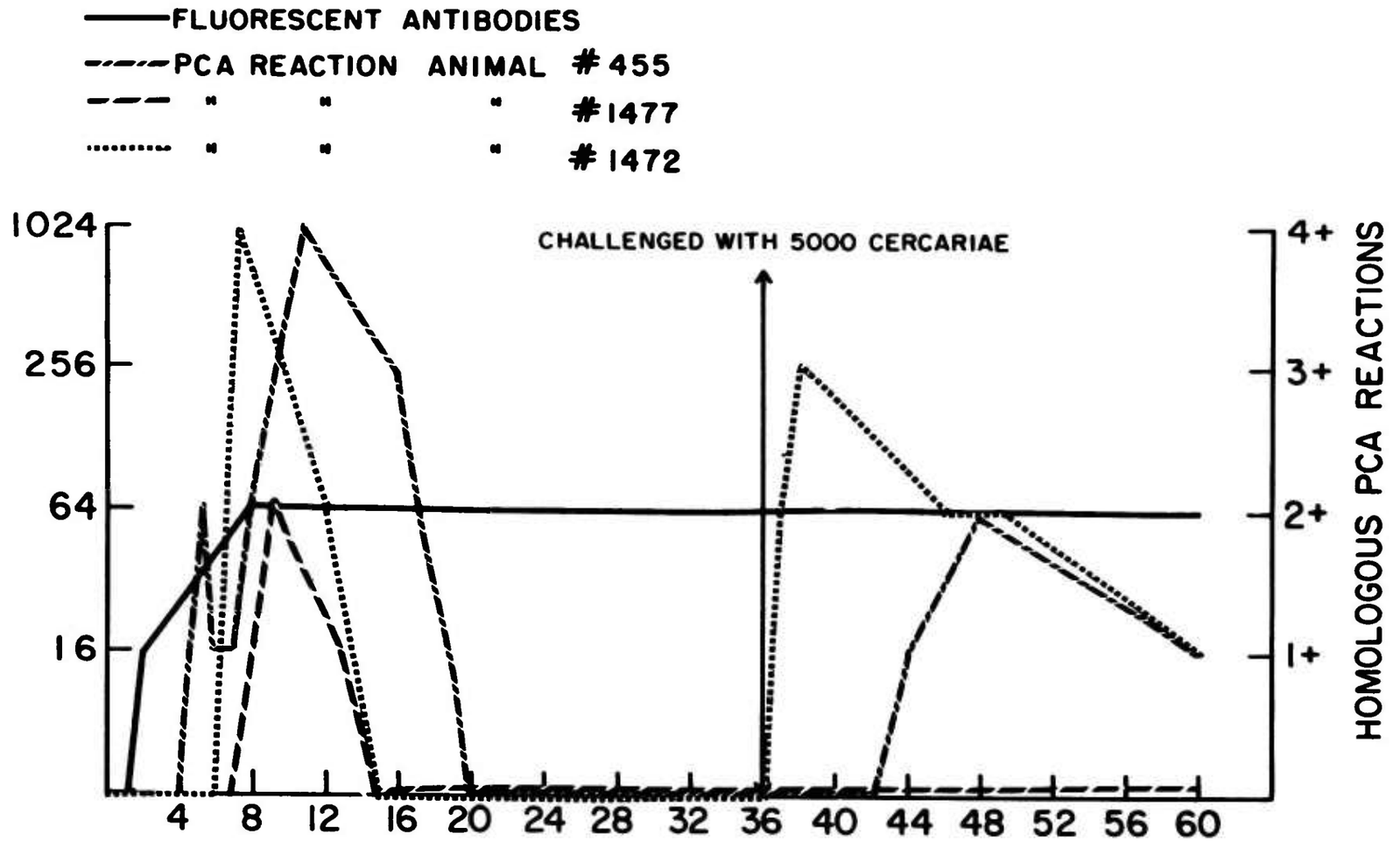

Fig. 5. Patterns of development of fluorescent and homologous PCA antibodies in rabbits exposed to 1,000 cercariae of S. mansoni and subsequently challenged with 5,000 each.

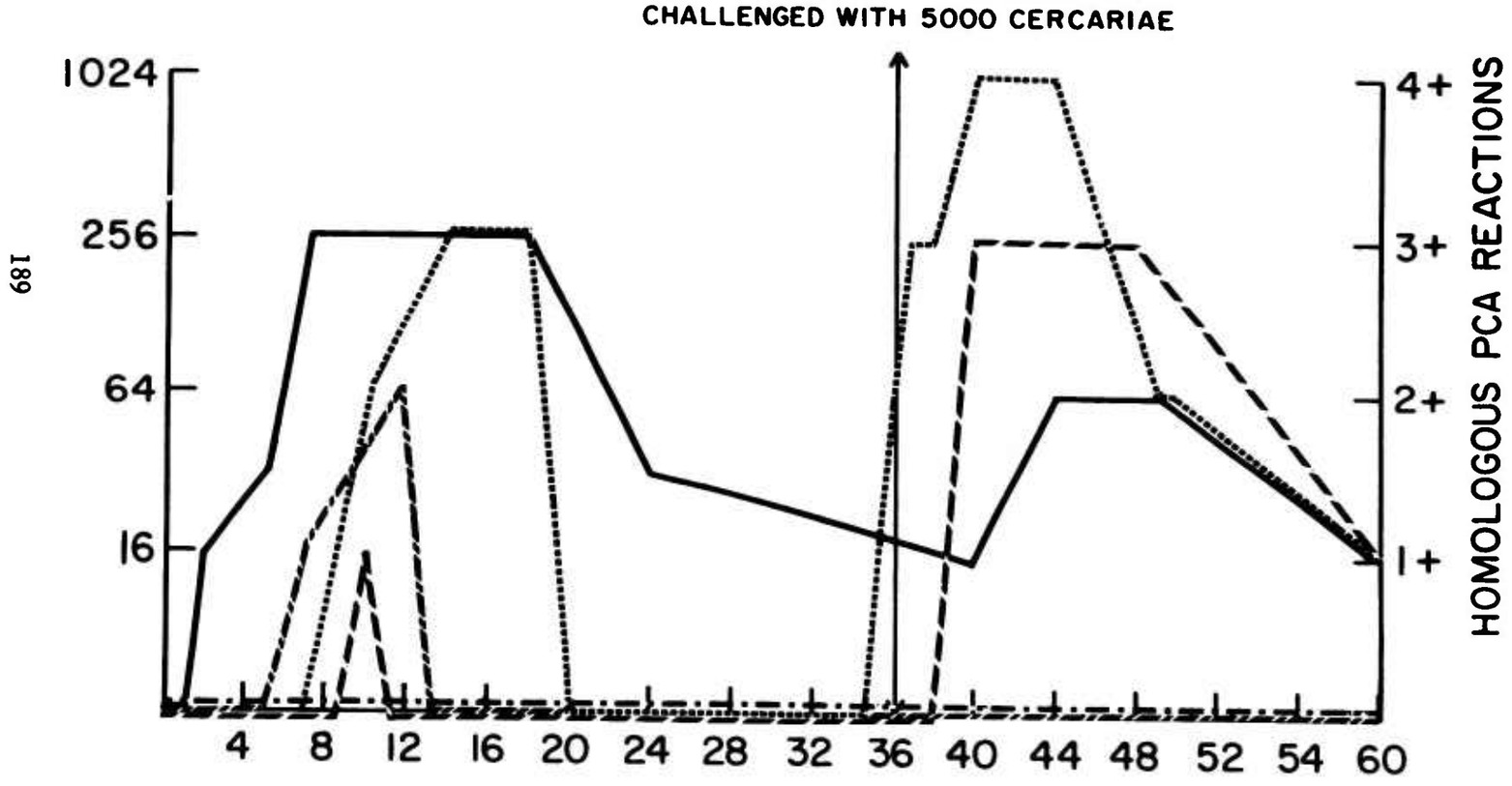

Fig. 6. Patterns of development of fluorescent and homologous PCA antibodies in rabbits exposed to 5,000 cercariae of S. mansoni and subsequently challenged with 5,000 each.

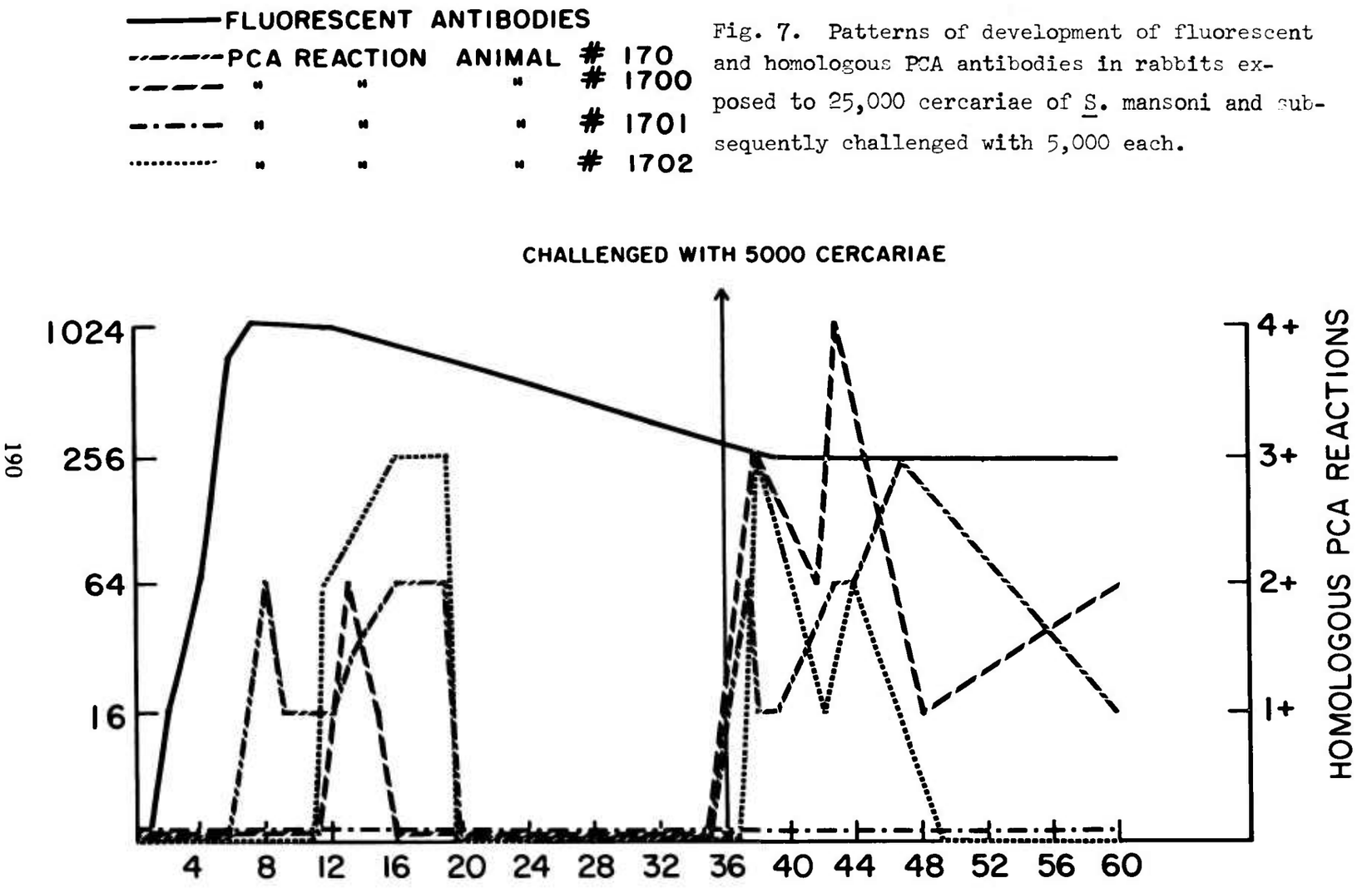

Fig. 7. Patterns of development of fluorescent and homologous PCA antibodies in rabbits exposed to 25,000 cercariae of S. mansoni and subsequently challenged with 5,000 each.

change in titers following the reinfection at the 36th week. There was no correlation of the anaphylactic antibody reactions with the flocculating antibody titers.

Table 3

Antibodies Detected in Sera from West Indian Green Face Monkeys (Cercopithecus sabaeus) Exposed Once to 100 Cercariae

Animal No.	Results at Given Times Following Exposure			
	11 Months		19 Months	
	PCA*	SF Titer	PCA	SF Titer
26	Neg	1:2	Neg	1:16
54	Neg	1:4	Neg	1:64
55	Neg	1:4	Neg	1:64
61	4+	1:64	4+	1:256
62	3+	1:128	2+	1:128
63	Neg	1:8	Neg	Neg
75	Neg	Neg	Neg	Neg
76	Neg	1:4	Neg	1:32
77	Neg	Neg	Neg	Neg
71 (uninfected)	Neg	Neg	Neg	Neg

*Serum was undiluted.

The appearance of reagin-like antibodies in guinea pigs exposed to 100 cercariae was compared with the development of fluorescent antibodies. Individual sera of each weekly bleeding were pooled and tested for the presence of fluorescent antibodies and for the ability to induce PCA reactions. As indicated in Figure 8, fluorescent antibodies were detectable 5 weeks after exposure to infection; they increased in titer and reached a peak by th end of the experiment. PCA reactions were detected at 9 to 10 weeks after exposure and persisted up to the end of the experiment. PCA reactions also occurred 8 days after injecting antiserum. Heat, reduction and alkylation did not prevent the 72-hour reactions (Table 4).

Similar experiments were conducted with mice exposed to 100 S. mansoni cercariae each. The mice were killed at 2 week intervals after exposure to infection. The individual sera of each weekly bleeding were pooled and tested for fluorescent antibodies and for their ability to induce PCA reactions. As indicated in Figure 9, fluorescent antibodies were detected as early as 2 weeks after exposure to infection and reached a peak titer 8 weeks after exposure. No PCA reactions were induced by any of the pools of sera obtained during the experiment. These experiments were repeated with different strains of mice exposed to either 200 cercariae or 10,000 irradiated cercariae. No reagin-like antibodies were ever detected in mice after single or multiple infections with S. mansoni in any of the mouse strains used. In order to

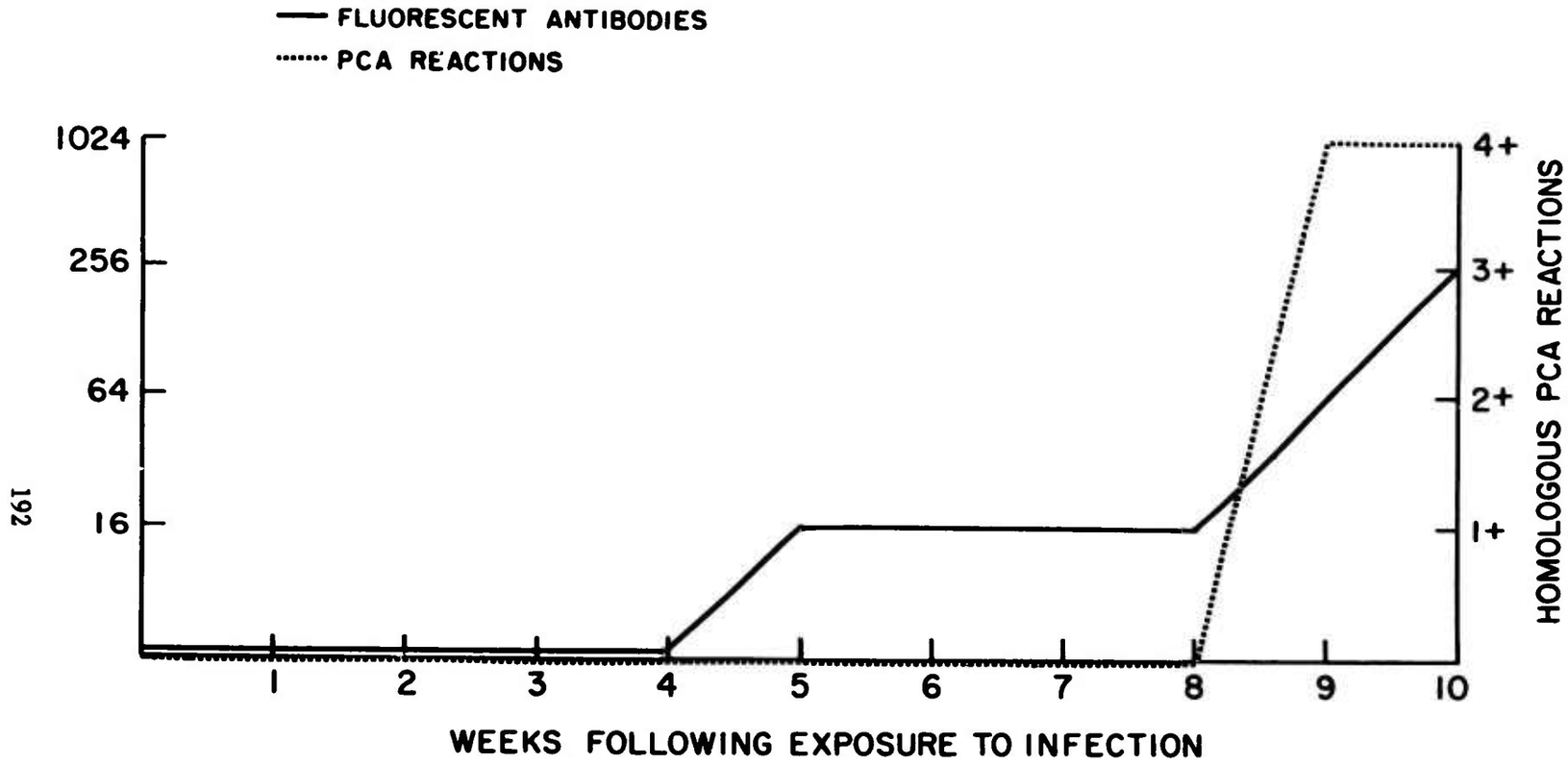

Fig. 8. Patterns of development of fluorescent and homologous PCA antibodies in guinea pigs exposed to a single dose of 1,000 S. mansoni cercariae (pooled sera).

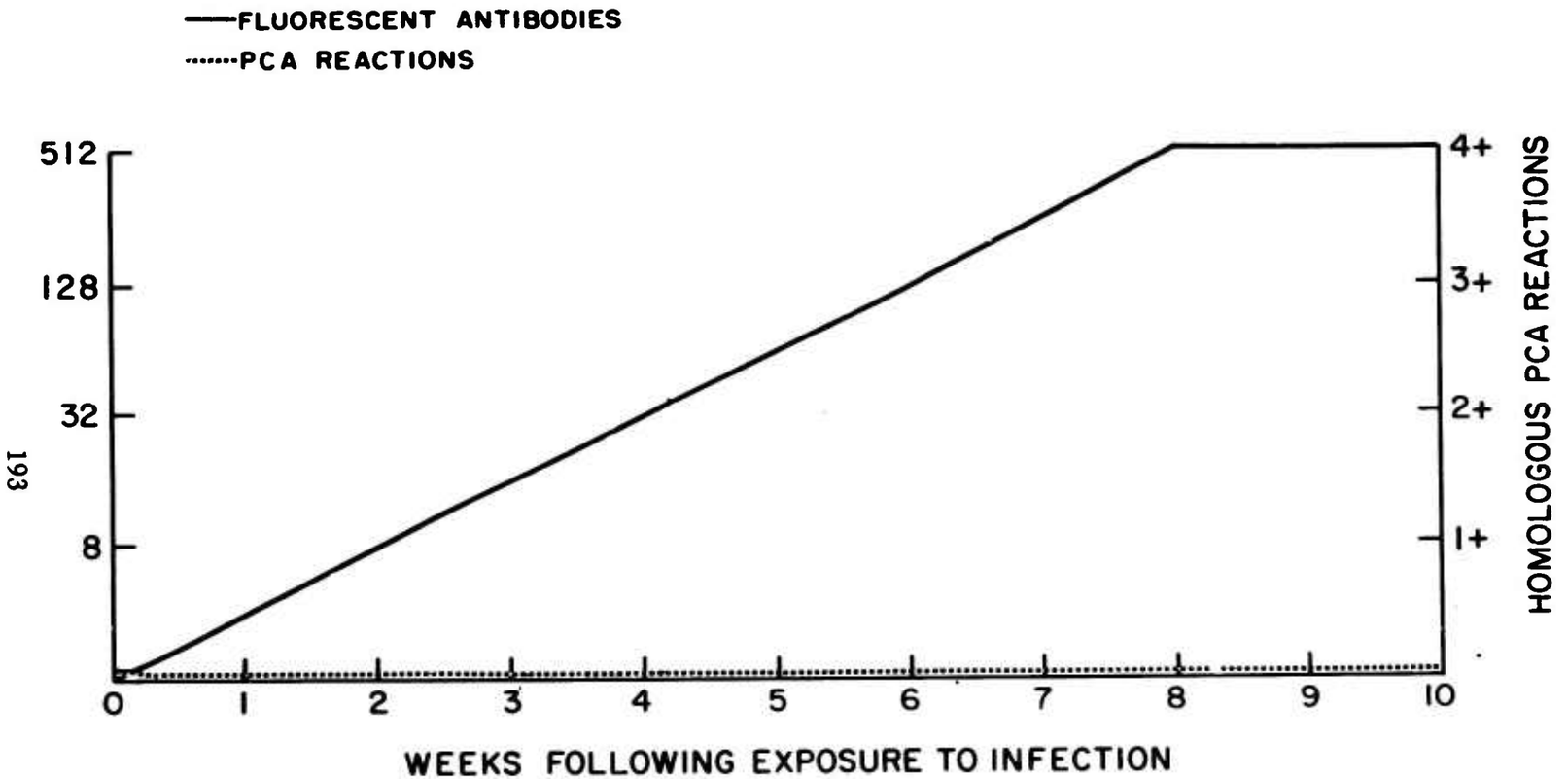

Fig. 9. Patterns of development of fluorescent and homologous PCA antibodies in mice exposed to a single dose of 100 S. mansoni cercariae (pooled sera).

determine whether these animals were capable of producing reagin-like antibodies, the mice which had been exposed to 200 S. mansoni cercariae were infected 4 weeks later with 100 Trichinella spiralis larvae. Reagin-like antibody was detected against T. spiralis but not S. mansoni 5 weeks after this infection.

Table 4

PCA Reactivity of Guinea Pig Sera After a Single Exposure to 100 Cercariae

Serum (weeks after exposure)	Passive Cutaneous Anaphylactic Titers				
	After 3 days latency		After 8 days latency		
	Unheated	Heated	Unheated	Heated	2-mercaptoethanol
12	1:50	1:1	1:8	Neg	Neg
16	1:64	1:8	Neg	Neg	Neg
23	1:16	1:16	ND*	ND	ND

*Not done

Characterization of anaphylactic antibody

In an attempt to define some of the properties of the PCA antibody, serum specimens from men and experimental animals were divided into aliquots and tested for heat stability at 56°C for 4 hours, and for susceptibility to reduction and alkylation by treatment with 2-mercaptoethanol and iodoacetamide. After heating or treating with 2-mercaptoethanol PCA activity was completely eliminated or the titer was markedly reduced in all except the guinea pig sera. Conversely, none of these procedures interfered significantly with the ability of these sera to react in the FA, SAFA or SF tests. Sephadex G-200 and DEAE ion-exchange chromatography were employed to separate and characterize some of the antibodies present in the various antisera. The fractions which contained the major amount of IgG immunoglobulin had either all or almost all of the detectable fluorescent or flocculating antibody activity. Conversely, electrophoretically faster immunoglobulins contained PCA activity but little or no FA and SF activity.

The results obtained from guinea pig sera 10, 12, 16 and 23 weeks after infection were not as clear cut as for the other host species (Table 4). Heat treatment did not destroy the sensitization demonstrated 3 days after injection (presumably due to gamma-1 antibodies), but by 7 days, heat-treated or 2-mercaptoethanol-treated sera had lost all sensitizing capacity. However, at that time untreated sera also gave a very diminished response to challenge.

Relationship of reagin-like antibody to susceptibility and pathogenesis

In order to determine whether any correlation existed between susceptibility of the various hosts and the production of PCA antibodies

all the animals were killed at the end of each experiment; the worms were perfused out and counted and various clinical and pathological observations were recorded. In addition, the fecal egg excretion pattern was determined throughout the course of the infection. No significant differences in worm burdens, degree of organ involvement and egg excretion patterns were observed between the animals within each species which produced detectable levels of PCA antibodies and those which failed to do so. The number of animals producing PCA reactions in different host species has been compared with the parasitological, clinical and pathological observations (Table 5). The percent of animals that produced PCA antibodies following a primary infection varied from zero (mouse) to 82 (rabbit). After reinfection, the percent of PCA positive animals varied from zero (Mouse) to 100 (rhesus monkey).

As indicated in Table 5, four levels of susceptibility were arbitrarily assigned to these animals based on the percent of worm recovery, the general appearance of worm development, the pattern of egg excretion and the clinical and pathological observations. The percent PCA reactivity correlated directly with the degree of susceptibility of the various species studied. When serum dilutions were tested for PCA, stronger reactions were usually observed with sera from rabbits and monkeys than with human or chimpanzee sera.

These investigations indicate that schistosomiasis patients develop homocytotropic (reaginic) antibodies which can be easily detected by testing the serum in the skin of rhesus monkeys. These results, which confirm and extend our early reports indicate that allergic manifestations may be an important component of schistosomal infection and that to avoid the risk of transferring infections such as viral hepatitis, testing for reaginic antibodies by the PCA reaction in monkeys should be favored over performing Prousnitz-Küstner procedures.

Seventy percent of the specimens from human patients gave positive PCA reactions, and no significant difference in reactivity was observed among the two species of human schistosome infections. Although obviously it could not be known whether the individuals were exposed to infection only once or repeatedly, it is likely that the latter was true for most of them since they lived in heavily endemic areas. Attention is called to the fact that all of these individuals with mixed infections reacted in the PCA test and that the percentage of patients with demonstrable reaginic antibodies increased with increasing fluorescent antibody titers.

The six animal species experimentally infected with schistosomes varied considerably in their degree of susceptibility, course of infection, and development of homocytotropic reagin-like antibodies. The results of these studies indicate that many features distinguish the fluorescent and flocculating antibodies from the anaphylactic antibodies which develop in various hosts with schistosomiasis. Fluorescent and flocculating antibodies were heat stable, not sensitive to 2-mercaptoethanol,

Table 5

Homocytotropic Reagin-like Antibody in Several Hosts Exposed to Schistosome Cercariae in Relation to Susceptibility and Pathogenesis

Host	Egg Excretion Pattern	Worm Recovery (primary infection) Percent	Worm Recovery (primary infection) Development	Percent PCA Reactors Primary infection	Percent PCA Reactors Re-exposure	Susceptibility Level
Mouse	Many eggs. Maintained throughout life.	34.0	Good	0	0	I
Green-faced monkey	Many eggs. Maintained for long time.	33.0	Good	22	ND	II
Chimpanzee	Intermediate. Maintained for long time.	19.8	Good	40	63	II
Rhesus monkey	Many eggs. Early peak, then decrease and disappearance.	23.6	Mostly good	50	100	III
Guinea pig	Occasional. No regular pattern.	17.0	Poor. Mostly stunted	(pooled sera)	ND	IV
Rabbit	Occasional. No regular pattern.	Only a few, stunted, hard to recover.		82	64	IV

ND = not done.

and were eluted from DEAE Sephadex with the gamma G globulins. Conversely, anaphylactic antibodies were destroyed by heating, were inactivated by reduction and alkylation and had a faster electrophoretic mobility than gamma G globulins. The time-course of development of anaphylactic antibodies also contrasted strikingly to that of flocculating and fluorescent antibodies. The flocculating and fluorescent antibodies appeared earlier and persisted longer than the anaphylactic antibodies.

Recently, Zvaifler and Robinson concluded from physicochemical characteristics and specific absorption studies that the homocytotropic antibody produced in rabbits in response to protein antigen is analogous to human gamma E. On the basis of the similar physicochemical and biological characteristics, the homocytotropic antibody detected in man and animals infected with schistosomes probably represents a different class of IgE-like immunoglobulin.

Sera from the guinea pigs in our study were retested for PCA antibodies after heating, reduction and alkylation with mercaptoethanol. Three days after injection the PCA antibodies were not destroyed by either heat or by reduction and alkylation. However, in at least one serum specimen obtained 12 weeks after infection the sensitizing capacity was lost 8 days after skin injection following heat treatment, but was retained in the untreated control. This could be interpreted as due to the fact that a heat labile and a heat stable antibody is present. Sensitization after a latent period of 3 days is probably due primarily to a heat stable gamma-1 antibody and sensitization after a latent period of 8 days is probably due to a heat labile homocytotropic reaginic antibody (IgE-like). However, we do not believe these results are sufficiently clear cut to be interpreted as a definite confirmation of the observations reported by Catty.

In general, four major susceptibility levels could be distinguished among the animals in this study: Group I. The percentage recovery of worms is high; worm development is good; many infective eggs are produced throughout the life of the animal; at necropsy the eggs are widely distributed throughout the intestine and liver. The mouse is an example of this group. Although fluorescent antibodies developed early in the infection in all of the mice and they rose rapidly to high titers, no reagin-like antibodies were detected after primary infection or reinfection with schistosomes. The same mice were capable, however, of producing reagin-like antibodies to Trichinella infections. Group II. The percentage recovery of worms is intermediate. Early worm recovery is lower than in Group I, but egg excretion is maintained over relatively long periods with little evidence of host resistance. Eggs are found principally in the colon with fewer eggs in the small intestine and liver. This group includes the chimpanzee and the cercopithecus monkey. Fluorescent and flocculating antibodies were detected early in the course of infection in most of these animals, whereas PCA antibodies were detected in a relatively small percentage of them after a primary infection, and they persisted for a comparatively short time at a low

level of reactivity. Group III. The percentage of recovery of worms is high, worm development is good, numerous viable eggs are produced and the eggs are widely distributed throughout the colon, small intestine and liver. This status, however, appears to be maintained for a limited time, then a strong tendency toward gradual self-cure and a resistance to reinfection ensues. The rhesus monkey is an example of this group. All of the infected animals in this group produced high fluorescent antibody titers early in the infection. One-half of them produced PCA antibodies after a primary infection and all of them produced PCA antibodies after a very heavy exposure to reinfection. Group IV. The animals are very resistant to infection. Worm and egg recoveries are low, miracidial infectivity for snails is low or absent and pathological features are irregular and atypical. This group includes the rabbit and guinea pig. The time-course development of fluorescent and flocculating antibodies was essentially similar to that of the other groups, but a large percentage of these animals produced PCA antibodies earlier and at much higher titers than the others. The PCA reactions observed in rats are in line with those obtained by us with the animals of Group IV. For obvious reasons, the length, size and number of infections in the men included in this study could not be determined. Therefore, the results obtained with this host were not included in Table 5 and could not be compared directly with those obtained in the six experimental hosts studied. However, it is believed that the degree of susceptibility of man to schistosome infections closely resembles that of the chimpanzee and of cercopithecus monkeys. The fact that 70 percent of humans reacted in the PCA test is consistent with this hypothesis.

A positive correlation between resistance to infection and production of reagin-like homocytotropic antibodies is apparent in comparing the reactivity of the different species studied. This striking correlation suggests that these antibodies play an important role in the host's natural susceptibility and response to schistosomiasis. However, no obvious explanation can be offered as to why some animals within each group gave strong reactions in the PCA test and others did not. Since the sera of nearly all animals infected with schistosomes had high titers of gamma G antibodies, which may act as blocking antibodies, the differences in PCA reactivity may be explained in part by the concentration of these antibodies or of other blocking mediators. However, other factors should be investigated under well controlled conditions before attempting to formulate pathogenetic and immunogenic theories about homocytotropic antibodies on the basis of observations in different species of animals. Prominent among these are 1) host factors such as the age of the host, the intensity of infection, and the greater ability of certain species to produce high levels of antibodies, and 2) factors related to the detection system such as skin differences in different species, and decreases in PCA reactivity observed when rhesus monkeys are used in testing for the presence of homocytotropic antibodies developed in chimpanzees and cercopithecus monkeys. Another important consideration is that natural resistance to S. mansoni in some animal species may not be determined primarily by immunological factors but

by morphological or biochemical differences in the host. Thus, in spite of the suggestive evidence presented here, it must be concluded that the exact role of homocytotropic antibodies in resistance to schistosomiasis has not yet been defined.

2. Experimental infection with Schistosoma haematobium in chimpanzees. Parasitologic, clinical and pathological observations.

Although rodents and monkeys can be infected with Schistosoma haematobium the resulting disease usually affects primarily the digestive system. Even in those animals in which urinary involvement has been described, the lesions observed usually contrast markedly with those observed in man. Experimentally infected African green monkeys (Cercopithecus aethiops centralis) passed eggs in the urine. African Sooty monkeys (Cercopithecus fuliginosus) developed extensive lesions in the bladder, including pedunculated growths characterized as papillomata. The lower third of the ureters was sometimes dilated and thick walled. Although extensive lesions of the bladder were produced in the baboon (Papio anubis), egg deposition occurred primarily in the mesenteric venules and, while infections of two-years duration produced lesions resembling early urinary schistosomiasis in man, hydroureter or hydronephrosis were not evident.

For over a decade studies have been conducted in our laboratories to determine the degree of susceptibility of various mammals to S. mansoni. The most suitable animal for pathologic and immunologic studies of Manson's schistosomiasis was found to be the chimpanzee (Pan satyrus). Parasitologic, clinical, serologic, pathologic and radiologic observations conducted following single and multiple exposures to varying doses of cercariae indicated that the progression of disease in this animal bears important similarities to that observed in infected humans.

A natural infection of the chimpanzee with S. haematobium was reported by de Paoli who found granulomas containing eggs in the urinary bladder, rectum, lungs, liver and appendix of a young male from Sierra Leone. After experimentally infecting 4 chimpanzees with S. haematobium in Liberia, Vogel suggested that this animal may provide a suitable model for studying the natural history of urinary bilharziasis.

The present experiments were conducted to study the course of S. haematobium infection in chimpanzees. Detailed parasitologic, clinical, serologic and pathologic studies were conducted in animals exposed to single or multiple doses of cercariae in an attempt to determine the pathophysiologic sequence and pathogenesis of urinary schistosomiasis. The results indicate that experimental infection in the chimpanzee shows significant similarities to urinary schistosomiasis in man as regards its course, the location of worms and eggs, the immunologic and biochemical findings and the development of lesions in various organs. By the use of this unique animal model it has been possible to study some of the relationships between the worm and egg

distribution, the degree of pathology and the time sequence of the lesions produced.

Eight young chimpanzees (Nos. 10, 20, 30, 50, 56, 60, 62 and 63) originating in West Africa were experimentally infected. The sex and weight of each animal at the time of exposure and at the end of the experiment as well as the number and magnitude of cercarial exposures are shown in Table 6. An additional animal (No. 59) was used as an uninfected control. Splenectomy was performed on all of the chimpanzees for use in an unrelated experiment on the susceptibility of chimpanzees to human malaria. These animals were utilized for the present experiment after termination of the malaria studies by therapy and were healthy when selected for the present work. At necropsy, it was found that four animals (Nos. 10, 30, 62 and 63) had developed an accessory spleen. Previous studies indicated that splenectomy has no demonstrable effect on the host response of primates to Schistosoma mansoni infection except for a reduction in the rate of "self cure" in Macaca mulatta.

Cercariae of an Iranian strain of S. haematobium were obtained from pools of 12 to 28 infected Bulinus sericinus and B. truncatus snails. After the abdominal hair had been removed with clippers and the skin had been washed with dechlorinated water, a suspension of cercariae was applied to the skin and the water was allowed to dry. Hamsters were exposed to cercariae from the same suspension and used as infection controls. Frequent examinations included determinations of changes in appetite, gross abnormalities in the feces and urine, alteration in behavior, temperature and weight of each animal throughout the course of the experiment.

The feces and urine of each chimpanzee were examined for schistosome eggs at weekly intervals. Although attempts were made to prevent mixing of feces and urine, a certain amount of contamination could not be excluded at all times. Fecal eggs counts were conducted by direct smear and AMS-III sedimentation techniques. Measured volumes of urine were centrifuged and the number of eggs in the sediment was determined and recorded as the number of eggs per 10 ml of urine. Egg hatching and miracidial infectivity studies were conducted. Beginning 7 days before exposure, blood was collected biweekly from each of the chimpanzees for leukocyte differential counts and hematocrit determinations. Serum specimens were obtained before exposure and at monthly intervals thereafter.

Bacteriologic examination of the urine was conducted on 4 chimpanzees (Nos. 10, 63, 62 and 56) on samples obtained by catheterization. In 3 chimpanzees (Nos. 20, 50 and 62) urine was collected in a sterile syringe by direct aspiration from the bladder at necropsy. Specimens of 1 ml or more were centrifuged and the pellets streaked on sheep-blood agar, eosin methylene blue agar, MacConkey's agar and SS agar plates. The plates were incubated at 37°C aerobically, with the exception of a blood agar plate which was incubated with carbon dioxide. Enteric media were incubated 48 hr before being discarded as negative.

Table 6

Exposures of Chimpanzees to Schistosoma haematobium Cercariae

Chimpanzee No.	Sex	Weight (lb.) at exposure	Weight (lb.) at necropsy	No. cercariae per exposure	No. of exposures	Total No. of cercariae	Duration of infection (months)
20	M	31	37	100	6	600	10
50	F	42	42	100	6	600	10
30	F	45	42	250	6	1500	7
60	M	33	36	250	6	1500	7
10	F	34	37	500	1	500	10
62	M	38	42	500	1	500	17-1/2
63	M	48	35	2000	1	2000	7
56	M	40	38	2000	1	2000	7

Blood agar plates were incubated a maximum of 4 days for bacterial growth. Any specimen with less than 1 ml was streaked on the above media without centrifugation.

At necropsy the pelvic veins, the portal tributaries and the pulmonary vessels were perfused separately. The worms recovered were separated by sex and counted. Worms from some animals were measured. The number of eggs in various tissues was determined after digestion in 4 percent KOH at 37°C for 12-18 hours. Approximately 90 percent of the intestine was digested. Smaller proportions of the liver, lungs, bladder and ureters were used. An attempt was made to select tissue from the bladder and ureters with lesions in proportion to the tissue which was not digested.

Perfusion of the veins of the pelvis was performed first. Ligatures were placed on the portal vein about 1 cm from the liver, on the femoral arteries and veins at each femoral triangle, on the abdominal aorta just caudad to the origin of the inferior mesenteric artery, on both renal arteries and on the inferior vena cava at the level of the origin of the renal veins. A plastic canula connected to a source of 0.85 percent saline through an automatic pipetting pump was inserted and tied into the abdominal aorta just caudad to the ligature on this vessel. A 0.5 cm opening was made in the inferior vena cava about 2 cm from the junction of the common iliac veins. As saline containing 325 mg of pentobarbital per liter was pumped into the abdominal aorta, the blood and perfusion fluids were aspirated at the opening in the inferior vena cava. Worms were collected on the stainless-steel screen of a filtration device. The initial rate of injection of saline was very slow, but was increased to 300 ml per minute when the perfusion fluid became colorless. The bladder and adjacent areas were greatly massaged during perfusion. Perfusion was continued until the vessels and tissues became blanched and edematous and worms were no longer being collected on the filter. The mesenteric circulation and the liver were thereafter perfused individually. The lungs were perfused in most of the animals, after evisceration, by pumping saline into the pulmonary veins and collecting the perfused fluids from the pulmonary arteries.

All major organs were dissected free at necropsy and observed for gross pathologic changes. Specimens were collected for organ egg assays and for histopathologic studies. All tissues collected for histologic studies were fixed in buffered 10 percent formalin and subsequently embedded in paraffin. Sections of various organs were cut at approximately 8 microns and stained with dilute Delafield's hematoxylin and eosin. In addition, multiple sections of some of the more important bladder and ureteral lesions were stained with Masson's trichrome, Verhoff's elastica stain and with alizarin red. Sections of the kidneys were also stained with Gram's stain.

Biochemical determinations included serum transaminases (SGOT and SGPT), alkaline phosphatase, blood urea nitrogen, total serum protein and paper electrophoresis of serum.

Cystoscopy was performed on female chimpanzee No. 10 using a pediatric instrument. This procedure was not possible in male chimpanzees because the instrument could not be inserted without excessive trauma. Cystograms were obtained 6 months after exposure in chimpanzees Nos. 10, 56, 62 and 63. Intravenous pyelography using 1 ml of methyl-glucamine diatrizoate 60 percent per pound of body weight was performed within one month after the first exposure to cercariae and at 4 to 6 month intervals thereafter.

Serologic determinations by the fluorescent antibody test (FAT) were made according to the described method. Passive cutaneous anaphylaxis (PCA) reactions using cercarial extracts as antigen were studied as described previously. Cercariae of S. mansoni were used both in the FAT and in the PCA tests, since insufficient S. haematobium cercariae were available. Previous investigations had indicated that cross reactivity exists between these two species in the above tests.

Parasitologic observations. None of the chimpanzees passed schistosome eggs before experimental infection or for a number of weeks afterwards. The onset of patency was determined by urinary or fecal egg excretion varied (Table 7, Figures 10 and 11). Eggs appeared in the feces between 9 and 16 weeks and in the urine between 8 and 25 weeks after exposure. In 6 of the 8 animals, eggs were detected in the feces before they were seen in the urine. As indicated in Figures 10 and 11, the number of eggs in the urine and feces increased rapidly after patency and reached a peak shortly thereafter. There was some correlation between the number of eggs excreted in the feces and the size of cercarial exposure, but little correlation between the latter and egg excretion in the urine. One chimpanzee (No. 10), which had been exposed to a single dose of 500 cercariae, passed relatively few eggs in the feces but had a relatively large number of the eggs in the urine. Eggs from feces and urine hatched normally and were infective for laboratory-reared snails.

Table 7

Patterns of egg excretion of chimpanzees infected with S. haematobium *in relation to the number of female worms recovered and the mean number of eggs per gram of tissue in various organs*

Chimpanzee No.	No. female worms	First eggs* detected		Peak egg* output				Mean No. eggs per gram of tissue × 10²				
		Feces	Urine	Feces	Urine	MNEF†	MNEU‡	Lungs	Liver	Small intestine	Large intestine	Bladder
20	45	16	8	28	8	2	1	1.9	17.9	0.4	21.8	82.7
50	22	16	25	17	34	1	<1	<0.1	3.4	0.1	4.6	71.3
30	52	15	9	18	26	2	4	2.9	7.5	0.1	15.4	138.3
60	56	11	17	22	28	18	20	1.2	2.8	0.1	36.0	141.8
10	43	11	12	20	20	2	48	4.9	2.6	0.1	7.5	591.5
62	80	10	13	55	33	11	13	14.8	0.1	0.1	73.4	102.0
63	218	10	11	17	20	28	33	5.4	18.7	0.7	36.1	483.9
56	229	9	15	15	23	54	12	25.8	36.6	0.8	108.6	1,545.7

* Week.
† MNEF, Mean no. eggs per gram of feces.
‡ MNEU, Mean no. eggs per 10 ml urine.

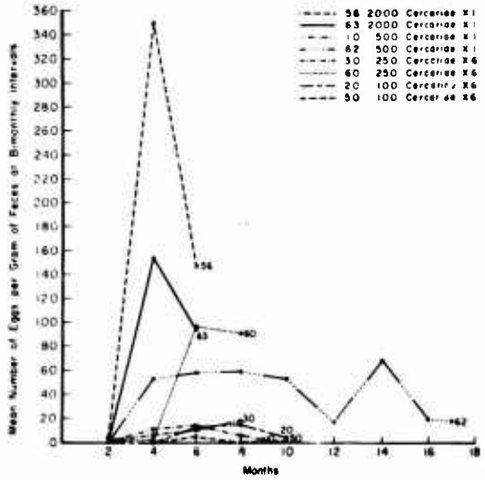

Fig. 10. Mean number of eggs per gram of feces plotted at bimonthly intervals.

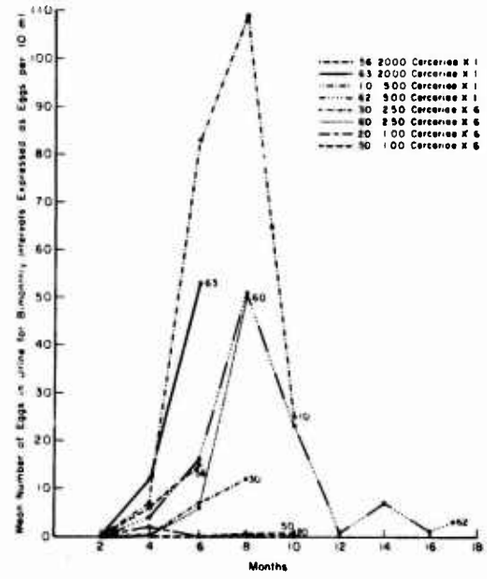

Fig. 11. Mean number of eggs per 10 ml of urine plotted at bimonthly intervals.

The number and location of adult worms recovered by perfusion are indicated in Table 8. The recovery of adult worms varied from 14 to 40 percent. Whereas in 3 of the 4 animals which received a single inoculation (Nos. 10, 62 and 63), most of the worms were found in the pelvic veins, in the remaining 5 chimpanzees a relatively large percentage was recovered from the portal tributaries. Only a few worms were recovered from the pulmonary vessels, and these were in 3 heavily infected chimpanzees (Nos. 60, 63 and 56). All the adult worms recovered by perfusion were active, well developed and of normal size. In 4 chimpanzees, 2 to 25 pairs of adult worms were identified in the tissues by gross inspection after perfusion (Fig. 12). It is probable that not all worms remaining in the vessels were found, but it is assumed that worm recovery was fairly complete. This is supported by the fact that the number of eggs in the tissues was closely related to the number of worms recovered. However, the number of worms found in histologic preparations exceeded the number expected from gross inspection. Eggs were found by digestion in all of the organs sampled, but in greatest numbers in the bladder, colon and liver (Table 9). The relative number of eggs obtained from the tissues of the bladder and ureters was generally related to the number of eggs found in the urine (Fig. 13). The number of eggs in the large intestine showed a better correlation with the number of eggs found in the feces (Fig. 14). Although eggs were uniformly distributed in the liver and lungs, their distribution in the bladder was very irregular. Therefore, the egg counts obtained from bladder tissue may have been influenced by the

area selected for digestion, although representative tissue samples were selected for examination.

Table 8

Number, location, and sex of adult S. haematobium *recovered by perfusion* in different systems in chimpanzees*

Chimpanzee No.	No. cercariae	No. live worms recovered in various venous systems: Pelvic M	Pelvic F	Pelvic Both	Mesenteric M	Mesenteric F	Mesenteric Both	Intrahepatic M	Intrahepatic F	Intrahepatic Both	Total† M	Total† F	Total† Both	Percent recovery	Percent recovered from pelvic system
20	600	3	3	6	48	41	89	40	1	41	91	45	136	23	4
50	600	13	15	28	10	7	17	37	0	37	60	22	82	14	34
30	1,500	32	28	60	129	22	151	33	2	35	194	52	246	16	24
60	1,500	40	25	65	43	6	49	177	24	201	264	56	320	21	20
10	500	33	41	74	11	2	13	20	0	20	64	43	107	21	69
62	500	69	68	137	18	12	30	33	0	33	120	80	200	40	69
63	2,000	111	115	226	140	94	234	59	8	67	318	218	536	27	42
56	2,000	75	73	148	179	151	330	21	0	21	289	229	518	26	29

* Histologic examinations revealed that some worms were missed by perfusion. A portion of these had been noted on gross examination of the tissues and are included in the worm counts.

† Including worms perfused from lungs as follows: No. 60, four male, one female; No. 56, 14 male, five female; and No. 63, eight male, one female.

Table 9

Number of eggs of S. haematobium *found in various organs in relation to the number of female worms recovered by perfusion and duration of infections*

Chimpanzee No.	Months infected	No. female worms	Eggs found × 10^3: Liver	Lung	Small intestine	Colon	Bladder	Ureters	Other*	Total	Total per female	Eggs in bladder and ureters/female from pelvic veins
20	10	45	857	22	13	833	198	Few	111	2,034	45	66
50	10	22	181	0	2	226	215	Few	34	658	30	14
30	7	52	731	101	11	993	594	Few	65	2,495	54	21
60	7	56	205	49	5	452	752	430	430	2,691	48	47
10	10	43	112	76	4	265	2,662	Few	Few	3,119	69	65
62	17½	80	4	222	4	1,965	408	1,746	29	4,378	55	32
63	7	218	1,234	99	31	2,724	4,839	†	4,787	13,714	63	40
56	7	229	1,730	597	31	4,665	4,637	†	1,843	13,503	59	64

* Mesentery with lymph nodes and retroperitoneal tissues.

† Included in bladder count.

Clinical observations. All animals gained weight in the early part of the experiment. This trend was later reversed. Following exposure to infection small red papules appeared at the site of cercarial penetration and disappeared after a few days. During the first 8 weeks after exposure no signs of illness were observed. During the 9th to the 11th week blood was observed in the stools of 4 animals (Nos. 10, 56, 62 and 63). One chimpanzee (No. 62) started losing weight rapidly one year after exposure. At that time the animal became lethargic, gaunt and dehydrated. Copious quantities of water and fruits were consumed by this chimpanzee which consistently refused dry food. From that time up to the end of the experiment, water intake by this animal was estimated at 2-3 liters per day as compared to its previous normal

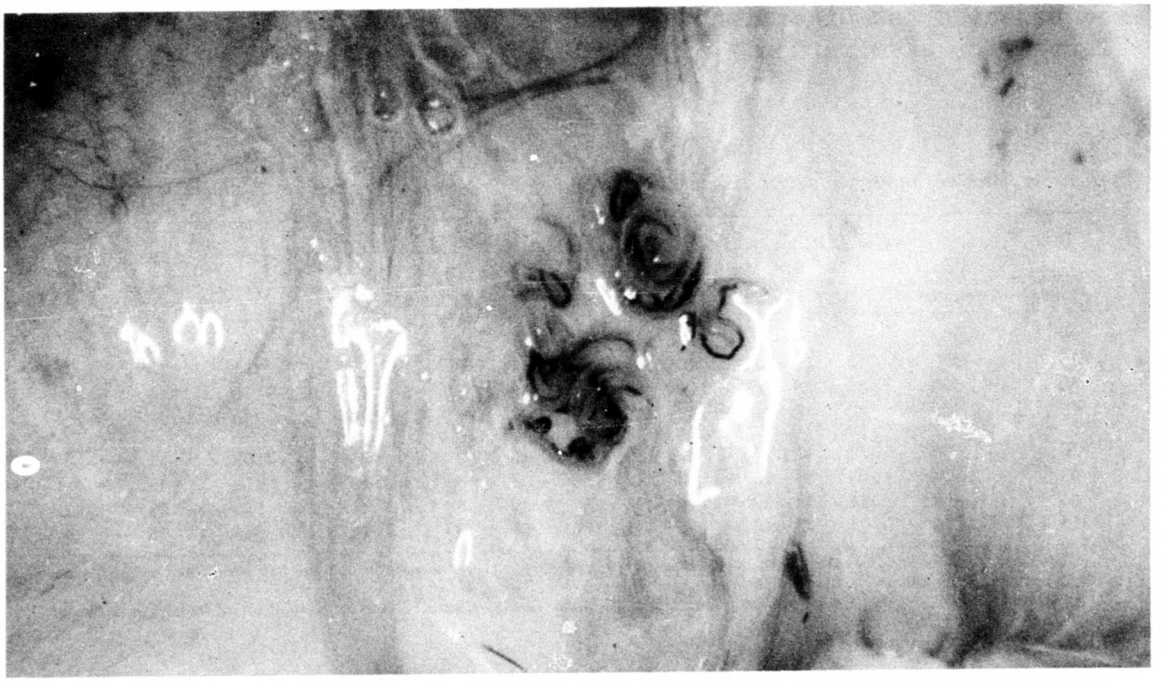

Fig. 12. Chimpanzee 56. Bladder serosa after perfusion; the vessels and tissues are blanched and reveal residual worm pairs in the subserosal veins underlying a polypoid patch.

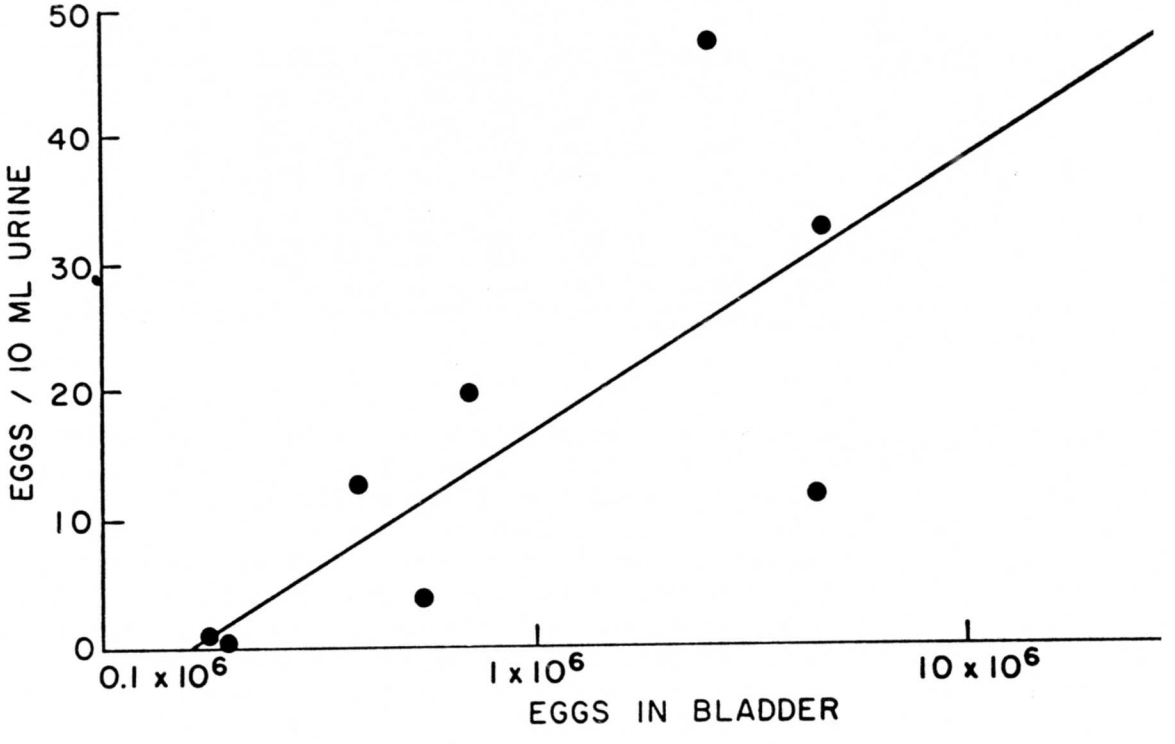

Fig. 13. Regression graph relating eggs per 10 ml of urine with the log of the number of eggs found by digestion of bladder tissue.

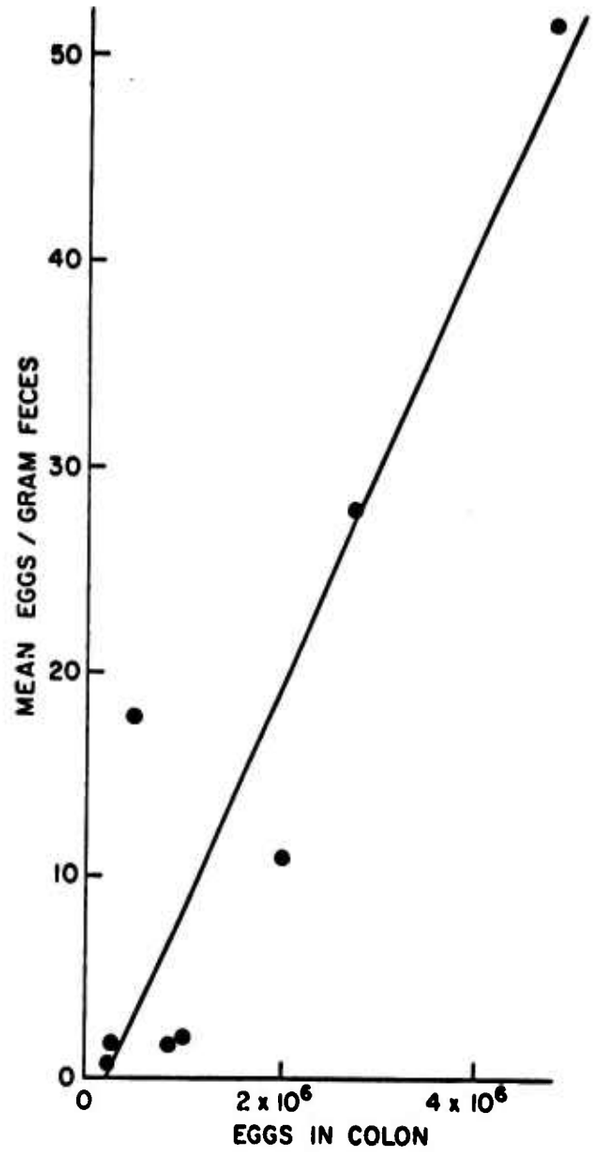

Fig. 14. Regression graph relating the mean number of eggs per gram of feces with the number of eggs found by digestion of colon tissues.

consumption of less than one-half liter per day. Gross hematuria was observed in only 3 of the infected animals (Nos. 10, 62 and 63). All urine samples collected for bacteriologic examination were negative on culture.

Three chimpanzees (Nos. 10, 30 and 60) died suddenly. Post mortem examination revealed that the cause of death for one chimpanzee (No. 10) was acute pneumococcal septicemia which also caused death or severe illness in 3 uninfected chimpanzees. During this outbreak all of the surviving animals were given penicillin, tetracycline and chloramphenicol.

No obvious abnormalities attributable to S. haematobium infection other than those reported were observed at any time.

Hematologic determinations in all chimpanzees were unremarkable except for a slight eosinophilia appearing in most animals during the third to fourth month after exposure.

A cystoscopic examination of chimpanzee No. 10 was conducted 6 months after exposure. Small papillomatous projections were observed over the base of the bladder. These were gritty and sandy in appearance. Most of the visible pathologic changes were on the floor of the bladder. These papillomata were nonulcerated, and in some, a whitish appearance suggesting flecks of calcification was noticeable. Few hemorrhagic patches were seen.

Biochemical observations. No significant changes were observed in the serum transaminase (SGOT and SGPT) and in the alkaline phosphatase levels. An increase in blood urea nitrogen was observed in 2 chimpanzees (Nos. 10 and 62) in the latter part of the infection (Table 10). These animals had shown the greatest clinical abnormalities resulting from infection. One of them (10) showed a sudden striking increase in BUN at the time it died of acute pyelonephritis. Consistent increases in total serum proteins occurred in all animals as infection progressed (Table 11). Greater increases were observed in the chimpanzees receiving a heavy single exposure or infection of long duration (Nos. 62, 63 and 56). No consistent significant changes were observed in the albumin and alpha globulin levels in any of the animals. Conversely, there was an increase in the beta globulins (Table 12) and in gamma globulin (Table 13). The greatest increases in gamma globulin levels occurred in the 2 animals with the heaviest infection (Nos. 63 and 56).

Serologic observations. Fluorescent antibody tests and tests for the passive transfer of immediate hypersensitivity from chimpanzees to rhesus monkeys were carried out. The time-course development of antibodies detected by the FA test was quite similar for all the chimpanzees (Table 14). Antibodies were detected one month following exposure. The titers reached a peak early in the infection and, in at least 3 animals (Nos. 62, 63 and 10), decreased after six months following exposure. In one animal (No. 62) antibodies were no longer detectable 16 months after exposure. No consistent differences in peak titers were observed between the chimpanzees given a single exposure and those given multiple exposures. However, the peak appeared earlier in the animals exposed repeatedly than in those with a single exposure.

Cutaneous reactivity was successfully transferred from 4 of the 8 infected chimpanzees to recipient tuberculin-negative rhesus monkeys (Table 15). These animals were tested 72 hours after intradermal injection of serum from infected chimpanzees. Thirty minutes after intravenous injection of the dye and S. mansoni cercarial antigen, positive PCA reactions characterized by local edema and blue cutaneous coloration were observed in the passively sensitized monkeys. This reaginic antibody

Table 10

Blood urea nitrogen (BUN) in chimpanzees at various intervals following infection with S. haematobium

Chimpanzee No.	Values (*mg per 100 ml*) at given times following first exposure (*months*)																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
20	10.4	10.4	15.6	10.4	10.4	9.5	11.3	12.1	—	—	—	—	—	—	—	—	—	—
50	9.5	9.4	12.1	9.4	7.8	9.7	6.9	11.4	—	—	—	—	—	—	—	—	—	—
30	13.8	10.4	13.8	14.7	10.4	10.1	10.1	—	—	—	—	—	—	—	—	—	—	—
60	8.5	10.2	10.4	11.1	11.3	10.1	10.4	—	—	—	—	—	—	—	—	—	—	—
10	12.2	13.3	12.2	12.9	13.3	11.1	15.6	21.8	24.9	24.5	118.0	—	—	—	—	—	—	—
62	10.3	13.9	15.0	13.9	9.8	11.1	12.9	12.9	13.9	12.9	9.8	11.8	11.8	11.8	16.1	16.1	23.6	22.5
63	18.8	16.5	14.2	11.3	15.8	11.1	13.3	15.5	—	—	—	—	—	—	—	—	—	—
56	13.3	14.7	14.4	13.3	12.4	11.1	12.7	12.2	—	—	—	—	—	—	—	—	—	—
Mean	12.1	12.3	13.4	12.1	11.4	10.4	11.6	14.3	19.4	18.7	63.9	11.8	11.8	11.8	16.1	16.1	23.6	22.5

Table 11

Total serum proteins in chimpanzees at various intervals following infection with S. haematobium

Chimpanzee No.	Values (*g per 100 ml*) at given times following first exposure (*months*)																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
20	7.3	7.3	8.0	7.8	7.8	8.1	8.3	8.5	—	—	—	—	—	—	—	—	—	—
50	7.3	7.3	7.7	7.4	8.0	7.4	8.1	8.1	—	—	—	—	—	—	—	—	—	—
30	6.2	7.4	7.8	8.1	7.5	8.1	8.3	—	—	—	—	—	—	—	—	—	—	—
60	7.5	8.0	8.7	8.9	7.1	8.3	8.7	—	—	—	—	—	—	—	—	—	—	—
10	7.6	7.0	7.9	7.9	8.4	8.0	7.5	8.2	8.0	9.6	8.8	—	—	—	—	—	—	—
62	7.7	8.2	8.1	8.5	8.8	8.3	8.9	8.7	8.4	8.3	8.3	8.3	8.7	9.7	9.1	9.4	9.3	9.4
63	7.3	7.0	7.9	7.3	8.1	8.5	9.8	9.5	—	—	—	—	—	—	—	—	—	—
56	7.6	8.0	8.6	8.8	8.8	9.1	9.2	9.2	—	—	—	—	—	—	—	—	—	—
Mean	7.3	7.5	8.1	8.1	8.0	8.2	8.6	8.7	8.2	8.9	8.5	8.3	8.7	9.7	9.1	9.4	9.3	9.4

Table 12

Serum beta globulins in chimpanzees at various intervals following infection with S. haematobium

Chimpanzee No.	Values (*mg per 100 ml*) at given times following first exposure (*months*)																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
20	1.7	1.9	2.1	1.9	1.6	1.7	1.8	2.0	—	—	—	—	—	—	—	—	—	—
50	1.6	1.8	1.7	1.7	1.8	1.9	2.0	2.2	—	—	—	—	—	—	—	—	—	—
30	1.8	1.7	2.0	2.0	1.8	2.0	2.3	—	—	—	—	—	—	—	—	—	—	—
60	1.5	1.6	2.0	2.0	2.0	1.7	1.9	—	—	—	—	—	—	—	—	—	—	—
10	1.9	1.8	2.2	2.2	2.4	2.2	1.9	2.3	2.1	2.1	2.5	—	—	—	—	—	—	—
62	1.8	2.0	2.2	2.3	2.2	2.1	2.3	2.2	2.1	2.2	2.2	2.2	2.4	2.8	2.7	2.9	3.0	2.8
63	1.9	1.8	1.9	1.9	2.2	2.0	2.2	2.0	—	—	—	—	—	—	—	—	—	—
56	1.7	1.8	1.9	2.1	2.4	2.2	2.2	2.5	—	—	—	—	—	—	—	—	—	—
Mean	1.7	1.8	2.0	2.0	2.0	2.0	2.1	2.2	2.1	2.1	2.3	2.2	2.4	2.8	2.7	2.9	3.0	2.8

Table 13

Serum gamma globulin in chimpanzees at various intervals following infection with S. haematobium

Chimpanzee No.	Values (*mg per 100 ml*) at given times following first exposure (*months*)																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
20	1.2	1.4	1.3	1.5	1.6	1.4	1.5	1.7	—	—	—	—	—	—	—	—	—	—
50	1.4	1.5	1.4	2.2	1.6	1.5	1.5	1.9	—	—	—	—	—	—	—	—	—	—
30	1.2	1.4	1.4	1.6	1.6	1.7	1.9	—	—	—	—	—	—	—	—	—	—	—
60	1.5	1.5	1.8	2.3	1.5	2.3	2.4	—	—	—	—	—	—	—	—	—	—	—
10	1.8	1.7	1.7	1.8	2.0	2.3	2.2	2.2	2.2	3.0	2.6	—	—	—	—	—	—	—
62	1.4	1.3	1.4	1.6	1.9	1.9	2.0	2.1	1.9	1.8	2.0	2.1	2.0	2.2	2.1	2.3	2.3	2.3
63	1.6	1.3	1.5	1.4	1.8	2.7	3.3	3.3	—	—	—	—	—	—	—	—	—	—
56	1.4	1.3	1.5	1.7	1.9	2.5	2.6	2.7	—	—	—	—	—	—	—	—	—	—
Mean	1.4	1.4	1.5	1.7	1.7	2.0	2.2	2.3	2.0	2.4	2.3	2.1	2.0	2.2	2.1	2.3	2.3	2.3

Table 14

Results of fluorescent-antibody (FA) tests with serum collected from chimpanzees at various intervals following infection with S. haematobium

Chimpanzee No.	Titers at given times following first exposure (*months*)																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
20	Neg.	64	256	1,024	1,024	1,024	1,024	256	—	—	—	—	—	—	—	—	—	—
50	Neg.	256	256	1,024	1,024	1,024	1,024	256	—	—	—	—	—	—	—	—	—	—
30	Neg.	64	1,024	1,024	256	64	1,024	256	—	—	—	—	—	—	—	—	—	—
60	Neg.	1,024	1,024	1,024	1,024	256	1,024	1,024	—	—	—	—	—	—	—	—	—	—
10	Neg.	16	256	256	256	256	256	1,024	64	64	16	—	—	—	—	—	—	—
62	Neg.	64	256	256	1,024	1,024	1,024	256	256	64	64	64	64	64	16	16	Neg.	Neg.
56	Neg.	16	16	16	1,024	1,024	1,024	1,024	1,024	—	—	—	—	—	—	—	—	—
63	Neg.	16	16	256	1,024	1,024	256	16	—	—	—	—	—	—	—	—	—	—
59C	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

Table 15

Results of passive cutaneous anaphylaxis (PCA) with serum collected from chimpanzees at various intervals following infection with S. haematobium

Chimpanzee No.	PCA (*Neg. to 4+*) at given times following first exposure (*months*)																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
20	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	—	—	—	—	—	—	—	—	—	—
50	Neg.	Neg.	Neg.	2+	Neg.	Neg.	Neg.	Neg.	—	—	—	—	—	—	—	—	—	—
30	Neg.	Neg.	Neg.	2+	1+	Neg.	Neg.	Neg.	—	—	—	—	—	—	—	—	—	—
60	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	4+	Neg.	—	—	—	—	—	—	—	—	—	—
10	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	—	—	—	—	—	—	—
62	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg	Neg.	Neg.	Neg.	Neg.
56	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	—	—	—	—	—	—	—	—	—	—
63	Neg.	2+	3+	4+	2+	1+	1+	±	—	—	—	—	—	—	—	—	—	—
59C	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

was largely destroyed by heating the reacting serum at 56°C for one hour before passive transfer experiments. The time-course development of reaginic antibodies varied considerably. In one animal (No. 63) dermal reactivity was present one month following exposure and persisted for the duration of the experiment. In 3 other animals reaginic antibodies were detected 3 and 6 months after exposure to infection and persisted for only a relatively brief time. In 4 animals, no reaginic activity was demonstrated at any time.

Radiologic observations. All intravenous pyelograms (IVP) made within one month after exposure revealed normal function and anatomy, except for slight, bilateral dilatation of the renal pelvis in chimpanzee No. 10. Six months after infection a cystogram revealed persistent filling defects in the dome and along the right lateral border of the bladder of one chimpanzee (No. 63). Persistent filling defects involving the entire dome of the bladder and one or two areas along the base, suggestive of papillomas, were seen in another animal (No. 56). The kidneys and the portions of the ureters visualized were normal in both animals, but in chimpanzee No. 63 the right ureter was not seen, and only the distal 10 cm of the left ureter were visualized.

A cystogram of chimpanzee No. 10 made 6 months after exposure demonstrated that the bladder was contracted with persistent filling defects involving almost the entire organ, and that there was reflux 2 cm up the right ureter. The IVP showed the left renal pelvis to be slightly larger than in the examination done 1 month after infection, with appearance of contrast medium delayed until 20 minutes on the left side. Cystoscopy of No. 10 showed papillary lesions with submucosal calcific spotting. This animal had not been catheterized or examined by cystoscopy prior to this examinatior

The most striking IVP abnormalities were seen as the disease progressed in chimpanzee No. 62. Six months after infection the kidneys, ureters and bladder were essentially normal (Fig. 15). There was, however, some suggestion of an intrinsic filling defect in the left upper quadrant of the bladder. By twelve months after infection, there was a Grade II-III bilateral hydronephrosis involving the majority of the collecting system, and hydroureter was also evident. The left ureter was markedly displaced laterally (Fig. 16). The hydronephrosis was increased on examination 4 months later, but the bladder outline remained within normal limits. The last IVP (17 months after infection), revealed Grade II to III hydronephrosis on the right side with dilatation of the ureter down to the level of the bladder. On the left side, excretion was retarded to 40 minutes, and a Grade III hydronephrosis with persisting dilatation and lateral deviation of the ureter were seen. The bladder contour remained essentially normal.

No significant changes were observed by IVP in the other four animals (Nos. 20, 30, 50 and 60).

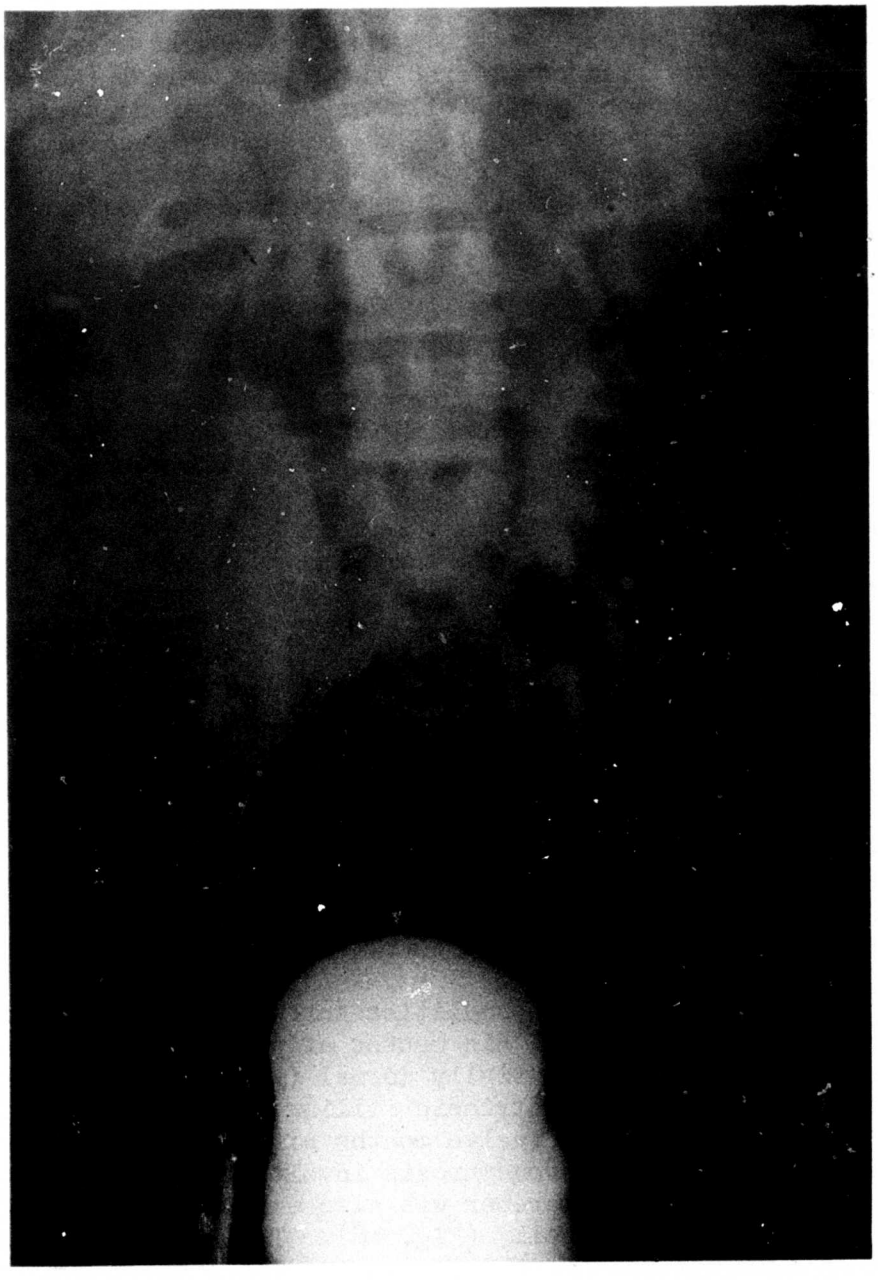

Fig. 15. Intravenous pyelogram of chimpanzee 62 made 12 months after exposure. After 20 minutes, a grade II to III bilateraly hydronephrosis was evident. Both ureters are displaced laterally.

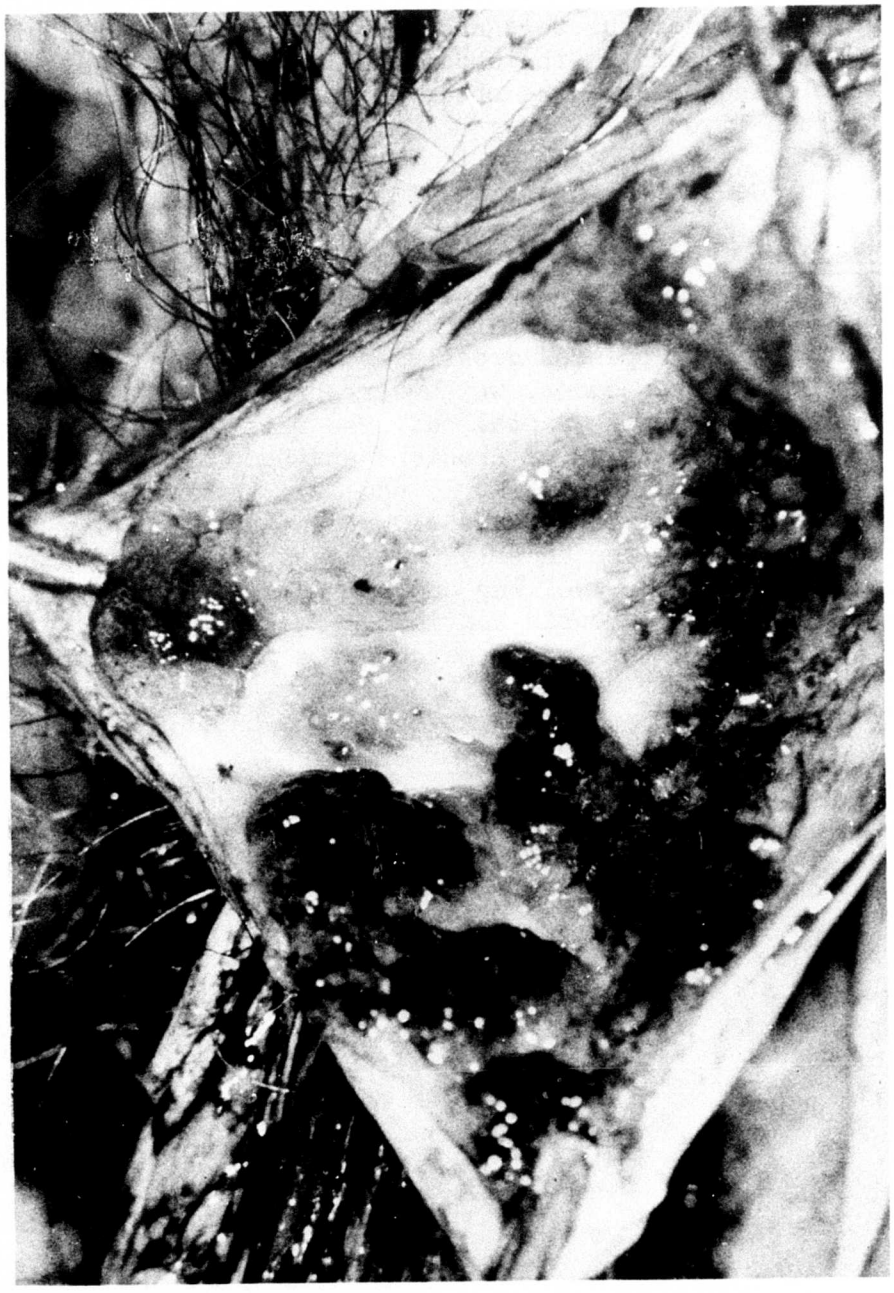

Fig. 16. Chimpanzee 56. Bladder opened before perfusion and demonstrating widespread polypoid patches of bladder mucosa. Some of them were hemorrhagic. Note the sharp delimitation of the patches against the smooth, whitish uninvolved mucosa, and their granular surface. Overhang of the margins is especially prominent on the more elevated patches.

Pathologic observations. Major alterations were found in the urinary system (bladder, ureters and kidneys), in the gut (chiefly the colon and appendix), and in the abdominal lymph nodes. Relatively smaller pathologic changes were observed in the genital organs, in the lungs and in the liver.

General Description of the Lesions

The bladder: The following three general categories of lesions were noted: polypoid, fibrous and sandy patches.

Polypoid patches appeared grossly as sharply-limited, raised, hyperemic mucosal excrescences, which were broad-based with slightly redundant margins rather than stalked. The raised mucosa was dark red, granular or fissured with small erosions and hemorrhages, and was raspberry-like in appearance (Fig. 17 and 18). After perfusion its color changed to mottled yellow and pink. The patches were soft and rubbery, and on section the underlying tissues were gelatinous and pink or hemorrhagic, with swelling and poor demarcation of all layers of the wall; the lamina propria was most affected reaching a thickness of up to 0.7 cm and in the larger patches the muscularis was thickened and delicately streaked, reaching a maximal total thickness of 1.2 cm in the involved bladder wall segment. In some cases, the subserosal

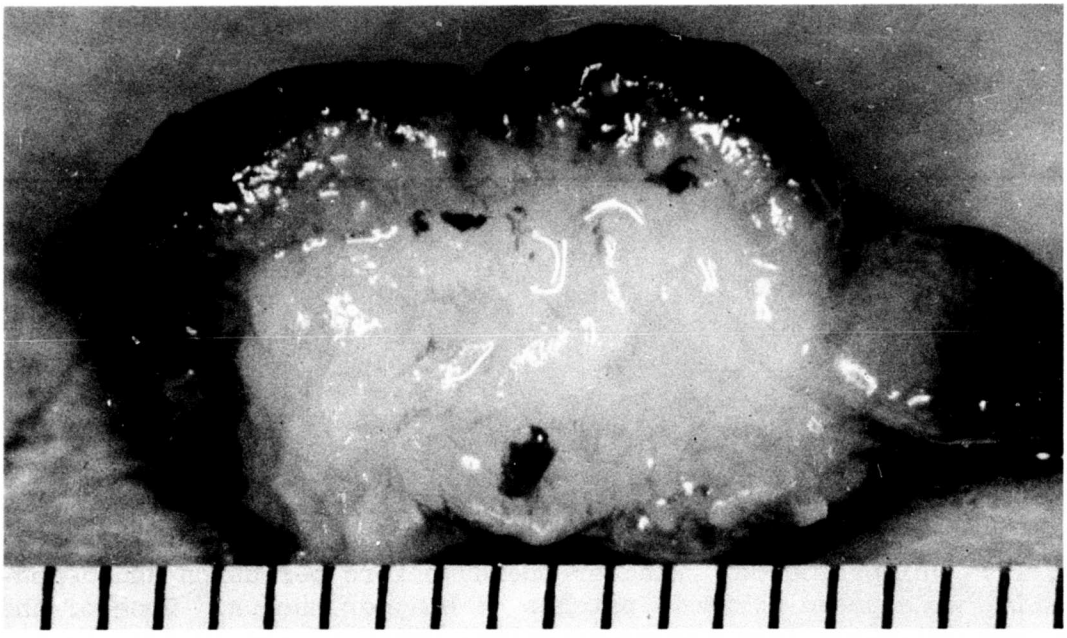

Fig. 17. Chimpanzee 56. Magnified cross-section of bladder wall at the site of two adjacent polypoid patches. The surface indentation toward the right marks a thin strip of normal bladder mucosa. Note the edema and thickening of all layers, particulary of the submucosa. The infiltrative streaking of the muscular layer is barely visible at its lower margin (cm scale).

veins and venules were dilated and prominent prior to perfusion (Fig. 19). Isolated patches were usually rounded or oval, while confluent patches were irregular in outline. They were separated from each other by smooth and glistening bladder mucosa with or without scattered inflammatory foci.

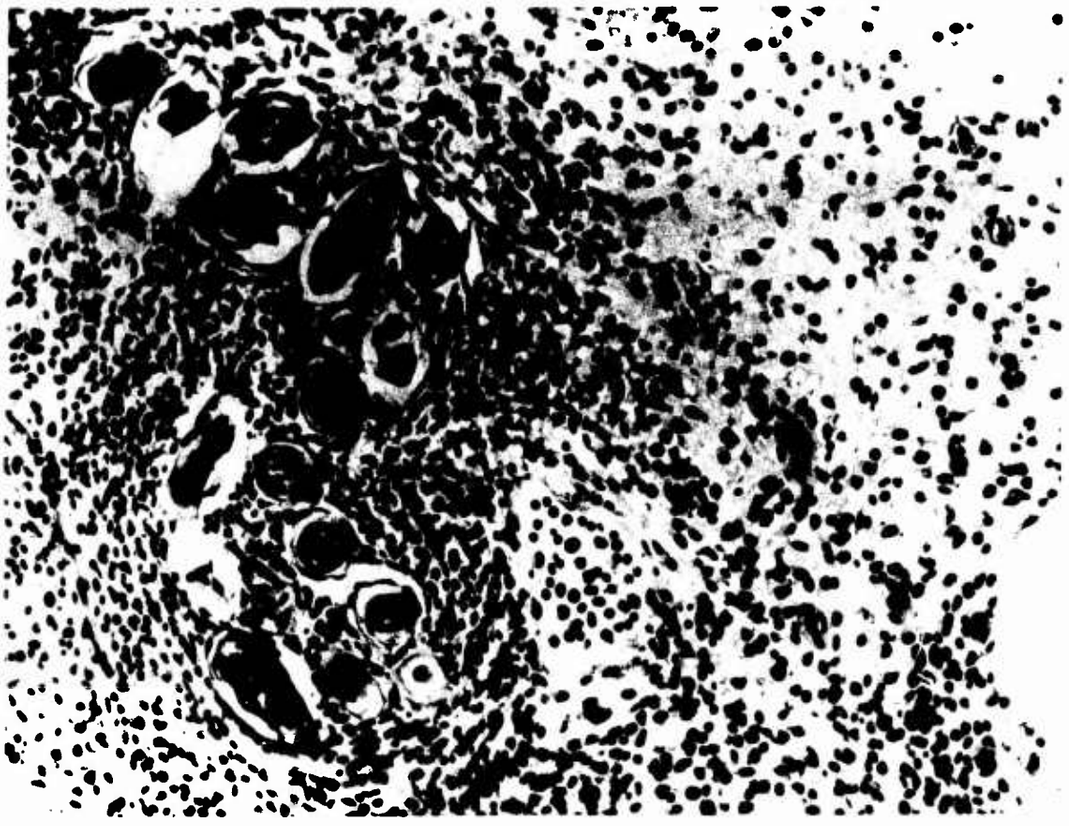

Fig. 18. Chimpanzee 56. Convoy of immature basophilic S. haematobium eggs, with destruction of the vein wall, converting to a composite granuloma. Many of the surrounding cells are eosinophils. On the right, there is marked edema, with dilated venules and lymphatic vessels. This is from a preperfusion biopsy specimen.

Polypoid patches contained large numbers of eggs which frequently aggregated in clumps forming intravascular "convoys" or egg masses occupying the center of composite granulomas (Fig. 20). Although some eggs were undeveloped and others immature, most were mature. A few were degenerate, but uncalcified. Eggs were found in all bladder layers including the lumen of mucosal nests and, rarely, the epithelium itself. The largest concentration was in the lamina propria. In the bigger patches, eggs were scattered throughout the muscular and subserosal layers. Some eggs and egg-clumps were surrounded by early granulomas with necrotic centers, which were poor in giant cells and rich in eosinophils and plasma cells, but most were embedded in a

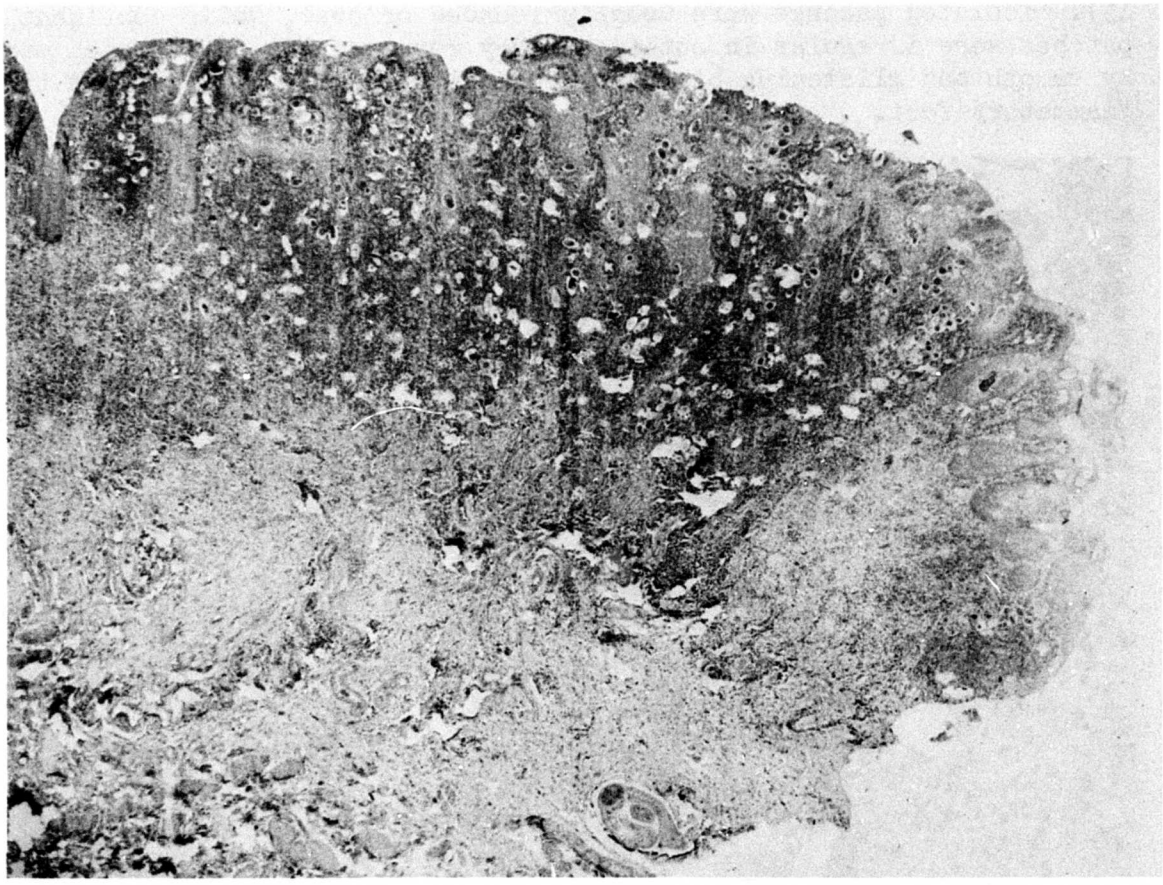

Fig. 19. Chimpanzee 56. Low-power survey picture of a polypoid bladder patch. A schistosome pair is seen within a deep submucosal vein (right lower corner). Note superficial hemorrhages, marked epithelial hyperplasia, abundance of eggs embedded within a mass of dense, diffuse inflammatory tissue that thickens the subepithelial layer, and streaky infiltration of the muscular interstices by eggs and inflammatory tissue. An areas of edema is seen toward the right margin. Preperfusion biopsy. (Hematoxylin and eosin, X 22).

diffuse and bulky inflammatory infiltrate or granulation tissue mass of similar cell composition. Only a few granulomas appeared discrete by virtue of concentric peripheral fibrosis; in most areas, the granulomatous and diffuse infiltrates simply merged and blended with each other (Fig. 21). The bulky granulation tissue in the lamina propria was permeated by proliferating capillaries and venules and was interspersed with zones of edema which were devoid of metachromasis on toluidine blue staining and were relatively cell free.

Superficially, the urothelium was thickened with microcystic Brunn's nests and epithelial pegs dipping into the lamina propria. There was

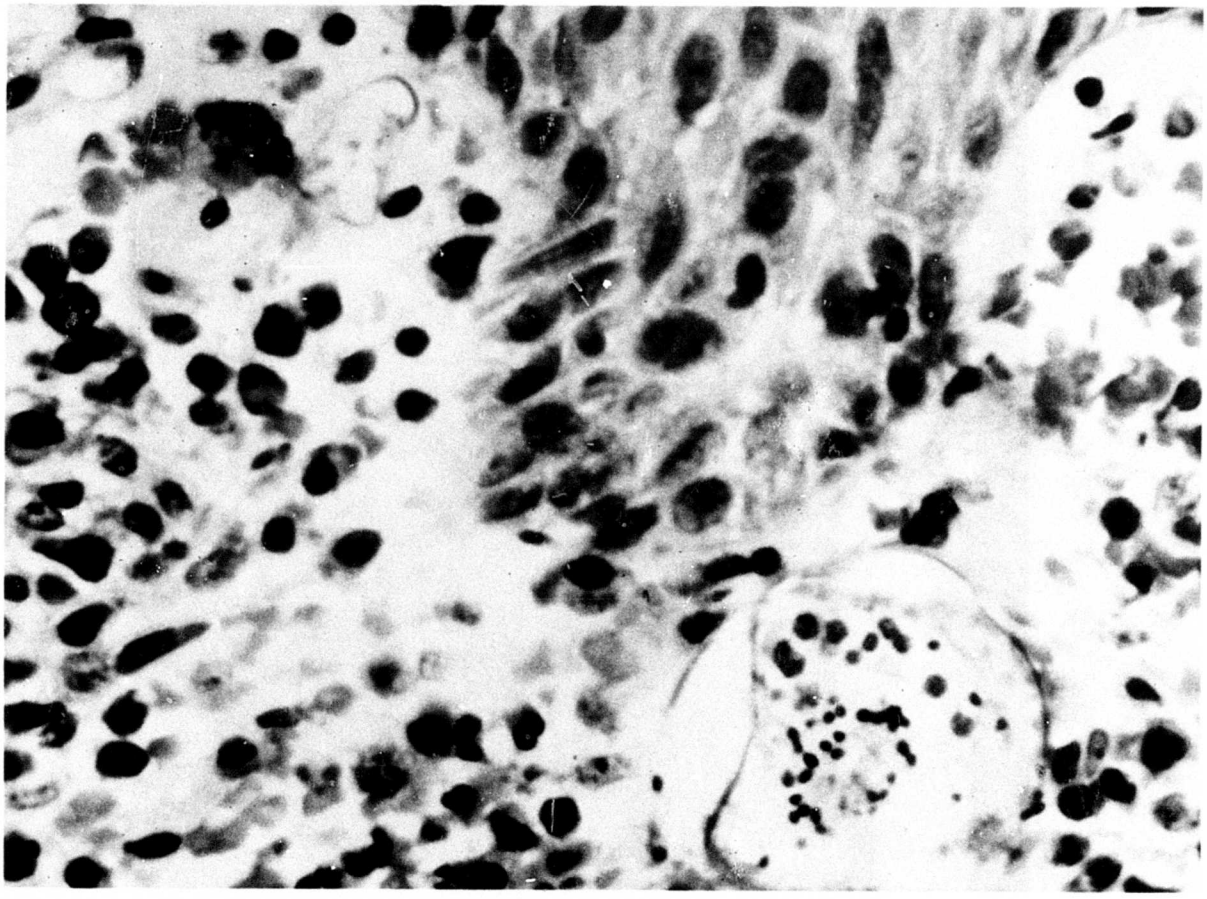

Fig. 20. Chimpanzee 56. Same specimen as Fig. 19, high power view near center of patch. The bladder mucosa shows squamous metaplasia, with intercellular bridges. Note dilated submucosal venule, plasma cells in the infiltrate and mature ovum abutting on an epithelial peg. (Hematoxylin and eosin, X 723).

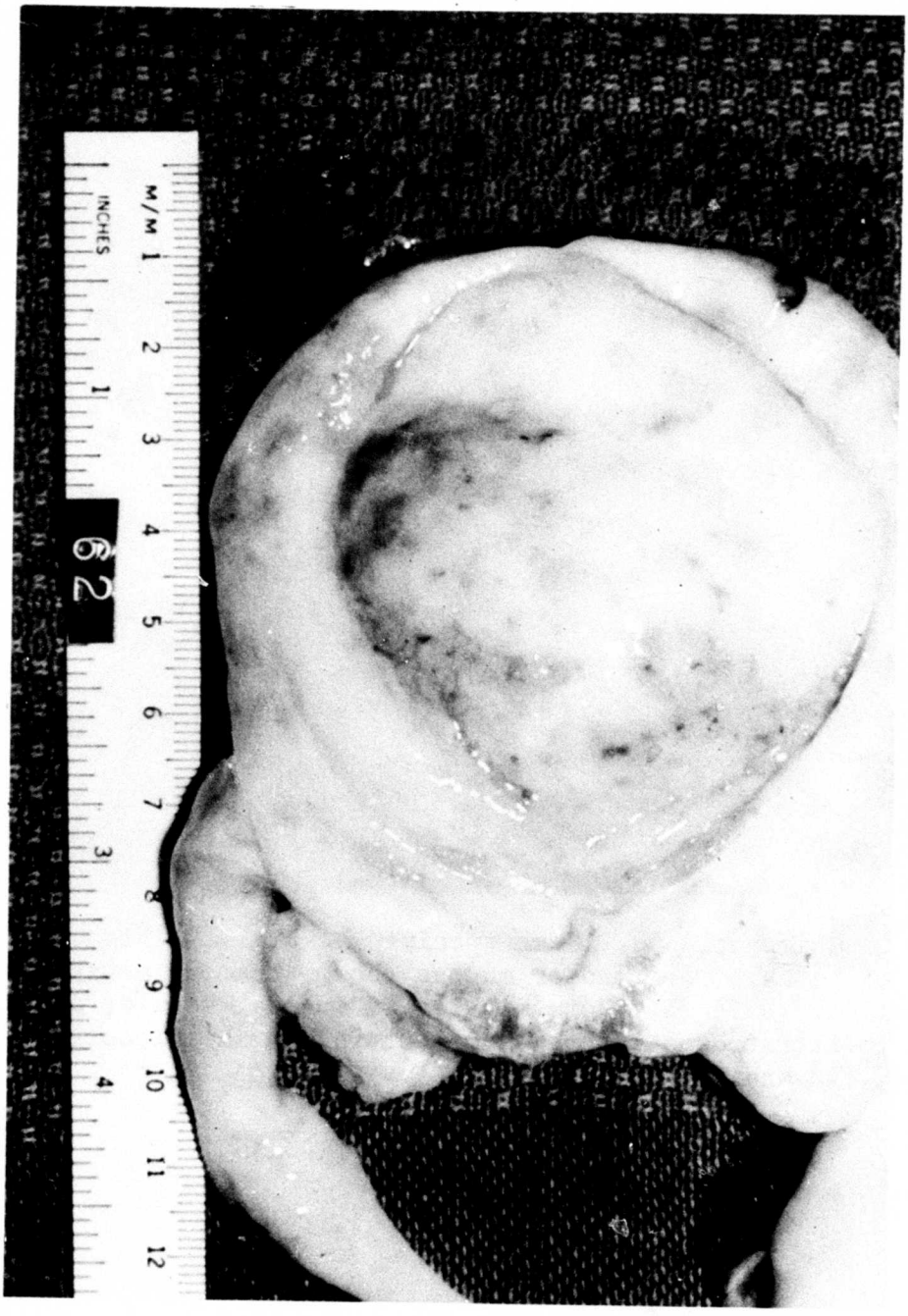

Fig. 21. Chimpanzee 62. Fibrous and sandy patches, and scattered mucosal lesions spread over most of the perfused bladder mucosa. Note the irregular and ill-defined margins of the lesions, their lesser bulge and their finely granular surface compared with the polypoid patches (Fig. 16). The thickened lower ureters have been turned downward.

patchy squamous metaplasia with intercellular bridging and with a thin, parakeratotic layer (Fig. 22). Focal mucosal erosions were noted, with hemorrhagic effusions around dilated vessels in eroded and non-eroded areas, and the epithelium was invaded by granulocytes. Toward the base of the zone of massive infiltration, strands of smooth muscle appeared; larger patches showed actual invasion and replacement of muscle bundles by granulomas, while smaller ones showed a zone of milder inflammation at their base, with only scattered eggs and inflammatory foci deeper in the muscular interstices. The edema of the lamina propria continued into the muscle layer where the connective tissue septa appeared widened and the vessels dilated. Throughout the lesion, there was little or no fibroblastic proliferation, and its bulk appeared largely due to the inflammatory cells, hypereima and edema, the last partly caused by perfusion.

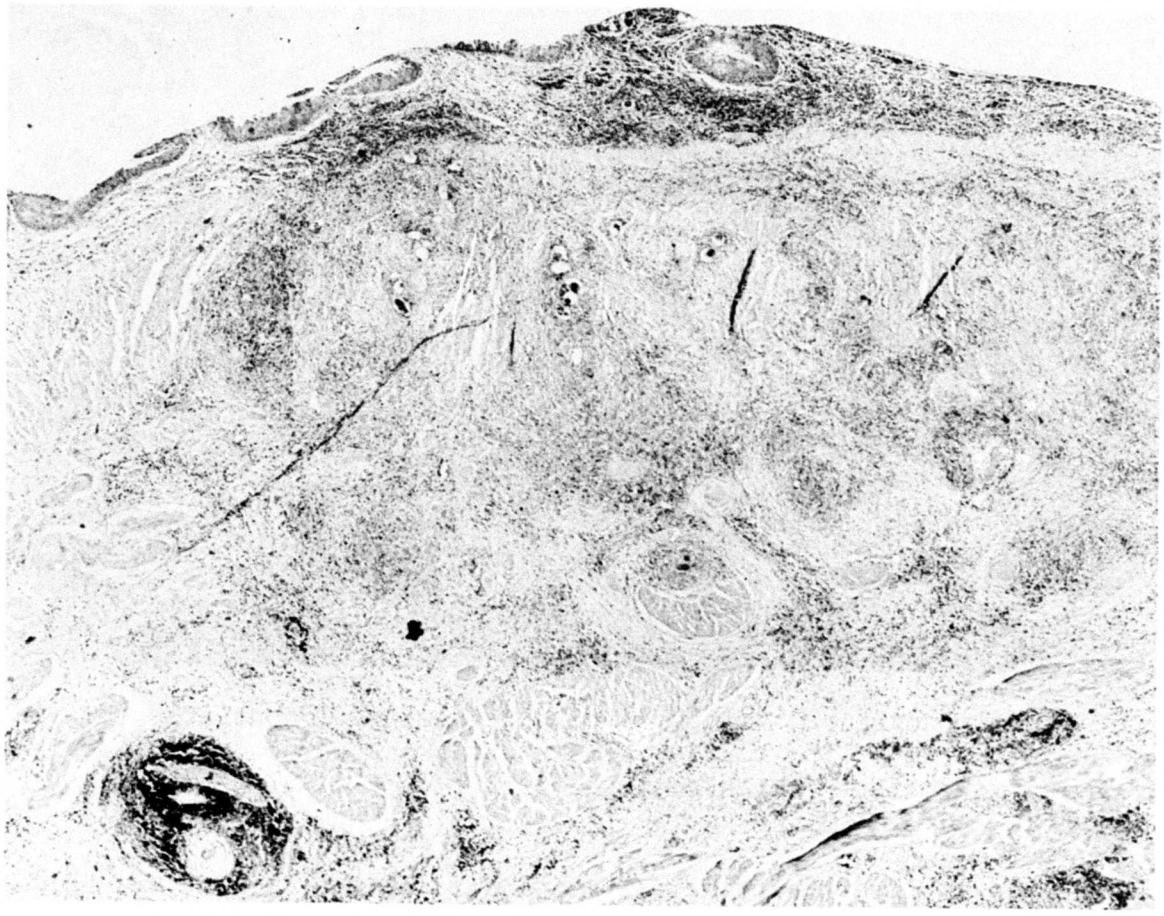

Fig. 22. Chimpanzee 62. Fibrous patch of bladder, panoramic view. Compare with Fig. 19. The thickened subepithelial layer is occupied by large, poorly defined granulomata with exuberant, concentric fibrous-tissue proliferation; the density of the inflammatory exudate has lessened considerably. There is persisting epithelial hyperplasia and nest formation. The muscle layers are infiltrated, with encroachment of granulomata on individual bundles, best seen near the center, and with streaky fibrous, inflammation, and egg deposition toward the serosal layer. (Hematoxylin and eosin, X 28).

Fibrous patches showed many of the same gross features seen in the polypoid variety, but differed by their paler, tannish-pink color, their lesser elevation and redundancy, and their tough, elastic consistency (Fig. 23). On section, the knife grated through firm, somewhat whorly fibrous tissue, which was pinkish-white to grey in color, with partial loss of the markings of the bladder layers. The mucosa at the level of the patch was fused with the muscularis and the whole bladder wall segment involved was less pliable. Sometimes the subserosal was obviously thickened and showed granularity of its peritoneal aspect.

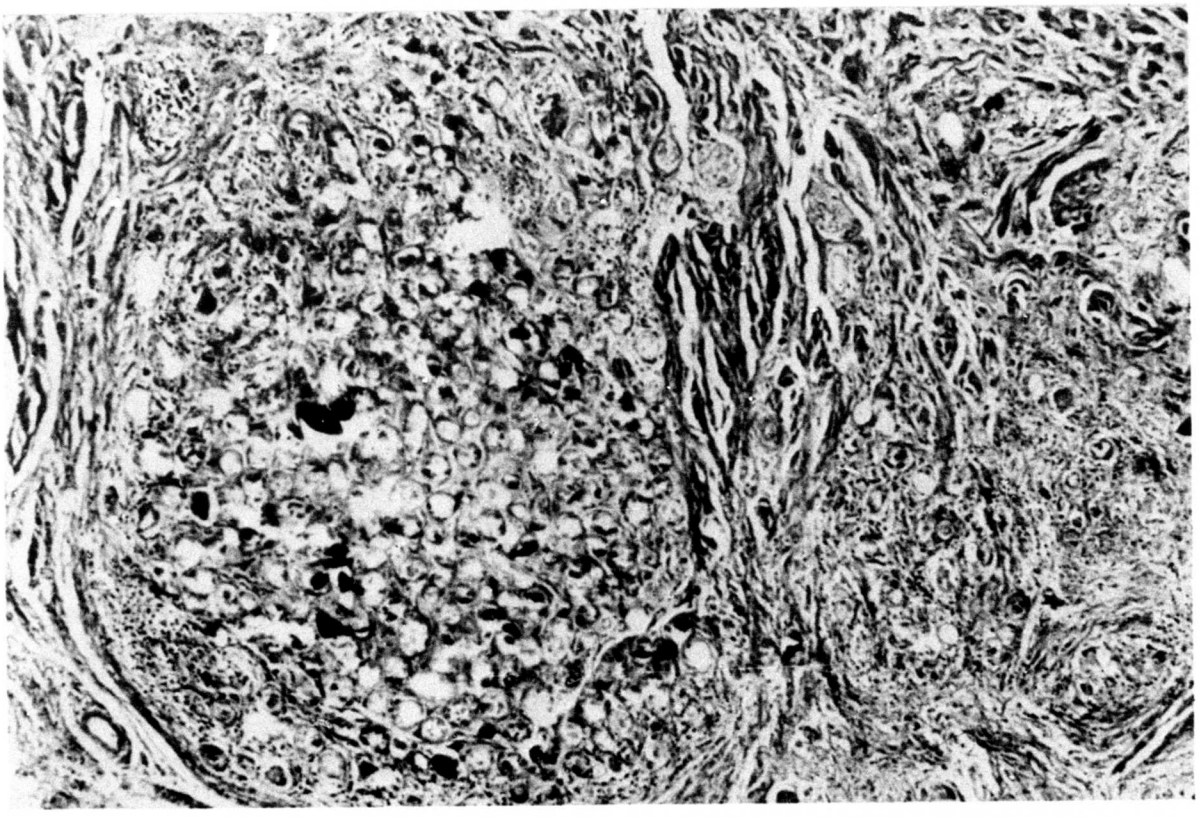

Fig. 23. Chimpanzee 62. Nidus of partly calcified, nonviable eggs in the subepithelium of the bladder within a fibrous patch. Note massive, peripheral fibrosis. (Trichrome, X 64).

Histologically, the principal components of the lesions were diffuse granulation tissue and focal granulomas, as observed in the polypoid patches. However, in the fibrous patches, the diffuse inflammatory infiltrate appeared generally less bulky and dense, and edema was replaced by fibroblastic proliferation and collagen deposition (Fig. 24). Usually, the subepithelial zone showed diffuse active inflammation with little fibrosis. Large composite granulomas were seen with exuberant, concentric fibrosis aggregated in the deeper lamina propria and superficial muscle layers, which they infiltrated and partially destroyed. These granulomas tended to form a confluent matrix of proliferating fibroblasts and hyalinized collagen oriented around clumps of eggs,

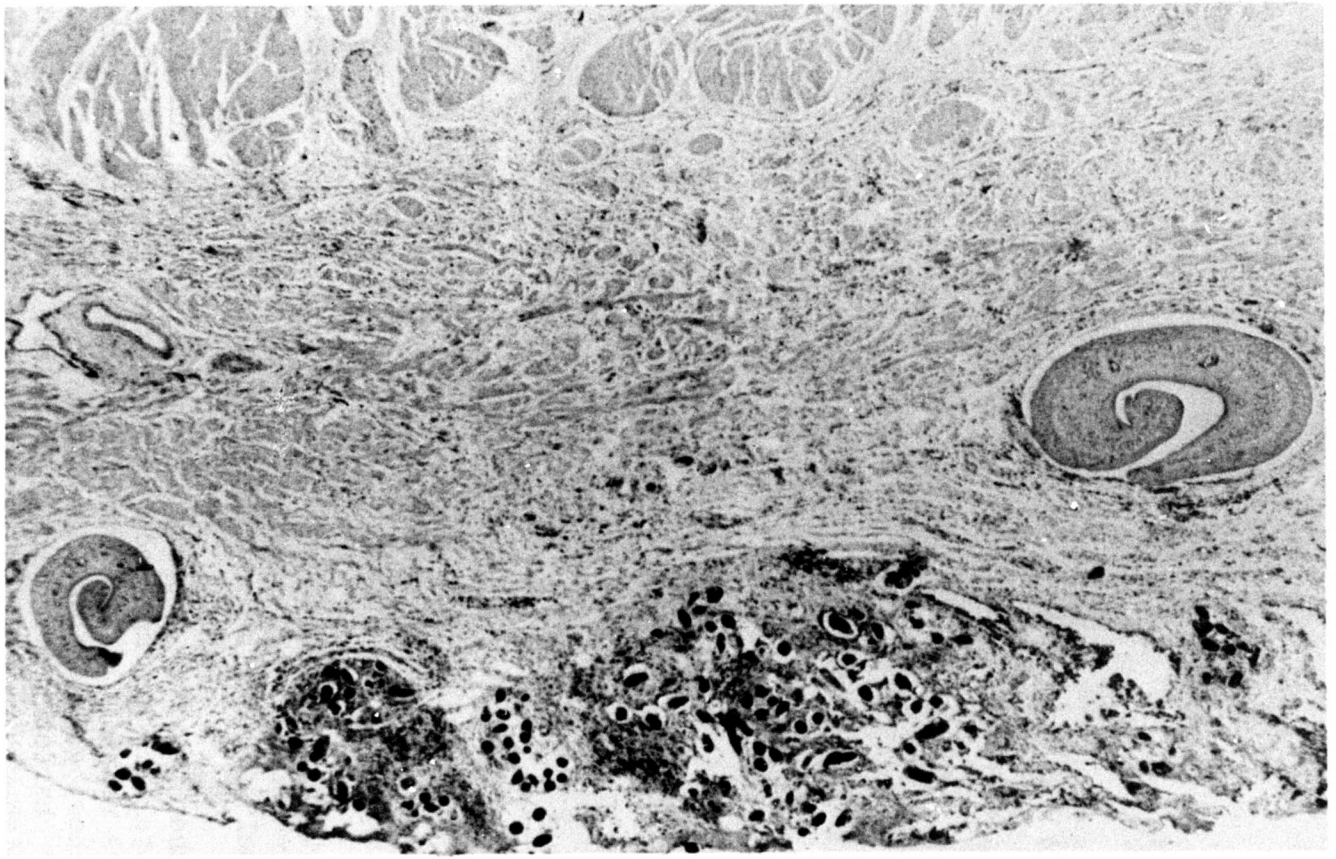

Fig. 24. Chimpanzee 10. Low-power view of a sandy bladder patch (compare with Figs. 19 and 22). Two male schistosomes in submucosal veins are seen in each side. Closely packed eggs, many of them calcified, are seen in the epithelium and superficial subepithelial layer, the deeper portion of which shows mild fibrosis. There is a decrease in the number of inflammatory cells, and little involvement of the deeper layers. (Hematoxylin and eosin, X 41).

most of which were degenerating, and a few calcified (Fig. 25). The fibrotic area was interspersed with granulation tissue and permeated by eosinophils and other inflammatory cells.

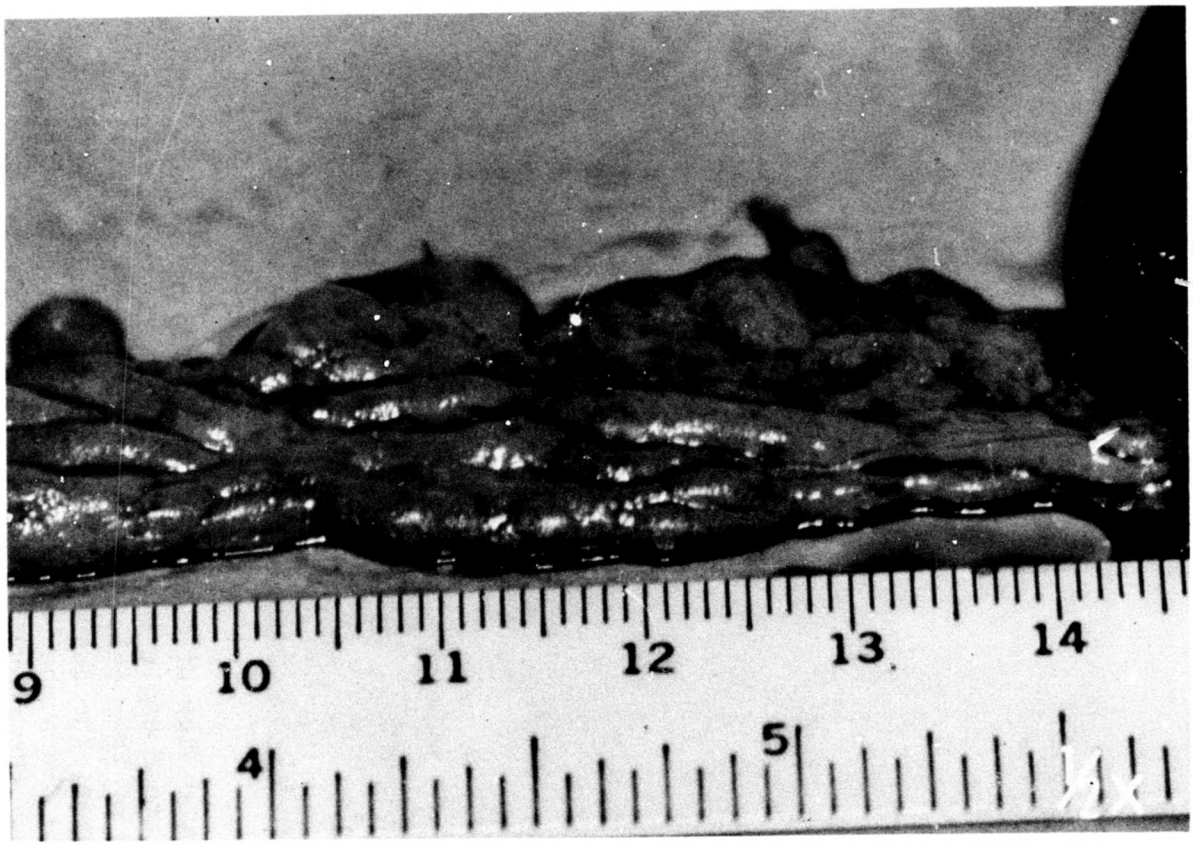

Fig. 25. Chimpanzee 56. Close-up of fusiform thickening, mucosal view. Note the logitudinal furrowing of the confluent polypoid patches typically seen in the ureter.

Both recent and involuting granulomas were present in many lesions. The muscle layer showed areas of fibrosis, and was invaded by eggs and inflammatory foci as described earlier, often down to the serosal level.

Sandy patches appeared grossly as slightly elevated and sharply demarcated mucosal thickenings and roughenings of irregular shape and size. They were relatively flat and small compared with the polypoid or fibrous patches, and were sometimes more easily felt than seen, by virtue of their characteristic finely granular, "sandy" surface. The mucosa was tannish-yellow to grey in color, sometimes red mottling and with numerous minute erosions visible on close inspection. Sandy patches were variably indurated, though usually more pliable than fibrous patches. Many had thin layers of calcification, which on cross-section cracked easily. Yellowish deposits in the superficial lamina propria and mild thickening of this layer were seen. Some larger

patches affected the entire thickness of the bladder wall. Although there was mild fibrosis, little or no edema occurred.

Histologically, as also seen in the polypoid and fibrous patches, adult schistosomes were sometimes found in underlying veins (Fig. 26). Sandy patches contained schistosome eggs which were primarily degenerate and calcified (Fig. 27). The eggs were aggregated in densly packed massive deposits just under the bladder epithelium, and appeared there in larger concentration than in either the polypoid or fibrous patches. The edema and dense granulation tissue seen in polypoid patches were absent. Focal reaction to viable eggs was usually granulomatous. Concentric fibrosis, and a sparse inflammatory infiltrate consisting mainly of lymphocytes and eosinophils condensed around some eggs and blood vessels were scattered throughout the patch. The fibrosis was limited and was concentrated around the massed deposits of degenerate and calcified eggs. Thin connective tissue septa and vascular spaces separated the clumps of eggs. Epithelial proliferation was less marked than in the polypoid patches, and areas of nest formation and metaplasia alternated with shallow erosions and atrophy. Sometimes groups of eggs were separated from the bladder lumen by only a thin, parakeratotic cornified membrane. Active and granulomatous lesions were at the base and periphery of calcified egg masses. Sometimes calcified egg deposits with sparse tissue reaction alternated with more active and recent lesions. The muscularis was relatively intact in the smaller sandy patches, but in the larger ones it contained scattered eggs, together with granulomatous or fibrotic foci, usually displaying only moderate trabecular distortion.

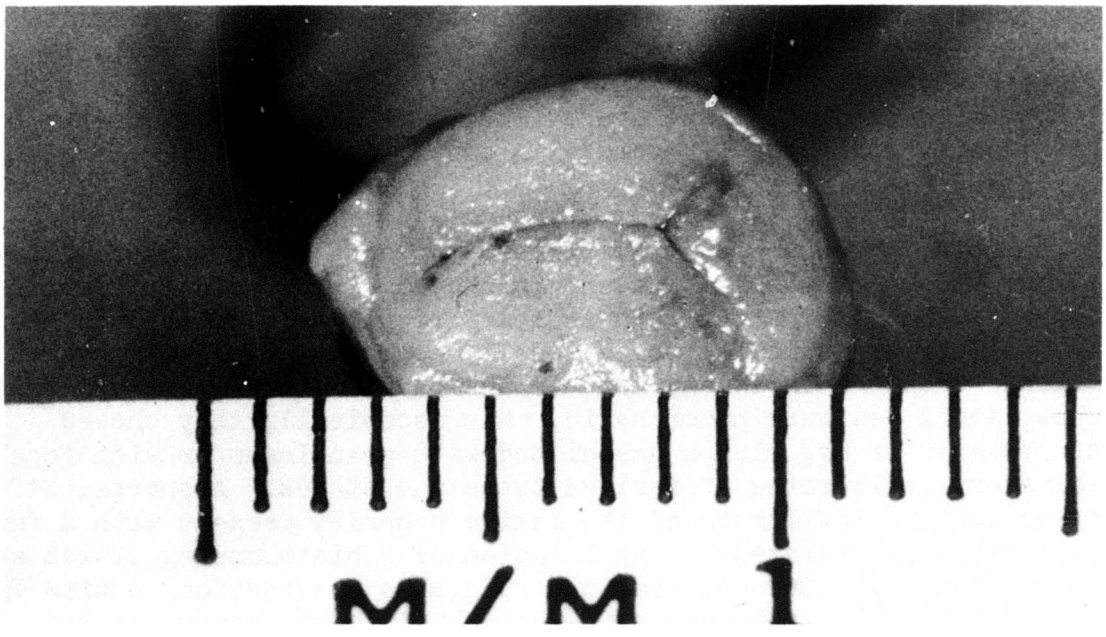

Fig. 26. Chimpanzee 63. Cross-section through a fusiform ureteral patch shows thinning and distention of the circular muscle layers, exuberant and confluent polypoid patches of the subepithelial layer, and slit-like compression and deformity of the lumen.

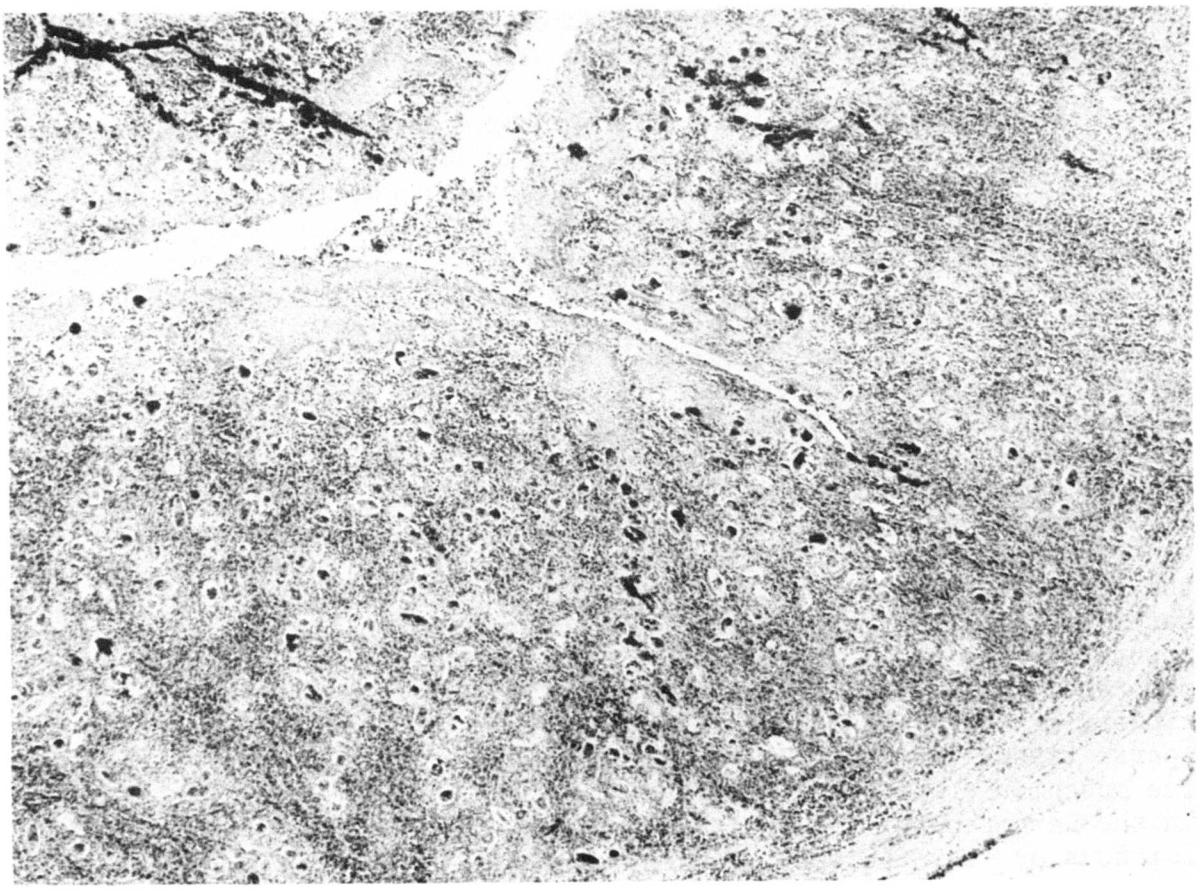

Fig. 27. Chimpanzee 63. Panoramic view of cross-section of ureter involved by confluent polypoid patches. Note the slit-like deformity of the lumen, exuberant, diffuse inflammatory tissue alternating with poorly defined granulomata in the submucosa, and streaky infiltration of the thin, distended muscular layer. The folds in the upper corner of the field are artifacts. (Hematoxylin and eosin, X 36).

Polypoid, fibrous and sandy patches appeared in various combinations, sometimes all of them being present in the same bladder. In addition, scattered inflammatory foci were visible grossly as minute (less than 1 mm), whitish or yellowish, occasionally confluent, mucosal dots and granules. These occurred anywhere in the bladder mucosa between the patches, and a few were hemorrhagic. Microscopically they showed scattered eggs or egg clumps associated with granulomas or with focal inflammatory infiltrates of various types and stages. A sparse, diffuse lymphocytic infiltrate of the lamina propria, present with a few eosinophils and unrelated to the location of schistosome eggs, was also seen occasionally. Our observations suggest a progression in time from the polypoid, to the fibrous, and eventually, to the sandy patches.

The ureters. The same types of lesions described in the bladder also occurred in the ureters. Polypoid patches involving the entire circumference of the ureter were the most frequent type. Seen from

the mucosal side, they often showed marked longitudinal ridging and furrowing (Fig. 28). On cross-section they involved all layers of the wall, with the heaviest inflammatory lesions in the lamina propria. The delicate normal folding pattern of the mucosa was severely distorted, and the normally thin and parallel muscle layers were infiltrated, resulting in considerable muscular disorder and disaggregation. In some affected segments these changes resulted in slit-like compression and stenosis of the lumen (Figs. 29 and 30), while in others with marked muscular stretching, there was fusiform dilatation of the lumen (Fig. 31). Worm paris in serosal veins showed a close spatial relationship to the patches. This relationship was more evident in the ureter than in the bladder. Fibrous patches characterized by marked thickening, stiffening and stenosis of the ureter occurred occasionally (Fig. 32). Sandy patches of the ureter tended to be shallower and more circumscribed than in the bladder, and were located in segments of either dilated or normal caliber and were not the cause of strictures (Figs. 33 and 34). Scattered inflammatory foci similar to those in the bladder were seen anywhere along the ureter, and occasionally in the renal pelvis.

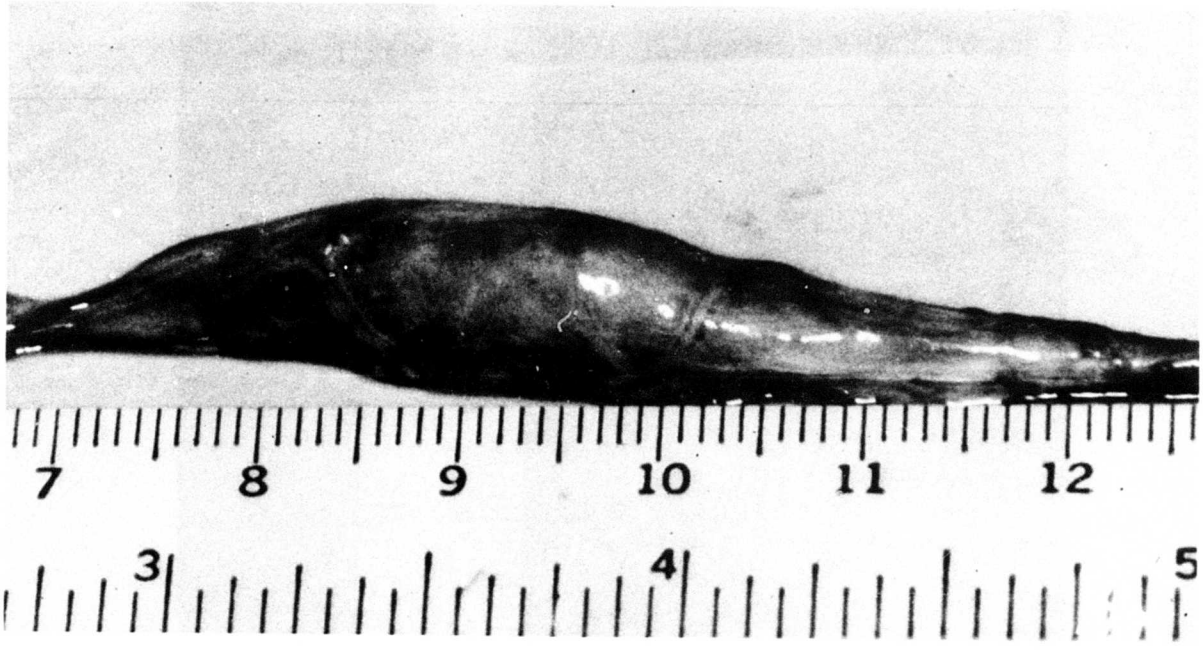

Fig. 28. Chimpanzee 56. Close-up of a fusiform ureteral thickening due to confluent polypoid patches; serosal view.

The kidneys. Groups of schistosome eggs were found microscopically in several kidneys, mostly near arcuate arterial branches at the corticomedullary junction; none were seen in glomeruli. Most eggs in the kidney were nonviable or calcified, with little tissue reaction. they occurred independently of any other significant parenchymal pathology.

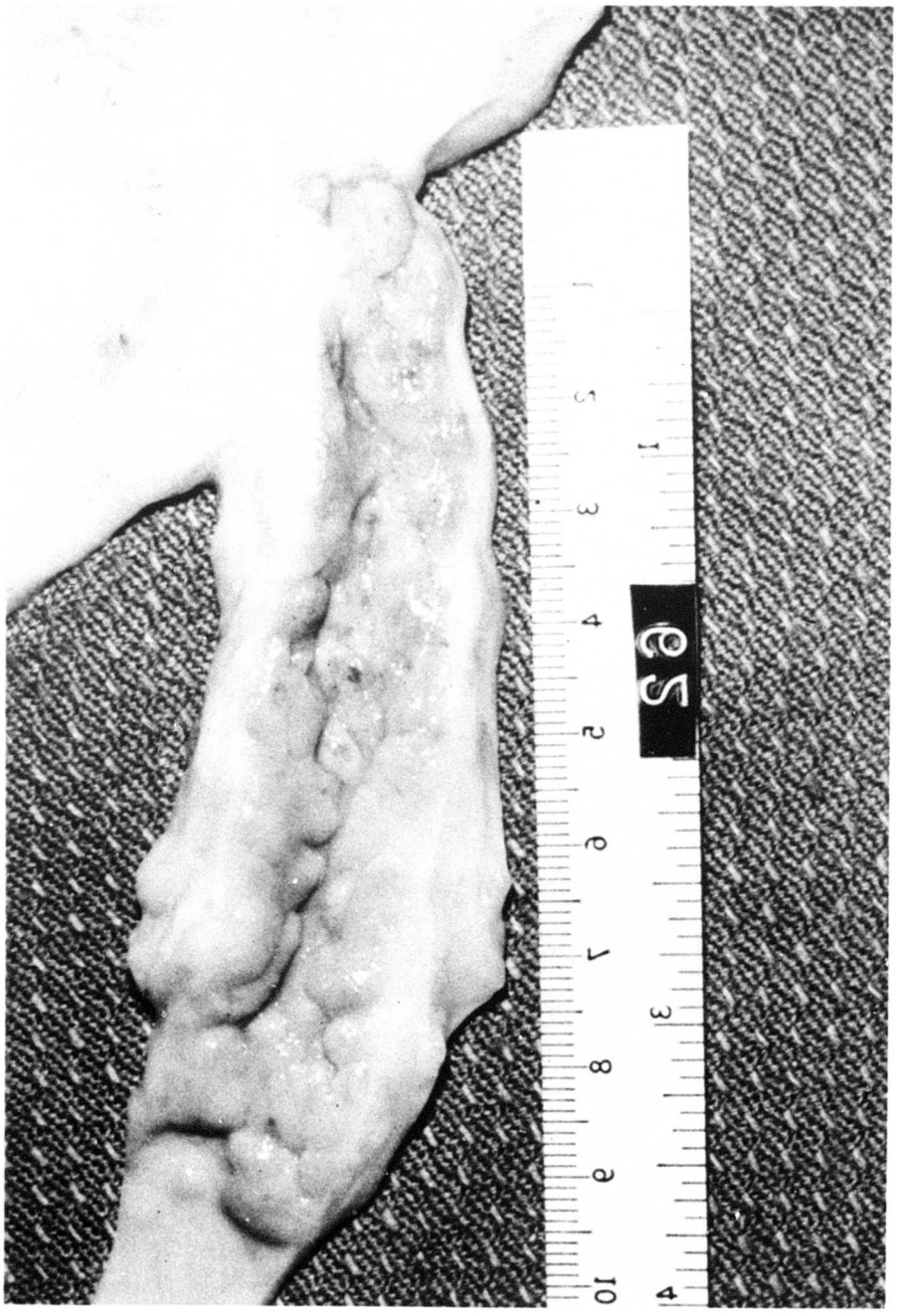

Fig. 29. Chimpanzee 62. Close-up of fibrous patches occupying the lower ureter close to its ostium. Note the thickening of the wall, best seen at the lateral-section lines, and the endophytic bulging of the mucosa. The proximal transition from the thin-walled, dilated portion of the ureter to the fibrous lesion is sharp. Sandy patches are seen in the bladder mucosa.

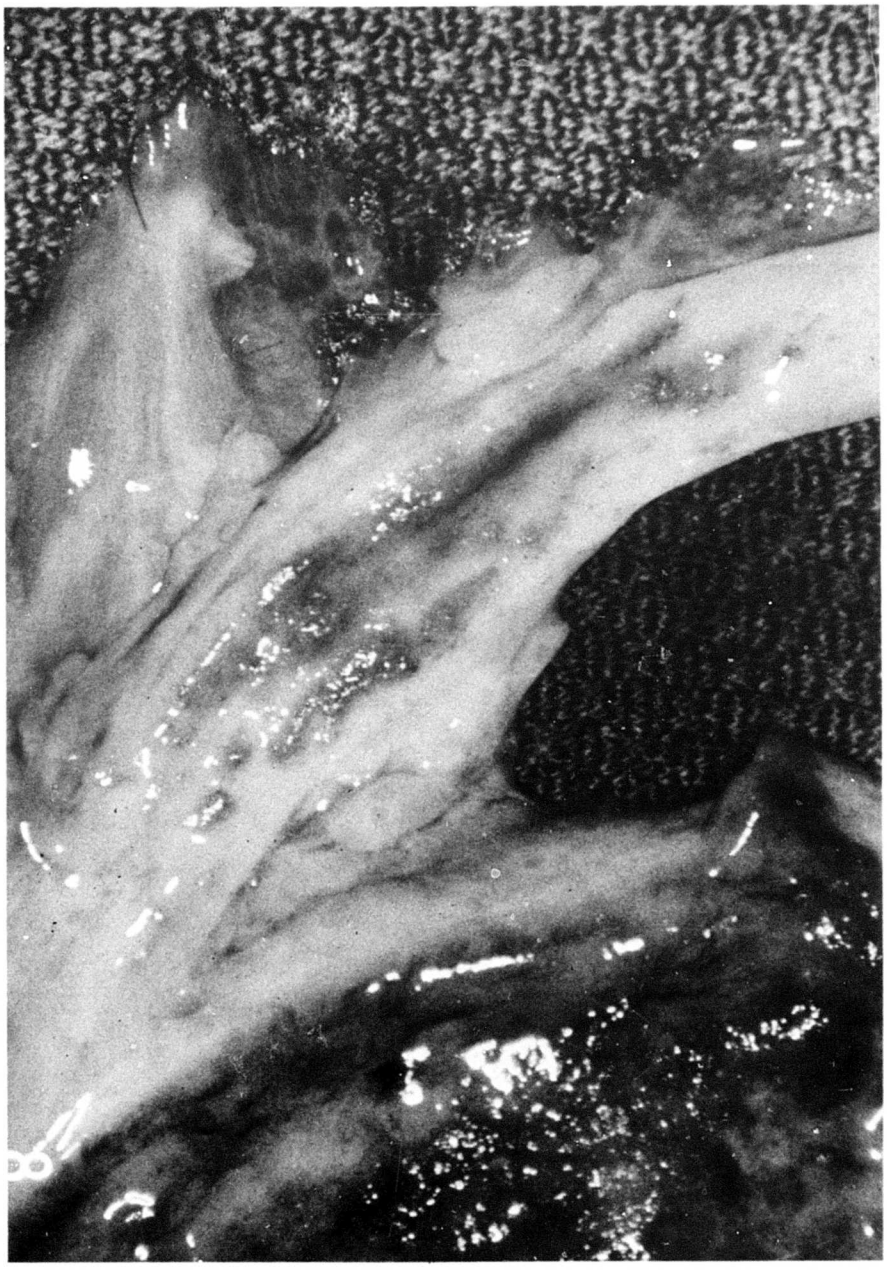

Fig. 30. Chimpanzee 10. Sandy patches and scattered mucosal granulomata of the distal portion of the ureter. The patches contrast with the normal mucosa by their tannish color. Note the relatively flat elevation of the patches and the normal thickness of the affected ureteral wall. The bladder mucosa shows sandy patches and diffuse inflammation.

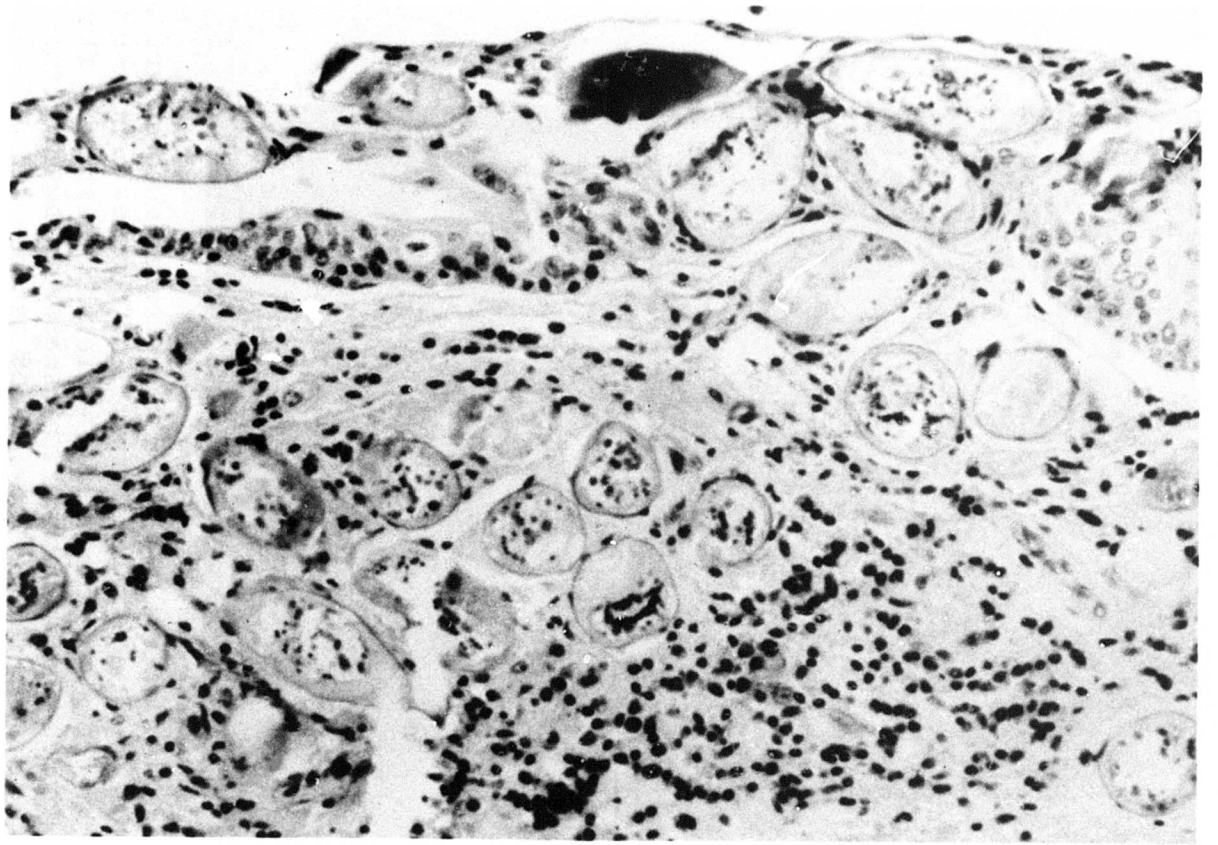

Fig. 31. Chimpanzee 62. Sandy patch in the ureter. The mucosa shows squamous metaplasia with a thin, superficial keratinized layer that barely separates the eggs from the ureteral lumen. The inflammatory infiltrate is sparse and predominately mononuclear. (Hematoxylin and eosin, X 256).

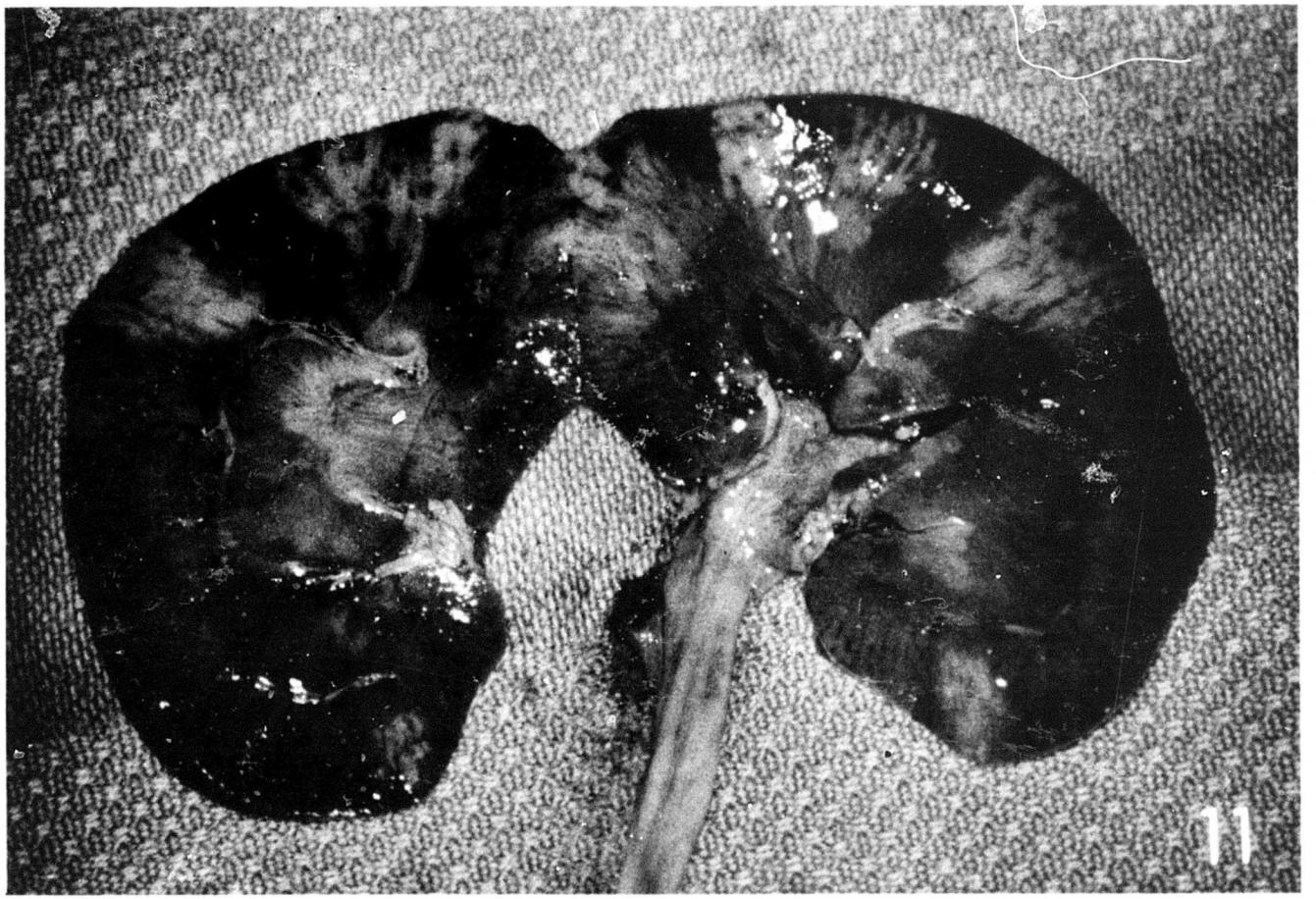

Fig. 32. Chimpanzee 10. The bisected kidney shows large, wedge-shaped cortical abscesses, with purulent medullary streaking and diffuse hyperemia. There is a patch of grayish discoloration of the cortex, and diffuse inflammation of the pelvic mucosa. A small sandy patch is seen in the upper ureter.

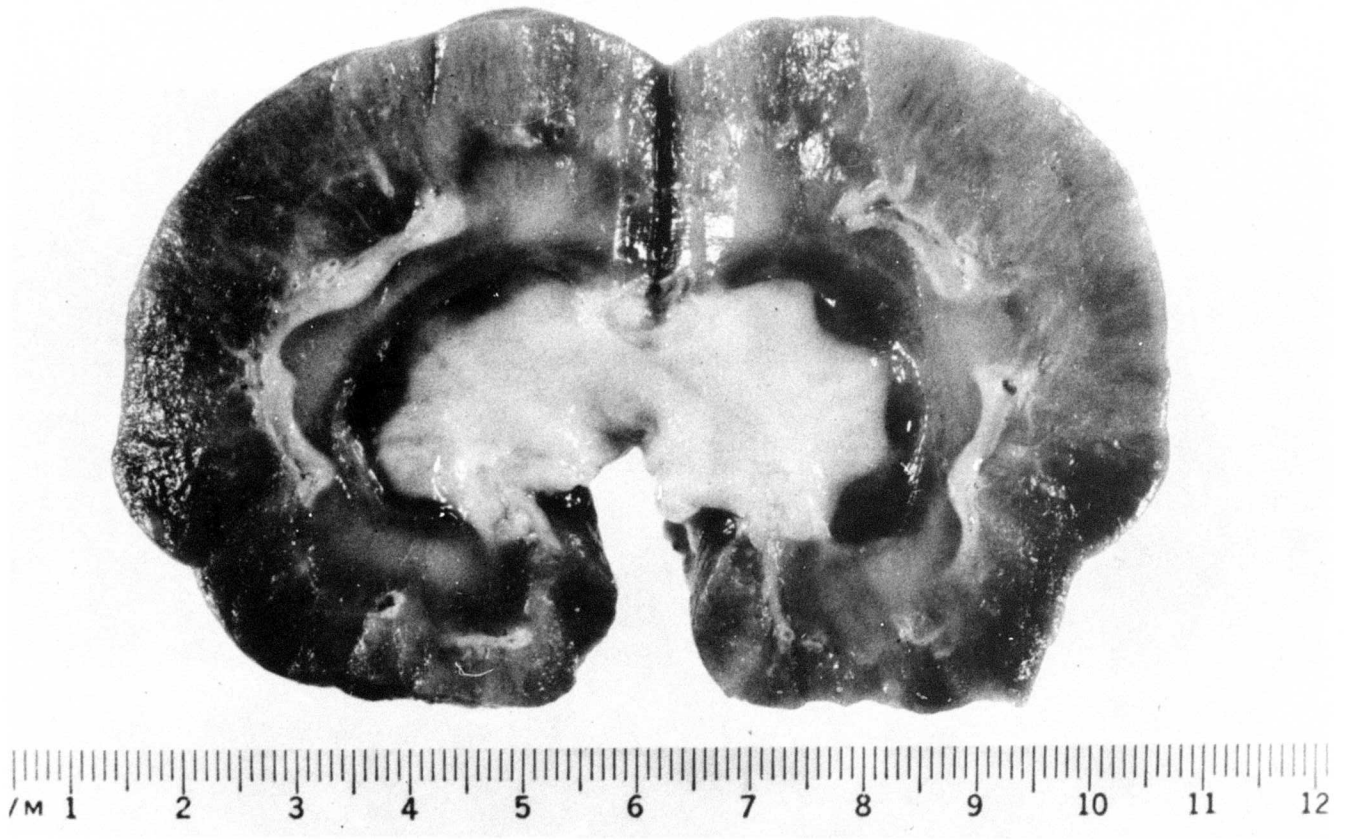

Fig. 33. Chimpanzee 62. Kidney detached from its ureter and opened through its outer margin to show the distended pelvis with blunting of papillae, characteristic of hydronephrosis. The corticomedullary junction is poorly defined, due to concomitant chronic pyelonephritis.

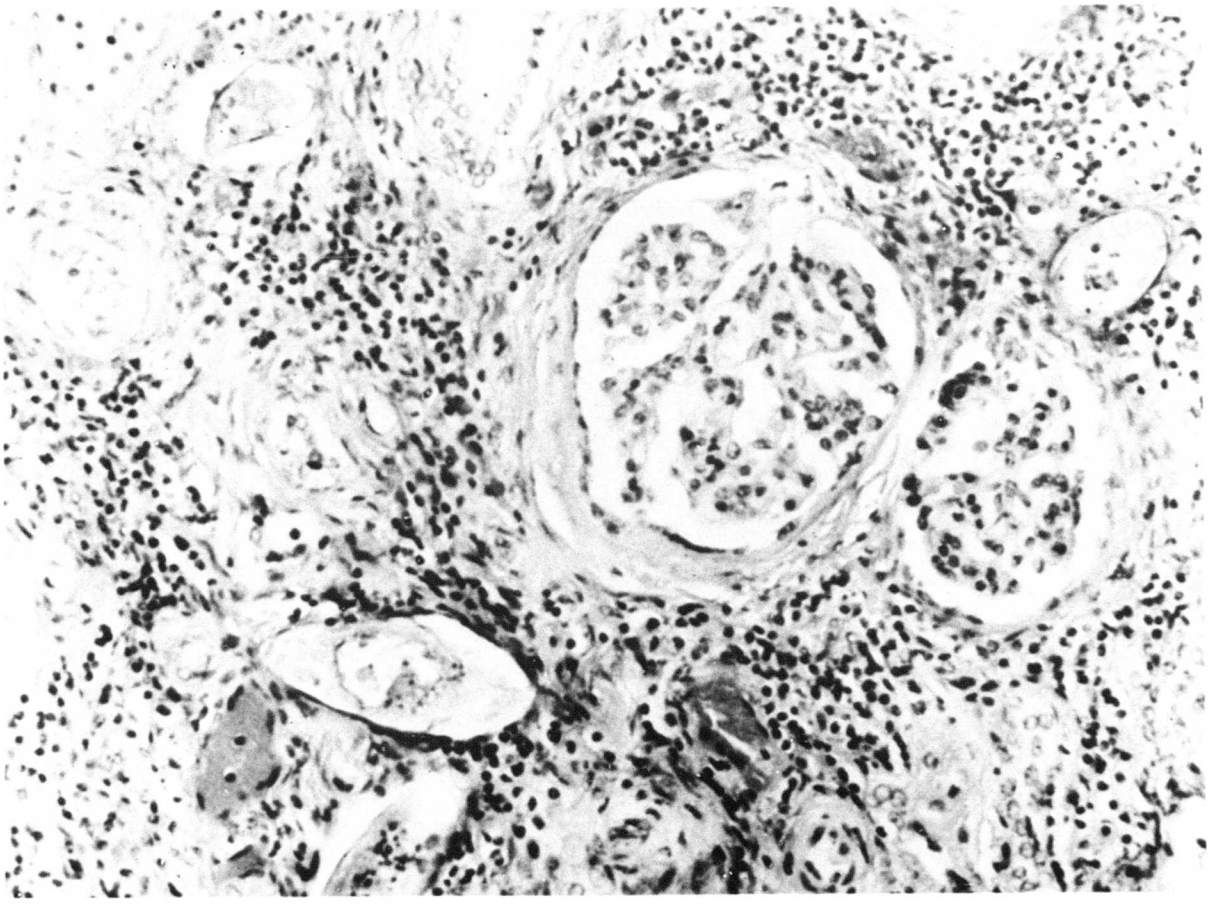

Fig. 34. Chimpanzee 10. Kidney showing chronic active pyelonephritis and schistosome eggs. Note capsular proliferation of glomeruli, diffuse interstitial infiltrate including plasma cells, and arteriolar thickening. Four schistosome eggs are scattered in this coritical focus.

Acute and chronic ascending pyelonephritis was found in two animals (Nos. 10 and 62, respectively). In one of them (No. 10), it was associated with unilateral renal atrophy due to scarring and tissue loss; the opposite kidney showed multiple cortical abscesses of pyramidal shape (Fig. 35); in the other (No. 62) the disparity between kidney sizes was less, inflammation was more diffuse and associated with bilateral hydronephrosis (Fig. 36). In both cases the pyelonephritic lesions were of the common type, consisting of streaked, mixed inflammatory infiltrates of the medulla and cortex, granular casts and abscesses in the collecting tubules, glomerular destruction, periglomerular capsular fibrosis, areas of severe scarring and "thyroidization" of tubules, and mild vascular sclerosis (Fig. 37). Clumps of bacteria were demonstrated by the Gram stain. In the other six animals of the series, kidneys were normal.

The gut. Polypoid patches grossly similar to those of the bladder were seen in the colon of most of the chimpanzees. A sandy patch was

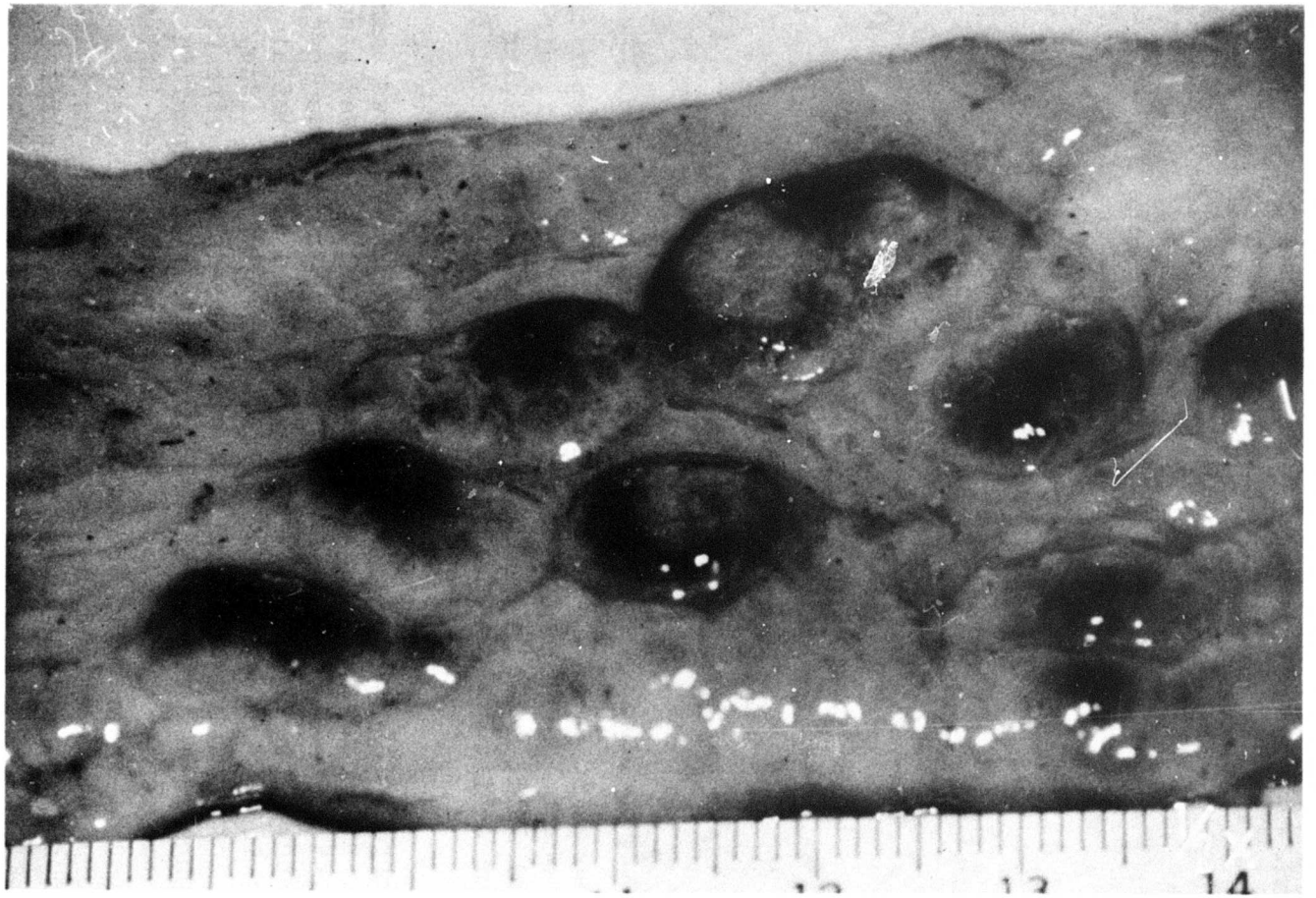

Fig. 35. Chimpanzee 56. Rectum showing polypoid patches; the pale areas topping some of the patches represent mucosal erosion, the darker areas, hemorrhage and hyperemia. Note the round-to-ovid shape of these discrete patches and their longitudinal orientation.

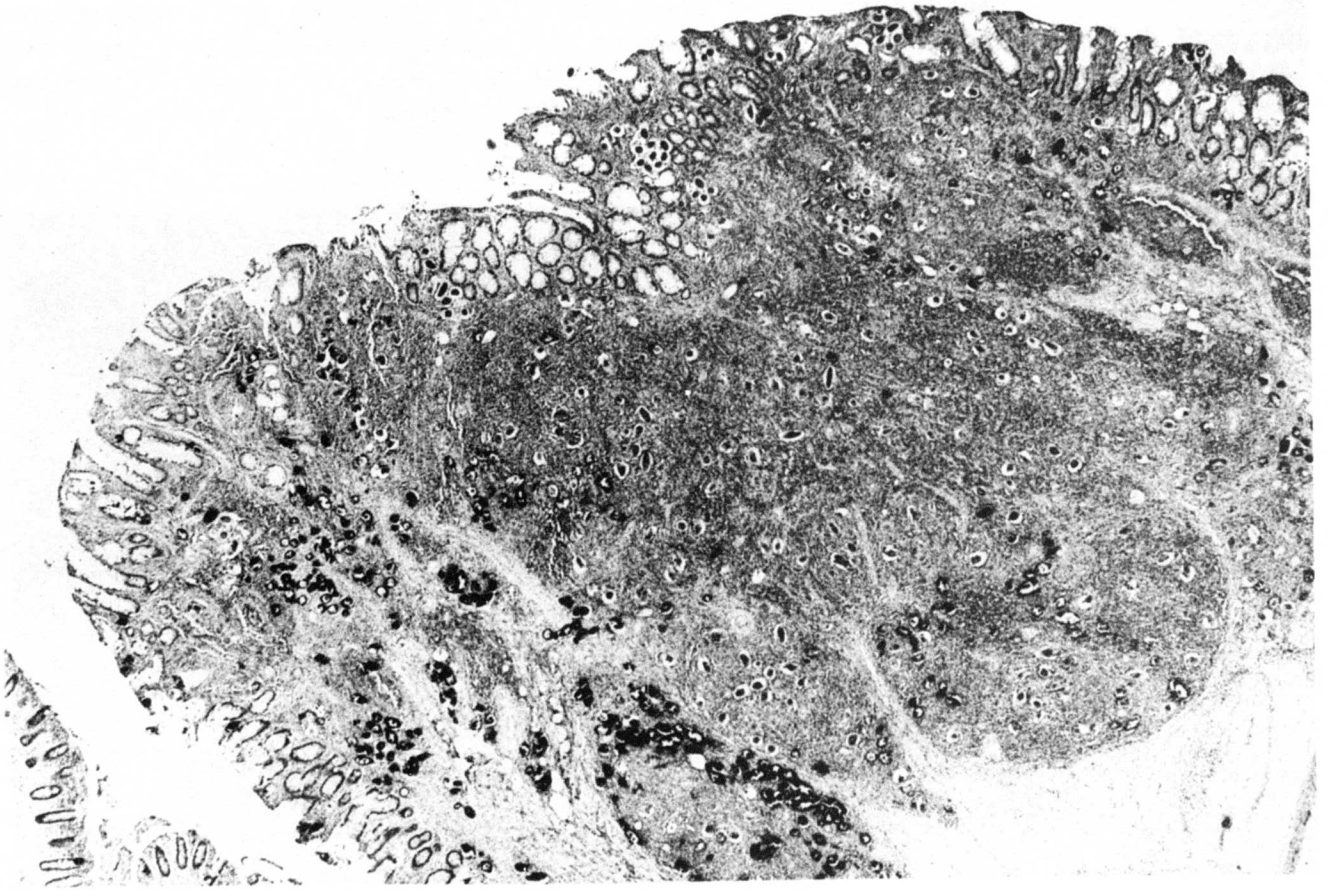

Fig. 36. Chimpanzee 56. Polypoid patch of sigmoid colon, panoramic view; compare with Figs. 19 and 27. Some of the eggs have undergone calcification and appear basophilis. Note irregularity of the mucosal crypts compared with the normal structure seen in the left lower corner.

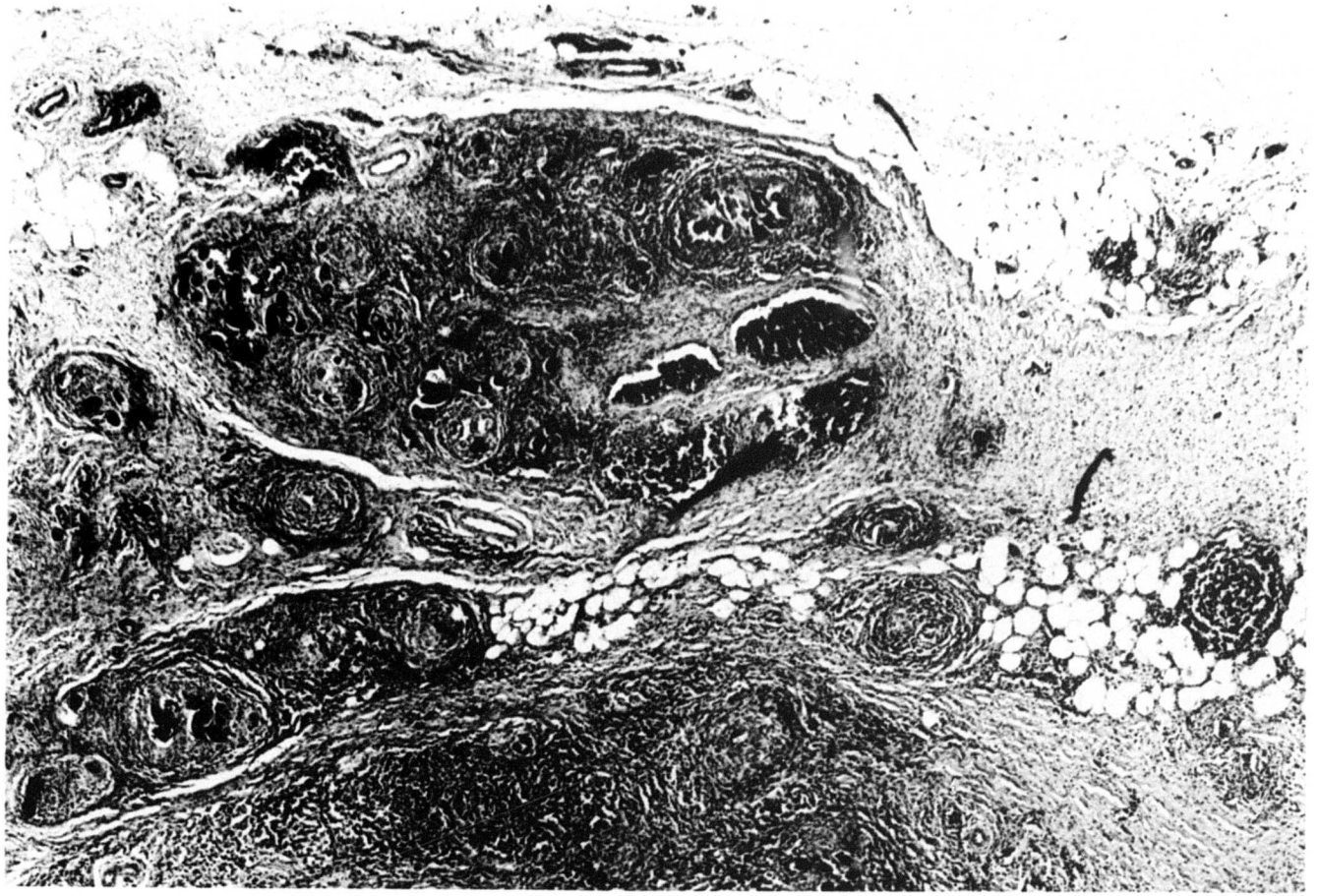

Fig. 37. Chimpanzee 63. Massive, fibrotic type of lumph-node involvement, showing granulomata lesions extending from the lymph node capsule (left) into the areolar fat tissue. The composite granulomata are large, fibrotic, and surrounded by proliferating fibrous tissue. All lymphatic structures have been obliterated.

visualized grossly in only one animal. Scattered inflammatory foci corresponding to the small mucosal teleangiectases seen grossly, focal hemorrhages or fine mucosal granularity were detected microscopically. The polypoid patches of the rectum and sigmoid tended to be discrete, 1-2 cm in size, ovoidal in the direction of the long axis of the gut and without overhang. They frequently showed mucosal erosion, ulceration or hemorrhage (Fig. 38). One large patch near the anus was quite bulky. Ulceration of rectal plaques was noted in two animals (Nos. 60 and 62). Schistosome eggs and granulomas (Fig. 39) and a number of abscesses and sinus tracts containing pyogenic exudate indicating fissuring and bacterial superinfection were seen histologically. The polypoid patches of the colon were similar to those of the bladder as regards the massing of eggs, granulation tissue and edematous changes. The scattered lesions outside the patches were often close to or at the site of lymphoid follicles of the lamina propria. In addition, there was a diffuse increase of eosinophils in the lamina propria throughout the colon, most noticeable near the areas of egg deposition. The colonic lesions were separated by wide areas of uninvolved mucosa. Marked pararectal fibrosis was present in one animal (No. 60).

Fibrous patches were found in the appendix in 5 chimpanzees. These consisted of hard, focally calcified masses with segmental enlargement of the appendix, usually with serosal granularity, and were histologically quite similar to what has been described in the bladder and ureter.

In general, no patches and only scattered foci were found in the ileum. No schistosomal lseions were observed in the upper small intestine.

Abdominal lymph nodes. Focal and massive forms of involvement of the lymph nodes were observed. In focal involvement, the nodes were conspicuously enlarged, but discrete, with intact capsules, and were soft and moist on sectioning. Histologically, eggs and granulomas were scattered in the sinuses, sometimes with coalescence into composite pseudotubercles (Fig. 40). Some of the granulomas were recent, with many eosinophils and central necrosis; in others, the eggs showed the Hoeppli phenomenon. Throughout the rest of the involved lymph node, and often even in lymph nodes apparently devoid of eggs or granulomas, there was lymphoreticular hyperplasia with marked eosinophil and plasma cell infiltration of the sinuses.

Massive involvement was seen primarily in the retroperitoneal and periaortic lymph nodes in one animal (No. 63). The lymph nodes contained indurated, tumor-like nodular masses and were hard and gritty on sectioning and composed of whorly, tannish-to-white fibrous tissue with focal calcifications. Microscopically this lesion was equivalent to the fibrous patches described earlier.

Genital organs. Only a single lesion was found in the female internal genitalia, namely a group of eggs in the mucosa of the lower

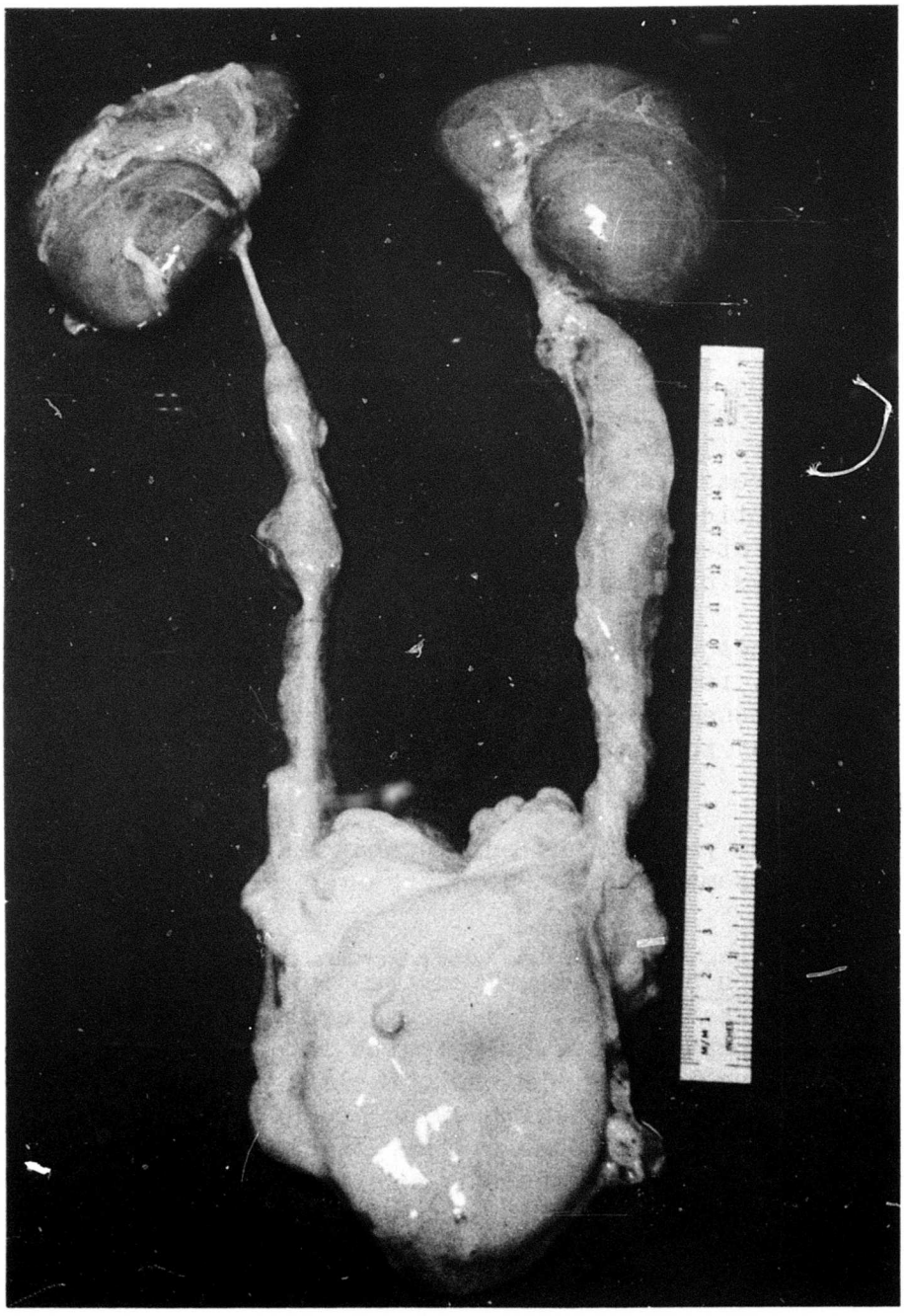

Fig. 38. Chimpanzee 63. Unopened urinary tract showing irregular bilateral ureteral thickening; note the contrast with a normal-caliber segment proximal to the kidney on the left. The involvement is asymmetrical. A Pentastomid larva is seen in the bladder serosa. The encapsulated kidneys as shown were normal.

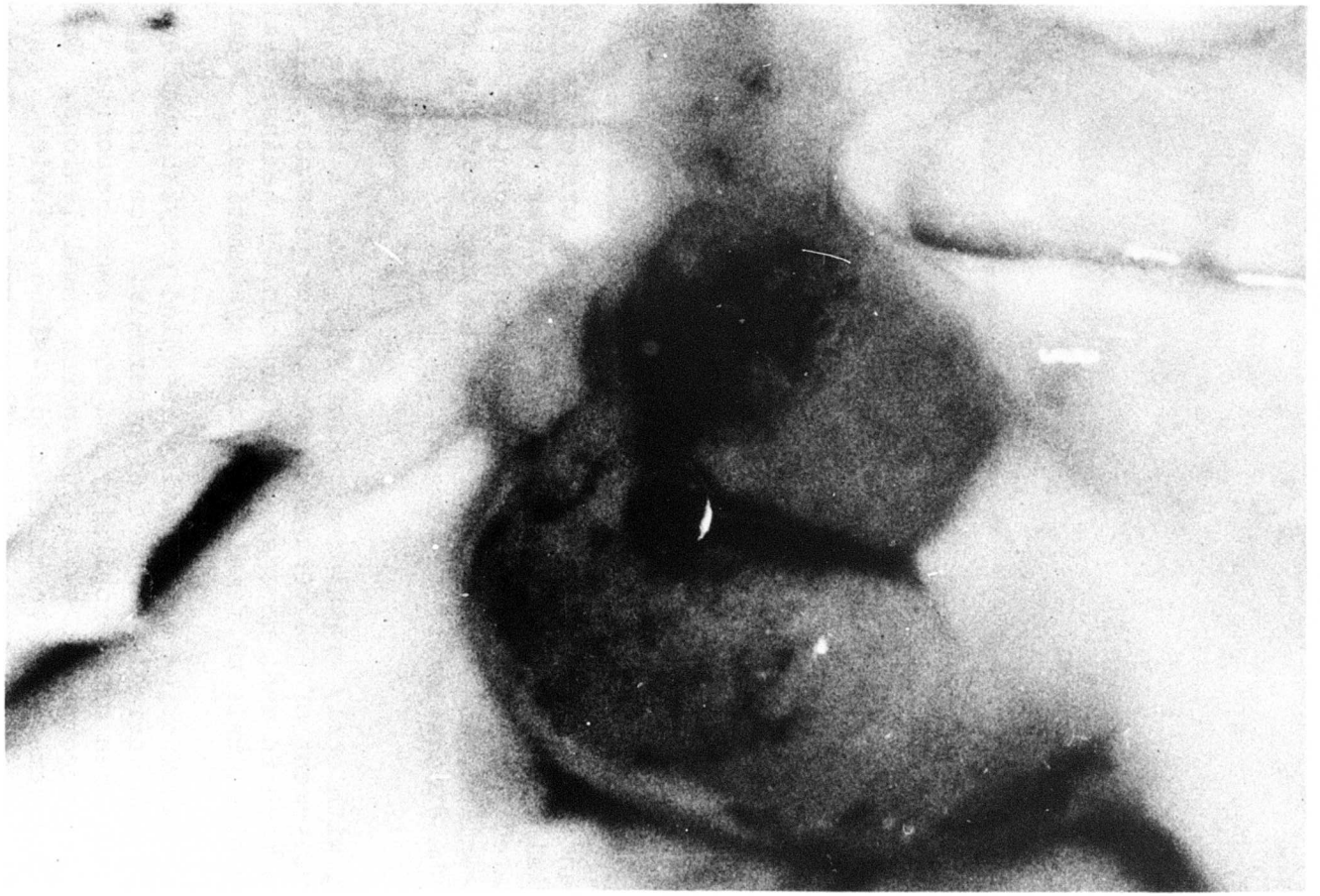

Fig. 39. Chimpanzee 63. Encroachment of a large polypoid patch on the right ureteral orifice, which is marked by insertion of a nail.

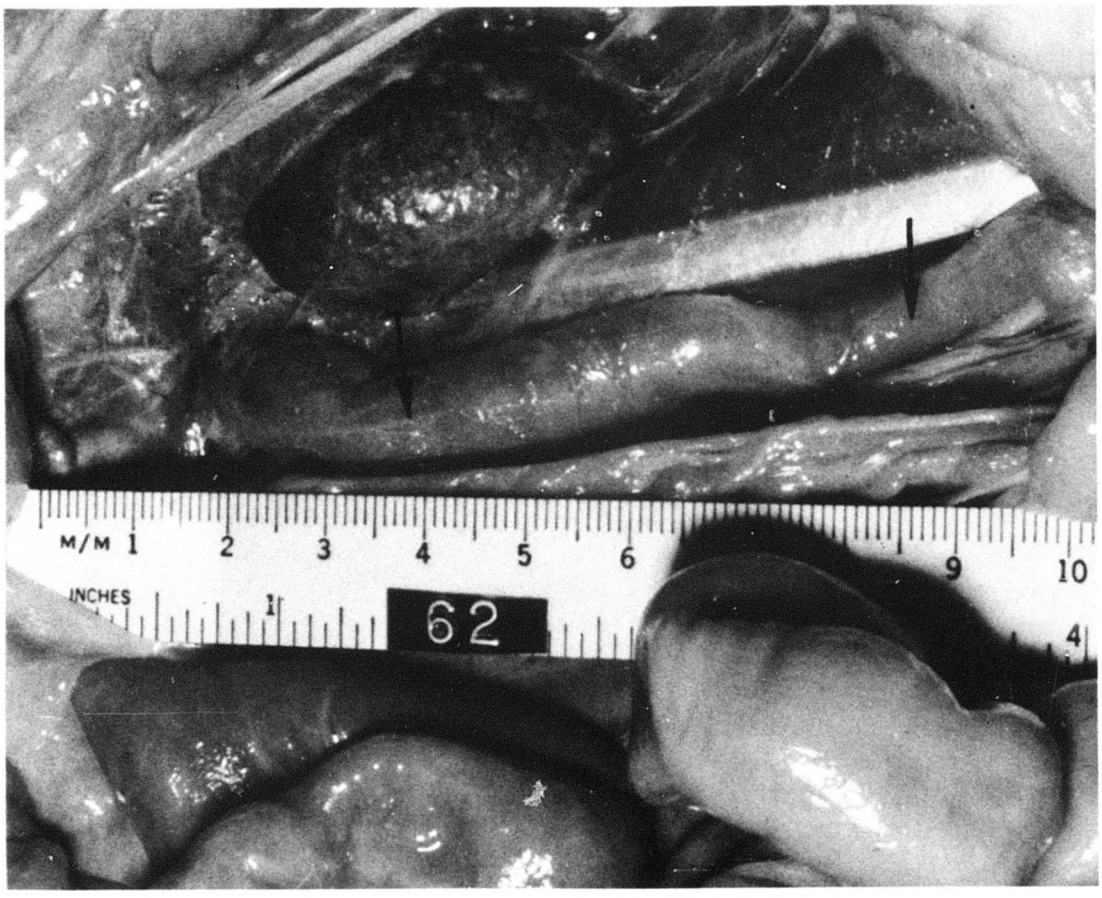

Fig. 40. Chimpanzee 62. Proximal portion of the left ureter in situ, shown lying medial to the iliopsoas muscle and left lower kidney pole, which was herniated forward during dissection. The ureter (arrows) is distended, thin-walled, and translucent. Note the loop near the lower margin, due to the elongation of the organ.

uterine segment, with early granulomas. Similarly, only a single case showed focal involvement of the veru-montanum of the prostate. In contrast to lesions seen in man, no eggs were found in the seminal vesicles, or in any of the other male or female genitalia systematically examined.

Lung. Gross examination occasionally revealed greyish subpleural specks, together with variable degrees of hyperemia. Scattered eggs and granulomas were seen microscopically and some were composite. These granulomas were not accompanied by diffuse parencyhmal lesions. One animal (No. 63) had some adult worms in middle-sized arteries. One pair was necrotic, causing a hemorrhagic inflammatory focus. In another chimpanzee (No. 62) a few thickened arterioles were seen adjacent to granulomas, but no intimal proliferation or fibrinoid necrosis was found. This animal showed the greatest lung involvement. The remainder had sporadic granulomas only. True schistosomal pulmonary arteritis was absent in this series of animals.

The liver. Subcapsular, widely-scattered pseudotubercles were seen at necropsy. In one animal (No. 60), mild accentuation of the portal fields was observed grossly. No excessive pigmentation of the liver tissue was found in any of the chimpanzees. Microscopic examination revealed scattered egg deposition and granuloma formation of variable degree with some composite granulomas. In addition, there were scattered diffuse infiltrates of the portal fields with lymphoid cells and eosinophils, occasionally with slight enlargement of the portal triad. In 3 animals, there was stellate fibrosis of portal fields found focally in the subcapsular area. This focal increase in fibrous tissue was not accompanied by alteration of the trabecular or lobular pattern.

Unrelated lesions. Strongyloid larvae or adults with focal eosinophilic infiltration of the lamina propria were found in the histologic sections of the upper small intestine or 4 animals. In some cases reddened patches were visible grossly in the small intestine. Oxyurid helminths were found at the level of the ileum or appendix with focal ulceration and severe inflammation in 3 chimpanzees. These lesions were accompanied by hypertrophy of Peyer's patches. One chimpanzee (No. 62) showed an unidentified helminth in a lymphatic of the submucosa, without any inflammatory response; two others (Nos. 20 and 63) showed Capillaria hepatica eggs in the liver, with focal subcapsular scarring. A tapeworm larva was seen in one animal (No. 20), and a Pentastomid embedded in fibrous tissue in the bladder subserosa in another. Scattered small granulomas with hemosiderin pigment and vacuolated giant cells were seen in the epicardium of one animal (No. 20).

Several chimpanzees showed focal pulmonary atelectasis, or focal acute bronchitis, and some degree of pulmonary congestion. Focal fatty change was occasionally noted in the liver. One chimpanzee (No. 30) had spotty, recent centrolobular necrosis of the liver associated with relatively marked acute enteritis; the kidneys were markedly congested with focal hemorrhage.

The only other noteworthy lesions were numerous hematomata of the portal fields in the liver of No. 50, characterized by cavernous vessels and multiple bile ductules in these enlarged portal fields.

Distribution and type of urinary lesions in individual chimpanzees. Polypoid patches were found in the bladder of 7 of 8 infected animals. Animal No. 10 had only sandy patches at the time of death. In two animals, only polypoid patches were seen at autopsy, while 5 showed a mixture of types of patches. The localization of these patches was irregular and their extent varied with the intensity of infection (Table 16). In general, the patches were irregularly distributed; however, in the animals with fewer lesions, the middle and lower thirds of the bladder were more affected. Patches in the vicinity of the ureteral orifices produced no apparent obstruction.

Table 16

Lesions of the bladder and ureters in chimpanzees infected with S. haematobium

Chimpanzee No.	Bladder lesions: % of surface involved*	Bladder lesions: Severity†	Ureteral lesions: Severity	Ureteral lesions: Local dilatation and thickening	Ureteral lesions: True hydroureter‡	Comment
20	15	1+	0	0	0	None
50	7	1+	0	0	0	None
30	18	1+	1+	0	0	Single 2 × 0.5 × 0.3 cm polypoid patch in left ureter 0.5 cm above ureterovesical junction
60	50	3+	2+	1+	0	Fusiform polypoid patch in middle of left ureter, with local dilatation; right ureter involved over 4 cm segment at ureterovesical junction with dilatation of 1.4 cm circumference
10	90	3+	1+	0	0	Bilateral involvement of ureterovesical junction; bilateral pyelonephritis
62	90	3+	6+	6+	3+	Voluminous fibrous patches extending 8–12 cm upward from ureterovesical junction with marked hydro-ureter and moderate hydronephrosis proximal to this; pyelonephritis
63	50	4+	4–5+	5+	1+	Polypoid patches as in No. 56 but with much more extensive tissue involvement (see Fig. 29)
56	50	4+	3–4+	3+	0	Polypoid patches involving most of both ureters

* Estimates of the percentage of surface involved varied with the degree of contraction of the bladder before and after perfusion and before and after fixation.

† A subjective rating, giving added weight to the presence of thickening of the bladder wall and the presence of large plaques, as opposed to petechiae and sandy patches.

‡ Thin-walled dilatation of the ureter proximal to apparent obstruction.

Direct ureteral involvement occurred early, and was noted in 6 of the 8 animals. In the two most heavily infected animals (Nos. 56 and 63) polypoid patches involved most of both ureters (Fig. 31) and produced thickening and local dilatation. In general, ureteral dilatation was confined to the thickened areas and did not involve the remainder of the ureter. It thus appeared to be related to inflammatory involvement of that portion of the ureter and not to obstruction. In animal No. 63 an areas of thin-walled dilatation of otherwise uninvolved ureter was present on the left side.

In the remaining animals, with one exception, dilatation was confined to the areas of direct tissue involvement, but not all schistosomal patches caused segmental dilatation. Chimpanzee No. 62, infected for 17-1/2 months, showed extensive fibrous thickening of the lower 8 to 12 cm of the ureters which measured over 2 cm in diameter and were extremely hard. The lumen was narrowed and distorted in this portion of the ureter (Fig. 32). The more proximal ureter was dilated (left 1.5 cm, right 0.8 cm in diameter), thin-walled and tortuous. Scattered sandy patches were seen in the thin-walled portions of these ureters.

These experiments indicate that the chimpanzee is well suited for studies on several aspects of S. haematobium infections. In all animals adult worms developed and produced eggs containing viable miracidia. After patency eggs were excreted in the feces and in the urine of these animals for the duration of the experiment. There was no significant decrease in the number of eggs excreted with time, nor was there any indication that the number of worms might be reduced by a process of self cure as occurs in S. mansoni infections in some hosts. In fact, the highest percentage of recovery of adult worms compared with cercarial exposures was obtained in a chimpanzee (No. 62) perfused 17-1/2 months after a single exposure. The ratio of worms recovered in the pelvic veins to those recovered from other systems was considerably higher in 3 of the 4 animals which had been exposed to cercariae only once. This suggests that a primary infection might have interfered with establishment of worms from subsequent exposures in the pelvic veins. Whether this was due to physiological crowding or a manifestation of acquired immunity is uncertain.

The figures for worm recoveries are considered only approximate since, in contrast to the observations with S. mansoni in chimpanzees, a significant number of worms remained in the pelvic venules after perfusion, as evidenced by histopathologic sections. Furthermore, since eggs in the bladder tissues were very unevenly distributed, bias occurred in spite of our attempts to select representative areas for digestion. Since eggs in the lungs and liver were distributed in a more uniform manner, the counts reflect a much closer approximation of the number of eggs in these organs. The distribution of lesions in the intestines was of no consequence for egg assays, since nearly all intestinal tissue was digested.

Schistosome eggs were found in all organs studied with the greatest numbers occurring in the bladder and in the large intestine, the organs in which the greatest pathologic changes occurred. As anticipated, the animals which developed extensive bladder pathology, hydroureter and hydronephrosis had the largest number of worms in the pelvic circulation.

Although the results varied greatly, a general correlation existed between the number of eggs in the colonic tissues and the number of eggs in the feces. Similarly, the number of eggs passed in the urine was generally related to the number of eggs in the tissues of the bladder and ureters. The total number of eggs in the tissue per female worm recovered (Table 9) was remarkably constant, considering the varied manner and duration of infections. A good correlation was found between the severity of bladder lesions, expressed as the percentage of bladder surface involved by schistosomal patches, the number of eggs found in the bladder and the number of eggs excreted in the urine during peak output. Although this peak varied from animal to animal, the greatest number of eggs found in the urine at any one time may reflect the maximal capacity of the worm pairs present in the urinary tract to produce eggs and consequently to produce tissue damage. As the infection progressed, the flow of eggs to the lumen of the bladder might have been influenced by factors other than the worm's rate of oviposition.

There was no obvious relationship between the number and intensity of lesions observed in the urinary tract and those found in the gut. The proportion of worms and eggs in the gut and urinary tract varied considerably. Therefore, it can be concluded that in the chimpanzee, as in man infected with this parasite, the mean number of eggs in the stools is a poor measure of the overall intensity of the infection. In some chimpanzees there was a predominance of the colonic over the urinary lesions; in others the converse was true. Thus, the chimpanzee may differ to some extent from man in the predominance of colonic over vesical lesions.

The serum biochemical findings in chimpanzees infected with S. haematobium contrasted in some important respects with the biochemical observations reported for mice and chimpanzees infected with S. mansoni. The increases in total serum protein and globulin concentrations recorded previously for schistosomiasis mansoni were more moderate in schistosomiasis haematobia. As in the chimpanzees infected with S. mansoni, there were no detectable increases in the levels of serum transaminases or alkaline phosphatases.

Antibodies reactive in the fluorescent antibody test were detected in the serum of all the chimpanzees shortly after exposure to infection. In contrast to what had been observed in the chimpanzees infected with S. mansoni, in the animals infected with S. haematobium there was a tendency for the antibody levels to decrease with time as the infection progressed. In one animal observed for 17-1/2 months, no antibodies could be detected after the 15th month of infection. The time course development of antibodies detectable by passive cutaneous anaphylaxis was unrelated to the magnitude or manner of exposure (whether single or repeated), to the intensity of infection or to the evolution of the disease.

One of the most striking findings in the pathology of schistosomiasis haematobia in the chimpanzee was the early appearance and patchy distribution of lesions and their relation to the sites in which the adult worms were found. The polypoid patches found in these chimpanzees were similar to those observed in the Tanzanian baboon, in clinical studies in children and in some human autopsies. It does appear that it also occurs early in human infections. The polypoid lesions observed in the colon of chimpanzees experimentally infected with S. mansoni and of infected humans in Egypt differe somewhat from those we observed with S. haematobium in that the patches seen here were scattered and more widely separated. This difference may result both because of the difference in the oviposition habits of the female worms of the two species (eggs are laid singly by S. mansoni, but in clusters by S. haematobium) as well as in the migrating habits of the adult worms. Since S. haematobium possesses a muscular sucker and resides deep in the complex venous plexus of the bladder it may be more difficult to dislodge. This may account for the failure in attempts to remove worms by extracorporeal circulation filtration technique in schistosomiasis haematobia. It also may explain why these worms often undergo necrosis in the bladder submucosa rather than embolize to the lungs.

The early tissue response to eggs of S. mansoni and S. haematobium in the chimpanzee are quite different. With S. haematobium the response is more exudative and less focal, and a smaller proprotion of eggs are found in the granulomas, whereas a larger proportion are in areas of diffuse inflammatory infiltration. Granulomas tend to be less uniform in size with fewer epithelioid and giant cells. At later stages, the minimal tissue reaction seen around massive calcified egg deposits in sandy patches is quite distinctive of S. haematobium. This difference in response may be related to the different nature of the egg shells of these two parasites. S. mansoni is acid fast while S. haematobium and intercalatum are not. The clustering of eggs in convoys and composite granulomas in S. haematobium infections was observed not only in the bladder and rectum, but also in sites such as the lung or liver where eggs are presumed to arrive by embolization.

The progression observed in the bladder lesions from the polypoid patches to either fibrous or sandy patches seems to follow a sequence similar to colonic inflammatory lesions. However, it should be pointed out that only sandy patches were observed in one animal at the time of death (No. 10) and that only polypoid patches were seen in two other less heavily infected chimpanzees. Moreover, one animal with the longest duration of illness presented a mixture of all three types of patches; this is consistent with a continual prolonged pattern of oviposition and indicates that the patches do not all involute or become fibrotic at the same time. This also suggests that worms might move into new sites of ovipoistion after a prlonged residence in one site, or that diminution of oviposition by certain worm pairs occurs, while other pairs in different locations continue their normal output. The change in morphology of the patches seems to be a gradual process and is similar to that observed in human autopsy material where the mean age of patients with exudative patches was significantly younger than that of patients with sandy patches. These observations might have a bearing on the clinical evolution of urinary schistosomiasis. One possible implication is that evolution of a patch from the polypoid to the sandy stage reduces its volume and might reduce or abolish attendant obstructive uropathy. Clinical evidence is accumulating that such changes occur in children with bladder lesions of the exudative type.

Ureteral dilatation was observed in 6 out of 8 of these infected chimpanzees. This is a greater proportion than has been observed in human cases with high tissue egg counts. Similarly, the frequency of lesions in the proximal and higher segments of the ureter was greater in the chimpanzee than in humans, where this type of lesion tends to occur in very heavy infections. It appears therefore that the habitat of S. haematobium in man is somewhat more circumscribed than in the chimpanzee. Two types of ureteropelvic dilatation were seen in this series. In two chimpanzees, portions of dilated ureter characterized by thin-walled widening proximal to areas of obstruction were found. In one of these chimpanzees the lower third of both ureters seemed uniformly narrowed; in the other, the obstruction was near the

ureterovesical junction. Similar lesions have been described for infected human. The other, more common type observed in our experimental animals was segmental hydroureter found where full-thickness schistosomal patches in the ureteral wall produced thickening and dilatation at the same time without the presence of mechanical obstruction. The relative frequency of this type of lesion and of obstructive hydroureter in human material remains obscure. Ibrahim and Sayegh indicated that obstruction was the most frequent cause of ureteral dilatation. Fam, in reviewing 312 cases with ureteral involvement, found many with strictures, but cases with dilated spindle-shaped and dilated atonic ureter were also very frequent. Gelfand's meticulous study of 110 consecutive necropsies in cases of schistosomiasis haematobia revealed 25 cases with dilated ureter without obstruction and only 2 cases with obstructive hydroureter. When no obstruction was present, dilatation was confined to the diseased segment of the ureter as it was in our chimpanzees. Gelfand emphasized that segmental ureteral dilatation could not be reasonably ascribed to obstruction, since this should produce dilatation of all of the more proximal portions of the involved ureter. In a recent series of autopsies involvement of the interstitial ureter was the predominant lesion associated with hydroureter, also without clear evidence of stenosis in most instances. High ureteral lesions were rare in this study. It therefore seems probable that the involved and functionally impaired portions of the bilharzial ureter might cause not only a local deformity, but also a physiologic obstruction with proximal ureteral dilatation, but this could not be ascertained conslusively in our short term study of chimpanzees, nor in Gelfand's series. However, this condition has been described in human material.

In the present study, intravenous pyelograms did not detect (1) the significant calcification of the bladder in one animal which was found after necropsy at 17-1/2 months; (2) the relatively flat sandy and polypoid patches in the same animal at later stages; (3) segmental dilatations of ureters in 2 chimpanzees (Nos. 56 and 63) found at necropsy shortly after the last pyelogram, and (4) the relatively mild polypoid patches in 4 animals. Thus, it appears that pyelograms may miss some significant anatomical lesions. A cystogram demonstrated reflux in one animal (No. 10) which was otherwise undetectable. In the most severely affected chimpanzee (No. 62) a filling defect at 6 months disappeared later. This is consistent with the postulated change from polypoid to sandy stage in the patches. Hydroureter became detectable at 6 months, with slow and progressive enlargement thereafter. It was described from the IVP as total ureteral dilatation down to the bladder in all cases seen, which is in marked contrast to the pathologic findings which showed segmental involvement with more or less dilated and narrowed fusiform areas. However, the involved segments of the ureters were generally not visualized in the intravenous pyelograms. Part of the distal left ureter of No. 63 showed extensive anatomic alteration but was normal in appearance on the IVP. Since the IVP was done one month before sacrifice, the disease may not have involved the visualized portion of the ureter at the time of the IVP.

Alternatively, the localized ureteral lesions may not greatly affect the luminal profile under conditions of functional urinary excretion.

Although it has been postulated that schistosome infections promote pyelonephritis in man, the evidence thus far available in outpatients does not confirm this hypothesis. It may be that this condition is more common among patients studied in hospitals and subjected to frequent catheterization or cystoscopy. Our chimpanzees which developed pyelonephritis had been catheterized repeatedly or examined by cystoscopy.

A surprising finding in our study was the total absence of involvement of the seminal vesicles. This is indeed different from what has been reported in man, where the seminal vesicles may be as heavily calcified and involved as the bladder itself. In a detailed documentation of an experimental infection of man, eggs appeared in the seminal fluid before they were excreted in the urine. Another difference between the findings with S. haematobium infection in chimpanzees and in man was the massive fibrous involvement of abdominal lymph nodes observed in the present series of animals, but very rarely in human schistosomal pathology.

Although the egg counts in the lung tissues with S. haematobium were much higher than with S. mansoni infections (as would be expected due to the caval distribution of many worms of S. haematobium), the diffuse pulmonary arteritis observed in the S. mansoni infections failed to materalize with schistosomiasis haematobia. The lesions consisted essentially only of focal granulomas and were both grossly and microscopically rather inconspicuous. Likewise, although the number of eggs observed in the liver with S. haematobium infections was sometimes high, fibrosis was absent, pigmentation was slight and no significant changes in the liver function tests were observed. Although the number of animals is small and the duration of infection was shorter for S. haematobium infected animals, our observations suggest that the amount of tissue damage produced by individual S. haematobium eggs is usually less than that produced by those of S. mansoni, except for the exuberant fibrosis seen in the bladder, ureters, appendix and lymph nodes, and provisionally regarded as an aberrant form of host response. Similar exuberant fibrous lesions are occasionally seen in persons infected with S. mansoni.

3. Effect of niridazole on muscle glycogen levels of monkeys.

One of the earliest detectable effects of the administration of the antischistosomal drug niridazole to the host consists of a reduction of the glycogen levels of the male Schistosoma mansoni. Degradation of glycogen is catalyzed by glycogen phosphorylase (alpha-1, 4-glucan-orthophosphate-glycosyltransferase, EC 2.41.1). In S. mansoni, as in a wide variety of species and tissues, glycogen phosphorylase is present in an active and an inactive form. The two forms are readily interconvertible. Active phosphorylase is converted to an inactive form by the action of phosphorylase phosphatase (phosphorylase phosphohydrolase, E.C. 3.13.17).

It has been found that the activity of phosphorylase phosphatase of male S. mansoni is inhibited as early as two hours after the oral administration of niridazole to the host. As a result of the inhibition of phosphorylase inactivation more active glycogen phosphorylase becomes available. This, in turn, brings about an increased degradation of glycogen in the worm and thus can account for the glycogen depletion of the parasite observed after the administration of niridazole. Attempts to determine whether this niridazole-induced inhibition of phosphorylase phosphatase was selective for the parasite revealed that oral administration of the drug to mice in daily doses of 200 mg per kg for 7 days did not affect phosphorylase phosphatase activities of mouse liver, brain and heart. However, the activity of the enzyme catalyzing the inactivation of phosphorylase in skeletal muscle was reduced significantly already 4 days after niridazole administration had been initiated. Therefore, administration of niridazole might reduce the glycogen content of the host's skeletal muscle. This problem was studied in rhesus monkeys receiving niridazole in a dosage schedule which had proved curative in these animals. Under these conditions a marked glycogen depletion in skeletal muscle was observed. Such an effect, at least in part, can be attributed to an inhibition of phosphorylase phosphatase activity produced by this drug.

A total of 57 monkeys (Macaca mulatta-rhesus) weighting between 1.7 and 5.5 kg at the beginning of the experiment was used in this study. Niridazole (100 or 40 mg per kg) was enclosed in gelatin capsules and was administered daily for 5 successive days. The capsule was placed in the back of the mouth of the hand-held monkey by means of a forceps. A small volume of water was added to stimulate swallowing. Thereafter the mouth was examined to ensure that the capsule was swallowed.

Once daily, weighed amounts of monkey food pellets were provided and daily food consumption of the drug-treated animals was determined by weighing any food remaining in the feeders and under the animal cage. Two control groups of pair-fed monkeys received no drug, but were fed the same amount of food as the average weight of pellets consumed each day by monkeys administered 40 mg or 100 mg per kg of niridazole each day.

Muscle biopsy samples were obtained as follows. The animals were anesthetized by the intramuscular injection of 1 mg per kg of phencyclidine hydorchloride. After removing the hair from the lateral thigh area with animal clippers and scrubbing the biopsy site with a hexachlorophene solution, an incision 1.5 to 2 cm in length was made through the skin and the underlying fascia. The latter was dissected back from the muscle and two muscle samples (each weighing 15 to 40 mg) were removed. The samples were blotted on cotton gauze to remove excess blood, placed in a glass dish surrounded by ice, weighed and transferred into calibrated centrifuge tubes containing a predetermined volume of 2N KOH. The tubes were covered with a marble, heated (within 1 to 2 min. after excision) in a boiling water bath for 20 minutes, cooled in ice

and neutralized to pH 7.0 with a predetermined volume of 2.5N H_3PO_4. After removal of the muscle samples, the fascia was closed with gut sutures, and the skin with synthetic polyfilament. Alternate thighs were used on successive biopsies. Animals usually had returned to normal activity within 2 to 3 hours after administration of the anesthetic. An interval of at least 2 days was allowed between the removal of the biopsy samples from the same monkeys.

Following centrifugation of the neutralized muscle extracts at 1000 x g for 10 minutes, glycogen concentration in aliquots of the resulting supernatant was determined by a specific enzymatic micromethod. Variations in the glycogen levels of duplicate samples rarely exceeded 5 and never 10 percent.

Analyses of samples removed from the same muscle and frozen immediately thereafter by immersion in methylbutane (cooled to 90° in liquid nitrogen) revealed that their glycogen concentration was the same as that of samples which had been placed in the ice-cooled dish. Therefore, no glycogen loss occurred when the latter procedure was used. Serum glucose concentration in blood obtained by venipuncture during biopsies was determined by the methods of Keston and Teller.

Two drug schedules were used: a. the minimal dosage regimen reported by Sadun et al. to produce a parasitological cure of schistosomiasis mansoni in rhesus monkeys; namely, the oral administration of 100 mg/kg of niridazole once daily for 5 consecutive days; b. a 60 percent lower dose of niridazole, i.e. 40 mg per kg given in the same manner.

In the early stages of this study it became evident that the administration of niridazole to the monkeys produced anorexia and a progressive reduction in food intake. This was much more pronounced in the animals receiving the higher dose of niridazole (Table 17). In both groups normal food consumption was resumed 24 hours after niridazole administration had been discontinued. Since prolonged starvation produces a reduction in muscle glycogen levels, the latter might be affected by decreased food intake. Therefore, two pair-fed control groups of monkeys were used. There was a progressive decrease in muscle glycogen levels in the control group of monkeys given the same amount of food as was consumed by those animals to whom 100 mg per kg of niridazole had been administered (Table 18). Two days after normal food intake was resumed, the glycogen concentrations had returned to their control levels. Subsequently, a significant increase in muscle glycogen was observed. A similar "rebound" phenomenon has been reported in man by Hultman and Bergstrom, who found that starvation produced a decrease in muscle glycogen and that subsequent feeding, especially of a carbohydrate-rich diet. resulted in a marked increase over and above the pre-starvation levels.

Following the oral administration of 100 mg per kg of niridazole, the decrease in muscle glycogen levels was more pronounced than in the

Table 17

Average 24-hour food pellet consumption by rhesus monkeys receiving a single daily dose of niridazole for 5 successive days

Dose of niridazole (*mg/kg body weight*)	Days after first dose					
	0–1	1–2	2–3	3–4	4–5	5–6
	Food pellets consumed (*grams*)					
100	130	86	17	13	7	130
40	130	105	94	85	76	130

Table 18

*Effect of the oral administration of niridazole (100 mg/kg daily for 5 days) on muscle glycogen levels of rhesus monkeys**

Time after initiation of drug administration or food restriction (*days*)	Time after last dose or cessation of food restriction (*days*)	Food-restricted controls				Niridazole (*5 × 100 mg/kg*)			
		No. of monkeys	Control level	Post-treatment level	% Change	No. of monkeys	Control level	Post-treatment level	% Change
1		8	0.80	0.63	−21	7	1.03	0.75	−27
2		5	0.84	0.63	−25	8	1.10	0.48	−56
3		8	0.81	0.56	−31	9	1.02	0.36	−65
4		6	0.67	0.45	−33	7	1.03	0.18	−82
5		6	0.62	0.37	−40	9	1.06	0.30	−72
	2	3	0.70	0.78	+12	—	—	—	—
	3	8	0.84	0.78	− 7	—	—	—	—
	4	3	0.70	1.23	+76	8	1.10	0.28	−75
	7	2	0.69	1.33	+93	—	—	—	—
	8	—	—	—	—	2	0.72	0.75	+ 4
	11	—	—	—	—	4	1.08	1.49	+38
	12	2	0.69	1.45	+110	—	—	—	—
	13	—	—	—	—	2	0.72	0.99	+37
	15	2	0.69	0.89	+27	—	—	—	—
	16	—	—	—	—	2	0.72	0.75	+ 4
	19	—	—	—	—	1	0.87	0.99	+13
	20	—	—	—	—	2	0.72	0.85	+18
	24	—	—	—	—	2	0.72	0.75	+ 4

* All figures for levels denote mg of glycogen per 100 mg of muscle (fresh weight) and represent the averages.

Table 19

Effect of the oral administration of niridazole (40 mg per kg daily for 5 successive days) on muscle glycogen levels of rhesus monkeys

Time after initiation of drug administration or food restriction (*days*)	Food-restricted controls				Niridazole (*5 × 40 mg/kg*)			
	No. of monkeys	Control level	Post-treatment level	% Change	No. of monkeys	Control level	Post-treatment level	% Change
2	3	0.52	0.58	+11	6	0.76	0.70	− 8
3	6	0.63	0.77	+23	9	0.77	0.71	− 8
4	6	0.57	0.61	+ 7	3	0.54	0.46	−15
5	9	0.62	0.55	−11	6	0.66	0.44	−33

pair-fed controls (Table 18). Already after two or three doses of the drug, a marked difference between the two groups was observable. Furthermore, after cessation of drug administration, there was no immediate reversal of glycogen depletion. The glycogen concentrations remained low for at least four days after the last dose of niridazole while the muscle glycogen stores of the pair-fed controls had risen already above their initial levels during this period.

A reduction in muscle glycogen concentration was detectable also when the lower dose od niridazole was used (Table 19). However, under these conditions, glycogen depletion was less pronounced and became significant only after the fourth and the fifth dose. The less drastic decrease in food consumption on this dosage regimen did not result in a reduction in the glycogen stores in pair-fed controls except possibly on the fifth day.

No significant alterations in serum glucose concentrations were detected in monkeys given niridazole or in pair-fed controls.

Two frequently clinically observed side effects of niridazole are anorexia and myasthenia. Since glycogen is a major source of energy for muscular contraction, it is conceivable that glycogen depletion produced by niridazole brings about muscular weakness.

The present study indicates a)that niridazole administration has a direct glycogen lowering effect in monkey muscle which can be accounted for by an inhibition of phosphorylase phosphatase activity and b) that glycogen depletion is aggravated by the reduced food intake brought about by the anorexic action of the drug. It remains to be determined whether niridazole administration brings about similar changes in the muscle of man, whether the decrease in food intake, as a result of anorexia, is sufficient to contribute toa further decrease in muscle glycogen and whether such changes should affect the management of patients to whom niridazole is administered as an antischistosomal drug.

4. Soluble antigen fluorescent antibody test for echinococcosis: comparison of hydatid fluid and scolex antigens.

Since ancient times the diagnosis of hydatid disease has been a major problem because specific clinical signs are scarce and the parasite cannot be recovered from the blood or excreta of the infected persons. For over 60 years attempts have been made to develop and evaluate immunological tests as aids in the diagnosis of this infection in man.

A complement fixation test was first developed by Chadini and subsequently improved by others. Shortly afterwards Casoni developed an intradermal test using hydatid fluid as antigen to detect delayed hypersensitivity in infected individuals. Subsequently, Fairley and Bryce et al. found that antigens extracted from scolices were superior to hydatid fluid in the complement fixation test. Attempts to increase

the sensitivity of this test have involved the use of conglutination of micromethods and of isofixation curves. In 1957 an indirect hemagglutination test was used for the serological diagnosis of this infection and shortly afterwards flocculation tests were developed by Norman et al. using bentonite and by Fischman using latex. In general, the bentonite flocculation test was more sensitive than the latex agglutination test but less so than the hemagglutination test. When human type O, Rh negative erythrocytes were used the hemagglutination test was found to be preferable to the latex and complement fixation test. Extensive studies conducted by Gonzales-Castro suggested that the bentonite flocculation is more sensitive than the complement fixation test but less so than the hemagglutination test in proven cases of hydatid disease. However, the latter given rise to a large number of nonspecific reactions than the complement fixation test. Cross reactions from other helminthic infections are eliminated by first absorbing the sera either with red blood cells or bentonite sensitized by extracts of liver from the same species of animal from which the hydatid fluid was prepared. The author considers the latex test less sensitive and as specific as the hemagglutination test.

A fluorescent antibody test for hydatid disease using unextracted whole scolex as antigen was developed by Azevedo and Rombert. Their encouraging results were confirmed by several investigators. Although this technic showed great promise when scolices or when forzen sections were used as a source of antigen, the indirect hemagglutination test appeared to be more sensitive and more specific.

Paronetto suggested using soluble antigens in the fluorescent antibody test by placing them on a matrix of cellulose acetate filter paper. This system was applied to parasitic infections by several investigators. The soluble antigen fluorescent antibody (SAFA) technic obviates the for maintaining the parasite in the laboratory where the tests are performed, permits the investigator to select and purify the antigen to be employed and provides means of objectively reading the test. In the present study the serological activity of antigens extracted from scolices and from hydatid fluid have been investigated in the development of a SAFA technic for hydatidosis.

Sera

Human sera from 307 documented cases were tested. These included 84 specimens from echinococcosis patients. Of these, diagnosis was confirmed surgically in 24 cases. Of the surgically proven cases 17 were due to Echinococcus granulosus and 7 to E. multilocularis. All other cases were diagnosed on the basis of clinical, epidemiological, radiological and serological evidence. A total of 109 control sera from individuals with proven viral, bacterial or parasitic infections other than echinococcosis was used to determine the specificity of the test. Normal sera were obtained from 114 healthy individuals who were undergoing physical examination as candidates for appointment to a military academy. All of the specimens were stored at -20°C until the

time of testing. Some specimens were lyophilized or preserved with merthiolate, since preliminary tests indicated that these procedures did not interfere measurably with test results. All sera were diluted 1:10 with 0.85 percent sodium chloride containing 2 percent Tween-80 in 0.05 M tris-2-amino-2(hydroxymethyl)-1,3 propanediol buffer (pH 8.0) and tested for reactivity. Four-fold dilutions of sera reacting at 1:10 were then tested to determine titers.

Antigen Preparation

Hydatid fluid from sheep infected with E. granulosus was passed through a 0.45 Millipore filter, dialyzed for 24 hours against several changes of distilled water, then freeze-dried and stored at -70°C until further processed for use as hydatid antigen. The scolices were washed 3 times with physiological saline, then freeze-dried and stored at -70°C until processed for scolex antigen.

In preliminary investigations these relatively crude antigens reacted with sera from a considerable number of uninfected individuals at relatively high titers. Therefore, the following procedures were performed in an attempt to eliminate some of these undesirable reactions: 1) inactivation of sera at 56°C for 30 minutes, 2) inactivation of sera at 56°C for 30 minutes and absorption with sheep red blood cells, 3) precipitation of crude antigens with 50 percent saturated ammonium sulphate, and 4) gel filtration with Sephadex G-200. Since none of the first 3 procedures eliminated the large number of nonspecific reactions, the gel filtration method was used to prepare antigens throughout this study.

Hydatid fluid was reconstituted to 10 mg of dry weight per ml of a 0.14 tris cationic (pH 7.2) buffer (Triethanolamine 2.8 ml, NaCl 7.5 gm, $CaCl_2$ 0.2 gm, $MgCl_2$-$6H_2O$ 0.2144 gm and 17.7 ml of 1 normal HCl and diluted to 1 liter with distilled water). Gel filtration was performed by applying 20 ml of antigen to a 2.5 cm x 100 cm Sephadex G-200 chromatography column and eluting with 0.14 M phosphate buffered saline. The eluate was collected in 5 ml aliquots and monitored with a Beckman D.U. Spectrophotometer at O.D. 280 mμ. The optical density was recorded on graph paper and aliquots within each peak were pooled and then concentrated by dialysis under negative pressure before testing with known reactive and nonreactive antisera. Since the first pool (Fraction I) gave fewer reactions with the nonreactive antisera, it was selected for further testing.

Freeze-dried scolices were weighed and processed in no more than 600 mg portions. The dried scolices were placed in a glass (Tenbroek) tissue grinder with 25 to 30 ml of absolute diethyl ether and homogenized in the cold for 20 minutes at a temperature between -20°C and -10°C. The ether was decanted and the sediment was allowed to dry at room temperature under a current of air. The dry sediment was returned to the tissue grinder in approximately 25 to 30 ml of tris cationic buffer not to exceed the volume required for 10 mg dry weight per milliliter

of buffer. The suspension was placed on a magnetic stirrer at 4° to 10°C for 18 to 24 hours. The extraction mixture was then centrifuged at 27,000 g for 20 minutes at 3° to 4°C. The supernatant fluid was further fractionated by passage through a chromatography column. The scolex extract was applied in 25 ml portions on a 2.5 cm x 100 cm Sephadex G-200 column and eluted with 0.14 M phosphate saline buffer (pH 7.2). This wluate was collected, monitored, cooled and concentrated in the same manner as the hydatid fluid antigen before it was tested with known reactive and nonreactive antisera. Since the pool from the first peak (Fraction I) reacted with all the known reactive antisera and none of the nonreactive antisera, Fraction I was chosen for further testing.

Test Procedure

The tests were conducted with strict adherence to the published method (Sadun and Gore, 1967). A negative control serum was used to set the fluorometer dial at zero. The optimal dilution of conjugate was determined by serial dilutions of serum and antiglobulin. In most instances, a 1:20 dilution of labeled antihuman globulin was used. On the basis of previous tests, arbitrary values were established for the interpretation of fluorometer dial reading. A reading of 7 or less was recorded as nonreactive; a reading of 8 or more was recorded as reactive.

Results obtained with the SAFA test for echinococcosis with antigens extracted from hydatid fluid or from scolices are summarized in Table 20. The findings with sera from echinococcosis patients illustrate the relative sensitivity of this procedure. The findings with sera from patients with other conditions and from healthy individuals provide an index of the specificity of the test. In tests with Fraction I of hydatid fluid antigen passed through a Sephadex G-200 column frequent cross-reactions were observed with sera from patients with parasitic, bacterial, mycotic or viral infections. Conversely, when an antigen consisting of delipidized saline extract of scolices of E. granulosus fractionated by Sephadex G-200 chromatography was used, no cross reactions occurred with sera from healthy individuals and only a few reactions at low titers (up to 1:10) were observed with sera from individuals with parasitic infections or diseases other than echinococcosis. Of the 7 specimens from patients with E. multilocularis, 1 failed to react with E. granulosus hydatid antigen and 2 failed to react with E. granulosus scolex antigen. All of the specimens from individuals with surgically proven E. granulosus infections reacted with both antigens.

Although both antigens frequently reacted at high titers (Table 21), the extract from scolices conferred a greater degree of specificity to the test.

Pooled serum specimens from infected individuals and uninfected controls were divided into aliquots and tested 14 different times with

Table 20

Results of SAFA test for Echinococcosis Using Antigens Extracted from Hydatid Fluids or Scolices

Diagnostic Status	No. Tested	Fraction I Sephadex G-200 Hydatid Fluid No. of Reactors	Hydatid Fluid Percent Reactors	Scolices Extract No. of Reactors	Scolices Extract Percent Reactors
Echinococcosis	84	73	87	69	82
Healthy controls	114	2	2	0	0
Ascariasis	15	6		0	
Hookworm	7	4		0	
Trichuriasis	5	3		0	
Schistosomiasis	11	3		1	
Taeniasis	2	2		1	
Trichinosis	17	4		0	
Filariasis	19	14		2	
Amebiasis	8	3		0	
Malaria	11	8		1	
Trypanosomiasis	6	2		0	
Toxoplasmosis	8	1		0	
Total parasitic infections	109	49	45	5	
Bacterial	36	10		4	
Mycotic	12	6		1	
Viral	7	0		1	
Lupus	16	1		1	
Total bacterial or degenerative diseases	71	17	24	6	8

the same lots of antigens and antiglobulin, to obtain information on the reproducibility of results with the SAFA test. None of the specimens from healthy controls reacted (Table 22). Conversely, all of the specimens from infected individuals were reactive in the tests. In all instances and with both antigens tested, the highest and lowest titers obtained in repeated tests of a given specimen were within a four-fold dilution.

No generalizations can be made on the reliability of serological tests for echinococcosis because many variables, including the choice of antigen employed, influence the sensitivity and specificity.

The precise nature of the antigens used and their chemical characterization has still not been determined. The most widely used antigen for routine serological testing of hydatidosis is fluid taken from the cyst. This is because hydatid fluid is readily available in relatively large quantities and does not require elaborate procedures for its extraction. This fluid, however, is a complex mixture of substances and contains several protein and carbohydrate fractions some of which are probably derived from the host. New technics for the purification and characterization of antigenic constituents have greatly increased the general interest in the serology of hydatid disease. The antigenic components of hydatid fluid have not yet been clearly defined. Fluid from different sources varies in its antigenic properties and the fluid from sterile cysts usually lacks antigenic activity. Antigens from tissue extracts of hydatid cysts appear to have greater specificity. Scolex extracts have been shown to increase the specificity of the various tests and Fischman has described a new complement fixation test using whole scolex as antigen. With this scolex antigen, the complement fixation test is at least as sensitive as the latex agglutination test utilizing hydatid fluid as antigen, and it is significantly more sensitive than the hydatid fluid complement fixation test. An agar double-diffusion technic using an antigen made from the scolices of E. granulosus was found to be superior to similar tests using hydatid fluid as antigen.

Our results obtained in the SAFA test with human sera indicate that this procedure possesses a satisfactory degree of sensitivity, specificity and reproducibility when an antigen extracted from scolices and fractionated by chromatographic methods using Sephadex G-200 is employed. Similarly obtained fractions of hydatid fluid did not permit a desirable degree of specificity, since a large percentage of sera from Echinococcus-free individuals with parasitic, baceterial and mycotic infections reacted frequently at high titers in the test. It is of interest to note that antigen extracted from E. granulosus permitted the detection of E. multilocularis infection as well. Since the few "false negative" reactions occurred among individuals whose diagnosis had not been surgically proven, it is possible that the sensitivity of this test might be even higher than indicated in these results. Therefore, the results suggest that the SAFA test using as antigen an extract of scolices provides a relatively simple and reliable procedure

Table 21

Serum Titers Obtained From Echinococcosis Patients with Hydatid Fluid Antigen or Scolex Antigen

Antigen	Diagnostic Status	No. of Reacting Sera at Given Titer <1:10	1:10	1:40	1:160	≥1:640	Total
Hydatid Fluid (Fract. I)	Healthy controls	110	2	0	0	0	114
	Echinococcosis	11	22	8	23	13	84
Sclices Extract (Fract. I)	Healthy controls	114	0	0	0	0	114
	Echinococcosis	15	25	20	16	8	84

Table 22

Results of Repeated SAFA Tests using the Same Sera, Antigen and Antiglobulin at Different Times

Antigen	Diagnostic Status	No. of Times Reacting at Given Titer <1:10	1:10	1:40	1:160	<1:640	Total
Hydatid Fluid (Fract. I)	Healthy controls	14	0	0	0	0	14
	Echinococcosis	0	0	2	6	6	14
Scolices Extract (Fract. I)	Healthy controls	14	0	0	0	0	14
	Echinococcosis	0	0	2	7	5	14

for the serological diagnosis of echinococcosis. The test would be of particular value to small laboratories if reagents could be made from better equipped laboratories or from commercial sources. Moreover, the accurate reading of the test is rapid and objective since it can be obtained by means of a fluorometer. A greater experience with this test and the use of more purified antigenic extracts should increase even further the test's sensitivity and specificity and offer possibilities of greater standardization. If subsequent comprehensive studies support the observations and delineate clearly the relative efficiency of the test, the SAFA technic using a scolex antigen of E. granulosus may be well suited as a screening procedure in investigations of the sero-epidemiology of hydatid disease.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 165, Parasitic diseases

Literature Cited

1. References

None

2. Publications

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6441	70 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 07 01	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	61102A	3A061102B71Q	00	166
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A (2)			

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(U) Viral Infections of Man (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 010100 Microbiology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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FISCAL YEAR PRECEDING 69	13	330
FISCAL YEAR CURRENT 70	13	340

19. RESPONSIBLE DOD ORGANIZATION

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(U) Virus Diseases; (U) Viruses; (U) Immunology; (U) Ecology; (U) Human Volunteer; (U) Respiratory Tract Infections; (U) Arbovirus Infections

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 etiology of acute infectious diseases of special hazard to military personnel, to determine and evaluate factors influencing occurrence, distribution, severity and medical result of human virus infections, and to develop means for reducing disability due to virus diseases.

24 (U) Contemporary virological and immunological methods are applied to disease problems occurring in troops or in susceptible civilian populations in strategically important areas. New conceptual approaches and methods are developed as needed for specific problems.

25 (U) 69 07 - 70 06 Acute Respiratory Disease (ARD) in basic combat trainees was caused by both adenoviruses Type 7 (ADV-7) and Type 4 (ADV-4) during Winter 1970. A field trial of the efficacy of live, oral, ADV-7 vaccine was undertaken at Fort Dix, N.J. beginning 6 Jan 70. One training brigade (3rd Bde) received ADV-4 and ADV-7 vaccines and the other (2nd Bde) only ADV-4 vaccine. A 96% suppression of ARD caused by ADV-7 occurred in the 3rd Brigade immunized with both vaccines. Simultaneous administration of ADV-4 vaccines to trainees at Forts Lewis and Wood in the face of an ARD outbreak due to both adenoviruses led to a reduction in the adenovirus-associated ARD rate from >4.0/100/wk and >7.0/100/wk, respectively, to <0.5/100/wk 5 weeks after immunization began. Immunization with ADV-7 vaccine alone at Fort Campbell significantly reduced the ADV-7 associated ARD rate but led to the emergence of ADV-4 associated ARD. The soluble complement-fixing antigen of dengue-2 has been shown to be nonstructural antigen by immunologic and biochemical methods. SCF antigen from the 4 dengue serotypes were found to have both group- and type-specific antigenic determinants. Using radioimmune precipitation, the slowly sedimenting hemagglutinin of dengue-2 was found to be antigenically closely related to but differentiable from the dengue-2 virion. Three structural and five nonstructural proteins of Japanese encephalitis virus have been identified. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 166, Viral Infections of Man

Investigators.

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Description.

To define the etiology and ecology of human virus infections, particularly those of military medical significance; to devise and evaluate means for precise diagnosis, control and/or prevention of disease. Studies have applied virological, immunological, epidemiological and physiological approaches to understanding disease caused by respiratory, arthropod-borne and other viruses, the factors influencing transmission among men, other vertebrates and invertebrates, and their survival in nature.

Progress.

I. Evaluation of Adenovirus Type 7, Live, Oral Vaccine (L-AV-7) in Man.

The initial studies of the safety and immunogenicity of adenovirus type 7, live, oral vaccine were reported in the previous Annual Report. During the past year, three additional studies of L-AV-7 were undertaken. In addition, laboratory studies were completed on a fourth study. These four studies are reported below. The following abbreviations will be used in this section: L-AV-7 (adenovirus type 7, live, oral vaccine), L-AV-4 (adenovirus type 4, live, oral vaccine), ADV-7 (adenovirus type 7), ADV-4 (adenovirus type 4), ARD (Acute Respiratory Disease), BCT's (basic combat trainees), and $TCID_{50}$ (Tissue Culture Infectious $Dose_{50}$).

Study #3, Basic Combat Trainees, Fort Dix, New Jersey, 21 Mar-22 May 69.

Preliminary results of this study, designed to evaluate the safety, antigenicity, and specific protective effect of L-AV-7 administered simultaneously with L-AV-4 to BCT's, were reported previously; only new results will be considered here.

The study was undertaken in BCT's at Fort Dix, N.J. at a time when ADV-7 was the predominant respiratory pathogen and the ARD rate was 6.4/100/week. The presence of ADV-4 on post necessitated the use of L-AV-4. One week's input of BCT's was immunized within 72 hours of their arrival on post. Trainees with serial numbers ending in 0 and 8 received L-AV-7 and L-AV-4 (type 7 group), and those with serial numbers ending in digits other than 0 and 8 received a placebo capsule and L-AV-4 (placebo group). L-AV-7 (Lot 16 CV-01101, Wyeth) contained $10^{5.4}$ $TCID_{50}$ per capsule and L-AV-4 (Lot 16 CI-00801, Wyeth) contained $10^{4.4}$ $TCID_{50}$ per capsule. Because of administrative problems in processing, about 5% of immunized trainees did not begin training in one of the six study companies formed. Together the type 7 and placebo group comprised 95% of all trainees in the study companies. The adenovirus vaccine immunization status of trainees in the six study companies is shown in Table 1.

Table 1. Adenovirus Immunization Status of Trainees in Study Companies

Company	Type 7 Group (L-AV-7 & L-AV-4)	Placebo Group (Placebo & L-AV-4)	Not Immunized
	(No. Men)	(No. Men)	(No. Men)
A-2-2	40	142	22
B-2-2	44	149	9
C-2-2	38	159	5
D-2-2	32	163	6
E-2-2	42	147	10
B-6-2	35	160	3
Total	231	920	55

Trainees from one platoon of each of the six study companies were bled at the beginning of the first and fourth week of training; these paired sera were used to determine antibody responses to immunization with L-AV-7 and L-AV-4. All trainees in the six study companies admitted to the hospital with symptoms of respiratory tract infection were seen within 12 hours of admission. A throat wash for viral isolation and acute blood samples for serology were obtained on all hospitalized trainees of the two study groups. A 2-week convalescent blood sample was obtained on all hospitalized trainees, except for those AWOL or those hospitalized after the middle of the 7th week of training.

A. Methods:

1. Isolation: Throat washings from hospitalized trainees were mixed with an equal volume of L-15 medium containing 2% Fetal Bovine Serum (FBS) and 1000 u/ml of penicillin and 1000 ug/ml of streptomycin. After

incubation at room temperature for 30 minutes, 0.3 ml of the throat wash mixture was inoculated into each of 3 HEK monolayer tissue culture tubes fed with L-15 medium containing 2% FBS. All cultures were observed for cytopathic effect (CPE) on alternate days for at least 21 days.

If cultures had bacterial contamination or had nonspecific cellular degeneration before 21 days of observation, a second sample of throat wash was filtered and reinoculated into fresh HEK tissue culture tubes. If Herpesvirus hominis or polioviruses were isolated from a throat wash, a second aliquot of throat wash was treated with chloroform or poliovirus pool antisera, respectively, prior to reinoculation for adenovirus isolation.

2. Identification of Isolates: Isolates with typical adenovirus CPE were typed in HEK tissue culture neutralization tests after preincubation with 20 antibody units of hyperimmune ADV-7 rabbit antiserum. ADV isolates which did not type with ADV-7 antiserum were tested with 20 antibody units of hyperimmune ADV-4 antiserum by tissue culture neutralization tests. Isolates with herpesvirus CPE or with enterovirus CPE were typed in tissue culture neutralization tests with hyperimmune Herpesvirus hominis antiserum or pooled antisera to poliovirus Types I, II, and III, respectively.

3. Determination of Serologic Response of Trainees to L-AV-7 and L-AV-4: Serum was obtained from trainees in one platoon of each study company at the beginning of the first and the fourth week of training. Paired sera of trainees with serial numbers ending in digits 0 and 8 (type 7 group) and in digits 4 and 7 (placebo group) were tested for ADV-7 and ADV-4 neutralizing (N) antibody in HEK monolayer tissue culture tubes. The type 7 vaccine virus strain 55142 was used in the ADV-7 neutralization tests and the type 4 vaccine strain CL68558 was used in the ADV-4 neutralization tests. After the sera were heated at 56°C for 30 minutes, serial 2-fold dilutions of each serum was made. 0.2 ml of each serum dilution was incubated with 0.2 ml of a dilution of vaccine virus at room temperature for one hour prior to inoculation of 0.1 ml of the virus-serum mixture into each of two HEK tubes. The neutralization tests were read at a time when the test dose of virus was 3-10 $TCID_{50}$.

4. Serologic Determination of Adenovirus Infections of Hospitalized Trainees: Adenovirus complement-fixing (CF) antibody titers of acute and convalescent sera of hospitalized trainees were determined by standard techniques. ADV-7 N antibody titers were determined by procedures described above with ADV-7 strain A-3-143 and read at a time when the test dose of virus was between 32-320 $TCID_{50}$.

B. Results:

Vaccine and placebo capsules were administered without difficulty under supervision. No unusual illnesses attributed to gastrointestinal infection with vaccine viruses were noted in the type 7 group; one trainee in the placebo group (placebo and L-AV-4) was hospitalized with febrile gastroenteritis during the first week of basic training. Illness

involving the genito-urinary tract or cardiovascular system attributable to the vaccines were not seen in trainees of either study group. With the exception of one trainee of the placebo group who died of meningococcemia with meningitis, none of the study trainees developed central nervous system disease during the 8 weeks of training.

1. Antibody Responses to Adenovirus Vaccine: All trainees with serial numbers ending in 0 and 8 (type 7 group) and 4 and 7 (a random sample of the placebo group) from one platoon of each of the six study companies were studied for ADV-7 and ADV-4 serum neutralizing (N) antibody response to immunization. Data on ADV-7 N antibody responses only was presented in last year's report. Consideration in this report is directed to ADV-4 and ADV-7 N antibody responses in those trainees who lacked N antibody at a 1:4 serum dilution to both serotypes at the commencement of training (and who are presumably susceptible to ARD from either adenovirus type). Twenty-three of the type 7 group trainees and 21 of the placebo group trainees were found susceptible to both adenoviruses; the frequency of N antibody response after immunization in these trainees is shown in Table 2. ADV-7 N antibody responses were detected

Table 2. Frequency of ADV-4 and ADV-7 N Antibody Responses in Susceptible* Trainees after Immunization with Adenovirus Vaccine(s)

Vaccine Group	No. Trainees	No. Trainees Developing ADV-7 N Antibody No.	(%)	ADV-4 N Antibody No.	(%)	ADV-4 & ADV-7 N Antibody No.	(%)
Type 7 (L-AV-7 & L-AV-4)	23	20	(86)	18	(78)	15	(65)
Placebo (placebo & L-AV-4)	21	2	(10)	16	(76)	2	(10)

* lacking N antibody to ADV-4 and ADV-7 at a 1:4 dilution

in 20 of the 23 susceptible trainees immunized with both adenovirus vaccines, but in only 2 of the 21 susceptible trainees immunized with the type 4 vaccine alone. The geometric mean ADV-7 N antibody titer of type 7 group responders was 41.

No difference in the frequency of ADV-4 N antibody response after immunization was evident between the two vaccine groups. ADV-4 N antibody was present in post-immunization serum of 18 of the 23 susceptible type 7 group trainees and of 16 of the 21 placebo group trainees.

Geometric mean ADV-4 N antibody titers were similar in both vaccine groups; 10 in the type 7 group and 15 in the placebo group. No evidence for interference of the antigenicity of L-AV-4 by L-AV-7 was found. Serum N antibody responses to both adenovirus types was found in 15 of the 23 (65%) susceptible type 7 group trainees.

2. Protective Effect of L-AV-7: The number of ARD hospitalizations and the rate for ARD hospitalizations for trainees in the two study groups are summarized in Table 3. A significant difference in the

Table 3. Febrile ARD Hospitalizations, Study Groups

Group	Placebo	Type 7
Vaccine	Type 4, Placebo	Type 4, Type 7
No. Immunized	920	231
No. ARD Hospitalizations	240	32
Rate/100/8 wk	26.1	13.9*

* $X^2 = 15.3$, $p < 0.0005$

ARD hospitalizations between the two vaccine groups was evident; the type 7 group had 47% less ARD hospitalizations than the placebo group. Figure 1 shows the cumulative ARD hospitalization rate by training week for the two vaccine groups. No appreciable difference was apparent until after the 3rd week of training, but a marked suppression of the type 7 group rate during the 4th through 6th week of training is evident.

The occurrence of ADV-7 associated ARD in the two study groups is detailed in Table 4. ARD in both study groups was considered ADV-7 associated if this serotype was isolated from a hospitalized trainee's throat washing and/or a 4-fold or greater rise in ADV-7 serum N antibody between acute and convalescent serum was detected. In 114 of the 124 hospitalizations thus considered ADV-7 associated, ADV-7 was recovered from throat washings. In only 3 of 124 cases was virus isolated from throat washing in the absence of an ADV-7 N antibody rise. In over 90% of hospitalizations considered ADV-7 associated, the diagnosis of infection was made by both viral isolation and ADV-7 N antibody rise. As shown in Table 4, a significant difference in ADV-7 associated ARD hospitalizations was found between the two study groups. This represents a 96% suppression of ADV-7 associated ARD hospitalizations in the type 7 group.

ADV-4 N antibody titers were not performed with sera of all hospitalized trainees. An ARD admission was considered to be ADV-4 associated if this serotype was isolated from throat washings of hospitalized trainees without considering ADV-4 N antibody rises. Eight

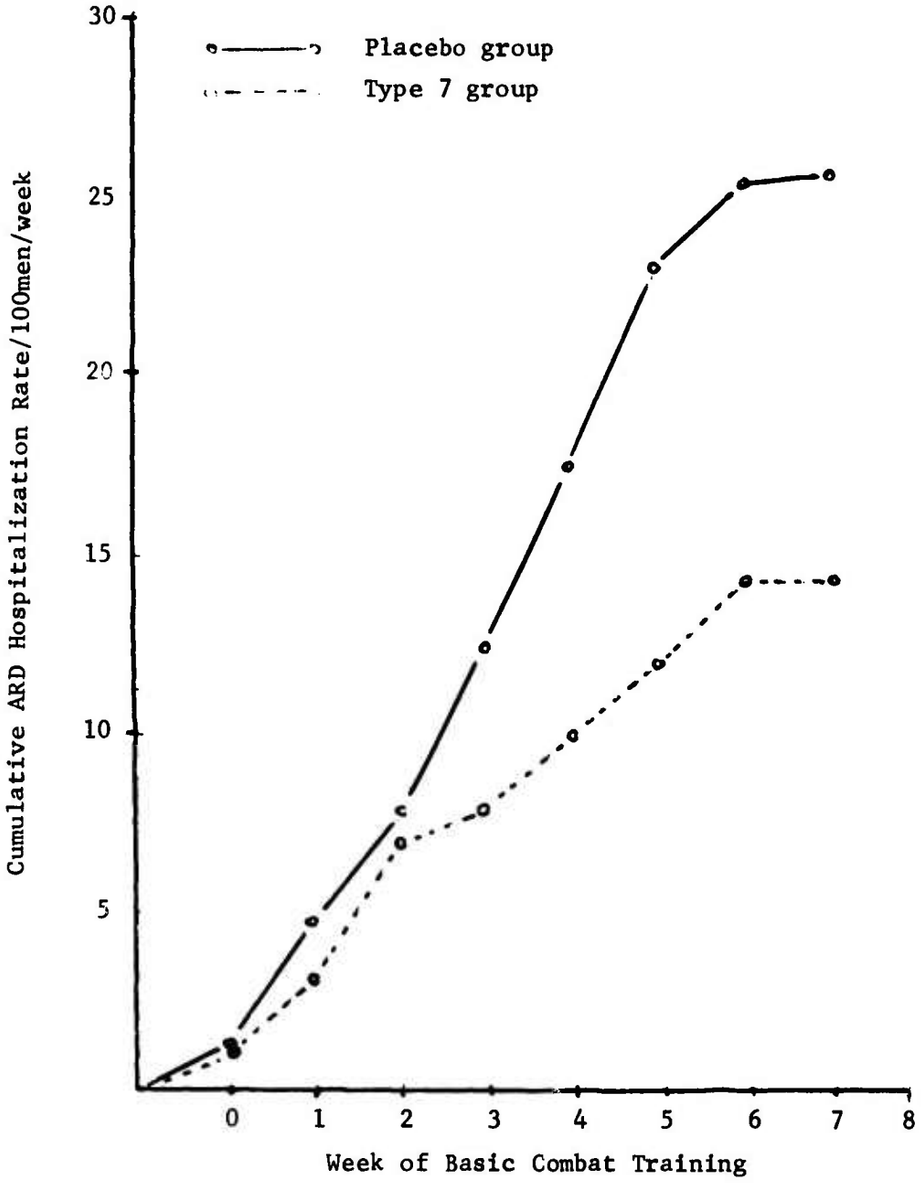


Figure 1. Cumulative ARD Rates, Study Groups, Fort Dix, Spring 1969.

Table 4. ADV-Associated ARD Hospitalizations, Study Groups

Group	Total No.	ARD Admissions: ADV-7 associated No.	ADV-7 associated rate/100/8wk	ADV-4 associated No.	ADV-4 associated rate/100/8wk	Non-ADV associated No.	Non-ADV associated rate/100/8wk
Type 7	231	1	0.4	2	0.9	29	12.6
Placebo	920	123	13.4*	8	0.9	109	11.8

* $X^2 = 32.6$, $p<0.0005$

placebo group trainees and two type 7 group trainees had ADV-4 associated ARD; ADV-4 associated ARD rates were identical, 0.9/100/8 wks, for both study groups. No significant difference in rates of ARD hospitalizations which could not be associated with adenovirus infection was evident between the two vaccine groups.

C. Discussion:

This study was designed to study the safety, antigenicity, and protective effect of L-AV-7 administered with L-AV-4 in BCT's. The design of this study differed from earlier field trials of L-AV-4 in that the protective effect of two vaccines (L-AV-4 and L-AV-7) administered simultaneously rather than the protective effect of one vaccine given alone (L-AV-4 and placebo) against ARD caused primarily by one adenovirus serotype (ADV-7) was determined. The use of L-AV-4 in this study was necessitated by the occurrence of ADV-4 associated ARD on post and the expectation that more than minimal amounts of ADV-4 associated ARD in the study population would obscure interpretation of ADV-7 associated ARD rates in the two study groups.

In addition, outbreaks of ARD in CONUS BCT's since 1966 have rarely been caused by one adenovirus serotype alone, but by both ADV-4 and ADV-7. It is expected that both L-AV-4 and L-AV-7 will have to be used together in trainee populations in order to achieve significant suppression of adenovirus-associated ARD. Thus, the experimental design of this study is of more practical significance than an evaluation of type 7 vaccine given alone.

Simultaneous administration of the two live adenovirus vaccines was safe. No trainee in the type 7 group (L-AV-7 and L-AV-4) developed gastrointestinal, cardiovascular, genitourinary, or central nervous system disease during basic training. Furthermore, ARD hospitalization rates for both vaccine groups were similar during the first three weeks of training (the time of expected vaccine virus intestinal infection), and neither ADV-7 nor ADV-4 was isolated from throat washings of type 7 group trainees hospitalized in the four weeks after immunization. Thus, no ARD attributable to vaccine virus was noted in this study which confirmed the safety of L-AV-7 previously shown in earlier studies.

ADV-7 N antibodies were induced after immunization in about 85% of sampled group 7 trainees who lacked N antibody to both ADV-7 and ADV-4 prior to immunization. The frequency of ADV-7 serologic response following immunization with both vaccines is similar to the frequency of ADV-4 N antibody response previously shown in susceptible trainees immunized with L-AV-4 alone.

ADV-4 N antibody developed in equal proportions of susceptible trainees receiving L-AV-7 and L-AV-4 and those receiving L-AV-4 alone. Little difference in post-immunization geometric mean antibody titers was apparent between the two groups. Thus, no evidence of interference with the antigenicity of L-AV-4 by L-AV-7 was found in this study. If

interference of a low order is to be detected, this must be studied in a much larger population. Of importance is the fact that no difference was found in ADV-4 associated ARD hospitalization rates between the two study groups.

A significant difference in the total ARD hospitalization rate and the ADV-7 associated ARD hospitalization rate was evident between the two study groups. L-AV-7, given together with L-AV-4, led to a 96% suppression of ADV-7 associated disease in this training population. This degree of protective efficacy is similar to that afforded by L-AV-4 against ADV-4 associated ARD in previously reported field trials.

Human Dose-Response to L-AV-7 Vaccines.
(Study #4, USAMEDTC Trainees, Sep-Oct 1969)

Previous studies of living adenovirus type 7 oral, enteric vaccine (L-AV-7) in susceptible human adults have employed capsules containing between $10^{4.9}$ to $10^{5.4}$ $TCID_{50}$. A capsule containing $10^{4.9}$ $TCID_{50}$ (Lot 16 CV-00101) infected 11 of 16 susceptible volunteers (See Study #1, WHITECOAT Volunteers, Annual Report 1969). A capsule containing but a slightly higher dosage, $10^{5.4}$ $TCID_{50}$ (Lot 16 CV-01101) infected 13 of 13 susceptible volunteers (Study #2, USAMEDTC Trainees, Annual Report 1969) and produced 96% suppression of ADV-7 ARD in a field trial (Study #3, Fort Dix Basic Combat Trainees above). The present study was undertaken to clarify the discrepancies in infectivity between the two vaccine lots of similar dosage and to determine the dose response curve for induced gastrointestinal infection with L-AV-7.

A. Design of Study:

1. The Study Group: Volunteers were obtained from enlisted trainees of Company D-3, The USAMEDTC, Fort Sam Houston, Texas. A comprehensive explanation of the study and its risks were given to trainees of this company by one of the responsible investigators. A consent statement, on file in the Department of Virus Diseases, WRAIR, was signed by each volunteer.

2. Vaccines: Adenovirus type 7 (ADV-7) strain 55142, propagated in human embryonic kidney (HEK) cells, was obtained from Wyeth Laboratories from Dr. Robert Chanock of NIAID, NIH. The strain was passaged three times in HEK cultures, then through 12 passages in human diploid fibroblast (WI-38) cultures, and lyophilized. Three separate lots of vaccine were prepared from this lyophilized virus--Lot 16 CV-01106A which was to contain 5 times (c. 6.5 $\log_{10}$ $TCID_{50}$) the amount of lyophilized virus in a standard vaccine preparation, Lot 16 CV-01107A which was to contain one tenth (c. 5.0 $\log_{10}$ $TCID_{50}$) the amount of lyophilized virus in a standard vaccine preparation, and Lot 16 CV-01108A which was to contain one one-hundredth (c. 4.0 $\log_{10}$ $TCID_{50}$) the amount of virus used in a standard vaccine. The three vaccine dosages were prepared into enteric-coated capsules. Enteric-coated capsules containing lactose filler, supplied by Wyeth Laboratories, were used as placebo capsules.

Titrations of the three vaccine capsules were performed in human embryonic kidney (HEK) tissue tube cultures; results of these titrations are shown in Table 5. Although the dosages of the higher two capsules were close to that anticipated, less than 10 $TCID_{50}$ of vaccine virus was found in Lot 16 CV-01108A capsules.

Table 5. Dose of L-AV-7 Capsules Used, Study #4

Vaccine Lot No., Wyeth	Tissue Culture Infectious Doses/Capsule
16 CV-01106A	$10^{6.8}$
16 CV-01107A	$10^{4.8}$
16 CV-00108A	$<10^{1.0}$

3. Immunization of Volunteers: One hundred and fifty-nine trainees volunteered for the study. Forty of the volunteers received Lot 16 CV-01106A, 40 received Lot 16 CV-01107A, 40 received Lot 16 CV-01108A, and 39 of the volunteers received a placebo capsule. No unusual reactions to the vaccines were related by the trainees.

4. Sampling: Blood samples for ADV-7 neutralizing (N) antibody determinations were obtained from all volunteers at the time of immunization and three weeks post-immunization. Stool samples for isolation of vaccine virus were obtained at 8, 10, and 12 days post-immunization from all volunteers who received one of the three L-AV-7 capsules.

B. Methods:

1. Determination of Serum ADV-7 N Antibody: Serum N antibody levels to ADV-7 vaccine virus (strain 55142) were measured in HEK tissue culture monolayer tubes. After the sera were heated at 56°C for 30 minutes, serial 2-fold dilutions from 1:2 to 1:256 were made. 0.2 ml of each serum dilution was incubated with 0.2 ml of a dilution of vaccine virus at room temperature for one hour prior to inoculation of 0.1 ml of the virus-serum mixture into each of two HEK tubes. Neutralization tests were read at a time when the test dose of virus was 3-10 $TCID_{50}$.

2. Adenovirus Isolation: Ten per cent stool suspensions were made in Hank's Balanced Salt Solution containing 0.4% bovine plasma albumin with 100 u/ml penicillin and 100 ug/ml streptomycin. After low speed centrifugation, the supernatant was centrifuged at 6000 rpm in a Sorvall centrifuge for one hour. The supernatant was collected and 0.3 ml inoculated into each of two HEK tissue culture tubes, maintained on L-15 medium containing 2% fetal bovine serum and 100 u/ml of penicillin

and 100 ug/ml of streptomycin. All cultures were observed for cytopathic effect (CPE) on alternate days for 21 days. All isolates obtained showed typical adenovirus CPE. One isolate from each volunteer was typed in HEK tissue culture neutralization test after preincubation with 20 antibody units of hyperimmune ADV-7 rabbit antiserum; all isolates tested proved to be ADV-7.

C. Results:

ADV-7 N antibody titers in pre-immunization serum of all volunteers was first measured to determine those volunteers lacking ADV-7 N antibody at a 1:2 serum dilution. Only those volunteers lacking ADV-7 N antibody are considered in the following results.

The results of ADV-7 stool isolation and ADV-7 serum N antibody response for the four groups of ADV-7 susceptible volunteers are shown in Table 6. Stool excretion of ADV-7 on at least one of the three study days (8, 10, and 12 days after immunization) was detected in 11 of 11 susceptible volunteers given the highest dose vaccine, 17 of 18 susceptible volunteers given the intermediate dose vaccine, and 10 of 18 susceptible volunteers given the low dose vaccine. Isolation of vaccine virus from susceptible volunteers given the placebo capsule was not attempted.

The development of ADV-7 serum N antibody three weeks post-immunization was detected in all 12 susceptible volunteers given the highest dose vaccine, 18 of 19 susceptible volunteers given the intermediate dose vaccine, and 10 of 18 susceptible volunteers given the low dose vaccine. Geometric mean titers of ADV-7 N antibody after immunization for the three vaccine groups are shown in Table 7. Only one of the 17 ADV-7 susceptible volunteers given the placebo capsule developed ADV-7 serum N antibodies. Significant transmission of wild ADV-7, thus, did not occur in the trainee population during the three weeks of the study, so that serologic rises detected in the immunized volunteers were due to immunization and not natural ADV-7 infections.

D. Discussion:

The intent of this study was to determine the proportion of ADV-7 susceptible trainees who developed serum ADV-7 N antibody after immunization with doses of L-AV-7 which ranged between $10^{6.5}$ to $10^{3.5}$ $TCID_{50}$. The highest dose represents the maximum virus titer which the manufacturer can produce and the lower titer represents the minimal titer of virus expected to afford protection against natural adenovirus acute respiratory disease in basic combat trainees (based on experience with L-AV-4). Thus, we intended to establish a dose-response curve for live adenovirus type 7 enteric-coated vaccine over a practical dose range.

This intent was compromised by the unexpectedly low virus dosage in Lot 16 CV-01108A. Repeated titrations of different capsules of this lot consistently showed that they contained less than 10 $TCID_{50}$ of vaccine virus. The initial titration, made 4 days after immunization

Table 6. ADV-7 Stool Isolation and Serum N Antibody Response in Susceptible Volunteers

Vaccine Lot	Virus Titer Vaccine (Log_{10} $TCID_{50}$)	No. Volunteers Immunized	No. ADV-7 Susceptible Volunteers		
			Immunized	with ADV-7 Stool Excretion	with ADV-7 N Antibody Response
16 CV-01106A	6.8	40	12	11*	12 (100%)
16 CV-01107A	4.8	40	19	17*	18 (95%)
16 CV-01108A	<1.0	40	18	10	10 (56%)
Placebo	---	39	17	not measured	1 (6%)

* One volunteer absent during stool collection period.

Table 7. Geometric Mean Serum ADV-7 N Antibody Titers of Volunteers with Antibody Response

Vaccine Lot	Vaccine Dose (Log_{10} $TCID_{50}$)	No. Volunteers	Geometric Mean Titer
16 CV-01106A	6.8	12	100
16 CV-01107A	4.8	18	89
16 CV-01108A	<1.0	10	55
Placebo	---		---

of volunteers, was designed to detect $\geq$3.0 logs of vaccine virus and no virus was detected. Subsequent titrations, which were begun about three weeks after the time of immunization, were performed by adding the entire constituents of two capsules to 2 ml of Hank's Balanced Salt Solution and inoculating 0.1 ml of the suspension after mechanical agitation to each of six HEK monolayer tissue culture tubes. In each of three such replications, only one of the six tubes inoculated showed adenovirus CPE. Thus, each capsule contained viable vaccine virus, but less than 10 $TCID_{50}$ per capsule.

The intermediate dose capsule (Lot 16 CV-01107A) contained $10^{4.8}$ $TCID_{50}$ per capsule. The low dose capsule (Lot 16 CV-01108A), made with an equivalent weight of a 1:10 dilution of the lyophilized virus contained in the Lot 16 CV-01107A, should have contained c. $10^{3.8}$ $TCID_{50}$ of vaccine virus per capsule. The three log difference in titer from that expected may have resulted from dilution of certain protein stabilizers contained with the lyophilized virus past a critical minimal level needed to maintain viable vaccine virus.

Despite a titer of less than 10 $TCID_{50}$ per capsule, the lowest dosage capsule infected 10 of 18 (56%) ADV-7 susceptible volunteers. Thus, the human enteric human infectious $dose_{50}$ of ADV-7 vaccine virus is less than 10 $TCID_{50}$ determined in HEK monolayer cultures.

The results of this study are similar to the data obtained in susceptible trainees immunized with L-AV-4 (1). The results of the two studies are compared in Table 8. The dose response of L-AV-7 is quite similar to that of L-AV-4 except for doses less than 10 $TCID_{50}$.

The results of this study indicate that a L-AV-7 dosage of $10^{4.8}$ $TCID_{50}$ is adequate for immunization of trainees. A satisfactory explanation for the disappointing infectivity of L-AV-7 found in Study #1 when a similar dosage was used ($10^{4.9}$ $TCID_{50}$) is not known but may have been related to formulation of the enteric capsule used in that study.

Table 8. Dose Response to L-AV-4 and L-AV-7

L-AV-4*		L-AV-7	
Titer of Virus (Log_{10} $TCID_{50}$)	No. Infected/ No. Immunized (%)	Titer of Virus (Log_{10} $TCID_{50}$)	No. Infected/ No. Immunized (%)
6.2-6.5	43/43 (100)	6.8	12/12 (100)
4.2-4.7	28/30 (93)	4.8	18/19 (95)
1.0-2.7	16/28 (57)	---	---
<1.0	1/28 (3)	<1.0	10/16 (56)

* Gutekunst, R.R., et al, Amer. J. Epidem. 86:341, 1967.

Simultaneous Administration of L-AV-4 and L-AV-7 Vaccines.
(Study #5, USAMEDTC Trainees, Oct-Dec 1969)

A potential problem in control of adenovirus ARD in military trainees by immunization with multivalent adenovirus live, oral vaccines is the problem of interference of gastrointestinal infection and antigenicity of one vaccine virus type by another. An earlier study (Study #2, Annual Report, 1969) of simultaneous immunization of 12 volunteers lacking serum N antibody to both ADV-4 and ADV-7 with L-AV-7 containing $10^{5.4}$ $TCID_{50}$ and L-AV-4 containing $10^{4.0}$ $TCID_{50}$ provided little evidence for interference between the two vaccine viruses. In another study involving 23 trainees susceptible to both ADV-4 and ADV-7 carried out at Fort Dix, N.J. (Study #3, this report), a L-AV-7 containing $10^{5.4}$ $TCID_{50}$ did not interfere with the antigenicity of a L-AV-4 containing $10^{4.4}$ $TCID_{50}$. The number of subjects in these two studies was too small to permit a confident conclusion that a practically significant degree of interference does not exist between the two vaccine viruses.

Approximately 75% of incoming BCT's in CONUS lack N antibodies to ADV-4 and approximately 50% lack antibodies to ADV-7. Historically, ADV-4 has been the principal adenovirus pathogen at CONUS training posts where L-AV-4 was not used. It is of considerable practical importance to know whether L-AV-7 in various dosages interferes with the immunogenicity and efficacy of L-AV-4. Reduction of the antigenicity of L-AV-4 by L-AV-7 so that only 2/3 of susceptible trainees developed protective antibody would entail that 25% of the trainee population would be susceptible to ADV-4 associated ARD. Therefore, it was deemed important to explore the question of whether a high dose or a moderate dose L-AV-7 would interfere with the antigenicity of a low dosage L-AV-4.

A. Design of Study:

1. The Study Group: Volunteers were sought from enlisted trainees of company A-1, The USAMEDTC, Fort Sam Houston, Texas. A comprehensive explanation of the study and its risks were given to all trainees of this company by one of the responsible investigators. After the briefing, trainees were given the opportunity to volunteer for the study. A total of 207 trainees volunteered.

2. Vaccines:

a) Type 7: ADV-7 strain 55142, propagated in human embryonic kidney (HEK) cells, was obtained by Wyeth Laboratories from Dr. Robert Chanock of NIAID, NIH. The strain was passaged three times in HEK cultures, then through 12 passages in human diploid fibroblast (WI-38) cultures, and lyophilized. Two separate vaccine lots were prepared from this lyophilized virus: Lot 16 CV-01106B which contained $10^{6.8}$ $TCID_{50}$ per capsule and Lot 16 CV-01107B which contained $10^{4.8}$ $TCID_{50}$ per capsule. The two vaccine dosages were prepared into enteric-coated capsules.

b) Type 4: Adenovirus type 4, live, oral vaccine (Lot 16 CI-00801, Wyeth) was used. This vaccine contained $10^{4.0}$ $TCID_{50}$ per tablet on titration in HEK cultures.

3. Immunization of Volunteers: Two hundred and seven trainees volunteered for the study. Volunteers were enrolled by order of entry into the study into three vaccine groups.

a) Group A: Immunized with L-AV-7, Lot 16 CV-01106B and L-AV-4.

b) Group B: Immunized with L-AV-7, Lot 16 CV-01107B and L-AV-4.

c) Group C: Immunized with L-AV-4 alone.

The first two of five men in line were enrolled in group A, the second two of five in group B, and the last of five in group C. No unusual reactions to the vaccine(s) were related by the trainees.

4. Sampling: Blood samples for determination of ADV-4 and ADV-7 serum neutralizing (N) antibody were obtained from all volunteers. Three and six week post-immunization blood samples were obtained from all volunteers found to lack ADV-4 and ADV-7 N antibody (groups A and B) or ADV-4 N antibody alone (group C) in their pre-immunization serum specimens.

5. ADV Serum N Antibody Determinations: Serum N antibody levels to ADV-7 vaccine virus (strain 55142) and ADV-4 vaccine virus (strain CL 68558) were measured in HEK tissue culture monolayer tubes. After sera were heated at 56°C for 30 minutes, serial two-fold dilutions from 1:2 to 1:256 were made. 0.2 ml of each serum dilution was incubated with 0.2 ml of a dilution of ADV-4 or ADV-7 vaccine virus at room temperature for one hour prior to inoculation of 0.1 ml of the virus-serum mixture into each of two HEK tubes. Tests were read at a time when the test dose of virus was 3-10 $TCID_{50}$.

Initially serum from each volunteer was tested for ADV-4 and ADV-7 serum N antibody at a dilution of 1:2. Thereafter, serum N antibody titers to both vaccine viruses was determined in pre- and post-immunization sera from all volunteers in group A and B who lacked serum N antibody at a 1:2 dilution to both vaccine viruses and from volunteers in group C who lacked ADV-4 serum N antibody at a 1:2 dilution.

6. Viral Isolations from A-1 Company Trainees: Since a small number of adenovirus infections had been determined in trainees during a study conducted at the USAMEDTC during late Fall of the preceding year, an attempt was made to monitor trainees from company A-1 who reported to the Dispensary with URI symptoms for adenoviral infections. Throat swabs were taken from trainees of company A-1, whether or not enrolled in the study, by Dispensary personnel and held at ambient temperature in viral transport medium for the duration of the study. Swabs were briskly

agitated in 2.0 ml of Hank's Balanced Salt Solution with 0.4% Bovine Plasma Albumin containing 1,000 u/ml of penicillin, 1,000 ug/ml streptomycin and 5 ug/ml Fungizone. After incubation at room temperature for 30 minutes, 0.2 ml of each specimen was inoculated into each of two HEK cultures fed with Leibovitz-15 medium with 2% Fetal Bovine serum. All cultures were observed every other day for 21 days for cytopathic effect (CPE). Isolates with typical adenovirus CPE were identified in tissue culture neutralization tests with hyperimmune ADV-4 and ADV-7 antisera.

B. Results:

Vaccines were administered to volunteers without difficulty and with the possible exception of URI's, no adverse reactions to either vaccine were related by the volunteers.

1. Viral Isolations from Trainees with URI's: Mild URI's were common in company A-1 during the course of the study. Twenty per cent of all A-1 trainees (116 of 585) visited the Dispensary with URI's during the three weeks after immunization, a proportion similar to that among study volunteers from company A-1, 17% (35 of 207). Both ADV-7 and ADV-4 were isolated from ill trainees; a similar proportion of immunized volunteers (10 of 35, 29%) and unimmunized company members (20 of 82, 24%) had ADV associated URI's. Both adenovirus types were first isolated from immunized volunteers after or simultaneous with their isolation from unimmunized trainees suggesting that disease was caused by wild adenovirus strains prevalent at the USAMEDTC rather than vaccine strains introduced by immunization. In the absence of a marker for the vaccine strains, this hypothesis could not be tested further.

Five per cent of immunized volunteers had isolation established adenovirus-associated URI's by three weeks post-immunization; it is likely that a much higher proportion experienced adenoviral infections without illness. This high incidence of wild adenoviral infections at the USAMEDTC obscured the data on serum N antibody responses since increases in serum N antibody might be associated with natural adenoviral infection, vaccine virus infection, or both.

The proportion of volunteers lacking N antibody to both adenovirus type in pre-immunization serum is shown in Table 9. An unexpectedly low proportion of trainees in vaccine group C were susceptible to both adenovirus types. An additional 12 trainees in vaccine group C were susceptible to ADV-4 but had N antibody to ADV-7. Since volunteers in this vaccine group received L-AV-4 alone; ADV-4 serum N antibody responses were determined in these 12 ADV-4 susceptible volunteers to obtain a larger sample for determination of the antigenicity of L-AV-4 in susceptibles.

ADV-7 serum N antibody was detected by three weeks post-immunization in 96% (27 of 28) of susceptible volunteers in group A, 94% (30 of 32) of susceptible volunteers in group B, and 56% (5 of 9) of volunteers in group C. The ADV-7 antibody rises in the last group, immunized with L-AV-4 alone, probably is due to natural ADV-7 infection.

Table 9

Vaccine Group	Total Number	No. Volunteers Susceptible* to ADV-7 and ADV-4
A	84	28 (33%)
B	82	34 (42%)
C	41	9 (22%)
TOTAL	207	71 (34%)

* Initial serum N antibody titer to ADV-4 and ADV-7 less than 1:2.

ADV-4 serum N antibody was detected by three weeks post-immunization in 79% (22 of 28) of susceptible volunteers in group A, 74% (25 of 34) of susceptible volunteers in group B, and in 86% (18 of 21) of ADV-4 susceptible volunteers in group C.

C. Discussion:

This study was designed to determine whether a high dosage L-AV-7 would interfere with the infectivity of a simultaneously administered L-AV-4. The evidence obtained suggested that no decrease in antigenicity of a low dosage L-AV-4 ($10^{4.0}$ $TCID_{50}$ per tablet) occurred when given with a high dosage L-AV-7 ($10^{6.8}$ $TCID_{50}$ per capsule) from that obtained when L-AV-4 was given alone or with a moderate dosage L-AV-7 ($10^{4.8}$ $TCID_{50}$ per capsule). Due to the presence of wild ADV-4 and ADV-7 infections in the training population studied, serum N antibody responses by three weeks after immunization could be due to wild adenovirus infection as well as vaccine virus infection. Since the immunized volunteers were not sequentially monitored for pharyngeal adenovirus infection, the proportion of volunteers in each vaccine group who developed serum N antibody after immunization in the absence of pharyngeal infection by a wild adenovirus strain is unknown. Consequently, the true rate of antibody acquisition after immunization with the three vaccine combinations could not be assessed by this study.

Field Efficacy of L-AV-7 Vaccines.
(Study #6, Fort Dix Basic Combat Trainees, 1970)

This study was designed to study the impact of L-AV-7 and L-AV-4 in a training population at risk of ADV-7 and ADV-4 ARD. Answers to the following questions were sought.

1) What is the protective effect of L-AV-7 given with L-AV-4 on ADV-7 associated ARD in a large trainee population?

2) Does L-AV-7 interfere with the protective effect of L-AV-4 against ADV-4 associated ARD?

3) Does an excess of respiratory illness not associated with ADV-7 or ADV-4 occur in a trainee population immunized with L-AV-7 and L-AV-4?

4) What is the role of respiratory pathogens other than ADV-4 and ADV-7 in trainees immunized with both adenovirus vaccines?

A. Design of Study:

The study was begun in trainees at Fort Dix, N.J. on 6 Jan 70. Prior to the Christmas leave, ARD at Fort Dix was due to both ADV-7 and ADV-4. Immunization was accomplished by a modified "pulse" technique with vaccine or vaccines given to all trainees in or before the 3rd week of training and thereafter to all incoming trainees in the Reception Center. Trainees in or expected to enter one of the two BCT brigades, the 2nd Bde, received L-AV-4 alone and those expected to enter the other BCT brigade, the 3rd Bde, received L-AV-4 and L-AV-7. As was anticipated, not all trainees expected to enter each of the two Bdes actually was assigned to that brigade. With the exception of one cohort in the 2nd Bde which was 30% filled with trainees who were expected to enter the 3rd Bde (and hence immunized with L-AV-4 and L-AV-7), less than 2% of all trainees entering cohorts of each Bde received the wrong vaccines. The same proportion of trainees of each cohort were not immunized with either vaccine for a variety of administrative reasons. As in Table 10, a total of 14 cohorts were enrolled in the study, 7 from the 2nd Bde (L-AV-4) and 7 from the 3rd Bde (L-AV-7 and L-AV-4). Trainees from the first two of the seven cohorts from each Bde were immunized in training while those of the last five cohorts of each Bde were immunized in the Reception Center. Each cohort constituted six companies of trainees beginning BCT during one week.

B. Vaccines:

1. L-AV-7: This is an adenovirus type 7 strain 55142, obtained by Wyeth Laboratories from Dr. Robert Chanock, NIAID, NIH, passaged 12 times in human diploid fibroblast cells (WI-38), lyophilized, and prepared into enteric-coated tablets (Lot 16 CV-02301, Wyeth). Tablets sampled during the course of the study contained between $10^{4.6}$ and $10^{4.7}$ $TCID_{50}$ per tablet.

2. L-AV-4: This is an adenovirus type 4 strain, CL 68578, obtained from Wyeth Laboratories from Dr. Robert Chanock, NIAID, NIH, passaged in WI-38 cells, lyophilized, and prepared into enteric-coated tablets (Lot 16 CI-00801, Wyeth). Tablets sampled during the course of the study contained between $10^{3.5}$ and $10^{4.3}$ $TCID_{50}$ per tablet.

C. Sampling:

Hospitalizations of all trainees in the 14 cohorts studied who were admitted to the ARD or Pneumonia wards of Walson Army Hospital were

Table 10. Immunization Status of Trainee Cohorts

Cohorts Immunized in Training	2nd Bde (L-AV-4) Cohort	2nd Bde (L-AV-4) Week of Training Immunized	3rd Bde (L-AV-4 & L-AV-7) Cohort	3rd Bde (L-AV-4 & L-AV-7) Week of Training Immunized
6-7 Jan	I	3		
6-7 Jan			II	2
6-7 Jan	III	1		
13 Jan			IV	1

Cohorts Immunized in Reception Center	Cohort	First Week of Training	Cohort	First Week of Training
9-15 Jan	V	19-24 Jan		
16-22 Jan			VI	26-31 Jan
23-29 Jan	VII	2-7 Feb		
30 Jan-5 Feb			VIII	3-14 Feb
6-12 Feb	IX	16-21 Feb		
13-19 Feb			X	23-28 Feb
20-26 Feb	XI	2-7 Mar		
27 Feb-5 Mar			XII	9-14 Mar
6-13 Mar	XIII	16-21 Mar		
13-19 Mar			XIV	23-28 Mar

recorded daily and used to calculate ARD rates for the study group. It is important that not all trainees admitted to these wards did, indeed, have ARD but that some had rubella, typhoid immunization reactions, or other illnesses. Since these trainees are normally recorded as ARD hospitalizations by the hospital administration and since no criteria for eliminating such admissions as ARD admissions could be derived which would not possibly bias the study sample, we elected to consider all trainees admitted to ARD or Pneumonia wards as having ARD.

One of the six companies constituting each cohort was selected as a study company prior to the company's beginning training. All trainees of these 14 study companies admitted to the URI wards of Walson Army Hospital were examined for respiratory disease by a member of the study team within 12 hours of admission. A brief history and physical examination limited to the upper respiratory tract was performed. A throat washing for viral isolation studies was obtained on all study company trainees admitted and acute and 2-week convalescent bleeding for serology were obtained on all study company trainees who were admitted before the middle of the 7th week of basic combat training.

In order to more thoroughly study respiratory disease occurring in a trainee population immunized with both L-AV-7 and L-AV-4, additional samples were obtained from study company trainees admitted from the 3rd Bde. In addition to the throat wash and bleedings mentioned above, a nasal swab for rhinovirus isolation, a throat swab for isolation of B-hemolytic streptococci, and a nasopharyngeal swab for isolation of meningococci were obtained.

D. Methods:

1. Virology:

a) Isolation: Throat washings, obtained by having each hospitalized trainee gargle ½ oz of Hank's balanced salt solution (HBSS) containing 0.4% bovine plasma albumin (BPA) were treated with penicillin (100 u/ml), streptomycin (100 ug/ml), and Fungizone (0.5 ug/ml). Treated 0.3 ml aliquots of each throat wash were inoculated into each of two tube cultures of primary human embryonic kidney (HEK) and primary Rhesus monkey kidney (MKR). HEK cultures were maintained with Leibovitz-15 medium containing 2% fetal bovine serum (FBS) and observed every other day for cytopathic effect (CPE) and observed for a minimum of 21 days before discarded. MKR cultures received with Eagle's minimal essential medium (EMEM) containing 2% FBS and SV-5 antiserum, were fed twice with medium 199 without protein prior to inoculation. MKR cells were observed every other day for CPE. Hemadsorption (HAD) with 1.0 ml of a 0.1% human O RBC suspension was performed at 7 and 14 days after inoculation. Cultures negative by CPE or HAD by 14 days after inoculation discarded.

Nasal swabs were agitated in 2.0 ml HBSS containing 0.4% BPA and the fluid was maintained at -60°C until isolation was attempted. An aliquot of nasal swab fluid was treated with penicillin and streptomycin

(1000 u/ml and 1000 ug/ml, respectively) and 0.2 ml aliquots inoculated into each of two WI-38 cell cultures. WI-38 cells were generally maintained on medium 199 containing 10% FBS. After incubation on stationary racks for 2 hours at 33°C, inoculated tubes were incubated in roller drums at 33°C and observed every other day for 14 days. If negative at that time, a blind passage in WI-38 was made which was challenged with Echo virus type II seven days after the blind passage was made.

b) Identification of isolates: Isolates with characteristic adenovirus CPE were identified in HEK tube neutralization tests using hyperimmune adenovirus typing antisera. Isolates with CPE characteristic of enteroviruses were identified by tissue culture neutralization tests using hyperimmune pooled poliovirus type antisera. Isolates with herpesvirus CPE were tentatively identified by their susceptibility to chloroform. Isolates obtained from MKR cells by HAD were passaged in embryonated eggs and identified as similar to A_2/HK/68 influenza strains by hemagglutination-inhibit on tests using hyperimmune antisera to A_2/HK/1/68 virus.

2. Serology:

a) Complement-Fixation (CF) tests: CF tests for adenovirus, Mycoplasma pneumonia, and influenza A were performed with all acute and convalescent hospitalization sera obtained by standard techniques. Due to standard immunization of BCT's with polyvalent influenza vaccines, a soluble CF antigen of A_2/HK/1/68 was used in these tests to determine influenza infection.

b) ADV-4 and ADV-7 neutralizing (N) antibody tests: ADV-4 and ADV-7 N antibody tests were carried out in HEK tube cultures and read at a time when the test dose of virus was between 80-320 $TCID_{50}$.

3. Bacteriology:

a) B-hemolytic streptococci: Throat swabs were plated and streaked on sheep blood agar plates which were incubated for 18-24 hours at 37°C. All were examined for B-hemolytic colonies with streptococcal morphology, and suspicious colonies were subcultured. All B-hemolytic streptococcal strains isolated were tentatively grouped by the bacitracin disk technique.

b) Meningococci: Nasopharyngeal swabs were plated directly onto plates containing modified Thayer-Martin media. After inoculation at 37°C for 18-24 hours in a candle jar, all cultures were read and, if positive, graded as to quantity. Colonies isolated were harvested by swabbing the plate with cotton swabs which were maintained in diagnostic transport medium until transportation to the Department of Bacterial Diseases, WRAIR for further characterization.

E. Results:

Both adenovirus vaccines were administered to a total of 14,670 Fort Dix trainees from 6 Jan to 1 May 70. No significant reactions to

adenovirus immunization were detected. Two trainees in the second BCT Bde died with pneumonia and intravascular coagulation. These illnesses were associated with ADV-7 by isolation of ADV-7 from throat washings and sputum upon hospital admission, a greater than four-fold rise in ADV-7 serum N antibody titer between admission and death and isolation of ADV-7 from lung tissue obtained post mortem. These illnesses were not vaccine related in that one trainee had received neither L-AV-7 nor L-AV-4 since he was hospitalized at the time his unit was immunized, and the second trainee received L-AV-4 alone.

Data will first be considered for the last five cohorts of the two study Bde's, the cohorts which were similar in that immunization of all took place in the Reception Center (Table 10). A significant difference in crude admission rates was found between the two brigades as shown in Table 11.

Table 11. Crude Admission Rates, Study Brigades

	Total Strength	Admissions to ARD Ward	Rate/1000/8wks
2nd Bde	5547	1641	295.8
3rd Bde	5795	1144	197.1

As mentioned previously, a significant proportion of trainees of one cohort expected to enter the 3rd BCT Bde and thus immunized with both adenovirus vaccines were assigned to the 2nd BCT Bde (trainees of which were to receive L-AV-4 alone). When these trainees are eliminated from consideration with the 2nd Bde, an increase in the difference in rates between the two training Bde's is seen (Table 12). This represents a 35% suppression of ARD admissions in the 3rd Bde.

Table 12. Crude Admission Rates, Study Bde's, Corrected for Immunization Status

	Total Strength	Admissions to ARD Ward	Rate/1000/8wks
2nd Bde (L-AV-4)	5350	1623	303.3*
3rd Bde (L-AV-4 & L-AV-7)	5795	1144	197.1*

* $X^2 = 168$, $p < .0001$

Table 13. Comparison of Crude ARD Rates of Study Companies and Entire Bde

	2nd BCT Bde			3rd BCT Bde		
	Strength	ARD Admissions	Rate/1000/8wk	Strength	ARD Admissions	Rate/1000/8wk
Study Companies	929	297	319.6*	911	911	166.8**
Entire Bde	5547	1641	295.8*	5795	1144	197.1**

* $X^2 = 2.1584$, $0.5 < p < 0.7$

** $X^2 = 4.7203$, $0.2 < p < .05$

In order to determine the effect of immunization on adenovirus type-specific ARD, all ARD admissions from one company of the six comprising each cohort were monitored. An estimate of whether this method of sampling yielded a study group which was representative of each brigade can be made by examining the crude ARD hospitalization rates for the study companies and their respective brigades. As seen in Table 13, there is no significant difference between the crude ARD rates of the 2nd BCT Bde study companies and the entire 2nd BCT Bde. A difference of borderline statistical significance ($0.02 < p < 0.05$) was found between the 3rd Bde study companies, and the Bde as a whole with a lesser rate in the study companies. This difference in rates between the study companies and the brigade is almost entirely due to a higher admission rate in the first three weeks of training in the companies not studied. Rates for the Bde study companies and entire Bde's for the first three weeks of BCT and the last five weeks of BCT are shown in Table 14. A significant difference in the 3rd Bde is found only in the first three weeks of training. As later described, ARD admissions during the first three weeks of training in this Bde were uncommonly associated with adenoviruses. It seems likely, therefore, that the 3rd Bde study companies did not differ significantly from the Bde as a whole at the time of training when ARD was caused principally by adenoviruses.

Total ARD rates for the study companies of the two Bde's is shown in Table 15; the difference between the Bde's is highly significant. The difference in the 2nd Bde rate shown from that previously described as crude ARD rate is due to the elimination of trainees inadvertently entering this Bde who were immunized with L-AV-7 and L-AV-4. Laboratory work on this project is sufficiently advanced to allow estimation of ADV type-specific rates for the two study groups based upon adenovirus isolation data alone. A hospitalization is considered adenovirus associated if an adenovirus type was isolated from the throat washing obtained on each trainee shortly after admission. ADV-4 and ADV-7 specific ARD rates are presented in Table 15. A significant difference in ADV-7 associated ARD rates was found between the two Bde's. As shown in Figure 2, the cumulative ADV-7 ARD rate for the 2nd Bde (L-AV-4 alone) rose steadily from the 3rd through the 7th week of training while very little ADV-7 associated ARD was found in the 3rd Bde (L-AV-7 and L-AV-4). The type 7 vaccine resulted in a 95% suppression of ADV-7 associated ARD.

In contrast, ADV-4 associated ARD was more common in 3rd Bde trainees. Figure 3 depicts the cumulative ADV-4 associated ARD rates in the two training Bdes. The difference is significant ($X^2 = 8.24$, $0.0001 < p < 0.01$).

Total rates for ADV-associated (and non-adenovirus associated) ARD are shown in Table 16. A significant difference, representing a 76% suppression of ADV-associated ARD in the 3rd Bde, is apparent, while ARD not associable with ADV occurred at a similar rate in both training Bde's.

Table 14. Comparison of Crude ARD Rates of Study Companies and Entire Brigade, 0-3 Weeks and 4-8 Weeks of Training

	2nd BCT Bde		3rd BCT Bde	
Weeks Training	Rate/1000 Entire Bde	Rate/1000 Study Companies	Rate/1000 Entire Bde	Rate/1000 Study Companies
1-3	105.4	104.4[a/]	85.2	63.6[c/]
4-8	190.3	215.2[b/]	112.1	103.1[d/]

[a/] X^2 = Not tested

[b/] $X^2 = 3.0489$, $0.05 < p < .1$

[c/] $X^2 = 4.8600$, $0.02 < p < 0.05$

[d/] $X^2 = 0.6453$, $0.3 < p < 0.5$

Table 15. ARD Rates/1000/8wk of Study Companies

Study Group:	2nd BCT Bde	3rd BCT Bde
Immunization Status:	L-AV-4	L-AV-7 & L-AV-4
Strength:	805	911
No. ARD Hospitalizations:	260	149
ARD Rate:	322.6	163.3[a/]
No. ADV-7 Hospitalizations:	145	8
ADV-7 ARD Rate:	179.7	8.8[b/]
No. ADV-4 Hospitalizations:	12	34
ADV-4 ARD Rate:	14.9	37.4[c/]

[a/] $X^2 = 126.6$, $p < 0.0001$

[b/] $X^2 = 177.2$, $p < 0.0001$

[c/] $X^2 = 8.2398$, $0.001 < p < 0.01$

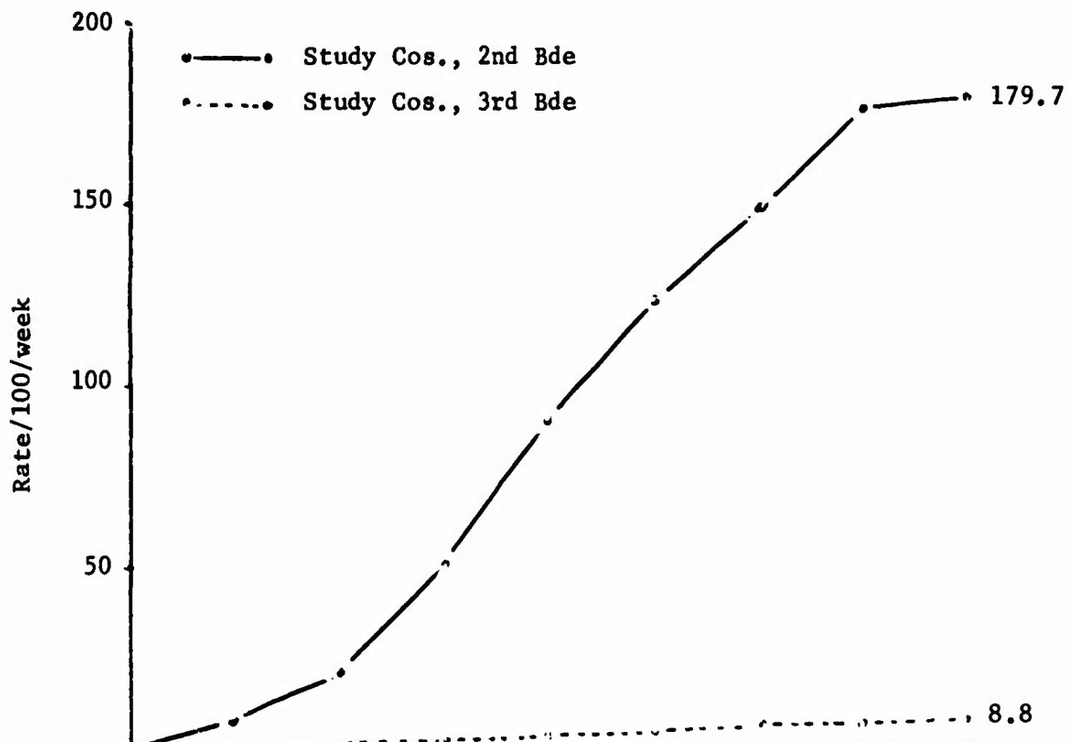


Figure 2. Cumulative Type 7 Adenovirus-associated ARD rates, study companies, Fort Dix 1970.

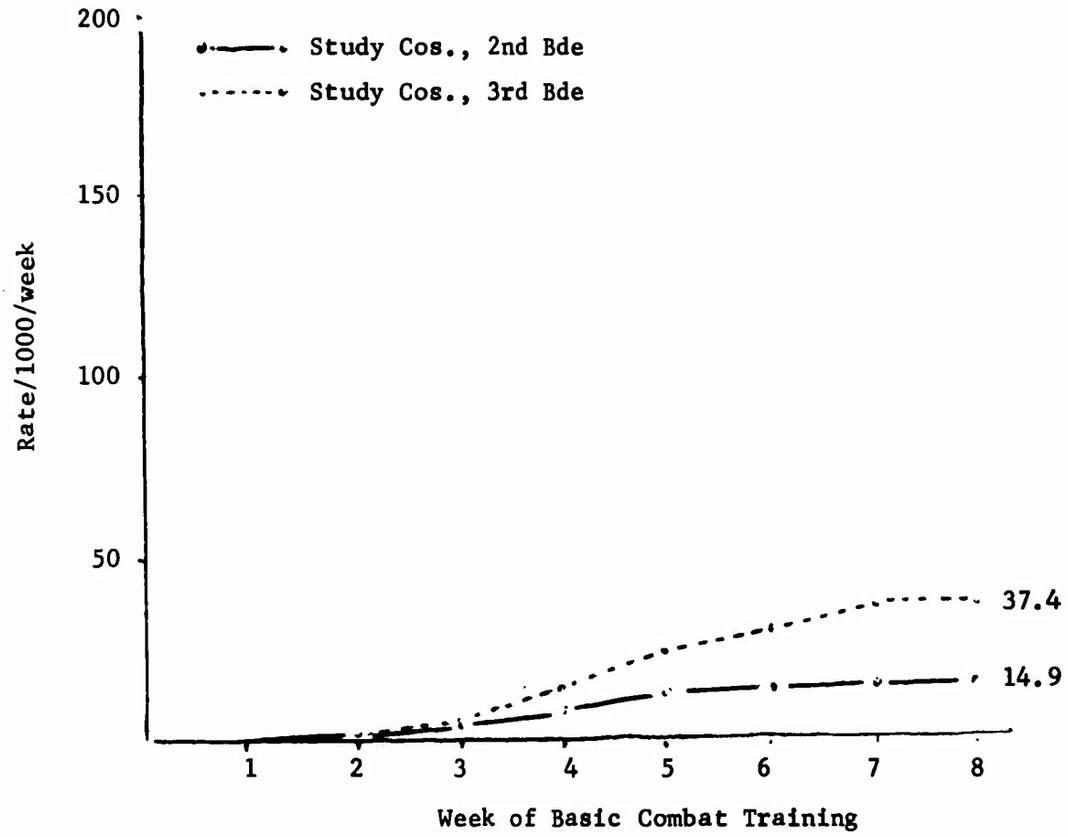


Figure 3. Cumulative Type 4 Adenovirus-associated ARD rate, study companies, Fort Dix 1970.

Table 16. ADV-Associated and Non-ADV-associated ARD Rates, Study Companies

	2nd BCT Bde	3rd BCT Bde
ADV-associated rate	195.0/1000/8 weeks	46.1/1000/8 weeks[a/]
Non-ADV-associated rate	127.6/1000/8 weeks	117.2/1000/8 weeks[b/]

a/ $X^2 = 92.34$, $p < 0.0001$

b/ $X^2 = 0.442$

F. Discussion:

This study was designed to study the impact of immunization with L-AV-7 and L-AV-4 in a basic training population at risk of ADV-7 and ADV-4 ARD. Although much of the laboratory data is incomplete at the time of writing, certain conclusions are evident from the data at hand.

The remarkable safety of L-AV-7 and L-AV-4 shown in previous studies by this department and other investigators was confirmed by this study. No illness attributable to the adenovirus vaccines was noted. The occurrence of two fatal atypical pneumonia cases at Fort Dix associated with ADV-7 during the study was a cause of some concern, but no relationship of these illnesses to L-AV-7 was apparent in that neither trainee received this vaccine.

Immunization with L-AV-7 and L-AV-4 resulted in a 95% suppression of ADV-7 associated ARD as compared with immunization with L-AV-4 alone. This study confirmed in a larger number of immunized trainees the findings of Study #3 previously reported that L-AV-7 was highly protective against type-specific disease. The degree of suppression of type-specific ARD by L-AV-7 is of the same order as that previously described for L-AV-4.

Simultaneous administration of L-AV-7 with L-AV-4 did decrease the protective effect of L-AV-4 against ADV-4 associated ARD. Approximately, 23% of ARD hospitalizations in the 3rd Bde were ADV-4 associated, as opposed to 5.7% of 2nd Bde admissions. The difference in ADV-4 associated ARD rates between the two Bde's was highly significant.

Firm evidence of interference by L-AV-7 on L-AV-4 was not obtained in past studies of these two vaccines (Study #2, Annual Report 1969, Studies #3 and #5 above), undoubtedly because numbers of immunized subjects in those studies were too small to show a difference of the magnitude found in this study. It should be emphasized that the difference in ADV-4 ARD rates between the two groups was small, 22.5/100/8 weeks, which represents five excess admissions per company per training cycle.

The dosage of the L-AV-4 used in this study was low, between $10^{3.6-4.3}$ $TCID_{50}$. It is possible that interference between the two adenovirus vaccines would be less manifest with a higher dosage L-AV-4, a hypothesis that should be amenable to test in the future. Whether near total suppression of adenovirus-associated ARD is more desirable than the level of suppression obtained in the 3rd Bde in this study is questionable since it is possible that some transmission of ADV-4 or ADV-7 in an immunized trainee population may serve to prevent less common adenovirus serotypes (ADV-21 or ADV-14) from emerging as a cause of ARD.

While immunization with L-AV-7 and L-AV-4 suppressed adenovirus associated ARD by 76% in the 3rd BCT Bde, only a 35% suppression of all ARD was achieved. This finding was related to the sizeable proportion of disease not related to adenovirus in both brigades. Preliminary data suggests that several other agents, among them Influenza A_2, Rhinoviruses, and Rubella virus, contributed significantly to disease causing ARD hospitalizations during this study.

II. Use of L-AV-7 on CONUS BCT Posts During 1970.

In addition to Fort Dix, L-AV-7 was used on three CONUS posts - Fort Lewis, Fort Wood, and Fort Campbell. Vaccine(s) were given under the supervision of these posts's Preventive Medicine Officers - MAJ Morgan, Fort Lewis; LTC Varela, Fort Wood; and CPT Levine, Fort Campbell. At each post, vaccine(s) were given by a modified pulse method, i.e. to all trainees in the first three weeks of BCT and thereafter to all incoming trainees until 1 May 1970 when adenovirus immunizations ceased at all posts.

Monitoring of ARD hospitalizations at each post and the collection of throat swabs for viral isolation studies were carried out as routinely for the Adenovirus Surveillance Study. Viral isolation was performed in HEK tube cultures by the 6th USAML, under the supervision of COL Albert Leibovitz, MSC and the area military laboratories responsible to the three posts.

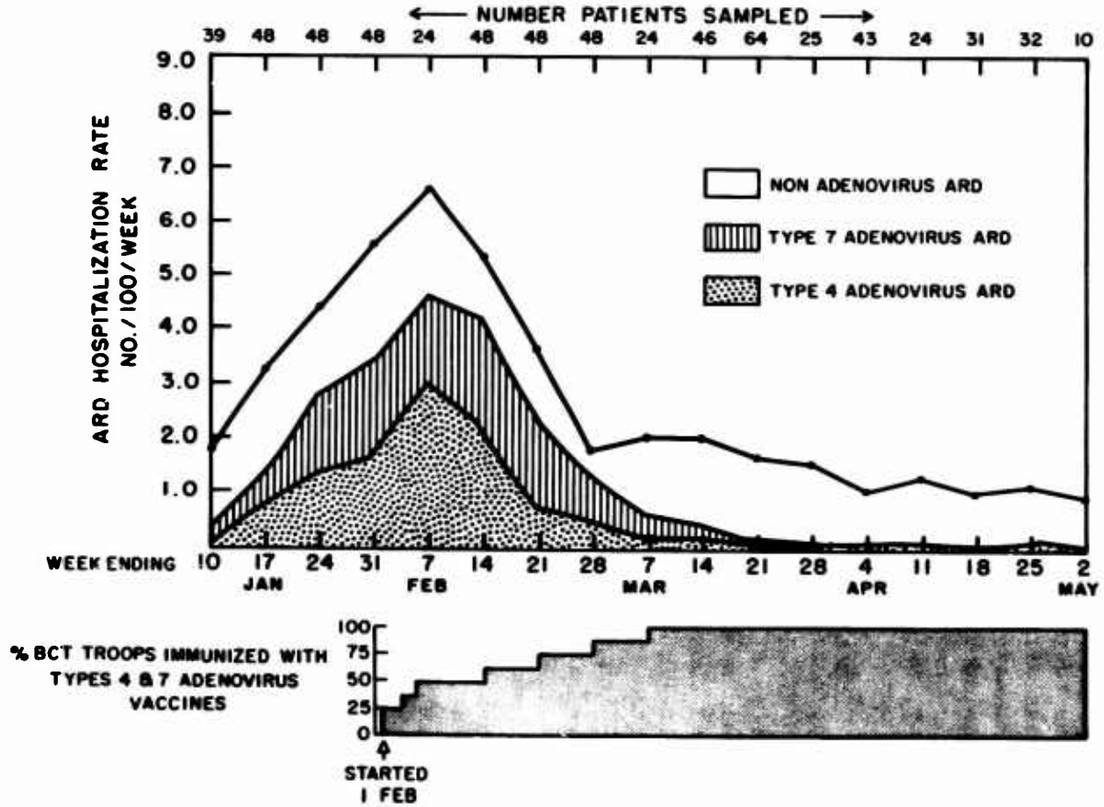
Crude BCT ARD rates were obtained weekly from each post. ADV-4 and ADV-7 type-specific ARD rates were determined by multiplying the weekly crude ARD rate by the proportion of ADV-4 and ADV-7 isolates, respectively, of the total throat swabs obtained from trainees hospitalized at that post during the week.

1. Fort Lewis: Simultaneous immunization with L-AV-4 and L-AV-7 was begun by the modified pulse method in Fort Lewis BCT's beginning 1 Feb. Prior to the Christmas leave period, ARD rates had exceeded 6/100/week at Fort Lewis, and disease was due to both ADV-7 and ADV-4. As shown in Figure 4, immunization with both adenovirus vaccines was commenced during an outbreak of ARD due to both ADV-4 and ADV-7. The crude ARD rate rose to 6.6/100/week during the first week of immunization, and then rapidly fell to below 2.0/100/week by four weeks after immunization began. The fall in the crude ARD rate was accompanied by

Figure 4

FT. LEWIS - 1970

ACUTE RESPIRATORY DISEASE HOSPITALIZATION RATE IN BASIC COMBAT TRAINEES



a similar decline in ADV-4 and ADV-7 type-specific rates. By the time all trainees were immunized (5 weeks after immunization), the adenovirus-specific ARD rate was less than 0.5/100/week below which it remained for the duration of the study.

2. Fort Wood: Simultaneous immunization with L-AV-4 and L-AV-7 was begun at Fort Wood on 9 Feb 70 by the modified pulse method. As shown in Figure 5, immunization was begun when the post BCT ARD rate was 3.7/100/week, and ARD was due principally to ADV-4 but also to ADV-7. The rate rose precipitously to 7.8/100/week and to 8.4/100/week one and two weeks after immunization began and then fell rapidly to 2.3/100/week by four weeks after immunization began. The fall in crude ARD rate was accompanied by a fall in ADV-4 and ADV-7 associated ARD rates, so that by six weeks after immunization was commenced, the ADV-associated ARD rate fell below 0.5/100/week where it remained.

3. Fort Campbell: Immunization of BCT's with L-AV-7 alone was begun at Fort Campbell on 22 Feb 70. Prior to the Christmas leave, only ADV-7 had been isolated at Fort Campbell and ADV-4 was not identified at Fort Campbell until well after the decision to use L-AV-7 alone had been made. Immunization was begun at a time when the ARD rate was above 7/100/week and disease was primarily due to ADV-7, as shown in Figure 6. The crude ARD rate began to fall during the second week after immunization but reached a plateau of 3/100/week two weeks later which was maintained until 25 Apr 70. A significant decrease in the ADV-7 associated ARD rate was first apparent by three weeks after immunization began and this rate remained below 0.3/100/week six weeks after immunization began. In contrast, after an unexplained fall in the ADV-4 associated ARD rate two weeks after commencing L-AV-7, the ADV-4 associated ARD rate rose to above 1.0/100/week where it remained through 25 April. Use of L-AV-7 at Fort Campbell resulted in the suppression of ADV-7 and the emergence of ADV-4 as the principal etiologic agent of ARD and resulted in little, if any, reduction in total ARD rates. A reverse situation (the emergence of ADV-7 as major respiratory pathogen) has been previously routine in posts using L-AV-4 immunization alone.

A total of 13,636 trainees received L-AV-7 and L-AV-4 at Fort Lewis, 12,933 trainees received L-AV-7 and L-AV-4 at Fort Wood, and 13,899 trainees at Fort Campbell received L-AV-7 alone. No untoward reactions to adenovirus immunization were reported.

III. Immunologic Response to Adenovirus Infection.

The role of local respiratory tract IgA antibody in protection against adenovirus-associated ARD remains unclear. Although L-AV-4 has been found to protect against ADV-4 associated ARD, attempts to detect ADV-4 neutralizing (N) antibody in nasal washes of volunteers previously immunized with L-AV-4 were unsuccessful (Annual Report 1967). This latter study would suggest that local IgA antibody is not required for protection against adenoviral ARD, as it is for protection against respiratory tract infections due to other agents such as myxoviruses

Figure 5

FT. LEONARD WOOD - 1970

ACUTE RESPIRATORY DISEASE HOSPITALIZATION RATE IN BASIC COMBAT TRAINEES

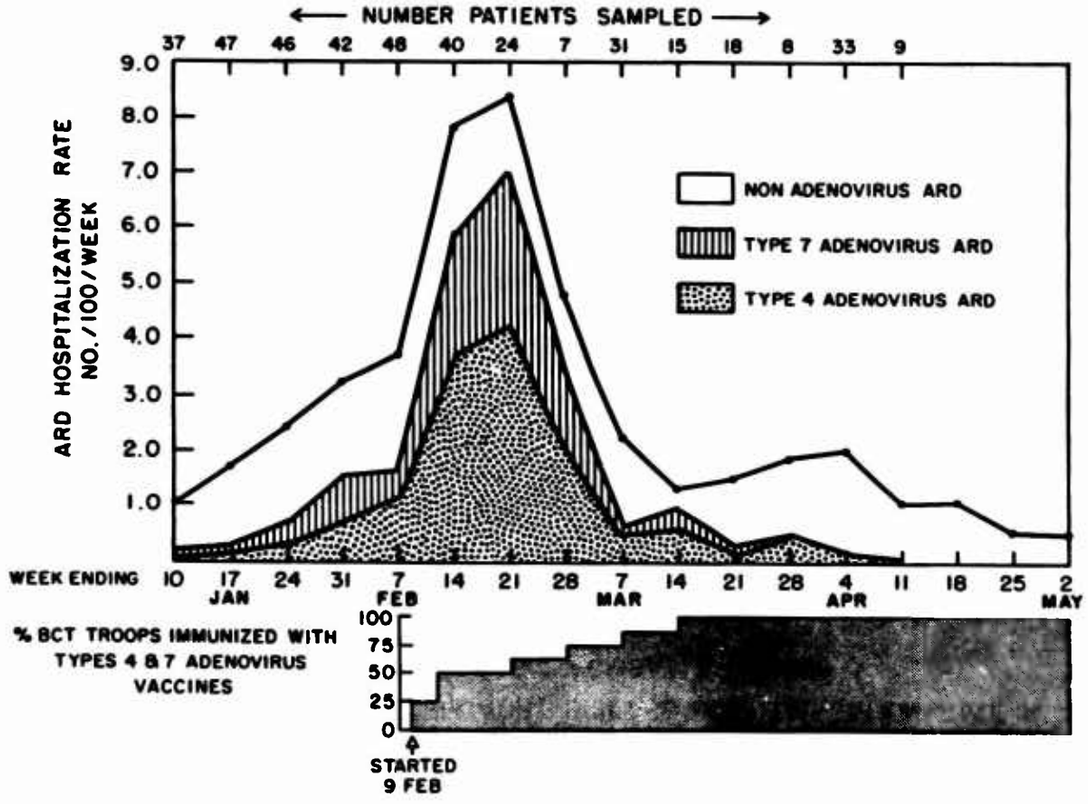
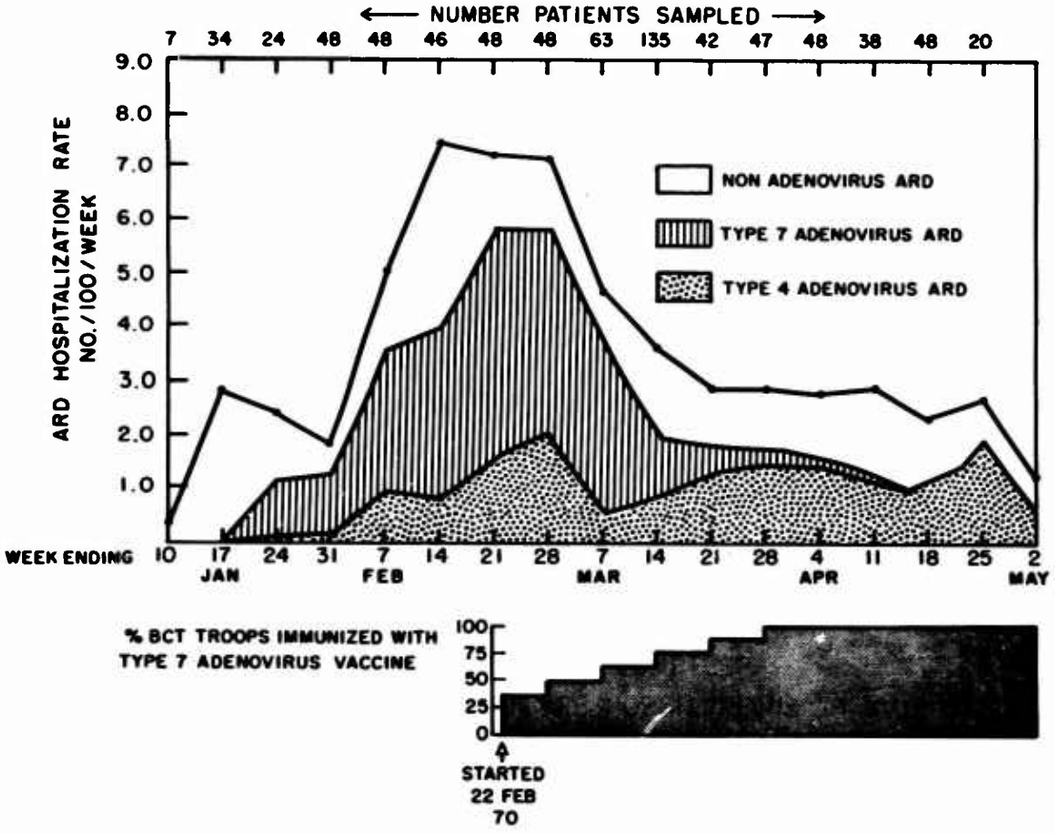


Figure 6

FT. CAMPBELL - 1970

ACUTE RESPIRATORY DISEASE HOSPITALIZATION RATE IN BASIC COMBAT TRAINEES



and rhinoviruses. Previously, N antibody was determined by conventional tube neutralization test which for viruses other than adenoviruses has been shown to be much less sensitive than the plaque reduction neutralization test (PRNT). It is possible that L-AV-4 induces only small amounts of protective nasal N antibody which could only be detected by the more sensitive PRNT. In order to investigate further the role of nasal antibody in protection against ADV disease, the development of an adenovirus PRNT was deemed essential, and it was pursued in this laboratory during the past year.

A variety of cell lines were tested for ability to plaque ADV-7 under conditions similar to those described below. No plaques were obtained with Hela cells, Hela-Rhino cells, Vero cells, Monkey Rhesus kidney, 9-day-old chick embryo cells, Chang's liver cells, or rabbit kidney cells. Plaques were obtained with HEK cells and KB cells. Plaque reduction neutralization tests were accomplished with HEK cells, but variation in the sensitivity of the test with different lots of this primary cell culture, the progressive loss of sensitivity to ADV-7 of the HEK cells on passage, and the expense involved made HEK cells less desirable than the continuous KB cell line. The following procedure was derived for adenovirus PRNT with KB cells.

A. Cell Husbandry:

KB cells (obtained from Flow Laboratories) were grown in 32 oz prescription bottles with growth medium (EMEM with 10% FBS, 1% glutamine and 100 u/ml penicillin and 100 ug/ml streptomycin-P&S). When confluent monolayers were obtained, growth medium was decanted and 5 ml of 25% trypsin solution was layered over the cells, agitated at room temperature for about 30 seconds, and decanted. An additional 5 ml of 25% trypsin solution was added and the bottle incubated at 37°C until cells floated off the wall. The cell suspension was harvested by aspiration with a propipette and centrifuged at 500-1000 rpm to remove the trypsin solution. The cell sediment accruing from one bottle was taken up in 50 ml of growth medium and after a uniform cell suspension was obtained, 5 ml of cells was inoculated into each of 10 2 oz Falcon plastic flasks. A complete monolayer of KB cells was formed usually three days after inoculation. In order for the cell sheet to remain viable for plaque production, it was necessary to use the cells on the day a complete monolayer was first apparent.

B. PRNT:

Serum was diluted 1:2 with Hank's balanced salt solution (HBSS) containing P&S and heated at 56°C for 30 minutes. Serial 2-fold dilutions were made in HBSS. Adenovirus type 7 strain 55142 (HEK 3, WI-12 HEK 4 2/6-2/11/70) was diluted to 10^{-5} in HBSS, 1 ml of the 10^{-5} virus dilution was added to 1 ml of serum dilution to give 2 ml of a $10^{-5.3}$ virus concentration which yielded 50-80 pfu/0.5 ml. The virus-serum mixture was incubated for one hour at room temperature. After decanting the growth medium from the KB monolayers in Falcon flasks, at least three flasks

per serum dilution were inoculated with 0.5 ml of the serum-virus mixture. In addition, three flasks each were inoculated with 0.5 ml virus at $10^{-5.0}$, $10^{-5.3}$, and $10^{-6.3}$ dilutions and two cell control flasks were inoculated with 0.5 ml HBSS containing P&S. All flasks were incubated at 37°C for 4 hours with gentle agitation every hour. After incubation, the virus-serum inoculum was removed by propipette and 5 ml of agar overlay was added to each flask. The composition of this agar overlay is as follows:

Nobles agar	1 gram
Distilled H_2O	75 ml
Earles Minimal Essential Medium (EMEM) 10X	10 ml
Fetal Bovine Serum (FBS)	10 ml
1-glutamine 100X	1 ml
Penicillin & Streptomycin (1X)	0.2 ml
$NaHCO_3$, 7.5%	4 ml

The agar and distilled water were autoclaved for seven minutes on slow exhaust prior to addition of the remaining materials. Final pH of this solution varied between 7.2-7.6. Flasks were left at room temperature for 15 minutes until the agar overlay solidified and then were incubated at 37°C.

On day 5, 4 ml of this agar overlay was added to each flask. On day 12, 5 ml of stain overlay was added to each flask. The stain overlay composition was as follows:

Nobles agar	1 gram
HBSS 10X	10 ml
Neutral Red (1:300)	5 ml
Distilled H_2O	85 ml
P&S	0.2 ml

Final pH of this overlay was 5.8-5.9. After the overlay solidified, flasks were incubated in darkness at 37°C for 3 hours and left in darkness overnight at room temperature. Plaques were counted on day 13.

Data on this ADV-7 PRNT are limited due to the recent development of the test, so that only three sera have been tested. In these tests, plaque reduction varied inversely with the logarithm_{10} of the reciprocal of the serum dilution between 15% and 85% reduction. ADV-7 neutralizing antibody titers by conventional HEK monolayer tube culture versus 100 $TCID_{50}$ ADV-7 and 50% plaque reduction endpoint titers using the KB cell system are compared in Table 17. The PRNT appears to be 8-10 times more sensitive than the conventional tube neutralization test. Further characterization of this test is felt indicated from this preliminary data.

Table 17. Comparison of ADV-7 Neutralizing Antibody Titers by Tube Neutralization Test and Plaque Reduction Neutralization Test

Sera	Titers	
	Tube Test	PRNT
X-1019	32	450
X-1101	256	1500
X-1141	8	70

IV. The Relationship of Hepatitis-Associated Antigen (Australia antigen) to Viral Hepatitis.

Sera from certain multiply transfused hemophiliacs have been found to react immunologically with sera from some patients with viral hepatitis (2). The immunological reactants are an IgG 7S gamma globulin in the hemophiliac serum and an abnormal protein containing a small amount of lipid in the hepatitis serum (3). This abnormal protein has been named Australia antigen, Serum Hepatitis antigen and, more recently, hepatitis-associated antigen (HAA). Because of the need for a reliable diagnostic test for viral hepatitis and a screening test for virus carriers among potential blood donors, studies were initiated to prepare purified HAA, antiserum to HAA (anti-HAA) and to develop sensitive tests for the detection of HAA.

A. Partial Purification of HAA:

HAA was obtained from plasma collected from a patient with serum hepatitis on the twelfth day of disease. Initial characterization of this antigen was carried out using reference antigen and antiserum kindly supplied by Dr. Robert Purcell of NIAID. The complement-fixing (CF) titer of HAA was 1:512.

Separation of HAA from plasma proteins was accomplished by a combination of equilibrium centrifugation in CsCl and rate-zonal centrifugation in sucrose. For the initial step, 5 ml of plasma was layered over a 25 ml pre-formed CsCl gradient with a density range of 1.112 to 1.334. After centrifuging at 25,000 rpm for 18 hours in a Beckman SW 25.1 rotor, fractions were collected dropwise from the bottom of each tube. HAA was regularly detected by CF in fractions with densities from 1.512 to 1.207 with the highest titers at densities from 1.168 to 1.202. HAA positive fractions were pooled and adjusted to a density of 1.203 with CsCl and centrifuged at 39,000 rpm for 40 hours in a Beckman SW 39 rotor. HAA was concentrated in a visible band between densities 1.184 and 1.201 (Figure 7). Initially, these fractions were collected from the bottom. Later, they were collected from the top to avoid mixing the HAA with higher density normal serum proteins.

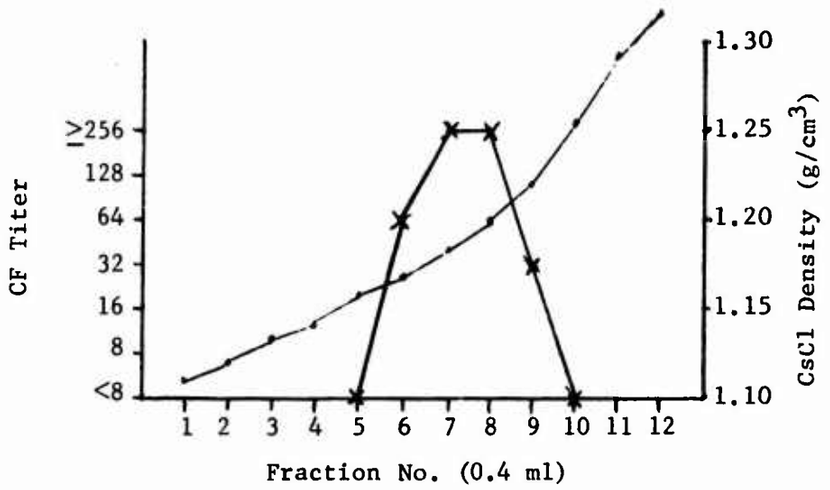


Figure 7. Distribution of HAA after isopycnic banding in a CsCl gradient. Antigen containing fractions collected after the first centrifugation were pooled, adjusted to a density 1.203 and centrifuged at 39,000 rpm for 40 hr in a Beckman SW 39 rotor. Fractions were collected from the top. CF titer is the reciprocal of the antigen dilution.

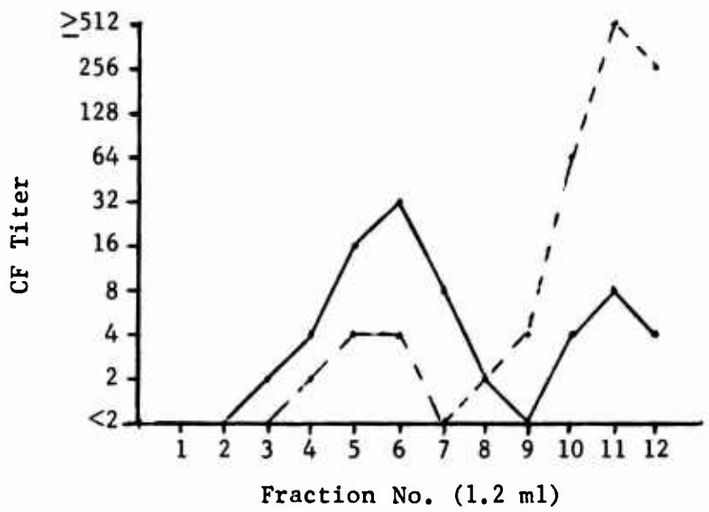


Figure 8. Distribution of HAA (solid line) and normal serum proteins (dashed line) in a 5-25% sucrose gradient following centrifugation at 50,000 rpm for 100 min in a Beckman SW 50 rotor. Fractions were collected from the bottom. CF titer is the reciprocal of the antigen dilution.

This HAA positive banded material was dialyzed in a 0.02 M Tris buffer solution and 0.25 ml was layered over 4.4 ml of a 5-25% sucrose gradient. Following centrifugation at 50,000 rpm for 100 min in a Beckman SW-50 rotor, 12 fractions were collected from the bottom of each tube. One fraction which reacted with anti-HAA by agar gel diffusion (AGD) was scanned under an electron microscope. Electron dense spherical particles of nearly uniform size and shape were seen. The mean diameter was approximately 24 nm. These particles were similar to those previously described as having HAA activity (4). Sucrose fractions containing HAA prepared in this manner were used as immunizing antigens fro producing hyperimmune antiserum in guinea pigs and rabbits.

Rabbit anti-human serum was prepared in order to detect normal proteins which remain in preparations of purified HAA. Rabbits were immunized with a pool of 1.0 ml aliquots of 14 HAA-negative sera from healthy adults. To the pool was added 0.5 ml of purified IgM and 0.8 ml of purified IgA obtained from two multiple myeloma sera by zone electrophoresis. This combined pool was diluted 1:2 with Freund's Incomplete Adjuvant and 2.0 ml of emulsion injected intradermally into each of four areas on the back of each animal. A booster dose of 1.0 ml into each area was given on the 25th day. Serum collected at six weeks had a CF titer of 1:3200 against human serum and was anticomplementary at 1:100. A dilution of 1:200 (16 antibody units) reacted with normal serum diluted from 1:64 to 1:262,144. This serum was used in AGD, CF and immunoelectrophoresis tests.

As a measure of the degree of HAA purification, the guinea pig antisera were tested for antibody against normal human serum proteins as well as anti-HAA. The antisera were found to have antibodies to human IgG by immunoelectrophoresis. The individual sucrose fractions were then tested by complement-fixation for normal serum protein using rabbit anti-human serum (Figure 8). The low concentrations of normal protein present in the HAA-positive fractions would not have been detected if only precipitin methods had been used.

In an attempt to accomplish complete separation of normal proteins from HAA, subsequent preparations have utilized a zone electrophoresis of HAA containing plasma in a Pevacon-Geon block before centrifugation. This eliminates all detectable IgG, but seems to reduce the HAA yield as well. Results of immunization with this material are incomplete.

B. Production of Hyperimmune Antiserum to HAA:

In an attempt to find a source of human anti-HAA, ten hemophiliacs were screened. Three had anti-HAA by AGD. Only one was available for repeated phlebotomy. His CF titer of anti-HAA fell from 1:4 to <1:2 by the time plasmapheresis was accomplished.

Hyperimmune animal anti-HAA was produced in guinea pigs and rabbits. Individual fractions collected from sucrose gradients containing partially purified HAA were kept separate. Aliquots of fractions with

low titers of normal serum proteins were diluted 1:2 with Freund's Complete Adjuvant. The initial immunizing dose was 0.25 ml of antigen-adjuvant emulsion intradermally into each of two sites on the back of each animal. An identical booster dose was given after four weeks. CF antibody titers of serum collected at 6 and 7 weeks from guinea pigs immunized with fractions shown in Figure 8 are given in Table 18. Because of the tendency for a rapid fall in antibody titer between the 6th and 7th week and insufficient antigen to permit a second booster dose, subsequent animals are sacrificed at six weeks. As stated above, the guinea pigs antiserum was found to contain antibodies to normal IgG by immunoelectrophoresis. This is represented in Table 18 by the antibody titers to normal human serum. In an attempt to determine if this antiserum could be used for detecting HAA without prior adsorption of the anti-NHuSP, block titrations were performed against HAA-positive plasma and HAA-negative serum. It was found that those guinea pig antisera with relatively high titers of anti-HAA ($\geq$1:1024) and low titers of anti-NHuSP (1:2) could be used for detecting HAA in CF tests. This was possible because the optimal dilution of antiserum for detecting HAA would not react with any dilution of normal serum protein from 1:2 to 1:8196. Thus, in routine tests of patient sera, the normal proteins are in relative antigen excess in relation to the anti-HuSP and the antiserum acts as though it is monospecific for HAA.

Rabbit anti-HAA was prepared in a similar manner except booster doses were given at four weeks and ten weeks. Although the anti-HAA CF titer was only 1:128, undiluted rabbit serum gave distinct monospecific precipitin lines in routine AGD screening tests.

C. Methods of Detecting HAA and Anti-HAA:

High concentrations of HAA or anti-HAA can be detected by AGD using the micro-Ouchterlony technique. Glass microscope slides, 3 x 1 inches, were covered with 3 ml of 1% agarose (L'Industrie Biologique, France) made up in 0.1 M Tris-EDTA buffer, pH 7.9-8.0, with 1:10,000 Thimerasol added for a preservative. Patterns of seven 3 mm diameter wells were punched so that each serum to be tested could diffuse against a HAA-positive plasma control and an anti-HAA serum. Thus, sera can be tested for antigen and antibody simultaneously. Wells are filled with untreated serum using 90 mm long, 0.8 mm internal diameter glass capillary tubes. Slides are incubated in moist chambers at room temperature for 18-24 hours before reading.

CF tests are performed with microtiter equipment. Sera to be tested are diluted in Triethanolamine buffered salt solution with 0.025 ml claw-shaped diluters. Sera are tested against four units of guinea pig anti-HAA or four units of HAA-positive human plasma. Testing for human serum protein requires 16 units of rabbit antiserum. Fresh guinea pig complement and sensitized sheep RBC are prepared daily by the Department of Serology, WRAIR. Complement is standardized to give five 50% hemolytic units per 0.3 ml solution. Each microtiter test well contains 0.025 ml each of diluted test serum, antiserum and complement plus 0.05 ml of sensitized sheep RBC. When standardizing reagent

Table 18. Representative CF Antibody Titers of Guinea Pigs Immunized with Partially Purified HAA in Individual Sucrose Gradient Fractions.

CF Antigen	Week Post Immunization	Fraction No. 1	2	3	4	5	6	7	8
HAA Positive Plasma	6	32*	256	512	≥2048	≥2048	1024	≥2048	4
	7	64	256	128	256	512	256	≥2048	N.T.
Normal Human Serum	6	<2	<2	2	2	8	2	<2	16
	7	<2	<2	<2	<2	8	2	2	N.T.

N.T. - Not Tested.

* Reciprocal CF antibody titer vs. optimal antigen dilution.

HAA plasma, antisera, or testing gradient fractions for antigen, all dilutions are made by pipette. When testing sera for HAA, the CF test is more sensitive than AGD (Table 19).

Table 19. The Sensitivity of Agar Gel Diffusion for Detecting HAA Compared to the CF Titers of 126 Sera.

CF Titer of HAA	Total No. Sera Tested	Agar Gel Diffusion	
		No. Positive	% Positive
≥1:256	34	34	100
1:128	16	15	94
1:64	5	2	40
1:32	21	6	29
1:16	12	2	17
≤1:8	38	0	0

D. Detection of HAA in Clinical Specimens:

Most sera received for testing for HAA were screened by both AGD and CF. CF screening tests required diluting the test serum to at least 1:16 since antigen excess sometimes prevents complement-fixation at lower dilutions. Anti-complementary activity has been observed in only a small number of fresh sera.

The frequency with which HAA was detected in random cases of acute hepatitis bore little relationship to the clinical diagnosis of serum or infectious hepatitis (Table 20). Such clinical diagnoses are notoriously inaccurate for differentiating the presumed two common types of viral hepatitis because of erroneous historical data. Furthermore, either virus may be transmitted orally as well as parenterally. However, in a carefully investigated epidemic of cholestatic hepatitis attributed to oral transmission which occurred at Fort Sam Houston, Texas in 1964, HAA was not found in any of the 15 patients tested. This is consistent with other reports of the very low incidence of HAA detection in epidemics of hepatitis. The low incidence of HAA in military personnel with acute hepatitis in Korea cannot be explained until further background information regarding these patients is available.

Table 21 shows that HAA was not detected in anyone without clinical hepatitis except for one patient with acute myelogeneous leukemia and one with chronic active hepatitis. At autopsy, the patient with leukemia

Table 20. Summary of Findings in 433 Patients with Acute Viral Hepatitis

Referral Diagnosis	No. Tested	No. HAA Positive	% HAA Positive
"Serum Hepatitis"	78	28	36
"Infectious Hepatitis"	54	18	33
Unspecified Hepatitis	41	13	32
Epidemic Cholestatic Hepatitis (FSH, Texas 1964)	15	0	0
Unspecified Hepatitis (Korea 1969-1970)	245	25	10.2
TOTAL	433	84	19.4

showed massive hepatic necrosis considered compatible with hepatitis. Blood collected at the time contained HAA. The man was suspected of parenteral self-administration of drugs. The patient who was thought to have chronic active hepatitis had HAA in serum collected 11 days prior to liver biopsy. The biopsy revealed post-necrotic cirrhosis. There was no history of blood transfusions or drug abuse. It appears even from such unreliable information as clinical diagnoses may sometimes be that HAA is quite specific for viral hepatitis.

The duration of antigenemia has been observed to range from less than 12 days to 153 days after the onset of symptoms in patients with serial serum samples. Four out of six patients were still HAA positive over six weeks after the onset of illness.

Generally, the CF titer of antigen closely parallels the abnormal serum transaminase and bilirubin levels. This is illustrated by the patient shown in Figure 9. Plasma from this patient, who had serum hepatitis acquired through drug abuse, has been the principal source of HAA for the partial purification procedures described above. Of 27 HAA-positive patients with serial serum determinations, four have demonstrated SGOT values of less than 100 u while maintaining antigenemia. Thus, the potential for transmission of HAA from blood of patients who have had hepatitis may persist beyond the period of abnormal liver function.

Although the incidence of HAA carriers in apparently healthy Americans is reported to be 0.1% (5), it has been found that asymptomatic carrier states may exist among contacts of hepatitis patients. An

Table 21. Summary of Findings in 133 Patients with Diagnoses Other Than Acute Hepatitis and 29 Normals.

	No. Tested	No. HAA Positive
Miscellaneous Liver Diseases		
Persistent Hepatitis	2	0
Chronic Active Hepatitis	9	1
Granulomatous Hepatitis	1	0
Halothane Hepatitis	4	0
Alcoholic Liver Disease	4	0
Cirrhosis	3	0
Reye's Syndrome	1	0
Infections		
Infectious Mononucleosis	3	0
Amebic Liver Disease	5	0
Malaria	4	0
Dengue	3	0
Toxoplasmosis	1	0
Influenza	1	0
Malignancies		
Leukemia: AML (2) ALL (1)	3	1
Lymphomas	3	0
Other Malignancies	3	0
Other Diseases		
Non-malignant Primary GI Disorders	8	0
Non-malignant Primary Hematologic Disorders	12	0
Renal Failure	3	0
Diabetes	1	0
Congestive Heart Failure	1	0
Lupus Erythematosis	2	0
Encephalitis	1	0
Drug Addiction	1	0
Upper Respiratory Infection	1	0
Past History of Hepatitis	11	0
Thrombophlebitis	1	0
Reiter's Syndrome	1	0
Diagnosis Unknown	40	6
Normal	29	0
TOTAL	162	8

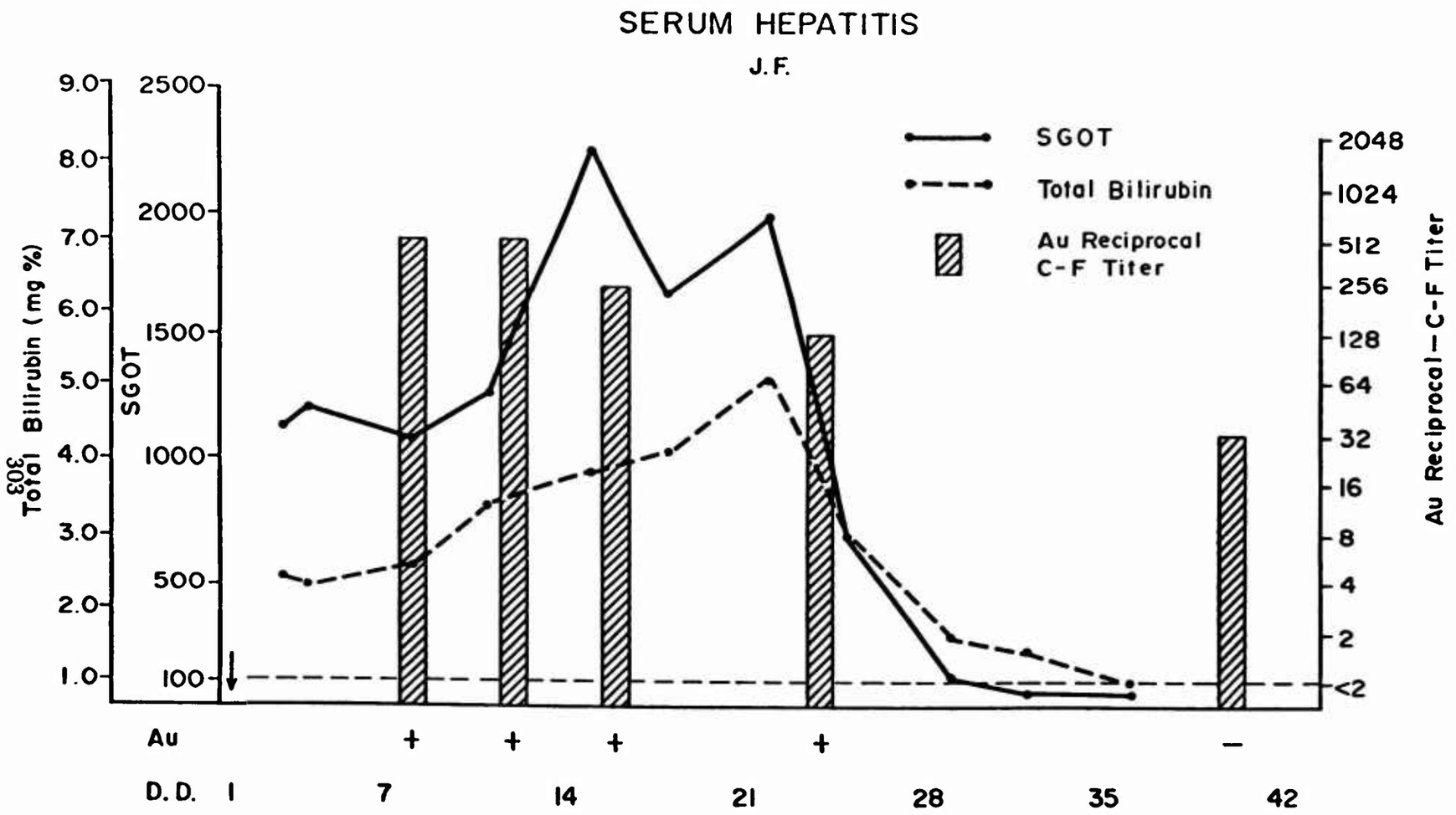


Figure 9. The relationship of the serum levels of HAA to abnormal liver function tests in a patient with serum hepatitis. Au refers to Australia antigen (HAA) D.D. represents day of disease.

infant who developed clinical hepatitis with jaundice and loose stools at age 3 months was found to have an HAA CF titer of 1:2 at age 6 months when his serum transaminase and bilirubin levels were waning. None of the four other family members reported having symptoms of hepatitis or known exposure outside the family. Nevertheless, serial serum samples have shown the mother and two sisters to be carriers of HAA while maintaining normal serum transaminase and bilirubin values for up to four weeks. One sibling has shown an unexplained elevation of her serum alkaline phosphatase. The father is also well and is HAA-negative. It is possible these three carriers may yet develop evidence of hepatitis or may be in a recovery period. However, the presence of 3 HAA carriers in the same family with apparently normal liver function is quite exceptional.

Since certain subhuman primates have been shown to carry HAA, interest has arisen in using monkeys for studying transmission of the agent. At the request of the National Center for Primate Biology, Davis, California, 85 primates representing 10 different species were tested for HAA by AGD and CF. All were negative. Low-titered anti-complementary (AC) activity was found in serum of 9/12 Pig-tailed Macaques, 3/11 Rhesus and 1/10 Cynomologous Monkeys. Sera from Stump-tail and Bonnet Macaques, Indian langurs, Mangabees, African Green monkeys and two species of baboons were not AC.

V. Studies of Dengue Viruses.

A. Molecular Size and Charge Relationships of the Soluble Complement-Fixing Antigens of Dengue Viruses:

The four closely related dengue virus serotypes can be distinguished on the basis of complement-fixation, neutralization, or hemagglutination-inhibition. The immunological separation suggests that biochemical and biophysical differences also exist among antigens of the four serotypes. Recently, a sensitive new approach to separation of protein "size and charge isomers" has been developed using disc gel electrophoresis in a series of polyacrylamide concentrations (6). When this method was applied to the soluble complement-fixing (SCF) antigens of each dengue serotype obtained from dengue-infected mouse brain, they were found to have similar molecular sizes but different electrophoretic mobilities.

To prepare dengue antigens, suckling mice were injected intracerebrally with 10^4 LD_{50} of the appropriate dengue serotype virus. Mice were sacrificed when moribund and their brains were homogenized (20% w/v) in 0.02 M Tris-HCl buffer at pH 7.2 in a Sorvall Omnimixer. Initial removal of excess brain tissue was achieved by precipitation with 2 mg/ml protamine sulfate, followed by centrifugation at 9000 x g; recentrifugation of the supernatant at 78,000 x g for 3 hours removed all detectable hemagglutinating viral antigens. The SCF antigen was then precipitated from the resulting ultracentrifuge supernatant with 60% ammonium sulfate. The SCF antigen was resuspended in Tris-HCl buffer

and applied to a 5 x 80 cm Sephadex G-100 column. Filtration was carried out in 0.02 M phosphate buffered saline at pH 7.2. Each column was standardized with bovine serum albumin, ovalbumin, and blue dextran. Fractions containing the SCF antigens were pooled and concentrated 50-fold by pressure dialysis.

Acrylamide monomer and N,N'-methylene-bis-acrylamide were each dissolved in acetone at 50°C, filtered, and recrystallized at -20°C. The acetone was decanted and the acrylamides were air dried. Stock solutions of acrylamide of concentrations varying between 4 and 16% were formulated according to the discontinuous system (6), so that stacking took place at pH 7.3 and separation at pH 8.5. Individual SCF antigens or mixed pairs were run in each gel concentration. At the completion of each run the gels were either quick-frozen in cold acetone and stored at -4°C until fractionation or were directly removed from the column tube and cut into 1 mm slices using a transverse gel slicer. Each slice was eluted in a test tube with normal saline and analyzed for the presence or absence of SCF antigens using a modified microtiter technique. Chymotrypsinogen, pepsin, ovalbumin, and bovine serum albumin, obtained from commercial sources, were used to establish slope values for a standard slope-molecular weight graph. Sufficient material was used during standardization runs to identify the protein after staining with naphthol blue black.

The peak concentration of each protein or peak activity of each antigen was determined and the log mobility relative to the bromphenol blue dye front was calculated. The slopes, y-intercepts (zero concentration polyacrylamide), and linear correlation coefficients were calculated for each experiment. All experiments described herein had linear correlation coefficients of greater than 0.990.

When the mobility of the antigen relative to the dye front was plotted against the concentration of polyacrylamide on a semi-log graph, a straight line was obtained (Figure 10). This linear relationship between log mobility and gel concentration (7) is independent of absolute mobility and provides a negative slope. The slope of any given protein is a function of the molecular size of that protein and, empirically, its molecular weight. The mean slopes of the four dengue SCF antigens ranged between 4.789 and 4.917, with an average slope of 4.860. This range is well within the reliability of the method and it is, therefore, unlikely that the molecular sizes of the four dengue antigens are significantly different. When these points are placed on the slope-molecular weight graph (Figure 11) based on the slope-molecular weight relationships of chymotrypsinogen, bovine serum albumin, ovalbumin, and pepsin monomer, the molecular weight estimates for the SCF antigens range from 38,000 to 40,000 with the average falling at 39,000.

Each of the four serotypes of dengue SCF antigens eluted from the Sephadex G-100 columns slightly behind ovalbumin (Figure 12). Using Determann's formula for gel filtration (Log M = 5.941 - 0.847 [Ve/Vo]), a molecular weight between 38,000 and 40,000 was estimated for each SCF antigen in comparison to ovalbumin. This corroborates the molecular weight estimates obtained by the Ferguson plots.

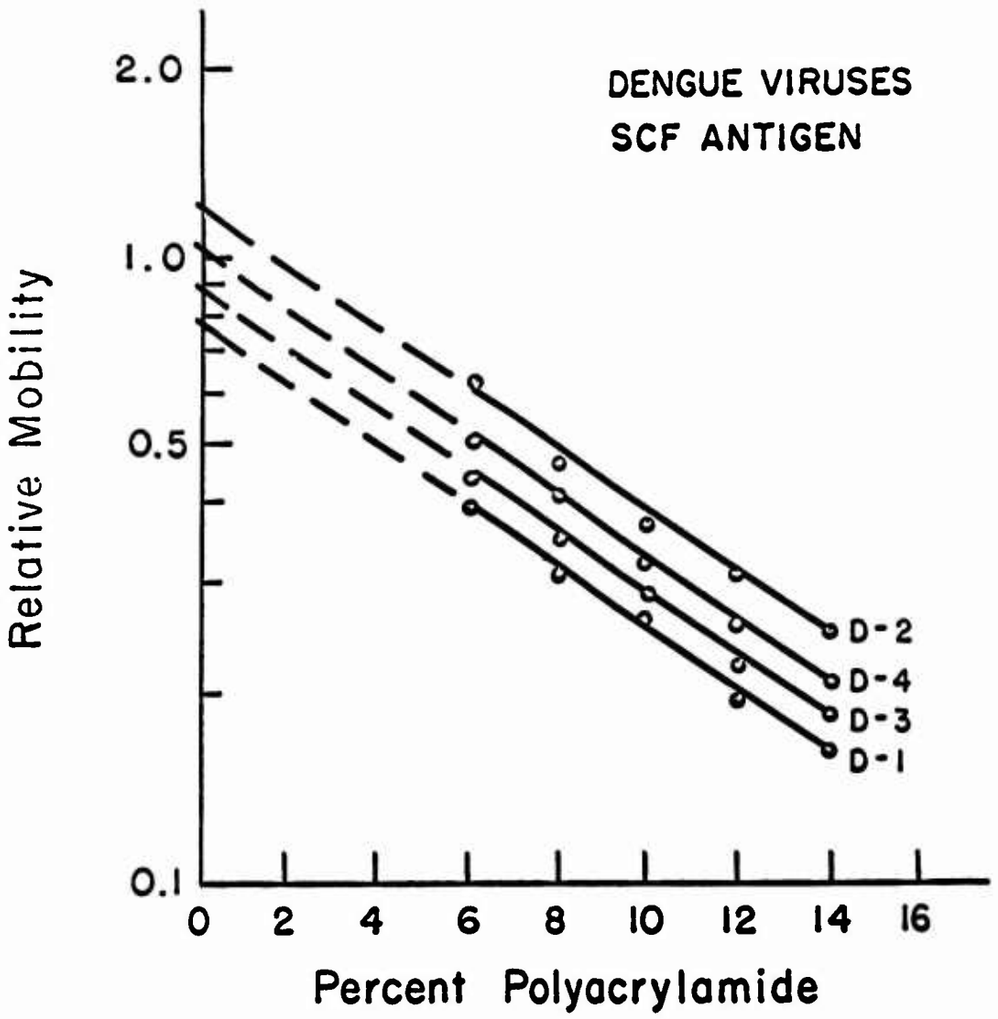
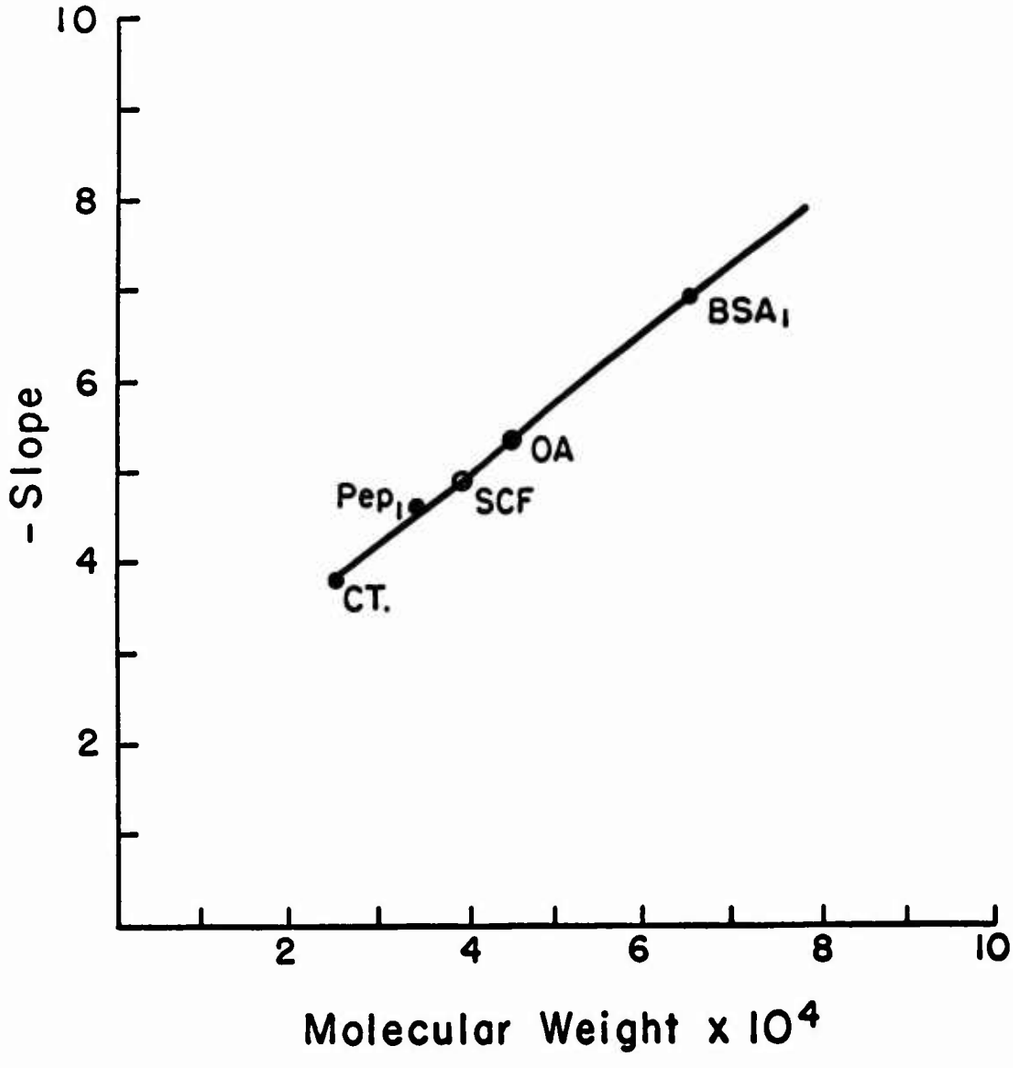


Figure 10. The effects of different polyacrylamide concentrations on the relative mobility of the SCF antigens of each of the four dengue virus serotypes. D-1, D-2, D-3, and D-4 designate the position of the respective dengue SCF antigens.

Figure 11. The negative slope-molecular weight relationship of chymotrypsinogen (CHT), pepsin monomer (PEP_1), ovalbumin (OA), and bovine serum albumin monomer (BSA_1). Relative position of dengue SCF antigens (SCF) is designated by an open circle.



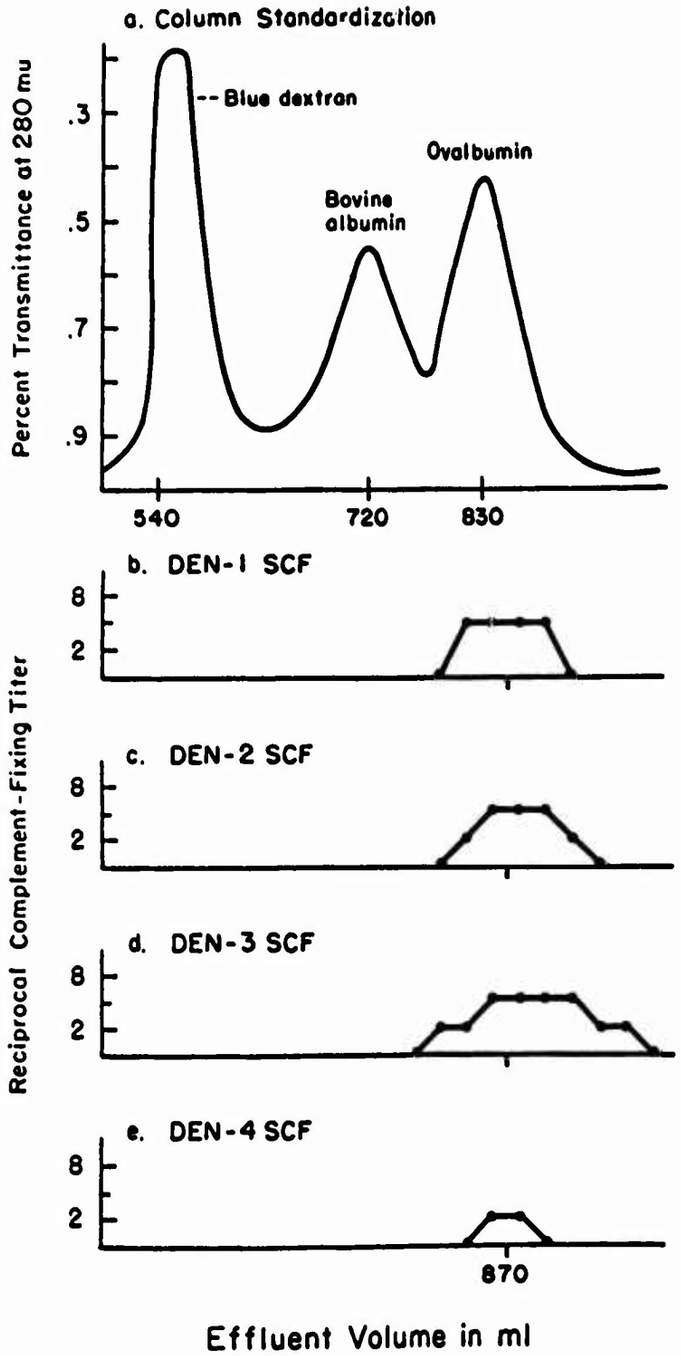


Figure 12. A comparison of standard macromolecules with dengue SCF antigens on Sephadex G-100.

Disc gel electrophoresis not only separates proteins on the basis of size but also on the basis of net charge. The Ferguson plot gives an estimate for the relative free solution electrophoretic mobility by extrapolation of the plot line for any protein to zero concentration gel (y-intercept). Under these experimental conditions, closely related proteins exhibit one of several plot patterns. Plots of "size isomers," such as the monomer, dimer, and trimer of bovine serum albumin, give lines with different slopes which intersect close to zero concentration gel. Plots of "charge isomers," such as the lactic acid dehydrogenase isozymes, give parallel lines with the same slope and, because of their different electrophoretic mobilities, different y-intercepts. Plots of unrelated proteins, on the other hand, tend to have different slopes, different y-intercepts, and intersect at concentrations of gel other than zero per cent polyacrylamide.

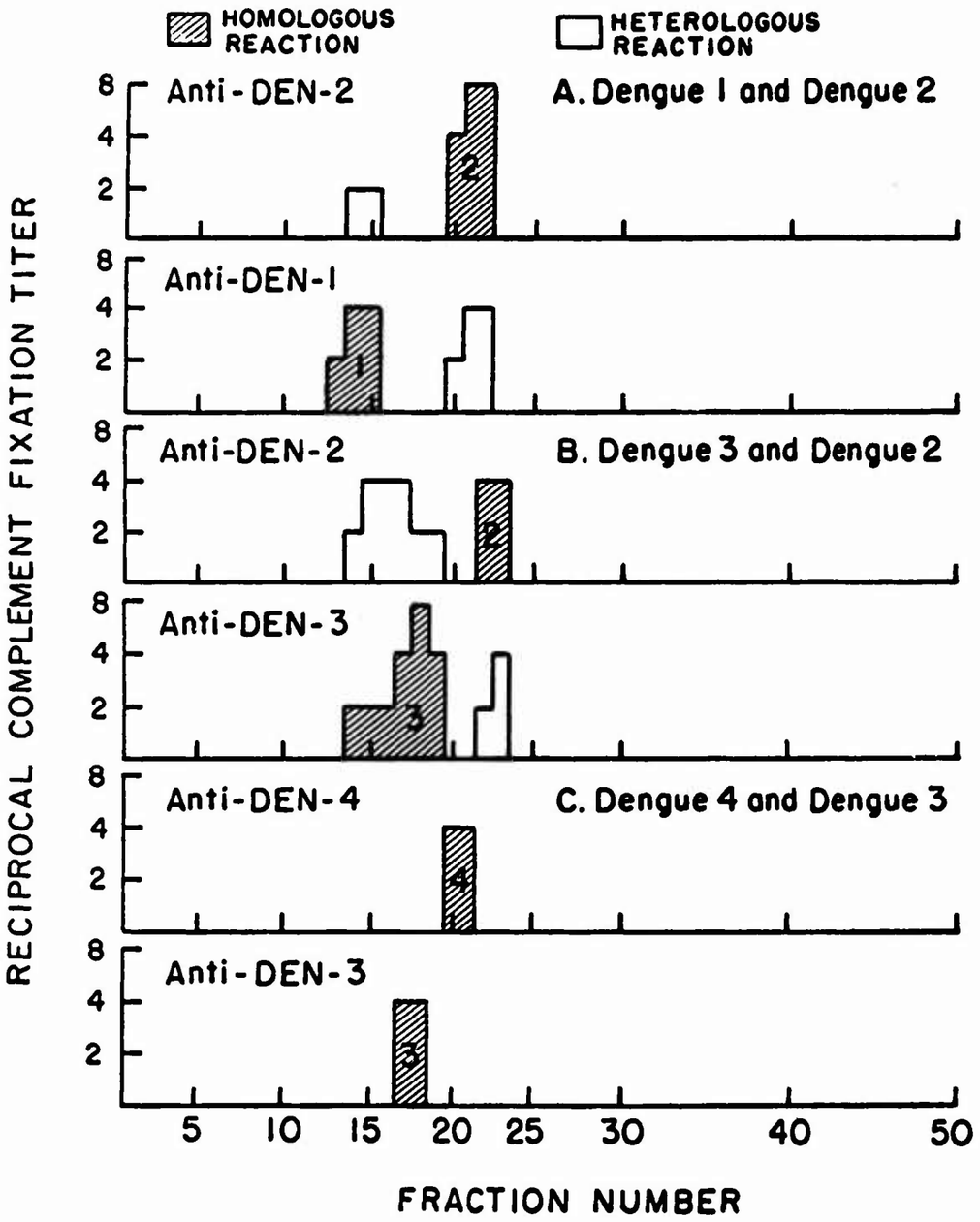
A Ferguson plot of all four SCF antigens gives four straight lines with different y-intercepts (Figure 10). The relative electrophoretic mobilities were, in descending order: dengue type 2, with a y-intercept of 1.122; dengue type 4, with a y-intercept of 1.001; dengue type 3, with a y-intercept of 0.866; and dengue type 1, with a y-intercept of 0.793.

Although such an analogy is not completely accurate, the four dengue SCF antigens resemble "charge isomers" of some enzyme families in that they have similar molecular sizes but different net charges. As would be predicted from such a relationship, the antigens were separable from each other when run in pairs in the same gel (Figure 13). The degree of separation of the different antigens in the co-runs corresponded to that which would be predicted on the basis of their relative y-intercepts (Figure 13).

The differences in SCF antigen net charges, as expressed by their relative y-intercepts, probably reflect differences in their respective amino acid sequences and/or configurations. In a like manner, the type-specific component of the serological reactions indicate that type-specific antigenic determinants exist. We do not, at this time, know the magnitude or the nature of these immunological and biophysical differences. As compared to the charge separations of enzyme "charge isomers," however, the charge difference between dengue SCF antigens appears to be small. On the other hand, the strong group cross-reactions (Figure 13), coupled with the similarities in molecular size and limited range of net charge (Figure 10), suggest that major portions of the dengue SCF molecule are common to all four serotypes.

As is the case with many arboviruses, the high degree of immunological cross-reactivity existing between the dengue serotypes make identification and classification a major problem. The techniques presented in this report allow, for the first time, definitive separation of these dengue serotype antigens on other than immunological basis. The ability to discriminate between serotypes on a biophysical basis may help to eliminate many of the ambiguities introduced by serological techniques.

Figure 13. Relative positions of SCF antigens in three separate coruns in 8% acrylamide, origin at the left.



It is possible that this sensitive approach to protein separation will be applicable to unresolved problems among other immunologically related viruses.

B. Immunoprecipitin Analysis of Dengue Soluble Complement-Fixing Antigens:

The basis of the observed antigenic interrelationships between dengue viruses is of considerable interest for taxonomic reasons and because of the suspected relationship of anamnestic antibody responses to the pathogenesis of the dengue shock syndrome. The anamnestic responses observed following a second dengue infection are presumably due to antigen-sharing between dengue serotypes. The dengue viruses have been shown to have at least three structural polypeptides in the virion and, in addition, at least three virus-specific non-structural proteins are thought to be produced during replication of group B arboviruses. Thus, there is a probability that several independent antigen-antibody systems exist.

Immunoprecipitation methods have demonstrated the presence of several antigenic components in crude antigens of group B arboviruses and such methods have been used by others for comparison and typing of dengue viruses and other group B arboviruses. However, use of unpurified antigens and hyperimmune sera results in the formation of a variable number of precipitin lines which are difficult to interpret. The inability to accurately quantify any single antigenic determinant and corresponding antibody limits the value of immunoprecipitation with unpurified antigens.

The finding and characterization of a soluble complement-fixation (SCF) antigen in dengue infected tissues and subsequent development of methods for purification of SCF antigens of each serotype allowed immunoprecipitin analysis to be carried out using a highly purified antigenic component. The biophysical characterization of these SCF antigens, described above, provided a unique opportunity to correlate physical differences between SCF antigens of the four serotypes with antigenic differences.

1. Methods: The antibody source for all serologic tests was hyperimmune ascitic fluids prepared in adult female mice. The soluble complement-fixation antigens were prepared from each strain of dengue virus by methods previously described. Briefly, 1-2 day old suckling mice were inoculated intracerebrally and virus-infected brains were harvested when the mice became moribund. A 20% suspension of infected suckling mouse brain was made in .02 M Tris-HCl pH 8.2 and protamine sulfate was added to final concentration of 2 mg/ml. The resulting precipitate was sedimented by centrifugation (9000 x g, 30 min) and discarded. Ammonium sulfate was then added to a final concentration of 60% saturation. The resulting antigen-containing precipitate was sedimented by centrifugation (9000 x g, 60 min) and redissolved in Tris-HCl buffer effecting a 10 X concentration. The antigen was applied to 5 x 80 cm column of Sephadex G-100 and eluted in phosphate buffered

saline. Fractions containing SCF antigen were concentrated by pressure filtration. A control antigen was made in a similar manner from uninfected mouse brains.

Crude antigens were prepared by making a 20% suspension of infected suckling mouse brain in normal saline, allowing the suspension to remain at 4°C for 24 hours and then clarifying by centrifugation at 9000 x g for 30 minutes.

Ouchterlony plates were prepared in 8.5 cm diameter plastic petri dishes using 1.0% agarose in 0.2 M Tris buffered saline, pH 8.2. Agar was poured to a depth of 3 mm; six mm diameter wells were cut with 12 mm center to center distance. When filled, each well held approximately 0.1 ml of reagent. Prior to use in immunodiffusion tests, ascitic fluids were diluted to a uniform CF titer of 1:128, a 1:2 to 1:4 dilution in most instances. Antigens were similarly standardized to a CF titer of 1:256. For absorption experiments undiluted SCF antigens were mixed in equal proportions with undiluted ascitic fluid and held at 4°C for 18 hours. Precipitates were removed by centrifugation prior to using the adsorbed ascitic fluids in gel diffusion experiments.

2. Results: The hyperimmune ascitic fluids had complement-fixing antibody titers of 1:512 to 1:1024 with homologous crude antigens. No reaction with control mouse brain antigens was observed. As seen in Figure 14, a significant reaction between each ascitic fluid and each heterologous crude antigen was observed. The CF antibody titers of the same ascitic fluids were two- to four-fold lower when tested against homologous purified SCF antigens (Figure 15) and an even greater difference in heterologous titers was noted. The dengue-4 ascitic fluid appeared specific when tested against SCF antigens, not reacting with heterologous SCF antigen preparations. Similarly, the dengue-3 ascitic fluid gave a much more specific pattern when tested against the purified antigens. Dengue-1 and dengue-2 ascitic fluids, however, exhibited a smaller difference in specificity between the crude antigens and the purified SCF antigens. The difference in the shape of the grids is striking. The SCF antigens usually produced a "post-zone" inhibition of complement-fixation in regions of relative antigen excess. With the crude saline antigens, however, inhibition due to antigen excess was noted only in the dengue-1 homologous reaction. In all other instances maximum antibody titers occurred with the highest concentration of crude antigen.

The SCF antigens of prototype strains of dengue-1, 2 and 4 gave a single line when tested by the immunoprecipitin method with homologous and heterologous ascitic fluids. The SCF antigen preparations of dengue-3 produced a faint but unmistakable second line in addition to the major precipitin line of the SCF antigen.

The relationship observed between dengue-1 and dengue-2 SCF antigens by immunoprecipitation is depicted in Figure 16-19. When antigen was placed in the center well and antibody in the peripheral wells, the precipitin lines fused, forming a line of identity between both homologous

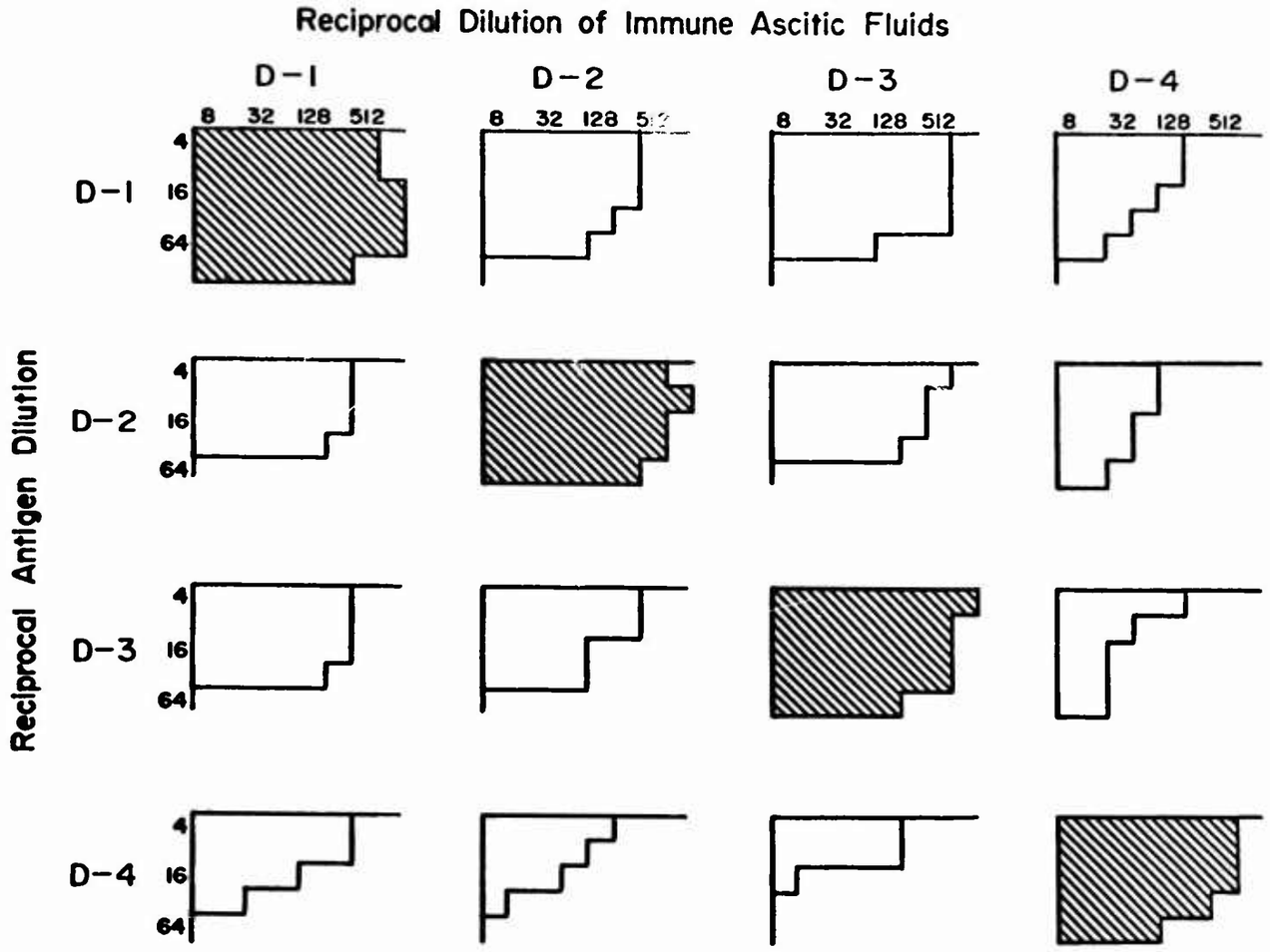


Figure 14. Reactions of crude complement-fixing antigens of dengue viruses with homologous and heterologous antibody in hyperimmune mouse ascitic fluid.

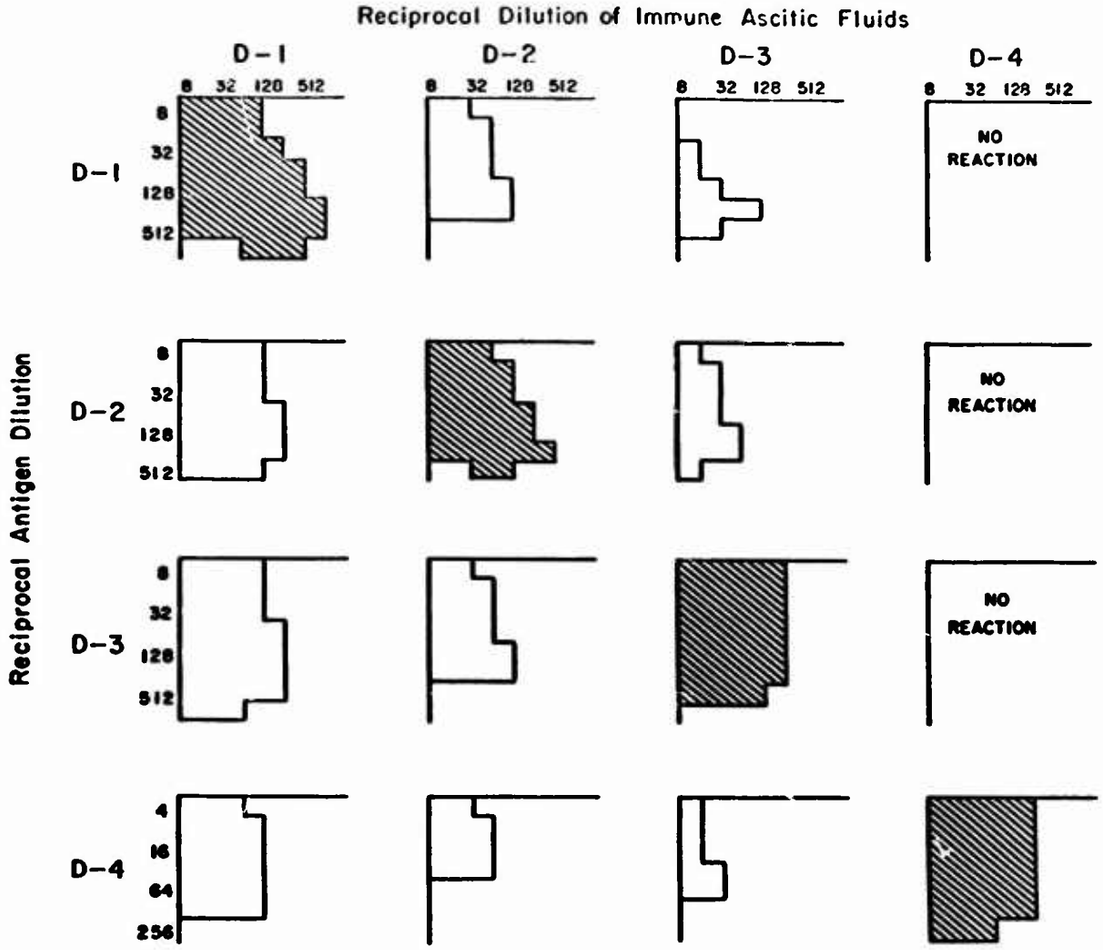


Figure 15. Reactions of sephadex purified soluble complement-fixing antigens of dengue viruses with homologous and heterologous antibody in hyperimmune mouse ascitic fluid.

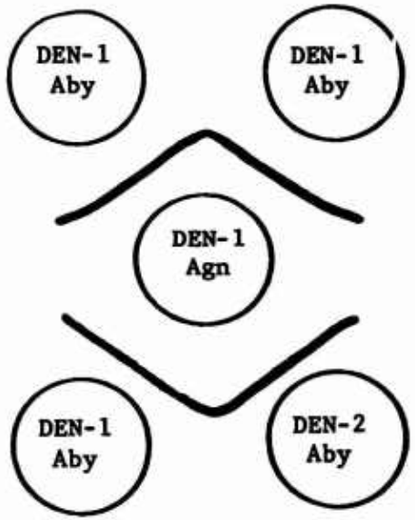


Figure 16. Relationship between dengue-1 SCF antigen (center well) and dengue-1 antibody (upper wells) and dengue-1 and dengue-2 antibody (lower wells). Note fusion of precipitin lines between homologous and heterologous antibody wells.

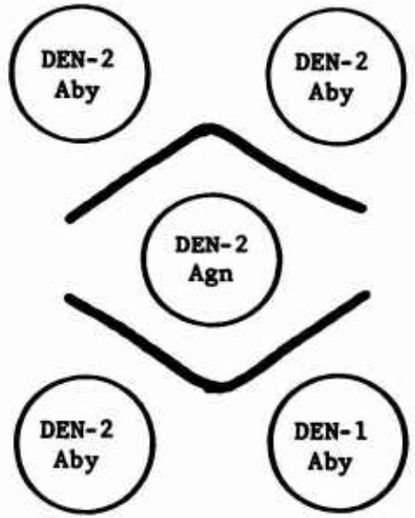


Figure 17. Relationship between dengue-2 SCF antigen (center well) and dengue-2 antibody (upper wells) and dengue-1 and dengue-2 antibody (lower wells). Note fusion of precipitin line between both homologous and heterologous antibody wells.

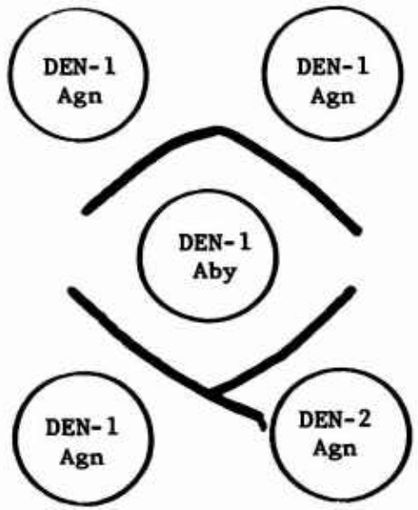


Figure 18. Relationship between dengue-1 antibody (center well) and dengue-1 SCF antigen (upper wells) and dengue-1 and dengue-2 SCF antigens (lower wells). Precipitin lines fuse between homologous antigen wells, whereas a spur is visible between heterologous antigen wells.

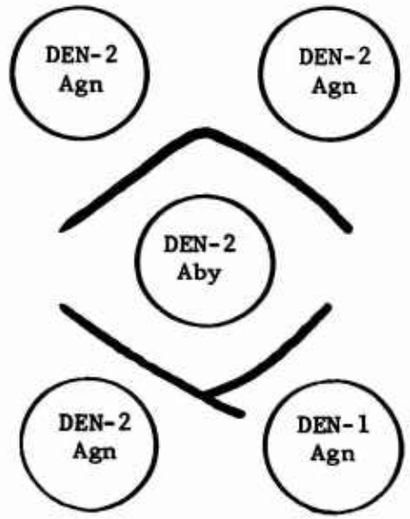


Figure 19. Relationship between dengue-2 antibody (center well) and dengue-2 SCF antigen (upper wells) and dengue-2 and dengue-1 SCF antigens (lower wells). Precipitin lines fuse between homologous antigen wells, whereas a spur is visible between heterologous antigen wells.

and heterologous antibody sources (Figures 16 and 17). However, when antibody was placed in the center well with antigen in the peripheral wells, a line of identity formed between adjacent homologous antigens, whereas, formation of a distinct spur was seen with heterologous antigens in adjacent wells, indicating partial identity (Figures 18 and 19). When dengue-1 and dengue-2 ascitic fluids were tested against homologous and heterologous pairs of all the dengue SCF antigens, a spur of partial identity was seen with each pair (Figures 20 and 21). This basic relationship was also true for dengue-3 and dengue-4. With dengue-3 or 4 antigen in the center well, homologous and heterologous antibody in adjacent outer wells formed lines of identity. Conversely, dengue-3 antibody in the center well resulted in spur formation when heterologous antigens were placed in wells adjacent to the dengue-3 antigen. The dengue-4 ascitic fluid, however, formed a precipitin line only with homologous antigen.

The hypothesis that a common identical antigenic determinant is present on the SCF antigen of each serotype was tested by diffusing heterologous antigens in pairs against ascitic fluid of a third serotype. Lines of identity formed between pairs of heterologous antigens are shown in Figures 22 and 23.

Dengue-1, dengue-2 and dengue-3 ascitic fluids reacted with all three respective heterologous dengue serotypes but dengue-4 ascitic fluid apparently lacked antibody to the common antigenic determinant. Absorption of dengue-1 ascitic fluid with dengue-2 SCF antigen eliminated the immunoprecipitin reaction of the ascitic fluid with dengue-2, 3, and 4 antigens leaving only homologous antibody. Similarly, absorption of dengue-2 ascitic fluid with dengue-1 antigen removed antibody against dengue-1, 3, and 4 SCF antigens leaving antibody which reacted only with the homologous SCF antigen.

3. Discussion: The ascitic fluids of mice hyperimmunized with antigen preparations known to be heterogeneous contain independent populations of antibodies to several different virus-specific antigenic determinants. Differences in degree of serologic cross-reactivity between serotypes as shown by different serologic methods which assay different antibody populations supports this assumption. Cross-reactivity among the dengue serotypes in serologic tests may result from either the presence of one or more identical antigens possessed in common by each serotype, or from immunologic cross-reactions due to antibodies reacting with analogous antigenic determinants of similar but not identical structure, or a combination of both mechanisms. Lower CF antibody titers were observed when immune ascitic fluids were tested with SCF antigens than when crude saline antigens were used. This may be explained by the presence of an antigenic determinant (or determinants) in the crude saline antigen which is not present in the SCF preparations if the antibody directed against this component is in higher titer than antibodies directed against SCF antigen. In the case of dengue-4 ascitic fluid, heterologous reacting antibodies appeared to react only with antigens other than those present in SCF. In addition to SCF, the two hemagglutinins present in crude suspensions of dengue-2 infectious mouse

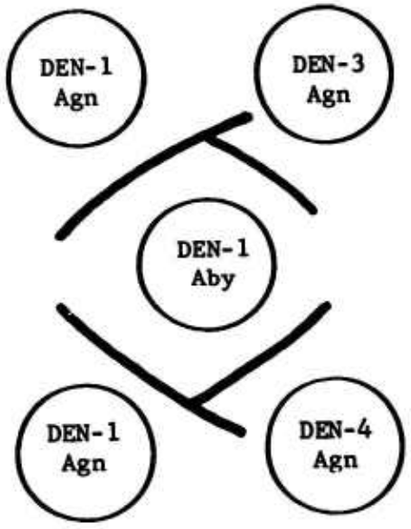


Figure 20. Relationship between dengue-1 antibody (center well) and dengue-1 and dengue-3 SCF antigens (upper wells) and dengue-1 and dengue-4 SCF antigen (lower wells). Spurs are visible between both pairs of antigens.

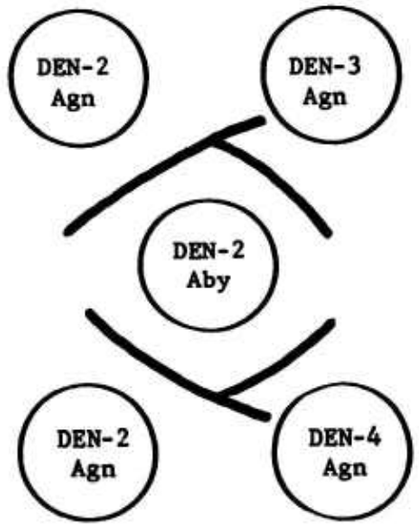


Figure 21. Relationship between dengue-2 antibody (center well) and dengue-2 and dengue-3 SCF antigens (upper wells) and dengue-2 and dengue-4 SCF antigens (lower wells). Spurs are visible between both pairs of antigens.

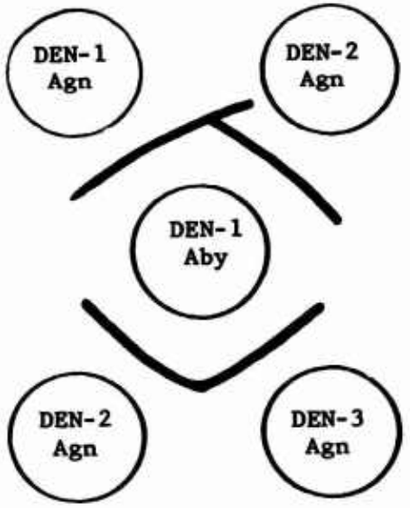


Figure 22. Relationship between dengue-1 antibody (center well) and dengue-1 and dengue-2 SCF antigens (upper wells) and dengue-2 and dengue-3 SCF antigens (lower wells). Fusion of precipitin lines without spur formation occurred between the two heterologous antigen wells.

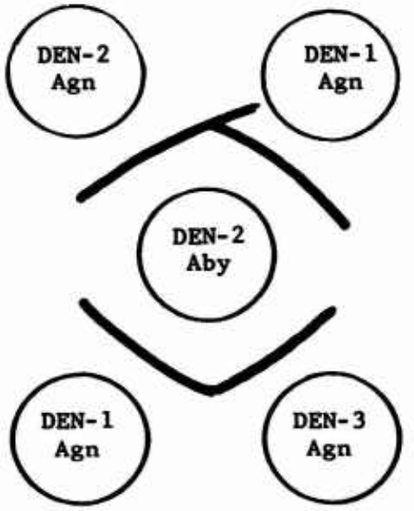


Figure 23. Relationship between dengue-2 antibody (center well) and dengue-2 and dengue-1 SCF antigens (upper wells) and dengue-1 and dengue-3 antigens (lower wells). Fusion of precipitin lines without spur formation occurred between the two heterologous antigen wells.

brain or cell culture have CF reactivity. It appears that one or more of the CF-reactive antigenic determinants present on the hemagglutinins are not present in the SCF antigens. The SCF antigen has been shown to be a nonstructural virus-specific antigen, thus it is not surprising that antibodies directed against SCF are separate from those directed against virion-associated antigens such as the hemagglutinins.

The SCF antigen of each serotype, although of relatively small size, has both group- and serotype-specific activity. SCF antigens of all four serotypes reacted with heterologous ascitic fluids when antigens were placed in the center wells. Fusion of the precipitin lines indicated the common identity of this group-specific antigenic determinant, as did formation of lines of identity with one ascitic fluid versus two heterologous antigens. The absorption experiments proved the presence of the common determinant.

Differentiation between SCF antigens of the four serotypes could only be demonstrated by spur formation when antibody was placed in the center well. This type of reaction, designated as Type IV reaction by Ouchterlony occurs when two antigenic determinants are linked together on the same molecule and separate populations of antibodies are produced against each determinant. Thus, the interpretation most consistent with the results of our immunoprecipitin analysis is that the SCF antigen of each serotype has two determinant groups, one of which is identical to the corresponding site on SCF antigens of the other serotypes; in addition, each has a type-specific antigenic determinant which is unique for the serotype. Serologic cross-reactivity of dengue SCF antigens, therefore, appears to be due to antigen-sharing rather than to true immunologic cross-reactions.

The immunogenicity of the common antigenic determinant on the SCF molecule appeared quite variable between serotypes. Dengue-4 ascitic fluid had little or no antibody directed at the common antigenic determinant of SCF in spite of the demonstrated presence of the common antigenic site on the dengue-4 SCF antigen by both CF and precipitin tests. The intensity of precipitin lines produced by dengue-3 ascitic fluids with common antigens was low compared to dengue-1 and dengue-2 ascitic fluids. Biologic variation may be the most likely explanation since variation of this nature was noted between lots of dengue-2 ascitic fluids. Differences in immunogenicity due to structural differences or differences in relative amounts of SCF antigen in the immunizing antigens are also possibilities.

The demonstration of differences in electrophoretic mobilities between the SCF antigens of the four dengue serotypes lead to the speculation that the structural differences which result in the electrical charge differences are related to the type-specific antigenic determinants.

The biologic role of SCF antigen and antibodies produced against it during infection remains to be evaluated. Since SCF antigen is a nonstructural virus-specific protein, antibodies against SCF do not affect

the virion and presumably can play no role in defense against infection. The failure of antibody against dengue-2 SCF to neutralize dengue-2 virus supports this concept.

SCF antigen is produced in both infected cell cultures and infected suckling mice and has been shown to be present in the blood of infected mice. It may contribute to in vivo antigen-antibody reactions and, thus, be involved in the pathogenesis of disease. The anamnestic antibody response which occurs in second dengue infections has been incriminated in the pathogenesis of the dengue shock syndrome. Because of the group-specific antigenic site on the SCF antigen, an anamnestic immunologic response to this determinant would be expected to occur during a second dengue virus infection and, hence, the SCF antigen may play a role in pathogenesis of the dengue shock syndrome.

The demonstration of a group-specific determinant on the SCF molecule only partially explains the antigenic relationships between dengue viruses since serologic cross reactions observed by tests dependent on virion-associated antigens, such as hemagglutination-inhibition, are unrelated to the SCF system. Similarly, the specificity of the neutralization test is unrelated to the type-specific antigenic determinant on the SCF antigen. Knowledge of the antigenic make-up of the SCF antigen emphasizes the requirement for similar data pertinent to the structural proteins of the virion.

C. Biophysical and Immunological Separation of Dengue-2 Antigens:

Rate-zonal centrifugation of infected tissue in sucrose gradients has led to the definition of two hemagglutinins (HA) which also fix complement (CF) and a soluble complement-fixing (SCF) antigen which does not HA. The most rapidly sedimenting HA (RHA) is the intact virus measuring about 50 nm in diameter and the slowly sedimenting HA (SHA) is a small ring form about 14 nm in diameter. Electron micrographs of SCF contained particles indistinguishable from the capsomeres of the virion initially suggesting that SCF was a part of the virion and could be derived from RHA. However, attempts to derive SCF from RHA by chemical or physical degradation yielded a soluble complement-fixing antigen which differed from SCF by its instability in urea; further, relatively high titers of SCF were found circulating in the blood of dengue-infected mice as compared to infectious virus/SCF ratios in the brain. Since these antigens are found in large quantities in dengue infected cells, they potentially play a significant role in the pathogenesis of dengue-associated diseases, particularly the hemorrhagic shock syndrome. It is, therefore, important to define the relationship of the two non-infectious antigens to the intact virus. The molecular weight of SCF was found to be 39,000 in this laboratory while the reported weights of the virion structural polypeptides were 59,000, 13,500 and 7,700 (8). These data suggested that SCF was a nonstructural antigen. The following experiments test this hypothesis by degrading radioactive virions to their constituent polypeptides and comparing them with SCF. In addition, radioactive RHA and SHA were used in radioimmune precipitation (RIP) tests in an attempt to differentiate all 3 antigens on an immunological basis.

1. Propagation of Radioactive Dengue-2 Virus in LLC-MK2 Cells: Highly radioactive dengue-2 virus was required for these experiments, however, dengue-2 grows slowly to a relatively low titer. Rather than concentrate seed stocks to allow for a high multiplicity of infection (MOI) the experimental conditions were designed using a low MOI and a late harvest time. The growth curve of dengue-2 virus propagated in LLC-MK2 cells under these conditions is illustrated in Figure 24. Using an MOI of 0.13, virus appears in the culture medium about 24 hours after infection and continues to increase in titer until it plateaus at 65 hours. Using this type of virus production, maximal specific activity and minimal host cell contamination was obtained by introducing tritiated amino acids at 48 hours and harvesting the culture medium at 72 hours post infection. The virus was concentrated into a pellet by ultra-centrifugation of the culture medium (M-199, no serum) at 78,000 x g for 3 hours. Sucrose gradient distribution of pelleted tritium-labeled dengue antigens corresponded to the distribution of RHA and SHA (Figure 25). The proportion of labeled SHA varied between preparations as can be seen by the higher number of counts in the SHA peak in Figure 26.

2. Separation of Dengue Virus Structural Polypeptides and SCF Antigen by Polyacrylamide Gel Electrophoresis: SCF has previously been estimated to have a molecular weight of 39,000, yet none of the dengue-2 structural polypeptides reported by Stollar had a molecular size estimate in the same range. To establish that the structural polypeptides degraded from the virion with SLS and the SCF antigen have different sizes, SCF was also treated with SLS to minimize charge differences and co-electrophoresed on the same gel and in parallel gels. Briefly, water washed polyacrylamide (Canalco Prep-Cryl) was recrystallized from acetone and made up to various percentages in 0.1 M phosphate buffer containing 0.1% SLS, 1% bis-acrylamide (cross-linking agent) and 0.28% ammonium persulfate as the chemical catalyst for polymerization. The density of the sample applied to the gel was increased with glycerol to confine it to a small region under the buffer head. Electrophoresis was carried out at 5 milliamps per gel for 14 to 16 hours (Canalco model 900 power unit). Gels were pressed into a block of razor blades held at 1 mm intervals with stainless steel spacers (WRAIR Instrumentation). For co-runs of labelled structural proteins and SCF antigen, each gel slice was cut in half, one portion placed in saline for elution of SCF antigen, the other portion in a scintillation vial. Water (0.05 ml) and NCS (Amersham Searle) (0.5 ml) was added to the scintillation vials and incubated overnight at 60°C. Ten ml of liquiflour-toluene scintillation cocktail was added to the vials, and the samples counted in a Packard Tri-carb Scintillation counter.

RHA had three distinct polypeptides when analyzed in this manner, virus specific proteins (VSP) 1, 2, and 3 (Figure 27); the smallest and fastest moving peptide in 10% gel was apparently retarded by a 16% gel plug, as evidenced by a repeat experiment on a 12% gel shown in Figure 28. In both experiments, SCF antigen migrated several fractions ahead of the largest and most radioactive polypeptide. Recovery of SCF antigen was difficult when it was mixed and diluted with radioactive degraded virions but more easily recovered from parallel gels electrophoresed at the same time.

Figure 24. Propagation of dengue-2 virus in LLC-MK_2 cells.

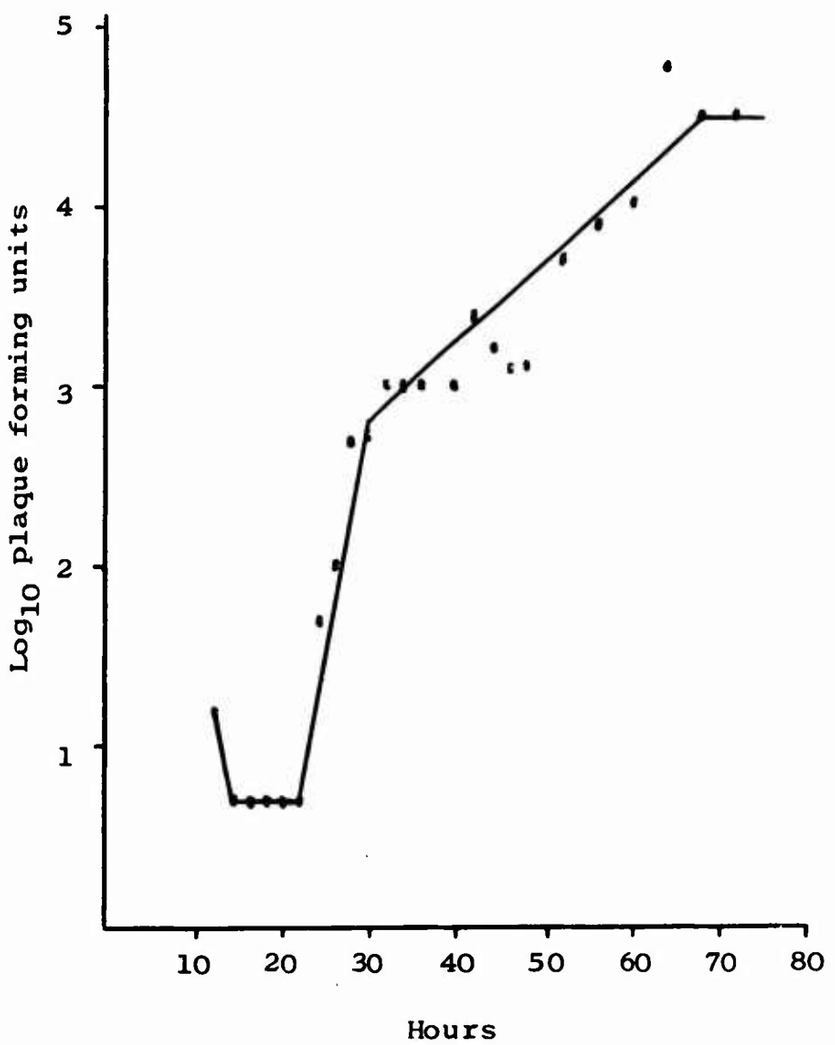


Figure 25. Rate zonal centrifugation in 5-25% sucrose gradients of radioactive dengue-2 virus propagated in LLC-MK2 cells.

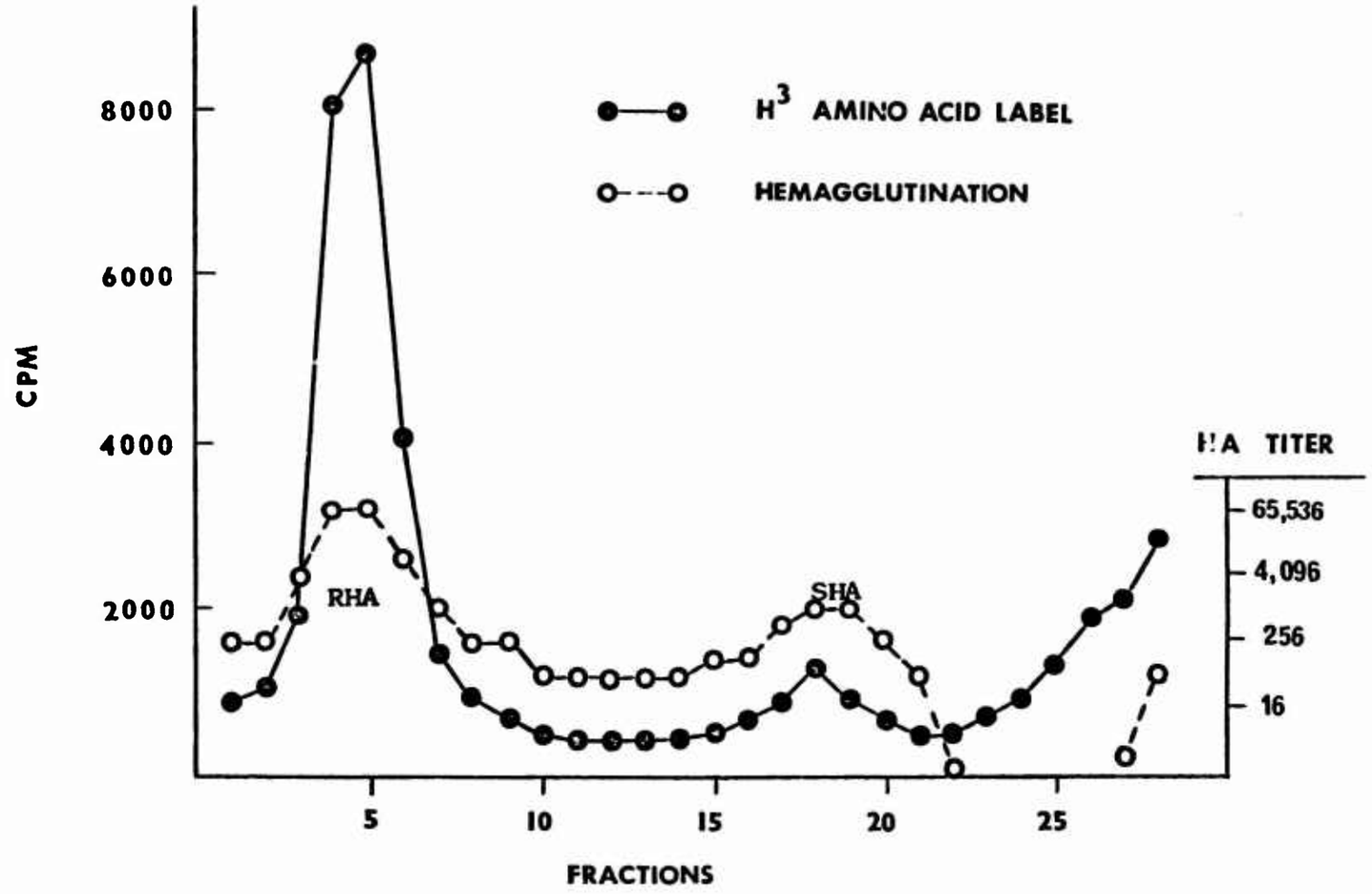


Figure 26. Rate zonal centrifugation in 5-25% sucrose gradients of dengue-2 virus labelled with tritiated amino acids in LLC-MK2 cell culture.

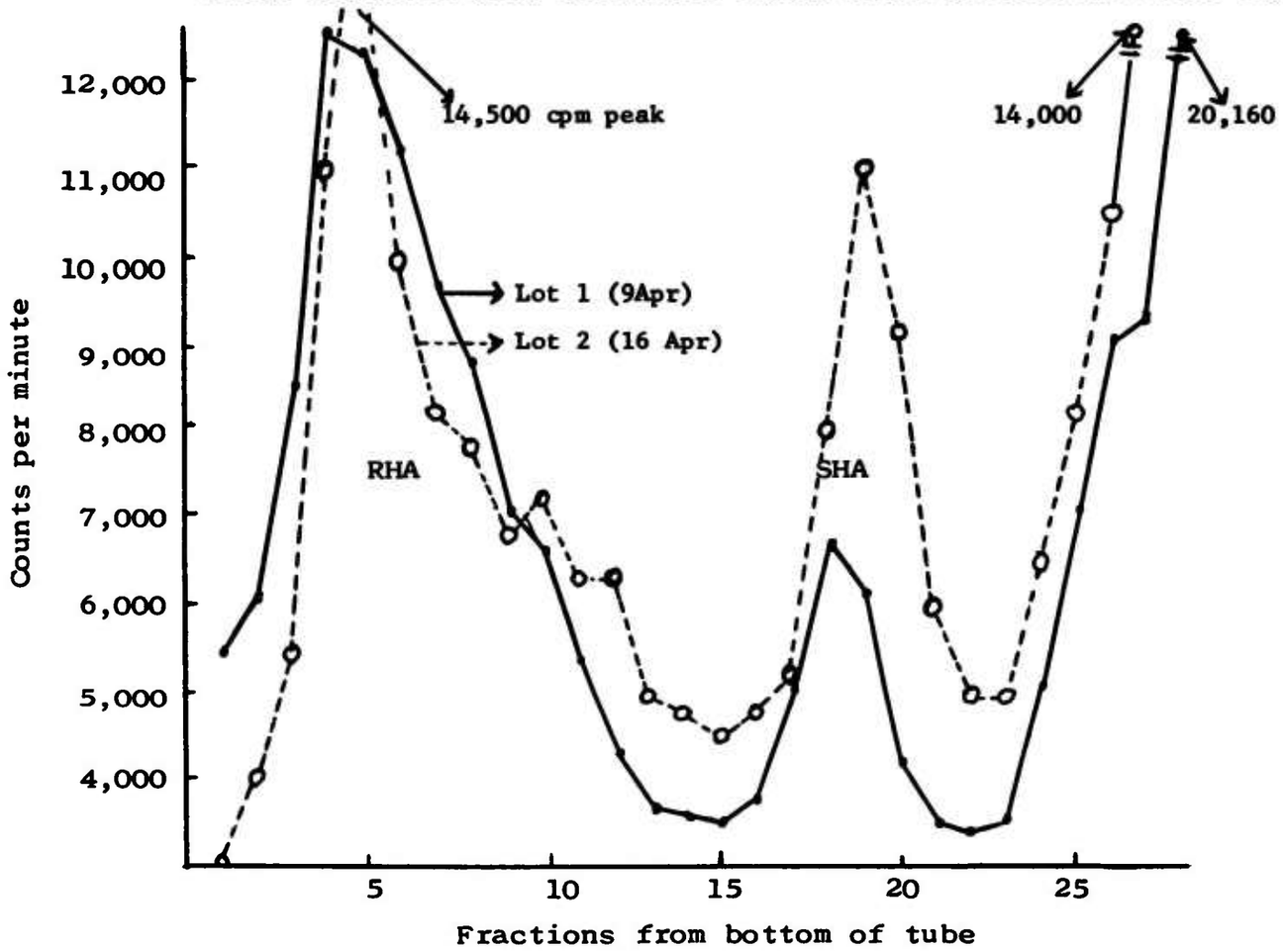
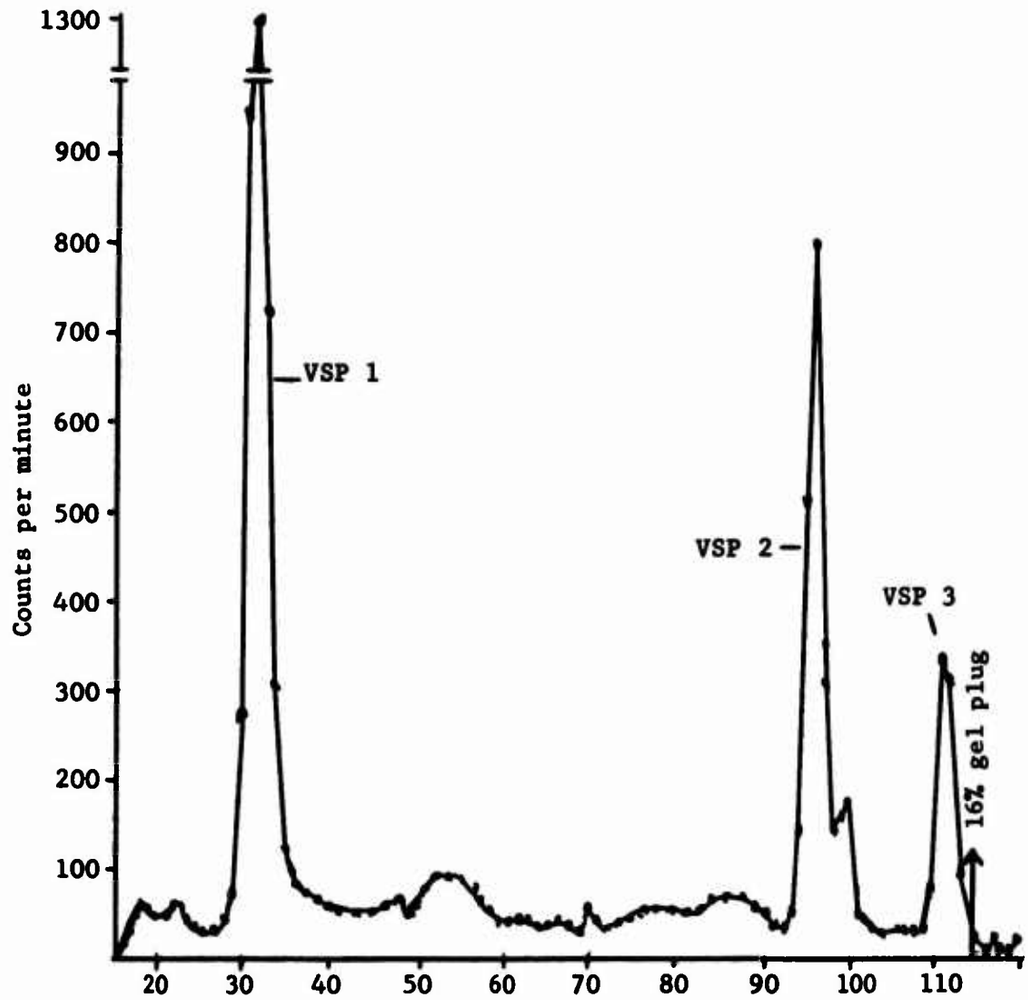


Figure 27. Dengue-2 structural proteins obtained by SLS degradation of virions (RHA) as compared to the soluble complement fixing (SCF) antigen on 10% polyacrylamide gels.

a. Structural proteins of dengue-2 virions.



b. Soluble complement fixing (SCF) antigen.

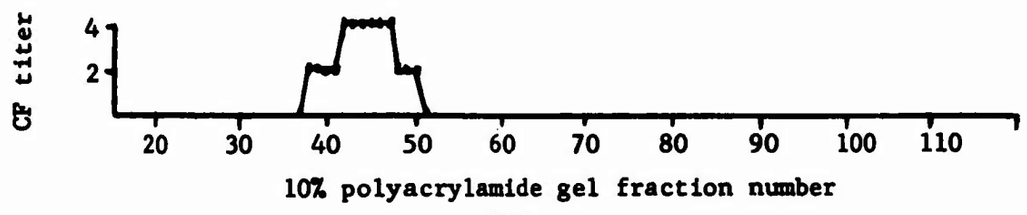
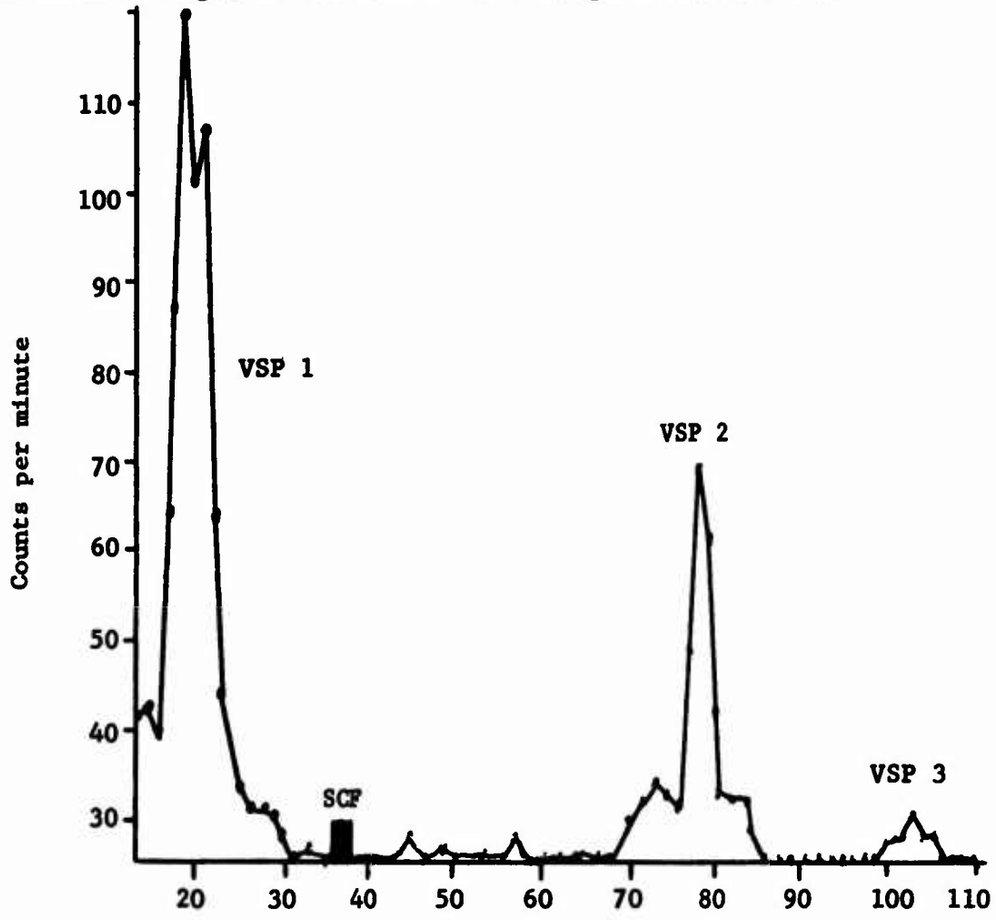
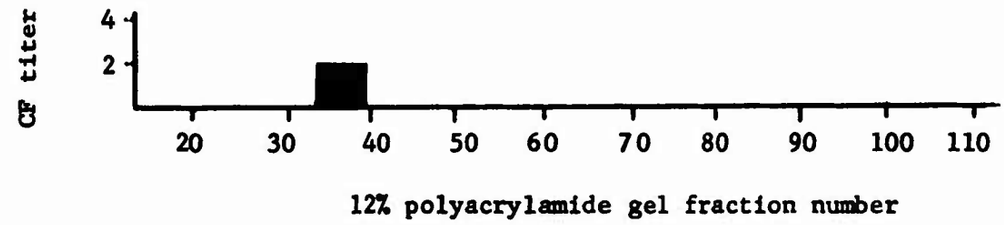


Figure 28. Comparison of dengue-2 virus structural proteins obtained by polyacrylamide gel electrophoresis of SLS degraded RHA (virions) and the soluble complement fixing (SCF) antigen.

a. Corun of degraded radioactive (H^3) dengue-2 RHA and SCF.



b. Parallel run of SCF only.



The molecular weights (MW) of each dengue structural polypeptide was determined by comparing their migration with known protein markers obtained from SLS-degraded C^{14} labeled Sindbis virions (Figure 29). The $\log_{10}$ MW of the two Sindbis structural proteins (MW 53,000 and 30,000) was plotted against polyacrylamide gel fractions number (Figure 29) to obtain a reference slope. Intersects of each dengue polypeptide peak fraction with the reference slope provided MW estimates of 56,000, 12,800 and 8,000; these compare favorably with Stollar's values of 59,000, 13,500 and 7,700. As stated above, recovery of SCF antigen after co-electrophoresis with structural proteins was not routinely accomplished, but co-runs are more reliable than parallel runs for determining MW. SCF has a MW of 39,000 when compared with standard proteins by both co-electrophoresis and parallel electrophoresis (Figure 28); this agrees exactly with SCF MW determinations on both Sephadex G-100 and precision discontinuous polyacrylamide gel electrophoresis (above).

Physical separation of the structural polypeptides and CF activity was further demonstrated by using the disc gel system of Hedrick and Smith with added SLS. The samples were co-electrophoresed at four different gel concentrations. When $\log_{10}$ mobility of each protein or antigen was plotted against gel concentration (7), a straight line is obtained (Figure 30). SCF is found between VSP 1 and VSP 2 in each of the four gel concentrations. These data were also used to obtain molecular weight estimations for the virus structural proteins and the SCF antigen. As described elsewhere in this report, the slope of any given protein is a function of the molecular size of that protein and, empirically, its molecular weight. SCF was again estimated at 39,000 but VSP 1 was found to be 68,000, VSP 2-10,000, and VSP 3 - 7,000. The estimates for the virus structural proteins vary only slightly from Stollar and the methods reported above utilizing C^{14} Sindbis protein markers.

It is possible that structural proteins of virions propagated in cell culture may not be properly compared to a soluble complement-fixing (SCF) antigen isolated from infected mouse brain; virions propagated in cell culture have previously been shown to be more dense than those isolated from mouse brain. Mouse brain derived virions containing a greater concentration of lipid may release proteins or lipoproteins in a different form or configuration (shape) when they are degraded with detergents. Thus, co-electrophoresis of structural proteins from mouse brain derived virions with the mouse brain derived SCF may produce different results than those obtained above. Until labeled virions and antigens can be obtained economically from mouse brain, an attempt was made to compare the virions and SCF as well as SHA on an immunological basis. The radioimmune precipitation (RIP) test was chosen as a sensitive indicator system for immunological comparisons; the experiments that follow indicate that SCF and SHA can both be differentiated immunologically from RHA (virion).

3. Control of the RIP Test: The general features of the RIP test have been defined by several investigators, but the specific conditions for the microsystem (9) were found applicable to the dengue-2 system used in this report.

Figure 29. Polyacrylamide gel electrophoresis and molecular weight determination of H^3 dengue-2 structural proteins by comparison with known C^{14} Sindbis structural proteins.

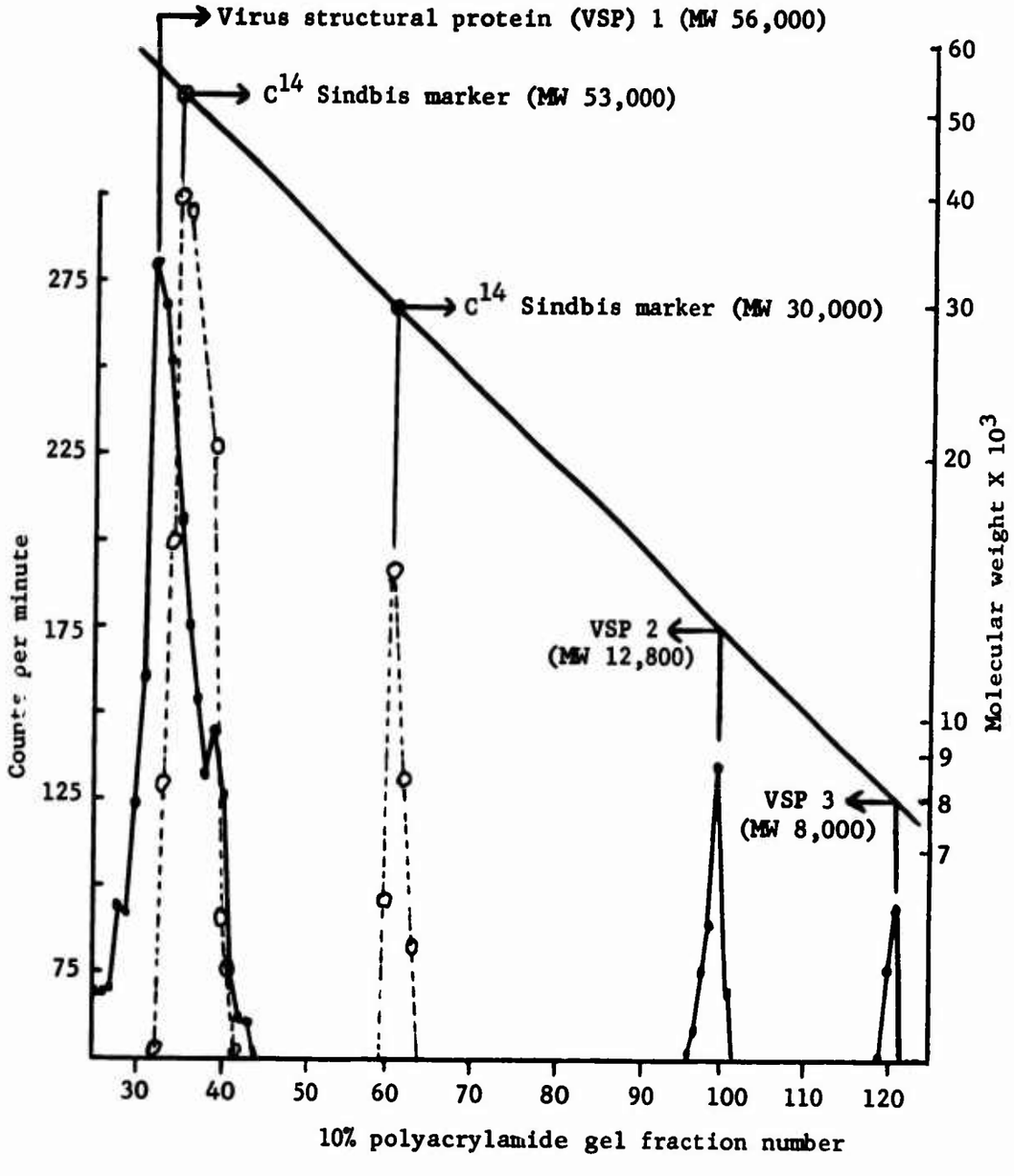
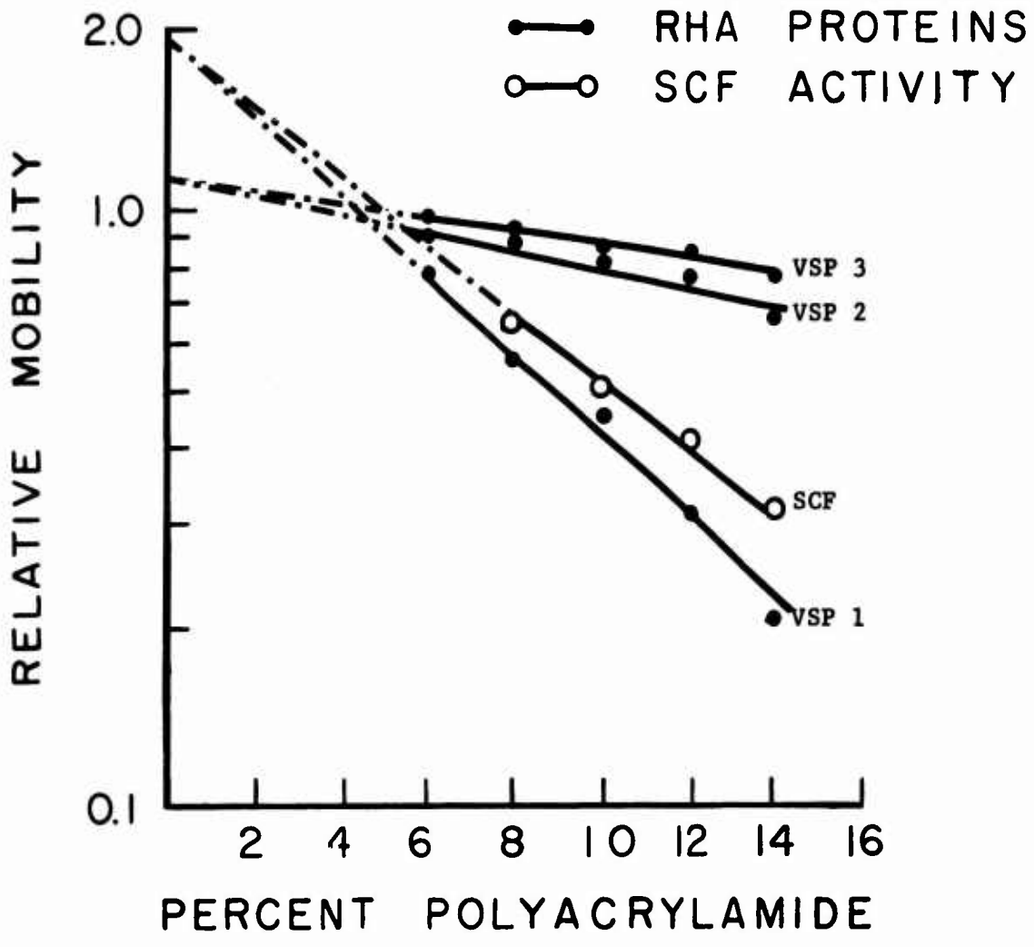


Figure 30. Mobility of the structural proteins of dengue virions (RHA) compared to the soluble complement fixing (SCF) antigen in increasing concentrations of acrylamide.



Controls using no dengue hyperimmune mouse ascitic fluid, normal mouse ascitic fluid, or no rabbit anti-mouse serum generally showed no precipitation. Occasional batches of radioactive RHA showed 10-20% nonspecific precipitation upon storage. Although adequate information could be obtained by adjusting baseline figures for nonspecific precipitation, the errant batches were generally discarded.

4. RIP using Anti-Dengue-2: Anti-dengue-2 represents a pooled mouse ascitic fluid against crude 20% homogenates of dengue-2 mouse brain and should have antibodies against most dengue-2 antigens. It had a neutralization titer of 1:1280, a complement-fixation titer of 1:512 and a precipitin titer of 1:16 (Table 22).

Table 22. Serological Comparisons of Dengue Hyperimmune Mouse Ascitic Fluids

	Anti-DEN-2	Anti-RHA	Anti-SCF
Neutralizing Antibody	1:1280	>1:160	0[a]
CF Antibody			
Crude antigen	1:512	1:64	0[b]
SCF antigen	1:256	0[b]	0
Precipitin vs SCF	1:16	N.T.	1:32

[a] = <1:10

[b] = <1:2

The effects of dilution of this antisera on the radioimmune precipitation of labeled RHA and SHA are illustrated in Figure 31. The maximal per cent of SHA precipitated in these experiments was always less than that for RHA. A 96% maximum was attained for RHA but only 86% maximum was attained for SHA. The maximum SHA precipitated varied with each batch of labeled SHA and seemed to be inversely related to the amount of overlap existing between the label on top of the gradient and the SHA peak.

Perhaps a more accurate representation of the RIP pattern is obtained by redrawing the dilution curve by plotting each point as the per cent of the maximum precipitation obtained (Figure 32). No matter how the curve is represented, a larger per cent of SHA is precipitable at the same dilutions of antibody required to precipitate RHA.

5. RIP Inhibition using Anti-Crude Dengue-2 Ascitic Fluid: Common antigens can be detected by inhibiting the precipitation of labeled antigens by preadsorption with an unlabeled test antigen. Preadsorption

Figure 31. Precipitation of radioactive (H^3) dengue-2 antigens by antibody against crude homogenates of infected mouse brain.

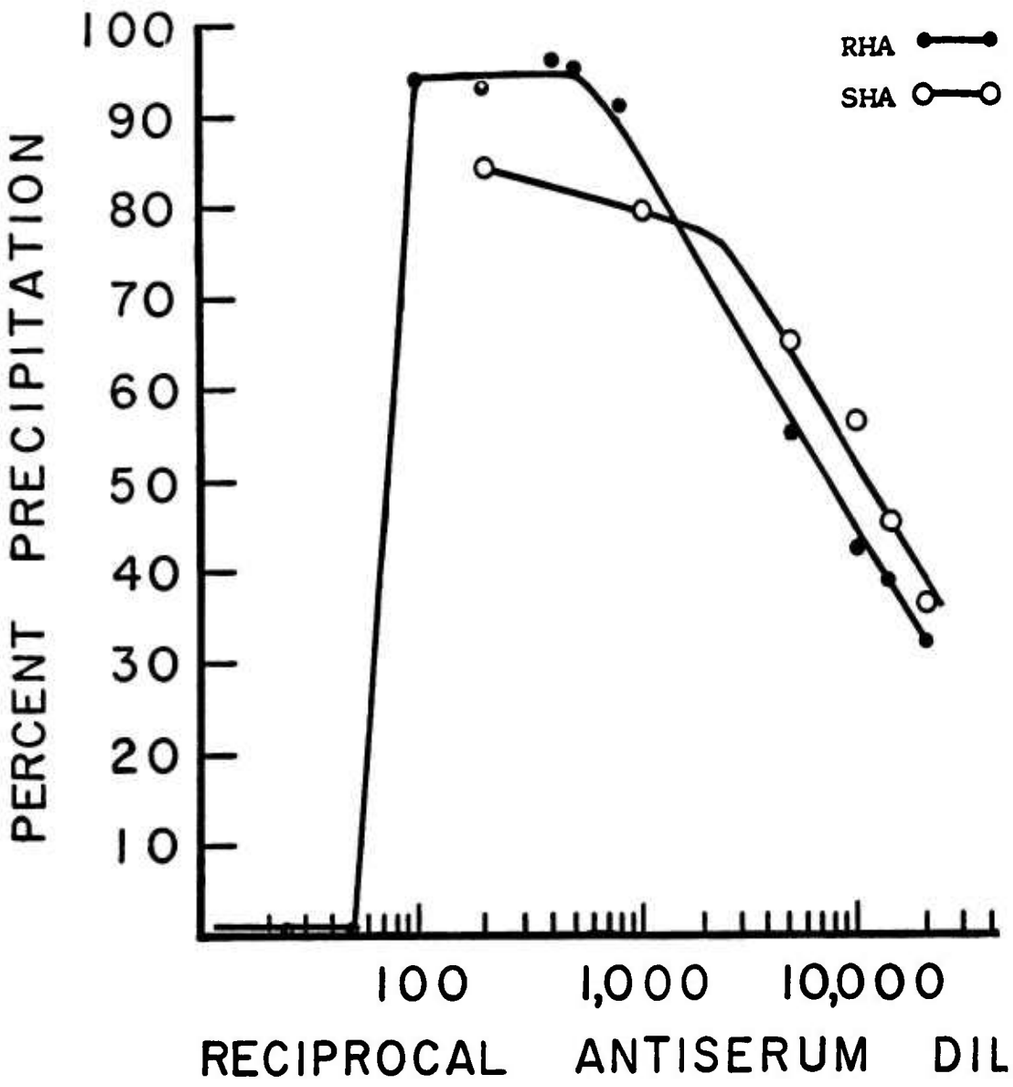
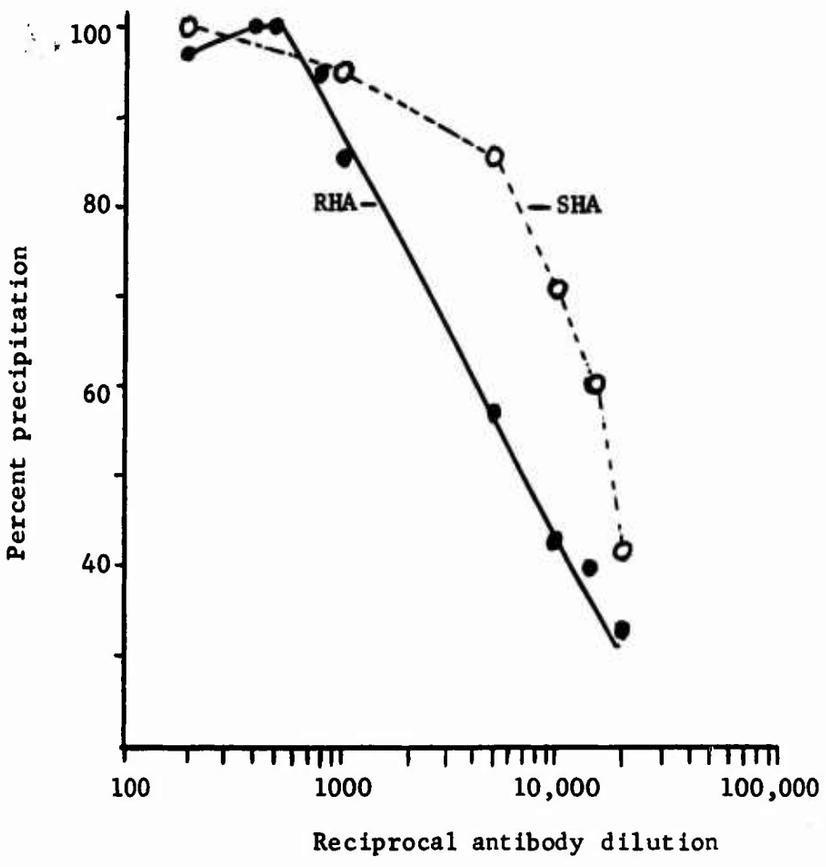


Figure 32. Precipitation of radioactive (H^3) dengue--2 antigens when each point is expressed as percent of the maximum precipitation obtained by any particular dilution of antibody against crude homogenates of infected mouse brain.



with the appropriate concentrations of either unlabeled mouse brain RHA or SHA completely inhibited the RIP of both labeled RHA and labeled SHA (Figure 33). This pattern of complete cross inhibition indicates immunological identity between RHA and SHA with the anti-dengue-2 ascitic fluid. Even higher levels of Sephadex purified SCF antigen failed to inhibit precipitation, indicating that no immunological cross-reaction existed between SCF and the two hemagglutinins.

6. RIP using Anti-RHA Ascitic Fluid: The anti-RHA mouse ascitic fluid was prepared by injecting into mice virions (RHA) obtained from infected mouse brain and purified on sucrose gradients. The immunization schedule has been previously reported (Annual Report 1969). The anti-RHA ascitic fluid had a neutralization titer greater than 1:160 and a CF titer of 1:64 when tested against crude antigen (Table 22); however, this ascitic fluid did not fix complement with Sephadex purified SCF. Precipitation of labeled RHA and SHA by anti-RHA ascitic fluid is shown in Figure 34. Again, precipitation of SHA did not reach 90% as did RHA, but when the curves were adjusted to per cent maximum as it was done for the anti-crude dengue-2 ascitic fluid, the curves for precipitation of RHA and SHA were indistinguishable (Figure 35). It can be seen by comparing figures 32 and 35 that anti-RHA and anti-crude dengue ascitic fluid have approximately equivalent potencies.

7. RIP Inhibition using Anti-RHA: Preadsorption of anti-RHA with unlabeled RHA completely inhibited the precipitation of labeled RHA (Figure 36). In the same manner, unlabeled SHA inhibited SHA precipitation (Figure 37). However, when unlabeled SHA was used to inhibit the RIP of labeled RHA, the slope was shallow and plateaued at 40% inhibition. Likewise, inhibition of the RIP of SHA with unlabeled RHA was incomplete with a plateau at 60%. These patterns indicate partial cross reactions between the antigens of SHA and RHA rather than immunological identity. SCF antigen, as with the anti-dengue-2 system, failed to inhibit the RIP of either SHA or RHA.

8. RIP using Anti-SCF: The mouse ascitic fluid against Sephadex purified SCF failed to neutralize, did not fix complement but had a high precipitin titer against SCF (Table 22). In contrast to the high levels of RIP with the other two antisera, anti-SCF precipitated only 4-10% of labeled SHA or RHA. This level of precipitation could be due to either low-levels of true immunological cross reaction or the presence of low levels of SHA and/or RHA antigens in the immunizing injections. Inhibition studies showed that the RIP of RHA and SHA were inhibited by unlabeled RHA and SHA but not by SCF. This suggests that the anti-SCF ascitic fluid contains a low level of activity against the hemagglutinating antigens and that there is no cross reaction between SCF and the hemagglutinins.

9. Neutralization Studies: Plaque reduction assays demonstrated the inability of the SCF antigen to combine with neutralizing antibody (Table 22). RHA and SHA were capable of inhibiting the effects of anti-dengue-2 ascitic fluid. SCF antigen had no such effect.

Figure 33. Inhibition of precipitation of labelled (H^3) RHA and SHA by preadsorbing anti-crude dengue-2 ascitic fluid with unlabelled antigens (inhibitors).

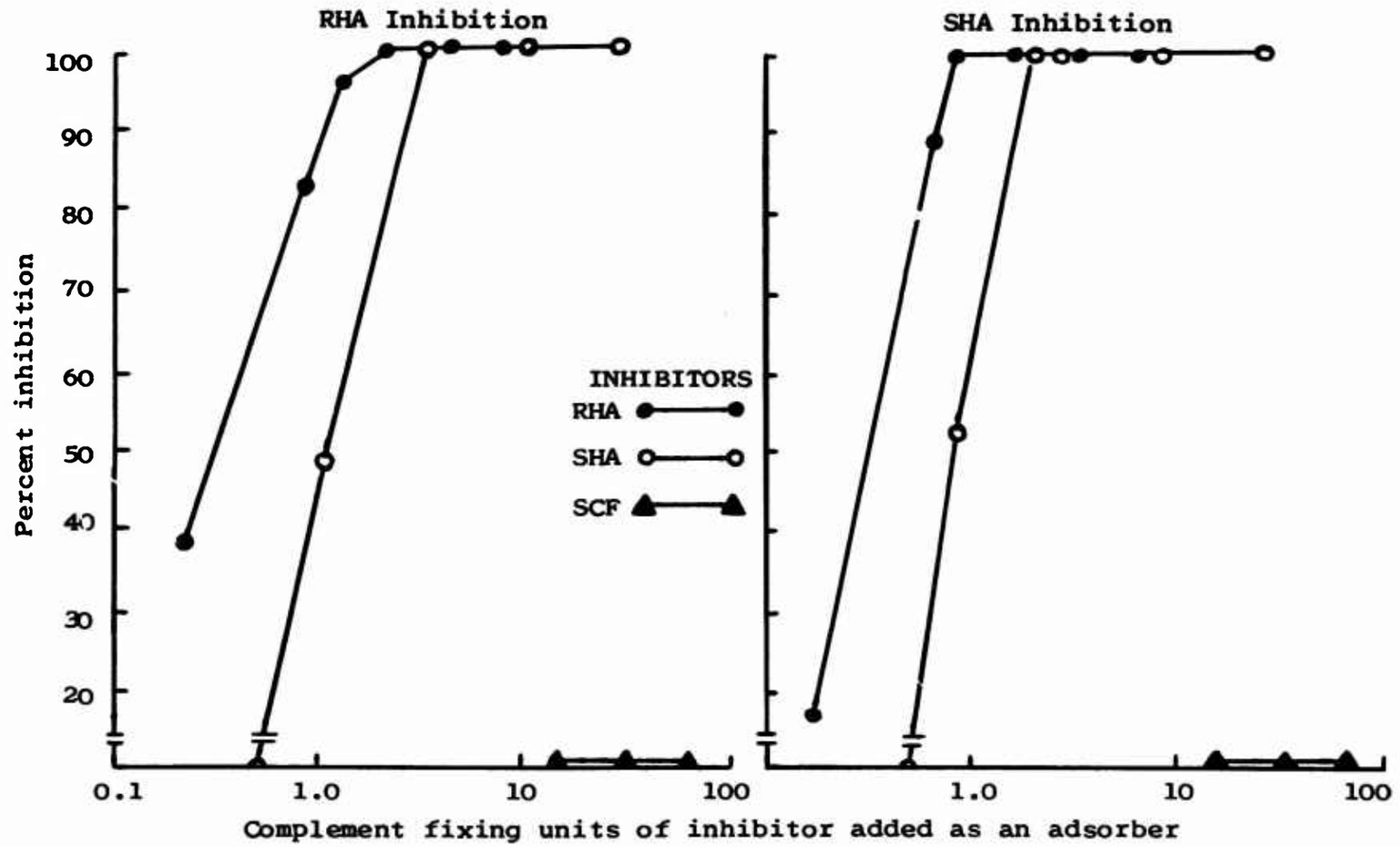


Figure 34. Precipitation of radioactive (H^3) dengue-2 antigens by antibody prepared against purified virions.

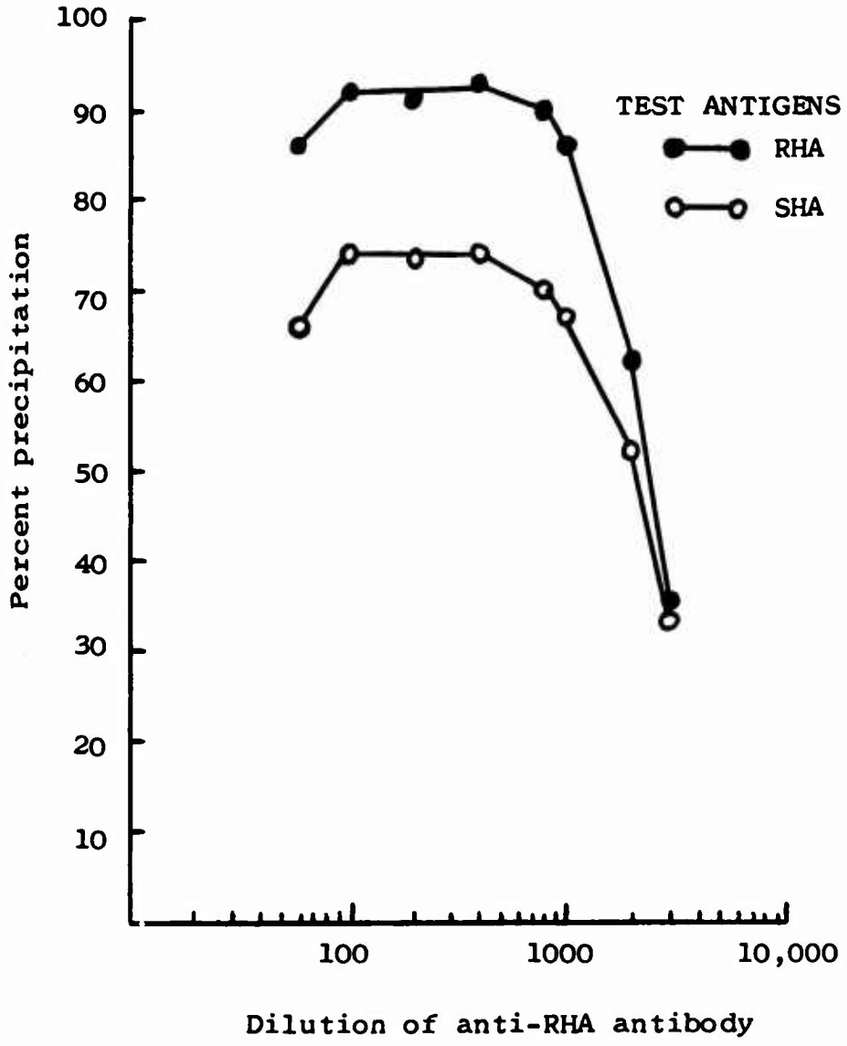


Figure 35. Precipitation of radioactive (H^3) dengue-2 antigens when each point is expressed as percent of the maximum precipitation obtained by any particular dilution of antibody against RHA.

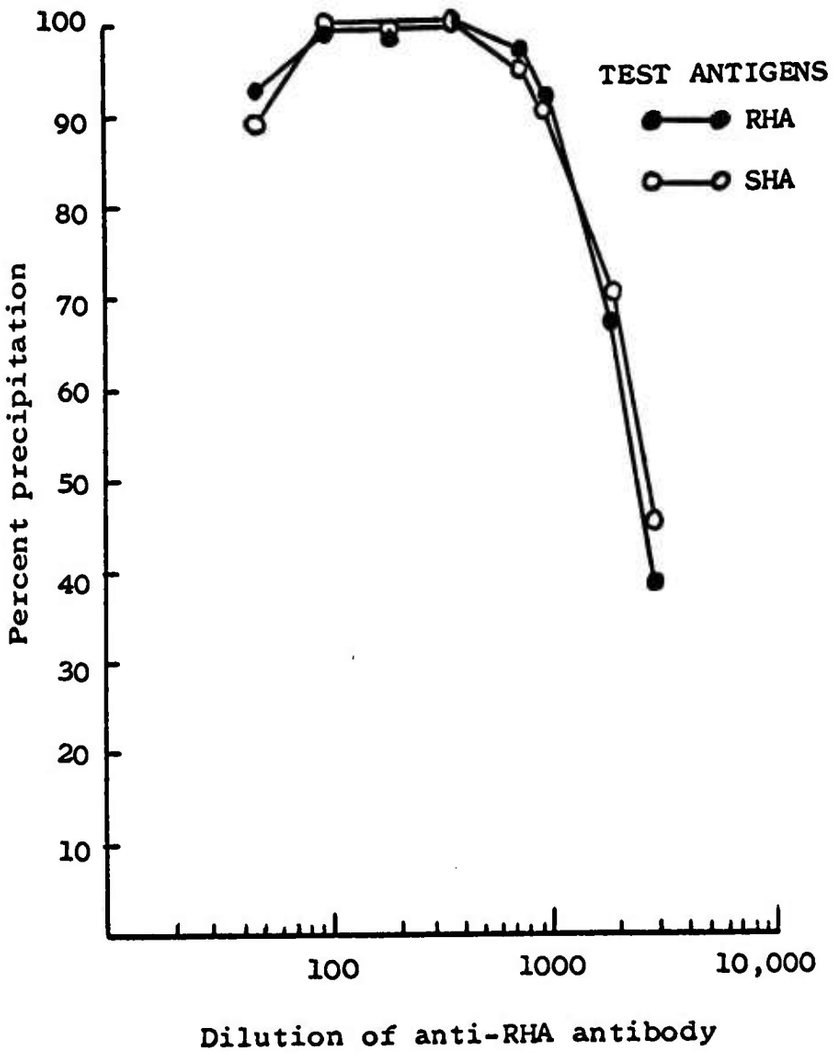


Figure 36. Inhibition of precipitation of labelled (H^3) RHA by preadsorbing antibody against purified virions with unlabelled inhibitors.

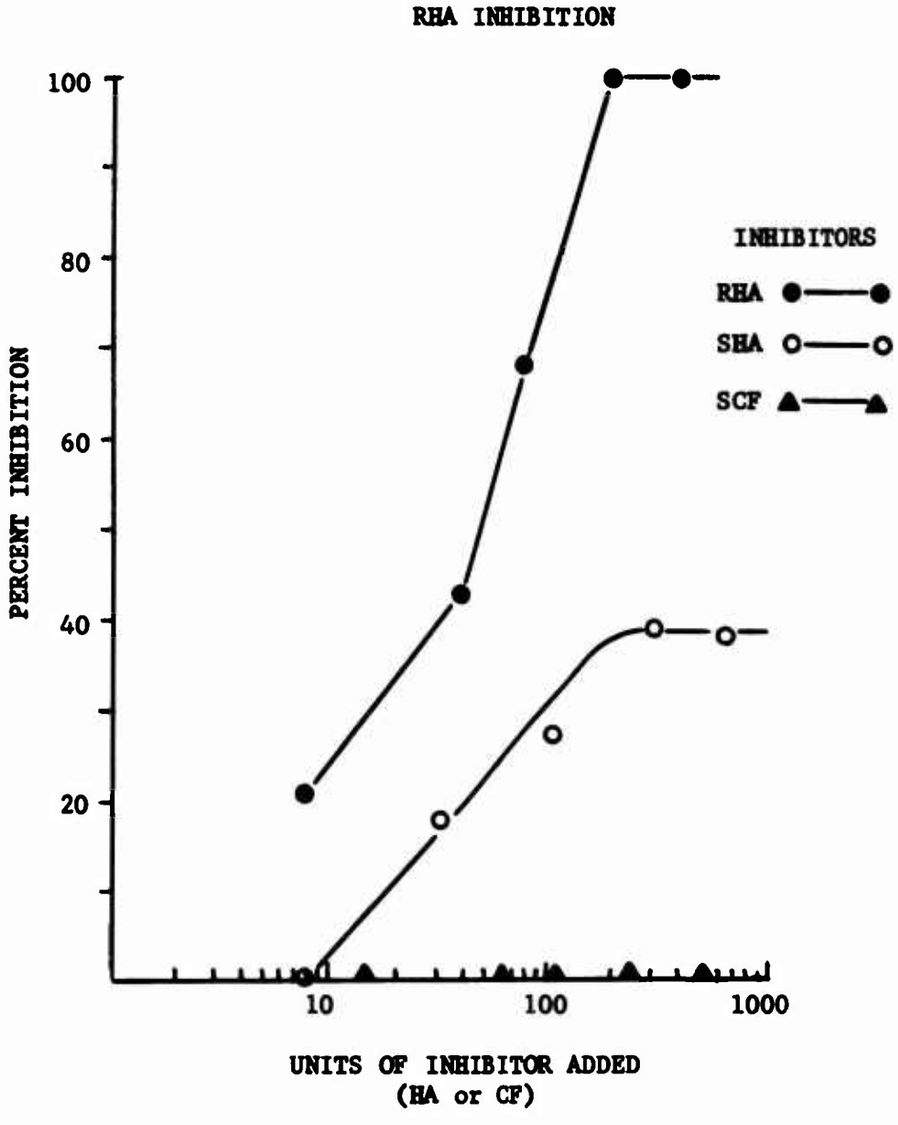
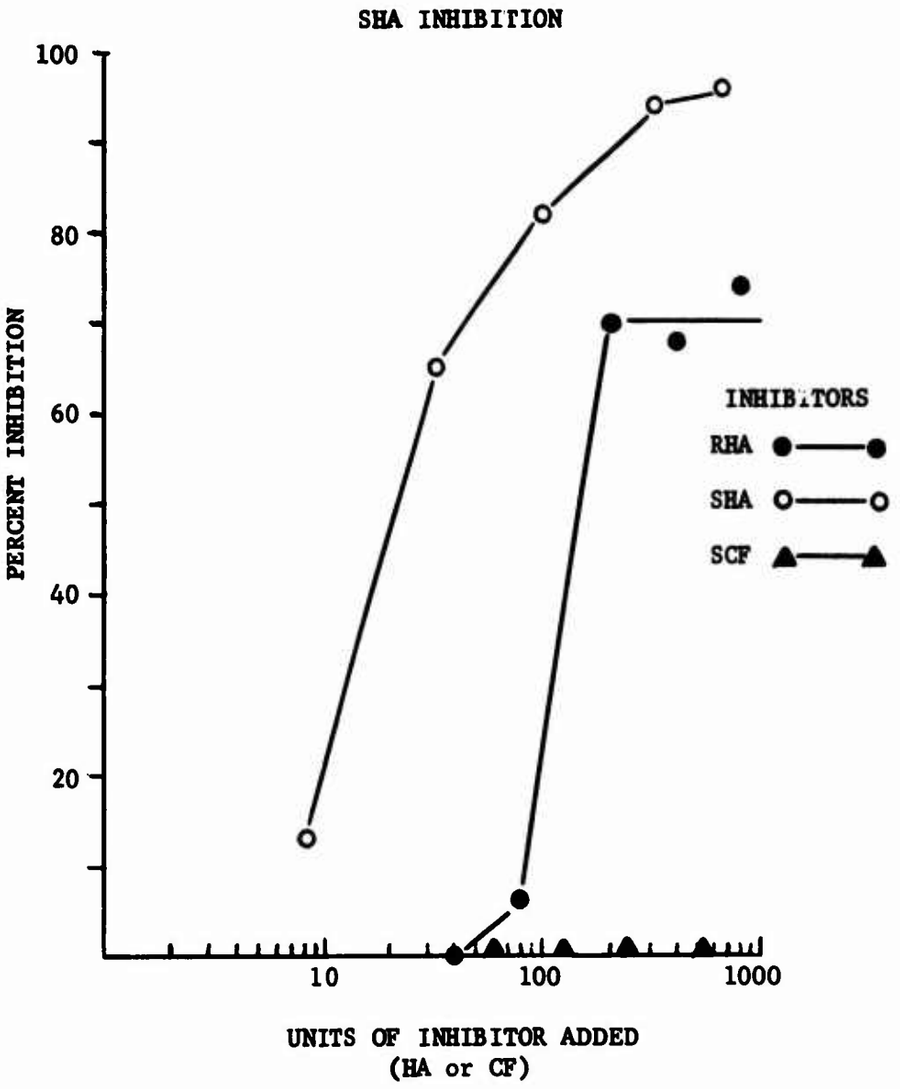


Figure 37. Inhibition of precipitation of labelled (H^3) SHA by preadsorbing antibody prepared against purified virions (RHA) with unlabelled antigens (inhibitors).



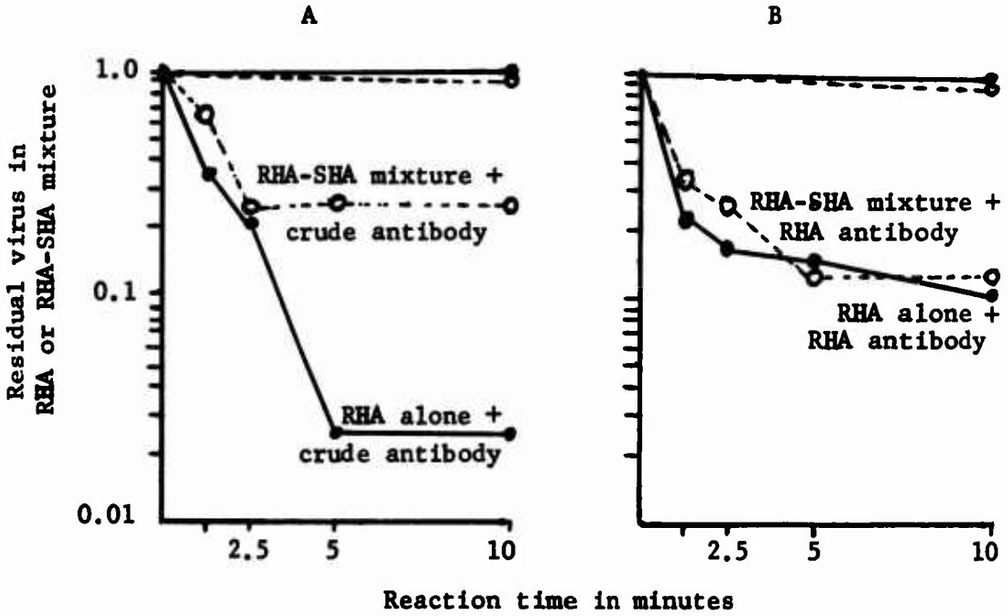
A kinetic neutralization assay was used to compare the effects of SHA inhibition on the anti-dengue-2 and anti-RHA ascitic fluids (Figure 38); details of this test have been described in the 1969 Annual Report. When anti-crude dengue-2 was used as the neutralizing antibody, SHA interfered with neutralization, that is, SHA was able to adsorb out a significant quantity of neutralizing antibodies. In contrast, when the same system was used with the anti-RHA ascitic fluid, no discernable effect on the kinetic neutralization of this antisera was observed. This corroborated the data obtained by RIP inhibition experiments. SHA could not block neutralization of RHA nor effectively inhibit precipitation of RHA. Thus, the anti-RHA ascitic fluid is capable of distinguishing between RHA and SHA.

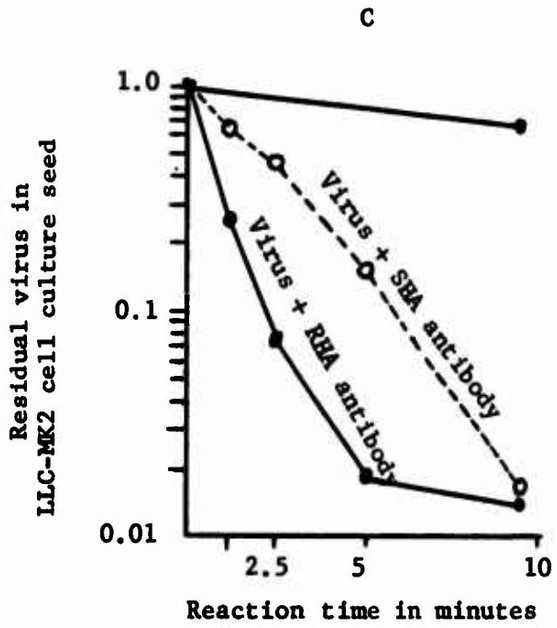
10. Discussion: SHA was first described as an incomplete virion. Our data and that of Stollar (8) indicates that it contains two polypeptides which have the same size as two of the polypeptides of RHA. Stollar's work suggests that they two polypeptides are coat proteins. Indeed, immunological identity between RHA and SHA was shown by hemagglutination-inhibition tests, most of our neutralization blocking experiments, and radioimmune precipitation inhibition tests. However, antibody prepared against purified virions (RHA) gave results suggesting only partial identity. SHA was unable to totally inhibit neutralization of dengue infectivity when anti-RHA antibody was used. Likewise, less than a 100% inhibition plateau obtained with RHA vs SHA systems when anti-RHA was used as the combining antibody in the RIP test indicated a partial immunological cross reaction between SHA and RHA. This does not necessarily mean that they contain different amino acid sequences but rather could indicate conformational differences between the two hemagglutinating antigens. Although a slowly sedimenting hemagglutinin can be derived from RHA by chemical degradation, the derived SHA differs from the native SHA in its buoyant density, stability in storage, and electron microscope appearance. If the naturally occurring SHA were "incomplete virion" in the sense of a coat without core, it should share many more of the characteristics of SHA derived from RHA. An alternative interpretation of SHA is that this unique particle represents membrane-associated coat protein micelles released not by budding but by breakdown of the cell membrane.

Co-runs of radioactive polypeptides of RHA or SHA with SCF on a variety of polyacrylamide gels demonstrate that the SCF antigen is biophysically separable from the polypeptides of the hemagglutinins. Separation was achieved in continuous and discontinuous buffer systems and at all gel concentrations tested. The estimated molecular weight of SCF under these conditions was again 39,000, while RHA contained polypeptides of 56,000, 12,800 and 8,000 by comparison with Sindbis proteins and 68,000, 10,000 and 7,000 by the slope-MW relationship. These results are in general agreement with previously published values and establish that the SCF can be separated from the structural proteins when electrophoresed under the same conditions.

Immunological experiments established the distinctive nature of SCF. A mouse ascitic fluid against partially pure SCF did not neutralize

Figure 38. Immunological comparison of RHA and SHA by kinetic neutralization tests. A. Ability of SHA to block neutralization of RHA by antibody against crude dengue-2 virus; B. Ability of SHA to block neutralization of RHA by antibody against RHA; C. Rate of neutralization of standard cell culture seed virus by RHA antibody and SHA antibody.





viral infection. Although a high SCF precipitin titer was established for this fluid, it did not precipitate significant levels of either radioactive RHA or SHA while ascitic fluids against crude brain preparations or purified virus precipitated at a 90-96% level. The precipitation that was present was probably due to low levels of contaminating RHA and SHA in the immunogenic preparations because the precipitation was inhibited by RHA or SHA but not SCF. Further, the radioimmune precipitation by anti-dengue infected mouse brain or anti-RHA of either radioactive RHA or SHA was not inhibited using highly concentrated sephadex purified SCF.

The SCF antigen, which is found in the brain and sera of dengue infected mice as well as dengue infected tissue culture cells, also has a different molecular size than any of the established dengue structural polypeptides and does not show any immunological relationship to the surface antigens of either RHA or SHA. Although none of our immunological systems exclude a possible relationship with internal antigens; the size relationships appear to preclude this possibility. Our experiments, therefore, establish SCF as a major nonstructural antigen produced by dengue infected cells.

It is premature to speculate on the functional significance of SCF. Because of its possible association with the complement consuming hemorrhagic shock syndrome, however, it will be of interest to establish whether or not the serum of dengue infected patients contains either the SCF antigen or antibodies against it. As a nonstructural, dengue-specific antigen with a cross reacting determinant, SCF could play a major role in the pathogenesis of the immunologically implicated hemorrhagic shock syndrome.

D. Alkaline Degradation of Dengue-2 Virus:

Investigation of methods for degrading dengue virions are being pursued as a means of producing subunits and as a tool for understanding the types of bonds necessary for dengue structural integrity. The work described herein involves the use of dengue-2 virions purified by rate-zonal centrifugation on sucrose gradients; the virus was propagated in either suckling mice or LLC-MK2 cell cultures and was concentrated by pelleting in the ultracentrifuge as previously described (Annual Report 1969).

Virus pellets were resuspended in 0.02 M Tris, 0.14 M NaCl, 0.001 M EDTA pH 8.2 (TNE) buffer, and sonicated for two minutes at 10 KC. Approximately 0.25 ml was layered onto a 4.8 ml linear 5-25% sucrose-TNE gradient and centrifuged in the SW 50 rotor at 50,000 rpm (204,000 x g) for 35 minutes at 4°C (Figure 39a). The major and most rapidly sedimenting peak of HA (RHA) contains intact virions and the slower sedimenting peak (SHA) contains small "ring" structures.

1. Degradation of Mouse Brain Derived Dengue-2 Virus by Changes in pH: Aliquots of RHA from a sucrose gradient were dialyzed against 200

volumes of either a) TNE pH 8.2, b) glycine-NaOH, ionic strength (IS) 0.05, pH 10.6 or c) glycine-HCl, IS 0.05, pH 3.0 at 4°C overnight, and then recentrifuged on 5-25% sucrose gradients for analysis of degradation products by hemagglutination assay (Figure 39b). At pH 8.2 in TNE buffer, RHA lost considerable potency; at pH 10.6 and low ionic strength, the RHA was converted to an "SHA-like" hemagglutinin; at pH 3.0, no HA activity was recovered. In other experiments, the effects of several alkaline pH's (9.0, 9.6 and 10.0, all IS = 0.05) on RHA were studied (Figure 39c). Degradation of RHA into SHA-like material occurred only at pH 10.0, the virion remaining intact at pH 9.6). Back dialysis of alkaline degraded RHA against TNE buffer in other experiments indicated that the product was stable and that the degradation process under these conditions was irreversible (Figure 39d).

2. Degradation of Cell Culture Derived Radioactive Dengue-2 Virus by pH: When a similar experiment to that described in Figure 39b was performed with radioactive (tritiated amino acids) RHA, it can be seen that the pH 10.5 alkaline-derived "SHA-like" product is composed of two peaks of radioactivity (Figure 40a). In a repeat experiment with higher counts per minute (cpm), the second alkaline-derived peak is seen only as a shoulder (Figure 40b). It also appears that even at pH 8.2 where the virion is generally stable, there is some dissociation of RHA into smaller structures. The above experiments employed 5-25% sucrose gradients in isotonic TNE buffer. Figure 40c shows that the results are the same when alkaline products are sedimented on a gradient in which the sucrose is dissolved in glycine-NaOH, IS 0.05, pH 10.6, and confirm that two radioactive peaks are obtained by alkaline degradation of RHA as shown in Figure 40a. Since Tween-80 and ether was shown to degrade RHA into an SHA-like peak (Annual Report 1969), an attempt was made to line up one of the alkaline-derived radioactive peaks with radioactive natural SHA (Figure 40d). The results were inconclusive in this first test; the gradients were in separate pH systems (10.6 and 8.2) and will be retested in a co-run in the same gradient.

It was observed that alkaline-derived radioactive hemagglutinating proteins were further degraded to soluble proteins when dialyzed against the standard pH 6.2 borate saline-phosphate buffer prior to using the same buffer for hemagglutination. It was also found that the alkaline dialysis could be omitted; direct dialysis of dengue hemagglutinins in pH 6.2 buffer prior to testing them for HA in the same buffer at the optimum pH of 6.2 destroyed their HA activity. The resulting soluble proteins are defined as those which remain on the surface of sucrose gradients. While these soluble proteins would not HA, they were shown to adsorb onto goose erythrocytes; an equal volume of packed red cells mixed with the labeled proteins at pH 6.2 and then centrifuged back out of suspension removed the radioactivity from the supernate.

The soluble proteins obtained by alkaline dialysis at pH 10.6 followed by acid dialysis at pH 6.2 could be differentiated into three discrete peaks when they were heated to 100°C for 2 min in 1% SLS-2-ME, dialyzed in a reservoir buffer of 0.1% SLS-0.01 M phosphate and

Figure 39.

a. Rate-zonal centrifugation of dengue-2 pellet in 5-25% sucrose gradient at 204,000 x g for 35 minutes (50L rotor).

b. Degradation of dengue-2 virions (RHA) by pH as analyzed on 5-25% sucrose gradients.

c. Alkaline pH degradation of dengue-2 virions (RHA) as analyzed on 5-25% sucrose gradients.

d. Back dialysis of alkaline degraded dengue-2 virions (RHA) to original pH analyzed on 5-25% sucrose gradients.

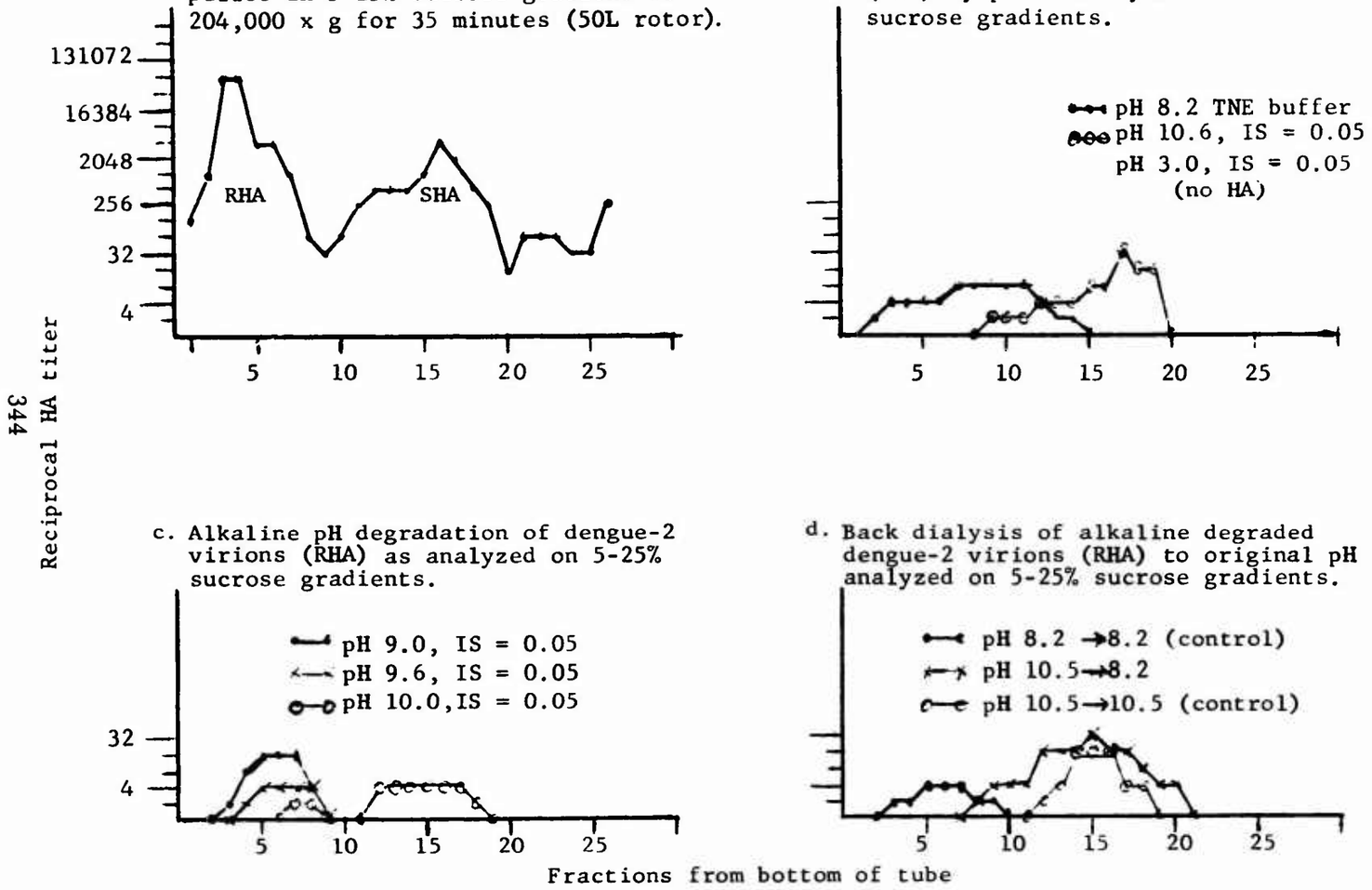
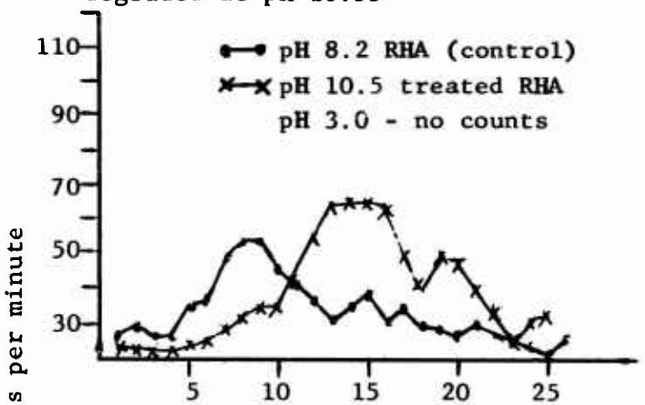
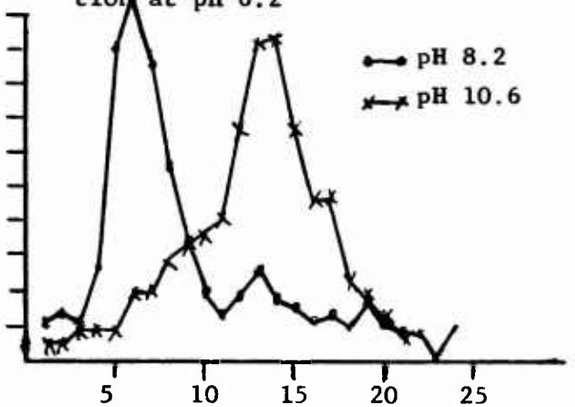


Figure 40.

a. Sucrose gradient (5-25%) analysis of radioactive dengue-2 virions (RHA) degraded at pH 10.55

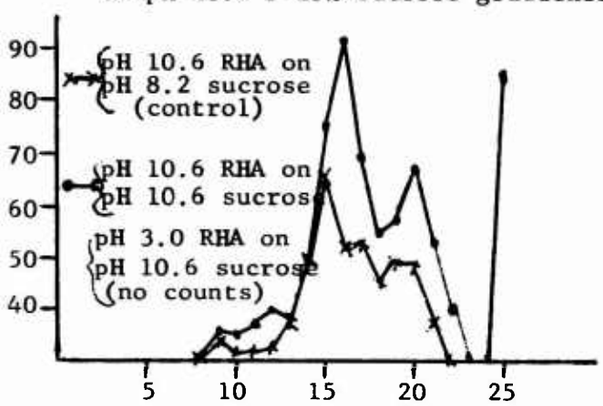


b. Same as a., higher activity with reproducible minor RHA fragmentation at pH 6.2

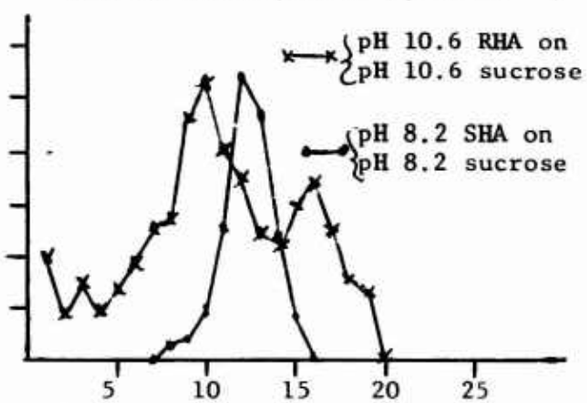


Counts per minute

c. Radioactive dengue-2 virions(RHA) degraded at pH 10.6 and analyzed on pH 10.6 5-25% sucrose gradients



d. Sucrose gradient (5-25%) analysis of alkaline degraded RHA and natural SHA. (RHA = ^{3}H; SHA = ^{14}C)



Fractions from bottom of tube

electrophoresed in 10% polyacrylamide gel containing 0.1% SLS as described below. The SLS may have allowed for differentiation of the three proteins and does not reflect the results of alkaline or acid degradation per se. Stollar found the same number of peaks when he degraded purified dengue virions by SLS treatment, and found that the center peak was associated with the viral "core," or an internal protein and that the outer peaks were external or "coat" proteins. If the same results (same number of peaks) apply to pH degraded dengue virions, then whether internal or external, all of the proteins in adsorbed onto the surface of goose erythrocytes.

When dengue-2 RHA labeled with H^3-uridine (RNA) and C^{14}-amino acids (protein) was alkaline degraded, an RNA-rich fragment was seen to sediment slightly ahead of the major alkaline fragment (Figure 41a). When the alkaline-derived material was subjected to isopycnic banding in a sucrose-D_2O gradient, three fractions were seen (Figure 41b); an RNA-rich fraction with a density of approximately 1.26; a protein rish fraction with a density of about 1.21 and a 3rd peak of undifferentiated lighter material (all peaks contained TCA-precipitable counts). The RNA-rich peak rebanded in a similar position when subjected to a recentrifugation. When H^3-amino acid labeled dengue-2 RHA derived from infected LLC-MK2 cells is mixed with dengue-2 RHA derived from infected suckling mice and subjected to isopycnic centrifugation, the same pattern of three radioactive peaks is observed (Figure 41c). The densest peak does not hemagglutinate, whereas the lighter peaks do.

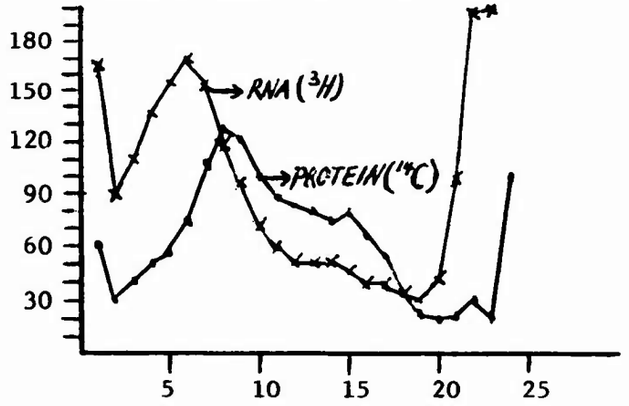
When the mixture is sedimented, again two peaks of radioactivity are observed (Figure 41d). It is not clear why the faster sedimenting "SHA-like" peak is decreased in this run compared to previous experiments. Hemagglutination appears to be predominantly associated with the faster peak. There is 50% complement-fixing activity associated with one fraction of the second peak.

Another alkaline pH degradation study of ^{3}H-amino acid labeled dengue-2 RHA was carried out with different buffers and ionic strength. The sucrose gradients were in 0.02 M Tris pH 8.2 without NaCl and EDTA as previously described. RHA remained intact at pH 8.2 under these condtions (Figure 42a). At pH 10.0 and 11.2, RHA was degraded into two more slowly sedimenting peaks (42b) that compare favorably with other alkaline degradation experiments shown in Figure 40. Since the pH 11.2 buffer was carbonate IS 0.1, instead of glycine-NaOH, it is unlikely that alkaline degradation results from a specific buffer effect. When the ionic strength is increased to 0.2 by the addition of NaCl to the pH 10.6 glycine-NaOH buffer (Figure 42c), the slowest moving protein becomes the dominant product.

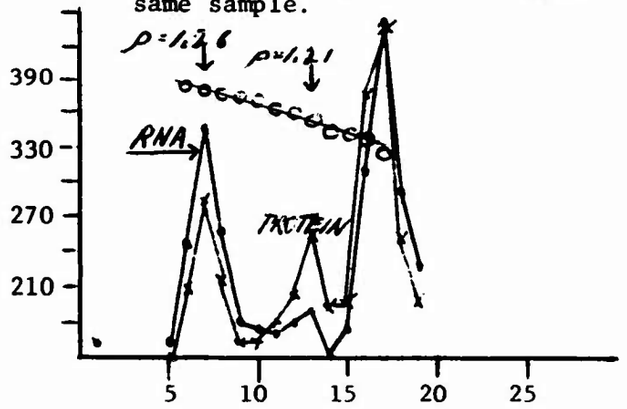
These results show that alkaline treatment can degrade dengue into three specific fragments: two protein-rich, RNA-poor fragments in the SHA region of the gradient and an RNA-rich protein-poor fragment which is apparently the ribonucleoprotein core. The latter point is not definitely established, although the particle has a density compatible with this interpretation and it does not hemagglutinate.

Figure 41.

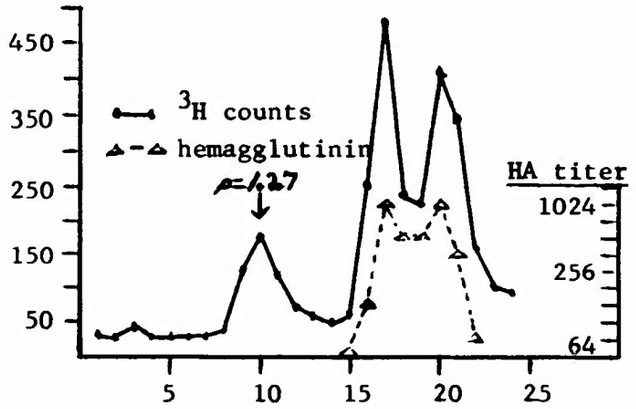
a. Rate zonal analysis of alkaline degraded double-label RHA in 5-25% sucrose.



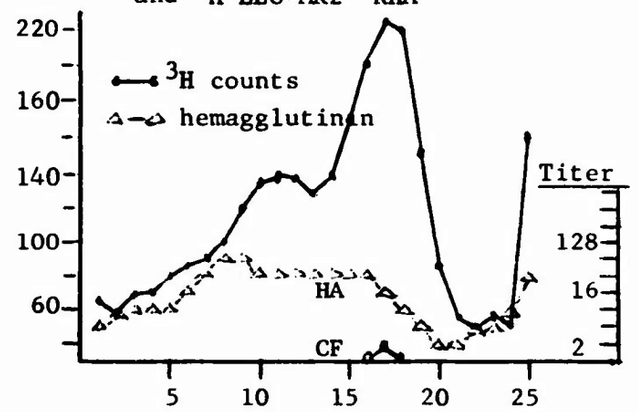
b. Isopycnic centrifugation in D_2O sucrose, 6 hrs, 300,000 x g, of same sample.



c. Isopycnic analysis of alkaline degraded mixture of mouse brain and ^3H LLC RHA.



d. Rate zonal analysis of alkaline degraded mixture of mouse brain and ^3H LLC-MK2 RHA



Counts per minute

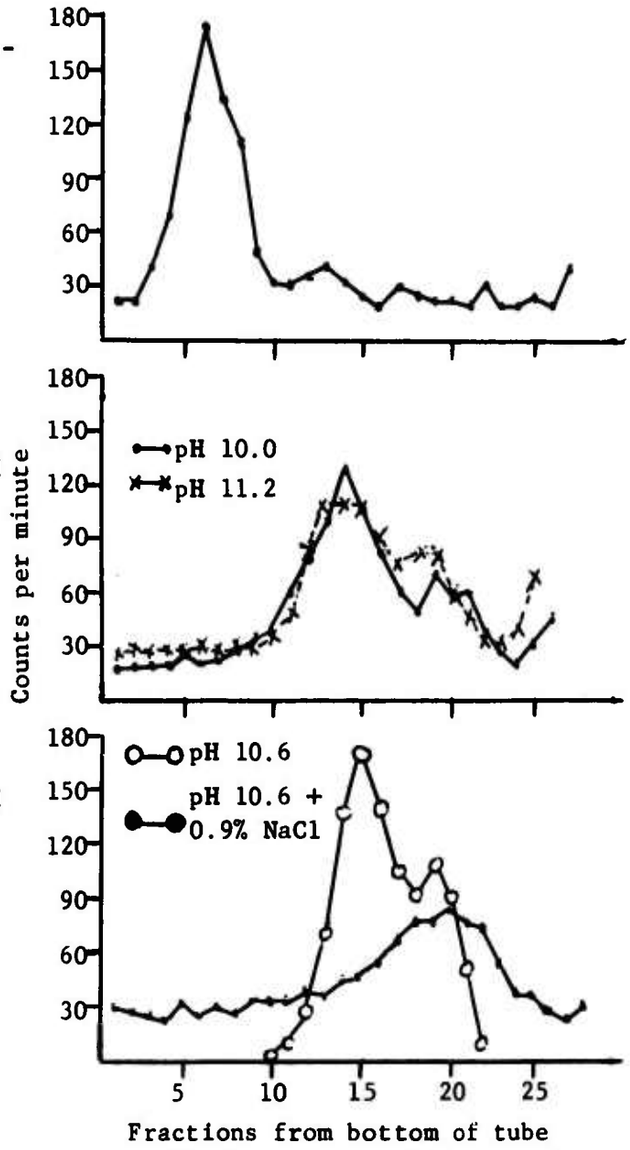
Fractions from bottom of tube

Figure 42. Rate zonal analysis in 5-25% sucrose gradients of alkaline degraded radioactive dengue-2 virions (^{3}H-RHA) in varying ionic strength and buffer composition.

a. RHA recycled in Tris-sucrose gradients without NaCl & EDTA

b. RHA dialyzed in pH 10.0 glycine-NaOH buffer and pH 11.2 carbonate buffer prior to gradient analysis.

c. RHA dialyzed in pH 10.6 glycine-NaOH buffer and the same buffer with 0.9% NaCl.



It is to be expected that pH affects virus structural integrity predominantly by influencing polar bonds and hydrogen bonds with relatively little effect on hydrophobic interactions. We, therefore, tend to attribute the breakdown of the virus by these procedures to disruption of polar protein-protein or protein-RNA interactions which would probably be greatly different in mechanism than disruption of virus by detergents. In fact, our proposed "core" particle sediments significantly slower than a dengue core isolated by deoxycholate treatment by Stollar.

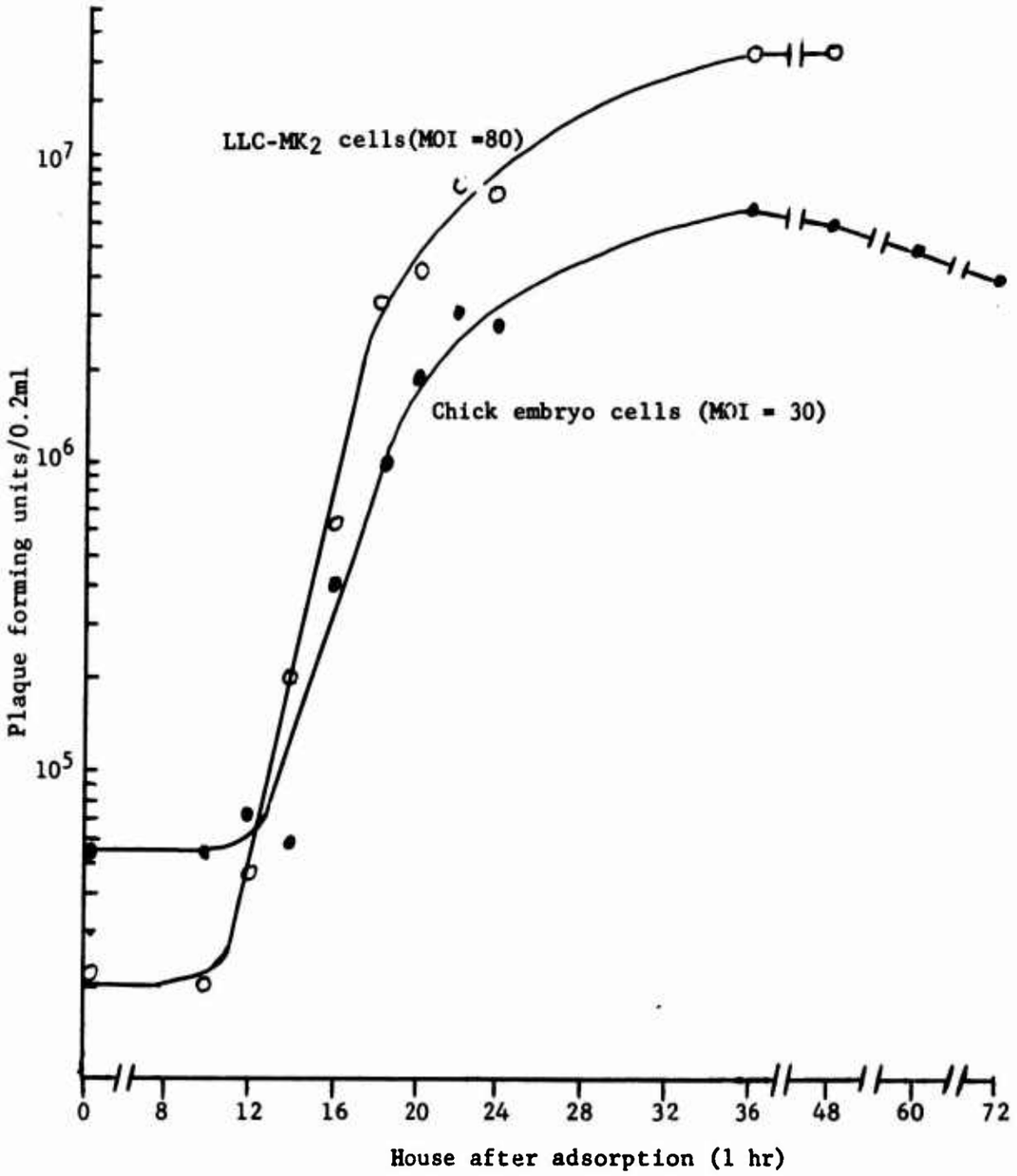
Of interest is the fact that the virus appears to be completely degraded at pH 6.2, the optimal pH for HA. The only amino acid which has a maximal pK in that region is histidine and it is tempting to speculate that lowering the pH breaks a critical imidazole^{+}-receptor^{-} bond. With this in mind, we attempted to see if exogenous histidine could compete for this hypothetical receptor and thereby inactivate dengue virus; the results were inconclusive since only 50% of the infectivity was lost in the presence of histidine. Histidine-specific agents may be able to degrade the virus, but further experiments along that tract would be premature without stronger evidence that histidine, either <u>in vitro</u> or <u>in vivo</u> is intimately associated with either virus stability or assembly.

E. <u>Structural and Nonstructural Proteins of Japanese Encephalitis Virus</u>:

During investigation of the intracellular proteins found in actinomycin D-treated, dengue-2 infected LLC-MK2 cells, new viral induced or specific proteins could not be detected. It was also found that actinomycin D had small and non-reproducible effects on protein synthesis in normal LLC-MK2 and pig kidney cells, even for treatments longer than 12 hours at concentrations of up to 5 micrograms/ml. It was felt that a more rapidly metabolizing cell such as chick embryo might have messenger RNA of shorter half-life than monkey or pig kidney cells and, therefore, protein synthesis would be more sensitive to inhibition by actinomycin D. Japanese encephalitis virus (JEV) strain M^{1}/311 was chosen because it allowed the use of high multiplicities of infection (MOI).

1. <u>Growth Curve of JEV in Chick Cells and LLC-MK2 Cells</u>: Growth curves at high multiplicity of infection (MOI) of Japanese B encephalitis virus in both chick cells and LLC-MK2 cells at 36°C are presented in Figure 43. The curves were obtained by plaque assay in chick embryo cells in the usual manner; the agar overlay medium consisted of 0.5% lactalbumin hydrolysate, 0.1% yeastolate, 2 mM glutamine in Hanks BSS, 4% of 7.5% $NaHCO_3$ and 0.8% agarose (Mann). Virus first begins to appear in the medium at about 10-12 hours after virus adsorption (11-13 hours after initial virus contact); the replicative cycle is over by 36 hours. The cells are dead by 36 hours. The yield of virus from LLC-MK2 cells is significantly greater than from chick cells. Because of varying cell numbers in flasks receiving equal virus inocula, the MOI for each cell

Figure 43. Growth of Japanese encephalitis virus in LLC-MK_2 and chick embryo cell cultures.



type was different; therefore, had the yield of virus been normalized to infective centers, the LLC-MK2 cells would probably be even a better relative host for JEV than suggested by the curves. Thus, propagation of radioactive virus was carried out in LLC-MK2 cells; the experiment below suggested that studies of intracellular proteins be carried out at 35° in chick cells. Viral inocula consisted of infected suckling mouse brain.

2. Effect of Actinomycin D on Protein Synthesis in Chick Cells: Since dose levels of less than 1 ug/ml of Actinomycin D generally do not maximally inhibit protein synthesis in a variety of cells, levels of 1 to 10 ug/ml were used to determine the dose required for maximum inhibition in uninfected chick embryo cells (Figure 44a). The medium was changed and the various dose levels were added to the cells at time 0 and radioactive (^{3}H) amino acids pulsed in for 1 hour at the indicated times. It can be seen that even at 1 ug/ml actinomycin D is maximally effective.

3. Effect of JEV Infection on Protein Synthesis in Normal Chick Cells: In order to detect new proteins in virus infected cells, normal host cell protein synthesis must be decreased either by drugs or by the infecting virus such that the proteins specific by the infecting virus become a significant proportion of the proteins synthesized by the infected cell. We, therefore, studied the effect of JEV infection at high MOI on protein synthesis in chick cells without actinomycin D treatment (Figure 44b). There is generally a suppression of protein synthesis (reduced rate of incorporation of radioactive amino acids) in the infected cell to a level of about 2/3 of that in the uninfected cell. In such a situation it would seem highly unlikely that polyacrylamide gel electrophoresis of radioactive infected cell extracts would reveal new proteins. This contention was confirmed when a radioactivity plot of gel fractions of infected and uninfected cells were compared.

4. Effect of Cycloheximide Pulse on Actinomycin D-Treated JEV Infected Chick Cells: Trent (10) has demonstrated that a brief cycloheximide pulse irreversibly inhibits host protein synthesis but not viral-directed synthesis in SLE infected actinomycin D-treated BHK-21 cells. Similar experiments were attempted with JEV-infected actinomycin D-treated chick cells and similar results were achieved (Figure 45a). For each point on the graph, cycloheximide was added for 30 minutes, then washed off, and 30 minutes later radioactive (^{3}H) amino acids added for 4 hours. Prior to the cycloheximide pulse, each flask was treated with actinomycin D (1 ug/ml) for 9 hours; the drug was readded with the isotope. The isotope was washed off and the cells disrupted with 1% SLS in TNE buffer and heated at 100°C for 10 minutes; 0.1 ml was precipitated with cold 10% TCA and washed 3 times with 5 or 10% cold TCA on a millipore filter before liquid scintillation counting. Control curve represents uninfected cells treated with actinomycin D but no cycloheximide. Other controls consisted of infected and uninfected cells not treated with either actinomycin D or cycloheximide

Figure 44.

a. Effect of actinomycin D on protein synthesis in chick embryo cells.

b. Effect of Japanese encephalitis virus on protein synthesis in chick embryo cells.

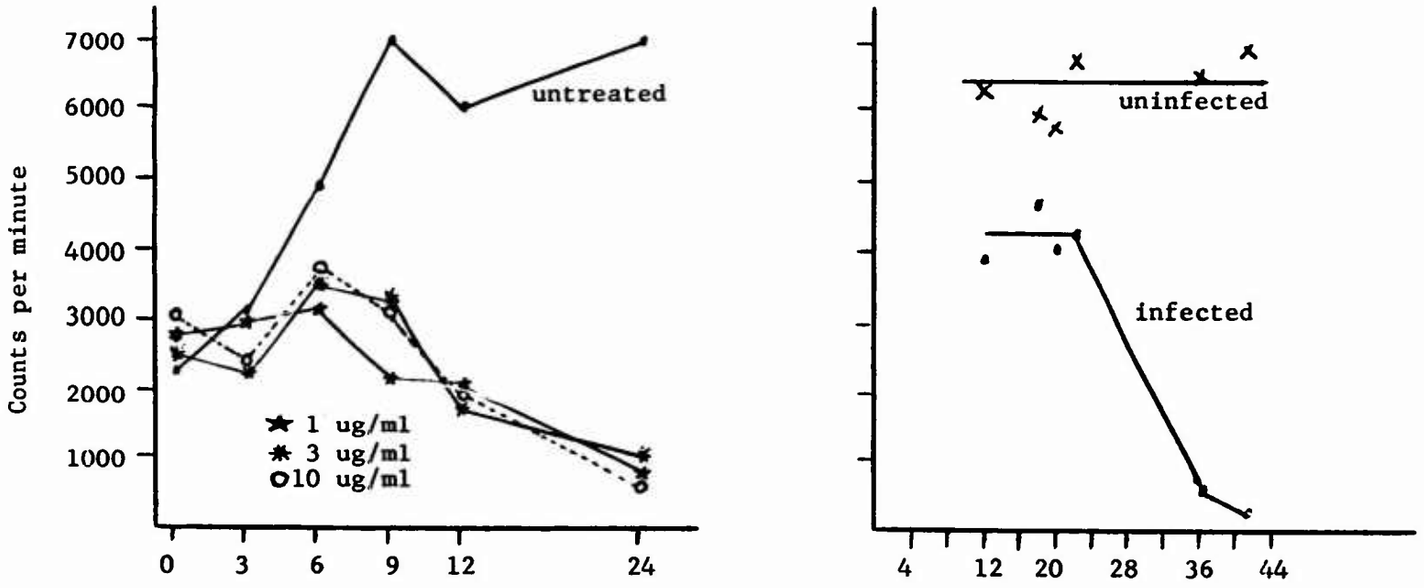


Figure 45a.
Protein synthesis as a function of time of cycloheximide pulse in uninfected and JEV infected chick embryo cells.

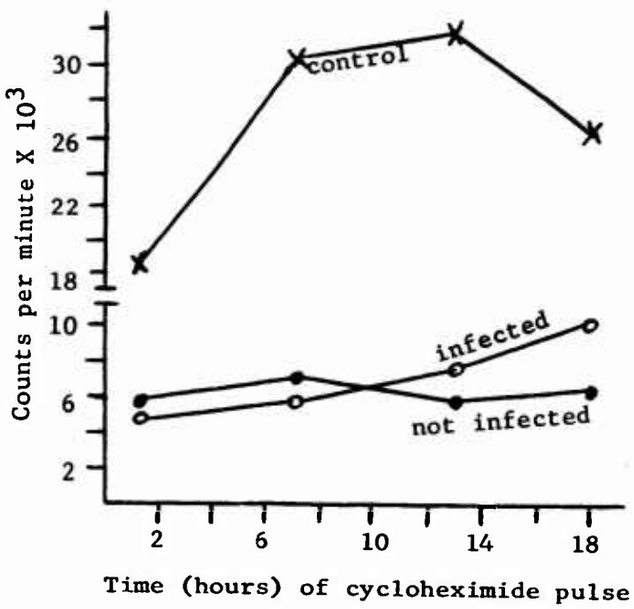
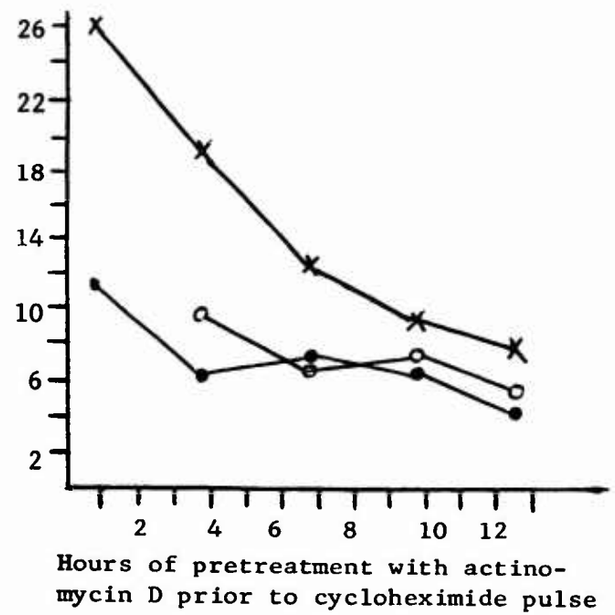


Figure 45b.
Effect of pretreatment time of actinomycin D on cycloheximide inhibition of protein synthesis in uninfected and JEV infected chick embryo cells.



but receiving a 4-hour radioactive pulse of amino acids beginning after the adsorption period of 1 hour: uninfected cell preparation - 40,000 cpm; infected cell preparation - 30,000 cpm. Other controls consisted of infected and uninfected cells treated for 5 hours with cycloheximide beginning after adsorption period and a 4-hour pulse of label beginning 1 hour after the adsorption period: uninfected cell preparation - 685 cpm; infected cell preparation - 764 cpm. It would appear that the cycloheximide must be added more than about 10 hours after infection; if added earlier, infected and non-infected cells remain irreversibly inhibited after cycloheximide removal.

5. Effect of Duration of Pre-Treatment with Actinomycin D: The cells in the above experiment were pretreated with actinomycin D for 9 hours before cycloheximide was added. In the following experiment, the duration of the pre-treatment with actinomycin D was varied, and the effects of protein synthesis following a pulse of cycloheximide 13½ hours after infection was studied. The results (Figure 45b) indicate that a 4-hour pre-treatment with actinomycin D is sufficient for irreversible inhibition of host cell protein synthesis by cycloheximide.

6. Intracellular Proteins in JEV-Infected Chick Cells: When the 18-hour sample of uninfected and infected chick cells shown in Figure 45a were subjected to polyacrylamide gel electrophoresis, it seemed seven new polypeptide peaks could be detected in infected cells (Figure 46). The number of peaks were reproduced in several other experiments, but there was variation in the height of the peaks relative to each other. Electrophoresis was carried out in 8% recrystallized acrylamide (60 mm) in 0.1% sodium lauryl sulfate (SLS); the reservoir buffers consisted of 0.1% SLS in 0.1 M phosphate, pH 7.2, and constant voltage (70V) was applied for approximately 2 hours. The 18-hour cell extract samples received 2-ME to 1% concentration, and were heated to 100°C again for 10 minutes and dialyzed in 0.01 M phosphate buffer containing 0.1% SLS, pH 7.3.

7. Effect of Early Actinomycin D Treatment of JEV Replication in Chick Cells: The dose of actinomycin D (1 ug/ml) that killed the cells in 24 hours was added immediately after absorption. The same seven intracellular proteins were found 12 hours after infection as were found 18 hours after infection above. Thus, no differences in terms of early and late proteins were found at this time. It is currently not possible to examine infected cells prior to 12 hours since the amount of labeled proteins is about the same as uninfected cells; no difference in distribution of radioactivity on polyacrylamide gels would be seen.

8. Structural Proteins of Japanese Encephalitis Virus: Radioactive (^{3}H) JEV was prepared in LLC-MK2 cells by adding 6.7 uc/ml of tritiated amino acids immediately after absorption. Supernatant fluid was harvested 40 hours after infection, and the virus pelleted from it by ultracentrifugation at 78,000 x g for 3 hours. Virus pellets were resuspended in TNE buffer, sonicated and purified on 5-45% sucrose gradients centrifuged at 63,000 x g for 2.5 hours (Figure 47). Purified

Figure 46. Intracellular proteins in uninfected and JEV infected chick embryo cells.

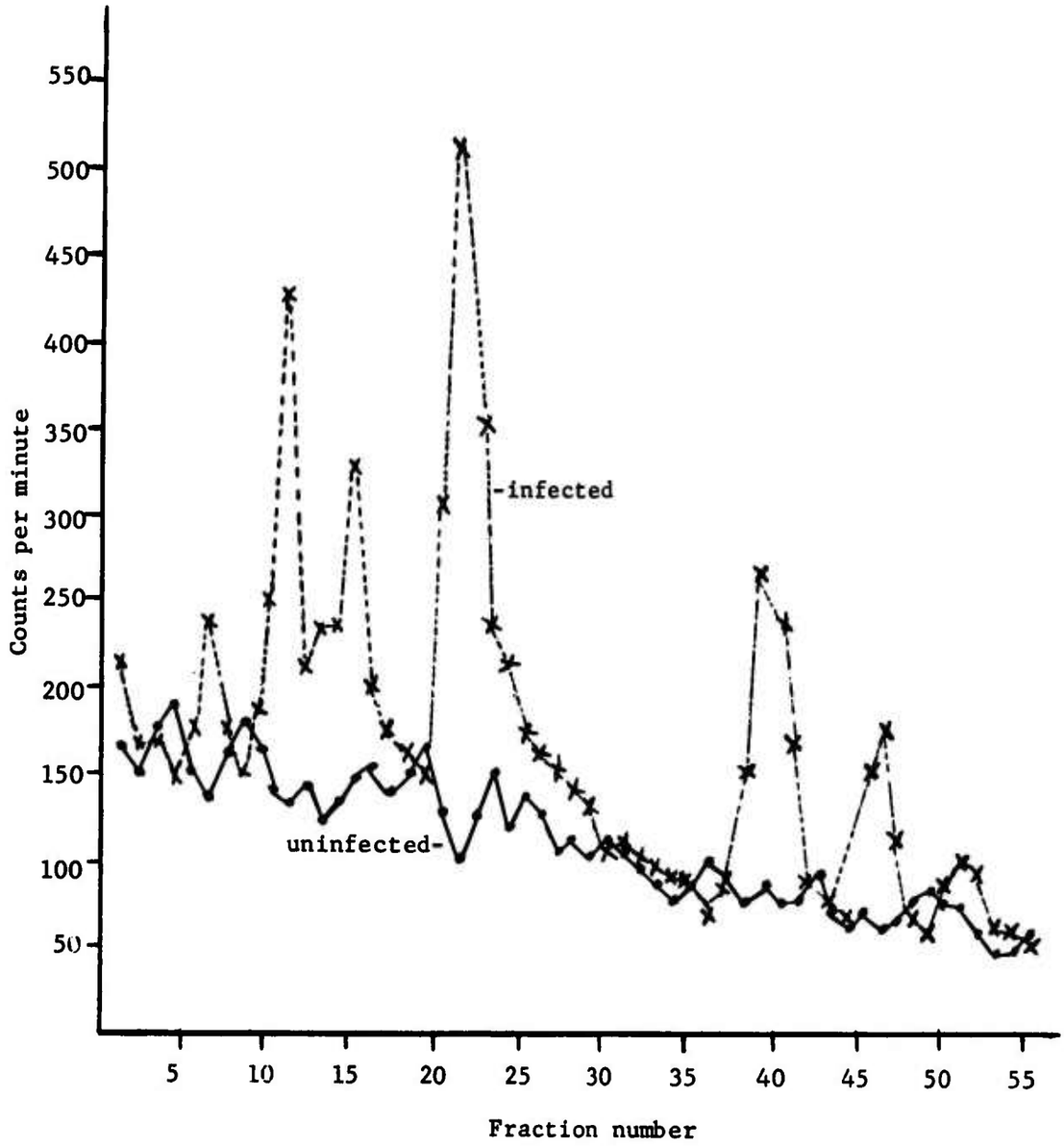
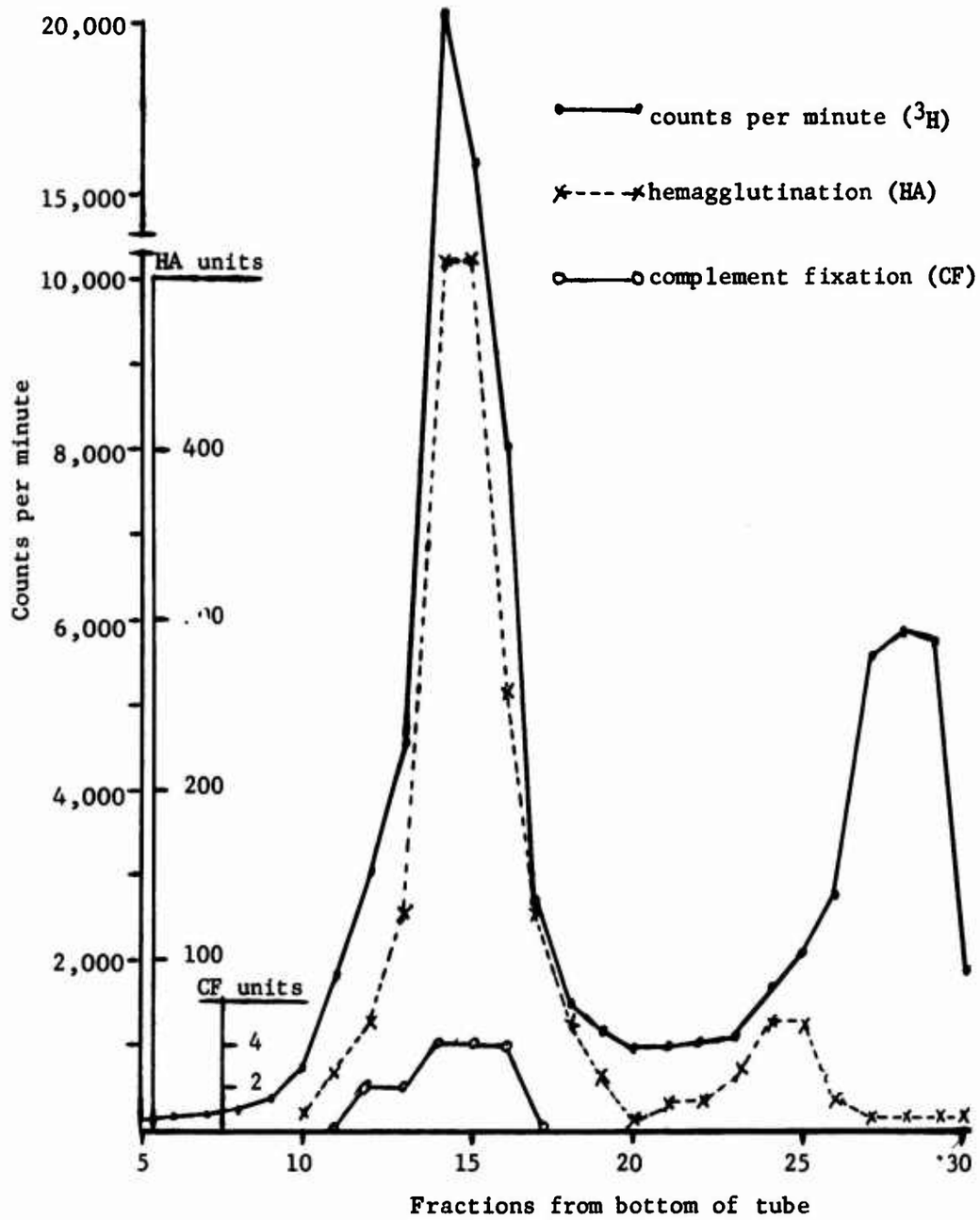


Figure 47. Rate zonal centrifugation in 5-45% sucrose gradients of radioactive Japanese encephalitis virus propagated in LLC-MK2 cells.



virions are in the center of the gradient as marked by overlaping HA, CF and radioactivity. Infectivity of mouse brain-derived JEV was also in this position in the same gradient system (11). The peak fractions were brought to 1% SLS and 1% 2-ME, heated for 10 minutes at 100°C, and subjected to polyacrylamide gel electrophoresis (Figure 48a); it can be seen that three separable proteins are derived from the virion in this manner. Known marker proteins from Sindbis virus (described elsewhere) were electrophoresed in parallel and co-run and are indicated by arrows. The co-run with C^{14} Sindbis produced an artifact peak of ^{3}H because of "spillover" into the ^{3}H channel which was left wide open to maximize counts and was left out of the graph for this reason. By plotting marker proteins on $\log_{10}$ arithmetic paper, molecular weights of 53,000, 13,200, and 8,300 respectively were obtained for the three structural proteins. By comparing them with intracellular proteins plotted in the lower panel (Figure 48b), it was possible to identify the nonstructural and structural proteins produced in the infected cell. By this method, only the smallest structural protein was not found inside the infected cell. The structural proteins of JEV are very similar in size to the structural proteins of dengue-2.

VI. Arbovirus Infection in Man.

A. Epidemic Dengue in the Caribbean, 1969:

During 1969 major outbreaks of dengue occurred in the Caribbean. Epidemic dengue was reported in Jamaica, Puerto Rico, Venezuela, and French Guyana; many of the small islands also reported disease. Through the collaboration of Dr. L.S. Grant, University of the West Indies, Kingston, Jamaica, Dr. J. Ehrenkranz, University of Miami, and COL J.B. Moyar, Commanding Officer, Rodriquez Army Hospital, San Juan, Puerto Rico, serum specimens were obtained from patients with dengue-like illnesses. Dengue virus strains from Jamaica, Haiti and Puerto Rico were isolated in LLC-MK2 cell culture and identified by plaque reduction neutralization test using hyperimmune mouse ascitic fluids.

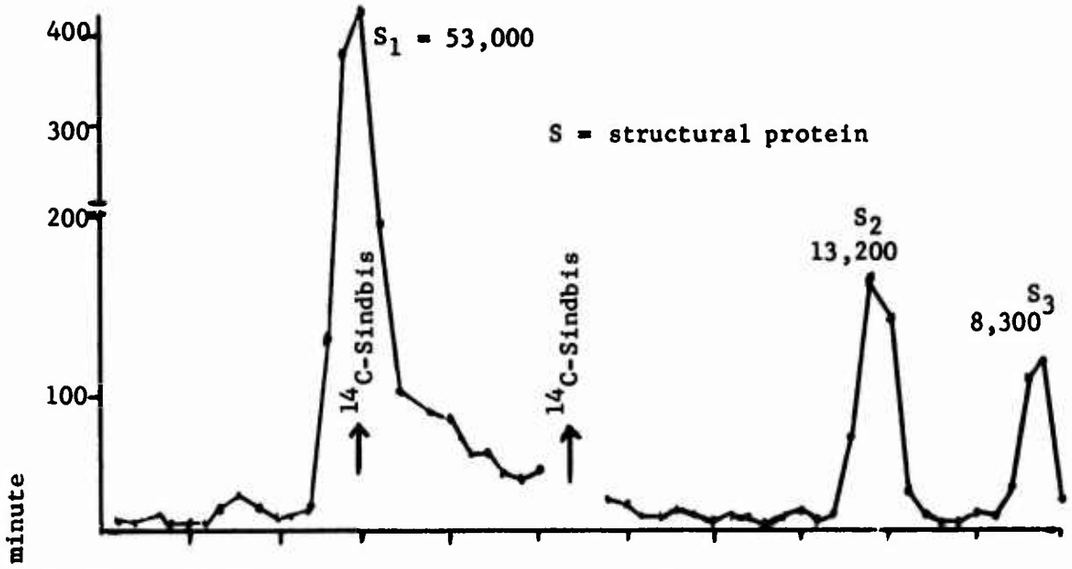
1. Jamaica: In 1968, epidemic dengue occurred in the Kingston-St. Andrews area. Several strains of dengue-3 were recovered from serum specimens collected in May through November 1968 and dengue-2 strains were recovered in December 1968 and January 1969 (Annual Report, 1969). Epidemic dengue continued during 1969 and spread to north coastal areas. Four viruses recovered in June, August and September 1969 were identified as dengue-2. The clinical syndromes associated with dengue infections included undifferentiated febrile illness and dengue fever. No cases of hemorrhagic fever or shock syndrome were reported.

2. Haiti: Two viral agents isolated from the serum of patients with dengue in Port au Prince in May 1969 were identified as dengue-2.

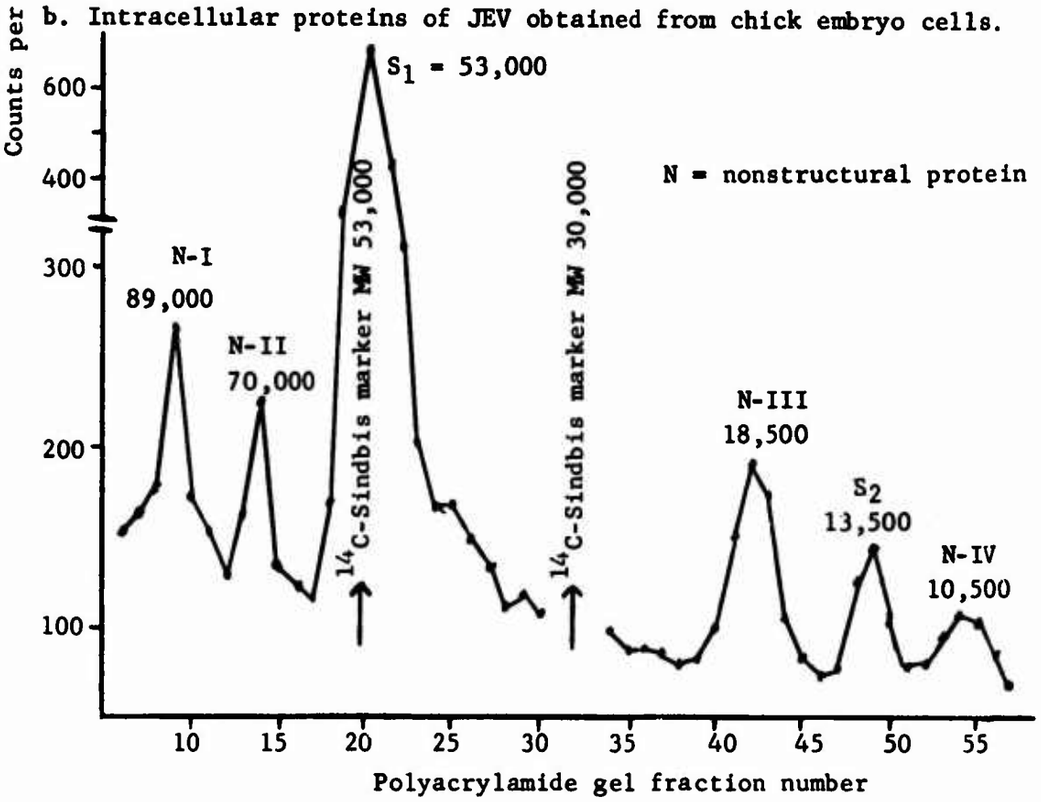
3. Puerto Rico: A major epidemic of dengue swept over the entire island of Puerto Rico between June and October 1969. Over

Figure 48. Comparison of Japanese encephalitis virus structural proteins with virus specific proteins inside the infected cell as analyzed on parellel polyacrylamide gels.

a. Structural proteins of JEV propagated in LLC-MK2 cells.



b. Intracellular proteins of JEV obtained from chick embryo cells.



16,000 cases were reported by the Department of Health and unofficial estimates are many times the official figure.

A clinical evaluation of 54 outpatients with serologically confirmed dengue infections was carried out at Rodriquez Army Hospital in July-August 1969. The clinical manifestations ranged from mild undifferentiated febrile illness to classical dengue fever illness to classical dengue fever with severe myalgias and typical rash. No hemorrhagic disease was observed in this study nor was any reported elsewhere on the island.

Thirty-five dengue viruses recovered from patients serum were identified as dengue-2

4. Characterization of Dengue Viruses from the Caribbean, 1968-1969: Neutralization (N) tests were carried out with hyperimmune mouse ascitic fluids made against prototype dengue viruses. N antibody titers against representative Caribbean strains are given in Table 23.

Table 23. Identification of Representative Dengue Strains from Caribbean 1968-1969

	HMAF			
Strain	DEN-1	DEN-2	DEN-3	DEN-4
Jamaica 837/68	10*	<10	110	<10
Jamaica 1007/68	<10	<10	120	<10
Jamaica 1203/68	<10	600	<10	<10
Jamaica 154/69	<10	>640	<10	<10
Jamaica 379/69	<10	250	<10	<10
Haiti 26/69	<10	500	20	<10
Haiti 100/69	<10	600	20	<10
Puerto Rico 109/69	<10	350	20	<10
Puerto Rico 152/69	10	800	20	<10
Puerto Rico 156/69	<10	500	10	<10
Puerto Rico 159/69	<10	450	<10	<10
(Homologous Viruses)	(300)	(1200)	(160)	(100)

* Reciprocal of 50% plaque reduction titer.

The dengue-2 strains from Jamaica, Haiti, and Puerto Rico all appear similar.

The 1968 Jamaican dengue-3 strains exhibited biologic characteristics similar to the 1963 dengue-3 strains from Puerto Rico. The new strains were successfully propagated only in cell cultures, attempts at mouse adaptation using standard methods were unsuccessful. The strains grew relatively poorly in LLC-MK2 cell culture. Maximum titers of 6×10^2 pfu/0.2 ml were observed after four passages. No CPE was

apparent even after 14 days incubation in cell culture. In contrast, the 1968-69 dengue-2 strains from the Caribbean grew rapidly in cell culture and produced visible CPE in 6 to 10 days. The dengue-2 strains were virulent for suckling mice producing paralysis and death on first intracerebral passage.

Two 1969 strains of dengue-2, PR-109 and PR-B, were compared with the prototype New Guinea "C" strain and the TR-1751 strain by plaque reduction neutralization tests using hyperimmune mouse ascitic fluids, a hyperimmune monkey antiserum and a human convalescent serum. The PR-109 strain was used at the 5th mouse passage, the PR-B strain was in the second LLC-MK2 passage. The New Guinea "C" and TR-1751 strains were in the 31st and 55th mouse passage respectively. The monkey antisera, obtained through the courtesy of Dr. C. Calisher, NCDC, Atlanta, Georgia, was made by inoculating a rhesus monkey 3 times with a 1969 strain of dengue-2 (P-28307) from Puerto Rico.

The results summarized in Table 24 provide strong evidence that the dengue-2 strains tested are identical. No differentiation of strains is apparent by this method in spite of a wide variation in time and place of origin and passage history.

Table 24. Comparison of Dengue-2 Viruses by Plaque Reduction Neutralization Tests

Virus Strain	Antibody NG-"C" (HMAF)	TR-1751 (HMAF)	PR-109 (HMAF)	P-28307 (Monkey)	PR-165 (Human)
New Guinea "C"	3000*	1700	2000	400	300
TR-1751	1300	1300	1200	350	200
PR-109	2800	1600	1400	500	400
PR-B	1800	1600	2600	400	180

* Reciprocal of 50% plaque reduction titer.

There is good evidence for significant strain variation within the dengue-3 serotype. The dengue-3 strains recovered in Puerto Rico in 1963 and Jamaica in 1968 can be readily differentiated from Asian strains of dengue-3 by biologic parameters, further, the Tahitian dengue-3 strain recovered by Rosen can be differentiated by biologic and antigenic parameters. This allows the tentative conclusion that the 1968 dengue-3 strain was a "native" of the Caribbean, at least since 1963, and not a recent import. However, this distinction is, as yet, impossible with dengue-2 strains. No technique has yet succeeded in differentiating Asian and American strains of dengue-2. The source of

the 1969 epidemic strain cannot be determined, the virus may have remained in an endemic focus in the Americas since the mid-50's or it may have been recently imported from elsewhere in the world.

B. Immunologic Response to Dengue Infection in Man:

It has been previously demonstrated that primary dengue infections cause an initial rise in IgM antibodies followed by a rise in IgG antibodies. Secondary infections with dengue differ from primary infections in that anti-dengue IgG is present at the time of infection and an anamnestic reaction occurs with a depressed IgM response and a rapid increase in levels of cross-reactive IgG. Because secondary infections with dengue virus are associated with an increased probability of severe disease, studies were undertaken to extend our knowledge of the immunological differences in human responses to primary and secondary dengue infections.

Preliminary studies utilized sera collected in the 1969 Puerto Rican dengue-2 epidemic. Six early convalescent sera from patients with primary dengue infections were fractionated using the sucrose density gradient ultracentrifugation technique. The fractions were tested for protein (Lowry's method), hemagglutination-inhibiting (HI) antibody, and complement-fixing (CF) antibody. The presence of IgM antibody was shown by its position in the sucrose gradient in fractions 1-3 (Figure 49). Antibody in these fractions was susceptible to reduction by 2-mercaptoethanol.

A similar technique was followed on sera from three individuals who had antibody titers which indicated a previous experience with group B arbovirus (Figure 50). Dengue antibodies may have been acquired by these individuals during the 1963 dengue-3 epidemic in the Caribbean. However, because of the nature of the military population from which the serum was obtained, it is impossible to tell the origin of this experience. All these military personnel and many dependents had been immunized with yellow fever vaccine; thus, making it extremely difficult to analyze previous exposure to dengue. Nonetheless, IgM antibodies were present in these sera.

To elucidate the immunoglobulin response to primary and secondary dengue and the interactions of the various anti-dengue antibodies, techniques were developed to isolate specific immunoglobulins. Once isolated, the anti-dengue activity was studied by HI, CF, and neutralization tests. Preliminary methodology was worked out using serum #PR-147, obtained on the 5th day following the onset of disease due to dengue-2 in a 20-year-old airman who had been in Puerto Rico only 4 months. He had no previous history of dengue infection but had received a yellow fever vaccination.

IgG was isolated by batch extraction with DEAE Sephadex A-25 at low ionic strength. After isolation of the IgG, the DEAE beads were washed with a higher ionic strength buffer causing the release of an IgM containing fraction. This IgM was further purified using a Sephadex G-200 column.

Figure 49. Protein Concentrations and Anti-dengue Titers From a Sucrose Gradient of Primary Dengue Infection. (Serum Pr.120)

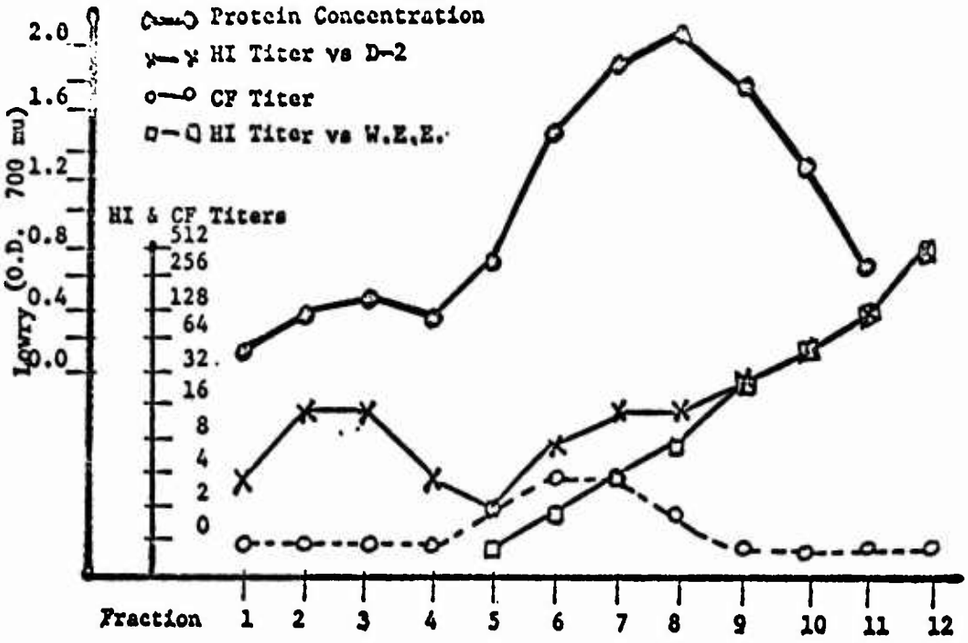
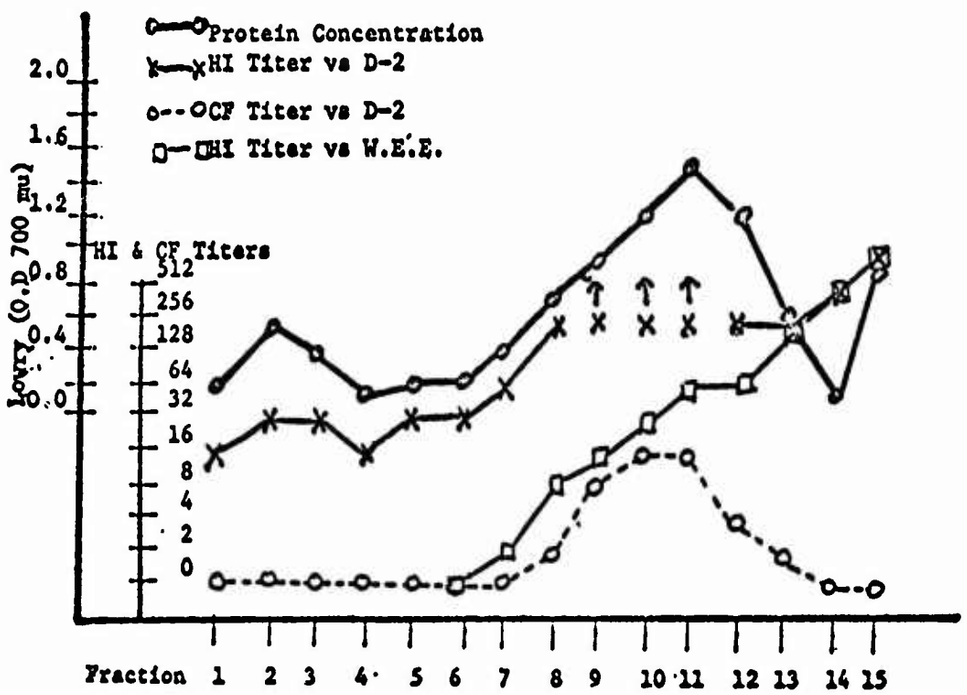


Figure 50. Protein Concentrations and Anti-dengue Titers From a Sucrose Gradient of Secondary Dengue Infection. (Serum Pr. 147)



Presence of immunoglobulins was determined by immunoelectrophoresis and concentrations of immunoglobulins were measured by the single radial diffusion technique. The immunoglobulin preparations were concentrated by pressure filtration to approximately the concentration in the initial serum.

Hemagglutination-inhibition tests on the PR-147 serum and on the purified immunoglobulin preparation were done using eight hemagglutinating (HA) units of dengue-2 SHA extracted from mouse brain. The same preparations were also tested against eight HA units of dengue-1, dengue-3, Japanese B encephalitis, yellow fever, and western equine encephalitis antigens. Dengue-2 HI antibody activity of whole PR-147 serum was due to both IgM and IgG (Table 25). The IgG fraction contributed most of the activity of whole serum because of the higher concentration of IgG.

Table 25. Immunoglobulin Content and Anti-Dengue-2 Hemagglutination-Inhibiting Activity of Whole PR-147 Serum and Its Isolated Immunoglobulins

	IgM Conc	IgG Conc	HI Titer
PR-147	80 mg%	1300 mg%	2560[a]
IgM	65 mg%	<4 mg%	256
IgG	<4 mg%	1400 mg%	4096
IgG & IgM	65 mg%	1400 mg%	4096

[a] Reciprocal titer vs. 8 units of dengue-2 SHA anti-dengue.

Table 26 shows the HI titers of the whole serum and its isolated immunoglobulins against several heterologous arboviruses. Of interest is the specificity of the IgM antibodies produced in response to dengue-2 infection compared to the broad cross-reactivity of the IgG antibodies. It is possible, however, that this may, in part, reflect a quantitative rather than a qualitative difference since the IgG titers are extremely high and endpoints were not determined.

Neutralization tests were done by plaque reduction versus prototype viruses. Initial studies showed that PR-147 and its isolated immunoglobulins all neutralize the homologous dengue-2 virus to relatively high dilutions (Table 27). IgG and the whole serum were found to have considerable cross-reactivity with dengue-1 and dengue-3. IgM, on the other hand, is again noted to be much more specific than IgG.

Table 26. Hemagglutination-Inhibiting Antibody Titers of PR-147 and Its Isolated Immunoglobulins

	Antigen					
	DEN-1	DEN-2	DEN-3	YF	JBE	WEE
PR-147	1024[a]	2048	512	>4096	2048	<2
IgM	8	256	<2	16	<2	<2
IgG	>4096	>4096	>4096	>4096	>4096	<2

[a] Reciprocal titer vs. 8 units of antigen.

Table 27. Neutralization Characteristics of PR-147 and Its Isolated Immunoglobulins Against Dengue-1, -2, and -3

	PR-147	IgM	IgG
Dengue-1	380[a]	<10	470
Dengue-2	810	150	290
Dengue-3	>20	<10	>20

[a] Reciprocal of 50% neutralization titers.

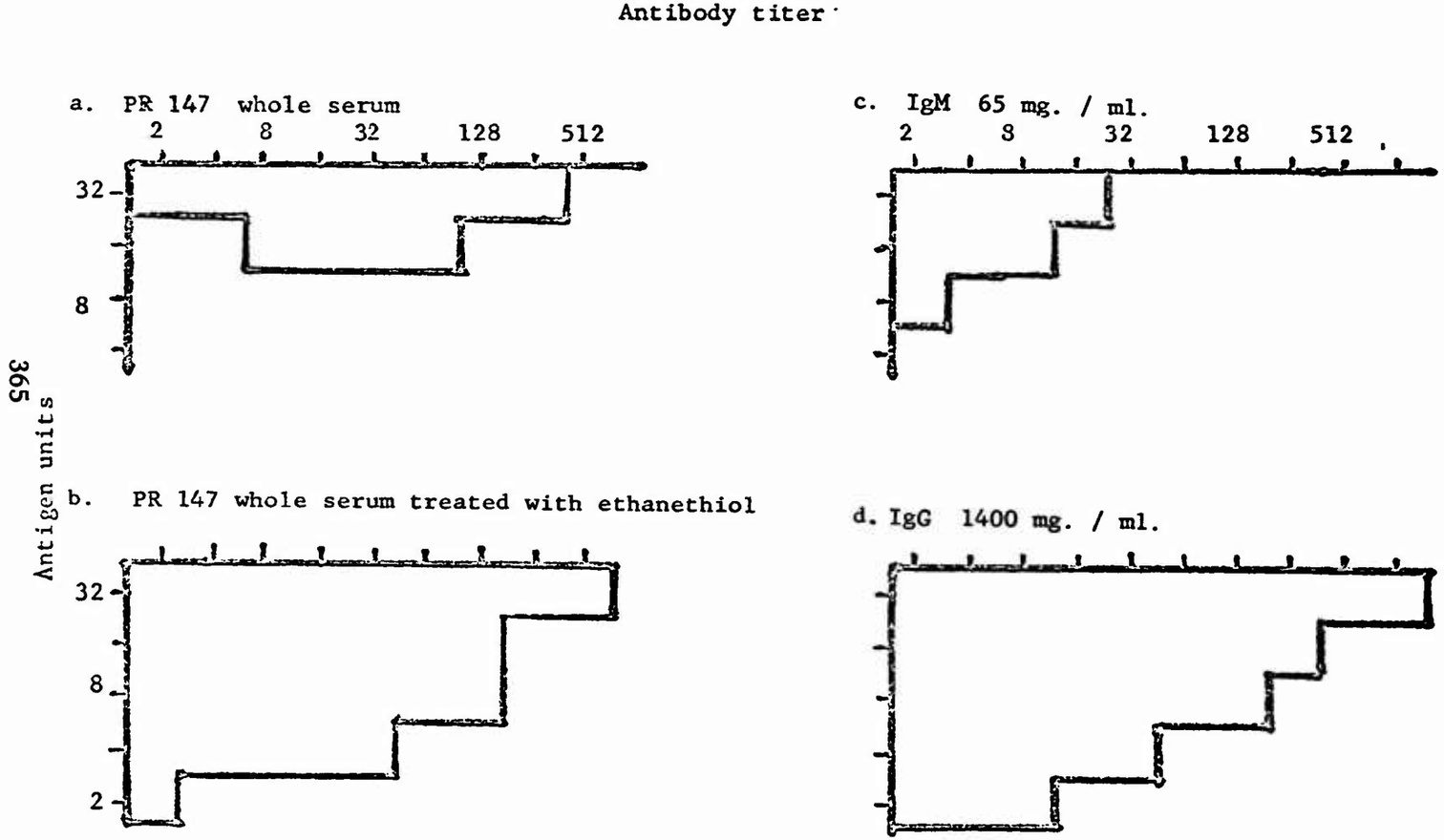
In the PR-147 serum, IgM anti-dengue activity differs from IgG activity by having greater specificity in both the hemagglutination-inhibition and neutralization systems.

It appears that different antigenic determinants may be involved, a type-specific antigen giving rise to IgM and a group-specific determinant stimulating an anamnestic IgG response.

Complement-fixation tests were carried out using crude saline CF antigens. Data are summarized in Figure 51; antigen units given are based on antigen titrations vs. homologous hyperimmune mouse ascitic fluid. Anti-dengue IgG fixed complement with 2 units of crude dengue-2 antigen while IgM antibodies required at least eight CF units and fixed complement only in areas of high antibody concentration (Figure 51 c & d).

With the PR-147 serum (and certain other sera as well), complete complement-fixation occurred when a large amount of antigen was used.

Figure 51. Complement fixation reactions of whole human serum and isolated immunoglobulins with dengue-2 crude saline antigens.



However, as the amount of antigen decreased a prozone developed (Figure 51a). Explanation was sought as to the nature of this prozone. Pretreatment of antigen with dilutions of IgM antibody below those necessary to fix complement have been shown to block the CF activity of whole serum. As there was a considerable amount of IgM antibody present in all the sera showing prozone reactions, the IgM activity was destroyed using a mercaptan, ethanethiol, to which IgM is sensitive. Complement-fixation of the ethanethiol-treated serum showed no prozone (Figure 51b) and a similarity was noted between the CF relation of the ethanethiol-treated serum and the IgG antibody extracted from that serum (Figure 51). This suggested that removal of IgM from the serum allowed the full expression of the CF activity of the IgG.

It appeared, therefore, that IgM fixes complement inefficiently and only with large concentrations of antigen. With lesser concentrations of reactants IgM still combines with the antigen but will not fix complement. However, because it combines, it does inhibit the action of IgG giving rise to large prozones and/or no complement-fixing reactions in standard systems. This might be one explanation for the late appearance of complement-fixing activity in the response to dengue infections.

The difference in the immunological activities of anti-dengue IgM and IgG in vitro gives rise to questions regarding their activity in vivo, which may in turn provide an immunological basis for the observed clinical differences between primary and secondary dengue infections.

VII. Ecological Studies of Western and Eastern Encephalitis Viruses in Maryland.

Repeated isolations of Western and Eastern Equine Encephalitis viruses (WEE and EEE) from a river swamp near Pocomoke City, Maryland, have been reported in detail in previous annual reports. During this year, a study of arbovirus ecology in the Pocomoke Cypress Swamp was conducted with the following parameters as principal objectives:

1) To measure virus transmission in the swamp habitat proper in comparison with nearby habitats exhibiting a different ecology but under the influence of the swamp.

2) To determine if mosquitoes other than Culiseta melanura are naturally infected with EEE or WEE viruses.

3) To capture and test sufficient numbers of resident and migrant wild birds such that the relative species involvement might be compared.

4) To investigate the possibility that certain of the larger, longer-lived mammals, reptiles and amphibians may become infected with either of these viruses.

5) To investigate the influence of the epizootic of the previous year on virus maintenance in the swamp during an inter-epizootic year.

A. Study Sites:

Habitats were selected in and around the swamp which, in some cases, were quite different from those previously studied in the swamp Five major areas representing different habitats were examined: a) swamp grid; b) peripheral swamp; c) upland; d) farmland; and e) marsh.

Figure 52 is a pictorial representation of the general study area. The Swamp Grid was essentially the same area as that studied in 1968. The peripheral swamp habitat consisted of two separate sites, Super Swamp and Slab Road, both within the swamp area but on the periphery and separated from the Swamp Grid. The upland habitat was well defined by characteristic timber and the absence of standing water other than temporary pools. The marsh was also easily distinguished but adjoined farmland at one of the study areas, Pasture Point.

B. Methods:

Mosquitoes were collected by several methods, including CDC miniature light traps, light traps with added carbon dioxide (as dry ice), resting boxes, vacuum sweeping, Shannon traps, and human biting collections. All traps were operated from approximately one hour prior to sunset until three hours after sunrise. Mosquitoes were frozen and stored at -70°C. All mosquito identification was performed by Department of Entomology personnel. Female mosquitoes were identified and placed in pools of 25 for virus isolation testing. Species for which fewer than 25 individuals were collected at a given site on a given date were also tested regardless of the number of specimens in that pool.

Mosquito pools were triturated for virus isolation by adding 5-10 glass beads (6 mm diameter) to a Wasserman tube containing the mosquitoes and mixing briskly on a Vortex mixer until a fine suspension was achieved. A 1.5 ml quantity of growth medium diluent (Basal Medium, Eagles, containing 20 per cent heated fetal bovine serum and antibiotics) was added to each tube, the tube mixed and then centrifuged to sediment mosquito debris. A 0.1 ml aliquot of the supernatant fluid was added to each of two primary hamster kidney cell culture tubes (Gibco, New York). Cultures were examined microscopically at 24 hours post-inoculation and again at 48-hour intervals for 5 days. Cell cultures exhibiting any cytopathic effect (CPE) were frozen and subsequently passed a second time. Positive second passage material was identified by neutralization tests employing specific rabbit antisera prepared against EEE (Cambridge) and WEE (Macmillan) viruses.

Wild birds were captured in mist nets for two days each week; although inclement weather often prevented completion of the full netting times. Consistency with respect to total netting hours was not an essential component of this year's study since all data presented have been expressed as percentage of total birds examined,

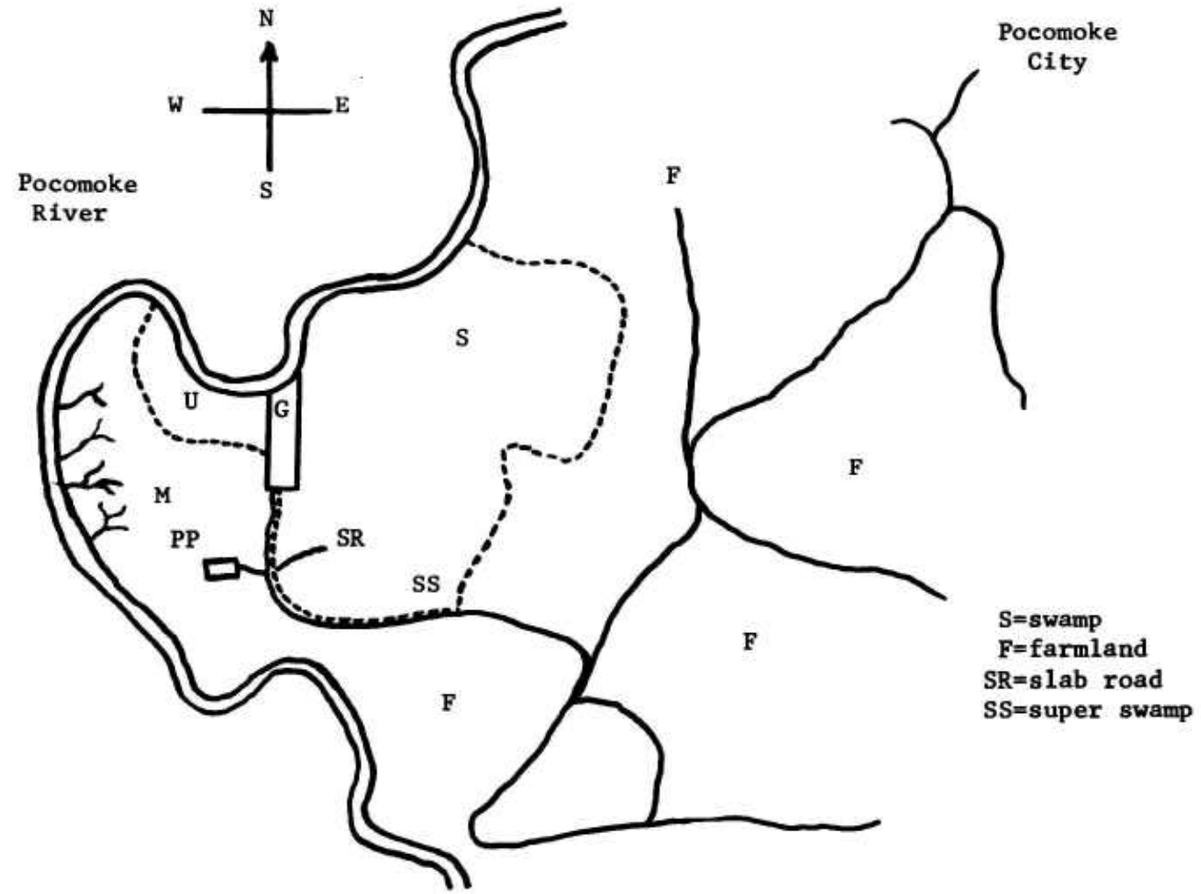


Figure 52. 1969 Pocomoke swamp study area

disregarding these values as an expression of total population densities. Birds were identified, bled by jugular venipuncture, recorded, banded and released. Heparinized blood specimens were centrifuged, the plasma and erythrocytes separated, and each stored at -70°C awaiting testing.

Virus isolation attempts were performed on the red blood cell portion of the blood sample in a manner similar to the testing of mosquito pools.

Plasma samples were heated at 56°C for 30 minutes, diluted 1:5 in growth medium containing 5 per cent fetal bovine serum, and tested for neutralizing antibody in a tube neutralization test utilizing two primary hamster kidney cell cultures per plasma sample and 30-70 tissue culture infective doses - 50 per cent ($TCID_{50}$) per test. Cultures were examined when virus test dose titrations first exhibited an optimal pattern and again after 5 days incubation. Samples exhibiting a definite retardation of CPE upon first reading but no effect after 5 days were considered suspicious, and those cultures with one or both tubes protected after 5 days incubation were considered positive for antibody against the particular virus being tested. Samples from negative, suspicious and positive sera were periodically selected and tested by plaque reduction neutralization testing. To date, all suspicious and positive sera have also been positive by this more precise method.

Eastern Bobwhite Quail (Colinus virginianus) were used in all sentinel studies. Quail were pre-bled prior to placing them in a mosquito-proof environment for an additional two weeks and a third blood specimen obtained. Blood specimens were tested for serologic activity and the presence of virus as described above for wild bird samples.

Reptiles, amphibians and some larger mammals were captured when opportunities arose, although no systematic trapping program was conducted. Animals were bled by cardiac puncture without heparin; in the case of some mammals, anesthetization with ether prior to bleeding was necessary. Serum specimens were tested for antibody using procedures described for wild bird plasma.

C. Results:

1. Mosquitoes: A total of 17 CDC miniature light traps baited with carbon dioxide were simultaneously operated in 17 different locations for one trap night each week. No attempt was made to select a night conducive to optimal mosquito capture based on advantageous weather conditions, i.e., cloud cover, temperature, humidity, etc.; however, those nights during which high winds or rains occurred were eliminated and the routine trapping performed on a more favorable night later in the week. The numbers of mosquitoes captured by this method are presented in Figure 53.

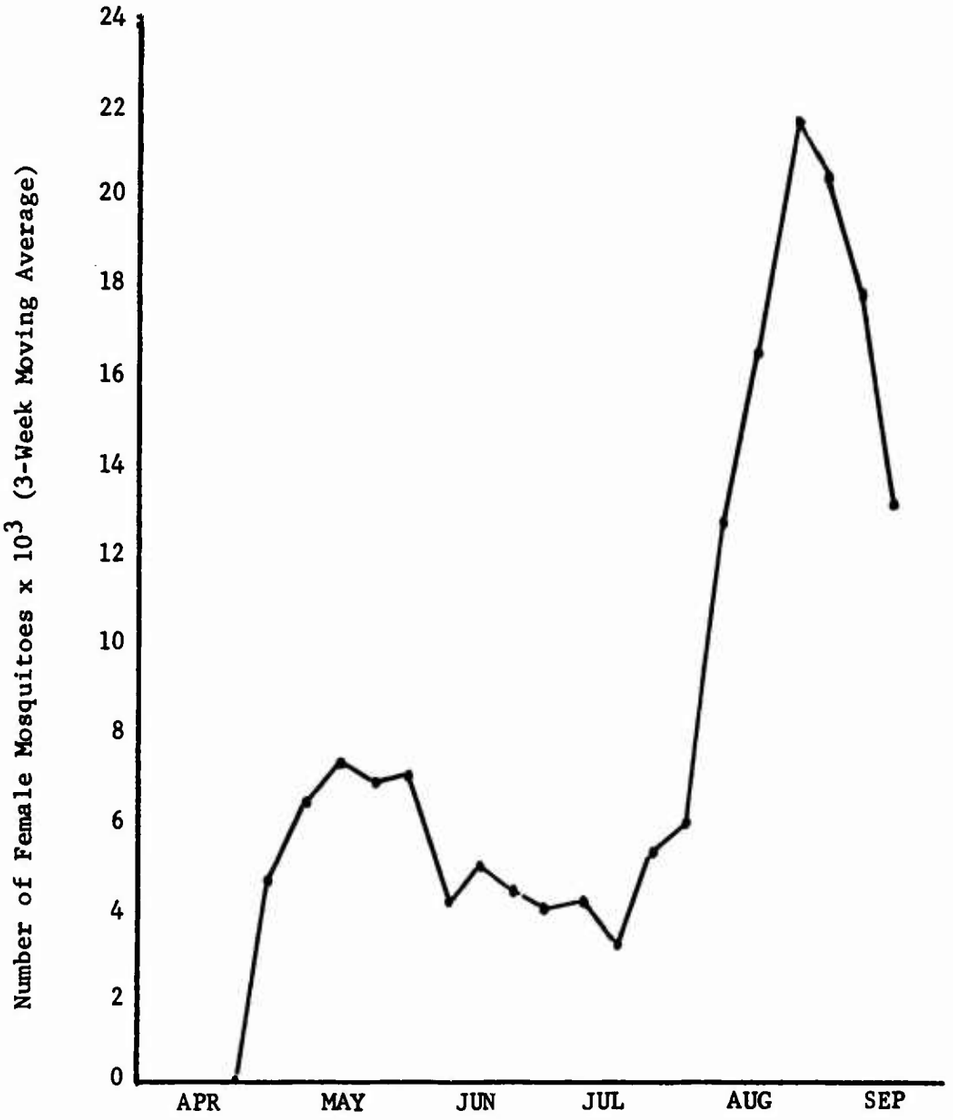


Figure 53. Total female mosquito captures from seventeen carbon-dioxide baited light traps.

The data are expressed as a 3-week moving average because of fluctuations in catch numbers attributable to variable climatic conditions such as cloud cover, temperature, humidity, etc. Mosquito populations increased during the latter part of April and exhibited a peak during the month of August. The abrupt onset of mosquito activity during late April is a valid observation, since mosquito trapping operations gave essentially negative results from early March until late April. The absence of data points beyond September reflects uncompleted laboratory work rather than the termination of collections.

The temporal relationships of the four major species collected by the light-CO_2 method are presented in Figure 54. These four species represented greater than 90 per cent of the total catch for the year. A single peak of Aedes cantator was observed early in the year, subsequently dropped to low levels in June and remained a minor consideration for the rest of the year. Aedes canadensis populations exhibited two peaks during the year, one in May and another during August. Culex salinarius were found only occasionally early in the year but numbers increased during July, and it became the most abundant species during late August. Culiseta melanura, the principal vector of EEE and WEE viruses in the Pocomoke Swamp, were collected in increasing numbers throughout May and June, and a maximum population density was attained during late July and early August. Based on the numbers of each of these four species collected during the year, it was anticipated that virus isolation attempts on all mosquitoes collected could provide a valid comparison of infection rates and probable importance in the transmission of EEE and WEE viruses in the study area.

Correlation of this year's findings with results of the 1968 study was attempted in order to detect any differences which might explain the alternating annual EEE and WEE virus cycles previously observed (Annual Reports, 1967 and 1968). Data from the two years could not be directly compared since 1968 studies employed only light trap mosquito collection and the 1969 study utilized light plus CO_2 as an attractant. An experiment designed to compare the different methods is described in Figure 55.

Five traps were placed at 200-foot intervals in the center of the swamp grid, such that each trap was in an equivalent area, but no two traps within sight of one another when observed at night with lights operating. All traps were operated on five consecutive nights during the month of June. Different conditions were employed on each of four of the traps, i.e., light plus CO_2, CO_2 only, light only, and suction only, the fifth trap being operated each night with light and CO_2 as a control for environmental conditions affecting trapping efficiency. Conditions at each of the four experimental traps were randomly rotated each night. The data are expressed as an average of the five nights for each of the four conditions.

As can be seen in Figure 55, light plus CO_2 resulted in the capture of more mosquitoes than any other method. Using light as the only

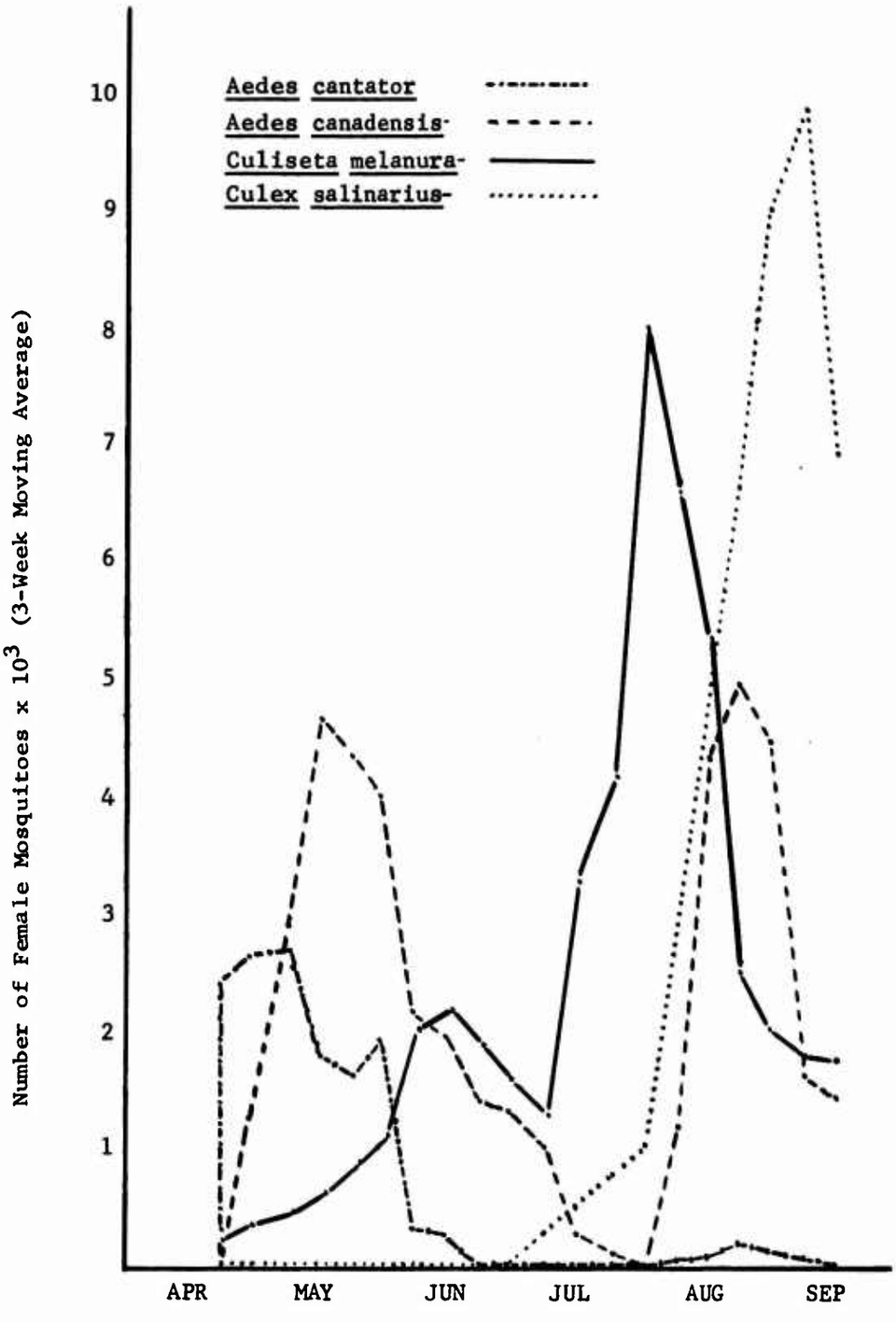


Figure 54. Temporal relationships of the four predominant mosquito species captured by carbon-dioxide baited light traps.

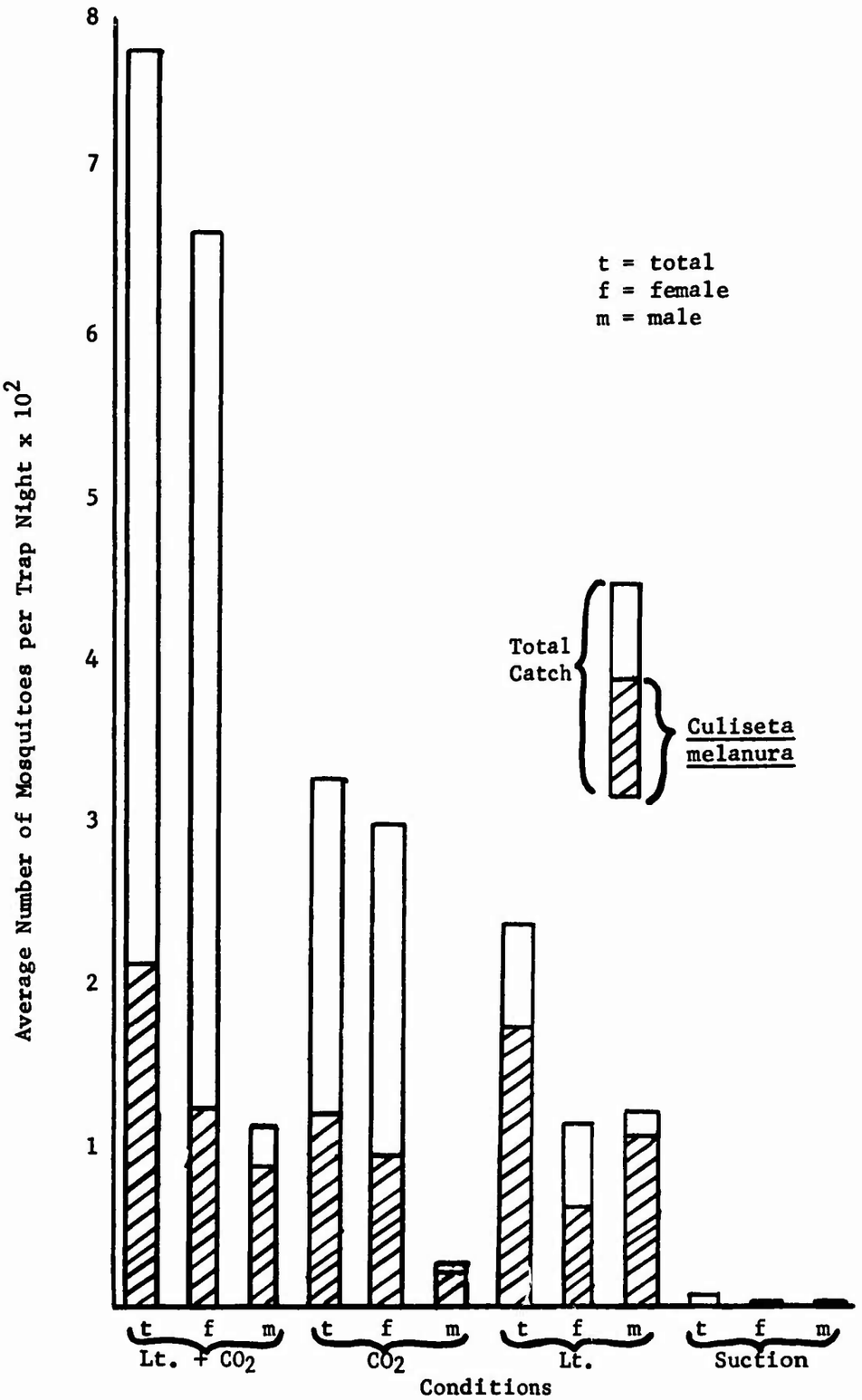


Figure 55. Comparison of Mosquito Trapping Attractants

attractant, total captures were reduced. It should be pointed out that essentially the same number of Culiseta melanura were collected from these traps as were captured using light plus CO_2.

In an attempt to compare the quantitative and temporal relationships of the 1968 and 1969 studies, three of the four sites used for for the 1968 study mosquito captures were again used during 1969, utilizing the same conditions as were employed the previous year, i.e., double traps, side-by-side and light only. Figure 56 depicts both total captures and Culiseta melanura captured by this collection method. Although greatly reduced in number, the temporal variation in these collections are quite similar to those described for light plus CO_2 collections (Figures 53 and 54).

A comparison of Culiseta melanura collections for 1968 (Figure 57) and 1969 (Figure 56) shows a comparable time of first appearance. The 1968 data suggest a more rapid numerical increase during May, June and July and a maximum peak slightly later than occurred during 1969; however, the differences observed between the two years are minimal.

A comparison of mosquito collections from six different habitats was made during 1969. In addition to the five distinct habitats previously described, i.e., swamp, peripheral swamp, upland, farmland and marsh, two traps were operated in areas representing transitions in habitats; however, each bordered on swamp and was possibly influenced by this close proximity (Figures 58-63).

Although most areas appeared similar with respect to total mosquito collections, both the farmland and upland habitats yielded comparatively few Culiseta melanura, while the total capture figures did not show these areas to be mosquito deficient. It was interesting to note that the marsh habitat yielded consistent numbers of Culiseta melanura throughout the year yet exhibited no July-August peak so characteristic of the other swamp-associated habitats.

Virus isolation was attempted on all female mosquitoes collected during the year. A listing of the 19 virus isolates obtained so far is presented in Table 28.

Over 12,000 mosquito pools have been tested for virus to date, representing all collections up to 8 September. Fifteen of the 19 viruses recovered have been identified. All agents isolated prior to 12 August were WEE virus, while three recovered between 18 and 26 August were EEE virus. All isolations were made from pools of Culiseta melanura, and it is of interest to note that two virus isolations were made from each of the upland and marsh habitats and three isolations made from farmland. These three areas exhibited reduced Culiseta melanura populations and consequently would tend to suggest elevated mosquito infection rates in these areas. The earliest virus isolation was made on 14-15 July, while the majority of the isolates (8 of 19) were obtained from 28-29 July collections - coinciding with the peak of Culiseta melanura captures during the year.

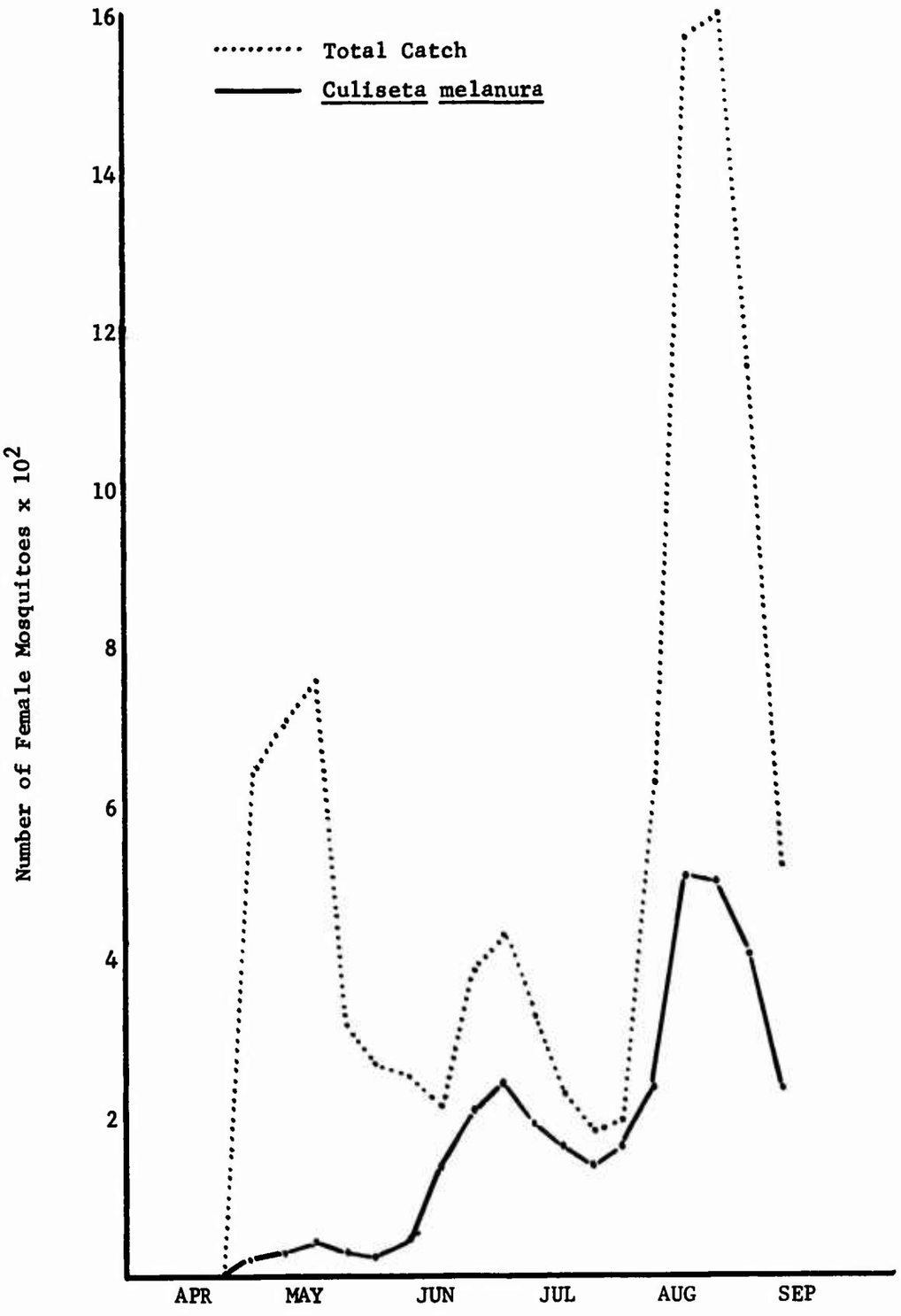


Figure 56. 1969 Light Trap Mosquito Captures

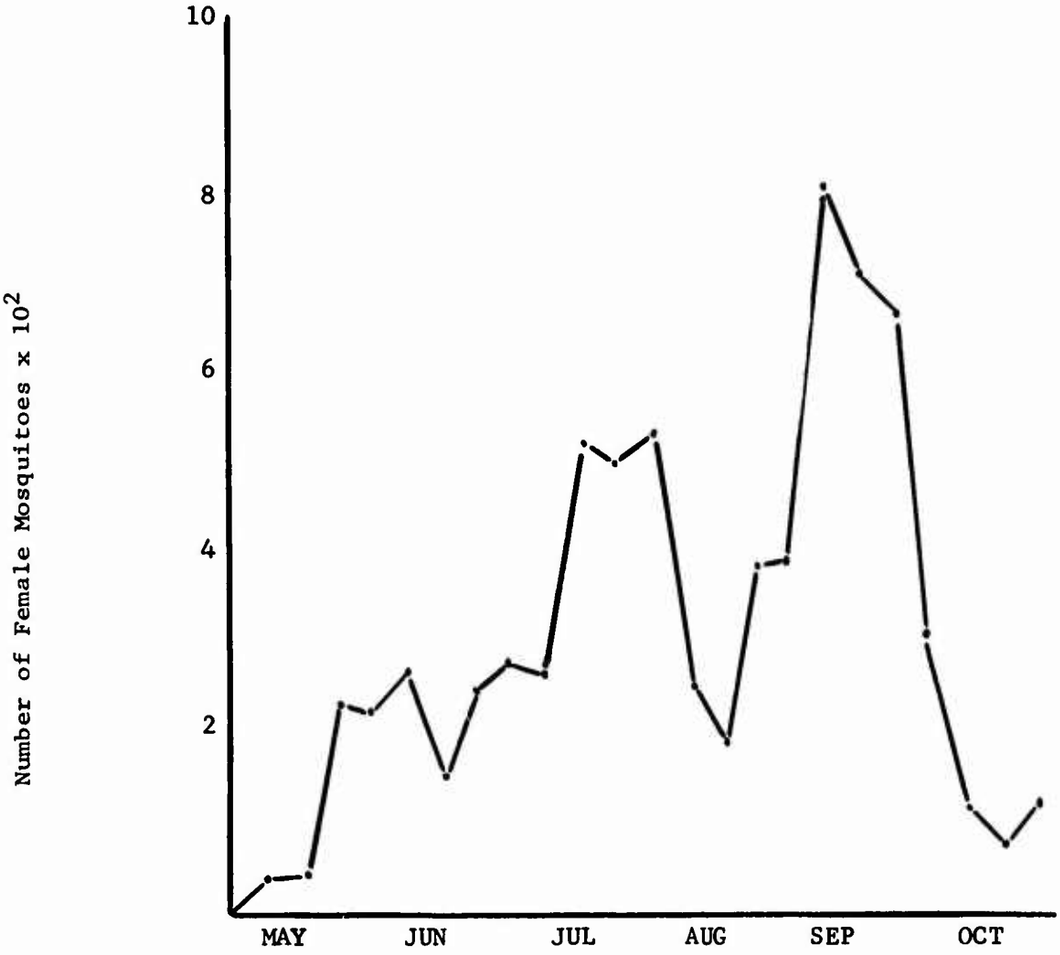


Figure 57. 1968 Light Trap Capture of Culiseta melanura Mosquitoes

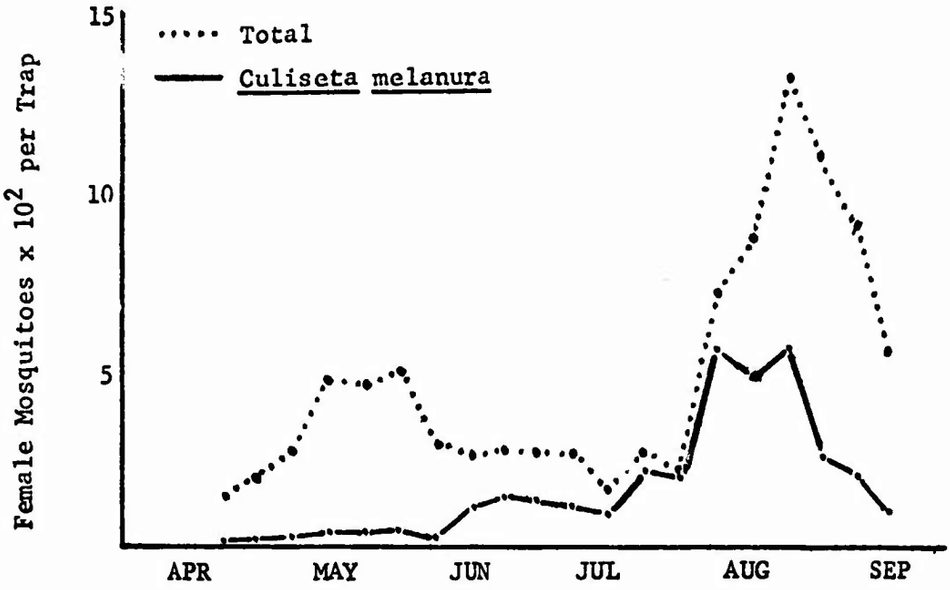


Figure 58. Swamp Grid Mosquitoes

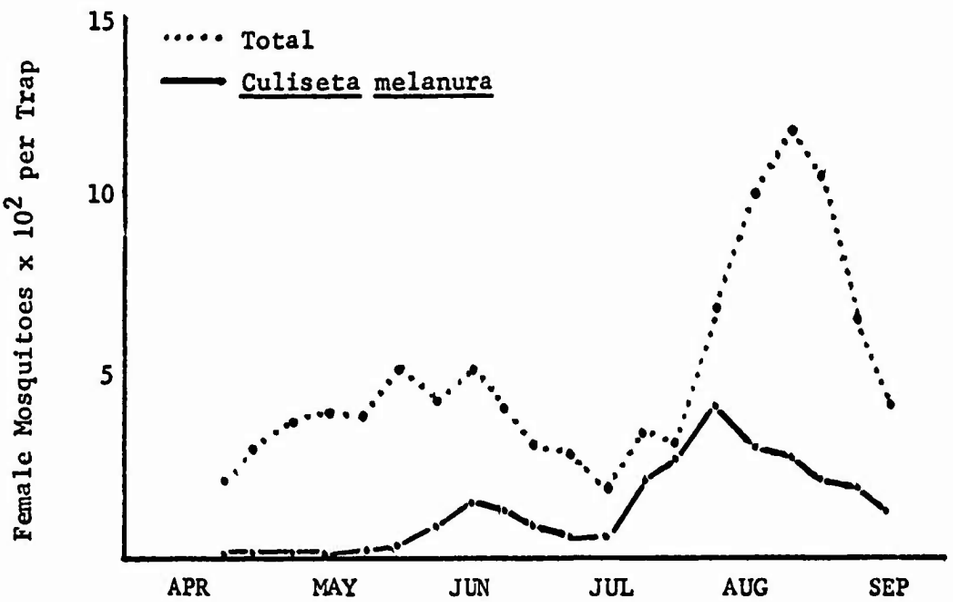


Figure 59. Mosquitoes of Transition Areas

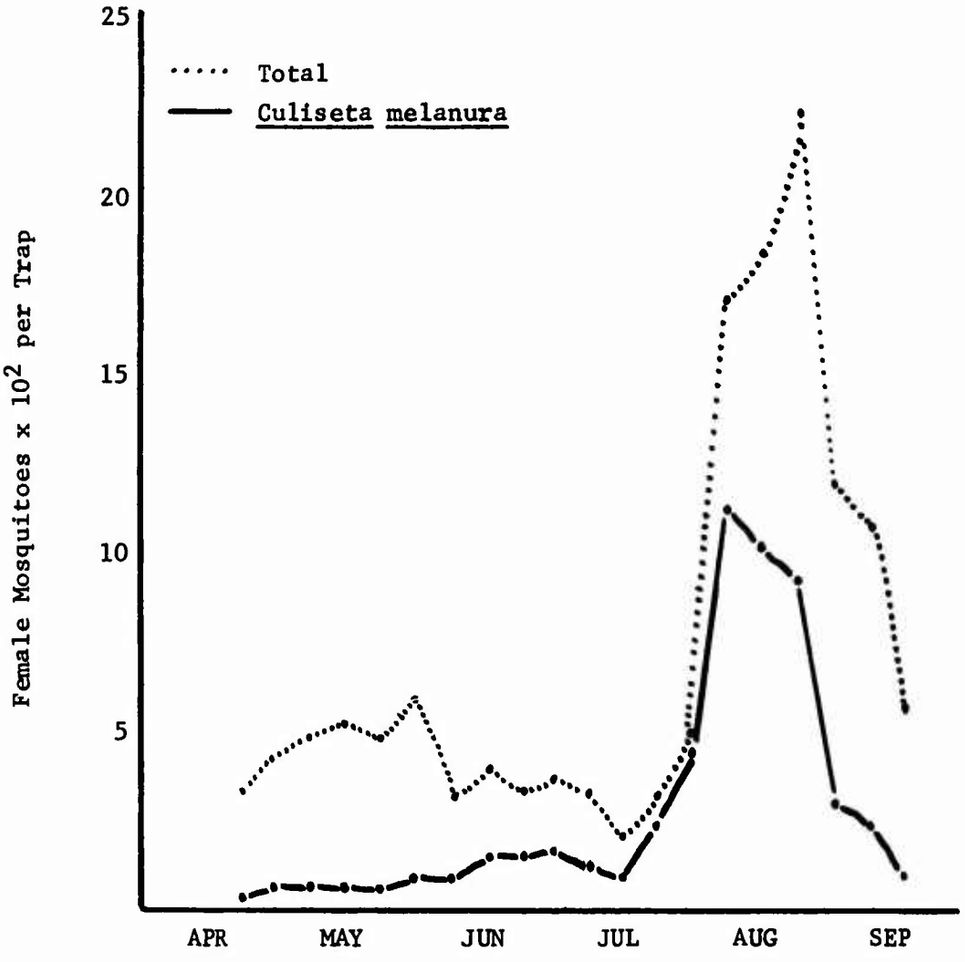


Figure 60. Peripheral Swamp Mosquitoes

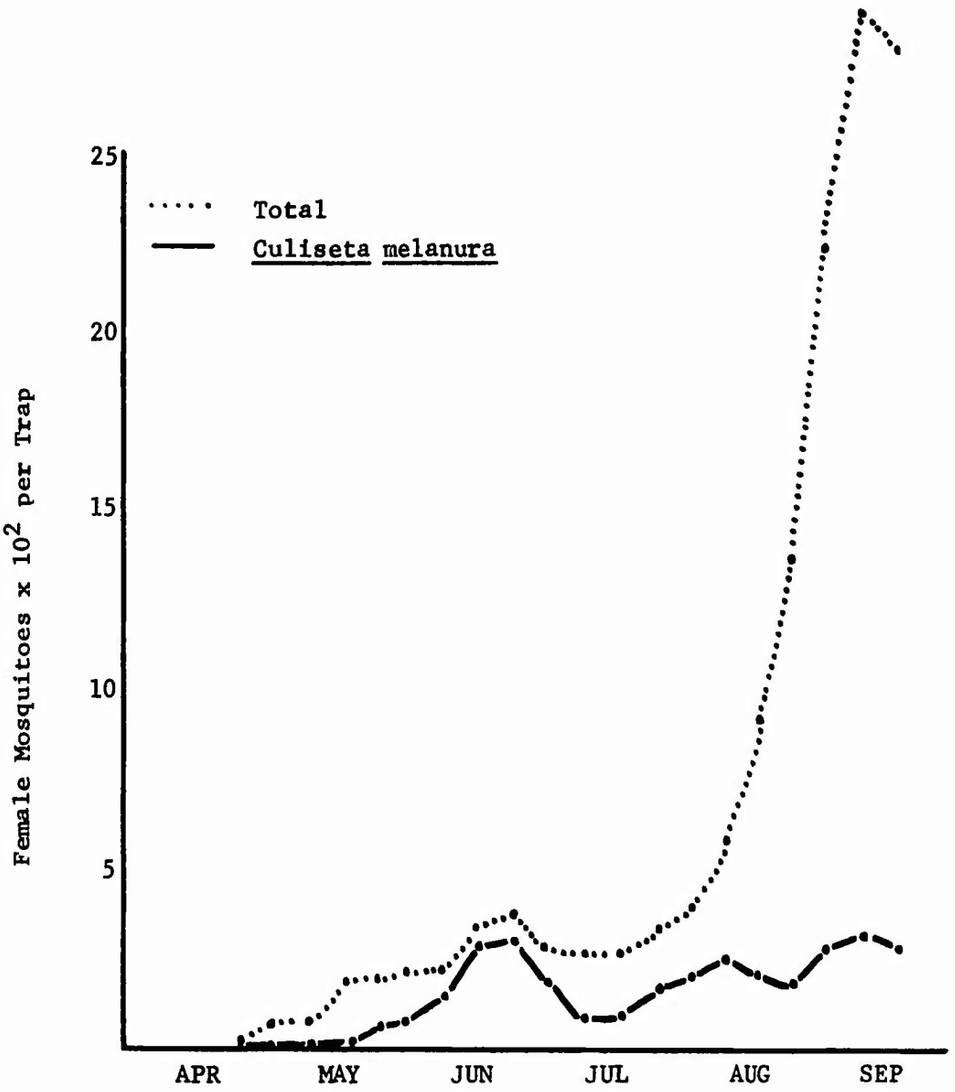


Figure 61. Marsh Mosquitoes

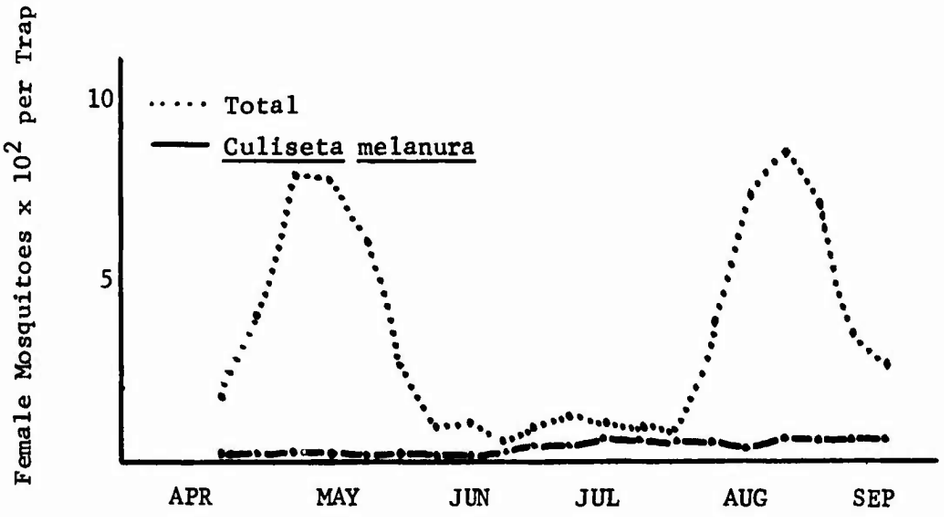


Figure 62. Upland Mosquitoes

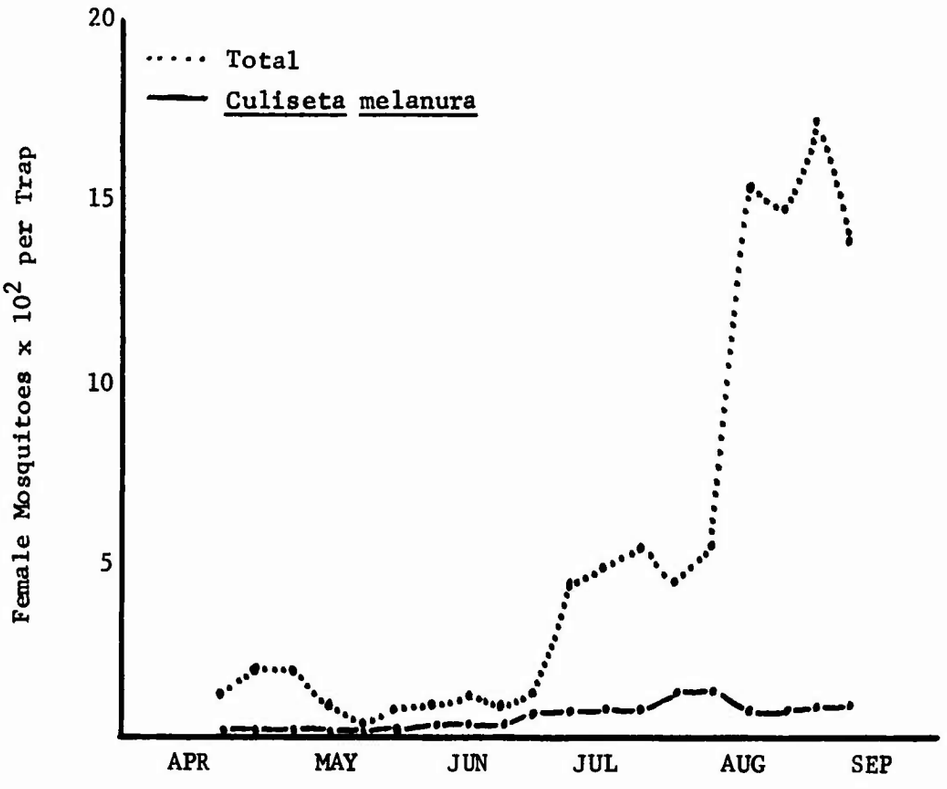


Figure 63. Farmland Mosquitoes

TABLE 28

VIRUS ISOLATIONS FROM MOSQUITOES

Pool No.	Mosquito Species	Date	Virus Identified	Habitat
5881	C. melanura	14-15 July	WEE	Marsh
5980	C. melanura	16-17 July	WEE	Swamp
6117	C. melanura	21-22 July	WEE	Peripheral Swamp
6695	C. melanura	28-29 July	WEE	Farmland
6701	C. melanura	28-29 July	WEE	Farmland
6751	C. melanura	28-29 July	WEE	Peripheral Swamp
6780	C. melanura	28-29 July	WEE	Transition
6816	C. melanura	28-29 July	WEE	Marsh
6894	C. melanura	28-29 July	WEE	Upland
6905	C. melanura	28-29 July	NT	Swamp
6950	C. melanura	28-29 July	WEE	Peripheral Swamp
7500	C. melanura	4-5 Aug	WEE	Swamp
8650	C. melanura	11-12 Aug	WEE	Swamp
10,815	C. melanura	18-19 Aug	EEE	Farmland
11,045	C. melanura	18-19 Aug	EEE	Transition
11,944	C. melanura	25-26 Aug	EEE	Upland
12,666	C. melanura	1-2 Sept	NT	Peripheral Swamp
12,878	C. melanura	1-2 Sept	NT	Transition
13,174	C. melanura	7-8 Sept	NT	Swamp

NT = Not Tested

C. Sentinel Quail:

Bobwhite Quail, Colinus virginianus, were used as indicators of virus activity in a bird population consistent with sentinel programs in previous years (Annual Reports, 1967 and 1968). One notable difference in the 1969 program, as opposed to those of previous years, was the addition of non-immune quail to the various areas every two weeks, providing a susceptible population throughout this year's study. All sentinel quail cages were placed at least 150-200 feet from any attractant used for capturing mosquitoes.

A total of 80 susceptible birds were employed at eight separate sites throughout the year. One cage of 10 birds was maintained on Chincoteague Island, Virginia, to serve as a control since few, if any, Culiseta melanura mosquitoes have ever been collected in this area (Annual Reports, 1960 and 1961). The remaining seven sentinel cages were distributed throughout the study area, three at separate sites on the swamp grid, two at the peripheral swamp study areas, one in the upland habitat and one on a pasture point bordering the marsh which shared a marsh-farmland habitat. The serological conversions of these birds from negative to positive for antibody to either EEE or WEE virus are summarized in Table 29.

The complete absence of any detectable antibody to either EEE or WEE virus in any of the birds maintained at Chincoteague, Virginia, allowed their exclusion from the total. During the latter part of the summer (September and October), a few birds were received from Willards, Maryland, which contained antibody in the pre-bleed specimens, and certain birds were re-used during the latter part of the study which also contained antibody in the pre-bleed specimens, explaining differences observed in the EEE and WEE virus susceptible bird totals. At no time did these positive birds constitute greater than 10 per cent of the total; consequently, their exclusion from the total did not significantly reduce the number of available susceptible birds at any one time. It should be pointed out that these data represent the cumulative total of the birds converting during the 2-week exposure in the study area and those converting during the subsequent 2-week period of maintenance in a mosquito proof environment, so that any animal infected on its last day of exposure would still record a positive conversion.

As can be seen from the table, a peak of WEE virus activity was observed during late July and early August, in which greater than 90 per cent of exposed birds subsequently contained antibody to WEE virus. Although EEE virus activity was demonstrated by these methods, it occurred at least 6-8 weeks later in the year and at a much lower level than WEE virus. Virus activity, as measured by this method, was first detected during June and was found to persist until late October or early November.

The three swamp habitat sentinel cages were designated Swamp 40Q, 20Q and 1Q, and were placed near the edge of the Pocomoke River on the swamp grid (40Q) and at 20 chain intervals south into the swamp grid

TABLE 29

ANTIBODY CONVERSIONS OF SENTINEL QUAIL

Exposure	Virus Antibody	Habitat: Swamp 40Q	Swamp 20Q	Swamp 1Q	PERI Swamp Slab Road	PERI-Swamp Upper Swamp	Pasture Point	Upland	Total	Chinco. Is. Control
19 Mar-26 May	WEE	0/10*	0/10	0/10	0/10	0/10	0/10	0/10	0/70	0/10
	EEE	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/70	0/10
27 May-30 Jun	WEE	5/10	9/10	0/10	2/10	1/9	0/10	1/10	9/69	0/10
	EEE	0/10	0/10	0/10	0/10	0/9	0/10	0/10	0/69	0/10
1 Jul-28 Jul	WEE	9/9	10/10	9/9	10/10	9/10	10/10	7/10	64/68	0/10
	EEE	0/10	0/10	0/9	0/10	0/10	0/10	0/10	0/69	0/10
29 Jul-11 Aug	WEE	10/10	7/7	9/10	10/10	3/7	10/10	5/10	54/64	0/10
	EEE	0/10	1/7	0/10	0/10	0/10	0/10	0/10	1/67	0/10
12 Aug-25 Aug	WEE	6/10	4/10	0/10	6/8	3/7	6/10	0/10	25/65	0/5
	EEE	0/10	1/10	0/10	2/9	2/10	1/10	0/10	6/69	0/10
26 Aug -8 Sep	WEE	3/6	2/10	5/10	6/9	1/6	2/10	0/10	19/61	0/4
	EEE	0/6	3/10	1/10	1/9	2/10	2/10	0/10	9/65	0/10
9 Sep-23 Sep	WEE	0/10	0/10	0/10	8/10	0/10	2/10	0/10	10/70	0/10
	EEE	0/10	1/10	0/10	1/10	0/10	0/10	0/10	2/70	0/10
24 Sep -7 Oct	WEE	2/10	0/10	0/10	1/8	0/9	4/8	0/9	7/64	0/10
	EEE	1/10	0/10	1/10	0/9	1/10	0/10	0/9	3/68	0/10
8 Oct - 20 Oct	WEE	1/10	0/10	1/10	3/10	2/10	1/9	3/9	11/68	0/10
	EEE	0/10	0/10	0/10	4/10	0/10	2/9	0/9	6/68	0/10
21 Oct - 10 Nov	WEE	0/10	0/10	0/10	1/10	0/3	0/10	0/9	1/62	0/10
	EEE	1/8	0/10	1/10	0/10	1/3	0/10	0/9	3/60	0/10
11 Nov - 15 Dec	WEE	0/10	0/10	1/10	0/10	0/10	0/10	0/10	1/70	0/10
	EEE	0/5	0/6	0/7	0/7	0/8	0/8	0/6	0/47	0/10

* No. of Birds Exhibiting Antibody Following Exposure / No. of Susceptible Birds Exposed

(20Q and 1Q). No pronounced differences were noted between any of the three areas in that they all reflected the July peak of WEE virus activity, however, data obtained near the river's edge (40Q) would suggest earlier virus activity during June and activity remaining later during September and October. The pasture point study area, previously described as sharing a farmland and marsh habitat, demonstrated, in addition to the July peak of activity, a second peak of WEE virus activity during October. Both representatives of the peripheral swamp habitat, Slab Road and Super Swamp, exhibited July activity peaks for WEE virus, although the Slab Road sentinels showed a longer peak of virus activity extending into October. The reduced activity found associated with the upland habitat was not unexpected in that reduced Culiseta melanura populations were also measured in that habitat. Sentinel conversions to positive antibody for EEE virus occurred relatively late in the year (Aug-Nov) but did not appear to be restricted to any given habitat. This late appearance of EEE virus activity is in good correlation with the three isolations of EEE virus from mosquitoes during 18-26 August. Further speculation relating sentinel conversions and mosquito populations must await data reduction at individual sites within each general habitat, i.e., Slab Road and Super Swamp mosquito collection data.

Virus isolation was attempted on all blood specimens taken from sentinel quail. Table 30 lists the six virus isolates obtained. All virus isolations were made from blood samples taken immediately following the 2-week swamp exposure period. Four WEE and one EEE virus isolate have been identified to date. The observation that three of the six isolates were made on 8 September would appear unexpected since most virus activity was measured earlier in the year. The serologic conversion data, however, from the peripheral swamp and farmland-marsh areas, areas from which these virus isolations were made, showed virus activity during this period. The isolation of EEE virus from a peripheral swamp habitat was also unexpected because of the low level of EEE virus activity measured by the sentinel program, however, 20 per cent of the quail at the peripheral swamp (Super Swamp) were positive on that date.

The serologic data on all quail from which isolations were made were examined as supportive evidence for each virus isolation. These data are listed in Table 31. All isolates except #1321 exhibited the same serologic profile of a negative pre-bleed, a negative post-swamp sample at the time of virus isolation, and a specific positive WEE antibody titer following two weeks in a mosquito proof environment. Although only four of these isolates have been positively identified as WEE virus, it is anticipated that identification neutralization testing will show #1309 to be a WEE virus isolation. Isolate #1321 illustrates an interesting case of a WEE positive bird, re-used as a sentinel, becoming infected with EEE virus and subsequently exhibiting EEE virus antibody titer. These data are presented as supportive evidence of the previously described virus isolations, as well as controls for the specificity of the neutralization testing routinely performed on all sentinel and wild bird sera.

Table 30. Virus Isolations from Sentinel Quail

Isolate No.	Virus Identified	Habitat	Date
812	WEE	Upland	1 Jul
1309	NT	Swamp (20Q)	25 Aug
1321	EEE	Peripheral Swamp (Super Swamp)	25 Aug
1820	WEE	Peripheral Swamp (Slab Road)	8 Sep
1821	WEE	Peripheral Swamp (Slab Road)	8 Sep
1852	WEE	Farmland-Marsh (Pasture Point)	8 Sep

NT = Not Tested

Table 31. Serologic Conversions of Virus Infected Quail

		Neutralizing Antibody Detection					
		Pre-Bleed		Post-Swamp		Post-Mosquito Proof	
Isolate No.	Virus	EEE	WEE	EEE	WEE	EEE	WEE
812	WEE	-	-	-	-	-	Pos
1309	NT	-	-	-	-	-	Pos
1321	EEE	-	Pos	-	Pos	Pos	Pos
1820	WEE	-	-	-	-	-	Pos
1821	WEE	-	-	-	-	-	Pos
1852	WEE	-	-	-	-	-	Pos

NT = Not Tested

- = Negative for antibody

D. Wild Bird Studies:

During 1969, several approaches to the study of bird populations and their relation to the virus transmission cycle were employed. The bird population of the study area was estimated by both observational and

netting methods. Several baited nets were operated at a central location during the winter months, both to sample wintering bird populations with a minimum amount of effort and to compare antibody levels in birds captured by the baited and non-baited netting methods. Thirty-seven mist nets (non-baited) were in operation in five major areas from 2 March to 15 December. Five areas were compared in an attempt to demonstrate differences in bird populations and degree of virus involvement between these areas. A breeding species exhibiting the greatest number of captures and the highest level of antibody was examined more closely in an attempt to determine whether a single species could provide a monitor of virus activity in the total bird population.

Routine observations were made throughout the year and a special breeding-bird census conducted; both procedures determined netting efficiency by correlating captured bird populations with an independent bird population estimate. These data are, at present, not fully analyzed but an initial study in 1968 indicated that the netting method captures all species observed but in different relative proportions. Bird species such as the Red-eyed Vireo, which regularly inhabit the upper canopy, were predictably captured at a much lower rate than would be estimated by observational methods. Species inhabiting the study area at a height less than eight feet, such as the White-eyed Vireo, were captured in relatively high numbers, perhaps representing more than 50 per cent of the observed population of this species.

The area surrounding two mist nets on the swamp grid was baited with bird seed during the winter months. This bait was a strong attractant for the ground-feeding species and observations seemed to indicate that these species preferred this baited area over all other net sites in the general area. These baited nets were in operation for eleven weeks between 5 January and 13 April and for the weeks of 25 November and 15 December 1969. During some of these weeks and at other times of the year, non-baited nets were in use at 37 sites. Table 32 shows the similarity in antibody level for three winter residents--White-throated Sparrow, Fox Sparrow and Slate-colored Junco captured in two different areas by two different methods. Concentrating ground-feeding winter residents by baiting in a specific area for subsequent capture required less effort than operating 37 nets throughout the study area, and the demonstrated antibody levels do not appear to be significantly different. The Cardinal is the only species listed that was present throughout the year, and it appears that winter sampling of at least the one permanent resident yielded approximately the same results as a continuous sampling throughout the year.

During 1969, 2550 birds of 76 species were captured by non-baited mist nets, bled, and tested for the presence of virus and antibody to either EEE or WEE virus. Table 33 lists all species captured, arranged in descending order with accompanying antibody data. Only 28 of the 76 species were represented by 20 or more specimens.

A rearrangement of the 28 most frequently captured species allows a comparison of the degree of virus involvement of these birds as measured

Table 32. Virus Neutralizing Antibody in Winter Residents

(Comparison of Baited and Non-Baited Nets)

Species	Baited Nets Positive/Tested	%	Non-Baited Nets Positive/Tested	%
White-Thr. Sparrow	17/70	24*	16/80	20
Slate-Col. Junco	11/111	10	5/53	9
Fox Sparrow	16/126	13	2/14	14
Cardinal	6/9	67	37/62	60

* Per cent of specimens positive for neutralizing antibody to either EEE or WEE virus.

by the percentages of each found positive for virus antibody. Table 34 describes three such groupings which upon examination were also found to have other common characteristics in addition to similar levels of antibody activity. The 13 species with greater than 25 per cent prevalence of EEE and/or WEE virus antibody were all typical breeding birds of the study area and consequently were those most likely to be in contact with infected mosquitoes, both with respect to location and time period of occurrence in the swamp area. The majority of the species with less than 24 per cent antibody were either transients or winter residents, groups that would not be in the swamp during period of peak virus transmission.

Table 35 divides the 76 species captured into four major temporal groups. Transient species were defined as those that passed through the area during migration in spring and/or fall, but did not winter or breed. Winter residents exhibited both fall and spring migration and stayed in the area during the winter months. Breeders usually showed a spring and fall migration peak and were present during the summer but not in winter. Permanent residents bred in the area but were also present during winter months and, in addition, may have shown some spring and fall migration activity. Table 36 shows the levels of antibody in the four temporal groups for the entire year.

Permanent residents and breeders in the swamp area represented approximately 60 per cent of the total numbers tested, but 84 per cent of those exhibiting antibody. These two groups have a higher level of antibody to WEE virus than to EEE virus, probably representing local WEE virus activity during the breeding season. The transient and winter resident groups have a higher EEE antibody level than WEE, possibly indicating EEE virus activity at their breeding areas further

TABLE 33

NEUTRALIZING ANTIBODY TO EEE AND/OR WEE VIRUS IN WILD BIRDS

Species Captured		Antibody					
		EEE		WEE		Both EEE & WEE	
	No. Tested	No.	(%)	No.	(%)	No.	(%)
Myrtle Warbler	499	38	(8)	9	(2)	1	(0)
White-eyed Vireo	162	28	(17)	60	(37)	44	(27)
Swamp Sparrow	120	21	(18)	5	(4)	2	(2)
Carolina Wren	114	18	(16)	38	(33)	7	(6)
Common Grackle	100	9	(1)	9	(1)	1	(1)
Worm-Eating Warbler	88	14	(16)	11	(13)	5	(6)
Hermit Thrush	84	5	(6)	6	(7)	2	(2)
Yellowthroat	83	5	(6)	22	(27)	5	(6)
Tufted Titmouse	83	7	(8)	31	(35)	26	(30)
White-Throated Sparrow	80	9	(11)	5	(6)	2	(3)
Prothonotary Warbler	77	4	(5)	4	(5)	0	(0)
Catbird	73	10	(14)	14	(19)	3	(4)
Robin	72	6	(8)	4	(6)	3	(4)
Ovenbird	71	9	(13)	19	(27)	6	(9)
Black & White Warbler	66	4	(6)	4	(6)	2	(3)
Carolina Chickadee	65	8	(12)	13	(20)	2	(3)
Cardinal	62	18	(29)	7	(11)	12	(19)
Slate-Colored Junco	53	5	(9)	0	(0)	0	(0)
Wood Thrush	46	6	(13)	8	(17)	22	(48)
Northern Waterthrush	41	3	(7)	0	(0)	0	(0)
American Redstart	38	3	(8)	0	(0)	0	(0)
Downy Woodpecker	35	2	(6)	3	(9)	0	(0)
Rufous-Sided Towhee	33	5	(15)	4	(12)	2	(6)
Redwing	32	0	(0)	5	(16)	0	(0)
Prairie Warbler	31	3	(10)	2	(7)	1	(3)
Song Sparrow	20	3	(15)	1	(5)	0	(0)
Red-Eyed Vireo	20	9	(45)	1	(5)	7	(35)
Pine Warbler	20	3	(15)	6	(30)	3	(15)

Table 33
(continued)

Species Captured	No. Tested	Antibody EEE No.	EEE (%)	WEE No.	WEE (%)	Both EEE & WEE No.	Both EEE & WEE (%)
Crested Flycatcher	17	1	*	1		1	
Yellow Shafted Flicker	15	4		5		2	
Fox Sparrow	14	1		1		0	
Acadian Flycatcher	14	1		3		1	
Yellow-Breasted Chat	13	1		2		3	
Yellow Throated Warbler	13	5		1		1	
Golden-Crowned Kinglet	11	1		1		0	
Blackpool Warbler	10	0		0		0	
Red-Bellied Woodpecker	10	1		3		0	
Hooded Warbler	10	0		0		2	
Brown Thrasher	10	2		3		0	
Blue-Grey Gnatcatcher	9	2		0		0	
Grey-Cheeked Thrush	9	3		0		0	
Hairy Woodpecker	9	2		2		0	
Brown-Headed Cowbird	9	0		1		0	
Black Throated Blue Warbler	9	0		0		0	
Kentucky Warbler	8	3		0		1	
Yellow Billed Cuckoo	8	0		1		0	
Parula Warbler	8	1		4		0	
Veary	7	1		0		0	
Brown Creeper	6	0		0		0	
Indigo Bunting	6	2		0		0	
Ruby-Crowned Kinglet	5	0		0		0	
Woodcock	5	1		1		1	
Swainson's Thrush	4	1					
Screech Owl	4	0		1		3	
Red Breasted Nuthatch	3						
Piliated Woodpecker	3	1					
Blue Jay	3	1					

Table 33
(continued)

Species Captured	No. Tested	Antibody EEE No.	EEE (%)	WEE No.	WEE (%)	Both EEE & WEE No.	Both EEE & WEE (%)
American Goldfinch	3						
Magnolia Warbler	3						
Sharp-Shinned Hawk	2						
Starling	2						
Field Sparrow	2						
Swainson's Warbler	2						
Palm Warbler	2						
Louisiana Waterthrush	2						
Winter Wren	2						
Bob-White	1						
Mourning Dove	1						
Turkey Vulture	1						
Barred Owl	1						
Saw-Whet Owl	1						
Belted Kingfisher	1						
Whip-poor-will	1						
Eastern Kingbird	1						
Summer Tanager	1						
Mockingbird	1						
Ruby Throated Hummingbird	0**						
Rusty Blackbird	0**						
Canada Warbler	0**						

*Species containing less than 20 specimens not calculated
**Species captured but blood specimens unavailable

TABLE 34

LEVELS OF EEE AND/OR WEE ANTIBODY IN SPECIES REPRESENTED BY AT LEAST 20 SPECIMENS

Highest %		Medium %		Lowest %	
Red-Eyed Vireo	85%	Yellowthroat	39%	Redwing	16%
White-Eyed Vireo	82	Catbird	37	Hermit Thrush	15
Wood Thrush	78	Carolina Chickadee	35	Black & White Warb.	15
Tufted Titmouse	77	Worm-Eating Warbler	34	Downy Woodpecker	14
Pine Warbler	60	R.S. Towhee	33	Prothonotary Warb.	10
Cardinal	60	Swamp Sparrow	23	Myrtle Warbler	10
Carolina Wren	55	White-Throated Spar.	20	Slate-Colored Junco	9
Ovenbird	48	Song Sparrow	20	Amer. Redstart	8
		Prairie Warbler	19	Northern Waterthrush	7
		Common Grackle	19		
		Robin	18		

Table 35

Temporal Status of Birds Captured in
Pocomoke Cypress Swamp

Transient	Breeding	Permanent Residents
Rusty Blackbird	Yel. Bil. Cuckoo	Woodcock
Summer Tanager	Ruby-Thr. Hummingbird	Bobwhite
Bl.-Thr. Blue Warb.	Eastern Kingbird	Mourning Dove
Magnolia Warbler	Crested Flycatcher	Turkey Vulture
Nor. Waterthrush	Acadian Flycatcher	Sharp-Shinned Hawk
Winter Wren	Br.-Headed Cowbird	Barred Owl
Swainson's Thrush	Indigo Bunting	Saw-Whet Owl
Grey-Ch. Thrush	Red-Eyed Vireo	Screech Owl
Field Sparrow	White-Eyed Vireo	Belted Kingfisher
Blackpoll Warb.	Bl. & Wh. Warbler	Hairy Woodpecker
Palm Warbler	Prothonotary Warb.	Downy Wdpr.
Canada Warbler	Swainson's Warb.	Pileated Wdpr.
Amer. Redstart	Worm-Eating Warb.	Red-Bellied Wdpr.
Ruby-Cr. Kinglet	Parula Warbler	Yel.-Sh. Flicker
Veery	Yel.-Thr. Warb.	Whip-poor-will
	Pine Warb.	Blue Jay
Winter Resident	Prairie Warb.	Starling
	Ovenbird	Redwing
	Louisiana Waterthrush	Common Grackle
White-Thr. Sparrow	Kentucky Warbler	Amer. Goldfinch
Slate-Col. Junco	Yellowthroat	Rufous-S. Towhee
Swamp Sparrow	Yel.-Br. Chat	Cardinal
Song Sparrow	Hooded Warb.	Mockingbird
Fox Sparrow	Blue-Grey Gnatcatcher	Catbird
Myrtle Warbler	Wood Thrush	Brown Thrasher
Hermit Thrush	Robin	Carolina Wren
Brown Creeper		Tufted Titmouse
Golden-Cr. Kinglet		Carolina Chickadee
Red-Br. Nuthatch		Robin

TABLE 36

EEE & WEE VIRUS NEUTRALIZING ANTIBODY IN FOUR TEMPORAL GROUPS OF WILD BIRDS

Group	No. Tested	(%)	Total EEE & WEE Virus Antibody		EEE Virus Antibody		WEE Virus Antibody	
			No. Positive	(%)*	No. Positive	(%)*	No. Positive	(%)*
Transient	133	(5)	11	(1)	11	(4)	0	(0)
Winter Resident	890	(35)	118	(15)	83	(29)	28	(8)
Permanent Resident	743	(29)	304	(39)	95	(33)	145	(45)
Breeder	784	(30)	355	(45)	102	(35)	149	(46)
TOTALS	2550	(100)	788	(100)	291	(100)	322	(100)

*Percent of Total Antibody Positives Represented by This Group

north. These data also support previous observations that breeding birds present during the summer months demonstrate a consistently higher level of antibody involvement than do birds present in the area only during the winter months.

Virus isolations made from wild bird specimens are listed in Table 37. Of considerable interest was the isolation of EEE virus from each of three Swamp Sparrows captured on 14 October in the same area. These Swamp Sparrows most probably represented early fall migrants into the swamp habitat. This species was first captured three weeks prior to these virus isolations; however, these specimens contained EEE virus antibody. Data of this nature would seem to indicate EEE virus activity further north, since the Swamp Sparrow usually breeds no further south than New Jersey.

Table 37. Wild Bird Virus Isolations

Date Captured	Species	Location
22 Jul 69	White-Eyed Vireo	Peripheral Swamp (Slab Road)
23 Jul 69	Tufted Titmouse	Swamp
22 Sep 69	Downy Woodpecker	Swamp
8 Oct 69	Red-Eyed Vireo	Farmland & Marsh (Pasture)
14 Oct 69	Swamp Sparrow	Farmland & Marsh (Pasture)
14 Oct 69	Swamp Sparrow	Farmland & Marsh (Pasture)
14 Oct 69	Swamp Sparrow	Farmland & Marsh (Pasture)

Five major areas were utilized for bird captures from 2 March to 15 December and a summary of virus antibody levels in these areas is presented in Table 38. The Super Swamp and Slab Road designations in the table were both representatives of the Peripheral Swamp habitat previously described, however, have been considered as separate habitats for wild bird data. The pasture area has also been described as sharing a farmland-marsh habitat.

A single transient species (Myrtle Warbler) represented 20 per cent of all captures for the year, yet yielded very few antibody positive

Table 38. 1969 Area Summary - Level of Antibody in Wild Bird Population

Netting Area	No. Captured	No. Tested	Pos. Ab.		Pos. EEE		Pos. WEE		Pos. EEE & WEE	
			No.	(%)	No.	(%)	No.	(%)	No.	(%)
Super Swamp	308	261	101	(39)	38	(15)	31	(12)	32	(12)
Slab Road	419	372	155	(42)	50	(13)	73	(20)	32	(9)
Pasture	501	456	124	(27)	60	(13)	48	(11)	16	(4)
Upland	67	62	20	(32)	9	(15)	7	(11)	4	(7)
Swamp Grid	1662	1406	393	(28)	132	(9)	169	(12)	92	(7)
TOTALS	2957	2557	793	(31)	289	(11)	328	(13)	176	(7)

specimens (Table 33). These data possibly caused an undue reduction in the virus antibody level in the area in which this species was most abundant - Swamp Grid. For this reason, all Myrtle Warbler data have been removed from the area summaries in Table 39. Once corrected in this manner, no significant differences were observed in the degree of antibody involvement of bird populations at any of the various sites.

On examination of the serological data presented in Table 39, it shows that 9 per cent of all birds tested were positive for both EEE and WEE virus antibody. This value was twice that of the expected based on the simple probability of any given bird acquiring these two separate and different virus infections within a given season. Although neutralization cross reactions have not been demonstrated in the only specific instances available for testing, i.e., sentinel quail from which virus was isolated, the possibility that they do occur cannot be dismissed. Detailed investigation of possible cross reactions are in progress; however, for purposes of these discussions, double reactors have been excluded from single virus antibody activity determinations.

The temporal distribution of virus antibody levels for the total bird population tested is shown in Figure 64. It was of interest to note that the level of antibody-positive specimens remained relatively constant throughout the year and in this relatively crude form would not suggest any appreciable increase resulting from virus transmission during the summer months. When the total antibody-positive birds were separated into specific EEE and WEE virus immune individuals, a different profile was observed, as shown in Figure 65.

Early in the year, antibody to EEE virus was more prevalent than to WEE virus. This observation could be interpreted as a reflection of the EEE virus epizootic which occurred in the swamp during the previous year. A drop in levels of all antibody was observed during late June, coincident with the appearnce of the year's immature population. A rise in the number of WEE virus antibody positives was found during the late summer which rapidly declined with a total antibody drop as fall migrants passed through the swamp. The peak of EEE virus antibody positive specimens observed during October could be partially attributed to the previously described EEE virus immune Swamp Sparrows which appeared in the swamp at that time.

The White-eyed Vireo was the most abundant breeding bird occurring in the study area, as determined by both observational and netting methods. Their period of occurrence in the area was one of the longest of the previously described breeding birds, usually from mid-April to early October. Previous serological data supported this year's observation that White-eyed Vireos were the most involved bird species found in the habitat (antibody level in 1969 of 82 per cent). The habits of this species tend to keep it at a height under eight feet, making it very prone to capture in mist nets and within the level of resident mosquito activity. They have a very high survival rate after bleeding, a high recapture rate during the season, and exhibit a marked tendency to return to the same specific area in subsequent years. The White-eyed Vireo is

Table 39. 1969 Area Summary - Level of Antibody in Wild Bird Population*

Netting Area	No. Captured	No. Tested	Pos. Ab.		Pos. EEE		Pos. WEE		Pos. EEE & WEE	
			No.	(%)	No.	(%)	No.	(%)	No.	(%)
Super Swamp	301	254	101	(40)	38	(15)	31	(12)	32	(13)
Slab Road	376	330	145	(44)	42	(13)	71	(22)	32	(10)
Pasture	361	322	111	(33)	49	(15)	46	(14)	16	(5)
Upland	67	62	20	(32)	9	(15)	7	(11)	4	(7)
Swamp Grid	1304	1090	368	(34)	113	(10)	164	(15)	91	(8)
TOTALS	2409	2058	745	(36)	251	(12)	319	(15)	175	(9)

* All Myrtle Warblers removed from totals.

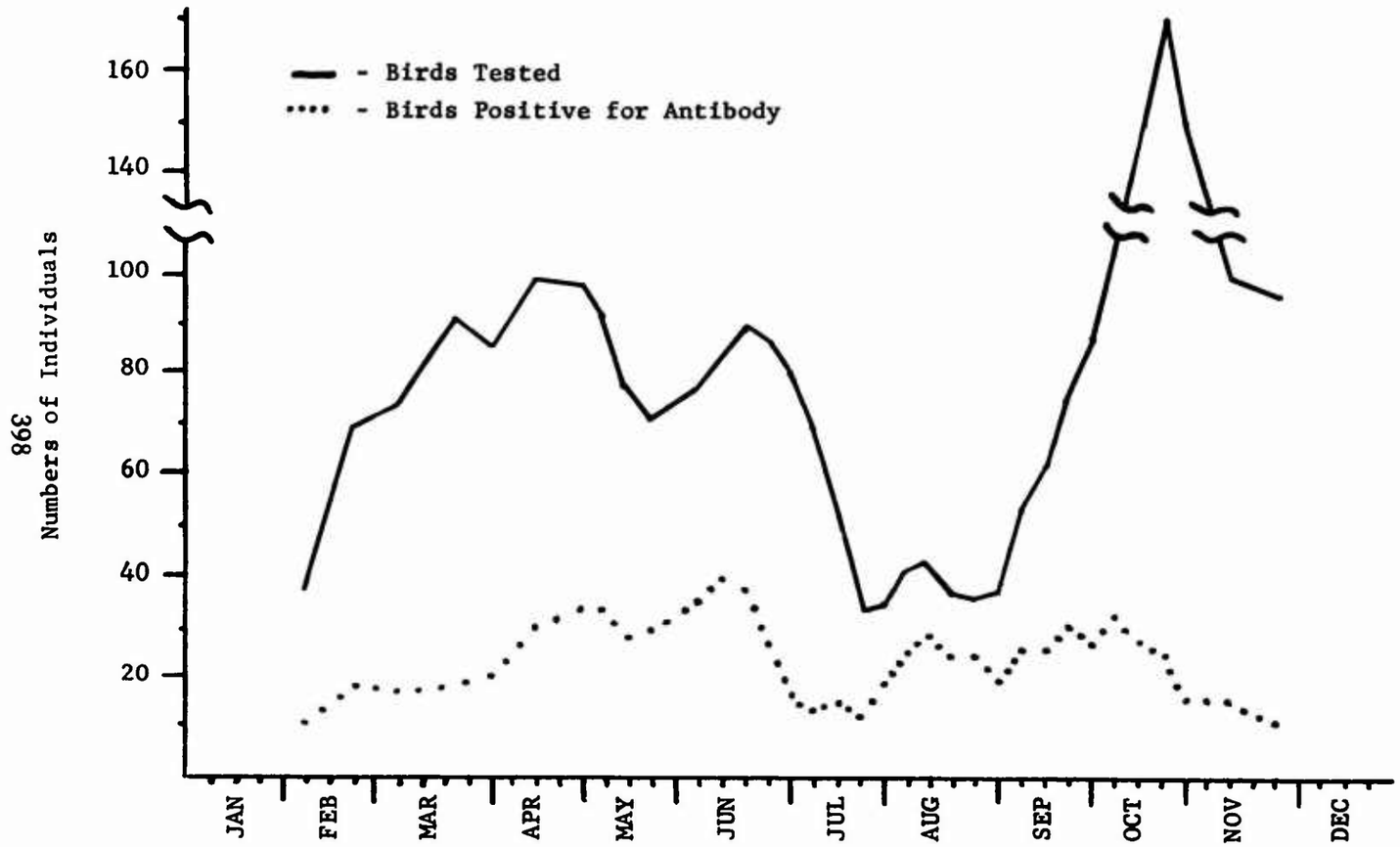


Figure 64. 1969 Antibody Survey of Wild Birds

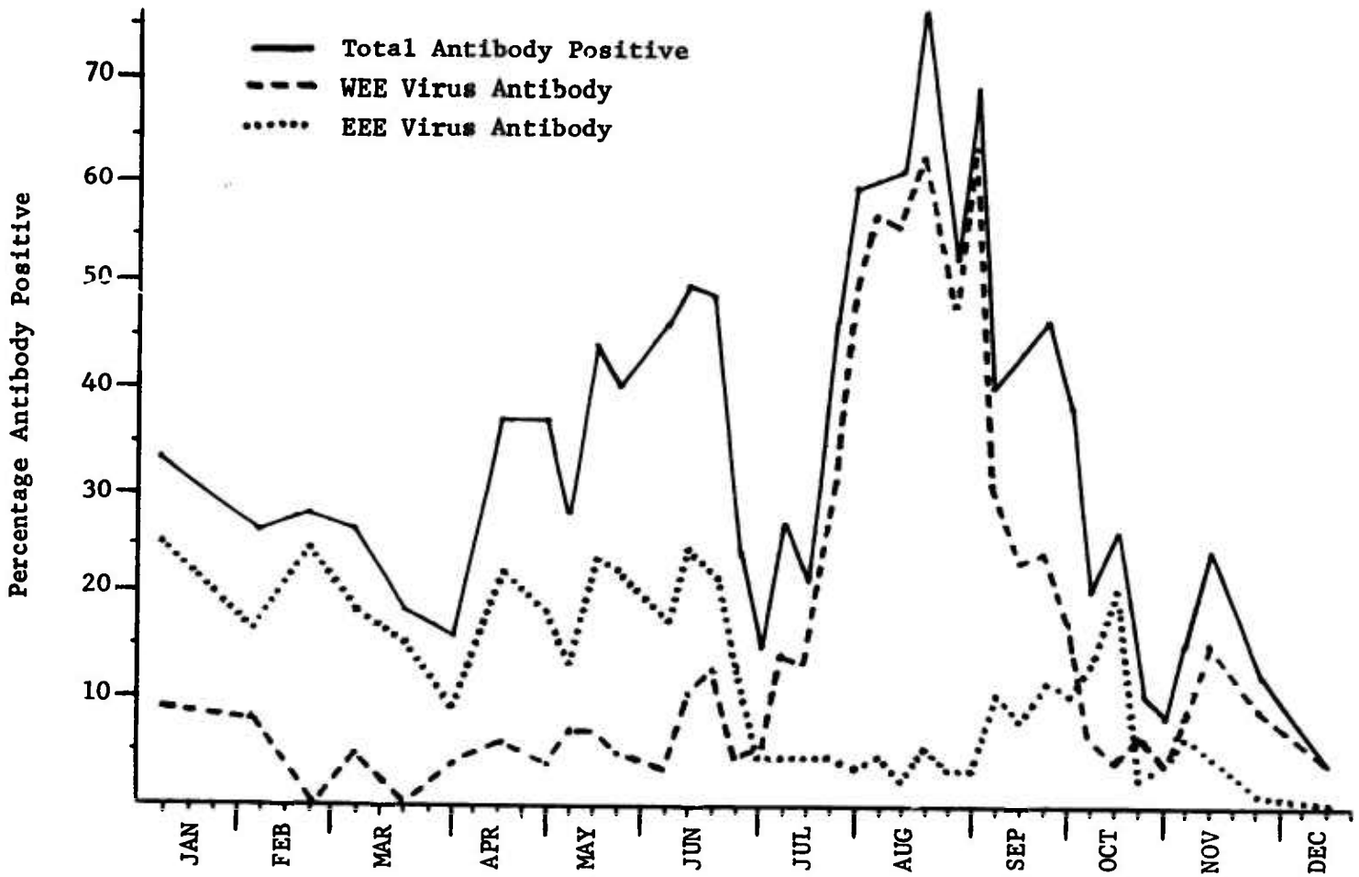


Figure 65. Temporal Relationships of Antibody Positive Wild Birds

also one of the few species in the study area in which an age determination is both simple and consistent. The iris of adult birds is white with immatures showing a brown or grey color. The adult eye color is not assumed until immatures are on their southern wintering grounds. It seems that White-eyed Vireos could be a species which might best demonstrate sequence, rate, and timing of antibody production in the total wild bird population of the area.

White-eyed Vireos raise only one brood in the study area and after the young fledge, the adults appear to leave the general area, the population captured in mist nets after 13 July 1969 being almost exclusively immature (Figure 66). There was only a slight increase in the number of adults during the fall migration period which is predictable since these birds are near the northern-most limit of the species' breeding range and a high incidence of transient northern breeding migrants in the fall would not be expected.

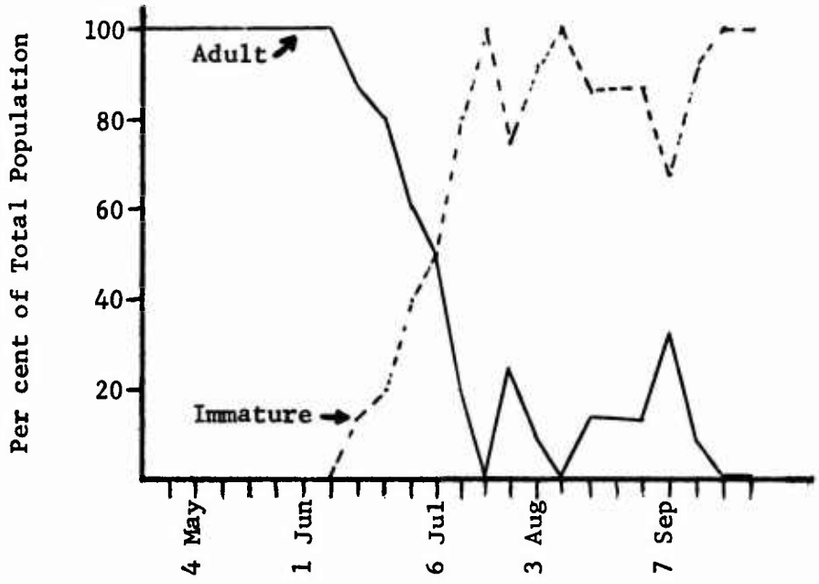
The entrance into the total wild bird population of a group of non-immune, susceptible individuals (immature White-eyed Vireos) and their subsequent capture in an endemic area of EEE and WEE virus during a period of potential mosquito transmission (after 15 June) should give some indication as to the virus activity at that particular time. A three-week lag period was demonstrated (Figure 67) between occurrence of the first immature and the first WEE positive antibody reactor. After an additional three weeks, all immatures captured were positive for WEE antibody and this continued for another three weeks before immature not containing WEE antibody was found. The first wild bird virus isolation of WEE virus for 1969 was obtained from an immature White-eyed Vireo during the period of the observed increase in WEE virus antibody (Table 37). Incidence of EEE antibody in this species after 6 June was minimal and confined almost exclusively to adult birds.

A comparison of seasonal levels of antibody in the total wild bird population and the White-eyed Vireo population demonstrates no real difference (Figures 68 and 69). Both groups contain higher levels of EEE antibody at the beginning of May, elevated levels of WEE antibody at the end of September, and the point in time when EEE virus antibody levels drop below those of WEE virus in both populations is the same (29 June). Although antibody activity in the White-eyed Vireo population was consistently higher than in the total wild bird population, the relationships between EEE and WEE virus antibody activity remained constant.

E. Reptiles, Amphibians and Larger Mammals:

Previous studies of the small mammal population in the swamp have demonstrated that these animals were rarely, if ever, infected with either EEE and/or WEE viruses (Annual Report, 1965-66). The suggestion that some of the larger mammals, reptiles and amphibians could be implicated with these viruses was also made during that study. These animals were captured during 1969 and tested for the presence of neutralizing antibody to EEE and/or WEE viruses. The results of these studies are presented in Table 40.

Figure 66. Seasonal Relationship of Adult and Immature White-eyed Vireo Populations



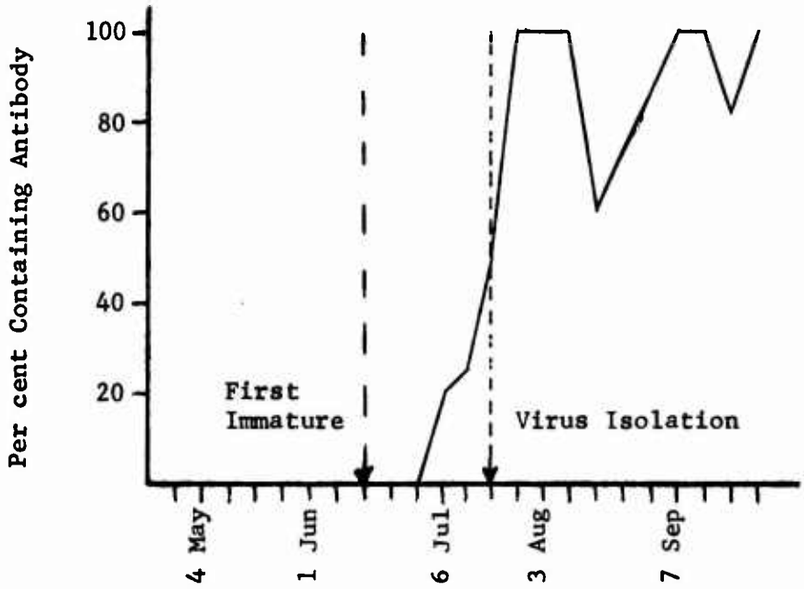
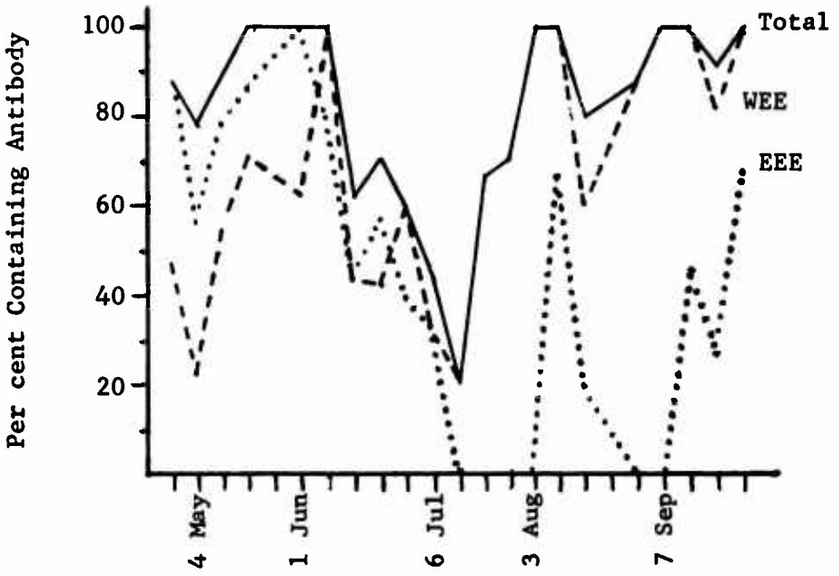


Figure 67. Level of Antibody in an Immature White-eyed Vireo Population

Figure 68. Seasonal Antibody Levels in Total White-eyed Vireo Population



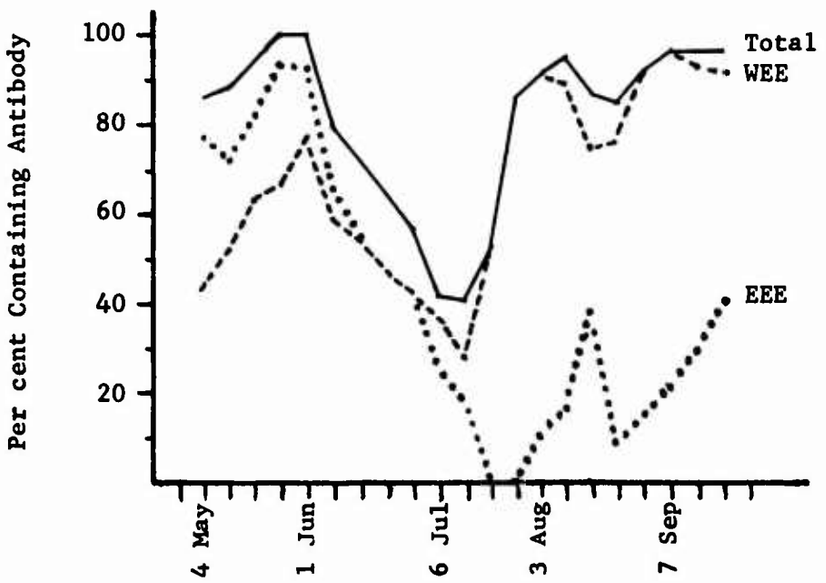


Figure 69. Seasonal Antibody Levels in Total White-eyed Vireo Population (Three-Week Moving Average)

Table 40. EEE and WEE Virus Antibody Distribution in Reptiles, Amphibians and Larger Mammals

Species	# Tested	Pos. EEE	%	Pos. WEE	%
Mammals					
Racoon (*Procyon lotor*)	76	23	30	0	0
Oppossum (*Didelphis virginiana*)	16	5	31	1	6
Skunk (*Mephitis mephitis*)	5	1	20	0	0
Squirrel (*Sciurus carolinensis*)	3	1	33	0	0
Reptiles and Amphibians					
Painted Turtle (*Chrysemys picta*)	60	5	8	7	12
Box Turtle (*Terrapene carolina*)	54	5	9	11	20
Mud Turtle (*Kinosternon subrubrum*)	49	6	12	4	8
Snapping Turtle (*Chelydra serpentina*)	26	3	12	0	0
Spotted Turtle (*Clemmys guttata*)	10	1	10	3	30
Black Rat Snake (*Elaphe obsoleta obsoleta*)	9	0	0	0	0
King Snake (*Lampropeltus getulus*)	8	1	12	1	12
Frogs (*Rana clamitans*)	7	2	29	1	14
Musk Turtle (*Sternotherus odoratus*)	4	0	0	0	0
Black Racer Snake (*Coluber constrictor*)	3	1	33	1	33

Table 40
(continued)

Species	# Tested	Pos. EEE	%	Pos. WEE	%
Hognose Snake (Heterodon nasicus)	3	0	0	0	0
Toad (Bufo sp.)	1	0	0	0	0
Diamond-Backed Terrapin (Malaclemys terrapin)	1	0	0	0	0
Copperhead Snake (Agkistrodon contortrix)	1	0	0	0	0
Common Water Snake (Natrix sipedon)	1	0	0	0	0

It was interesting to note that a greater number of mammals were positive for antibody to EEE virus than to WEE virus. Many of these positives were suspicious positive reactors in the tube neutralization test but have subsequently been confirmed by plaque reduction testing. The presence of neutralizing antibody to EEE virus in the mammal population most probably reflected EEE virus activity in the swamp during previous years, since most of these specimens were obtained in early 1969 prior to any virus transmission. The lack of WEE virus antibody even in late 1969 specimens collected after the observed WEE virus activity previously described would suggest the absence of whatever transmission elements are necessary to put the WEE virus into the mammal population.

Further evidence for this hypothesis was obtained by antibody surveillance of local pigs. Pigs raised near the swamp during late summer 1969, a year of high WEE virus activity, were bled at slaughter; however, only a single positive WEE virus antibody specimen was found in the 40 pigs tested. No positive EEE virus antibody-containing pigs were found.

Reptiles and amphibians also yielded positive antibody specimens but lower percentages than mammals and were approximately equally distributed between EEE and WEE viruses. These data must be considered suspect until they can be verified by plaque reduction neutralization tests. Previous studies describing high titered, non-specific inhibitors of arbovirus hemagglutination in reptile and amphibian sera (Annual Report, 1965-66) also suggest additional experiments necessary prior to any critical interpretation of these data.

Summary.

I. In the initial field trial of L-AV-7 at Fort Dix, N.J., the vaccine group immunized with L-AV-7 and L-AV-4 had 96% less ADV-7 associated ARD hospitalizations than did a group immunized with L-AV-4 alone. No difference in the ADV-4 associated ARD rate was evident between the two vaccine groups, but the number of trainees studied was insufficient to detect a lower order of interference of L-AV-4 by L-AV-7 in this study.

In a study undertaken in susceptible volunteers at the USAMEDTC, FSH, the dose response of L-AV-7 was found to be quite similar to that of L-AV-4. The human infectious dose of L-AV-7 was less than 10 $TCID_{50}$.

A study to determine whether a high dosage L-AV-7 would interfere with the infectivity of a simultaneously administered L-AV-4 was undertaken. The data suggested that no decrease in antigenicity of a low dosage L-AV-4 ($10^{4.0}$ $TCID_{50}$ per tablet) occurred when given with a high dosage L-AV-7 ($10^{6.8}$ TCID50 per capsule) from that obtained when L-AV-4 was given alone or with a moderate dose L-AV-7 ($10^{4.8}$ $TCID_{50}$ per capsule). The presence of wild ADV-4 and ADV-7 infections in the study population invalidated this conclusion, however, since the true rate of antibody acquisition after immunization could not be assessed.

A second study of the field efficacy of L-AV-7 vaccines was undertaken in Fort Dix BCT's in January 1970. Trainees entering one training brigade (the 3rd brigade) were immunized with L-AV-7 and L-AV-4 within 72 hours after arrival on post, while those entering the 2nd brigade received L-AV-4 alone. One of the six companies filling one of the two brigades each week was selected as a study company from which all ARD admissions were studied for viral and bacterial pathogens. ADV-7 associated ARD was suppressed by 96% in the 3rd Bde. ADV-4 associated ARD rates were significantly higher, however, in the 3rd Bde, although the difference (22.5/1000/8 weeks) was small. Trainees in the 3rd Bde (immunized with L-AV-7 and L-AV-4) experienced 75% less ADV-associated ARD hospitalizations than did 2nd Bde trainees immunized with L-AV-4 alone.

II. Immunization of BCT's with L-AV-7 and L-AV-4 during outbreaks of ARD due to both adenovirus types in 1970 result in a rapid decline in ARD rates and ADV-associated ARD rates. Immunization of trainees at Fort Campbell with L-AV-7 done during an outbreak of predominantly ADV-7 associated ARD led to the suppression of ADV-7 and the emergence of ADV-4 as the principal respiratory pathogen and resulted in little, if any, reduction in crude ARD rates.

III. A plaque reduction neutralization test for ADV-7 was developed with KB cells. This test shows promise of affording more sensitive quantification of neutralizing antibody to ADV-7

IV. Studies were initiated on Hepatitis Associated Antigen (HAA). Antigen was prepared by centrifugation procedures from the plasma of a

patient with acute serum hepatitis. The partially purified HAA obtained, which contained a small amount of normal IgG by immunoelectrophoresis, was immunologically identical to that of other laboratories. Antiserum to HAA was prepared in guinea pigs and rabbits. These reagents were used in routine agar gel diffusion and complement-fixation tests to detect HAA and antibodies to HAA (anti-HAA). The complement-fixation test could detect HAA in serum dilutions 16 to 64 times greater than the agar gel diffusion test.

In routine tests of patient sera, it was found that HAA is highly specific for viral hepatitis. However, it was detected with equal frequency in nonepidemic cases of serum and infectious hepatitis. Furthermore, antigen was detected in three asymptomatic family contacts of an infant with HAA positive giant cell hepatitis. Anti-HAA was found in a few hemophiliacs but no patient with clinical hepatitis. Sera from ten species of healthy subhuman primates did not contain HAA or anti-HAA. Screening sera for HAA appears to be a specific diagnostic test for viral hepatitis which will open new paths of investigation into the epidemiology of this disease.

V. Disc gel electrophoresis of dengue virus soluble complement-fixing antigens (SCF) demonstrated that the four SCF antigens have similar molecular sizes but differ in their electrophoretic mobility. SCF antigens were obtained from infected mouse brain suspensions and purified by Sephadex gel filtration. The antigens were placed either individually or in pairs on varying concentrations of polyacrylamide gel. Following electrophoresis, the antigens were allowed to elute from 1 mm slices of the gel into Kolmer saline where they were detected by complement-fixation. The log of the mobility of each antigen relative to the dye front was plotted against the concentration of polyacrylamide and the slope-Y-intercept functions for each antigen were calculated. Plots of the four serotypes on the same graph provided a series of four parallel straight lines, indicating that the dengue virus SCF antigens had similar molecular weights but different electrophoretic mobilities. Electrophoretic separation of two SCF antigens were achieved with co-runs of both antigens on the same gel. The relative electrophoretic mobilities were, indescending order, types 2, 4, 3, and 1.

Soluble complement-fixing antigens obtained from suckling mouse brain infected with each of the four dengue virus serotypes were purified and compared by complement-fixation and immunoprecipitation. SCF antigens formed discrete precipitin bands with hyperimmune mouse ascitic fluid. An antigenic determinant common to all four dengue serotypes was demonstrated as was a type-specific antigenic determinant which is unique for each serotype. Cross absorption experiments confirmed the presence of the group antigen. Immunoprecipitin patterns indicated that both group- and type-specific determinants are on the same molecule and separate antibodies are formed to each determinant.

Antigenic compositions of slowly sedimenting dengue-2 hemagglutinin (SHA) and soluble complement-fixing antigen (SCF) were compared

with the virion (RHA) by radioimmune precipitation (RIP), RIP inhibition, kinetic neutralization, and neutralization blocking tests using hyperimmune mouse ascitic fluids (HMAF). RHA and SHA were unable to inhibit completely the RIP of each other by anti-RHA; neutralization by anti-RHA was not blocked by SHA. This indicated that SHA is serologically related, but not identical, to RHA and when considered with other evidence, suggests that SHA is a special form of "incomplete virus."

SCF was unable to inhibit the RIP of SHA or RHA; further, anti-SCF HMAF did not neutralize RHA or precipitate significant levels of SHA or RHA. Polyacrylamide gel electrophoresis separated SCF from structural polypeptides by molecular size; this evidence suggests that SCF is a nonstructural antigen.

Dengue virions (RHA) could be degraded into an SHA-like antigen by treatment at alkaline pH. The virions remained intact up to pH 9.6, but at pH 10.0, it lost its physical integrity and was recovered as a slowly rather than rapidly sedimenting HA antigen on sucrose gradients. Back dialysis at pH 8.2 did not result in reassembly of the virion coat. Dengue virus required a slightly acid pH (6.2) before it will HA; this adjustment in pH is done at the time erythrocytes are added to the test. It was unexpectly observed that prior dialysis at pH 6.2 18 hours before the test destroyed the ability to HA. When the same experiment was carried out with radioactive virions, all the radioactivity was converted to a soluble form (defined by no sedimentation in sucrose gradients) but the soluble proteins were found to adsorb onto red cells. In contrast, alkaline dialysis resulted in two protein peaks that sedimented in the SHA region of sucrose gradients and an RNA rich-low protein peak (nucleoprotein "core") was found between the RHA and the SHA region. This provides the first method of isolating nucleoprotein free of detergents usually used to degrade virions.

The structural proteins of Japanese encephalitis virus (JEV) consists of two surface proteins having molecular weights (MW) of 53,000 and 8,300 and a 13,200 MW protein associated with an RNA rich fragment ("core" protein). Seven virus specific proteins were found in infected cells of which two were structural proteins (the largest surface protein and the "core" protein). Dengue virions were also found to be composed of three structural proteins which are very similar in molecular weight to the three JEV proteins.

VI. During 1969, major outbreaks of dengue occurred in the Caribbean. Epidemic dengue was reported in Jamaica, Puerto Rico, Venezuela, French Guyana and many smaller islands. There were 35 strains from Jamaica, Haiti and Puerto Rico that were isolated in LLC-MK2 cell culture and identified by plaque reduction neutralization test as type 2 virus.

Dengue-2 IgM antibody was identified in the serum of individuals suffering from dengue-2 infection in the 1969 Puerto Rican dengue epidemic. Sera from individuals recovering from primary dengue infection and from secondary exposure to group B arbovirus contained anti-dengue

IgM. Dengue-specific IgM and IgG antibodies were isolated from serum obtained from an individual undergoing secondary group B arbovirus infection. HI and neutralization studies revealed a specificity of IgM antibodies for the homologous dengue-2 virus. IgG showed considerable cross-reactivity among several group B arboviruses. CF by IgM occurred only in high concentrations of IgM antibody and CF antigen. Below these levels, the IgM effectively blocked the CF activity of IgG under experimental conditions. CF activity of whole serum could be rescued by removal of native IgM antibody by means of mercaptan reduction.

VII. During 1969, extensive arbovirus ecological investigations were conducted in and around the Pocomoke Cypress Swamp, Maryland. The use of CO_2 baited light traps allowed the collection of many mosquito species with numbers sufficient for virus isolation testing, however, all virus isolates were obtained from Culiseta melanura mosquitoes. Differences in the density of this species were observed in the various habitats, but Culiseta melanura mosquitoes did not appear to be restricted to a swamp habitat, and virus isolates were obtained from all major areas studied. Twelve isolates of WEE virus and three isolates of EEE virus have been identified from the 19 strains isolated. In addition to the remaining virus isolates awaiting identification, numerous mosquito pools remain to be tested for the presence of virus.

Sentinel quail, placed in representative habitats in and around the swamp, reflected a peak of WEE virus activity during late July and early August, which coincided with peak densities of Culiseta melanura mosquitoes. Some EEE virus activity was detected but was much lower than WEE virus levels and occurred much later in the summer. Area differences in virus transmission were not pronounced but appeared to reflect the distribution of the Culiseta melanura mosquito.

A study of the wild bird populations inhabiting the swamp showed a high level of EEE virus antibody in birds moving into the swamp in the spring, a reduced level of all antibody positive specimens following the appearance of nonimmune immatures, and a rapid and high rise in WEE virus antibody attributable to the conversions exhibited by young birds raised in the swamp. A detailed examination of a single species with a high degree of virus involvement (White-eyed Vireo) exemplified the virus activity pattern of all birds in the swamp. These data suggest certain wild bird species could be used as virus activity monitors in preference to extensive survey-type studies.

Serological surveillance of mammals in the study demonstrated EEE but not WEE virus activity in these animals. The possibility that these data reflect virus activity of previous years was suggested.

Based on the data obtained, a hypothesis of virus activity in the Pocomoke Cypress Swamp during 1969 can be presented. Wild birds migrating into the swamp in the spring dictated that WEE virus would predominate because of the high number of birds immune to EEE virus. Amplification of WEE virus activity increased proportionate to the increases in Culiseta melanura mosquitoes and susceptible immature birds.

As the young birds became immune and Culiseta melanura populations decreased, WEE virus activity also diminished, concluding a theory of virus maintenance during an inter-epidemic year in an endemic area. Speculations on the alterations of this pattern necessary for the creation of an epidemic situation would be premature at this point.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 166, Viral Infections of Man

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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	8. DISP'N INSTR'N	8A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	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	61102A	3A061102B71Q	00	167
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Rickettsial Diseases of Military Personnel (09)

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010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING		
	70	3	150
	CURRENT		
	71	4	200

19. RESPONSIBLE DOD ORGANIZATION

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(U) Rickettsial Infections; (U) Laboratory Diagnosis; (U) Vaccines; (U) Epidemiology; (U) Ecology; (U) Vectors and Reservoirs

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

23. (U) 1. Prevention of scrub typhus in troops in Southeast Asia. 2. Evaluation of military importance of R. canada, a new member of the Typhus Group of Rickettsia.

24. (U) 1. (a) Formulation of a polyvalent scrub typhus vaccine to immunize against the many different strains causing disease. 1. (b) Develop fluorescent antibody methods for rapid identification of wild strains of scrub typhus. 2. (a) Identification of the causative agent(s) and arthropod vector(s) responsible for rickettsial disease in the Fort Bragg area. 2. (b) Detect additional geographic locations of human disease.

25. (U) 69 07 - 70 06 1. (a) Tissue culture methods for large scale production and for cloning vaccine candidate strains are being developed. 1. (b) Strain specific antirickettsial fluorescein cinjugates are being prepared for direct staining of R. tsutsugamushi. 2. (a) At Fort Bragg, Rocky Mountain spotted fever caused one case last year and serologic evidence of enzootic infection in wild animals was found. 2. (b) Additional cases of suspected R. canada infection found were from Fairfax, VA, Cherry Point, NC, and from Michigan, in a recent returnee from Vietnam. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 Jun 70.

*Available to contractors upon originator's approval.

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Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 167, Rickettsial diseases of military personnel

Investigators.

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Description.

For most of the past year the laboratory facilities of the Department of Rickettsial Diseases were being remodelled. Assigned personnel were relocated in temporary quarters where work with any infectious material was precluded. Research activities during this period were limited to serologic procedures employing noninfectious antigens. Full scale operation was not reinitiated until the middle of January, 1970. Consequently, the degree of progress achieved in the various research projects reflects the amount of effort that could be devoted to the problem and does not indicate a change in priority. During the current reporting period research activities have been concerned with: (1) the scrub typhus vaccine development program including (a) application of tissue culture technics for the preparation of complement-fixing antigens of newly recognized prototype strains and cloning of candidate vaccine strains; and (b) the preparation of type-specific antirickettsial serum conjugated with fluorescein for identification of wild strains of scrub typhus by direct immunofluorescent staining; and (2) evaluation of the existing and potential military importance of Rickettsia canada, a newly recognized member of the Typhus Group of rickettsia by: (a) attempts to isolate and identify the agents causing rickettsial disease among military personnel and their dependents at Fort Bragg, North Carolina, and to define the components of the infection cycle in the environment; and (b) to detect additional geographic locations of human disease by retesting of specimens from patients previously diagnosed as having Rocky Mountain spotted fever or murine typhus.

Progress.

During the period 21 October 1968, and 15 January 1970, the Department of Rickettsial Diseases had to suspend operations in its laboratories because the facilities were being remodelled. The alteration of the physical plant included the installation of infectious hoods, the establishment of a ventilation system to provide a negative pressure gradient and controlled air flow, and provisions for decontamination of exhausted air with high efficiency particle air filters and the fluid wastes by heat treatment. These changes were necessary to reduce the hazard of accidental infection for laboratory personnel within the departmental

area and to protect other individuals in the vicinity of the department, both inside and outside of the building. When activities were resumed all of the effort was devoted to checking the potency of stocks of seed suspensions of all established rickettsial strains and replacing those materials that had deteriorated during storage for more than 15 months. In addition it was necessary to replenish the stocks and standardize all diagnostic complement-fixing and indirect immunofluorescent test antigens and control immune sera that had been exhausted during the unexpected prolonged period of construction. These reagents were needed for use in-house and to supply to military laboratories in Southeast Asia.

1. Scrub Typhus Vaccine Development. Over the past several years we have been evaluating the feasibility of developing a killed polyvalent vaccine that would protect man against the antigenically diverse strains of Rickettsia tsutsugamushi encountered in nature. An antigenic analysis of 78 strains of scrub typhus isolated in Thailand from patients, wild mammals and Leptotrombidium 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 tests were comprised of partially purified suspensions of 3 different strains established by Japanese workers as prototype strains and other distinctive Thai strains as they were recognized. Previous reports have presented the evidence that demonstrated the presence of at least 8 antigenically diverse strains in the Thai collection. At the present time, in addition to the Japanese prototype strains, Karp, Gilliam and Kato, the distinctive Thai strains that are candidates for prototype status and possible inclusion in a polyvalent vaccine include: TA763, TA716, TA678, TA686, and TH1817. Many of the Thai strains of R. tsutsugamushi irrespective of the source are suspected of being mixtures of 2 or more different antigenic types. Therefore, it is important to establish that each of the 5 candidate prototype strains are pure and do not contain other as yet unrecognized antigenic variants. After this is accomplished the major and minor antigenic components of each of the distinctive types must be characterized in order to determine the fewest number of strains to be incorporated into a polyvalent vaccine to provide the broadest degree of protection against scrub typhus in nature.

The difficulties encountered in obtaining the concentrations of organisms in yolk sacs infected with the Thai strains that are needed for the preparation of complement-fixing antigens to evaluate purity have been reported previously. Attempts to enhance the growth of these scrub typhus rickettsiae in the yolk sacs of embryonated eggs were unsuccessful. Similarly, attempts with physico-chemical methods for molecular separations to obtain purified heavy suspensions of the organisms from lightly infected yolk sacs failed (1).

a. Propagation of Candidate and Established Prototype Strains in Cultured Cells. Last year, the first method for a plaque assay for

several species of Rickettsia using primary chick embryo fibroblasts was published (2). Earlier trials to develop such a system in our laboratory and by other workers were uniformly unsuccessful. Attempts are now in progress to duplicate the results reported and to determine if scrub typhus organisms also will produce plaques with the same technic. The procedure, when developed, will be used to clone the candidate prototype strains in order to insure purity. Furthermore, the method will be used to confirm the existence of mixtures in nature and to determine the number of different strains in selected Thai agents that are suspected of being composed of 2 or more variants.

Other workers have reported previously methods for the production of Karp, Gilliam and Kato complement-fixing antigens in tissue culture (3,4). The antigens derived from cultured cells required greater effort and space to produce, were more costlier, and were not as type-specific as those prepared from yolk sacs infected with the 3 established prototype strains. However, over the past several years intensive efforts to develop means for the preparation of potent complement-fixing antigens from eggs infected with the 5 Thai candidate prototype strains have produced irregular and unpredictable results. Therefore, use will be made of recent improvements in cell culture methods for the cultivation of scrub typhus strains in order to obtain the heavy suspensions of organisms needed. Rickettsiae harvested from monolayers of cells grown in large roller bottles will be concentrated and type-specific as well as broadly reactive antigens will be prepared so that the antigenic analyses of the different strains can be completed. Similar production methods will be employed in the preparation of experimental vaccines.

b. Preparation of Type-Specific Antirickettsial Serum Fluorescein Conjugates. The current method used for the classification of newly isolated strains of scrub typhus based upon the complement-fixing reactivity of immune sera from infected guinea pigs is dependent upon the availability of type-specific antigens for all of the prototype agents. These antigens are difficult to prepare and it has been impossible to keep stocks of them on hand at all times. Prior attempts had been made to use direct immunofluorescence for typing of Karp, Gilliam and Kato strains with immune guinea pig serum conjugated with fluorescein (5), and with immune mouse ascitic fluid. But, these reagents had low potency and the degree of heterologous reactivity precluded their use for identification of unknown strains. Recently it was reported that serum collected from rabbits early after immunization containing antibodies of the IgM class of immunoglobulins could be used to distinguish among closely related antigens by a variety of serologic procedures. Serum collected later, that contained both IgM and IgG, or only IgG antibodies, would not differentiate the antigens. Initial efforts to produce direct staining conjugates with antirickettsial rabbit sera obtained from rabbits on the 25th day after infection with either Karp, Gilliam or Kato have been highly promising. The titer of each of these reagents with the homologous organism was 1:128 or higher. Heterologous reactivity of the

anti-Gilliam conjugate titered 1:4 or less. The anti-Karp and anti-Kato conjugates did not stain Gilliam rickettsiae when used at a 1:2 dilution, and the difference between the homologous and heterologous reaction with the other organism was at least 8-fold. Attempts are in progress to improve the specificity of the anti-Karp and anti-Kato conjugates by adsorption with heterologous rickettsial antigens. Also, serum will be collected earlier after infection from the additional rabbits currently being immunized with each of the established and candidate prototype strains. When sufficient stocks of reagents are produced that can be used to rapidly identify homotypes of R. tsutsugamushi, similar reagents will be prepared with species belonging to the Typhus and Spotted Fever Groups, as well as with Coxiella burneti.

2. Evaluation of the Existing and Potential Military Importance of Rickettsia canada. Last year the serologic evidence indicating that 4 patients had experienced a severe febrile illness caused by R. canada was presented (1). The epidemiologic and clinical aspects of these diseases were indistinguishable from Rocky Mountain spotted fever. Three of the patients were dependents of military personnel assigned to Fort Bragg, North Carolina, and the other was a pilot at Laredo Air Force Base, Texas. R. canada is the newest member of the Typhus Group of rickettsia. It was recovered from Haemaphysalis leporispalustris ticks removed from indicator rabbits near Richmond, Ontario, Canada (2). Information about the pathogenicity of this rickettsia for man had not been reported previously.

a. Investigation into the Etiology of Tick-Borne Rickettsial Disease in Fort Bragg - Fayetteville Region of North Carolina. In June 1969, a coordinated clinical and field study carried out with the collaboration of Womack Army Hospital and the Preventive Medicine Activity, Fort Bragg, was initiated. The aim of the project was to establish a causal relation between R. canada or other unknown agents with human disease, to characterize the clinical features and pathophysiologic changes of the illness, as well as to identify the tick vectors and vertebrate hosts involved in the infection cycle in nature.

In 1967 and 1968, 3 patients with Rocky Mountain spotted fever and 3 patients with R. canada infection were treated at Womack Army Hospital. During 1969, 5 cases suspected of having rickettsial infections were admitted. However, serodiagnostic studies showed that only 1 patient had been infected with R. rickettsi and no evidence of R. canada was found. Surveillance for possible rickettsial infections will be continued at Womack Army Hospital during the present year and concerted effort made to isolate the etiologic agents causing disease.

The field studies were undertaken to identify areas where R. canada was enzootic so that more extensive investigations could be carried out later when the full support of the laboratories of the Department of Rickettsial Diseases were available. Live-trapping for small-and medium-sized mammals was carried out in the areas where the patients with

R. canada infection most likely acquired the tick transmitting the agent. All animals trapped were identified, blood was collected for serum for serologic tests, and all ectoparasites removed. During the period August through October 1969, 31 animals were trapped in the area of exposure of the index cases; viz., 16 opossums, 5 raccoons, 1 cottontail rabbit, 5 cotton rats, and 4 harvest mice. Complement fixation tests with spotted fever and typhus group, R. canada and Q fever antigens were negative. Identification of all of the arthropods removed from the animals has yet to be completed. Tick species recognized thus far include: D. variabilis, A. americanum, H. leporispalustris and I. texanus.

In addition, serum from animals trapped during the 1969 and 1970 annual zoonoses survey of the Fort Bragg reservation were examined for evidence of rickettsial infection. The results are summarized in Table 1. Spotted fever antibodies were found in some of the specimens from raccoon, rabbit and gray fox. No evidence of murine typhus, R. canada or Q fever infection was found in tests of the 59 animals included among 9 different species collected. Trapping in the area of index cases will be continued during the present season of suspected vector tick activity.

b. Antigenic Interrelationship Among Typhus and Spotted Fever Group Rickettsiae. Two of the 4 patients suspected of having been infected with R. canada reported last year had spotted fever complement-fixing antibodies in addition to R. canada antibodies. Investigations were carried out to gain a better understanding of the cross-reactivity that may occur when sera from patients who were infected with members of the Spotted Fever Group or Typhus Group are tested with group-reactive and specific antigens representative of the agents in both groups. Knowledge of the expected serologic responses is a prerequisite to the recognition of R. canada infections in areas where other members of the Typhus Group and Spotted Fever Group are endemic. Consequently, the serial serum specimens from 59 cases previously diagnosed as Rocky Mountain spotted fever on the basis of serologic reactivity with a spotted fever group antigen were reexamined. Complement fixation tests were carried out with spotted fever group-reactive and typhus group-reactive (epidemic and murine typhus) antigens, and specific R. prowazeki, R. mooseri and R. canada antigens. The reactivity of these antigens with the homologous and heterologous immune guinea pig sera has been described previously (1). The results with the spotted fever group antigens confirmed the previous serologic diagnoses. Antibodies that reacted with antigens of the Typhus Group were found in sera from 18 cases. Twelve were military personnel or veterans, who most likely had been immunized with epidemic typhus vaccine, and 3 were military dependents. No information was available about the immunization history of the other 3 patients who were elderly male civilians. In all of these cases the specific and group-reactive typhus antibodies were markedly lower than the spotted fever group antibodies. In general, specific R. prowazeki titers were higher than those of R. mooseri. Antibodies to R. canada developed only in 4 veterans and 1 civilian. In 3 of the veterans the

TABLE 1

FORT BRAGG ANNUAL ZOONOSES SURVEY

	MARCH 1969		MARCH 1970	
ANIMAL	NUMBER TESTED	NUMBER WITH SF ANTIBODY+	NUMBER TESTED	NUMBER WITH SF ANTIBODY+
Raccoon	7	1	10	1
Rabbit	1	1	11	7
Gray Fox	2	1	4	0
Opossum	6	0	8	0
Deer	2	0	0	-
Skunk	1	0	0	-
Bobcat	1	0	0	-
Cotton rat	0	-	1	0
Domestic cat	1	0	4	0

+ Presence of SF antibody indicated prior infection with R. rickettsi or other members of the Spotted Fever Group. Complement fixation tests with Typhus Group, R. canada and Q fever antigens were negative.

R. canada titers were essentially the same as the levels with R. prowazeki and R. mooseri. In the remaining veteran and the civilian, the specimens did not react with the typhus group-reactive or specific epidemic and murine typhus antigens. These last 2 cases may have had either a dual infection with R. canada and R. rickettsi, or had an infection with another strain of rickettsia that shared antigens with both of the species. The veteran contracted his illness in Cherry Hill, North Carolina and the civilian was exposed in Fairfax County, Virginia.

Serologic tests of specimens from 36 murine typhus patients with the same antigens are in progress. The origin of these cases was as follows: 25 from South Vietnam, 6 Department of Rickettsial Diseases laboratory infections, 2 soldiers returning from Vietnam, 2 from Malaysia and 1 from Louisiana. Spotted fever group antibodies developed in only 3 patients. These individuals were infected accidently in the laboratory and all had received Rocky Mountain spotted fever vaccine prior to the onset of murine typhus. None of the other laboratory personnel who had also received the Rocky Mountain spotted fever vaccine developed spotted fever antibodies after their R. mooseri infection. Considerable degree of cross-reactivity was found with the specific R. prowazeki, R. mooseri and R. canada antigens. Only 1 soldier who became ill in Michigan 1 week after his return from South Vietnam had levels of R. canada antibody that were 4-fold or greater than with the other specific antigens. Although it is suspected that he was infected with R. canada, it is not known whether the exposure occurred in Vietnam or in the USA. In all of the other patients the R. canada titers were equal to or significantly lower than the R. prowazeki and R. mooseri titers. Where it was known that the patient had been immunized previously with epidemic typhus vaccine it was not possible to identify the etiology of the disease since there were 2-fold or less differences in the titers obtained with the 3 specific complement-fixing antigens. Additional studies are planned to evaluate the usefulness of agglutination and indirect immunofluorescent tests for differentiating among infections caused by the members of the Typhus Group.

Summary and Conclusions.

1. Scrub Typhus Vaccine Development.

a. Tissue culture methods are being perfected that should provide heavy suspensions of the 3 established prototype strains, Karp, Gilliam and Kato, as well as the 5 Thai candidate prototype strains, TA763, TA716, TA678, TA686 and TH1817. These suspensions are required for production of complement-fixing antigens to complete characterization of the dominant and minor antigenic components of the strains and for the preparation of experimental vaccines. In addition, a procedure for plaquing strains of R. tsutsugamushi is under development that will be used to clone candidate vaccine strains in order to insure purity.

b. Antirickettsial serum fluorescein conjugates are being prepared

that can be used for the rapid identification of the antigenic type of scrub typhus strains by direct immunofluorescent staining.

2. Evaluation of Existing and Potential Military Importance of Rickettsia canada.

a. Surveillence of patients admitted to Womack Army Hospital, Fort Bragg, North Carolina, last year uncovered only 1 case of Rocky Mountain spotted fever. In the 2 prior years 3 cases of R. canada infection and 3 with R. rickettsi infection were recognized. Serologic tests with serum from small and medium-sized mammals trapped in the Fort Bragg - Fayetteville area showed that some of the raccoons, cottontail rabbits, and gray foxes had been infected with R. rickettsi. Enzootic foci of R. canada have not yet been identified.

b. Serum specimens from patients previously diagnosed as having Rocky Mountain spotted fever or murine typhus on the basis of serologic reactivity with the corresponding group-reactive antigen were retested with the following complement-fixing antigens: typhus and spotted fever group-reactive antigens, and specific R. prowazeki, R. mooseri and R. canada antigens. After infection with either R. rickettsi or R. mooseri, the patient does not develop antibodies that will fix complement with the heterologous group-reactive antigen unless he has been immunized previously with a vaccine prepared with a member of the other group of rickettsiae. Thus, 12 of 22 cases, or 55%, of patients with Rocky Mountain spotted fever who had received epidemic typhus vaccine, developed antibodies that reacted with Typhus Group antigens. Correspondingly, 3 of 6 patients with murine typhus, who had received Rocky Mountain spotted fever vaccine developed Spotted Fever Group antibodies. Serologic evidence of coincident R. canada infection was found in 2 of the Rocky Mountain spotted fever patients. One of these patients became ill in Cherry Point, North Carolina and the other in Fairfax County, Virginia. Tests with specific antigens showed that a soldier who became ill in Michigan 1 week after returning from South Vietnam had been infected with R. canada and not with R. mooseri as had been presumed previously.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 167, Rickettsial diseases of military personnel

Literature Cited.

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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
69 07 01	D. Change	U	U	NA	NT	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A061102B71Q	00	168
b. CONTRIBUTING				
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21. GENERAL USE

Foreign Intelligence Not Considered

DA

22. KEYWORDS (Precede each with Security Classification Code)

(U) N. meningitidis; (U) Bacteria; (U) Mycoplasmas; (U) L-Forms; (U) Immunology; (U) Endotoxin; (U) Air Sampling; (U) Antibiotics; (U) Viral Diagnosis; (U) Adenovirus

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Precede individual paragraphs identified by numbers, preceded first by appropriate 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, gonococcal and mycoplasma infections in military forces.

24 (U) - Development of bacteriologic techniques - isolation, identification, antibiotic sensitivity tests, etc. - for study of various infectious diseases. Field studies on prophylactic regimens, spread and persistence of organisms in various military populations.

25 (U) - 69 07 - 70 06 Group C meningococci have continued to be the most common cause of meningitis in recruits. Five different serologic tests for meningococcal infection have been under study. The hemagglutination test is group specific; the complement fixation and newly developed soluble antigen fluorescent antibody (SAFA) tests are species reactive. Bactericidal antibodies are directed against group specific polysaccharides as well as strain specific antigens and species-common antigens. The fluorescent antibody test using whole organisms measures antibodies against all antigens. Mycoplasma studies have been directed towards methods for isolation and identification of organisms isolated from respiratory and genital tract infections. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1498-1 (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 shown that group C sulfadiazine resistant strains have caused 95 percent of the disease seen in Army personnel in the current year. Serologic responses of cases, carriers and vaccinated volunteers have been measured by five different assays. The CF test measures species reactive antibodies which develop following natural infection, but are not induced by polysaccharide vaccines. The HA test is group specific and is very useful in vaccinated persons. A new SAFA test has been developed for meningococcal serology. An influenza diagnostic SAFA test has been shown to differentiate type A and B infections. Mycoplasma studies of respiratory and genital infections have centered on techniques for recovery and identification of these organisms.

Progress.

1. Meningococcal meningitis.

a. Prevalence of serogroups causing disease in U. S. Army personnel, 1964-1970.

Strains of N. meningitidis from patients in Army hospitals throughout the world have been submitted to this laboratory for study

since 1964. Over 98 percent of these patients are Army personnel, the remainder being military dependents. Each year over 90 percent of strains have been found to belong to serogroups B or C (Table 1).

Table 1. Prevalence of meningococcal serogroups (1964-1970) (Blood or spinal fluid isolates).

Serogroup	1964	1965	1966	1967	1968	1969	1970*
A	5	3	1	1	0	1	1
B	259	153	226	53	37	37	5
C	29	22	40	81	275	309	150
Y	2	3	12	11	10	7	0
29E	1	0	0	0	1	0	0
135	0	0	2	2	2	0	0
NT	4	0	0	0	0	0	0
Total	300	181	281	148	325	354	156

*Through 15 May only

Group A strains have been rare during this period and of 12 such strains, only one was isolated from an illness acquired in the continental U. S. Only four nongroupable strains were noted in this series. These occurred during the first year of the study; none have occurred in the last 1400 strains examined. Group B strains predominated in the years 1964 through 1966 (Table 2).

Table 2. Changing prevalence of serogroups B and C as causes of disease.

Serogroup	1964	1965	1966	1967	1968	1969	1970**
B	86.4*	84.5	80.4	35.8	11.4	10.4	3.2
C	9.7	12.3	14.2	54.7	84.6	87.3	96.2
Other	4.0	3.3	5.3	9.4	4.0	2.2	0.6

* % of total strains

** Through 15 May only

Subsequently group C strains have increased in number to the current ratio of 96 percent. Sulfadiazine resistance among group B strains has varied slightly during the seven years of study, from 48 to 70 percent of strains being resistant to concentrations of 1 mcg/ml of the drug (Table 3).

Table 3. Sulfadiazine resistance in meningococcal case strains.

		1964	1965	1966	1967	1968	1969	1970*
Group B	No. received	259	153	226	53	37	37	5
	No. SR	167	87	160	34	18	18	1
	% SR	64.5	56.9	70.8	64.7	48.6	48.6	20.0
Group C	No. received	29	22	40	81	275	309	150
	No. SR	2	2	25	70	264	303	144
	% SR	6.9	12.3	62.5	86.4	96.0	98.0	96.0

SR = Resistant to 1 mcg/ml sulfadiazine or greater

* Through 15 May

Almost all group C strains isolated in the past two years have been resistant to this concentration of sulfadiazine.

It is of some interest that despite the rarity of groups Y, 29E and 135 as causes of systemic illness certain of these serogroups are frequently identified in the nasopharynx of symptom free carriers.

b. Carrier surveys in basic training centers.

These sporadic surveys show the variation in carrier prevalence and dominant serogroup which occurs in different companies sampled on the same day at a given post (Table 4). The survey at Fort Bragg showed a totally different pattern in each of three basic training companies. At Fort Bragg, the three companies tested were in the same brigade and at Fort Knox, three companies in the same brigade and the same battalion showed entirely different patterns of carriage. Such tremendous variation will seriously hamper interpretation of vaccine or chemoprophylaxis experiments unless large sample size is utilized. These data also illustrate the fact that results of once monthly routine carrier surveys of 100 men in one company do not reflect the true prevalence of carrier rates for that post.

Table 4. Meningococcal carrier rates* in basic trainees.

Post	Date	Unit	% Pos.	Serogroup				
				B	C	Bo	29E	NG
Dix	15 Jul 69	A-2-3	20	2	3	1	8	6
		E-2-3	16	1	1	0	6	8
	28 Aug 69	D-1-3	41	2	23	8	3	5
		E-1-3	33	3	7	4	8	11
	7 Oct 69	A-3-3	44	0	2	0	18	19
		C-3-3	50	0	0	0	23	27
		E-3-3	42	10	6	4	4	18
	2 Dec 69	D-2-3	45	5	14	8	3	14
		B-2-3	38	0	23	4	1	9
	20 Jan 70	C-4-3	41	0	15	1	13	18
		E-5-2	33	1	15	5	4	8
		B-5-2	46	5	11	8	6	16
Knox	7 Jan 70	E-18-5	40	1	16	12	9	12
	28 Jan 70	C-16-4	32	2	15	3	2	10
		A-16-4	29	1	2	1	16	9
		D-16-4	24	3	2	8	4	7
Bragg	3 Feb 70	A-4-1	21	0	0	8	6	5
		C-3-1	11	1	2	0	4	4
		C-2-1	32	2	19	3	3	5

*Sample of approximately 100 men in 6th or 7th week BCT

c. Serological responses to meningococcal infections or vaccines.

Serological studies to detect meningococcal antibodies have been carried out by a variety of methods in this laboratory (Table 5).

Table 5. Serologic assays for meningococcal infection.

Test system	Antigen characteristics	Specificity of antibody
Complement fixation	protein	cross reactive for species
Indirect hemagglutination	polysaccharide	group specific (anti polysaccharide)
Immunofluorescence	whole organism	cross reactive for species also antipolysaccharide
Bactericidal	whole organism	1) antipolysaccharide 2) strain specific when absorbed with polysaccharide 3) cross reactive for species also
SAFA	protein, polysaccharide	cross reactive for species
Precipitation	polysaccharide	group specific

In addition, other tests such as opsonization, mouse protection and radioactive antigen binding capacity have found use in specific investigations by others. In this report a brief description of the methods and a comparison of the various assays will be presented.

Indirect hemagglutination:

This technique involves sensitization of erythrocytes with polysaccharide antigens and observation of agglutination titers of sera using microtiter apparatus. Purified polysaccharides have been used primarily (method of Gotschlich) although crude preparations (method of Edwards) are also satisfactory. It has been found necessary to vary the pH of the system and to determine the optimum antigen concentration for sensitization for each of the polysaccharide used. Human group O erythrocytes have been used for testing of human sera although sheep red cells have been found satisfactory. Erythrocytes have been treated with fixatives (formaldehyde, glutaraldehyde, pyruvic aldehyde) but such cells have

been useful only with the C polysaccharide human rbc system. In addition, the group B test has required heparin-manganese chloride treatment of sera to achieve satisfactory hemagglutination patterns.

One very important aspect of the HA test is that 1-2 tube variation occurs frequently and for comparisons to be valid sera should be tested on the same day.

Results of HA testing have shown the antibody responses to be group specific, that is, patients with group C meningitis show HA antibody response to group C antigen only, patients with group B or Bo disease do not show anti-C response. No sera from patients with group A diseases have been studied. After vaccination with A or C polysaccharides only group specific HA antibodies have developed.

Following natural infection with group C meningococci HA titers rise rapidly to a peak at 6-9 days and begin to fall to lower levels by 27-32 days after onset of illness. By two months an average decrease of four tubes (1:256 to 1:16) has occurred (Table 6).

Table 6. Hemagglutination antibody titers following group C systemic infection.

Days after admission	No. of samples	Geometric mean HA titer ± SE (LOG_2 units)
0-2	40	1.60 ± .35
3-5	3	6.00 ± 1.16
6-9	40	8.75 ± .28
10-12	9	8.11 ± .49
13-15	7	8.14 ± .71
21	1	8.00
27-32	16	6.31 ± .36
34-36	3	5.67 ± .91
40-56	6	4.00 ± 1.44

Comparison was made of anti-C polysaccharide titers in vaccinees, carriers and meningitis cases, Table 7 shows that systemic infection results in higher titers than vaccination or the natural carrier state. Previous work from this laboratory by Goldschneider has shown a similar situation using bactericidal and immunofluorescence tests when cases and carriers were studied, but by his methods vaccination gave very high titers.

Table 7. Comparison of hemagglutination titers following immunization and natural infection with group C meningococci.

	No. tested	Mean max. HA rise*
Vaccinees	10	4.30
Carriers	13	4.85
Cases	48	6.83

*No. of 2-fold dilutions

It should be pointed out that HA antibody consists of both immunoglobulin M (IgM) and immunoglobulin G (IgG) whereas bactericidal antibody has been found to be IgG exclusively. The HA data suggests that systemic infection provides a greater stimulus to antibody development, either because the amount of antigen is greater than carriers or vaccinees receive, or because more antibody producing cells are stimulated by the bacteremia.

Complement fixation (CF):

The standard WRAIR 50 percent hemolysis, microtiter system was utilized. Antigens were prepared by the ammonium sulfate technique of Edwards.

Results of tests on patients with meningitis due to groups B or C meningococci are shown in Table 8.

Table 8. CF antibody rises in meningitis patients.

Meningococcus isolated	CF antibody rises		
	A Antigen	B Antigen	C Antigen
Group C	5/13*	13/13	8/13
Group B	2/5	5/5	5/5

*No. pos./No. tested

The cross reactivity of the antigens is apparent. From the data it appears that the B antigen is the most sensitive of the three antigens used. Of nine individuals vaccinated with group C polysaccharide,

serum obtained two weeks after immunization failed to show CF antibody response to all three antigens.

Thirty-five volunteers who received group A vaccine provided serum specimens at two week intervals through six weeks. None showed a CF antibody response to a group A CF antigen.

Several attempts to use purified group C polysaccharide as a CF antigen have failed to demonstrate CF with immune human sera.

Immunofluorescence:

This test utilizes agar grown meningococcal organisms as antigen. Human antibody which binds to the bacteria is detected by means of a fluorescein conjugated anti-IgG antiserum prepared in rabbits. Preparation of the bacterial suspensions requires meticulous attention. Young (5-6 hour) cultures are essential; it has been found that slides can be fixed and stored for up to one week with reproducible results.

The fluorescent antibody (FAB) test has been used to detect natural infections (carriers) as well as vaccine induced antibody. Carriers who develop antibody demonstrate cross reactivity between A, B and C meningococcal antigens (Table 9). Polysaccharide vaccine induced antibody, however, is group specific by this test (Table 10).

Bactericidal antibodies:

This test measures the ability of serum to kill meningococci. In addition to antibody (IgG) the complement system is required. Unheated, antibody-free human serum is used as a source of complement. Inoculum size, age of culture, media and other variables must be carefully controlled to assure reproducible results. Previous studies by Goldschneider have shown that lack of bactericidal antibody is correlated with susceptibility to disease. Also, he showed that natural infection with a specific serogroup results in the development of bactericidal antibody which is cross reactive with other serogroups of meningococci. Polysaccharide vaccines also induced bactericidal antibodies which were group specific. Table 11 shows the antibody response of seven volunteers two weeks following a 50 microgram dose of group C vaccine.

Discussion:

A variety of serologic tests are now available for measuring anti-meningococcal antibodies. Some are highly specific; others are cross reactive among the various meningococcal serogroups. Antibodies directed against the polysaccharide capsular antigens are quite specific when measured by HA whether antibody is acquired by natural infection or immunization. The bactericidal and FAB methodology detects not only antipolysaccharide but also other bacterial antigens. When antibody is induced by purified polysaccharide vaccine the FAB and bactericidal

Table 9. Fluorescent antibody response in recruits who became meningococcal carriers.

Name	Week of specimen	Pharyngeal culture	Reciprocal of serum FAB titer against indicated antigen		
			A	B	C
Bus.	0	Neg	8	8	4
	2	Bo	128	256	256
	4	Bo	64	256	128
Cos.	0	Neg	<2	2	4
	2	Bo	<2	8	2
	4	Bo	16	8	256
Rod.	0	Neg	4	2	<2
	2	Nongroup.	8	8	2
	4	Rough	4	8	<2
	6	Bo	4	64	32
Aus.	0	Neg	-	-	-
	2	Neg	<2	8	2
	4	Bo	<2	8	2
	6	Neg	16	64	128

Table 10. Antibody response following immunization with group C polysaccharide.

Vol. No.	Gp C-HA titer	Gp C-FAB titer	Gp B-FAB titer	Gp A-FAB titer
1.	<2*	8	16	<2
	128**	128	32	<2
2.	4	4	16	<2
	64	128	16	<2
3.***	4	8	16	2
	64	16	64	2
4.	<2	8	64	2
	128	256	128	2
5.	<2	8	32	2
	64	32	16	2
6.	<2	4	16	2
	32	16	16	2

Titers expressed as reciprocal of serum dilution.

* preimmunization serum

** 2 weeks post immunization serum

***carrier of nongroupable meningococcus at 2 week bleeding

Table 11. Hemagglutination and bactericidal antibody titers before and two weeks following immunization with group C polysaccharide.

Volunteer	Serum	Group C antibody titer	
		HA*	Bactericidal (% killing)**
1	A	4	0
	C	64	99
2	A	2	0
	C	128	99
3	A	2	0
	C	64	99
4	A	0	0
	C	128	95
5	A	4	0
	C	128	99
6	A	4	0
	C	128	92
7	A	0	0
	C	64	>99

* Reciprocal of serum dilution

** 1:10 dilution of serum

responses are group specific. Following natural infection, however, cross reactive antibodies are detected. This phenomenon was previously documents by Goldschneider et al.

The CF test is broadly reactive following natural infection but antipolysaccharide antibody has not been detected by CF test following vaccination. Thus, it may be possible to differentiate carrier induced antibody from that stimulated by vaccine by utilizing both CF and HA tests. Such a procedure could prove useful when vaccine field studies are undertaken in geographic areas where bacteriologic cultures cannot be performed.

d. A SAFA test for serologic studies of meningococcal infection.

A variety of serologic techniques have been used to detect meningococcal antibodies in man. Of these, some antibodies are group specific, others react with other members of the genus Neisseriae. The soluble antigen fluorescent antibody (SAFA) technique has been found to offer many advantages for serologic diagnosis of parasitic and viral diseases and, thus, was examined in relation to problems in meningococcal serology.

(1) Materials and methods: Meningococcal strains A_1; B (99-Misc), and C (9-Misc) were isolates from patients with meningitis and are standard organisms in use in this laboratory. Several types of antigens were prepared for the SAFA test. An alcohol extracted antigen (ETOH) was produced using the method of Edwards and Driscoll. Briefly, overnight Mueller-Hinton broth cultures were inactivated by beta propriolactone and sedimented by centrifugation (600 g). The sediment was washed, resuspended in saline, dissolved in 1N NaOH, and after readjusting pH to 6.5 with 1N HCl the antigen was precipitated with 5 volumes of absolute ethanol. A "genus" antigen was obtained by the method of Edwards and Devine. This antigen was prepared by using the culture supernate from the previous method, precipitating the antigen with saturated ammonium sulfate ($(NH_4)_2SO_4$) and dialyzing the precipitated material. The antigen was clarified by centrifugation just prior to use in the SAFA test. Fluorescein conjugated horse anti-human globulin (AHG) (Lot 3132, Progressive Laboratories, Inc., Baltimore, Md.) was used at a 1:15 dilution.

(2) Technique of the SAFA test: The improved method of Toussaint was used. Briefly, discs are prepared by filtering the antigen through a 0.45 micron millipore membrane and, after overnight drying, 1/4 inch diameter discs are punched out. Heat inactivated sera are incubated with antigen discs in wells in plastic trays; after suitable washing, conjugated AHG is added. Following further washing and drying discs are mounted on black electrical tape and readings are performed in a fluorometer.

(3) Serum specimens: Sera were obtained from patients with proven meningococcal meningitis at the time of admission to the hospital and at intervals thereafter. Sera from volunteers immunized with polysaccharide vaccine were drawn at two week intervals and stored frozen between tests. Meningococcal nasopharyngeal carrier status was determined by methods standard in this laboratory.

(4) Results: Optimum concentrations of serum and antigen were determined by box titration with several patient's sera. On the basis of these results a serum dilution of 1:10 and antigens diluted 1:4 were used. A comparison of the two types of antigens is shown in Table 12.

Table 12. SAFA test results on group C cases using two group C antigens.

Case	Serum date	Antigen	
		ETOH	$(NH_4)_2$ SO_4
Ka	0 wk	147*	48
	1 wk	774	114
	4 wk	495	150
La	0 wk	83	22
	1 wk	846	83
	5 wk	414	68

*Fluorescent Dial Reading

These data are representative of 12 patients in whom the $(NH_4)_2$ SO_4 antigen gave fluorescent dial readings (FDR) consistently lower by 3 to 10 fold and showing no rise (<100 FDR) between acute and convalescent sera when compared to the ETOH antigen. The specificity of the SAFA ETOH antigen was tested by comparing results in four patients whose meningitis was caused by group B and eight individuals whose illness was caused by group C meningococci. Representative results are shown in Table 13.

These results show the cross reactivity of the two antigens with sera from infection due to both serogroups. In general, the group B antigen gave somewhat higher readings than the group C antigen, but both produced marked increases in FDR in sera obtained 7-10 days after onset of illness in all 12 patients. All these individuals also showed FDR increases against a group A SAFA antigen.

Table 13. Specificity of SAFA test.

Case	Serogroup isolated	Serum date	FDR using indicated ETOH antigen	
			B	C
Be	C	0	165	90
		10 days	513	495
		4 wk	324	234
Bl	C	0	138	96
		1 wk	828	>900
		4 wk	577	312
Co	B	0	231	156
		8 days	819	603
		4 wk	549	186
Ki	B	0	138	46
		1 wk	>900	494
		2 wk	693	186
		5 wk	378	123

Sera obtained before and two weeks following immunization of volunteers with meningococcal polysaccharides were tested with SAFA antigens. Under the conditions shown in Table 14 this test was not as sensitive as the HA test in detecting vaccine induced antibody. Another experiment was performed in which serum specimens were used without dilution. These results are shown in Table 15.

Table 14. SAFA test to detect vaccine induced antibody.

Vaccine administered	SAFA antigen	Serum dilution tested	
		1:2	1:10
A	A	9/12*	5/12
C	C	10/19	3/19

*No. pos./No. tested (Pos. = 80 or more FDR units). All volunteers had HA titer increases.

Table 15. Results of SAFA test with undiluted serum of 30 men who received group C vaccine*.

SAFA antigen	No. men pos.** at indicated week following immunization	
	2 weeks	6 weeks
Group C	26	4
Group B	7	4

*100 micrograms of Lot C-4 polysaccharide administered; serum obtained at 0, 2 and 6 weeks; nasopharyngeal cultures obtained at 0, 2, 4 and 6 weeks. All men had hemagglutinating antibody rise.

**Rise in FDR of 80 units or more over baseline serum.

Twenty-six of 30 volunteers had SAFA-C antibody increase within two weeks of immunization; seven men had SAFA-B rise in two weeks. (One of these latter seven was not positive by SAFA-C but he had a very high initial SAFA-C titer.) Five of the seven group B SAFA rises occurred in recruits who were meningococcal carriers. Of the men who had late SAFA rises all four SAFA-C and 3/4 SAFA-B responders were men who had become pharyngeal carriers during the study.

Thus, when serum concentration in the test was increased vaccine induced specific antipolysaccharide antibody could be detected in 85 percent of volunteers by the SAFA test. However, cross reactive antibody related to a carrier infection by a different meningococcal serogroup was also detected.

(5) Conclusions: The SAFA test offers advantages such as ready standardization, objective quantitation, stability of reagents and no requirement for serial dilutions of serum. The test is cross reactive within the species, N. meningitidis, using the antigen described. Further studies are in order to determine if group specific purified polysaccharides can be used to detect vaccine responses.

e. Antibiotic prophylaxis: in vitro studies of rifampin.

The new antibiotic, rifampin, has demonstrated a high degree of in vitro activity against N. meningitidis and a recently reported clinical study has shown that prophylactic treatment with rifampin eliminated the nasopharyngeal meningococcal carrier state. The potential widespread use of rifampin to eliminate the nasopharyngeal carrier state in high risk populations prompted this laboratory to study the in vitro response of large populations of meningococci to this antibiotic.

Fourteen recently isolated strains of meningococci, 11 carrier strains and 3 strains from cases of meningococcal meningitis were studied (1) to determine their rates of mutation to different concentrations of rifampin and (2) to learn if the level of rifampin resistance in mutants was limited to the concentration of rifampin used in the selection procedure.

Briefly, the selection of resistant mutants was done by plating 10^8 or 10^9 viable cells of each strain of meningococcus onto different concentrations of rifampin in solid media. Resistant clones developing at low concentrations of rifampin were then tested for their actual level of rifampin resistance by replication onto solid media containing higher concentrations of rifampin. Representative mutant clones were tested by gram stain and oxidase reagent. The results are described below.

Resistant mutants were usually apparent as micro colonies on the selection media after 24 hr incubation. At 48 hr to 72 hr the colonies were large enough for further work. As can be seen in Table 16, 3 strains, 6, 12 and 40, grew well on 0.4 mcg rifampin, ml and were considered

Table 16. Mutation to rifampin resistance (0.4γ/ml) among 14 strains of N. meningitidis.

Strain	Serotype	Source	No. cells plated	No. mutants / 10^8 cells	Strain	Serotype	Source	No. cells plated	No. mutants / 10^8 cells
1	C	NP*	1.5×10^8	3	42	29E	NP	2.2×10^8	15
6	29E	NP	5.6×10^7	10**	64	29E	NP	3.5×10^8	6
12	29E	NP	1.7×10^8	3**	94	C	NP	1×10^9	2.5
16	C	NP	3.8×10^8	2	97	C	NP	1×10^8	2
28	C	NP	2.6×10^8	13	69-4857	C	CSF	3.8×10^8	14
39	C	NP	3×10^8	1	69-4860	C	CSF	1.7×10^8	20
40	29E	NP	1.9×10^8	10	69-5319	C	CSF	4×10^8	12

* NP = nasopharynx

** Mutants present at 1.6γ/ml rifampin: Strain considered resistant to 0.4γ/ml.

resistant at this concentration. Concentrations as high as 0.4 mcg/ml have been reported in the saliva of volunteers following administration of a prophylactic course of rifampin. The numbers of mutants listed for each strain that could be expected to appear per 10^8 cells correspond to mutation rates which range from 1×10^{-7} to 1×10^{-8}. These rates are 100 to 1000 fold greater than the rates described for mutation to streptomycin resistance among the Enterobacteriaceae.

Increasing rifampin concentration had little effect upon the numbers of mutants which were recovered. In Table 17 it can be seen that the numbers of mutants observed at high concentrations for a given strain either remained the same as that seen at low concentrations of rifampin or were slightly decreased as seen in strain 69-4860. The findings presented in the above tables indicate that mutation to rifampin resistance is a spontaneous, random event occurring at frequencies which vary slightly between strains. The findings also suggest that mutation to high levels of rifampin resistance occurs in one step rather than in a stepwise fashion.

The observation that 3 of 14 (21%) of the strains tested were apparently resistant to 0.4 mcg/ml rifampin is in contrast with the observations of other investigators who found only rare strains resistant to as much as 0.1 to 0.2 mcg/ml. Our findings indicate that one of the three strains would have had sufficiently large numbers of resistant cells to have been detected by routine procedures using overnight broth cultures for the inoculum. However, it is estimated that 0.4 mcg/ml resistant cells comprises less than 0.01 percent of the total populations in the other two strains. With such small numbers the possibility of detecting resistant cells in an overnight broth culture become remote.

Table 18 describes the results of replicating mutants selected at 0.4 mcg/ml onto media containing higher concentrations of rifampin. While a great deal of variation is apparent between strains the data demonstrate that the resistance levels of mutants selected at 0.4 mcg/ml rifampin is not limited to 0.4 mcg/ml but that substantial numbers in most of the strains tested were resistant to 25 and 100 mcg rifampin/ml. These data demonstrate that mutation to rifampin resistance occurs as a one step event; that exposure of meningococci to low concentrations of rifampin could result in the selection of mutants resistant to at least 100 mcg/ml.

The introduction of new antibiotics, such as rifampin, with demonstrable activity against meningococci will offer to many a new hope in breaking the cycle of transmission of nasopharngeal carriage in high risk populations and thereby drastically reduce the incidence of meningococcal meningitis. While the prophylactic use of rifampin will probably meet with initial success, the exposure of large numbers of meningococci to such pressure is likely to result in the selection of mutants resistant to rifampin levels much greater than that which can

Table 17. Effect of cell concentration and drug concentration on mutation to rifampin resistance in strains of Neisseria meningitidis.

Strain	Serotype	Source	No. cells plated	No. rifampin resistant mutants observed					Mutation rate at 0.4γ/ml
				0.4	1.6	6.25	25	100	
NP-1	C	NP	1.5×10^9	38	30	34	30	23	
			1.5×10^8	4	1	2	2	2	2.6×10^{-8}
NP-40	29E	NP	1.9×10^9	TNTC	250	272	255	235	
			1.9×10^8	TNTC	36	25	30	25	1.5×10^{-7}*
69-4857	C	CSF	3.8×10^9	TNTC	221	182	174	152	
			3.8×10^8	57	23	14	14	0	1.5×10^{-7}
69-4860	C	CSF	1.7×10^9	150	146	123	112	69	
			1.7×10^8	33	27	25	23	14	2.0×10^{-7}

*Based upon mutants appearing at 1.6γ/ml rifampin.

Table 18. Rifampin resistance levels of mutants selected at 0.4γ rifampin/ml.

Strain	No. of mutants tested	Rifampin concentration		
		6.25γ	25γ	100γ
NP-1	75	55 (73%)	50 (67%)	18 (24%)
NP-16	206	50 (24%)	40 (19%)	0 (0%)
NP-39	126	89 (71%)	68 (54%)	10 (8%)
NP-64	252	22 (9%)	16 (6%)	9 (4%)
NP-94	146	98 (67%)	56 (38%)	44 (30%)
69-4857	100	24 (24%)	22 (22%)	18 (18%)
69-4860	51	47 (92%)	46 (90%)	42 (82%)

be achieved in the saliva. The number of mutants which could be expected to occur and the eventual fate of these mutants is clouded by such imponderables as the numbers of meningococci in the nasopharynges of carriers and the ability of resistant mutants to multiply and establish the carrier state.

f. A new classification of meningococci by means of bactericidal reactions.

A bactericidal assay has been developed which allows identification of distinct serotypes within a serogroup of N. meningitidis. Antisera produced in rabbits against seven group C strains by two intravenous inoculations of live organisms were found to have two types of antibodies. One, directed against group specific polysaccharide, caused varying degrees of killing of all strains. Absorption of this antibody by purified group C polysaccharide revealed the presence of the second bactericidal antibody. This antibody was directed against antigenically distinct factors associated with serotype specificity. Extensive cross absorptions yielded antisera with activity directed against four separate factors. The presence of a factor in a strain was indicated by its susceptibility to killing by antisera containing antibody to that factor. A serotype was defined by the particular combination of factors. Eight different serotypes, containing one to three factors, have been identified among 49 group C strains examined.

g. Hemagglutination-Inhibition test for serogrouping N. meningitidis cultures.

At present isolates of N. meningitidis are grouped by agglutination of bacterial suspensions by specific rabbit antisera. This method has proven to be less than satisfactory for several reasons: (1) some cultures agglutinate in more than one serum (multiply agglutinable), (2) some agglutinate in all sera (rough), and (3) others do not agglutinate in any sera (nontypable). Because of these inadequacies the hemagglutination inhibition (HI) test is being investigated as an alternative. The HI test identifies the specific polysaccharide antigen produced by a strain by its ability to inhibit hemagglutination of sensitized erythrocytes by specific antisera.

(1) Methods.

(a) Antigens: Hemagglutinating antigens are prepared according to the method of Edwards and Driscoll. Strains used for production of the antigens are the A_1 strain of group A, group B (99 Misc), group C (9 Misc), Bo (135-M), 29E, 135 WRAIR, and the Slaterus strains, X and Z. Cells grown in Mueller-Hinton broth are collected and extracted by mild alkaline hydrolysis followed by ethanol precipitation. The suspension is centrifuged and the clear supernatant is the group specific antigen. Occasional antigens

prepared by this method lack the required group specificity and must be discarded. Optimal concentrations for sensitization of red blood cells (RBC) vary according to antigen batch and serum used. Therefore, optima are determined for each system.

Purified polysaccharide was used in the A, B and C systems, but it was not available for the other serotypes. Therefore, it was decided to use similar crude antigens for all serogroups.

(b) Red blood cells: Fresh sheep red blood cells (RBC's) are fixed with pyruvic aldehyde according to the method of Ling.

(c) Sensitization of RBC's: Fixed RBC's are washed three times in phosphate buffered saline (PBS) and adjusted to 4% in PBS. The cells are sensitized by mixing equal volumes of the washed 4 % RBC's and antigen diluted to its predetermined optimal concentration in PBS. The mixture is incubated with frequent mixing for one hour at 37°C; then washed three times in PBS. The sensitized cells are then diluted to a final concentration of 0.5% in PBS containing 0.5% bovine serum albumin. Sensitized cells can be stored in the cold for at least one week.

(d) Antisera: Specific rabbit antisera prepared against whole organisms are used. Prior to use with each batch of sensitized cells the serum titers are determined. For the HI test four units of antisera are used.

(e) Bacterial suspensions: Living bacterial cells from one day old cultures on chocolate LP medium are heavily suspended in physiological saline. The cells are spun out at 3000 rpm for 10 min. and the resultant clear supernatant is separated and frozen. Attempts at killing the cells with formalin and β-propiolactone prior to extraction failed because the formalin caused all the RBC's to settle and the β-propiolactone caused all the cells to agglutinate. Extraction of cells was tried at 37 C and room temperature for various time periods. All suspensions gave the same reactions. Thus, time and temperature of extraction are not critical.

(f) HI test: For the test 0.05 ml bacterial supernatant is mixed with 0.05 ml 4 unit serum in "U" bottom microtiter plates. These are incubated at 37 C for 30 min. The 0.05 ml 0.5% sensitized cells are added, the plates are sealed with transparent tape, and are incubated on a vibration damping platform at room temperature for two hours. At the end of this time reactions are read.

(g) Description of results: Results of the HI tests are described as typable when agglutination is totally inhibited for one or more serotypes, and nontypable (NT) when agglutination is not inhibited for any serotypes. Results of the bacterial agglutination (BA) test are described as typable when agglutination occurs in only one serotype, multiply agglutinable (MA) when agglutination occurs in more than one but not all serotypes, rough (Ro) when agglutination

occurs nonspecifically in all serotypes as well as saline, and nontypable (NT) when agglutination does not occur in any serotype.

(2) Results. Meningococcal carrier surveys were performed in two basic training companies at Fort Bragg, North Carolina on two separate occasions. Results of serogrouping of 396 meningococcal isolates are given in Table 19. Two hundred and seventy, or 69 percent of the total, were typable as a single serogroup by the HI test. One hundred and ninety, or 71 percent, of these were confirmed by the BA test, 74 or 27 percent were not typable by the BA test, and six or 2 percent of these were grouped as different by the BA test.

Twenty-one isolates or 5 percent of the total 396 were typable in two groups by the HI test. Only 3/21 (14%) of these were confirmed by the BA test, 11/21 (52%) were typed as one of the two groups included in the HI test, six or 27 percent were nontypable in the BA test and one was typed as a different group by the BA test.

(3) Conclusions. Examination of the results suggests that the HI test may be superior to the BA test for typing meningococcal isolates. Increased specificity is suggested by isolates that were multiply agglutinable or rough in the BA test and typable in the HI test, and increased sensitivity is suggested by the fact that 74 percent of the isolates were typable by HI and only 57 percent were typable by the BA test. These results must be confirmed, however, by experiments to determine the reproducibility of the HI test and by reexamination of those strains which were nongroupable and those in which identification of the serogroup by the two methods differed.

The HI test appears to offer a number of general advantages over the BA test in regard to objectivity of readings and using smaller amounts of grouping antisera. If the sensitivity and specificity of the HI test is confirmed by further study it will replace bacterial agglutination as our definitive diagnostic procedure.

2. Investigations on mycoplasmas and L-phase variants of bacteria.

a. Characterization studies on Streptobacillus moniliformis and its L-phase variant.

The classification and characterization of Streptobacillus moniliformis have remained subjects of controversy and ambiguity for many years. Frequent requests from hospital laboratories for identification of a wide variety of pleomorphic organisms suspected of being S. moniliformis have emphasized the need for clarification of the diagnostically significant features of this species. The occasion for a thorough reexamination and characterization of extant strains of S. moniliformis was provided during the past year by the need to revise the chapter on the genus Streptobacillus for the impending Eighth Edition of Bergey's Manual of Determinative Bacteriology.

Table 19. Comparison of HI and BA serogrouping of meningococci.

	Serogroup	No. of isolates	% of group	% of total
Results of HI and BA agree	A	1	0.5	
	B	9	4.7	
	C	34	17.9	
	Bo	136	71.6	48%
	29E	6	3.2	
	135	0	0	
	X	3	1.6	
	Z	1	0.5	
	Total	190	100.0	
Not typable by HI and BA	Nontypable	46	59.7	
	Mult. agglut.	17	22.1	19%
	Rough	14	18.2	
	Total	77	100.0	
Typable by HI Not typable by BA	A	1	1.4	
	B	4	5.4	
	C	50	67.6	
	Bo	9	12.2	19%
	29E	7	9,5	
	135	2	2.7	
	X	0	0	
	Z	1	1.4	
	Total	74	100.2	
Double HI		21		5%
Nontypable-HI Typable BA	A	0	0	
	B	1	3.6	
	C	8	28.5	
	Bo	10	35.7	
	29E	3	10.7	7%
	135	2	7.1	
	X	3	10.8	
	Z	1	3.6	
	Total	28	100.0	
Disagree		6		2%
	Total	396		100%

Requests for S. moniliformis strains were sent by the Swiss International Center for Information on and Distribution of Type Cultures to culture collections and private investigators throughout the world. Of 10 cultures obtained, 1 was nonviable, 3 were contaminated and revealed no streptobacilli, and 1 has not yet been opened because no information about its source or culture history is available. Of the remaining 5 strains, 3 are bacillary phase and 2 are L-phase variants of S. moniliformis.

It was noted with interest that the above contaminated cultures labeled S. moniliformis were Gram variable, pleomorphic bacilli, members of the genus Corynebacterium. The fact that Gram variable corynebacteria are frequently mistaken for streptobacilli may account for the false notion reiterated in the literature that S. moniliformis can appear as weakly and irregularly Gram positive.

The morphologic and cultural characters of the bacillary phase of S. moniliformis were determined using the reference strain, ATCC, and 2 other strains, WRAIR and 120-jt. The species consists of rods 0.3-0.7 μm by 1-5 μm long, with rounded or pointed ends, frequently in chains, and filaments 0.5-0.9 μm by 10-150 μm long. Rods may show a central thickening; filaments often have a series of oval to elongated bulbous swellings 1-3 μm in diameter, giving the appearance of a string of beads. True branching does not occur. The species is consistently Gram negative. The organisms are nonencapsulated, nonmotile, nonspore-forming and nonacid-fast.

S. moniliformis is a facultative anaerobe that requires serum, ascitic fluid, or blood for growth, as well as an atmosphere containing CO_2 and moisture, an optimal temperature of 35-37°C, and an optimal pH of 7.4-7.6.

The morphology of the cells varies with the cultural conditions, age, and source of the organism. On 20% (v/v) horse serum infusion agar, 1-4 hour old cultures of laboratory strains consist of filamentous single cells that may or may not contain granules. After 6-21 hours, filaments elongate and develop multiple rounded or fusiform swellings. The filaments lie in wavy loops and often in a parallel or twisted arrangement. After 12-18 hours, the filaments fragment into segments forming chains of rods and round, oval, or fusiform bodies. Between 24 and 30 hours, tiny coccoid and ring forms appear accompanied by masses of bubbles and large irregular-shaped droplets of cholesterol or cholesterol-like material. After 3-5 days, the cells consist of very small, pale granulated rods.

In 20% (v/v) horse serum infusion broth cultures, the growth for the first 24 hours consists of chains of short rods with rounded ends and 1-4 granules located centrally, subterminally, or terminally. Longer granulated rods with tapered ends and long, tangled filaments with granules may or may not be present. After 48-72 hours, small,

slender, granulated rods predominate, or, if filaments were abundant earlier, the tangled filamentous form tends to predominate. Masses of bubbles are numerous. Tiny coccoid forms and round bodies may or may not be present.

Colonies on serum or ascitic fluid agar are 1-3 mm in diameter after 48-72 hours of growth. They are circular with a smooth or slightly irregular edge; convex or cone-shaped with flattened surface facets; grey to whitish-grey; slightly translucent to opaque with a glistening surface; and have a butyrous consistency.

In serum or ascitic fluid broth, growth after 24 hours consists of whitish granules or flocculent balls, which sediment on the bottom and along the side of the tube leaving a clear supernatant fluid. Occasional strains produce a slight turbidity. Growth produces no odor.

On sheep blood agar, colonies are barely visible at 24 hours, but after 48-72 hours are smooth, grey, translucent, and nonhemolytic. Slight alpha hemolysis of sheep and horse erythrocytes can, however, be demonstrated by blood cell-agar overlay techniques.

Acid but no gas is produced from various carbohydrates as shown in Table 20. The other significant biochemical activities of S. moniliformis are also listed in the table. In addition, the bacillary phase on 20% (v/v) horse serum agar produces film and spots similar to those produced by Mycoplasma strains.

All strains of S. moniliformis undergo a reversible conversion to transitional-phase variants. These variants have weakened or defective cell walls and during serial culture show alterations in cellular and colonial morphology and biology. In addition S. moniliformis can undergo conversion to the L-phase variant spontaneously in media containing serum or ascitic fluid. (The term "L-phase" is applied to bacterial variants that reproduce in the form of very small cells that lack rigid walls, and that produce colonies with central cores, which penetrate the agar.) The proportion of cells that undergo conversion varies from strain to strain and with the cultural conditions. The L-phase variant differs from the bacillus in cellular and colonial morphology, and in degree of resistance to those antibiotics that act primarily on the bacterial cell wall.

The individual L-phase cells vary from granule-like bodies 0.3 μm to large bodies 3.0 μm in diameter, averaging 1 μm. The smallest bodies are generally coccoid in shape. The larger bodies may be distorted from the normally round or spherical configuration to highly pleomorphic shapes and protoplasmic threads. True branching does not occur. The cells are nonmotile and Gram negative.

On serum or ascitic fluid agar, colonies of the L-phase variant range from 10 to 2000 μm in diameter, averaging 300 to 500 μm after

Table 20. Biochemical activities of bacillary and L-phase strains of Streptobacillus moniliformis.

Phase	Bacillary			L	
Strain	ATCC	WRAIR	Turkey	L_1 Rat 30	MLS
Source	ATCC	S.G.Cary	Yamamoto	E.K.-Nobel	R.M.Lemcke
ATCC No.	14647			14075	
Arabinose	±	±			
Dextrin	+	+		+	
Dulcitol	-	-			
Fructose	+	+		+	+
Galactose	+	+		+	+
Glucose	+	+		+	+
Glycerol	-	-			
Inositol	-	-			
Inulin				-	
Lactose	-	-		-	
Maltose	+	+		+	+
Mannitol	-	±		-	+
Mannose	+	+		+	+
Raffinose	-	-			
Rhamnose	-	-			
Salicin	+	+		±	-
Sorbitol	-	-			
Sucrose	±	-		+	+
Trehalose				-	
Xylose	±	±		-	-

Table 20. (Continued)

Phase	Bacillary			L	
Strain	ATCC	WRAIR	Turkey	L_1Rat 30	MLS
Source	ATCC	S.G.Cary	Yamamoto	E.K.-Nobel	R.M.Lemcke
ATCC No.	14647			14075	
Oxidase activity	-	-		-	
Catalase activity	-	-		-	
Phosphatase activity	+	+	+	+	+
Arginine hydrolysis	+	+	+	+	+
Urea hydrolysis	-	-		-	-
Esculin hydrolysis	±	±	±	±	±
Gelatin hydrolysis	-	-		-	-
Phenylalanine deamination	-	-	-	-	
Nitrate to nitrite reduction	-	-		-	
Tetrazolium reduction aerobic/anaerobic	+/+	+/+	+/+	±/±	+/+
Tellurite reduction aerobic/anaerobic	+/+	+/+	+/+	+/+	+/+
Methylene blue reduction aerobic/anaerobic	-/+	-/+	-/+	-/+	-/+
Casein digestion	-	-	-	-	-
Coagulated serum digestion	-	-		-	-
AMC production	-	-		-	
Indol production	-	-		-	
H2S production	+	±	±	±	
Film and spot production	+	+	+	-	-
Benzidine test	-	-		-	
Gluconate oxidation	-	-		-	
Hemolysis (sheep RBC)	α	α	α	α	α
Oxidation-Fermentation	F	F		F	F

24 hours of growth. Viewed at magnifications of 10 to 200 times, 24-48 hour-old colonies show coarse surface tracings due to the presence of large oil-like droplets of cholesterol or cholesterol-like substance. The peripheral portion of the colony appears translucent and lacy due to the accumulation of oil-like droplets as large as 30-40 μm in diameter, and to the presence of large, swollen bodies and amorphous material interspersed with small coccoid bodies. The central portion of the colony penetrates the agar to a depth of 30-50 μm, and has a round or irregular shape and a deep brownish color. It is composed of densely packed large and small bodies, granules, amorphous material, and smaller oil-like droplets. In very young, actively growing colonies, the predominant cell form, both in the core and at the edge, is the small coccoid body, 0.3-1.0 μm in diameter; bubbles and oil-like droplets are located near the edge.

In serum or ascitic fluid broth, growth of the L-phase variant resembles that of the bacillus. Nutritional requirements for the L-phase variant are less exacting than for the bacillus. Biochemical characters are generally similar to but somewhat weaker than those of the bacillus and are recorded in Table 20. The L-phase variant does not produce film and spots on 20% horse serum agar. Temperature, pH, and environmental requirements are similar to those of the bacillary phase. The L-phase variant is resistant to approximately 10,000 times higher concentration of penicillin than is the bacillus.

b. Biochemical reactions of Mycoplasma and Acholeplasma species.

The 1969 WRAIR Annual Report noted biochemical tests that were useful in identification of Mycoplasma species as well as several tests that were unsatisfactory for application to mycoplasmas. Because of the need for further tests for differentiating Mycoplasma and Acholeplasma (a new genus of a new family, Acholeplasmataceae, in the Mycoplasmatales) species from stable L-phase variants of bacteria, starch hydrolysis and production of hydrogen sulfide were reexamined. In addition, hydrolysis of aesculin, found useful for identifying L-phase variants by R. L. Cohen (Appl. Microbiol. 16:1655-1662, 1968), was also tested for differentiating members of the Mycoplasmatales.

Starch hydrolysis was tested on 31 mycoplasma or acholeplasma strains, a positive control organism (Bacillus cereus), and a negative control organism (Streptococcus faecalis). Method 1 given by Cowan and Steel (Identification of Medical Bacteria, Cambridge Univ. Press, 1966) was used. Hydrolysis of starch was detected for the positive control organism but not for the negative control organism nor for the uninoculated medium. Faint clearing could be seen for some of the strains indicating that hydrolysis might be occurring, but reactions were not strong enough to be considered satisfactory.

One of the problems in testing for hydrogen sulfide production has been finding a suitable medium. For these experiments, the medium was heart infusion broth supplemented with 0.5% (w/v) glucose, 0.02% (w/v)

lead acetate, 0.2% (w/v) agar, and 20% (v/v) inactivated (30 minutes at 56°C) horse serum. Thirteen strains (6 mycoplasmas, 5 acholeplasmas and 2 bacterial controls) were examined in this medium and on the same medium to which 0.01% (w/v) cysteine hydrochloride had been added as a source of sulfur. Six of the strains were also examined on a similar medium containing 0.01% (w/v) sodium thiosulfate as the sulfur source. Positive reactions were detected for the positive control organisms and for 1 mycoplasma strain. Reactions were best in the medium containing cysteine hydrochloride although growth of all strains tested was good in all 3 of the media used. Additional strains will be examined, since the results thus far would seem to indicate that this test may yet become useful for differentiating Mycoplasmatales from stable L-phase variants.

The third biochemical reaction studied, aesculin hydrolysis, proved to be a very useful test. In all, 23 mycoplasma and 32 acholeplasma strains were tested. Streptococcus faecalis was used as a positive control organism. Of these only S. faecalis and all but 4 of the Acholeplasma laidlawii strains gave a positive reaction. This result is important for two reasons. First, it provides a possible means of distinguishing mycoplasma strains usually found in clinical isolates from stable group D streptococcus L-phase variants which might also be found. Second, it provides a means other than serologic to distinguish the 2 sterol non-requiring species, A. laidlawii and A. granularum, from one another.

Biochemical characterization of 4 more strains recently submitted to the American Type Culture Collection are summarized in Table 21. The methods used were those of B. B. Aluotto (Int. J. Syst. Bact. 20: 35-58, 1970). The aeculin test has been included.

c. Identification of isolates from clinical material.

Various studies have been carried out from time to time by this laboratory and others to provide (1) the methods for isolation of Mycoplasma from clinical material and (2) the biochemical characteristics by which Mycoplasma can be identified. Actual identification of isolates, especially when composed of more than one species, remained to be tried. Preliminary experiments were run using a laboratory-made mixture containing approximately equal numbers of each of the 3 species usually isolated from the respiratory tract of man, ie. M. orale 1, M. orale 2, and M. salivarium. Separation of the 3 species was attempted using a cloning procedure. A filtration step was added before each of 3 clonings to insure that colonies growing on plates would originate from single organisms rather than from clumps of organisms which might contain more than one species. Filtration was done with Millipore Swinnex-25 filter holders fitted with various pore size membranes. Experiments showed that the 0.22 μm pore size membrane retained large numbers of organisms, and that a minimal quantity of 6 ml. of culture was needed for filtration to allow maximal numbers to pass through that pore size. By this technique, M. orale 1 and 2 were cloned from the

Table 21. Biochemical reactions of recently acquired strains of Mycoplasmatales.

Species	*M. agalactiae* var. *bovis*	*M. anatis*	*A. laidlawii* var. *inocuum*	*A.* species
Strain	Donetta	1340		S743
Test:				
glucose breakdown	-	+	+	+
O-F		F	F	F
arginine hydrolysis	-	-	-	-
aesculin hydrolysis	-	-	+	+
tetrazolium reduction aerobic/anaerobic	+/+	±/+	-/+	±/+
tellurite reduction aerobic/anaerobic	+/+	+/+	+/+	-/+
methylene blue reduction aerobic/anaerobic (24 hour reading)	±/+	-/-	-/±	-/-
phosphatase	+	+	±	-
film and spots	+	-	-	-
gelatin hydrolysis	ND	ND	-	ND
casein digestion	-	-	-	-
serum digestion	-	-	-	-
optochin sensitivity	-	-	-	-
hemolysis (sheep cells) (4 day reading)	alpha prime	beta ring	alpha prime	alpha prime
preferred gaseous environment	aerobic	aerobic	aerobic	aerobic

mixture and identified by the growth inhibition test of Clyde (J. Immunol. 92:958-965, 1964), but M. salivarium was missed. Subsequent experiments were set up to determine the percentage of each organism passing the filter. Each species was cultured by itself, filtered and colony forming units counted before and after filtration. Results showed that the percentage of M. salivarium organisms that passed the filter was markedly lower than the percentage of M. orale 1 and 2 organisms that passed. Thus, when M. salivarium was in the same culture as M. orale 1 and/or 2, dilutions of the filtrate which provided colonies isolated enough for cloning of M. orale 1 and 2 no longer contained M. salivarium. Therefore, a technique was tried using growth inhibiting antiserum discs to all 3 organisms on the plates to be cloned. Discs were arranged so that any 2 given organisms would be inhibited in a zone which allowed the third organism to grow. Theoretically, all 3 organisms could then be cloned from their respective zones. Using the combination of cloning from multiple overlapping growth inhibition zones followed by filtration of each cloned line, all 3 species were isolated from the laboratory-mixed culture, each free from contamination by another species, and identified by the Clyde growth inhibition test.

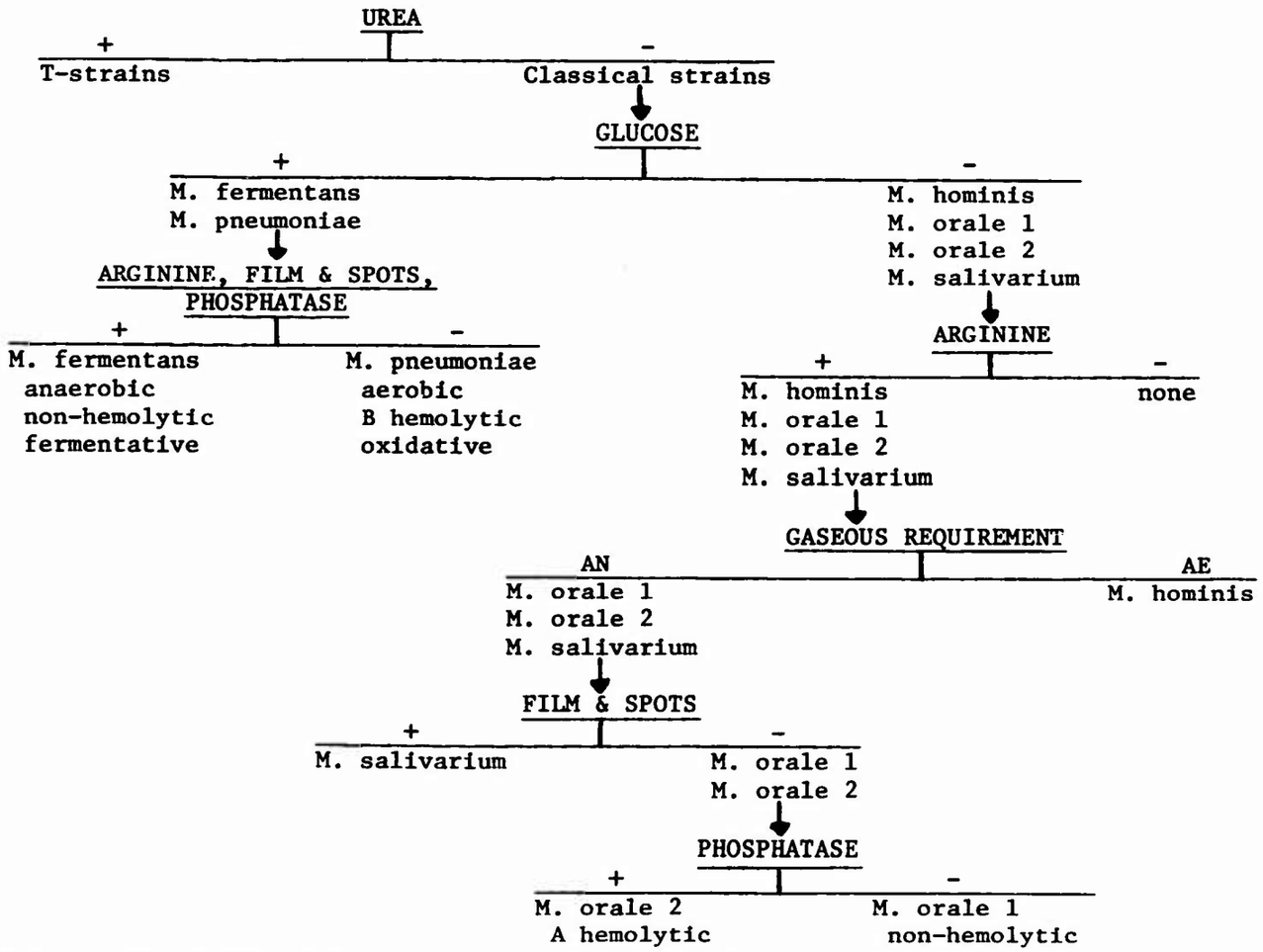
Next, these techniques were applied to a clinical isolate obtained from the sputum of a patient with chronic bronchitis. As a result, M. salivarium was isolated from the material, free of contamination by any other species, and identified by biochemical reactions according to the scheme given in Diagram 1. Identification was rechecked by the Clyde growth inhibition test.

Similar techniques will be employed with isolates from urogenital material. Antisera to M. hominis and M. fermentans will replace those antisera used for respiratory isolates in the cloning by the multiple disc growth inhibition technique. T-strains will be cloned by a specific staining technique developed by Dr. Maurice Shepard. Identification will be based on biochemical reactions, again according to the scheme given in Diagram 1, and confirmed where possible by the growth inhibition method of Clyde.

d. Investigation into the role of infection in chronic bronchitis.

Chronic bronchitis is a progressive debilitating disease. It is diagnosed in an individual who has had a productive cough nearly every day for 3 or more months over 3 or more consecutive years. Excluded are other conditions which can give a chronic productive cough, such as congestive heart failure. On the other hand, emphysema, an anatomical diagnosis made at postmortem, can coexist with chronic bronchitis. Chronic bronchitis, which is up to 45 times more prevalent in England, is seen quite frequently in the United States, and 1 percent of people admitted to Walter Reed General Hospital have this diagnosis. It has been shown that chronic bronchitics lose airway function 3 times faster than their age matched control population, and their death rate is 2.6 times higher. The excess deaths are nearly all from respiratory causes.

Diagram 1. Biochemical differentiation of Mycoplasma strains common to man.



The etiologies are probably multiple and interdependent. The anatomical finding in chronic bronchitis is an increase in the size of the mucous glands in the bronchi, the site of increased sputum production. The mucous gland hyperplasia, according to current theory, is in response to irritants such as tobacco smoke and air pollutants. However, it has been shown repeatedly that there is a high frequency of positive cultures of Hemophilus influenzae and Diplococcus pneumoniae in the sputum of chronic bronchitics. It is also well known that these individuals are prone to a higher rate of acute lower respiratory infections to which they not infrequently succumb unless appropriate antibiotics are administered. The point at issue is whether the infecting bacteria cause a persistent smouldering infection that damages the bronchial mucosa and gradually erodes these vital airways.

Microbiological assessment of the ordinary sputum specimen is unsatisfactory, because pharyngeal organisms almost invariably coat the sputum as it passes through the upper airway. When one realizes that the lower respiratory tree is usually devoid of microorganisms, it is important to be certain whether organisms claimed to originate from the lungs do, in fact, derive from this site. The recently developed transtracheal method, which enables more accurate specimen collection from the lower respiratory tract, offered a valuable approach to the clarification of this problem.

An investigation was undertaken to study the microbial flora in a carefully selected group of chronic bronchitics. In addition to the previously mentioned criteria of sputum production, candidates had to show at least mild to moderate symptoms of breathlessness and mild airway obstruction by pulmonary function tests. (Interestingly, most of those who were referred and who met the chronicity of sputum production requirement also had these functional findings.) Additionally, they were studied only if in their usual baseline state without an acute flareup, and if they had been off antibiotics for at least 30 days.

Fourteen patients were admitted to Walter Reed General Hospital for the studies. At a designated time the patient was taken to the treatment room and several throat swab specimens were taken. Then he was placed in the reclining position and a large needle was introduced under local anesthetic, through the cricothyroid membrane. A catheter was fed through the needle down into the trachea and the specimen obtained. All of these specimens were cultured for viruses, bacteria and mycoplasmas. Later, bronchoscopies and bronchial biopsies were performed on most of these patients.

The results are listed in Table 22. All specimens were negative for the following viruses: adenovirus, herpesvirus, myxovirus, enterovirus, respiratory syncytial virus, and rhinovirus. Mycoplasma species were recovered in the expected frequency from throat and sputum

Table 22. Microorganisms isolated from chronic bronchitis patients.

Patient		Virus	Mycoplasma†			Bacteria†		
No.	Name	All sites	Throat	Trans-trach.	Sputum	Throat	Transtracheal	Sputum
1	J.V.	0	0	0	M.sp.	Mix	Pne	Pne,Mix
2	R.H.	0	M. sp.	0	M.sp.	Mic,Nei,Str a	0	Mix
3	E.E.	0	0	0	M.sal	Nei,Sta,Str a Str D	0	Kle,Nei,Sta,Str a, Str D
4	S.G.	0	0	0	0	Sta,Str a,Str D	0	Kle,Sta,Str a, Str sp.
5	C.C.	0	0	0	>1 M.sp.	Str a,Str b, Nei,Str D	0	Hem,Kle,Nei,Str a, Str D
6	S.J.	0	0	?	2 M.sp.	Dip,Nei,Str a	Dip,Nei,Str a	Dip,Nei,Str a, Str D
7	C.M.	0	0	?	0	Dip,Nei,Str a	Nei,Sta,Str a	Dip,Nei,Str a, Str D
8	C.K.	0	M.sal	0	M.sal	Pne,Esc,Ser, Str a	Ser	Pne,Kle,Nei,Ser, Str a
9	R.P.	0	0	0	0	Hem,Mix	Hem	Hem,Esc,Mix
10	P.H.	0	M.sal	0	M.sal			Hem,Pne,Mix
11	W.B.	0	0	0	0	Mix	0*	Mix
12	C.W.	0	0	0	0	Mix	Pne,Mix	Pro,Mix
13	S.E.	0	M.sal	0	M.sal	Mix	0	Pse,Mix
14	R.M.	0	0	0	0		Dip,Mor	
Total positive/ Total cultured		0/16	4/14	2(?)/14	8/14	12/12	7/13	13/13

Table 22. (Continued)

0 = Negative for growth

† Dip = Diphtheroids
Esc = Escherichia coli
Hem = Hemophilus sp.
Kle = Klebsiella-Aerobacter
Mic = Micrococcus sp.
Mix = Mixed flora
Mor = Moraxella sp.
M. sal = Mycoplasma salivarium
M. sp. = Mycoplasma sp.
Nei = Neisseria sp.
Pne = Diplococcus pneumoniae
Pro = Proteus sp.
Pse = Pseudomonas sp.
Ser = Serratia sp.
Sta = Staphylococcus epidermidis
Str a = Streptococcus, alpha hemolytic
Str b = Streptococcus, beta hemolytic
Str D = Streptococcus, group D
St- sp = Streptococcus sp.

* Bronchoscopic culture: Nei,Sta,Str a, Str d

specimens, but in only two specimens of the transtracheal aspirates were possible isolations made. Original samples of these specimens, which were frozen and stored immediately after collection will be retested. Only Mycoplasma salivarium was definitely identified, but other species may well be present. The bacteriologic data reveal a much lower frequency of isolation and less variety of bacteria in the transtracheal than in the sputum specimens. Diplococcus pneumoniae, Hemophilis influenzae, and several other species of bacteria were isolated from the transtracheal specimens. The bronchial biopsy results are not listed on the table, but were compatible with chronic bronchitis.

In summary, this study demonstrates the value of transtracheal aspiration to ascertain the true flora of the lower respiratory tract. The project to date includes a limited number of patients, but already it tends to confirm the findings of other studies, which have usually been on less well defined groups of patients. There definitely are bacteria in the lower respiratory tree of chronic bronchitis patients, and these organisms presumably persist there. Much further work is needed in this area, but already the implications are suggestive. A possible analogous situation, chronic bacterial infection in the kidney, inexorably leads to chronic debility and death. This may well be the case in chronic bacterial infection in the lung.

e. Studies on gonococcal associated urethritis.

Urethritis of undetermined etiology (whether termed post-gonococcal, non-gonococcal, or non-specific urethritis) poses clinical problems in patient management, because its response to antimicrobial therapy is poor or lacking. This type of urethritis also offers a challenge to the laboratory, because of the difficulty of establishing as etiologic agent one microorganism from among the many that are present as part of the "normal" flora of the urogenital tract.

Over the past several decades numerous investigators have tried to determine whether mycoplasmas were etiologic agents of post-gonococcal urethritis, and have generally concluded that classical mycoplasmas are not. A similar conclusion has not yet been reached on the T-strain mycoplasmas, first described by Shepard in 1954. The T-strains are small-colony varieties, metabolically and serologically distinct from the classical mycoplasmas, and are as yet not fully characterized. This study was performed to obtain basic data on the biology of mycoplasmas present in genital specimens, particularly on T-strain mycoplasmas.

Specimens were collected from a group of patients coming for treatment in the Venereal Disease Clinic of the D. C. General Hospital, Washington, D. C. Material was collected on cotton-tipped swabs from the internal meatus in males and the cervical os in females. Swabs were placed immediately in 1-dram vials of a holding medium (final pH 7.6) consisting of heart infusion broth, horse serum, and yeast

extract, to which 500 units/ml of penicillin, were added. The vials were immediately frozen with dry-ice and acetone and maintained at -35°C until needed. This technique proved suitable for collection and maintained the viability of both classical and T-strain mycoplasmas for several months.

Twenty specimens that had been frozen for from 6 days up to 7 months were thawed and cultured for classical mycoplasmas. Of these 70 percent were positive for typical colonies, presumably Mycoplasma hominis. The patients from whom these specimens were obtained ranged from 15 to 52 years in age, were equally divided as to sex, and were predominantly Negro. Twenty-one specimens similarly stored were also cultured for T-strain mycoplasmas; 86 percent of these were positive. The 11 male and 7 female patients providing these specimens ranged from 17 to 39 years and were predominantly Negro.

A comparison of mycoplasma colony counts was made between frozen and nonfrozen specimens from 4 patients. A standard volume of specimen from each patient was plated on the day of collection before freezing and again after being frozen and stored for 28 days. The numbers of classical mycoplasmas counted were significantly reduced (by 2 to 10 times) in the frozen and stored specimens.

A comparison of colony counts of classical mycoplasmas was also made on specimens that were placed in a holding medium that either did or did not contain penicillin. Paired specimens from the above 4 patients were used for the study. Slightly lower counts were obtained from specimens in the nonpenicillin-containing medium. There is no immediate explanation for this finding and further tests are indicated.

A medium (Shepard's A medium) modified to contain 25% horse serum and 5% fresh yeast extract designed for growing T-strain mycoplasmas in primary culture was substituted for the usual HIB holding medium. After freezing and storage in the T-strain growth medium, T-strain mycoplasmas could not be grown from specimens known to contain them originally. It would appear, then, that reduction of numbers of mycoplasmas can be expected not only following freezing and storage, but particularly when the proper holding medium is not used.

Reports in the literature regarding the optimum gaseous atmosphere for initial cultivation of T-strain mycoplasmas give conflicting information. It was felt that a logical first step in the successful and consistent isolation of T-strain mycoplasmas from clinical specimens would be to determine the behavior of specimens known to contain T-strains in primary culture under a variety of gaseous conditions. Up to this point, all studies had been conducted by growing mycoplasmas empirically in stationary culture, both with and without increased carbon dioxide tension, depending on the medium used.

Three clinical specimens were selected. The relative amount of growth was estimated based on the assumption that a rise in pH in a

urea-containing medium (U9) was proportional to the number of urea-splitting units present in a standard volume of the specimen. A further assumption was that a urea-splitting unit was equivalent to a colony forming unit of T-strain mycoplasma.

Flasks (50 ml Erlenmeyer) containing 10 ml of U9 medium were inoculated with 0.2 ml of each specimen. Incubation was at 35-37°C under three separate conditions: on a shaker rotating at 150 rpm; in static culture in a candle jar; and, under anaerobiosis, using the BBL Gaspak in a polycarbonate anaerobic jar. At the end of each 24 hour period of incubation, an aliquot was removed from each flask for pH determination and for titration, using serial 10-fold dilutions in tubes of U9 broth.

There was an expected variation among the different specimens in terms of numbers of T-strain mycoplasmas present. However, it was generally clear that although shaken cultures reached as high a population of T-strain mycoplasmas (or sometimes higher) as cultures grown under static or anaerobic conditions, the population seemed to lose viability fairly rapidly. On the other hand, anaerobic cultures required a longer period to reach a given population level but maintained that level longer. Incubation under static conditions produced cultures that reached population levels intermediate between the other two. Thus, cultures could theoretically be grown slowly or rapidly according to individual requirements. However, incubation in static culture, with increased carbon dioxide tension, would seem to be preferable for initial isolation from specimens.

From these studies, a better understanding of the conditions necessary for successful isolation of T-strain mycoplasmas has been gained. Future studies must be aimed at characterizing the types of T-strain mycoplasmas present in clinical specimens during various clinical manifestations of urethritis.

f. Investigations of T-strain mycoplasmas.

Studies with classical Mycoplasma species have demonstrated that biochemical techniques may be used to characterize and differentiate the various species (Aluotto et al., Int. J. Syst. Bact. 20:35-58, 1970). An attempt was made to characterize 10 serologically distinct T-strain mycoplasmas in terms of their biochemical activities.

The T-strains used were 960, Cool C, 7, 23, 27, 354, Pi, 17, 24 and 58. All 10 strains were supplied by W. A. Clyde of the University of North Carolina.

The basic constituents of the growth and test media consisted of a base of Bacto Heart Infusion broth or agar, 10-20% (w/v) horse serum, 5% (v/v) of a 25% (w/v) fresh yeast extract solution, and 0.1% (w/v) urea. The final pH of the media was 6.0. The growth medium included

0.004% (w/v) phenol red as an indicator of pH change due to hydrolysis of urea during growth.

The biochemical tests employed were modifications of those described by Aluotto et al. (1970). Details of the test media and methods were as follows:

Media for testing for the breakdown of xylose, sucrose, and lactose were prepared by adding the sugars in a concentration of 1% (w/v) to growth broth containing 10% (v/v) horse serum and the usual concentrations of urea, yeast extract, and phenol red.

The method used to test for arginine and glucose breakdown (including the oxidation-fermentation test) were the same as the agar vial method employed by Aluotto et al. (1970) except that the media were adjusted to pH 6.0.

Plates for tetrazolium reduction were prepared by adding 1 ml of stock 2% (w/v) 2,3,5-triphenyltetrazolium chloride to 99 ml phenol red free growth agar having a pH of 6.0. Twenty percent (v/v) horse serum was used. Plates were inoculated in duplicate with 0.02 ml of an actively growing broth culture of organisms. One plate was incubated in a candle jar, and the other was incubated in a GasPak CO_2-H_2 anaerobic jar. It was necessary to pass the organisms at least twice on phenol red free medium so that residual indicator would not give a false positive reaction.

Reduction of potassium tellurite was tested for by including 0.5 ml of stock 1% (w/v) potassium tellurite in 100 ml growth agar containing 20% (v/v) horse serum. Phenol red was included in the medium. Plates were inoculated with 0.02 ml of an actively growing broth culture of the organism and were incubated under both aerobic and anaerobic conditions.

The ability to reduce methylene blue was tested for by the method of Aluotto et al. (1970). Organisms for the inocula were cultured in phenol red free growth broth and passed at least twice in this media to eliminate any color interference from the indicator.

Plates for determining the presence of phosphatase activity were prepared by adding 1 ml of stock solution of 1% (w/v) sodium salt of pheneolphthalein diphosphate to 99 ml growth agar from which phenol red was omitted. The plates were inoculated with 0.02 ml of an actively growing phenol red free broth culture. At least two passages on phenol red free media were carried out to eliminate false positive reactions that would be given by the indicator.

Gelatin liquefaction was tested for by including 8.5% (w/v) gelatin in growth broth. The test medium was dispensed in 5 ml quantities in screw capped tubes and inoculated with 1 ml of a growing broth culture of the organism. After 48 hours the tubes

were refrigerated to observe for liquefaction. Tubes were not considered negative until liquefaction failed to be observed after 2 weeks of incubation.

Casein hydrolysis was tested for by overlaying a section of an inoculated growth agar plate with a casein-agar mixture in such a manner that part of the inoculated area and part of the uninoculated area were covered with the overlay. The casein-agar mixture consisted of 4% (w/v) Difco Skim Milk and 1.0% (w/v) Difco agar.

Growth inhibition of the organisms by optochin was tested for by inoculating growth agar plates with 0.02 ml of a growing broth culture, allowing them to dry, and placing an optochin disc (BBL, Taxo P, Lot No. 701014) on the agar surface. Since T-strain colonies are not discrete when the organisms are present in high concentrations, dilutions of 10^{-3} and 10^{-4} were made, and inocula of these dilutions were used.

Hemolysis of sheep erythrocytes was tested for by the overlay technique of Aluotto et al. (1970). Growth agar plates were inoculated with organisms and allowed to incubate for 24 to 48 hours before the blood cell-agar overlay was applied.

Catalase activity was tested for by flooding growing agar cultures with 30% hydrogen peroxide and observing for the evolution of gas bubbles.

The ability of the organisms to deaminate phenylalanine was tested for by including 0.2% (w/v) D-L phenylalanine in phenol red free growth agar. After 48 hours of incubation, the plates were flooded with 10% (w/v) ferric chloride. A positive test was indicated by the presence of a green color upon addition of the ferric chloride due to the presence of the deamination product of phenylalanine, i.e., phenylpyruvic acid.

Medium was prepared to test for the oxidation of gluconate to 2-ketogluconic acid by including 4% (w/v) sodium gluconate in the growth broth. The medium was dispensed in screw capped test tubes in 2.5 ml quantities and was inoculated with 0.5 ml broth culture. After 24 and 48 hours, 1.5 ml of the test broth was boiled for 10 minutes with 0.25 ml Benedict's solution. A positive test was indicated by formation of a red precipitate.

The primary biochemical characteristic that distinguishes all T-strains from classical Mycoplasma species is the ability to hydrolyze urea, which substance is required for growth by all of the T-strains. This characteristic was confirmed in this study.

The sugars tested, xylose, sucrose, lactose, and glucose did not appear to be fermented by strain 960, thus the oxidation-fermentation

test was also negative. Other strains were not tested. The test for arginine hydrolysis appeared to be negative with strain 960.

A positive arginine test would be indicated by a more rapid pH shift toward alkalinity in a tube of test medium than in a control tube. The expected pH shift would be demonstrated by a color change from yellow to red in the phenol red containing medium. A positive test for carbohydrate breakdown would be indicated by an acid shift. Since the T-strains hydrolyze urea and, therefore, cause an acid to alkaline shift regardless of their action on arginine or carbohydrates, the apparent results may not be entirely satisfactory and could lead to false negative readings. However, the method of comparison of extent of pH shifts between test and control cultures was employed satisfactorily by Mϕller to test for amino acid decarboxylases (Act. Path. Microbiol. Scand. 36:158-172, 1955).

Collaboration with Dr. Marvin Rogul of the Veterinary Division of WRAIR was undertaken to determine if T-strains might be capable of attacking carbohydrates if the medium were to contain a higher concentration (up to 5%, w/v, of carbohydrates) than in ordinary test media. Preliminary experiments on strain 960 did not indicate that carbohydrates were fermented under these conditions.

All 10 T-strains gave negative test results for reduction of tetrazolium, tellurite, and methylene blue under both aerobic and anaerobic conditions.

Tests for phosphatase activity gave dubious results due to the interference by phenol red in the inocula. Tests will be repeated using phenol red free inocula and test media.

None of the 10 strains appeared to liquefy gelatin.

Strains 960, 7, and 23 were tested for casein digestion, and all 3 gave negative results.

Inhibition by optochin was not demonstrated in strains 960, 7, 23, and 27, the only strains tested.

Strain 960 was tested for sheep red blood cell hemolysis, and a negative test resulted. Hemolysis of guinea pig erythrocytes by T-strains has been reported, but since the purpose of these experiments was to compare the activities of T-strains with those of classical Mycoplasma species, as well as to determine differences among T-strains, sheep erythrocytes were used as described by Aluotto et al. (1970) for classical Mycoplasma species.

Four strains tested for catalase, 960, 7, 23, and 27 gave negative results.

Phenylalanine deamination was not demonstrated in any of the 4 strains tested, 960, 7, 23, and 27.

Gluconate oxidation tests yielded negative results with all 10 strains.

Other authors have reported that the T-strain mycoplasmas are sensitive to erythromycin and are inhibited by thallium acetate (M. C. Shepard, Ann. N. Y. Acad. Sci. 143:505-514, 1967). The T-strains appear to be a group of organisms distinct from the classical Mycoplasma species. However, studies on the DNA base composition of T-strains have demonstrated that their values for % G+C (ca. 28%), are similar to those of the classical Mycoplasma species (Bak and Black, Nature 219:1044-1045, 1968). To date, serological techniques are the sole means of distinguishing among the various T-strains.

g. Morphological variants of Treponema pallidum.

The purpose of this study is ultimately to determine the production, biology, maintenance and reversion of wall-defective variants of treponemes in vitro. Studies related to the production, persistence, reversion and pathogenicity of such variants in vivo are also planned.

A prerequisite to the study of treponemal wall-defective variants is the definition of the characteristics of normal, unaltered treponemes as a basis of reference. A group of cultivable Treponema pallidum strains were selected for the initial morphologic and cultural studies.

These strains included Kazan 2, Kazan 5, Kazan 8, Nichol, and Reiter treponemes. Their morphology was investigated by phase contrast, darkfield and electron microscopy, and by various staining techniques, including acridine orange. Some interesting observations, made at the ultrastructural level, showed certain morphological differences between the classical forms of the nonpathogenic (cultivable) and the pathogenic treponemes. The extremities of cultivable treponemes were blunt. The fibrils were 6-12 in number and were inserted into basal granules which were very close to the extremity. In pathogenic treponemes obtained from the Nichol rabbit strain, fibrils were usually 6 in number and were attached to basal granules that were further from the extremity than in the nonpathogenic strains. The terminal extremity was spongy in appearance and tapering in the pathogenic strain. In all the treponemes studied, the fibrils were located between the outer cell wall and the inner plasma membrane. The outer envelope measured 70-90 A thick. Cytoplasmic mesosome-like bodies were observed in both the pathogenic and the nonpathogenic organisms. These findings confirm the work of others in the field (Ovcinnikov and Delektorskij. Brit. J. Ven. Dis. 44:1-34, 1968; Ryter and Pillot. Ann. Inst. Past. 104: 496-508, 1963).

The cultivable treponemes are being studied for their growth characteristics both in broth cultures and as surface colonies on solid media. In order to check for possible contaminants, each fresh broth culture transfer was accompanied by inoculations of the same suspension into thioglycollate media and blood agar plates. On a special formula of enriched solid media treponemal growth occurred in the form of pearly gray-white colonies, which could be transferred easily from plate to plate. Some strains exhibited a variation in colonial morphology showing more pigmented colonies. To isolate pure strains, colonies were isolated as single clones and propagated on solid media. The clone transfers were studied by Dienes stained preparations and Gram stains.

Morphologically altered treponemal organisms were observed to appear spontaneously in old treponemal cultures and also following the addition of penicillin to fresh broth cultures. Their identity as wall-defective variants is being studied by various staining techniques, enzyme digestion methods and by electron microscopy.

h. Type culture collection of the Mycoplasmatales and L-phase variants of bacteria.

The initial results of an investigation on the use of sucrose for preserving mycoplasmas during freeze-drying were presented in the WRAIR Annual Report for 1969. During the current year this joint project conducted by WRAIR and ATCC has been completed.

Fifteen Mycoplasma strains (representing 12 or more species) were subjected to a freeze-drying (lyophilizing) program with and without sucrose added to concentrated cell suspensions in growth medium. As shown in Table 23, there is a variation between the strains in their response to the addition of sucrose. Whereas M. felis, M. hominis, and M. pneumoniae showed markedly improved recovery, M. anatis, M. bovimastitidis, and Mycoplasma sp. 67-166 were apparently unaffected. The rest of the strains showed moderate improvement. At present the freeze-drying method employing sucrose gives the best recovery not only in terms of total cell counts but also in that it permits the recovery of all strains submitted to the procedure.

Mycoplasma strains are also frozen and stored in liquid nitrogen at the ATCC. In this method the culture is diluted 1:1 with 20% (v/v) sterile glycerol in distilled water, mixed and dispensed into ampules. The ampules are subjected to a cooling rate of 1°C/min to -40°C and thereafter at an uncontrolled rate to the temperature of liquid nitrogen. They are stored in a liquid nitrogen refrigerator at -150 to -196°C. A study similar to the freeze-drying project described above was made using the 15 strains listed in Table 23. All showed good recovery from liquid nitrogen storage, but there was a varying degree of sensitivity to the method of preservation. For example, M. orale showed 7.9% survival, and M. arthritidis showed 95% survival.

Table 23. Effects of adding sucrose to the suspending medium on the freeze-drying of Mycoplasma strains.

Mycoplasma species	Strain	ATCC No.	Suspending medium without sucrose			Suspending medium with sucrose		
			Before freeze-drying	After freeze-drying	Survival	Before freeze-drying	After freeze-drying	Survival
			Cfu/ml*	Cfu/ml*	%	Cfu/ml*	Cfu/ml*	%
M. agalactiae var. bovis	Donetta	25523	6.8×10^9	6.2×10^8	9.1	6.8×10^9	1.7×10^9	25.0
M. anatis	1340	25524	5.3×10^9	4.7×10^8	8.9	6.8×10^9	3.5×10^8	5.1
M. arginini	G230	23838	2.6×10^9	1.5×10^8	5.8	2.8×10^9	4.8×10^8	17.1
M. arthritidis	H606	13988	9.0×10^{10}	1.2×10^9	1.3	6.0×10^{10}	2.5×10^9	4.2
M. bovimastitidis	01	25025	2.6×10^9	2.7×10^7	1.0	2.4×10^9	1.4×10^7	0.6
M. felis	CO	23391	1.4×10^7	0	0.0	4.8×10^7	3.6×10^5	0.8
M. hominis	PG 21	23114	1.7×10^9	1.2×10^6	0.1	3.5×10^9	6.7×10^8	19.1
M. hyorhinis	PG 29	25026	1.5×10^{10}	1.3×10^9	8.7	1.3×10^{10}	1.0×10^{10}	76.9
M. hyorhinis	BTS 7	17981	3.3×10^9	1.7×10^7	0.5	4.1×10^9	2.0×10^9	48.8
M. laidlawii	Laidlaw A	14089	1.4×10^{10}	1.6×10^9	11.4	1.3×10^{10}	3.0×10^9	23.1
M. leonis	LL	25528	9.0×10^9	1.4×10^8	1.6	1.0×10^{10}	3.2×10^8	3.2
M. meleagridis	17529	25294	4.7×10^9	2.4×10^9	51.1	4.7×10^9	2.9×10^9	61.7
M. orale, type 1	CH 19299	23714	2.9×10^9	5.0×10^7	1.7	3.4×10^9	2.6×10^8	7.6
M. pneumoniae	Mac	15492	1.5×10^8	7.7×10^6	5.1	2.0×10^8	1.8×10^8	90.0
Mycoplasma sp.	67-166	23243	4.4×10^9	4.5×10^8	10.2	4.4×10^9	2.9×10^8	6.6

* Colony forming units per ml. Average of triplicate determinations on contents of each of two ampules.

The even higher survival rates after storage in liquid nitrogen of strains M. agalactiae var. bovis, M. bovimastitidis, and M. pneumoniae can be explained at this time only by possible disaggregation of cell clumps in the freeze-thaw procedure.

A preliminary longevity study was performed on 20 strains of Mycoplasma stored in liquid nitrogen for 2 weeks and 3 years. There was a variable survival rate between strains; with some strains a higher colony count resulted after storage in liquid nitrogen. The majority of strains at the 3-year period retained the same degree of viability as at the 2-week period.

Starting this year and extending into 1971, a 10 year viability study will be carried out on the few strains which were processed at the ATCC in 1960.

The collection of mycoplasmas and L-phase variants now contains 76 strains of mycoplasmas (30 of which are named species), 21 strains of L-phase variants, 20 strains of their parent bacteria, and 9 strains of their revertant bacteria. The new policy for the acquisition of authentic strains by the ATCC has resulted in a greater interest among investigators in providing the collection with early sublines of valid strains.

The number of vials of mycoplasmas and L-phase variants supplied by ATCC to investigators throughout the world has increased over 50% (mycoplasmas: 179 vials in 1969 to 290 vials in 1970; L-phase variants: 16 vials in 1969 to 27 vials in 1970). Seventy-one vials of mycoplasmas and L-phase variants were supplied to investigators at WRAIR or other military installations.

It has been an established and profitable routine procedure to perform colony counts on all ATCC strains at the time of submission and as soon as possible after processing. This enables requesting investigators to be aware of the state of the strain on delivery. Three hundred and nine vials were used for such colony counts this year.

The majority of mycoplasma strains prior to submission to the collection have been kept in passage by the original investigator in media containing penicillin and/or thallium acetate. Due to the fact that all of the Mycoplasma strains are submitted by WRAIR to the collection on medium without these additives, a thorough sterility investigation was felt necessary. Routine sterility testing resulted in no contamination on blood agar plates, on agar used for performing Mycoplasma or L-phase variant colony counts or in the Bacto A C medium (stab method) used at ATCC. A pilot study was arranged between the two institutions to investigate the incidence of contamination using a highly enriched medium, which would support the growth of aerobic, microaerophilic and anaerobic organisms. BBL thioglycollate + 20% (v/v) inactivated horse serum was used not only for testing the

sterility of the strains at the time of each serial passage but also to detect the presence of possible contamination in the freeze-dried and frozen ampules. The results of a preliminary study showed the presence of a gram positive, pleomorphic rod in 37.7% of the freeze-dried vials, in 10% of the liquid nitrogen vials, and in 5% of the control tubes. The control tubes containing thioglycollate-serum medium received no inoculum but were briefly opened and recapped at the time the test series of tubes received inocula.

Following institution of sterilization of all systems involved in the processing of the strains, a marked decrease in the percentage of contaminants was obtained. The final results of this project are not yet available and will be reported at a later date.

3. Diagnostic microbiology.

a. A soluble antigen fluorescent antibody (SAFA) test for influenza virus infection.

SAFA test procedures have been developed at WRAIR for certain parasitic infections and tuberculosis and have been shown to be useful for diagnosis of adenovirus infection. The current studies report the development of a SAFA test using influenza type A and B antigens and present results of comparisons of this test with standard CF and HAI procedures.

Materials and methods:

Antigens: For the preliminary studies 4 strains of influenza virus were used: A2/Jap/305/57, A2/HK/19/68, B/Va/301/55, and B/Mass/3/66. In early studies the viruses were grown in the allantoic sac of 9 day old chick embryos at 37°C. After 2 to 3 days the allantoic fluid was harvested and the virus was purified by adsorption-elution on human "O" red blood cells and ultracentrifugation (Annual Report 1968-69). In subsequent experiments the viruses were grown in primary rhesus monkey kidney (RMK) monolayers. The RMK cells were grown in 32 oz. prescription bottles at 37°C in Medium 199 supplemented with 10% inactivated fetal bovine serum with penicillin and streptomycin added. When the monolayers were confluent the growth medium was removed from the bottles and each culture was infected with 2.5 ml of stock virus containing 10^2-10^3 cell culture ID_{50}/ml. After 30 minutes at 25°C, 30 ml of maintenance medium (Medium 199 with penicillin and streptomycin) was added to each bottle. The cultures were incubated at 37°C for 3-5 days or until cytopathic effect was 75% or greater, after which the cells were mechanically harvested into the maintenance medium. The virus was released from the cells by 3 freeze-thaw cycles and the medium was clarified by a low speed centrifugation (500 G for 15 min.). The medium plus virus was further clarified by a 30 minute centrifugation at 12,000 G, after which the virus was pelleted by centrifugation at 56, 573 G for 3 hours. The virus pellet was

resuspended in 1/10 the original volumn in pH 7.2 PBS.

SAFA Test: The improved SAFA test described by Toussaint was performed with one modification, that was in the preparation of antigen discs (Annual Report, 1968-69). The discs were prepared by filtering a PBS solution of influenza virus through a 0.45 μ millipore membrane. The membranes were allowed to dry overnight and the ¼ inch diameter discs were punched just prior to doing the test.

Readings of the test were performed in a fluorometer equipped with a 4 watt ultraviolet lamp, a No. 7-54 primary filter, and a secondary filter system consisting of a No. 2-12 sharp cut filter and a 10% neutral density filter. The fluorometer was adjusted to zero with an antigen disc treated with the negative serum pool.

Serum Specimens: Acute and convalescent sera from adults with influenza infections were used to evaluate the SAFA test. These sera represent A, A1, A2 and B influenza cases from an Army population and were collected over the past 18 years. All of the cases were documented by viral isolation and/or a hemagglutination inhibition titer rise and/or a complement fixation titer rise. A negative control serum pool was prepared by pooling sera that had a complement fixation titer of less than 4 and which showed very low immuno-fluorescent activity. All sera were stored at -20°C until tested.

Hemagglutination Inhibition Test: The microhemagglutination inhibition test was performed basically as described by Sever. All sera tested by the SAFA test were evaluated for HAI antibodies versus 4 units of the following viruses: A/PR/8/34, A1/FM/1/47, A2/Jap/305/57, A2/HK/19/68, B/Lee/40, B/Va/301/55, B/AA/1/62, and B/Mass/3/66. All sera were treated with receptor destroying enzyme prior to testing. Group "O" human red blood cells were used.

Complement Fixation Test: Sera were tested in microtiter for complement fixing antibody rise by the 50% hemolytic method. Four units of commercial soluble antigen were used in the test.

Results:

Initially a box titration was used to determine the relation of the concentration of antigen to the antibody concentration. Repeated titrations with several antigens revealed that a 1:15 dilution of patient's serum could be used effectively. Subsequently antigen preparations were titered with a 1:15 serum dilution. Several serum pairs were chosen for the titrations; these sera ranged in reactivity from moderate to strongly reactive. The concentration of antigen chosen was the highest dilution of antigen which gave a change in fluorescent dial reading (FDR), between the acute and convalescent sera, of at least 100 with a moderately reactive serum pair.

Using a serum dilution of 1:15, 29 paired sera from type A influenza cases which showed a 4-fold or greater complement fixation (CF) or hemagglutination (HAI) rise or both were tested in the SAFA test with 2 A influenza antigens, A2/HK/19/68 and A2/Jap/305/57. The 29 serum pairs tested represented A, A1 and A2 infections, of these, all were positive in SAFA test with the A2/HK antigen whereas only 76% were positive with the A2/Jap antigen. To evaluate the reactivity of 2 type B antigens, a similar experiment was performed. Twenty serum pairs with both CF and HAI rises were found to be positive with both antigens. The B/Va/301/65 antigen gave slightly lower acute serum readings and slightly larger changes in the FDR between the acute and convalescent sera than the B/Mass/3/66 antigen. The difference between the 2 B antigens was not very striking, however, the B/Va antigen was chosen for use on the basis of these differences. The remainder of the study was done using the A2/HK/19/68 and B/Va/301/55 antigens.

The technique of the SAFA test is briefly as follows: a single dilution of patient's serum is applied to the antigen impregnated discs, after incubation and thorough washing a fluorescein conjugated anti-human-globulin serum (equine) is applied. Again unbound serum is removed by washing. Discs are then dried, mounted on tape and the degree of fluorescence is read objectively in the fluorometer. Sample test results are shown in Table 24.

Table 24. Type A influenza SAFA test*.

Diagnosis	Case No.	CF Test Result	HAI Test Result		SAFA FDR**		
			A2/Jap	A2/HK	Acute	Conval.	Change
A Flu	A251	+	-	+	49	294	245
	A309	+	+	+	102	468	366
	A491	+	+	+	77	639	562
B Flu	976	-	-	-	333	249	-84
	1006	-	-	-	207	228	21

* Antigen A2/HK used 1:8, serum 1:15.

** FDR = fluorescent dial reading.

Comparison of the SAFA-A influenza test with CF and HAI tests is shown in Table 25. Of 77 cases of Type A influenza, the SAFA test was positive in 67 (87%) and CF and HAI were both positive in only 64 (83%). SAFA was positive in 69/73 diagnosed by positive CF and 64/67 diagnosed by positive HAI.

Table 25. Comparison of type A SAFA test with CF and HAI tests.

	CF +	CF -	HAI +	HAI -	HAI + CF +	HAI + CF -	HAI - CF +	HAI - CF -
SAFA +	69	4	64	7	62	4	7	0
SAFA -	4	0	3	1	2	1	1	0

Ninety-three percent of the B cases were positive by the SAFA test (Table 26). All the sera tested in the B SAFA test were positive by both the CF and the HAI test. One of the 2 SAFA negative B cases was weakly reactive in that it was positive in one test and negative in another. The other case was non-reactive.

Table 26. Comparison of type B SAFA test with type B CF and HAI tests in 28 influenza B infections.

	CF +	CF -	HAI +	HAI -	HAI + CF +	HAI + CF -	HAI - CF +	HAI - CF -
SAFA +	26	0	26	0	26	0	0	0
SAFA -	2	0	2	0	2	0	0	0

Thus, in patients with clinical infections, the SAFA test was positive in over 93%. For type A infections, in which the number of tested cases was largest, the SAFA test was as sensitive as the CF test and more often positive than the HAI used alone.

Specificity of the SAFA test antigens was examined by testing non-influenza illnesses (mumps, rubeola, rubella) as well as heterologous influenza infections. These results are shown in Tables 27 and 28.

Table 27. Influenza A SAFA test specificity.

	Flu B cases	Other
SAFA +	3	0
SAFA -	25	18

Table 28. Influenza B SAFA test specificity.

	Flu A cases	Other
SAFA +	3	0
SAFA -	74	19

Apparent false positive SAFA tests were observed in 6.5% of patients using the A antigen and 3.1% using the B antigen. Studies with sera representing A_1 and A_2 influenza infections showed that the SAFA antigens prepared as described in the Methods Section were not capable of showing strain differences. In this respect they resemble the CF antigens which are "group" specific.

Studies of vaccine induced antibody were undertaken using pre- and 2 week post immunization sera from recruits who received standard military formula influenza vaccine in 1970. These results are shown in Table 29.

Table 29. Comparison of 3 serologic tests following influenza vaccination.

	CF +	CF -	HAI +	HAI -	HAI + CF +	HAI + CF -	HAI - CF -
SAFA A +	7	5	10	2	6	5	1
-	2	2*	3*	1	2	1	1
SAFA B +	3	3	6	0	3	3	0
-	2	6	6	2	2	4	2

*Includes 2 patients who received monovalent A/HK vaccine.

It is apparent from these data that the SAFA test is negative in a large proportion of vaccinated individuals but it is somewhat more sensitive to vaccine induced antibody than is the CF test. The large proportion of HAI failures in this series of patients may be due to the fact that only 2 week post vaccination sera were studied.

Conclusions:

The SAFA test seems to be sufficiently sensitive and specific for influenza A and B infections to warrant its use as a routine

diagnostic procedure. This test would offer a number of advantages such as requiring very small amounts of serum and antigen and objective determinations of fluorescence. Preliminary studies indicate that antigen discs can be stored for many months, thus standardization of reagents is simplified.

As other viruses are added to the SAFA technique it may be possible to provide a battery of antigens for use in routine respiratory virus serology.

b. Isolation of bacteria from an Alaskan mummy.

Lung tissue from an Alaskan mummy, at least 300 years old, was submitted by Dr. Zimmerman, Department of Experimental Pathology. Possible bacteria were observed in smears of heart tissue. Special media for the isolation of Clostridia, Mycobacteria, fungi and other pathogens were used. The surface of the frozen lung tissue was burned and a portion from the interior was carefully broken up in broth. This suspension was used as inoculum. After 30 days incubation at 37°C, slight gas and slight turbidity was observed in the deep meat culture and thioglycollate medium. Upon subculture, anaerobic, slender gram positive rods with filaments and gram negative rods with granules and bulbous swellings were obtained. All isolates have been preserved for further study.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 168, Bacterial Diseases

Publications.

Aluotto, B. B., Wittler, R. G., Williams, C. O., and Faber, J. E. Standardized bacteriologic techniques for the characterization of Mycoplasma species. Int. J. Syst. Bact. 20:35-58, 1970.

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Williams, C. O., Wittler, R. G. and Burris, C. Deoxyribonucleic acid base compositions of selected mycoplasmas and L-phase variants. J. Bact. 99:341-343, 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
69 07 01	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	61102A	3A0611102B71Q	00	170
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(U) Militarily Important Diseases Transmissible Between Animals and Man (09)

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FISCAL YEAR CURRENT 71	1.5	185

19. RESPONSIBLE DOD ORGANIZATION

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

23. (U) Biological characteristics of zoonotic agents of diseases of real or potential military importance are studied to develop more suitable diagnostic procedures, treatment and control measures. Current studies are in leptospirosis and melioidosis.

24. (U) Conventional microbiological and chemical technics are used. New procedures are developed as needed.

25. (U) 69 07 - 70 06 Two major genetic complexes, each comprising 3 distinct genetic groups of leptospiras were disclosed. One strain was genetically unrelated to 6 genetic groups and may represent a new major grouping. The genetic groups may have a significant bearing on cross-immunity and other biological attributes of different types. Viability and virulence of leptospiras can be maintained for at least 4 years by liquid N freezing and storage. In 1969, 5 cases of melioidosis occurred in 3 separate outbreaks in non-human primates. The enzootic occurrence of melioidosis in imported monkeys was further established by seroepidemiological surveys. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 170, Militarily important diseases transmissible between animals and man

Investigators.
Principal: A. D. Alexander, Ph.D.
Associate: M. Rogul, Ph.D.; L. B. Evans, B.S.; J. Brendle, B.S.; A. Warner, Jr.

1. Leptospirosis.

a. Preservation of leptospiras. Observations on the viability and virulence of a strain of canicola after storage over a 2 year period in a liquid nitrogen (vapor phase) refrigerator were presented in the WRAIR Annual Report 1966-1967. Additional tests were made on the fourth year of storage. Six samples were removed from the liquid nitrogen refrigerator, rapidly thawed at 37°C, pooled, and then serially diluted by half-log increments with Stuart's medium. Dilutions from 10^{-1} to $10^{-6.5}$ were inoculated i.p. each into 10 hamsters using a 0.5 ml dose. The concentration of viable organisms was determined by direct microscopic counts with the use of a Petroff-Hauser counting chamber and by cultural tests in Fletcher's medium. The determined viability and LD_{50} virulence titers of 8×10^6/ml and $10^{-6.25}$ respectively, were approximately the same as those seen after 1 day of storage.

b. Genetic characteristics. On the basis of DNA base composition and annealing tests conducted on a selected number of leptospiral strains, 4 genetically distinct groups of leptospiras were demonstrated (Haapala et al., J. Bact. 98: 421, 1969). The genetic grouping appeared to be related to cross-immunity, pathogenicity, and other biological characteristics of leptospiras. To elucidate further the genetic relatedness of leptospiras, additional strains with differing phenotypic characteristics were studied. The previous DNA annealing experiments were done in agar matrix prepared with a double strength sodium citrate saline solution (2 X SSC; SSC = 0.15 M NaCl and 0.015 M Na citrate). For the additional studies, the Denhardt method (Biochem. Biophys. Res. Commun. 23: 641, 1961) using membrane filters was selected for DNA annealing tests. The Denhardt procedure had considerable advantages over the agar matrix method in time and material requirements for tests. As originally described, the Denhardt method allows a certain amount of leeway in the final concentration of salt in the annealing incubation fluid. For this reason, incubations were carried out in 6 X SSC and 3 X SSC. Assays were performed with homologous DNA and nonrelated DNA preparations to determine optimum temperature of specific binding. In both 6 X SSC and 3 X SSC, the desired temperature

was 65°C. The results of annealing tests are listed in Table 1, which also gives previous findings (J. Bacteriol. 98: 421-428, 1969) obtained with use of agar - 2 X SSC matrix.

It can be seen from Table 1 that the assessment of nucleic acid relatedness varies somewhat depending on the method used. This was most obvious when radioactive bataviae DNA was reacted with strain javanica DNA and radioactive patoc DNA was reacted with strain CDC DNA. Disregarding the physical matrix, immobilized javanica DNA reacted to 13% in 2 X SSC, 35% in 3 X SSC and 47% in 6 X SSC with bataviae DNA fragments. Immobilized CDC DNA reacted not at all in 2 X SSC, 30% in 3 X SSC and 20% in 6 X SSC. In general, there was a tendency for greater binding among DNA's of partial homology when the salt concentration was increased. This was not usually true of DNA from organisms which were definitely not genetically related. In fact, it may be advantageous to manipulate these salt concentrations and annealing conditions for the purpose of emphasizing partial homology among nucleic acids.

Our overall results have led us to the following conclusions. There are at least two major genetic complexes of leptospiras. One complex has been classically called the biflexa or water leptospiras, the other comprises the pathogenic leptospiras. Each complex in turn contains the two distinct genetic groups which were previously described. It now appears that a portion of the nucleic acid sequences are shared among strains within each complex. In addition, findings serve to indicate the presence of 2 additional genetic groups, one each within the pathogen (strain Iowa frog) and biflexa (strain A-183) complexes. Strain 3055 which was isolated from bovine urine but which has attributes of biflexa strains appears to have distinct nucleotide sequences and may represent a new group or complex. Further differentiation of genetic groups will probably be best served by thermal elution profiles of annealed DNA, DNA competition experiments, and the testing of more strains.

2. Melioidosis in non-human primates.

Episodes of melioidosis occurring during 1959 in primate colonies at Holloman Air Force Base, New Mexico; Veterans Administration Hospital, Albuquerque, New Mexico; National Institutes of Health, Bethesda, Maryland, and at the University of Washington, Seattle, were jointly investigated with Dr. A. Kaufmann (Communicable Disease Center, Atlanta, Ga.) and professional personnel at respective facilities where cases occurred (Dr. A. M. Allen, Dr. R. T. Cronin, Dr. A. Dillingham, LTC J. D. Douglas and Dr. T. D. Moore).

The first case occurred in a stump-tailed macaque monkey (Macaca arctoides) that was being used for renal clearance studies at VAH, Albuquerque. The diseased animal was one of 19 which was obtained by Holloman AFB from Thailand 10 months earlier, and was one of 4 sent to VAH. Hemagglutination (HA) and complement-fixation (CF) tests for

Table 1. Comparative Annealing of Leptospiral DNA by Different Methods*

Immobilized DNA from serotype (strain)	Radioactive reference DNA and incubation conditions											
	bataviae			javanica			patoc I			CDC		
	Agar 2 X SSC	Filter 6 X SSC	Filter 3 X SSC	Agar 2 X SSC	Filter 6 X SSC	Filter 3 X SSC	Agar 2 X SSC	Filter 6 X SSC	Filter 3 X SSC	Agar 2 X SSC	Filter 6 X SSC	Filter 3 X SSC
bataviae (V.T.)	100	100	100		50	34	0	3	9		6	5
australis (Ballico)	78						0					
pomona (Pomona)	70						0					
muenchen (90C)		98	98		62	33		3			4	
ictero. (RGA)		79	87		51	29		2	5		4	2
kabura (Kabura)		75	64		54	33		3	9		6	4
undet. (Iowa frog)		44	30		55	38		2	6		4	3
javanica (V.B.)	13	47	35	100	100	100	6	2	6		4	3
celledoni (Cell.)				78								
tarassovi (M.J.)	0			100								
semaranga (Patoc)**	0	4	3		4	0	100	100	100		35	28
semaranga (Sao Paulo)**	0						81					
undet. (A-183)**		5			4			32	29		52	48
undet. (CDC)**		6	2		6		0	20	30		100	100
undet. (3055)**		0			0	3		0	0		0	0

* Ratio of immobilized DNA to radioactive DNA was 30:1 in all tests.

** Strains have cultural and biochemical properties of biflexa types. Strain 183 was isolated from cloaca of turtle, strain 3055 from urine of bull, others from natural or tap water.

melioidosis were done on 36 monkeys (5 at VAH, 2 at Lovelace Foundation, and 29 at Holloman AFB) and also on 33 persons who had contact with the sick animals. Five monkeys had HA titers of 1:80 or greater or CF titers of 1:8 or greater in one or both tests. One of the monkey suspects (at Holloman) was from the same shipment as the index case and had subcutaneous abscesses at the site of an implanted catheter. Pseudomonas pseudomallei was isolated from abscesses. Infection was not demonstrated in the other suspects.

A few weeks after the second case was found, melioidosis was demonstrated in a young chimpanzee in the same colony and which had an electrode device implanted in its skull. The animal was imported 3 years earlier from Africa. Infection was probably spread from monkey to the chimp by investigators or animal handlers.

The case at NIH occurred in a rhesus monkey, one in a shipment of 50 received 9 months earlier from India. The animal had undergone a craniotomy for experimental purposes. P. pseudomallei was isolated from abscesses which subsequently developed beneath the craniotomy scar and on the skin of chest and leg.

Melioidosis was diagnosed in a 9 year old pigtail monkey (Macaca nemestrina being used for behavioral research in Seattle. The animal developed multiple subcutaneous abscesses and granulomatous lesions which resulted in death. It had been imported 3 years earlier.

Serologic tests were done at CDC on 20 other monkeys in the same behavioral group. Seven had significant HA titers but no infections could be demonstrated by cultural tests.

To obtain additional information on the occurrence of melioidosis in imported monkeys and also to evaluate the significance of low titer reactions obtained in epizootiological investigations of cases at Holloman, serum samples from 284 monkeys (250 imported animals and 34 born in the United States) were tested. The sera were obtained from various large primate centers and other facilities with primate colonies. The imported group included 135 Macaca nemestrina, 93 M. arctoides and 22 M. irus. The animals born in this country were all M. nemestrina. Sera were tested with hemagglutination (HA) and complement-fixation (CF) procedures. A laboratory error voided most of the CF results. Therefore, only the HA findings are presented. The distribution of HA titers in imported monkeys and in monkeys born in the United States is shown in Table 2.

Fifty percent of the imported animals had low titer reactions of 1:20 to 1:40. Eight (3.2%) had titers of 1:80 and 3 (1.2%) at 1:160. Only low titer reactions of 1:20 (14.7%) and 1:40 (8.8%) were elicited in 34 monkeys born in the United States. The distribution of titers had no apparent correlation with year of importation or age in the case of animals born in the United States.

Table 2. Distribution of Hemagglutination Test Titers for Pseudomonas pseudomallei in Normal Macaque Monkeys

Source	Species	Distribution of sera with HA titers					
		Neg.	1:20	1:40	1:80	1:160	Total
Imported	M. nemestrina	60	41	29	4	1	135
	M. arctoides	42	33	15	3	-	93
	M. irus	12	7	-	1	2	22
	Total	114	81	44	8	3	250
Born in U.S.	M. nemestrina	26	5	3	-	-	34

HA titers were determined for 4 of the monkeys with proved melioidosis. These ranged from 1:320 to 1:10:240. In 2 of the outbreaks, 24 contact animals were examined at necropsy for evidence of melioidosis. Nine had HA titers ranging from 1:80 to 1:2560, 5 had 1:80, one 1:160, one 1:320, one 1:1280 and one 1:2560. The others had titers of less than 1:80. None of the contact animals had lesions compatible with melioidosis, nor was the organism recovered.

On the basis of the foregoing data, a conservative interpretation of HA titers in non-human primates was adapted. Titers of 1:80 and greater were considered to be significant.

Serological tests on sera from human contacts elicited a titer in one person with HA but not CF test. Active infection in this person could not be established.

Summary and Conclusions.

1. Leptospirosis.

a. By the use of liquid N refrigeration, leptospiras can be preserved for at least 4 years without loss of viability and virulence. Liquid N storage provides a more practical and time-saving method for long term maintenance of stock cultures and for maintaining virulence of strains.

b. Additional genetic studies of leptospiras with the use of newer DNA annealing tests have affirmed the existence of two major genetic complexes of biflexa (e.g., saprophytic) and pathogenic leptospiras. Within each complex, there are at least three more genetic groups which share different degrees of genetic relatedness with strains chosen as reference types. One of the strains studied cannot be related to any

of the reference strains and may represent a new genetic group. The genetic groupings may be important in cultural, biochemical, immunological and other phenotypic characteristics of strains.

2. Melioidosis.

In 1969, five cases of melioidosis in 3 separate outbreaks were diagnosed in non-human primates in the United States. Cases appeared in 4 Macaque monkeys which had been imported 6 months to 3 years previously from Southeast Asia. One case in an chimpanzee was contracted through contact with a diseased monkey. History of cases provide evidence of occurrence of inapparent melioidosis in primates imported from Southeast Asia. Inapparent infections may fulminate when animals are stressed, particularly by surgery. In a serological survey of 150 imported monkeys, 4.4% had relatively high hemagglutination test antibody titers and approximately half had low titer hemagglutinins of questionable significance. The Public Health significance of melioidosis in imported monkeys merits additional study.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 170, Militarily important diseases transmissible between animals and man

Literature Cited.

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6448	70 07 01	DD-DR&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 07 01	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	61102A	3A061102B71Q	00	171
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Provide with Security Classification Code)

(U) Development of Biological Products of Military Importance (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
58 05	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: 70	6	170
FISCAL YEAR CURRENT: 71	6	170

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide Key Technical Terms with Security Classification Code)

(U) Biological Products; (U) Eastern Equine Encephalomyelitis; (U) Freeze-Drying; (U) Plague; (U) Q Fever; (U) Vaccines

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

23. (U) This work unit is concerned with the development of manufacturing methods and the production of new effective vaccines for military use, and with the modification of 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 07 - 70 06 Investigations have continued on the development of new and improved biological products for military use. - 1. Zonal centrifugation of Genetron-extracted rickettsial suspensions yielded purified phase 1 and phase 2 Q fever vaccines, which were equal in immunogenicity to vaccines prepared by the standard ether-extraction procedure. 2. Two additional attenuated strains of the plague bacillus have been prepared in the freeze-dried form for use as potential vaccines. 3. Significantly more potent vaccines and greater yields of HA and CF antigens were obtained when EEE virus was grown in suspension cultures of chick-embryo cells than in monolayers in stationary or roller bottles. 4. A new experimental lot of freeze-dried Shigella vaccine was prepared from Shigella flexneri 2a, x-130. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

* Available to contractors upon originator's approval.

DD, FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 171, Development of biological products of military importance

Investigators.

Principal: Joseph P. Lowenthal, ScD
Associate: Sanford Berman, PhD; Patricia L. Altieri, BS; Arthur White, PhD; Doria Dubois, BS; Albert Groffinger

Description.

This work unit is concerned with the development of manufacturing methods for the production of new effective vaccines for military use, and with the modification of existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.

Progress.

1. Q Fever.

During the past year the investigation on the use of the continuous flow zonal centrifuge for the preparation of purified Q fever vaccines was continued.

a. Previous studies (Annual Report, 1969) have demonstrated that Genetron (trichlorotrifluoroethane) extraction of crude yolk sac membrane suspensions containing formalin-inactivated Coxiella burneti, Henzerling strain, phase 2, followed by passage through a density-gradient zonal centrifuge, yielded fractions containing concentrations of rickettsial bodies. Vaccines prepared from this material were as immunogenic in guinea pigs as the standard ether-extracted phase 2 Q fever vaccine, but had significantly lower levels of nitrogen, protein and fat.

b. Additional studies were carried out during this period to determine whether the Genetron-zonal centrifugation procedure can be employed for the preparation of a phase 1 Q fever vaccine. The procedure described for the

preparation of the phase 2 Q fever vaccine (Annual Report, 1969) were applied to yolk sac membrane suspensions of inactivated Henzerling strain, phase 1, rickettsiae. A 10% yolk sac membrane suspension was extracted with Genetron, and was then fed into a sucrose-gradient in the zonal centrifuge. A series of 50 ml fractions was collected from the rotor. 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 Phase 1 Rickettsial Suspension by Density-Gradient Zonal Centrifugation

Fraction	% Sucrose	Density	CF Titer*
1	18.5	1.074	0
2	24	1.099	0
3	28	1.118	0
4	32	1.137	0
5	38	1.166	0
6	44.5	1.200	16
7	51	1.235	128
8	55.5	1.260	512
9	60	1.287	128
10	63.5	1.307	32
11	64	1.310	8

*CF Titer against guinea pig serum collected 42 days post-immunization with phase 1 vaccine. All fractions were negative when tested against 14 day post-immunization serum.

The results indicate that the phase 1 antigen, as measured by CF titer, was concentrated in fractions 7, 8 and 9 (densities 1.235-1.287), the same fractions in which the phase 2 antigen was found. Vaccine prepared from the Genetron-extracted zonal centrifuged phase 1 material proved to be as immunogenic in guinea pigs as was the standard ether-extracted phase 1 vaccine. The results are given in Table II.

TABLE II

Immunogenicity Test on Phase 1 Vaccines

Vaccine	Vaccine Dilution	21 Day Serum	42 Day Serum	
		Phase 2 Antigen	Ph 1 Ag	Ph 2 Ag
Genetron-Zonal	1:4	5/6*	5/6	5/6
	1:16	5/6	4/5	4/5
	1:64	6/6	3/6	6/6
Ether-Extracted	1:4	5/6	4/5	4/5
	1:16	5/5	3/5	4/5
	1:64	4/6	1/5	4/5

*No. of guinea pigs converted to CF positive/total no. of guinea pigs immunized.

c. All of the above studies with the continuous flow zonal centrifuge have been performed with one-liter volumes of rickettsial suspensions. In order to determine whether similar results can be obtained with larger volumes of material, five liters of a Genetron-extracted yolk sac membrane suspension, containing phase 2 rickettsia, were processed through the rotor. The bulk of the antigen was again found in fractions 8 and 9, but this time antigen was more widely distributed through the other fractions. A comparison of the nitrogen, protein and fat content of vaccines prepared from one liter and from five liter volumes, and diluted to a CF liter of 6, is given in the table below, along with the figures for the standard ether-extracted reference vaccine, lot DP-7.

TABLE III

Comparative Assays on Vaccines with Equivalent Antigen Content (CF Titer = 6)

Vaccine	Nitrogen (mg/ml)	Protein (mg/ml)	Fat (mg/ml)
Genetron-Zonal (from 1 liter)	0.0078	0.044	0.17
" " (from 5 liters)	0.011	0.042	0.078
DP-7 (Ether-Extracted ref.)	0.016	0.22	0.7

The results indicate that approximately the same degree of purification can be achieved when one liter or five liters of a Genetron-extracted rickettsial suspension is processed through the zonal centrifuge. Both Genetron-zonal preparations had considerably lower levels of extraneous protein and fat than the reference ether-extracted vaccine.

2. Plague.

During the past year the Department of Biologics Research has continued to provide production and freeze-drying support to the investigations on the development and evaluation of a stable living plague vaccine prepared with attenuated strains of Pasteurella pestis.

a. Two additional attenuated strains of the plague organism were received from Dr. K. F. Meyer, University of California Medical School, San Francisco, California. A supply of freeze-dried seed material and a lot of freeze-dried vaccine suitable for human use were prepared with the EV 76-Girard strain. A single lot of freeze-dried material was prepared for animal studies, using the EV 76-GF strain.

b. Stability studies of four freeze-dried living attenuated plague vaccines, prepared previously and stored at various temperatures, have continued.

(1) Long term surveillance of the viability of the freeze-dried EV 76-Saigon strain, prepared in November 1965, was continued during this period. The results indicate no significant loss of viability in those samples

stored at -20°C for a period of 4.5 years. As previously reported (Annual Report, 1969), maximum viability was maintained for 41 weeks for vials of the freeze-dried vaccine stored at 4°C, and for less than two weeks for material stored at higher temperatures.

(2) Stability studies were also continued on freeze-dried vaccines prepared in early 1968 with two other attenuated P. pestis strains, EV 76-Devignat and EV 76-51f, and on a vaccine prepared in December 1968 with an additional 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 -20°C	EV 76 Devignat Strain +4°C	EV 76 51f Strain -20°C	EV 76 51f Strain +4°C	NPM-23V Strain -20°C	NPM-23V Strain +4°C
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	[illegible]	5.8×10^9	2.0×10^9
24	6.1×10^9	1.8×10^9	4.9×10^9		5.6×10^9	
52	5.6×10^9	1.9×10^9	5.3×10^9	4.3×10^9	[illegible]	
65	5.7×10^9		5.6×10^9		2.8×10^9	5.7×10^9
106	2.7×10^9		2.9×10^9			

These results are similar to those obtained with the EV 76-Saigon strain, and indicate that, for maximum stability during long-term storage, these preparations should be stored at -20°C. At 4°C storage, maximum viability was 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 occurred within 2 weeks.

3. Eastern Equine Encephalomyelitis.

Studies on modifications of usual tissue culture methods for the production of viral antigens and vaccines were continued, employing Eastern Equine Encephalomyelitis

(EEE) virus in chick embryo fibroblast cell cultures as a model system.

a. A comparison of three tissue culture systems, stationary culture, roller bottle culture and suspension culture, was made to determine the optimum system for production of viral antigens. In all three systems the supernatant culture fluids were harvested after 18 hours incubation of the infected cells at 36°C. Results, given in Table V below, show that significantly higher yields were obtained in the suspension cultures.

TABLE V

Effect of Tissue Culture System on Yields of EEE Virus and Antigens

Culture System	Infectivity Titer (Logs per ml)	Hemagglutination Titer	CF Titer
Stationary	10.2	1,280	2
Roller	9.5	640	4
Suspension	11.5	10,240	16

Inactivation of the virus was accomplished by incubation of the culture fluids at 41°C for 72 hours. This inactivation procedure had no demonstrable effect on the hemagglutination or complement fixation titers of the preparations.

Stability tests on the antigens are currently in progress. The results to date indicate that the HA antigens are stable for at least 9 months when stored in the fluid state at 4°C.

b. EEE vaccines were prepared from stationary and suspension cultures by inactivation of the virus in the culture fluids with formalin by the method of Lowenthal et al. (Science, 1961, vol 134, p 565-66). In addition, a vaccine was prepared from the suspension culture by inactivation at 41°C for 72 hours. The potencies of these vaccines were determined in 350-400 g Hartley strain guinea pigs by the antigen-extraction-type assay of Cole and

McKinney (Appl Microbiol, 1969, vol 17, p 927-28). Animals were vaccinated subcutaneously with 0.5 ml of appropriate vaccine dilution on days 0 and 7, and were challenged intracerebrally with 0.1 ml of a virus dilution containing 200 LD_{50} doses on day 21. The results are given in Table VI.

TABLE VI

Potency Assays of EEE Vaccines in Guinea Pigs

Vaccine Preparation	Vaccine Dilution	Resp to Chall Surv/Total	ED_{50}(ml)*
Suspension cult-	1:5	10/10	
Heat inact	1:25	10/10	.0064
	1:125	3/10	
Suspension cult-	1:1	10/10	
Formalin inact	1:5	10/10	.032
	1:25	3/10	
Stationary cult-	1:1	10/10	
Formalin inact	1:5	0/10	.22
	1:25	0/10	

*ED_{50} calculated by method of Reed and Muench (Amer J Hyg, 1938, 27:493-97).

These results show that the suspension culture vaccines were significantly more potent than the standard stationary culture vaccine. In addition, the heat-inactivated suspension preparation was significantly better than the formalin-inactivated suspension preparation.

4. Shigella.

During this period the Department of Biologics Research has continued to provide production and freeze-drying support to Dr. S. B. Formal of the Department of Applied Immunology, WRAIR, in his work on the development of a live attenuated oral Shigella vaccine. A lot of

freeze-dried vaccine, suitable for human use, was prepared in March 1970, from another candidate strain, Shigella flexneri 2a, x-130. This vaccine has been provided to Dr. Formal for evaluation in animals and man.

Summary and Conclusions.

1. Studies on the use of the zonal centrifuge for the preparation of purified Q fever vaccines were continued. The method can be applied to the production of phase 1 vaccine as well as to phase 2 vaccine, yielding purified products which are equal in immunogenicity to vaccines prepared by the standard ether-extraction procedure.

2. Surveillance of the stability of vaccines prepared from four different attenuated strains of the plague bacillus has continued. No significant loss in viability was observed after storage at -20°C for 4.5 years. Two additional attenuated strains of the plague organism have been prepared in freeze-dried form for evaluation in animals and man.

3. Studies on modifications of tissue culture procedures for the production of viral antigens and vaccines, employing EEE virus in chick embryo cell cultures as a model system, were continued. Significantly higher yields of virus, hemagglutinating antigen and complement-fixing antigen were obtained in suspension cultures than in stationary or roller cultures. Vaccines prepared from suspension culture fluids had a higher level of potency than vaccine prepared from stationary culture fluids. Hemagglutinating antigen, prepared by inactivation of suspension culture fluids at 41°C for 72 hours, retained its high level of activity after storage in the fluid state at 4°C for 9 months.

4. Continued support to the development of a live attenuated oral Shigella vaccine was provided by the production and freeze-drying of a lot of vaccine, suitable for human use, with a new candidate strain, Shigella flexneri 2a, x-130.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 171, Development of biological products of military importance

Literature Cited.

1. References.

None.

2. Publications.

Altieri, P.L., Berman, S., Groffinger, A., and Lowenthal, J.P.: Preparation of Cholera Vaccines by fluorocarbon extraction of Cholera vibrio suspensions. Inf. and Immun. 1:334-337, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6449	70 07 01	DD-DR&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
69 07 01	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	61102A	3A061102B71Q	00	172
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Sero-recognition of Microbial Infections (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	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 70	6	120
	CURRENT 71	6	120

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS

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NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Antibody; (U) Antigens; (U) Complement Fixation Tests; (U) Fluorescent Antibody Technics; (U) Parasitic Diseases; (U) Serodiagnosis; (U) Serology; (U) Tuberculosis

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

23. (U) During military operations in Asia, troops regularly are exposed to a variety of infectious diseases not encountered in U.S. Serodiagnostic tests for many of these infections either nonexistent or unreliable. Present efforts directed to overcome these deficiencies by development of new serodiagnostic technics and/or improvement of existing methods. Procedures critically evaluated for diagnostic ability, guide for therapy, and relationship to course of disease. Serological procedures urgently required in diseases in which causitive agent is difficult to demonstrate.

24. (U) CF, FA and HA technics used to determine efficacy of antigen fractionation procedures and to evaluate the specificity and sensitivity of the purified products. Also, new serologic technics are developed (e.g. SAFA). Technical problems include at times, limited availability of organisms and their separation from host tissues. Improved technology in one case often facilitates research even in unrelated areas.

25. (U) 69 07 - 70 06. Work accomplished thus far has provided specific, sensitive serodiagnostic tests for variety of important diseases (various parasitic, treponemal, bacterial and mycotic). Tests for other diseases within these categories currently being critically evaluated. SAFA test continues to show promise for detection of active simian tuberculosis, consistently being more sensitive and specific than standard tuberculin tests. Innovation of methodology makes possible performance of CF tests on dog sera which become AC with heat inactivation. Adaptation of quantitative CF procedures for critical, objective evaluation of antigens accomplished. CF tests for malaria, developed in other work unit, to be evaluated for efficacy for detection of occult infection in returnees. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061102B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 172, Sero-recognition of microbial infections

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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 infectious diseases. Details of the development and progressive technical improvements of the SAFA test have been presented in previous reports on this Work Unit. The procedure continues to show considerable promise for the serodiagnosis of a variety of infectious diseases. In addition to the applications of the SAFA procedure reported from this Department, other investigators in the WRAIR have successfully applied the SAFA methods for serodiagnosis of amebiasis, filariasis, trichinosis, malaria, meningococcosis, adenovirus infections, and influenza. The potential of the SAFA test for other viral diseases is being investigated. Continued experience has shown that the methods for fixing the antigen to the test discs and the procedures for minimizing nonspecific fluorescence vary from antigen to antigen. However, once the proper conditions are established, performance of the test is technically simple and can readily be accomplished even by personnel with minimum experience. Furthermore, it has been observed that the majority of antigens are quite stable after fixation on the test disc; the T. cruzi protein antigen and adult S. mansoni antigen have been fixed on the discs and stored under vacuum at 3°C for more than 8 months without evidence of deterioration. Thus, it is conceivable that a

central laboratory could prepare antigen-sensitized discs for distribution to smaller satelite laboratories and provide a diagnostic capability in laboratories unable to perform the more intricate standard serologic procedures.

a. Tuberculosis. The need for a reliable immunodiagnostic test for early detection of tuberculosis in non-human primates and the limitations of the currently recommended intrapalpebral tuberculin tests have been discussed in detail in previous reports on this Work Unit. Initial studies on experimentally infected monkeys (1) conducted in collaboration with investigators at the Industrial Health and Safety Directorate, Fort Detrick, Md., have been completed and are reported herein. In addition, preliminary studies on the potential of the SAFA test for detection of tuberculosis in humans and for appraising the efficacy of therapy are reported.

Thirty tuberculosis-free Rhesus monkeys were selected for the initial studies on simian tuberculosis. Twelve of the group were exposed to an aerosol of M. tuberculosis administered in a manner in which each animal inhaled ca. 5000 viable tubercle bacilli. The remaining 18 monkeys were used to study the mechanisms of natural transmission of the disease, and served as a cagemate of an aerosol-exposed animal or were placed in adjacent cages receiving air only from a cage housing an exposed monkey. At 2-week intervals during the first 4-month post-exposure period and at monthly intervals thereafter, blood was collected for the SAFA test and the animals then were tranquilized, radiographed and tuberculin tested. For the latter, an intradermal injection of 0.1 ml (25 mg) of Koch's Old Tuberculin was administered intrapalpebrally and in the abdominal area. The tuberculin tests were read 24, 48 and 72 hours after injection.

Complete necropsies were performed on the monkeys that died during the course of the study and on those that were sacrificed at the conclusion of the investigations. Efforts were made to demonstrate the presence of tubercle bacilli in all animals, regardless of whether they expired or were sacrificed. Representative samples of tissues were triturated in Ten Broeck tissue grinders and plated on appropriate culture media. Other portions of the tissues were fixed in phosphate-buffered formalin and processed for histopathological examination, using hematoxylin-eosin, carbol fuchsin and fluorescent antibody stains.

Results of these investigations are summarized in Table 1. The 12 monkeys that were exposed to the aerosol of tubercle bacilli all became infected and died. However, the incidence of cross infection was unexpectedly low; only 4 of the cagemate or adjacent cage controls became infected. The SAFA test detected 13 of the 16 infected animals and no false positive reactions were observed. This was in contrast to the results obtained with the tuberculin tests. Twelve of the infected monkeys ultimately gave positive tuberculin reactions, but 4 of control

Table 1

Summary of Simian Tuberculosis Experiments

Monkey No.[a]	Tuberculin test SAFA[b]	IDP[bc]	IDA[bc]	X-ray[b]	Necropsy
1-A	+ (42)	+ (42)	-	+ (28)	+
1-B	-	+ (134)	+ (163)	-	-
1-C	-	-	-	-	-
2-A	± (28);+(42)	+ (56)	+ (70)	+ (28)	+
2-B	± (98);+(105)	+ (179)	-	+ (105)	+
2-C	-	-	-	-	-
3-A	+ (42)	+ (70)	+ (70)	+ (28)	+
3-B	± (126);+(162)	-	-	+ (134)	+
3-C	-	-	-	-	+[d]
4-A	-	-	-	+ (28)	+[e]
4-B	-	-	-	-	+[d]
4-C	-	+ (162)	+ (179)	-	-
5-A	+ (42)	+ (56)	-	+ (28)	+
5-B	-	-	-	-	-
5-C	-	-	-	-	-
6-A	± (28);+(42)	+ (70)	+ (70)	+ (28)	+
6-B	-	-	-	-	-
6-C	-	-	-	-	-

Table 1 (Continued)

Monkey No.[a]	Tuberculin test SAFA[b]	IDP[bc]	IDA[bc]	X-ray[b]	Necropsy
7-A	+ (42)	+ (56)	+ (70)	+ (28)	+
7-CI	-	+ (240)	-	-	-
8-A	± (28);+(42)	+ (56)	+ (56)	+ (28)	+
8-CI	-	-	-	-	-
9-A	+ (84)	+ (134)	-	+ (28)	+
9-CI	-	-	-	-	-
10-A	+ (42)	+ (56)	+ (56)	+ (28)	+
10-CI	-	-	-	-	-
11-A	+ (42)	+ (84)	-	+ (28)	+
11-CI	-	-	-	-	-
12-A	+ (42)	+ (134)	+ (134)	+ (28)	+
12-CI	-	+ (270)	-	-	-

a - A, aerosol-exposed monkey. (Dose: 5,000 viable M. tuberculosis)
B, cagemate of A.
C, monkey in cage adjacent to A and B
CI, same as C, but monkey treated with isoniazid

b - Figures in parentheses indicate days after exposure that test became positive.

c - IDP, intrapalpebral tuberculin test.
IDA, intradermal tuberculin test on the abdomen.

d - Tubercle bacilli only in hilar lymph node.

e - Fulminating, disseminated tuberculosis.

animals developed tuberculin hypersensitivity in the apparent absence of active disease. Analysis of the data from the necropsy-positive monkeys revealed that SAFA-reactive antibodies appeared before tuberculin hypersensitivity was manifested. With the single exception of monkey 1-A in which the tests became reactive on the same day, the SAFA test was reactive 14 - 74 days before either of the tuberculin tests was positive. Thus, the SAFA test was superior to the tuberculin tests for early detection of infected animals, and this feature could be an important factor in more effectively controlling tuberculosis in non-human primate colonies. It was further observed that the polysaccharide antigen was more sensitive than the A- or C-protein fractions for detection of simian tuberculosis.

Results of repeated radiographs of the monkeys in this study suggest that roentgenograms also provide an excellent method for early detection of simian tuberculosis, at least in animals challenged with a large number of organisms. Fourteen of the 16 infected animals showed positive radiographs. Moreover, in monkeys receiving the aerosol, pulmonary lesions usually were detected before the SAFA or tuberculin tests became positive. This apparent superiority of radiologic methods, however, was not observed in the few animals that acquired their infections by exposure to an aerololized monkey; with monkeys 2-B and 3-B who were in the latter category, the SAFA tests were reactive before the radiographs became positive (Table 1).

Review of the data obtained from the 3 infected monkeys that failed to react in the SAFA test (Table 1) revealed that monkey 4-A developed a fulminating infection and died 45 days after aerosol exposure, at approximately the time detectable antibodies would have been expected to appear. This animal also remained tuberculin-negative until death and it is possible that a severe anergic condition prevailed. Pulmonary lesions, however, were detected in radiographs taken 28 days after exposure to the aerosol. Monkeys 3-C and 4-B had very mild infections and exhibited no clinical evidence of active tuberculosis. They appeared to be healthy, were uniformly negative in all diagnostic tests, and exhibited normal weight gain throughout the observation period. Necropsy revealed no gross or microscopic pathology other than granulomata of the hilar lymph nodes. Cultures of these lymph nodes revealed the presence of M. tuberculosis, but cultures of other organs and tissues were negative. Thus, by the usual diagnostic criteria, these monkeys did not have active tuberculosis even though they were harboring tubercle bacilli. These findings and those obtained in current ancillary studies on human tuberculosis, suggest that the presence of SAFA-reactive antibodies is indicative of current or recent active tuberculosis, and that these antibodies do not reach detectable levels in sub-clinical infections.

In this study, 6 of the control monkeys regularly received isoniazid (10 mg/kg body wt orally, twice daily) throughout their period of exposure

to infected animals. These experiments were conducted in an effort to determine whether this drug was an effective prophylactic against naturally acquired simian tuberculosis. Although none of the treated animals became infected, the results were inconclusive because of the extremely low rate of cross infection in the untreated groups.

It is realized that exposure of the monkeys to 5000 tubercle bacilli represents a highly artificial condition. Thus, the results of the various diagnostic tests performed on these animals must be interpreted with care. The massive challenge was employed to assure infection in all of the aerosolized monkeys and permit investigations on the mechanisms of natural transmission. Unfortunately, the incidence of cross infection in the cagemate and adjacent cage control animals was much lower than had been anticipated, suggesting that airborne transmission probably is less common than had been previously surmised. Because of these unexpected developments, the present study provided little opportunity to evaluate the various diagnostic procedures in monkeys receiving a minimum infective dose, such as that which would be involved in most naturally acquired infections. Investigations using graded doses of viable tubercle bacilli for challenge are in progress.

It is believed that the unexpectedly low rate of transmission of tuberculosis to cagemates was due primarily to the design of the cages, which prevented contamination of the food with urine and feces. All of the aerosol-exposed monkeys regularly passed viable M. tuberculosis in their feces and urine, and direct or indirect contact with these contaminated excreta surely would have resulted in more infections among the control monkeys. Elimination of this possible source of infection, and better diagnostic methods, as represented by the SAFA test and/or serial radiographs, could reduce the risks of serious outbreaks of tuberculosis in non-human primate colonies.

The practice of performing SAFA tests for tuberculosis on all subhuman primates during their quarantine period at the WRAIR have been continued. A total of 1216 monkeys were screened with 3 M. tuberculosis antigens (A-protein, C-protein and polysaccharide) during the present reporting period. Of this group, 5 reacted in SAFA tests with one or more of the antigens. These animals were considered suspect for tuberculosis and were removed from quarantine, placed in isolation at the USAMRIID, Fort Detrick, Md., and held for further observations. In the initial tests performed on these monkeys, 2 reacted in the SAFA test but gave negative intrapalpebral tuberculin reactions. Although they ultimately developed tuberculin hypersensitivity, these animals would have been overlooked in the quarantine screening if only the tuberculin test had been employed. The remaining 3 monkeys initially reacted in both tests. One of the animals expired soon after transfer to Ft. Detrick and tuberculosis was confirmed by culture and histopathological methods. Three of the group recently died and showed

gross evidence of tuberculosis at necropsy. However, the culture studies and detailed histopathological examination of the tissues have not been completed. The remaining monkey is still alive and continues to show positive reactions in the SAFA and tuberculin tests. Observations on this animal are being continued.

Additional monkeys housed at Ft. Detrick also were studied during the present reporting period. Six monkeys showing positive tuberculin reactions and negative SAFA tests were sacrificed and examined for tuberculosis. All proved to be infected. The apparent superiority of the tuberculin test to the SAFA test in these cases is in marked contrast to previous experiences, and the clinical histories of these animals are being examined in an effort to determine what factor, possibly anergy, might account for these unexpected findings. Seven additional monkeys showing positive tuberculin reactions and negative SAFA tests currently are under observation.

Ancillary studies to evaluate the potential of the SAFA test for the serodiagnosis of human tuberculosis also have been conducted. Results of these preliminary investigations have been reported [1]. Three soluble antigens derived from M. tuberculosis (a concentrated culture-filtrate antigen, an A-protein preparation, and a C-protein fraction) were evaluated in SAFA tests on 42 human sera collected at various intervals during therapy. The C-protein fraction proved to be the most specific, sensitive antigen, and in contrast to intradermal tests, antibodies detected in the SAFA test appeared to indicate current or recent active disease. The majority of patients showed a significant rise in antibody titers following initiation of therapy and maximum levels were reached 60 - 90 days post treatment. The antibodies generally remained at these higher levels for 4 - 5 months and then began to gradually decline. Although individuals with advanced or far advanced disease showed considerable evidence of anergy, some being seronegative prior to initiation of therapy, it is believed that this would not pose a serious problem in detection of early infection. These preliminary findings indicate that the SAFA test has potential for early detection of active tuberculosis in humans and may be of value for appraising the efficacy of therapy.

The potential of the SAFA test for mass screening for tuberculisis in humans also is being investigated. These investigations are being conducted in collaboration with members of the Preventive Medicine Division, U.S. Army Hospital, Fort Ord, Calif. Recruits undergoing routine screening tests for tuberculosis on assignment to the Training Center, Fort Ord, comprise the subjects for this study. Following collection of a serum sample for the SAFA tests, each subject is tested for tuberculin hypersensitivity with the Tine test. The sera are packaged in dry ice and shipped in the frozen state to the Department of Serology, WRAIR, for evaluation in the SAFA tests. Three M. tuberculosis antigens (A-protein, C-protein and polysaccharide) are employed in SAFA tests on

each serum. Individuals showing a reaction in the SAFA and/or Tine tests are examined more critically for evidence of active tuberculosis. A serum sample is collected for repeat SAFA tests and the patient is tested for tuberculin hypersensitivity with IPPD, PPD-B and PPD-G antigens. In addition, a chest X-ray is taken and efforts are made to demonstrate mycobacteria in the sputum by microscopic and culture methods.

It is proposed to screen ca. 1000 recruits in this manner and to date more than 700 individuals have been examined. During the course of these studies it was observed that certain sera exhibited an unusually high level of nonspecific fluorescence with the control disc impregnated with bovine serum albumin. This had not been previously encountered in extensive studies on monkey sera or in the limited number of human sera examined. Although the factors responsible for excessive nonspecific fluorescence are unknown at this time, it was recognized that this could lead to erroneous interpretation of test results. Subsequent experiments on such sera revealed that this was the case. All sera initially giving a reaction with the antigen-sensitized discs were re-examined for nonspecific fluorescence with BSA impregnated discs, and 8 exhibited a high level of nonspecific reactivity. Subsequent sera from these patients, however, did not show excessive nonspecific fluorescence and tests with the antigen-sensitized discs were unequivocally nonreactive. Thus the reactions observed in the initial tests were solely nonspecific in nature. In view of these findings, all human sera tested with M. tuberculosis antigens also are tested with a BSA control disc to provide for adjustment of the fluorometer to zero before reading the test with the antigen-sensitized disc, and to identify sera giving high levels of nonspecific fluorescence. Sera in the latter category are considered unsatisfactory for the SAFA test and no attempt is made to interpret the results.

To exclude any bias in interpreting the results, the SAFA tests are being performed without knowledge of the status of any of the subjects. Results of the tuberculin and other laboratory tests and pertinent clinical findings will be provided at the conclusion of the study. These results will be compared with those obtained with the SAFA tests and the findings included in a subsequent report on this Work Unit.

b. Echinococcosis. Studies on the suitability of the SAFA procedure for the serodiagnosis of echinococcosis in humans, dogs, and sheep were continued in collaboration with Dr. J.F. Williams, Centro Panamericano de Zoonosis, Ramos Mejia, Argentina. Dr. Williams recently traveled extensively in areas hyperendemic for hydatid disease and collected a large number of sera from documented cases of echinococcosis. In each instance, the diagnosis was confirmed by demonstration of a hydatid cyst or Echinococcus scoleces. It is noteworthy that all human sera were collected prior to surgery. It is well

established that surgical removal of a hydatid cyst causes a rapid rise in circulating antibodies even though the cyst is removed intact. Therefore, tests on post-surgery serum samples would not provide a reliable index of the efficacy of the SAFA test for differential diagnosis of untreated cases. Hydatid fluid of sheep origin was used as antigen in these continued studies.

Attempts to employ the SAFA test for the serodiagnosis of echinococcosis in the sheep have been unsuccessful thus far. Control sera from uninfected sheep uniformly gave a high level of nonspecific fluorescence with the antigen-sensitized test discs. Moreover, fractionation of the hydatid fluid to remove sheep tissue components failed to reduce the nonspecific reactivity. Efforts are being made to overcome this problem by employing soluble antigen prepared from scoleces or using hydatid fluid from an animal other than sheep. Although simple air-drying of the hydatid fluid on the test discs provided the best antigen for SAFA tests on human sera, it was observed that the specific reactivity with dog sera was enhanced by treating the antigen-sensitized discs with a chemical fixative (1% acetic acid in 95% ethanol) prior to performing the tests. Thus, the fixative was used in preparing the discs for the tests on dog sera reported herein.

Results of studies on canine echinococcosis are summarized in Table 2. In initial experiments, the SAFA test was compared with the indirect hemagglutination (IHA) and Latex agglutination (LA) procedures in tests on dogs experimentally or naturally infected with Echinococcus granulosis. The latter tests have been employed by other investigators for the serodiagnosis of human echinococcosis and it was deemed worthwhile to evaluate their efficacy in canine infections. It was readily apparent that the sensitivity of the IHA and LA tests was very poor. The IHA test was uniformly nonreactive in tests on sera from 38 infected animals and the LA test gave reactions with only 6 of the group. In view of the obvious deficiencies of the IHA and LA tests, the procedures were not included in further studies on dog sera. The SAFA test, on the other hand, reacted well with sera from both experimentally and naturally infected animals. A total of 151 infected dogs were examined with the SAFA test and 115 (76%) gave reactions. The initial tests for specificity were conducted on dogs from the beagle colony maintained by the Division of Veterinary Medicine, WRAIR, and no false reactions were observed. However, subsequent tests on mongrel street dogs residing in areas known to be free from Echinococcus infestation revealed considerable nonspecific reactivity. This was first apparent in tests on dogs from Buenos Aires and Azul, Argentina. Ten (38%) of the 26 dogs examined reacted in the SAFA test. Moreover, the absence of Echinococcus infection was confirmed in each animal by necropsy. It was of immediate interest, therefore, to determine whether North American street dogs evidenced a similar pattern of false reactions. A group of 40 mongrels collected in the Washington, D.C. area were tested and 15 (38%) reacted. It is believed that the reactions in Echinococcus-free dogs

Table 2

Serologic Tests for Canine Echinococcosis

Clinical Status		Total	Serologic Test Results* SAFA R	SAFA NR	IHA R	IHA NR	LA R	LA NR
Experimental Infection	A	8	7	1		8		8
	B	27	20	7				
Natural Infection	A	30	23	7		30	6	24
	B	86	65	21				
non-infected (Walter Reed)		20		20				
non-infected (Buenos Aires)		5	4	1				
non-infected (Azul)		21	6	15				
non-infected (Washington)		40	15	25				

*SAFA = Soluble antigen fluorescent antibody

IHA = Indirect hemagglutination

LA = Latex agglutination

Test reported Reactive (R) or Nonreactive (NR)

are due to infections with other helminths that produce antibodies which cross react with the hydatid fluid antigen. Although the SAFA test is superior to the IHA and LA tests for the serodiagnosis of canine echinococcosus, the relatively high frequency of false reactions in street dogs compromises the value of the SAFA test for routine use. Efforts will be made to improve the specificity of the test by employing a purified soluble antigen from E. granulosis scoleces.

The SAFA, IHA, LA and immunoelectrophoresis (IE) procedures were evaluated in tests on sera from human cases of echinococcosis. It was noted earlier that all cases were confirmed by demonstration of a hydatid cyst and/or Echinococcus scoleces in the organs. In addition, all serum specimens were collected prior to surgical removal of the cyst. Results of these tests are summarized in Table 3. The SAFA test was the least sensitive of the procedures evaluated, detecting antibody in only 54 (61%) of the 88 sera examined. However, it was the most specific; no false positive reactions were observed in the tests with sera from healthy donors. The IHA procedure showed moderate sensitivity, giving reactions with 64 (73%) of the sera, and exhibited relatively high specificity. The LA showed a still higher level of sensitivity, reacting with 31 (86%) of the sera tested, but also showed considerable nonspecific reactivity in tests with the uninfected controls. The IE test was the most sensitive of the procedures evaluated. Reactions were observed with 34 (94%) of the 36 sera examined, and incidence of nonspecific reactivity was low. Thus it appeared that the IE test was superior to the others for detection of echinococcosis in humans. However, it is realized that the IE test is somewhat sophisticated and is not suited for testing large numbers of specimens. Efforts therefore are being continued to improve the conventional serologic procedures, particularly the SAFA test, by employing antigens of better quality.

c. Histoplasmosis. In the previous report on this Work Unit, it was noted that the results obtained with SAFA tests employing "Histoplasmin" antigen (mycelial phase exoantigen) showed no correlation with results obtained with the standard CF or precipitin tests. During the present reporting period, efforts were made to improve the SAFA test for histoplasmosis by employing a soluble antigen prepared from the yeast phase of Histoplasma capsulatum. Yeast phase cells suspended in tris buffered saline were passed two times through a French pressure cell at 20,000 psi. The effluent was centrifuged in the cold (3°C) for 1 hr at 1000 rcf to sediment gross debris and the few intact yeast cells not ruptured by the French press. The supernate contained the soluble antigens and was tested for reactivity in the SAFA test with sera from histoplasmosis patients that had given reactions in the standard CF test. Freshly prepared extracts of yeast phase cells processed in this manner gave strong reactions with the histoplasmosis sera. However, it was observed that the antigenic components precipitated during storage at 4°C or at -20°C. This precluded use of the material for sensitization of test discs for the SAFA test. Attempts to stabilize the soluble yeast phase antigen during storage thus far have been unsuccessful. In view of

Table 3

Serologic Tests for Human Echinococcosis

Clinical Status		Number	Serologic test results*							
			SAFA		IHA		LA		IE	
			R	NR	R	NR	R	NR	R	NR
Hydatid Disease	A	36	20	16	27	9	31	5		
	B	36	25	11	22	14			34	2
	C	16	9	7	15	1				
Healthy		50		50	2	48	6	44	1	49

* SAFA = Soluble antigen fluorescent antibody

IHA = Indirect hemagglutination

LA = Latex agglutination

IE = Immunoelectrophoresis

these findings and the persistent lack of correlation between the SAFA test with Histoplasmin antigen and the standard serodiagnostic tests for histoplasmosis, performance of the SAFA test has been temporarily discontinued until a more satisfactory, stable antigen becomes available.

2. Serodiagnosis of American trypanosomiasis (Chagas' disease). The importance of Chagas' disease as a major public health problem in Central and South America has been discussed in detail in previous reports on this Work Unit. The principal efforts during this reporting period have been directed toward the improvement of methodology for critical assay of antigens (see other section of this report) and participation with six laboratories in North, Central and South America that currently are evaluating selected T. cruzi antigens. These evaluations are being conducted in connection with the activities of a Study Group on Chagas' Disease Antigens organized by the PAHO. The initial phase of the evaluation, dealing with the non-specific reactivity of the various candidate antigens, has been completed. On the basis of these findings, three antigens were deemed unsatisfactory because of excessive nonspecific reactivity, and were dropped from further consideration as potential candidates as a reference antigen. The remaining five candidate antigens currently are being evaluated for specific reactivity in each laboratory. It is believed that these studies will permit selection of a standard antigen for universal use in serodiagnosis of Chagas' disease, and for reference in evaluating new antigens and methods.

3. Development and improvement of serologic methods and reagents. Further efforts to improve and evaluate serologic methods have been made during this reporting period.

a. A new method for critical evaluation of antigens. Recent innovations in the methodology of the quantitatively standardized complement fixation (CF) procedure developed in the Department of Serology have provided a new and critical method for objectively evaluating antigen preparations.

It has been established that there is a linear relationship between the logarithms of the amounts of immune complexes (in terms of serum or in terms of antigen) and the probits of hemolysis. From these linear relationships, the amounts of immune complex required for 50% hemolysis with each amount of complement (C') used in the tests (i.e. 5 and 7.5 units) can be determined. There is also a linear relationship between the amounts of immune complexes so determined and the amounts of C' used. Thus, the specific reactivity of a serum and/or antigen may be expressed as the slope of the line representing this relationship.

For the titration of serum, a maximally reactive dose of antigen is used in tests with the reactive serum serially diluted in nonreactive

serum. Conversely, for the titration of antigen, a low dilution of antiserum (representing excess antibody) is used in tests with serially diluted antigen. It should be realized that each antigen preparation in reality is a mixture of a number of different antigen entities, and that each serum contains a variety of discrete antibodies. Since the relative concentrations of the major antigen or antibody components usually are considerably greater than the minor components, reactions obtained with the higher dilutions of antigen or serum primarily reflect the characteristics of the respective major components. Under these conditions, the minor components will have been diluted beyond their effective concentrations, and the characteristics of the major components which may be masked in tests with higher concentrations of antigen or serum become readily apparent. Therefore, in titrations conducted according to the proposed procedures, the serum titer expresses the capacity of the major antibody components to react with the several components of the antigen preparation. The antigen titer reflects the capacity of the major antigen components to react with the variety of antibodies present in the serum.

Table 4 illustrates the titration of a single Chagasic serum with two T. cruzi antigens. The serum concentrations are expressed as the absolute amount of serum contained in 0.05 ml (the test dose) of the respective dilutions, and a logarithmic progression of dilutions (dilution factor = 1.33') was employed to obtain a number of tests giving partial hemolysis. The importance of diluting the reactive serum in nonreactive serum rather than saline should be emphasized. It has been established that alteration of the nonantibody components of the serum causes both qualitative and quantitative changes in the specific reactivity. Therefore, it is essential that this variable be controlled by diluting the reactive serum with nonreactive serum to eliminate changes in the total serum concentration as the antibody is diluted.

The volume of serum giving 50% hemolysis with each amount of C' used can be determined by plotting the volumes of serum giving partial hemolysis versus the corresponding percentages of hemolysis on log-probit graph paper, and fitting a straight line to the experimental points as illustrated in Figure 1. Thus, it can be seen that with antigen "A", 0.0027 ml of serum gives 50% hemolysis in tests with 5-unit C', and 0.0050 ml in tests with 7.5-unit C'. Similarly, the titration with antigen "B" revealed that the amounts of serum giving 50% hemolysis with 5 and 7.5 units of C' respectively were 0.0021 ml and 0.0040 ml. The serum titer with each antigen then could be determined by plotting on arithmetic coordinates the units of C' used versus the respective volumes of serum giving 50% hemolysis, constructing a straight line through these points, and determining the slope as illustrated in Figure 2. Inspection of the curves obtained with antigens "A" and "B" shows that there is little difference in the respective slopes. This is also reflected in the numerical values of the serum titers which may be calculated from the relation $T_S = \frac{\Delta C'}{\Delta S}$, where $\Delta C'$ is the difference

Table 4

SERUM TITRATIONS

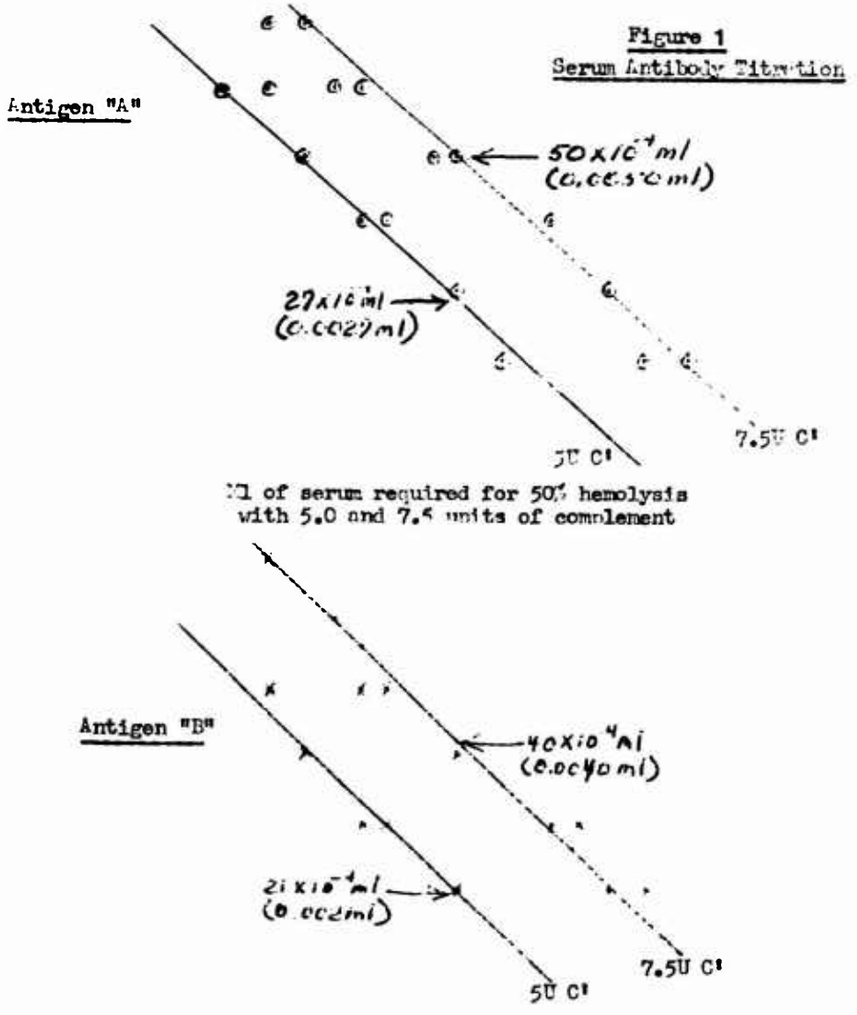
Serum: 043069 Antigens: "A" and "B". Complement n° BBL 9041247
Hemolysin n° D-490975 Dil. 1/3000 Sheep cells n° 22-092969
Human normal pool serum n° 1. Diluted 1/5
Dilutions of Chagasic Serum: 1:1 , 1:1.3 , 1:1.8 , 1:2.4.....(dilution factor = 1.33)

	Chagasic serum dilutions											
	1	2	3	4	5	6	7	8	9	10	11	12
	ml x 10^{-4} of Chagasic serum in 0.05 ml											
	500	375	281	211	158	119	89	67	50	38	28	21
Reactions with Antigen "A" (diluted 1/16)												
	% Hemolysis											
Compl.												
5U	0	0	0	0	0	0	5	15	20	30	50	60
	0	0	0	0	0	0	5	10	20	35	50	60
7.5U	0	0	0	0	0	5	15	30	45	70	80	85
	0	0	0	0	0	5	20	25	50	70	80	90
Reactions with Antigen "B" (diluted 1/64)												
	% Hemolysis											
Compl.												
5U	0	0	0	0	0	0	0	5	15	20	30	50
	0	0	0	0	0	0	0	5	15	20	35	50
7.5U	0	0	0	0	0	0	15	25	35	50	70	80
	0	0	0	0	0	0	15	25	30	50	75	85
	Serum controls											
Compl. 1U	0	0	0	0	10	15	20	25	20	20	20	25
2U	0	0	0	0	20	25	35	40	50	65	65	65

Complement control	Normal serum pool	Antigen controls	
		"A"	"B"
1U 20-20	1U 15-20	1U 20-20	25-20
2U 70-75	2U 55-60	2U 65-70	65-65

Cells control 0%

Figure 1
Serum Antibody Titration



ml of serum required for 50% hemolysis with 5.0 and 7.5 units of complement

Figure 2

Serum Antibody Titration

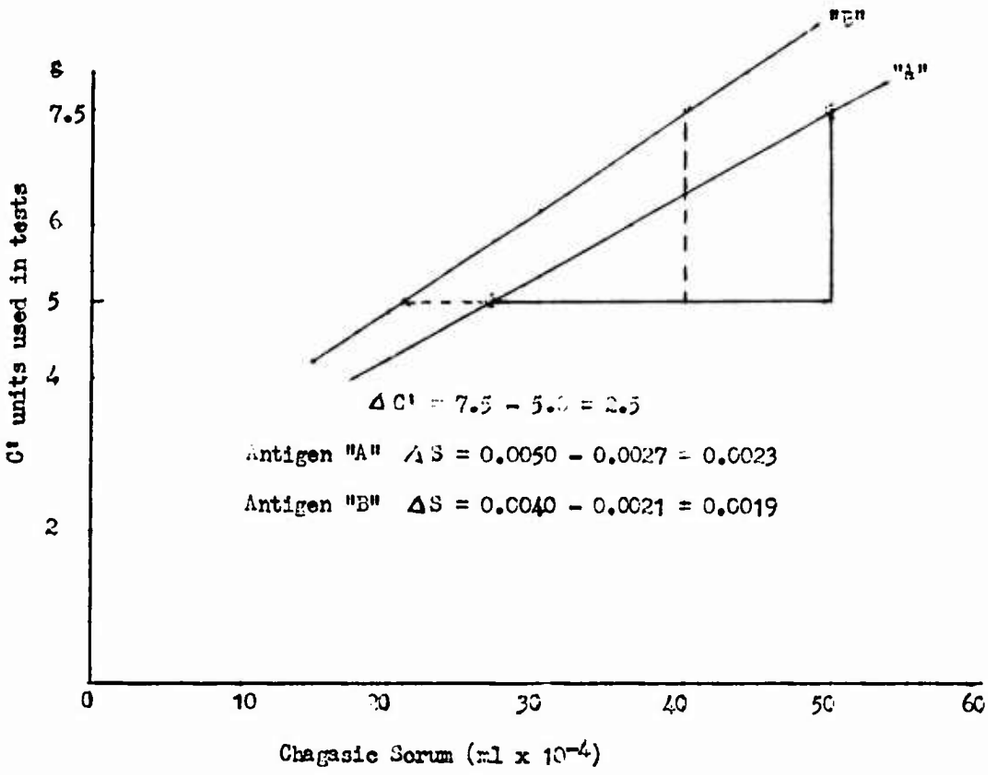
$$T_S = \frac{\Delta C'}{\Delta S}$$

Serum titer with antigen "A"

$$T_S = \frac{2.5}{0.0025} = 1000 \longleftarrow$$

Serum titer with antigen "B"

$$T_S = \frac{2.5}{0.0019} = 1316 \longleftarrow$$



$\Delta C' = 7.5 - 5.0 = 2.5$

Antigen "A" $\Delta S = 0.0050 - 0.0027 = 0.0023$

Antigen "B" $\Delta S = 0.0040 - 0.0021 = 0.0019$

between the amounts of C' used, and the ΔS is the difference between the amounts of serum giving 50% hemolysis. Thus, the serum titers obtained with antigens "A" and "B" respectively are 1090 and 1316. By this criterion, viz. reactivity with homologous antibody, the two antigens appear to be quite similar.

The antigens are titered by the same methods used for the serum titrations except that the volumes of antigen required for 50% hemolysis with excess antibody are determined. Table 5 illustrates the protocol for the titrations of antigens "A" and "B" with a single Chagasic serum, and Figures 3-A and 3-B show the log-probit plots of these data. From Figure 3-A, it can be seen that 0.0038 ml of antigen "A" gives 50% hemolysis with 5-unit C' and 0.0170 ml is required with 7.5 units of C'. Likewise, Figure 3-B shows that 0.00027 ml and 0.00063 ml of antigen "B" respectively give 50% hemolysis with the 5 and 7.5 units of C'. The antigen titers are determined by plotting on arithmetic coordinates the units of C' used in the titrations versus the volumes of antigen giving 50% hemolysis, constructing a straight line through the points, and determining the slope. The antigen titer, expressed as the slope of this line, can be calculated from the relation $T_A = \frac{\Delta C'}{\Delta A}$, where $\Delta C'$ is the difference in the units of C' used in the titration, and ΔA is the difference in the volumes of antigen giving 50% hemolysis. Figure 4 illustrates the plotting and calculation of the titers of antigens "A" and "B". It is readily apparent that the slopes obtained with the two antigens are markedly different. Antigen "A" gave a titer of 190 whereas antigen "B" had a titer of 6940. Thus, in marked contrast to the comparisons based on the serum titrations, by this criterion the antigens were not at all comparable. These examples graphically illustrate the requirement to employ both parameters (i.e. serum antibody titers and antigen titers) in critical evaluation of antigens; use of one parameter alone could lead to serious erroneous conclusions.

Additional valuable information can be obtained by further analysis of these data. An example of such analysis is given in Table 6. It is recognized that variations in the amount of C' that deteriorates during the fixation period will result in variations of the numerical values of the serum titer (T_S) and antigen titer (T_A). However, this variation due to C' can be eliminated by expressing the results in terms of the Index of Reactive Capacity (I. R. C.) of the antigens in question. The I. R. C. is the ratio of the antigen titer and the serum titer (i.e. T_A/T_S). Since the antigen titer is represented as $T_A = \frac{\Delta C'}{\Delta A}$, and the serum titer is $T_S = \frac{\Delta C'}{\Delta S}$, the ratio (I. R. C.) in reality is

$$\text{I. R. C.} = \frac{\frac{\Delta C'}{\Delta A}}{\frac{\Delta C'}{\Delta S}} = \frac{\Delta S}{\Delta A}$$

since the $\Delta C'$ is constant (2.5) and cancels out. Thus the I. R. C.

Table 5

ANTIGEN TITRATIONS

Chagasic serum n° 0430975 Diluted 1/2.5 date 10/16/69
Hemolysin n° D-490975 Dil. 1/3000 Complement n° BBL 9041247 Cells 22-092969

ANTIGEN "A" Maximally reactive dose - 1/16 for 7.5 U C'
Dilutions; 1/16 , 1/32 , 1/64 .....1/2048.

	Dilutions of antigen "A"							
	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
	ml of antigen X 10-5 in 0.1 ml							
	625	313	156	78	39	19	9.8	4.9
Compl.	Hemolysis %							
5U	35	55	70	75	85	90	100	100
	35	60	70	80	85	95	100	100
7.5U	70	85	90	100	100	100	100	100
	70	80	90	95	100	100	100	100
	Antigen controls							
1U	15	15	20	20	25	25	25	25
2U	60	65	700	75	75	75	75	75

ANTIGEN "B" Maximally reactive dose: 1/64 for 7.5 U C'
Dilutions 1/64 , 1/128, 1/256 .....1/8192

	Dilutions of antigen "B"							
	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	1/8192
	ml of antigen x 10^{-5} in 0.1 ml							
	156	78	39	19	9.8	4.9	2.4	1.2
Compl.	Hemolysis %							
5U	10	15	35	60	80	85	95	100
	10	20	35	65	80	90	95	100
7.5U	25	40	60	85	90	100	100	100
	25	45	60	90	95	100	100	100
	Antigen controls							
1U	20	20	25	25	25	25	25	25
2U	70	70	75	75	75	75	75	75

Chagasic serum control
1U 20-25
2U 45-55

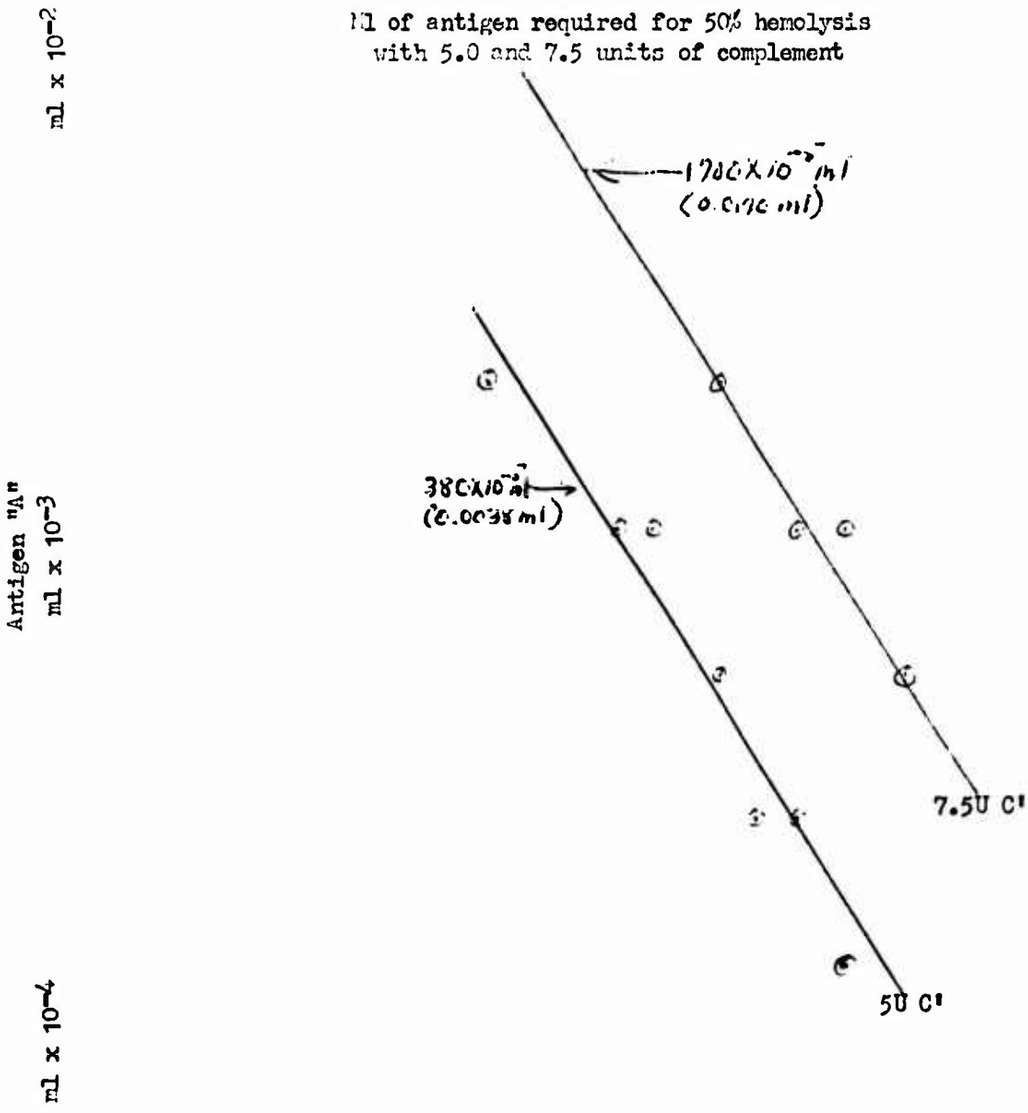
Complement control
1U 20-25
2U 70-75

Cells control
0%

Per cent hemolysis

Figure 3-A

Titration of Antigen "A"



Per cent hemolysis

Figure 3-B

Titration of Antigen "B"

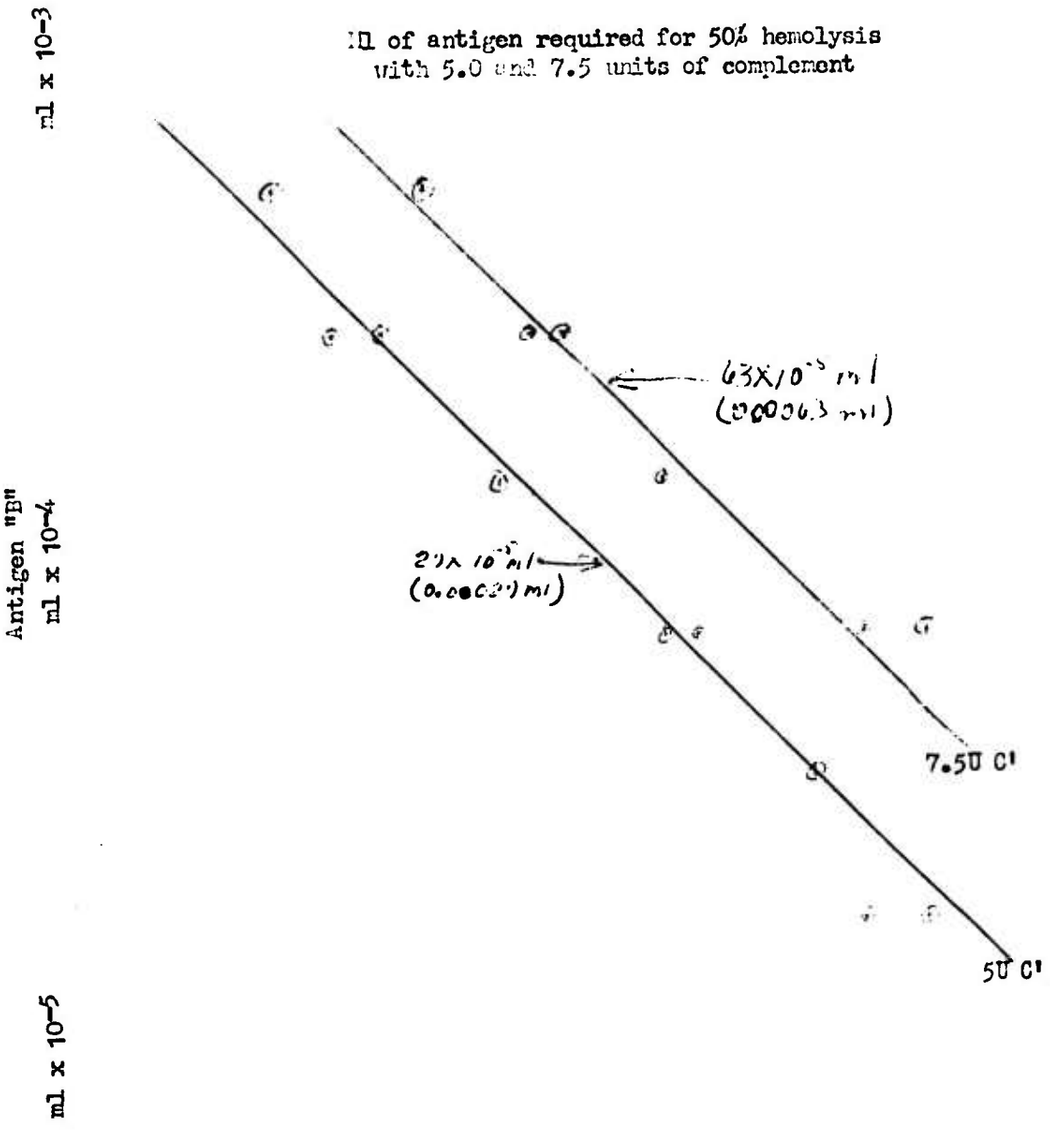


Figure 4

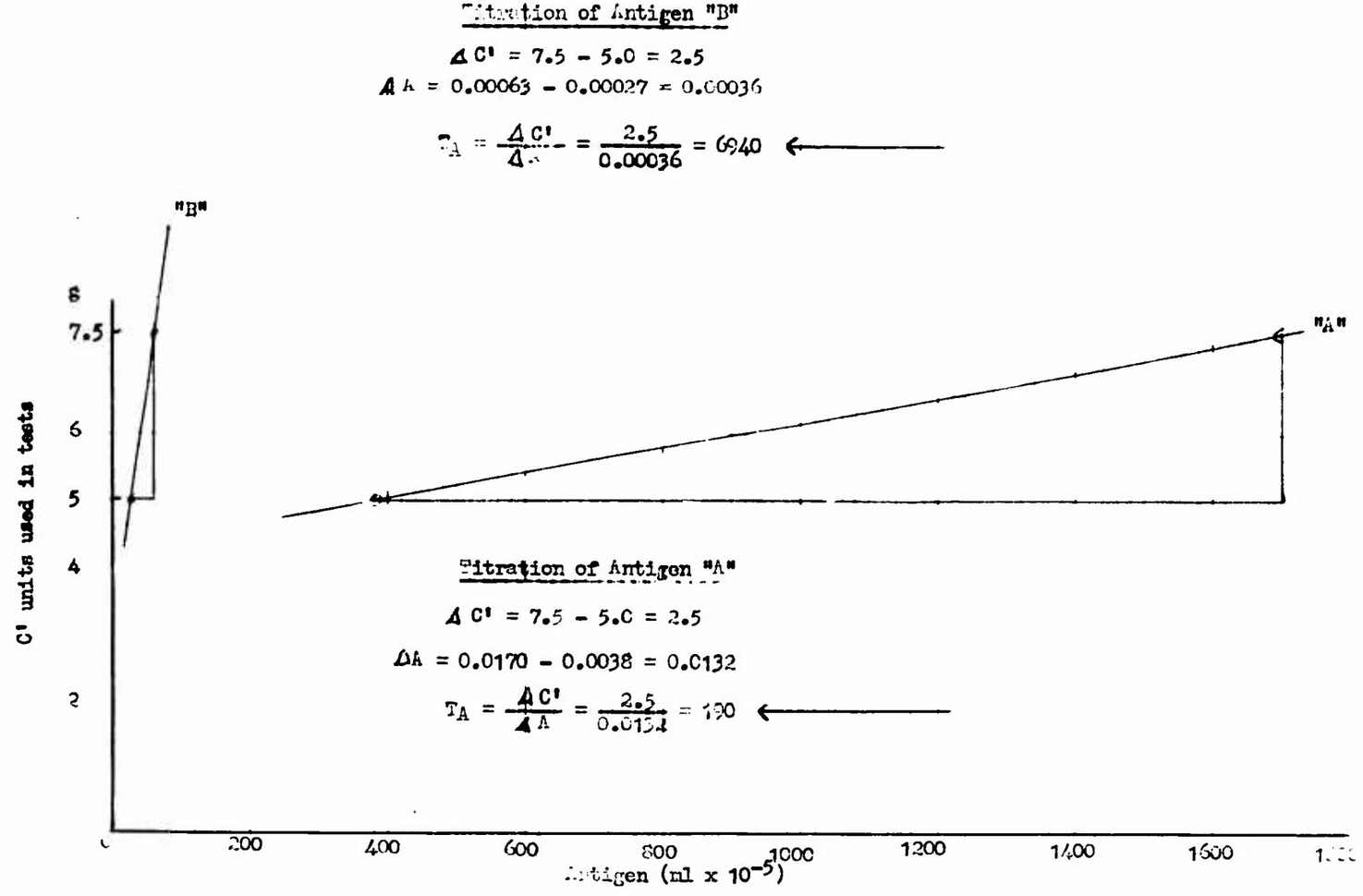

Titration of Antigen "B"
Δ C' = 7.5 - 5.0 = 2.5
Δ A = 0.00063 - 0.00027 = 0.00036
$T_A = \frac{\Delta C'}{\Delta A} = \frac{2.5}{0.00036} = 6940$
"B"
"A"
8
7.5
6
5
4
2
C' units used in tests
200
400
600
800
1000
1200
1400
1600
Antigen (ml x 10^{-5})
Titration of Antigen "A"
Δ C' = 7.5 - 5.0 = 2.5
ΔA = 0.0170 - 0.0038 = 0.0132
$T_A = \frac{\Delta C'}{\Delta A} = \frac{2.5}{0.0132} = 190$

Table 6

Index of Reactive Capacities of Two Different
T. cruzi Antigens Tested With the Same Chagasic Serum

Antigen	M.R.D.[1]	M.R.D. Ratio	Titer[2]		I.R.C.[3] (T_A/T_S)	I.R.C. Ratio
			Antigen (T_A)	Serum (T_S)		
A	1:16	4.0	190	1090	0.17	31.0
B	1:64		6940	1316	5.27	
Relative discrepancies between Antigens "A"&"B"[4]			189.3%	18.9%		

(1) Dilution representing maximally reactive dose of antigen.

(2) Data from Figures 5 & 7.

(3) I.R.C. = Index of reactive capacity.

(4) Relative discrepancy (R.D.) = $\frac{\text{Titer difference}}{\text{Mean titer}} \times 100$

R.D. tolerance due solely to variations within the test procedure $\leq$ 16%.

expresses the specific reactive capacity of the antigen-antibody system independently from the influence of C'. This application is of particular value in determining the stability of a given antigen preparation. Under these conditions, the antigen in question will be repeatedly titrated over a period of time with a lyophilized standard reference antiserum. Obviously, the numerical values of the antigen and serum titers will vary from titration to titration, depending on the stability of the C' on a given day. The question then is whether variations observed on a given day are significant. This problem is obviated by evaluating the results in terms of the T_A/T_S ratio. The ratios will remain constant, provided that the specific reactivity of the antigen has not changed.

The relative discrepancies ($R.D. = \frac{\text{Titer difference}}{\text{Mean titer}} \times 100$) between the antigen and serum titers obtained with two antigen preparations provide further valuable information. R. D. values due to technical variables within the test procedure should not exceed 16%. Thus, R. D. values greater than 16% in the antigen titrations indicate that the two preparations differ quantitatively, and R. D. values greater than 16% in the serum titrations indicate qualitative differences. In the example given in Table 6, it can be seen that antigens "A" and "B" differ significantly from the quantitative standpoint, but the apparent qualitative differences are only slightly greater than those that could be attributed to variables within the test procedure.

Comparison of the M. R. D. (maximum reactive antigen dose) ratio and the I. R. C. ratio of two antigens also provides insight concerning the quantitative and qualitative similarities and differences between two antigen preparations. Table 7 illustrates the results obtained when two dilutions of the same antigen are titrated as unknowns. Antigen "D" is actually a 1:5 dilution of antigen "C". As with the example given in Table 6, the relative discrepancy of the antigen titers was quite high (133.3%), indicating significant quantitative differences between the antigens. However, the relative discrepancy of the serum titers was 0%, suggesting that the antigens qualitatively were identical or very similar. These observations were verified by the fact that the M. R. D. and I. R. C. ratios were identical (5.0) Thus, these antigens ("C" and "D") differed only from the quantitative standpoint. These findings are in contrast to those obtained with antigens "A" and "B" (Table 6) in which the M. R. D. and I. R. C. ratios differed by a factor of 8, indicating that there were qualitative as well as quantitative differences in the antigens.

These procedures currently are being employed by a PAHO Study Group on Chagas' Disease Antigens to provide a basis for selecting a standard T. cruzi antigen for routine serodiagnosis. Although the examples given above are those obtained with T. cruzi antigens, the methods can be applied to any antigen-antibody system that fixes complement.

Table 7

Index of Reactive Capacity with Two Concentrations
of the Same Antigen Tested as Unknowns with the Same Antiserum

Antigen[1]	M.R.D.[2]	M.R.D. Ratio	Titer		I.R.C.[3] (T_A/T_S)	I.R.C Ratio
			Antigen (T_A)	Serum (T_S)		
C	1:100	5.0	1200	100	12.0	5.0
D	1:20		240	100	2.4	
Relative discrepancies between Antigens "C" & "D"[4]			133.3%	0%		

(1) Antigen "D" is a 1:5 dilution of Antigen "C".

(2) Dilution representing maximally reactive dose of antigen.

(3) I.R.C. = Index of reactive capacity.

(4) Relative discrepancy (R.D.) = $\frac{\text{Titer difference}}{\text{Mean titer}} \times 100.$

b. A modified complement fixation procedure for tests on dog sera. It has long been known that dog sera frequently become anticomplementary during the heat inactivation (56°C for 30 min) required to destroy the native complement. Although the reason for this phenomenon is unknown, it constitutes a serious problem when attempts are made to use such sera in diagnostic complement fixation tests. These problems have been overcome by decomplementing the serum by absorption with immune complex (sensitized erythrocyte stromata), thus avoiding the need for heat inactivation. Valid complement fixation tests can be obtained with the unheated decomplemented serum.

Various methods for preparing sheep erythrocyte stromata were investigated and the procedure providing the largest yield was that described by Parpart (2). In brief, washed, packed sheep erythrocytes were lysed with an equal volume of distilled water, and 80 volumes of cold (10°C) CO_2 -saturated distilled water was added to the mixture. The stromata were allowed to sediment and were repeatedly washed (6-10 times) with cold CO_2 -saturated water until the sediment was essentially white. It was observed that after the first 3 washings, addition of 1 volume of 20% NaCl solution to each 20 volumes of stromata suspension significantly increased the yield. However, the 2 final washings were carried out with salt-free CO_2 -saturated water.

Aliquants (0.5 ml) of the washed stromata were transferred to test tubes, and then were sensitized by adding 0.5 ml of 1:600 hemolysin to each, and shaking on a mechanical shaker for 15 min at room temperature. The contents finally were centrifuged, the supernates aspirated, and the sensitized stromata washed once to remove excess hemolysin.

Initially, dog sera were decomplemented by combining 1 volume of serum with 1 volume of sensitized stromata and incubating at 37°C for 30 min. However, it was observed that residual complement was present in all sera treated in this manner and thus it was necessary to reabsorb the sera. Subsequent studies revealed that the efficacy of absorption was improved by increasing the incubation period to 60 minutes. The 60 minute incubation period therefore was adopted as standard procedure and the majority of sera treated in this manner showed no residual complement. The decomplemented dog sera evidenced no anticomplementary activity and were suitable for use in diagnostic complement fixation tests. On the other hand, these decomplemented sera became anticomplementary when heat inactivated at 56°C for 30 min.

c. Stabilization of sheep erythrocytes for use in serologic tests. In the previous report on this Work Unit, it was noted that the storage life of preserved sheep cells could be increased 4-fold by incorporating progesterone with the cells suspension. The greatest stability was observed with cells treated with 0.024 μM of progesterone/50 ml of cells suspension.

During this reporting period, an opportunity to further evaluate

the efficacy of progesterone-stabilized cells was presented. Dr. Robert Anderson, The Johns Hopkins School of Public Health, returned to the Republic of Tchad to resurvey a village that had been included in an epidemiological study two years previously. It was desired to perform assays for circulating complement levels on fresh sera collected from these subjects, but it was doubtful whether the sheep cells would remain satisfactory during the 6-week period required for the survey. The village was located in a remote area and receipt of additional supplies was not feasible. It was suggested that this problem might be resolved by employing progesterone-stabilized cells. Immediately prior to departure, Dr. Anderson was supplied with three 50-ml bottles of untreated sheep blood and three bottles of progesterone-treated blood. The cells from the untreated blood became excessively fragile within 2 weeks after arrival at the village and could not be used for the C' assays. In contrast, the progesterone-treated cells remained stable throughout the entire survey period.

It remains to be determined whether progesterone-treated erythrocytes can be used in indirect hemagglutination (IHA) tests. Initial attempts to utilize progesterone-treated cells in the IHA test for amebiasis were unsuccessful. Apparently the progesterone altered the receptor sites on the cells and rendered them incapable of reacting with the antigen. This must be confirmed in further experiments. In addition, the effect of progesterone-treatment on the ability of the cells to adsorb other antigens will be investigated. Nevertheless, regardless of the outcome of the IHA studies, progesterone-treatment unquestionably extends the storage life of erythrocytes used in tests involving immune hemolysis.

d. Preservation of Treponema pallidum in the frozen state. A recent failure of the air-conditioning system in the animal room resulted in loss of the Nichols-strain T. pallidum used as antigen for the TPI and FTA-ABS tests for syphilis. Fortunately, a satisfactory inoculum was obtained from investigators at the Johns Hopkins University School of Public Health. Nevertheless, this accident emphasized the need to devise a method other than serial passage in rabbits to assure the availability of virulent treponemes. Unfortunately, this organism cannot be cultivated in vitro. A previous report by Hollander and Nell (3) suggested that freezing in glycerine and storage at low temperature might fulfill this requirement. These authors reported that T. pallidum in 15% glycerine, could be stored at -70°C for 2 months without detectable loss of virulence. On the other hand, aliquants of the same suspension deteriorated rapidly when stored at -40 and -15°C.

Initial attempts to confirm these observations were unsuccessful. Treponemes placed in 15% glycerine, rapidly shell-frozen in a CO_2-methyl cellosolve bath, and stored at -70°C rapidly deteriorated, and showed no motility or infectivity after storage for 2 weeks. In an attempt to account for these divergent experiences, review of the procedures employed revealed that U.S.P. grade rather than Reagent grade glycerine inadvertently had been used. The studies were repeated with

Reagent grade glycerine and excellent results were obtained. In investigations currently in progress, the treponemes showed no loss of motility or infectivity during storage for 6 weeks. At the end of this period, 98% of the organisms were motile, and when used for inoculum into a rabbit, firm orchitis appeared in 7 days. This is the period usually required when freshly harvested treponemes are employed for inoculum. These studies are being continued to determine the maximum time the motility and virulence can be maintained under these conditions of storage.

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 detection of a variety of parasitic, bacterial and viral diseases. Although the basic technic for performing the SAFA test usually can be employed regardless of the antigen-antibody system involved, the methods best suited for fixing the antigens to the test discs vary from antigen to antigen.

a. Studies on the use of the SAFA test for serodiagnostic of tuberculosis have been continued. Initial investigations on simian tuberculosis in experimentally infected monkeys revealed that the SAFA test was superior to the standard tuberculin tests for early detection of infection. Not only did the SAFA test show greater specificity than the tuberculin test, it consistently became reactive earlier (14 - 74 days) in infection than did the tuberculin reaction. The SAFA test for tuberculosis also is being routinely used to screen monkeys during their quarantine period. The test has detected some infected animals that would have been overlooked with the tuberculin test.

Investigations on the use of the SAFA test for serodiagnosis of human tuberculosis also were continued. Preliminary studies on selected cases suggest that the procedure may be of value for appraising the efficacy of therapy. In addition, the potential of the SAFA test for mass screening for human tuberculosis is being investigated.

b. Studies on the suitability of the SAFA test for echinococcosis in humans, dogs and sheep were continued. Although results of preliminary studies were encouraging, more extensive evaluation revealed that sheep hydatid fluid probably is not the antigen of choice. Efforts are being made to improve the specificity and sensitivity of the tests by employing soluble antigens obtained from scoleces of E. granulosis.

c. Efforts were made to improve the SAFA test for histoplasmosis by employing soluble antigen from the yeast phase of H. capsulatum. Although the freshly prepared yeast phase antigen reacted well with sera from cases of histoplasmosis, the reactive components of the antigen precipitated during storage. Attempts to improve the stability of

the antigen thus far have been unsuccessful. In view of these findings and the persistent lack of correlation between the SAFA and standard serologic tests with "Histoplasmin" antigen, performance of the SAFA test temporarily has been discontinued until a more satisfactory, stable antigen is available.

2. Studies on improvement of serodiagnostic tests for American trypanosomiasis (Chagas' disease) were continued. This laboratory is participating in an evaluation designed to select a standard antigen for universal use in routine serodiagnosis and for reference in evaluating new antigens and methods.

3. Development and improvement of serologic methods has been the object of continued investigations.

a. Innovations in methodology of the quantitatively standardized complement fixation procedure have provided a new and critical method for objectively evaluating and comparing antigen preparations. The procedure is based on theroretically sound principles and utilizes the linear relationships between the amounts of immune complex formed and the amounts of complement fixed. The methods readily detect antigen differences that would be inapparent in conventional assay procedures and are particularly suited for evaluation of antigen stability.

b. A modified complement fixation procedure for tests on dog sera has been developed. Prior to testing, the dog sera are decomplemented by absorption with immune complex (sensitized erythrocyte stromata). This obviates the need for heat inactivation which causes dog sera to become anticomplementary. The decomplemented dog sera show no anticomplementary activity and are suitable for use in diagnostic complement fixation tests when tested without heat inactivation.

c. Studies on the use of progesterone for stabilizing sheep erythrocytes were continued. Progesterone-treated erythrocytes were used in field studies in Africa and were suitable for performing C' assays during the entire 6-week period required for the evaluation. Untreated cells became unsatisfactory for use soon after arrival in the field. Progesterone-treated cells, however, may not be suitable for use in hemagglutination tests. The latter aspect is under further investigation.

d. Methods for the preservation of T. pallidum in the frozen state have been developed. Treponemes placed in 15% glycerine, rapidly shell-frozen, and stored at -70°C, showed no loss of motility or infectivity after storage for 6 weeks. At the end of this period, 98% of the organisms were motile and when used for inoculum into a rabbit, a firm orchitis developed in 7 days. The studies are being continued to determine the maximum time motility and virulence can be maintained under these conditions.

Project 3A061102B71Q, COMMUNICABLE DISEASE AND IMMUNOLOGY

Task 00, Communicable Disease and Immunology

Work Unit 172, Sero-recognition of microbial infections

Literature Cited

1. References.

(1). Fife, E.H., Kruse, R.H., Toussaint, A.J., and Staab, E.V. Serodiagnosis of simian tuberculosis by soluble antigen fluorescent antibody (SAFA) tests. Lab. Animal Care, in press.

(2). Parpart, A.K. Preparation of red cell membranes. J. Cell. & Comp. Physiol., 19 : 248, 1942.

(3). Hollander, D.H. and Nell, E.E. Improved preservation of Treponema pallidum and other bacteria by freezing with glycerol. Appl. Microbiol., 2 : 164, 1954.

2. Publications.

1. Toussaint, A.J., Fife, E.H., Parlett, R.C., Affronti, L.F., Wright, G.L., Reich, M., and Morse, W.C. A soluble antigen fluorescent antibody test for the serodiagnosis of Mycobacterium tuberculosis infection. Am. J. Clin. Path., 52 : 708, 1969.

PROJECT 3A061102B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 01
Surgery

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6465	70 07 01	DD-DR&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 07 01	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	61102A	3A0[illegible]1102B71R	01	091
b. CONTRIBUTING				
c. [redacted]	CDOG1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Metabolic Problems Associated with Injury and Disease (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 003500 Clinical Medicine 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	Cont	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 70	6	170
FISCAL YEAR CURRENT 71	6	170

19. RESPONSIBLE DOD ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

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NAME: Sleeman, H. K. Ph.D.
NAME: DA

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Trauma; (U) Shock; (U) Hemorrhage; (U) Enzymology; (U) Lipid Metabolism

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

23. (U) 1. To determine factors responsible for morbidity and mortality associated with trauma and shock. 2. To relate metabolic alterations and tissue damage produced by trauma and shock to morbidity and mortality. 3. To evaluate therapeutic agents in the treatment of shock in respect to altered biochemical, hemodynamic and physiological parameters. 4. To investigate metabolic problems associated with oxygen transport.

24. (U) 1. Establish and study animal models for hemorrhagic, endotoxin and bacterial shock, and other problems of military medicine. 2. Study biochemical, histological and physiologic parameters to evaluate altered metabolic processes and tissue damage in trauma and shock. 3. Surgical removal or isolation of tissue to evaluate the role of the tissue in the endotoxin shock syndrome. 4. Evaluate pharmacological doses of synthetic corticosteroids in the treatment of bacterial shock. 5. Determine levels of organic phosphates in erythrocytes and their effect on oxygen transport.

25. (U) 69 06 - 70 06 1. Elevated levels of serum enzymes (isoenzymes) indicated sequential tissue damage in shock which probably was related to morbidity and mortality; quantitative differences were found between endotoxin and hemorrhagic models. 2. Adrenalectomized, spleenectomized, and pancreatic duct ligated dogs treated with endotoxin showed modified tissue damage when compared with endoxotin controls. 3. Pharmacological doses of dexamethasone increased survival in bacterial shock possibly by maintaining hemostasis. 4. Preliminary studies on the control of oxygen transport by organic phosphates were initiated. 5. Models suitable for the study of stress ulcers and pulmonary fat embolism formation are being investigated. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 091, Metabolic problems associated with injury and disease

Investigators.
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H. K. Sleeman, Ph.D.

Description.

The major objective of this work unit is to define and characterize the metabolic and biochemical changes as a consequence of either trauma or disease. Included within this unit are studies concerned with:

1. Effects of endotoxin, hemorrhagic and bacterial shock on tissue injury.

2. Effect of a synthetic cortico steroid, dexamethasone on metabolic, hemodynamic and physiologic factors in experimental peritonitis.

3. Evaluation of the role of organic phosphate on oxygen transport after trauma.

4. Stress ulcer production and its treatment as well as pulmonary lipid embolism and its consequences.

Progress.

1. Effect of endotoxin shock on metabolic processes and tissue damage.

Employing male beagle dogs as the experimental model in endotoxin-induced shock, the effects of splenectomy, pancreatic duct ligation and adrenalectomy on mortality and the progressive metabolic changes following endotoxin administration were studied. Adrenalectomy and splenectomy significantly increased the mortality of these animals to endotoxin shock. Serum lactate, LDH isoenzymes, CPK isoenzymes served as indicators of the progression of tissue damage. In all instances, serum lactate concentrations became elevated. LDH isoenzymes 2 and 3 were the first to become abnormal, followed by isoenzymes 4 and 5. In adrenalectomized and pancreatic duct-ligated animals, LDH isoenzyme 1 was also consistently elevated suggesting progressive damage to heart disease. Serum CPK isoenzyme analysis indicated injury initially to organs other than heart and skeletal muscle, but the latter isoenzymes rose during the later stages of shock. Thus it would appear that both the spleen and the adrenals function to protect the animals against endotoxins. The

isoenzyme results are in accord with previous studies that the lung and the arteries are the organs first involved in endotoxic shock. Progressive damage to other tissues may be related to or result from this initial injury.

2. Effects of Dexamethasone on the metabolic, hemodynamic and physiologic factors in experimental peritonitis.

Studies on the therapeutic effects of dexamethasone on bacterial shock in rats, produced by a standardized peritoneal injection of E. coli bacteria and hemoglobin were continued. The results confirmed that dexamethasone conferred significant protection by reducing mortality even when given 16 hours after the initial infection. In the dexamethasone treated animals, there was significantly lessened hemoconcentration, and it prevented the hypoglycemia seen in the late stages of shock in the untreated controls. There was also a more rapid return to normal of plasma and adrenal corticosterone levels and a more rapid recovery of adrenal ascorbic acid content. These studies indicate a protective action of dexamethasone in maintaining normal vascular permeability and in adrenal function.

3. Evaluation of the role of organic phosphates on oxygen transport in trauma.

Recent reports have shown that the organic phosphate content of erythrocytes, in particular, D-2,3-diphosphoglycerate (2,3-DPG), directly affects the oxygen binding properties of hemoglobin. This compound, as well as other organic phosphate compounds, e.g., adenosine di- and tri-phosphate, profoundly lowers the affinity of hemoglobin for oxygen, thus promoting the discharge of O_2 from its carrier molecule and low pO_2 levels. Since tissue hypoxia is an important contributor to the shock syndrome, studies have been initiated to examine the 2,3-DPG concentrations of whole blood both in man and in experimental animals under a variety of conditions of acute injury, hyporemia and hemorrhagic and bacterial shock.

The fluorometric method of Kiett (Am. J. Med. 41: 762, 1966) for the analysis of 2,3-DPG was employed. The assay measures enzymatically the conversion of 2,3-DPG to 3-phospho glyceraldehyde by following the concomitant conversion of DPNH to DPN. Over 400 samples were analyzed in this manner in support of the Department of Human Studies, Division of Surgery, in their studies of hemorrhagic shock and oxygen toxicity and over 100 samples in support of the Department of Anesthesiology, Division of Surgery in their air-evacuation studies. The results of these analyses are incorporated in the reports of the Division of Surgery.

Preliminary studies aimed at the examination of the role of 2,3-DPG in O_2 transport and tissue hyporemia during bacterial shock in rats and hemorrhagic shock in dogs have also been initiated. In addition to

2,3-DPG, the levels of other organic phosphates in erythrocytes are being examined, since 2,3-DPG, ATP, ADP all act as feedback regulators of red cell glycolysis and hence contribute to erythrocyte function in these stress states.

4. Problems associated with the production and treatment of stress ulcers and pulmonary fat embolisms in experimental animals.

Consultations and technical assistance were provided in these areas of research to the Department of Experimental Surgery, Division of Surgery.

Summary and Conclusions.

The above studies indicate that shock, the spleen and the adrenals play an important role in endotoxin shock. The organs initially affected are probably the lung and the vascular system. Dexamethasone administration significantly decreases the mortality due to bacterial shock by decreasing the hemoconcentration and hypoglycemia that occur. It also apparently protects the adrenal glands against injury. Methodology was developed for the study of the role of erythrocyte 2,3-DPG in O_2 transport during hypoxic states and during acute injury and hemorrhagic shock. Experimental animal models were developed to study these problems in detail.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 091, Metabolic problems associated with injury and disease

Literature Cited.

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2. Sleeman, H. K., Diggs, J. W., Hayes, D. and Hamit, H. F.: Value of antibiotics, corticosteroids, and peritoneal lavage in the treatment of experimental peritonitis. Surg. 66: 1060, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6527	70 07 01	DD-DR&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 07 01	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	61102A	3A061102B71R	01	092
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Procede with security Classification Code)
(U) Clinical evaluation of responses of the body to combat injury (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical Medicine 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 07	CONT	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	6	170
FISCAL YEAR CURRENT 71	6	170

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with security Classification Code)
(U) Infection (U) Stress Ulcer (U) Pulmonary insufficiency (U) Coagulopathies and (U) Metabolic Wasting (U) Physiology at altitudes

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

23. (U) To define the principal physiologic and metabolic derangements of the human body to combat injury. Identification and quantitation of these problems clinically will allow establishement of more significant and comprehensive research programs in the laboratory.

24. (U) Various studies have been and are being undertaken on wounded and sick patients in Vietnam during Air Med evacuation and in Japan in an attempt to quantitate the degree of stress and morbidity following trauma and disease. The role of ACD stored blood in pulmonary insufficiency and the displacement of the oxygen dissociation curve following transfusion of ACD stored blood are being evaluated.

25. (U) 69 07-70 06 Clinical studies during the past year in these areas have produced the following results: (a) Strong implication now exists that stored banked blood, given in large quantities, contributes in large part to the development of pulmonary insufficiency. (b) Infection is usually due to Gram-negative organisms and most commonly associated with massive soft tissue injury or intra-abdominal injury. (c) Stress ulcer may be due to alterations in the composition of gastric mucus. Excellent data from the first prospective study of this subject is currently being analyzed. (d) Intravenous hyperalimentation may significantly reduce metabolic wasting in combat casualties. (e) Coagulopathies are probably most commonly due to qualitative platelet defects. Nevertheless, regardless of mechanism, such coagulopathies in combat casualties are always correctable by sequentially administered units of fresh whole blood. Air medical evacuation revealed a pulmonary dysfunction in wounded and seriously ill soldiers which is inapparent before flight because it is compensated at sea level. During flight the marked hypoxemia which develops, constitute an additional severe stress on the cardiopulmonary system of the patient. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

* Available to contractors upon originator's approval.

DD, FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

pressure of O_2 falls from 149 mm Hg to about 112 mm Hg in flight.

In January 1969, MAJ John N. Henry, MC, of the Division of Surgery at the Walter Reed Army Institute of Research, measured the blood gases of these patients before and during flight from Saigon to Japan and found that a marked hypoxemia developed in flight. The data revealed that seriously ill combat casualties develop a more pronounced hypoxemia in flight than walking wounded or than normal volunteers taken to comparable simulated altitudes at the School of Aerospace Medicine in San Antonio.

c. Approach to the problem: Forty-six combat casualties were studied during six flights from Tan Son Nhut Airport in Saigon to Yokota Air Force Base in Japan. The flying time was about five hours and the altitudes varied from 28,000 to 37,000 feet with cabin pressure equivalent of 3,000 to 7,400 feet. Pre-flight or baseline measurements were done 12 hours prior to departure and were repeated three hours in-flight at crusing altitude and, again, 24 hours post-flight at the 249th General Hospital in Japan. An informed consent was obtained from all patients.

An elaborate methodolgy was employed. All instruments tested worked well at altitude and on the ground. These are the results obtained.

d. Results: The patients are grouped according to the cabin altitude of their particular flight. This analysis does not allow separation of different responses to aeromedical evacuation of the various types of injury; however, it does show the directions and magnitude of the physiological changes as they relate to the cruising altitude. The patients were all previously healthy young men who had a wide variety of injuries.

At cruising altitude these patients sustained a mean fall in pO_2 of 5 mm Hg for every one thousand feet ascended. This change in arterial pO_2 is reflected at the higher altitude by a fall in per cent saturation of arterial hemoglobin and an actual narrowing of the $A\text{-}VO_2$ difference. The oxygen consumption and caridac output which were normal at baseline were significantly increased at altitude. On arrival in Japan this increase in oxygen consumption and caridac output in the higher flights did not return to baseline but persisted until the next day.

Baseline determinations of respiratory parameters revealed low pCO_2 values, above normal minute ventilation and a moderate alkalosis in these patients. Thus, these patients were already hyper-ventilating before the flight. They did not further increase their venilation at altitude.

The lactate, pyruvate ratio and concentration of red cell 2,3 diphosphoglycerate did not change in these patients. We also measured the per cent carboxyhemoglobin which represents the per cent of the patient's oxygen carrying capacity neutralized by his or his buddies' desire to smoke. The smokers all had at least a 5 per cent level and one went up to 14 per cent.

Another group of patients who did as poorly as the seriously injured patients at altitude are the hepatitis patients. From the data being analyzed there are indications to believe that the hepatitis patients have some degree of pulmonary insufficiency which becomes evident at altitude.

e. Discussion of the results: The results are consistent with what is known about man's adaptation to acute exposure to high altitude. However, the striking finding is that these patients react as though they had been exposed to much higher altitudes than they actually were. The basic insult of high altitude is hypoxia and the subsequent physiologic alterations are compensatory mechanisms invoked to maintain adequate tissue oxygenation.

Normal man reacts to altitudes below 10,000 feet primarily with an increase in alveolar ventilation and a small increase in cardiac output, whereas our patients were already hyperventilating prior to flight and were thur unable to utilize this mechanism. The reason for their hyperventilation is probably due to intrapulmonary shunting and other metabolic derangement which are poorly understood. Thus, in order to deliver an adequate supply of oxygen to the tissues these patients increase their cardiac output. This required an increase workload on the heart and the increase in oxygen consumption during flight probably was cardiac. The profound effect of the flight on the patient was evident from the fact that cardiac output and oxygen consumption were both increased on the day after the higher flights; which would suggest that the patients had incurred an oxygen debt.

The implications of the measurements are difficult to assess. Most clinicians would not look favorably upon exposing their ill patients to the degree of stress which we have documented; the fact that so few patients die in flight suggests that this is only exceptionally a lethal insult. The fact that the patients exposed to the higher altitudes did not return towards baseline values 24 hours post flight indicates that the alterations are not minimal.

f. Conclusions: These studies have revealed a pulmonary dysfunction in wounded and seriously ill soldiers which is inapparent before flight because it is compensated at sea level. During flight as the partial pressure of oxygen in the atmosphere falls, these patients develop a

marked hypoxemia. This constitutes an additional severe stress on the cardiopulmonary system of the patient.

g. Recommendations:

(1) A similar study should be undertaken on longer flights back to the continental United States with additional studies of hemodynamic parameters, neuro-endocrine responses and enzyme-isoenzyme determinations to attempt to quantitate the degree of stress and morbidity.

(2) Patients with an arterial oxygen tension of 70 mm Hg should not fly unless supplemental 0_2 is provided during flight.[1]

(3) Smoking should be discontinued on medevac flights.

(4) Volume-cycled respirators should be provided on the C-141 -- the pressure-cycled respirators currently available are incapable of adequately ventilating a patient who is hyperventilating.

(5) The policy of transfusing patients up to an accepted hematocrit in order that he can be evacuated should be modified such that following transfusion they be held in-country for two days before evacuation.

This recommendation stems from the fact that ACD-banked blood is not as good as we believed in the past. Banked blood, apart from the fact that it contains a larger number of debris, is low in 2,3 DPG. This stored blood actually shifts the 0_2 dissociation curve to the left, where it probably is because of the alkalosis already present in these patients. This shift to the left results in an increased affinity of hemoglobin for 0_2. We know that it takes about 24 hours to shift the curve back to normal.

2. Prospective Study of Gastric Juice and Possible Related Factors Following War Wounds in Vietnam: Pathogenesis of Acute Gastrointestinal Erosions.[2]

a. Statement of problem: To study the gastric juice and factors which have been implicated from experimental studies in a prospective manner before onset of acute gastrointestinal hemorrhage.

b. Background: In recent years, acute gastrointestinal ulcers following trauma, burns, shock, and infection have become known as the stress ulcer syndrome. The implication being that hormonal factors elevated in the stress syndrome cause changes in gastric physiology which leads to acute ulceration.

c. Approach to the problem: Fifty wounded men were studied from the time they entered the 24th Evacuation Hospital, Vietnam. Nine subsequently hemorrhaged from acute gastric lesions documented at surgery or autopsy. Beginning on the day of injury the total 24 hour gastric juice was collected by intermittent suction under ice through nasogastric tubes placed at laparotomy. An aliquot was filtered, fractionated, dialyzed, lyophilized and weighed. Another aliquot of gastric juice was titrated with 0.1 N NaOH to pH 2.8 for hydrogen in concentration and to pH 7.4 for total titratable acidity. A sample was used for gastric juice acid phosphatase, initial pH, qualitative gastric juice culture and electrolytes. Arterial and venous blood was drawn daily for determination of pH, pO_2, pCO_2, lactate, glucose, viscosity, hematocrit, acid phosphatase, electrolytes, prothrombin time, partial thromboplastin time and platelet count by standard methods and blood aggregation by the screen filtration technique. Twenty-four hour urine collection was used to measure 17-hydroxycorticosteroid, 17-ketosteroid, vanilmandelic acid, and electrolyte excretion.

d. Results: Gastric juice pH of the first 24 hour collection after injury was 4.48 ± 0.98 in those that developed AGH. The pH decreased after the first day in all groups and by day three was 2.06 ± 0.49 in those that bled. Total titratable acidity of those that had AGH was 58.8 ± 24.7 mEq/l. and those with minor injury 52.5 ± 15.0 mEq/l. during the first day after trauma. Total titratable acidity increased to 70.7 ± 13.9 mEq/l. on day four in those that bled. Hydrogen ion concentration in those that bled was 24.0 ± 14.8 mEq/l. initially and increased to the highest levels on days three and five of 36.5 ± 14.1 mEq/l. and 37.6 ± 21.3 mEq/l. Those with minor injury reached 55.5 ± 6.5 mEq/l. on day four and were always higher than all other groups. Hydrogen ion output was very low and followed the change in total 24 hour gastric volume after trauma. The first day following injury, those that developed AGH had a hydrogen ion output of 12.5 ± 7.6 mEq. The output was greater than all other groups on all days prior to bleeding in those that developed AGH (peak day five - 56.6 ± 31.9 mEq). Total concentration of gastric mucus in the first day following injury was not significantly different in those that had AGH (748.3 ± 209.5 mg/100 ml) and those with minor injury (667.5 ± 209.4 mg/100 ml). The concentration decreased in all groups and was lowest on day three (285.0 ± 20.6 mg/100 ml) in those that bled. This decrease was due to a decrease in the dissolved mucus fraction. Total output of gastric mucus did not parallel the change in gastric volume. There was no significant difference in total output of those that had AGH and those with minor injury as well as all other trauma types in the first day following trauma (AGH-2.5 ± 0.7 gm, Type I-1.72 ± 0.3 gm). However, the total output of gastric mucus increased by the second day after trauma in those that had AGH (6.0 ± 2.3 gm) and remained higher than those with minor injury. This increase was due to an increase in output of the dissolved mucus fraction. Gastric culture showed no growth in three patients that developed AGH, bacteria in three, and fungi in four, and was not related

to acidity or AGH. Gastric juice acid phosphatase was low and not significantly different in the first day following trauma (AGH 0.32 ± 0.14 s.u./ml, Type I-0.49 ± 0.3 s.u./ml) and declined to the low of 0.10 ± 0.03 s.u./ml on day five. Blood acid phosphatase paralleled this decline to a low of 0.17 ± 0.12 s.u./ml on day five.

During the first day after injury, 17-OH excretion was below the normal range and significantly less in those that bled (7.3 ± 1.6 mg/24 hr) than those with minor injury (14.8 ± 0.8 mg/24 hr). Excretion of 17-OH decreased in all groups but especially those that had AGH to a low of 2.0 ± 0.8 mg/24 hr on day five. VMA excretion was not significantly different in the first day following trauma and within the normal range in those that had AGH (7.8 ± 1.3 mg/24 hr). By day five it had decreased to 4.0 ± 1.6 mg/24 hr. Katosteroid excretion paralleled that of 17-OH and decreased in those that had AGH to 8.8 ± 4.6 mg/24 hr.

That PT was higher initially in those that bled and decreased to control values. There was an initial decrease in platelet count in those that bled and this decrease continued to a low of 77,500 ± 35,000 on day six post injury compared to 299,500 ± 49,739 in those that did not bleed. After the first day, in which there was no difference between groups, PTT was increased in those that bled.

There was no significant difference in blood pH, pCO_2, pO_2, sodium, chloride, potassium, glucose, lactate, fibrinogen, blood aggregation, viscosity, hematocrit, or gastric sodium, chloride, and potassium.

Changes related to the amount and type of trauma, as well as in gastric mucus chemistry, will be reported.

e. Discussion of results and conclusions: Postulated theories for the cause of acute gastrointestinal ulcers and hemorrhage from experimental animal models related to the stress syndrome do not seem to be applicable to man following trauma since hormonal parameters are not elevated. That the acid secretion is extremely low in the first 24 hours after trauma and then increases to relatively normal levels seems to be related to onset of acute gastrointestinal hemorrhage.

f. Recommendations: The initiation of the acute ulcer probably occurs within the first 24 hours after trauma and a closer examination of changes in gastric physiology during this important period seems to be in order to define the etiology of the lesion.

3. The Role of Collateral Circulation in Arterial Injuries

a. Statement of the problem: The role of collateral circulation in arterial injuries was studied.[4]

b. Background: Almost sixteen centuries ago Antylus noted that ligation of an artery did not necessarily result in the loss of the parts served by that vessel. We have been impressed by the remarkable nature of collateral circulation to frequently sustain life and function of a limb deprived of its normal blood supply. Extensive damage created by high velocity missiles employed in Vietnam has occasionally required the surgeon to ligate a major vessel or to attempt reconstruction under local conditions which are far from ideal. Infection and secondary disruption or thrombosis have been complications precluding satisfactory arterial repair.

c. Approach to the problem: This study includes follow-up examination of selected patients among the 4500 listed in the Vietnam Vascuarl Registry at the Walter Reed General Hospital. The management of those patients who had undergone ligation or throbmosis of major vessels was analyzed. From this analysis, factors which determined the adequacy of the collateral circulation have been elucidated, particularly, with reference to the axillary, brachial, superficial femoral and popliteal arteries. Using the authors background and experience with the anatomy of the vascular system as obtained from both cadaver and operative dissections, drawings of the major collateral pathways of both the upper and lower were developed. Clinical cases were selected because they examplified the role of collateral circulation in arterial injuries and because the arteriograms from these cases correlated well with the drawings. In addition, methods of assessing collateral artery function, including the use of the plethysmogram and ultrasonic sounding device (Doppler), were also investigated.

d. Results and discussion of results: In the group of selected patients all of whom had failure of the primary vascular repair involving the major arterial supply to the upper or lower extremity, it was noted that despite the failure of the initial procedure, more than half of these patients required no further therapy. When thrombosis was the principal cause of failure of the vascular procedure, only one-third of these patients required re-operation. These facts attest to the remarkable nature of the collateral circulation to preserve not only life but function in a limb deprived of direct arterial flow. With injuries of the extremities, nerve damage rather than arterial occlusion is frequently the limiting factor.

e. Conclusions: The collateral circulation consists of two major components. The first are the pre-existing pathways which are the main distributing branches of large and medium-sized arteris. These serve as stem and re-entry vessels. The second are the multiple muscular branches. These are the vast macromesh of unnamed segmental muscular branches which proliferate and anastomose with one another to serve as mid-zone vessels. The development and function of the collateral circulation is dependent upon several factors. Included among these are the magnitude of the pressure gradient across the collateral vessels, the site of the occlusion, the length of the occlusion, and the functional

capacity of the collateral circulation. Additional factors determining the function and development of the collateral circulation are the presence of secondary thrombosis, both proximal or distal, histopathological changes in the arterial wall, the presence of arterial spasm, and the metabolic demands of the distal part.

f. Recommendations: Continued careful followup of traumatic vascular injuries.

4. Cardiac Injuries in Vietnam

Cardiac injuries are a somewhat unique combat problem in that the serious injuries rarely arrive at the hospital. Those that are seen in the hospital usually present in dire straits and require immediate surgical intervention. A review of 12 cardiac wounds treated in a military evacuation hospital in Vietnam over a period of a year revealed that all but one patient presented with symptoms and signs of cardiac tamponade. Massive hemorrhage from large wounds is not compatible with survival for a sufficient length of time to allow evacuation to a hospital. The only patient that did present in massive hemorrhage was one that had been injured only two blocks from the hospital and arrived in extremis. This, furthermore, was the only patient in the series to die. A survival rate of 90% in cardiac wounds from the present series represents an improvement over a World War II and Korean War experience in which approximately 60% of patients survived. This is primarily attributable to more rapid evacuation and earlier treatment of the patient's injury and cardiac tamponade.

Recommendation: The importance of early open thoracotomy is stressed and repeated thoracocentesis and clinical observation are discouraged in the combat situation.

5. Flechette Wounds of the Heart

Penetrating cardiac injuries pose problems in the clinical management both due to the unique manifestations of cardiac injury and the type of wounding agents. A new type of cardiac wound was seen in evacuation hospitals in Vietnam and is the object of a report. This is the type of missile made up of a small metal dart of flechette contained in a variety of exposed warheads. The flechette has great penetrating power and the three patients seen all had penetration of the myocardium by a flechette and in all three the flechette was still within the confines of the pericardium. One was actually within the myocardial substance itself.

The management of this type of cardiac injury is no different from any penetrating myocardial wound. The defects are usually small and massive blood loss is not a problem but signs and symptoms of tamponade are characteristic.

Recommendation: Immediate thoracotomy is the treatment of choice and removal of the flechette when possible is recommended. In the case when it is intramyocardial and, with all intramyocardial lesions, after a period of stabilization evacuation to a facility with cardiopulmonary bypass capability is recommended.

6. Coronary Artery Disease in Combat Casualties in Vietnam

Post mortem coronary angiography and dissection of hearts from 105 U.S. soldiers killed in Vietnam demonstrated that (1) 47% have some evidence of atherosclerosis, (2) 5% have gross evidence of severe coronary arteriosclerosis, (3) no patient had angiographic evidence of severe coronary narrowing and only one patient with any degree of stenosis was observed. From this data it appears that coronary artery disease in the young age group is presently less than that reported in a similar series in the Korean War.[4]

Recommendation: It is recommended that further work in this area be undertaken to confirm this observation.

7. Surgical Management of Traumatic Injuries of the Colon and Rectum

Management of traumatic injuries of colon and rectum remained a major problem to the combat surgeon in that they are associated with subsequent intra-abdominal sepsis, most frequently of any intra-abdominal visceral injury. Two hundred and twenty patients with colon and rectal injuries in an evacuation hospital in Vietnam were reviewed to find the most effective method of treatment in those most commonly associated with subsequent complication. Right colon lesions should be treated primarily with right colectomy and ileocolic anastomosis except when the associated visceral injuries were present. Transverse left and sigmoid colon lesions are best treated by exteriorization or repair and proximal colostomy. Intraperitoneal rectal injuries should be repaired and protected by a proximal divided colostomy. Extraperitoneal rectal injuries should be repaired, if possible, protected by a proximal divided colostomy and drained widely with or without coccygectomy depending upon the anatomical situation. When possible, distal rectal lavage to remove retained feces should be part of the initial operative procedure. Further definition as to the most efficacious treatment of right colon injuries remains to be determined. It appears that ileostomy represents a conservative form of treatment when other intra-abdominal visceral injuries are present. Furthermore, the principle of wide presacral drainage in extraperitoneal rectal injuries is to be stressed. It is essential in the proper treatment of patients with this injury. The place of distal colonic lavage and the defunctionalized distal loop, in the treatment of rectal injuries need to be defined experimentally.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 092, Clinical evaluation of responses of the body to combat injury

Literature Cited:

References:

1. Luff, Ulrich C.: Physiologic alterations of altitude in Aviation. Clin. Cardiopulm. Physio. 1969.

2. Henry, J. N., Matsumoto, T., Hayes, G. J.: Obstacles in oxygen transport observed during aeromedical evacuation. Presented at the Aerospace Medical Association's 41st Annual Scientific Meeting, 27-30 April 1970, St. Louis, Mo., and also at the 1970 Biennial Army Science Conference, 16 - 19 June 1970, West Point, N. Y., and to be published in their proceedings.

3. Stremple, J. F., Molot, M. D., and McNamara, J. J.: Prospective study of gastric juice and possible related factors following war wounds in Vietnam: Pathogenesis of acute gastrointestinal erosions. Surgical Forum (accepted for publication in 1970 volume).

4. Enos, W. F., Holmes, R. H., and Beyer, I.: Coronary disease among U.S. soldiers killed in action in Korea. JAMA 152:1090, 1953.

Publications:

McNamara, J. J., Molot, M. D., and Stremple, J. F.: Coronary artery disease in combat casualties. Submitted to JAMA

Doty, D. B., and Moseley, R. V.: Reliable Sampling of Arterial Blood. The problems of venous admixture. Surg. Gynec. & Obstet. 10 Feb. 69.

Ganchrow, M. I., Lavenson, G. S., and McNamara, J. J.: Surgical Management of Traumatic Injuries of the Colon and Rectum. Arch Surg, 100:515-520, April 1970

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6461	70 07 01	DD-DR&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
	A. New	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A061102B71R	01	093
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Response of Cells Derived from Subjects Injured by Physical Trauma, Radiation or Infectious Agent Exposure. (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 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 70	6	125
FISCAL YEAR CURRENT 71	5	125

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21. GENERAL USE

Foreign Intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Wound healing; (U) Cytogenetics; (U) Radiobiology (U) Cellular hypersensitivity; (U) Host resistance; (U) Inflammatory response;

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

23. (U) To determine the reaction of cultured white blood cells (lymphocytes) as it pertains to antigenic, infectious, radiobiological, drug, biochemical and physical injuries encountered in military operations.

24. (U) Cytogenetic, histological, radiotracer, and serologic methods are used to examine proliferating lymphocytes cultured in the presence of well characterized stimuli. This lymphocytic response, in a system where non-experimental variables are well controlled, are used to test hypotheses concerning the nature and therapeutic modification of whole body responses.

25. (U) 69 07 - 70 06 - Cytogenetic studies on cultured white blood cells have been completed and appropriate reports submitted on an Advanced Research Project Agency sponsored project involving prolonged Rhesus monkey exposure in a microwave environment. Complementary studies were done on twenty Navy personnel. These studies clearly demonstrated that no cumulative or transitory chromosomal damage to the cells studied, resulted from exposure in this particular microwave environment. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

* Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 093, Response of cells derived from subjects injured by physical trauma, radiation, or infectious agent exposure

Investigators.
Principal: COL Helmuth Sprinz, MC
Associate: LTC Merrill Johnson, MC, MAJ Joshua Miller

Description

This Department was organized in December 1969 and currently studies include wound healing in irradiated and bone marrow transplanted rats and the proliforative and metabolic attributes of cultured canine lymphocytes after renal transplantation.

Summary and Conclusions

Wound healing, which is faster in rats receiving syngeneic marrow infusions after LD 50/30 X-irradiation is unaffected by infusion of syngeneic marrow after LD 50/30 neutron irradiation. The interval between neutron irradiation and wounding was found to importantly affect both survival and wound healing. The antibiotic, gentamycin, used as adjunctive treatment with bone marrow infusion, was found to significently slow wound healing when 50 mgm/kilo was given daily, but not when 5 mgm/kilo was the daily dose.

A plasma factor has been found in dogs receiving unmatched kidneys which depresses lymphocyte proliferation during the period of rejection and for several weeks thereafter.

3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 093, Response of cells derived from subjects injured by physical trauma, radiation, or infectious agent exposure

Literature Cited

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION: DA OB 6462	2. DATE OF SUMMARY: 70 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636

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

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A061102B71R	01	094
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Healing and Repair of Combat Inflicted Injury. (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

017100 Weapons Effects; 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 01	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

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 70	2	65
	CURRENT 71	4	125

19. RESPONSIBLE DOD ORGANIZATION

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TELEPHONE: 202-576-5284

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Bartos, E.M.,PhD; Vail, J.M., PhD

NAME: Werrlein, R.J., M.S.

DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Weapons Energy; (U) Combat Injury; (U) Wound Healing; (U) Tissue Repair; (U) Continuous Flow Analysis.

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

23. (U) Combat inflicted injury is due to the penetration of the energy released by various types of weapons into the body of the soldier. While the type of energy involved may vary, the result is invariably damage and loss of living tissue, which when compatible with life, is followed by tissue regeneration, healing and repair. As the restoration of the soldier's health and combat capability depends on these repair processes it is the objective of this study to uncover the underlying mechanisms and to develop means for increasing their effectiveness.

24. (U) Since living tissue is built from cells and these in turn from molecules, the attainment of this objective requires the analysis and mapping out of the sequence of molecular and cellular events which are initiated by the release of the energy of the weapon and lead first to the development of tissue injury and subsequently to its repair and healing. Model systems using animal material are being developed for this analysis and for designing new means for the enhancement of the repair processes, with clinical applications benefiting the injured soldier following.

25. (U) 70 01-70 06 The analytical systems developed permits fractionation, identification and quantitation of micro to picomoles amounts of diverse cellular constituents with minimal disruption when changing from one type analysis to another. The system is composed of; 1) a liquid chromatograph; 2) a recording spectrophotometer; and 3) a beta particle liquid scintillation counter which can be interconnected to allow the isolation and the assay of desired molecular species in a continous fluid stream. The advantage of this configuration is that it can increase greatly analytical sensitivity thereby reducing the amount of sample necessary for accurate quantitative results while the total amount of time needed for each individual assay and the likelihood of procedural errors are also reduced significantly. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 June 70.

Available to contractors upon originator's approval.

DD FORM 1498, 1 MAR 68 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 094, Healing and repair of combat inflicted injury

Investigators.
Principal: Andre D. Glinos, M.D.
Associate: E.M. Bartos, Ph.D.; J.M. Vail, Ph.D.; R.J. Werrlein, M.S.

Problem and Background

Combat inflicted injury is due to the penetration of the energy released by various types of weapons into the body of the soldier. While the type of energy involved may vary, the result is invariably damage and loss of living tissue, which, when compatible with life, is followed by a set of phenomena known collectively as tissue regeneration, healing and repair. As the restoration of the soldier's health and combat capability depends on these repair processes it is the objective of this study to uncover the underlying mechanisms and to develop means for increasing their effectiveness. Since living tissue is built from cells and these in turn from molecules, the attainment of this objective requires the analysis and mapping out of the sequence of cellular and molecular events which follow tissue injury and lead to its eventual repair and healing. It has been decided to begin this analysis by considering one of the most essential sequences following injury: the proliferation of fibroblasts in the early phase of the wound followed later by arrest of cell growth and elaboration of collagen. The significance of this sequence for the clinical course of wound healing in the injured soldier becomes readily apparent when it is considered that the first process, fibroblast proliferation, is an important determinant of the speed of healing, while the second, elaboration of collagen, is responsible for the development of the tensile strength of the wound. From this point of view the problem of wound healing can then be restated in the form of the following two questions: 1) what is the nature of the changes in the cellular environment which following injury induce fibroblasts to proliferate and later signal them to stop dividing and to begin the synthesis of collagen; and 2) what is the nature of intracellular molecular interactions which occur in response to the extracellular signals and result in the early phase of DNA replication and cell division followed later by the synthesis and secretion of specific macromolecules?

Approach

The reason that a great number of clinical and experimental studies have failed to provide the answer to the two questions defined above lies in

the great complexities of the clinical situation in man and the experimental conditions in the whole animal. To overcome these difficulties by simplifying the experimental situation, a well defined *in vitro* cell culture system shown to parallel the evolution of a wound in the body is used in this department (cf. Glinos, A.D. in *Control of Growth in the Adult Organisms*, edit. by Teir, H., and Rytomaa, T., 41, Academic Press, London, 1967; Glinos, A.D., Vail, J.M., and Bartos, E.M., High Density Suspension Cultures of Mammalian Fibroblasts: Increased Levels of Hydroxyproline in the Presence of Rapidly Declining Oxygen Tensions. In preparation). The system lends itself well to detailed kinetic analysis of the metabolism of nucleic acids, proteins and carbohydrates and of cellular energy production and utilization in conjunction with the determination of associated changes in the environment of the cells. Its unique feature lies in the fact that these determinations may be carried out before, during and after the transition of a population of fibroblasts from a phase characterized by a high rate of cell division and minimum collagen content to a phase charaterized by minimal cell division and high collagen content, these phases thus being the functional counterparts of the early and the late wound. Accordingly, our activities during the first six months of this new work unit have been devoted to acquiring, developing, modifying, adapting, and standardizing the equipment and methods necessary to carry out these analyses.

Results and Discussion

The analytical system developed permits fractionation, identification and quantitation of micro to picomoles amounts of such diverse cellular constituents as amino acids, proteins, nucleotides and carbohydrates with minimum disruption of the apparatus when changing from one type of analysis to another. The system is composed of: 1) a liquid chromatograph; 2) a recording spectrophotometer; and 3) a β-particle liquid scintillation counter which can be interconnected as one unit to allow isolation and assay of desired molecular species in a continous fluid stream. The advantage of such a methodological configuration is that it can increase greatly analytical sensitivity thereby reducing the amount of sample necessary for accurate quantitative results the total amount of time needed for each individual assay and the likelihood of procedural errors are also reduced significantly.

Fractionation of cellular extracts is accomplished primarily by the use of ion-exchange and gel permeation column chromatography although provisions for future use of ion retardation and liquid-liquid column chromatography has been made. Precise delivery of eluting buffer and complete gradient generation are provided by digitally-driven piston pumps capable of operating at pressures exceeding 2000 lb/in^2. This high pressure capability allows the use of small diameter resins resulting in chromatograms produced in less time and in addtion having

greatly increased resolution. Reproducible and accurate applications of samples to the columns is insured by the use of a sample injection loop connected between pump outlet and column inlet.

Difficulties were experienced during the initial operation of the system due to excessive pump leakage occurring under pressure and corrosion of stainless-steel pump cylinders during gradient development because of a "concentration cell" effect. These problems were solved by the use of Viton U cup seals mounted on the pistons and use of true-bore glass cylinders replacing those of stainless steel.

The effluent from the column may be: 1) diverted to a fraction collector for later, individual analysis; 2) sent directly to a detection device able to monitor moving fluid strains or alternatively; 3) mixed with specific reagents streams, reacted and assayed by 1) or 2) above.

Compounds eluted from the column having absorption spectra in either the U.V. or visible range can be measured at two wavelengths by a recording spectrophotometer equipped with a series of flow-through cuvettes, one of which was modified to withstand the operating pressure of the column.

Cellular constituents containing radioactive labels which have been fractionated by column chromatography are assayed, in addition to optical density or other measurements, in a four channel β-liquid scintillation counter capable of measuring two isotopes simultaneously and providing external channel ratios for quench correction. Attempts to measure radioactivity by non-destructive means directly in effluent streams using a commercial flow-through scintillation cell filled with anthracene crystals have not been very successful due to the extremely high flow resistance offered by the cell packing. To overcome this problem, an insoluble scintillator was developed which would exhibit acceptable liquid flow rates at low operating pressures of the column effluent and still retain adequate efficiency in the conversion of radioactive disintegrations to light pulses. A formulation which appears to meet these requirements was produced by dissolving 2,5 diphenyloxazole (PPO) in methyl methylacrylate and performing a suspension polymerization which yielded small (∿100μ), clear spherical beads. When placed in a solution containing radioactivity, these beads emit light at a wavelength compatible with phototubes currently used in liquid scintillation counters. Various formulations of these beads are currently under investigation regarding efficiency, long-term stability and resistance to liquid flow. The most suitable will be used as an insoluble scintillator for our system.

The use of these analytical tools for measuring metabolic kinetics of cells in our culture system is illustrated by the following experiment designed to determine the rate of collagen synthesis by assaying the conversion of proline to hydroxyproline. Logarithmically-growing fibroblasts maintained in suspension were incubated with medium containing 3,4 ^{3}H L-proline. High molecular weight components of the cells were isolated, washed and digested with acid. The hydrolysate was applied to a 1 X 93 cm column containing a cationic exchanging resin (5-80 μ particle diameter) and eluted from the resin by a sodium citrate buffer. Fractions of the column effluent were individually assayed for radioactivity and true disintegations/minute of the samples computed by a data reduction system programmed for liquid scintillation analysis. Fig. 1 shows that small amounts of radioactive bound hydroxyproline can be easily detected and resolved from a relatively large quantity of labelled proline incorporated into a cell and following mathematical integration of the peaks, precise quantitative data concerning rates of collagen synthesis is readily obtainable.

Conclusions and Recommendations

It is concluded that the analytical system under development is ideally suited to the requirements of the kinetic analysis of cellular metabolism in the *in vitro* cell culture model used in our department to study wound healing. Accordingly, its rapid expansion and application to problems of wound healing control is highly recommended.

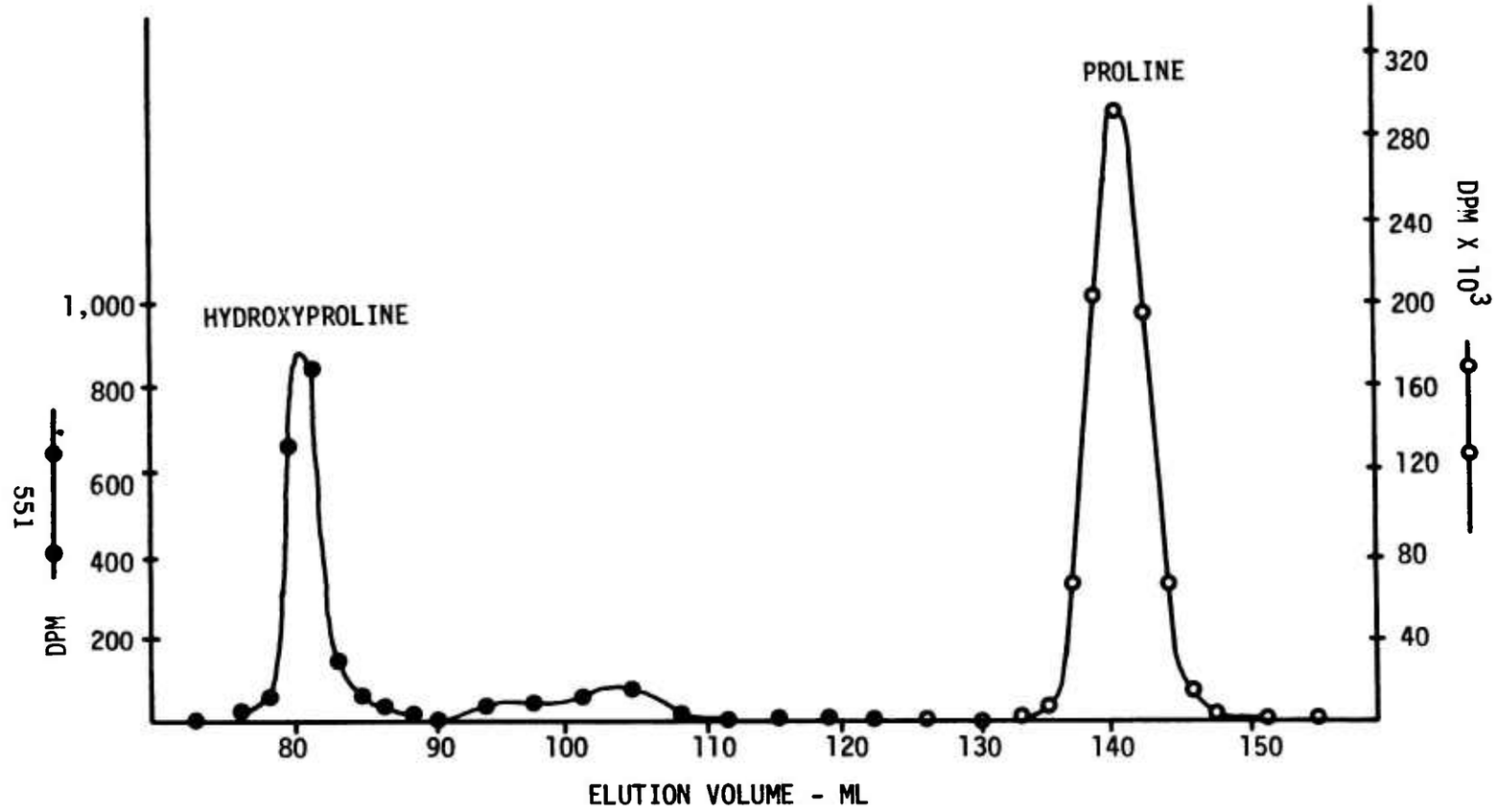

FIGURE 1

Resolution of radioactive hydroxyproline and proline in the protein hydrolysate of a logarithmically growing culture labelled with 3,4, tritiated proline for 2 hours. The system described in the text was used. True desintegrations per minute for hydroxyproline should be read against the left ordinate, those for proline against the right.

3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 094, Healing and repair of combat inflicted injury

Literature Cited.

No publications during the first six months of this new work unit.

PROJECT 3A061102B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 02
Internal Medicine

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6451	70 07 01	DD-DR&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 07 01	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	61102A	3A061102B71R	02	085
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Procede with Security Classification Code)

(U) The Heart Under Abnormal and Pathological Stresses (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012900 Physiology 002400 Bioengineering 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	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 70	9	240
FISCAL YEAR CURRENT 71	11	275

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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

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TELEPHONE: 202-576-5121

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Olsson, LTC, R. A.

NAME: Elliot, Dr. E. C. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

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

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

23. (U) Research is devoted (1) to studies of the hemodynamic and biochemical controls of the normal heart and its coronary circulation under a variety of normal and abnormal stresses, and (2) to studies of the natural history of development of the coronary arterial collateral circulation in the presence of induced coronary insufficiency and of ways to improve such collateral compensation.

24. (U) The major research is based on two experimental models developed for long term study of the coronary normal and coronary collateral circulations in the conscious dog.

25. (U) 69 07 - 70 06. Energy metabolism of the heart continues to be studied at the sarcosome level. Preliminary experiments indicate the feasibility of semiconductor beta ray devices for estimating myocardial blood flow distribution. The sizeable myocardial:blood adenosine gradients together with the rate of adenosine uptake suggest that in situ metabolism is the major mechanism for regulating tissue levels of this vasodilator compound. Reduction of blood flow in a coronary branch to zero in as short a time as two days causes immediate development of a coronary collateral circulation sufficient to prevent ventricular fibrillation and infarction. This large collateral circulation becomes nonfunctional within 24 hours after vessel release but with reocclusion up to 90 days later, it is quickly re-established (within 1 hour) at a high level. In the heart with coronary insufficiency, Xylocaine does not dilate the ischemic coronary bed, the heavily stressed normal coronary bed nor the coronary collateral bed. Nitroglycerine in the presence of well-developed collaterals, dilates the first two beds and to a much less extent the collateral vessels. In the presence of chronic pulmonary hypertension induced by hydraulic occluders, right coronary blood flow is unchanged or decreases. For technical reports see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 69 - 30 Jun 70.

* Continue to complete as applicable.

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Project 3A061102B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 085, The heart under abnormal and pathological stresses

Investigators.
Principal: Donald E. Gregg, Ph.D., M.D.
Associates: LTC Ray A. Olsson, MC; Eric C. Elliot, M.D., Ph.D.; LTC George M. McGranahan, MC: MAJ Jerry A. Snow, MC; Colin M. Bloor, M.D.; Arthur S. Leon, M.D.; Stanislaw Pasyk, M.D.; Bertram Pitt, M.D.; H. L. Green, M.D.; Y. Sugishita, M.D.; Darrell W. Haas, Ph.D.; Edward M. Khouri; Howard S. Lowensohn; Charles E. Cain; Mary K. Gentry; R. Richard Gray; William J. Mitchell.

Description.

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

Progress and Results.

1. Development of Instruments and Methods for Cardiovascular Research.

A prototype implantable, transcutaneous sliding obturator valve of minimal dead space (0.004 ml) has been tested. The original units made of epoxy resin and used in conjunction with a catheter implanted in the circumflex branch of the left coronary artery, functioned well for three and one-half months. A stainless steel version has been constructed.

Lithium-drifted semiconductor devices for beta ray detection are being evaluated for the possible study of myocardial blood flow distribution, coronary insufficiency, and coronary collateral development. Encapsulation techniques have been developed and a cable of adequate flexibility, low noise, low capacitance has been achieved. Two prototype units have been made and have helped to overcome further difficulties. To reduce the noise level further, a special stainless steel implantable coaxial connector has been designed and tested. The use of a bias amplifier has been found to help in further rejecting artifact noises.

2. Metabolic Control of Coronary Blood Flow.

Studies of the metabolic control of coronary blood flow rate have been extended to estimates of the myocardial:blood gradients of

adenosine, inosine, and hypoxanthine in oxygenated and ischemic hearts, studies of the kinetic parameters of adenosine uptake by heart muscle, and to estimates of the tissue levels and kinetic parameters of certain purine-metabolizing enzymes in dog heart. There appear to be sizeable tissue:blood gradients for all three nucleosides, and these decrease only slightly under ischemic conditions, which suggests that washout of adenosine by coronary blood may not be the major determinant of the tissue levels of this vasodilator compound. The uptake of adenosine by beating, oxygenated dog hearts has an apparent Vmax and apparent Km of 2.5 nmole/g/min and 4.8 μM, respectively. In mathematical model studies, these parameters were shown to account for the variation in the rate at which coronary blood flow rate returns to control values during reactive hyperemia following coronary occlusions of different duration. Adenosine uptake was not influenced by pharmacologic concentrations of dipyridamole, ouabain, uridine or deoxyadenosine, but appeared to be noncompetitively inhibited by 6-mercaptopurine riboside. These findings suggest that adenosine uptake may involve active transport, that ouabain-sensitive membrane ATPase is either not involved or is at least not rate-limiting. The studies of myocardial purine metabolizing enzymes indicated that there is no xanthine oxidase in canine heart. Some investigators have reported that allopurinol, a xanthine oxidase inhibitor, enhances survival in experimental canine hemorrhagic shock, and attribute this to an ATP-sparing effect of the drug. The present study suggests that this is an unlikely explanation for the cardiac deterioration which is a prominent feature of this shock model.

3. Myocardial Metabolism.

Continuing studies on site-specific inhibitors of oxidative phosphorylation in heart sarcosomes have been focused on the alkylbiguanides. It has been reported from another laboratory that long chain alkylbiguanides have a dual action on the respiratory chain. On the one hand, they appear to inhibit the phosphorylation reaction which occurs between NAD^+ and flavoprotein, while on the other hand uncoupling electron flow from the phosphorylation which occurs between cytochrome b and cytochrome c_1. We have shown that this conclusion is in error. The single action of the long chain alkylbiguanides is to block the site 2 phosphorylation reaction (thus inhibiting respiration in a tightly coupled system). Any uncoupling action can be accounted for by the introduction of small amounts of aliphatic alcohols and/or amines which can occur as contaminants in the preparation of the corresponding biguanide.

A new submitochondrial particle has been isolated from beef heart sarcosomes. This particle is unique in that it carries out the reduction of endogenous NAD^+ in the presence of exogenous succinate to an equilibrium of 70%-80% reduced. This compares favorably with

the best mitochondrial preparations and is twice the value reported for other submitochondrial particles. Since we are able to prepare a whole spectrum of subsarcosomal particles by a new technique, we have been able to compare their biochemical make-up to various physiological parameters. In preliminary findings, we observe that the cytochrome a/cytochrome b ratio of the particles is related to the extent of reduction of pyridine nucleotide by succinate. Since both cytochrome b and cytochrome a are firmly bound to the sarcosomal membrane, the variations in the ratio of these two pigments indicates a change in the proportion of inner to outer membrane of the intact sarcosome. It is to be hoped that the morphological studies presently being carried out will shed some light on this problem.

4. Experimental Models for Study of the Coronary Circulation.

Recently two experimental models have been developed to study in the trained unanesthetized dog: (1) the regulation of the normal coronary circulation in response to various stresses, and (2) the natural history of the development of the coronary arterial circulation following gradual reduction in circumflex flow to zero (11 to 2 days) and following abrupt reduction of circumflex flow to zero. In the first model, implantation of tubes in the aorta and coronary sinus and of appropriate flowmeters permitted measurements of aortic pressure, myocardial metabolism, cardiac output and blood flow in the major branches of the left coronary artery. In the second model, for the study of coronary insufficiency and the coronary collateral circulation, in addition to the preceding, placement was made on the circumflex branch of the left coronary artery of an externally adjustable hydraulic occluder for gradual or abrupt reduction of coronary flow to zero, and a tube was inserted into the artery to permit measurement of two collateral indices, xenon clearance and residual coronary pressure following temporary and/or permanent closure of the circumflex branch. A third collateral index has been added, namely, measurement of collateral flow arising from the nonoccluded coronary arteries and appearing in the circumflex branch distal to its point of closure. These models have been used extensively in studies of the normal heart and of the heart with an insufficient coronary circulation.

5. Regulation of the Coronary Collateral Circulation.

a. Progressive Circumflex Occlusion. Progressive occlusion of the circumflex branch of the left main coronary artery was carried out in chronic dogs by means of an adjustable Hg constrictor. This permitted constricting the vessel over a precisely known interval which was not possible with the previous ameroid experiments (Circulation Res. 22:237, 1968). A series of six such experiments has been completed with progressive closure of the circumflex branch

being completed within 2 to 11 days. A surprising finding has been that the collateral circulation can increase in 2 to 5 days so that (1) the xenon collateral flow reaches the control xenon blood flow level in the unoccluded circumflex, (2) peripheral coronary pressure rises to within 15 to 25 mm Hg of the aortic pressure, (3) the heart can escape infarction as evidenced by electrocardiogram and post-mortem examination, and (4) terminal retrograde flows are elevated to levels above expected levels for controls. Although this evidence is convincing, the number of experiments is still small. Therefore, these initial findings should be cautiously interpreted.

b. Regression and Re-establishment of the Coronary Collateral Circulation Following Coronary Artery Release and Reocclusion. In the 1969 annual report, preliminary results were given on the study of the function of coronary collateral channels following release of an occlusion and subsequent reocclusions. Four more experiments have been done in which the blood flow in the circumflex branch of the left coronary artery was gradually (2-3 days) reduced to zero in two dogs, or abruptly reduced to zero in the remaining two. Coronary collaterals developed from the other major coronary branches to supply the myocardial bed penalized by the occlusion, as evidenced by an increase in left descending coronary flow, residual circumflex pressure, and retrograde circumflex flow. There was little or no myocardial infarction in the gradual occlusion preparation, while abrupt occlusion resulted in massive myocardial damage, at times transmural. In both cases, following the development of the collateral circulation, the occlusion was removed. Total occlusion had been maintained for a period as short as 24 hours in one dog. The collateral indices returned to control levels within 24 hours. The circumflex was then reoccluded abruptly at various lengths of time from its release. Up to three reocclusions were performed in the same preparation, at up to 30 day intervals. These always resulted in the collateral indices increasing within one hour to levels obtained only after days following the first occlusion. These experiments suggest that following removal of a flow obstruction, coronary collateral channels quickly become nonfunctional, but up to 90 days following release of the first occlusion remain ready to resupply the myocardium should a subsequent occlusion occur.

6. Effect of Drugs in the Normal Heart and in the Coronary Insufficient Heart.

Preliminary tests were made of the effects of the coronary vasodilator, nitroglycerine, and of the effect of the local anesthetic, Xylocaine, on the coronary circumflex collateral indices during the control state, during vessel constriction, and after complete occlusion.

a. Xylocaine. Xylocaine in doses of 25-100 mg intravenously had no effect on the normal coronary circulation prior to circumflex branch occlusion. Intracoronary injections (1-4 mg) and intravenous injections (8-160 mg) of the drug also had no effect during circumflex occlusion. The larger intravenous doses made the dogs sleepy but did not affect systemic or coronary dynamics or the prevailing cardiac rhythm.

b. Nitroglycerine. Considerable controversy exists as to the mechanism by which nitroglycerine improves the heart with coronary insufficiency, and especially whether its beneficial effect is associated with an increased coronary flow. To test the mechanism of its action, the drug was given in amounts that did not significantly depress systemic dynamics. Nitroglycerine could affect three vascular beds -- the ischemic circumflex bed, the circumflex collateral bed, and the heavily stressed normal descending bed. The effect on the descending bed could be observed from changes in its flow. The crudeness and inadequacy of present methodologies made it difficult to separate out the drug effects on the other two vascular beds. However, a start has been made from consideration of the directional trends of the collateral indices that followed the two basically different routes of entrance for the drug, namely (1) entrance by direct injection into the circumflex branch, (2) entrance into the circumflex bed from the descending bed following sublingual or iv administration, or by direct injection into the descending branch. If the drug passes through all three vascular beds that it might affect, and dominant dilatation of the collateral bed occurs, then this should increase residual circumflex pressure, circumflex collateral flow, and retrograde circumflex flow. If the drug is made to pass through only the ischemic bed (from its artificial injection into the circumflex branch), and dominant dilatation occurs, it should decrease residual pressure, increase circumflex collateral flow, and not affect retrograde flow. If both routes of nitroglycerine administration give the latter responses, the drug would appear to cause only ischemic bed dilatation. Different combinations of these responses would occur if both beds were affected. Although experimentation is incomplete, some general trends have been established.

Following abrupt or gradual circumflex occlusion, nitroglycerine by either entrance route increases largely descending blood flow and with only moderate or no systemic changes. The increased flow continues as long as the drug is administered. During abrupt occlusion and in the early hours of gradual circumflex closure, especially if the latter is relatively rapid, nitroglycerine by both entrance routes has no effect on the collateral indices. However, after collateral indices are well-developed, nitroglycerine by either route of entry decreases coronary residual pressure but increases mildly

retrograde flow. Thus, when collaterals are well-developed in left circumflex coronary insufficiency, nitroglycerine dilates all three vascular beds, especially the heavily stressed normal descending bed and the ischemic bed, and to a lesser extent the collateral bed.

Although an earlier report from this laboratory (Federation Proc. 28:780, 1969) had indicated a decrease in the peripheral coronary pulse pressure following intracoronary nitroglycerine and a marked rise in peripheral coronary pressure after intracoronary or intravenous administration of Lidocaine, this could not be substantiated in further experiments. It is now realized that the instance found of decreased peripheral coronary pulse pressure with nitroglycerine was due to technical difficulties, and it is now clear that the rise in peripheral coronary pressure following Lidocaine administration could not be separated from the spontaneous transient improvement in systemic dynamic condition that occurred simultaneously.

7. Effects of Pulmonary Artery Hypertension on Right Coronary Artery Blood Flow.

An experimental model has been devised for study of the effect of elevated pulmonary artery resistance on right coronary artery blood flow in the unanesthetized dog. Successive right and left thoracotomies under pentobarbital anesthesia were performed about two weeks apart. In the first stage, implantations were made of 1.5 cm diameter plastic hydraulic occluding cuffs about the right and left pulmonary arteries, and of a pressure tube in the infundibular region of the right ventricle. In the second stage, a pressure tube was placed in the central aorta and electromagnetic flowmeters were placed on the right coronary artery and ascending aorta. In each stage, for cineangiographic purposes, two "figure 8" stainless steel markers were placed at strategic spots on the right epicardium. After recovery from surgery, and recording for a number of days of control data, right ventricular pressure was elevated by hydraulic inflation of the pulmonary artery cuffs. Studies included short-term inflations with subsequent releases of constriction, and long-term (3 weeks) chronic constriction.

Two dogs have been studied. Initially, right coronary blood flow increases from control values of 12-16 ml/min to values of 17-27 ml/min when peak systolic right ventricular blood pressure is raised from initial values of 34-60 mm Hg to 74-90 mm Hg, and right ventricular end-diastolic pressure rises from 1.5-6.5 mm Hg to 8.0-10.5 mm Hg. The degree of right coronary flow augmentation appears to be greater with abrupt increases in pulmonary resistance. With continued maintenance of elevated pulmonary resistance, right coronary flow diminishes somewhat and toward the end of the three week period this flow is down to or less than the control flow. Further elevation of right ventricular pressure at this time suppresses further right coronary flow.

Conclusions

There are sizeable myocardial:blood adenosine gradients in canine heart which, together with quantitative studies of adenosine uptake, suggest that in situ metabolism is the major mechanism for regulating tissue levels of this vasodilator compound. Xanthine oxidase is not found in dog heart so that the beneficial effects of allopurinol in hemorrhagic shock cannot be due to the postulated ATP-sparing effect of this drug on the heart.

The long chain alkylbiguanides have a single action -- to block the site 2 phosphorylation reaction. A new submitochondrial particle has been isolated which is similar to the reduction of endogenous pyridine nucleotide by succinate.

When blood flow in the circumflex branch of the left coronary artery is reduced to zero in as short a time as two days, development of the coronary collateral circulation is sufficiently rapid to prevent ventricular fibrillation and myocardial infarction.

A large collateral circulation develops within 2 to 3 days following abrupt or gradual (2 day) coronary artery closure, but this becomes nonfunctional within 24 hours after the occlusion is removed. Following reocclusion up to 90 days later, this protective circulation is quickly re-established (within 1 hour or less) and without changes in systemic dynamics or cardiac function.

In the heart with coronary insufficiency, vasodilator drugs could affect the ischemic coronary bed, the heavily stressed normal coronary bed, and the collateral bed. Xylocaine does not dilate these beds. Nitroglycerine, in the presence of well-developed collaterals, dilates the first two beds and to a much less extent the collateral vessels.

An unanesthetized canine model has been devised to study the effect of chronic elevation of pulmonary artery blood pressure on right coronary blood flow. Right coronary flow initially increases and then falls to or below the control level.

Project 3A061102B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 085, The heart under abnormal and pathological stresses

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION: DA 0A 6450	2. DATE OF SUMMARY: 70 07 01		REPORT CONTROL SYMBOL: DD-DR&E(AR)636
3. DATE PREV SUM'RY: 69 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	61102A	3A061102B71R		02	086		
b. CONTRIBUTING							
c. [redacted]	CDOG 1412A(2)						

11. TITLE (Precede with Security Classification Code)

(U) Military Hematology (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

00800 Life Support 002600 Biology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
58 05	CONT	DA			C. In-House

17. CONTRACT/GRANT: NA

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b. NUMBER:

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FISCAL YEAR	PRECEDING 70	7	130
	CURRENT 71	7	130

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21. GENERAL USE

Foreign Intelligence Not Considered

DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Coagulation; (U) Malaria; (U) Blood; (U) Blood Transfusion; (U) Anemia

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

23. (U) Documentation of the etiology of hematologic disorders in soldiers and the investigation of those which occur because of their occupation. Improvement of the Army Transfusion Service.

24. (U) Studies of hematologic abnormalities produced by chemicals, drugs and diseases encountered primarily in military populations and in natives of geographic areas of potential military operations. Studies of blood, blood products and blood substitutes used for the treatment of casualties and the prevention and diagnosis of diseases in soldiers.

25. (U) 69 07 - 70 06 Quantification of the methemoglobinemia induced by various chemoprophylactic antimalarial drugs with investigation of the etiology for variable host susceptibility, the decrement in performance caused by low levels of methemoglobinemia and the importance of drug degradation produced by storage. Studies of the etiology of hemolysis in malaria to establish the cause for relatively prof and anemia with mild parasitemia. Investigation of the coagulation defect caused by hperbaric and hypobaric environments to prevent decrements in performance observed in diverse and at high altitudes. Studies of blood additives which will minimize the reduced capability of banked red blood cells to transport oxygen to tissues. Subspecialty training of medical and laboratory officers in hematology. Establishment and maintenance of standards for distribution to military laboratories and to act as a reference laboratory for clinical and area laboratories and contractual agencies. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

* Available to contractors upon originator's approval.

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 086, Military Hematology

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Description.

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

Progress and Results.

Central in the pathogenesis of many blood diseases, including malaria, is hemolysis. Investigation has continued into the mechanisms by which diseased as well as normally effete red cells are recognized in the body and destroyed. Reduction of the negative surface charge may be the most important biologic signal. Red cell surface charge is mainly dependent on its N-acetyl-neuraminic acid (NANA) content. Reduction of surface charge can be demonstrated in cells which have been treated with neuraminidase (RDE), an enzyme which selectively destroys NANA. Aged red blood cells were tagged with ^{55}Fe and young cells with ^{59}Fe; both were treated with RDE and injected intravenously into rats. The disappearance curves of these cells indicate that the reduction of surface charge results in early recognition and destruction by the reticuloendothelial system. Even in this experimental system, however, the older cells were more rapidly destroyed. Data suggest this is due to the initially higher NANA content of the young cells. Work is underway to rule out a suggestion that transferrin on the reticulocytes may protect them from the action of RDE.

Clinical studies of the effect of chemoprophylactic doses of chloroquine and primaquine in G6PD deficient Caucasians are continuing. Hereditary G6PD deficiency of red blood cells is proving to be more complicated than just a reduction in quantity of the enzyme. An increasing number of qualitative differences in various parameters of

enzyme function is being demonstrated. In recent months three Caucasian soldiers have been studied, each demonstrating a new variant of enzyme deficiency. Comparisons with the typical G6PD deficiency in Negroes are being studied.

A number of soldiers developing methemoglobinemia while on chemoprophylaxis in Vietnam have been referred to Walter Reed for study. Four of these have had normal assays of red cell NADH methemoglobin reductase despite convincing evidence that they had methemoglobinemia at the referring installations. Among the questions arising in this connection are: can methemoglobin reductase be induced following an oxidative stress? can deficiencies of enzymes other than methemoglobin reductase unexpectedly explain the findings in such individuals? There is, further, a need to assess the incidence of deficiency of methemoglobin reductase in the normal population. Automated methods have been developed at the WRAIR for this assay, and thus far 600 soldiers and about 2,500 civilians have been assayed. Pending final collation of the data, the incidence of this enzyme deficiency appears to be between 0.5 - 1.0 per cent.

Tropical Canine Pancytopenia (Tracker Dog Disease) has become a serious veterinary problem in Vietnam. Investigation into the hematological aspects has continued in the Coagulation Laboratory. Clumping of red cells, white cells and platelets on peripheral blood smears suggested the presence of agglutinins. Such an agglutinin has been definitely demonstrated against the platelets but dog blood group incompatibilities in the test system have not yet been ruled out. Serial bone marrow examinations during experimentally induced infections have not yet demonstrated hypoplasia or maturation arrest, which supports the present belief that pancytopenia results from peripheral destruction. Coagulation assays in serial studies thus far reveal elevation of fibrinogen and factors V, VIII and IX and no evidence of disseminated intravascular coagulation.

Immunoglobulin studies have led to several observations with apparently significant research and clinical implications:

a. In a four year old child with repeated infections there was a markedly decreased plasma gamma G globulin level and reversal of the normal kappa/lambda light chain ratio. The latter was demonstrated to be due to defective synthesis of kappa chains. Family studies indicated a genetic basis for this light chain abnormality. Thus a kind of "thalassemia" of the immunoglobulins has been defined for the first time.

b. A 46 year old Caucasian woman was also found to have a dysgammaglobulinemia of a type (II) previously reported but associated with factor XI deficiency and antibodies directed against gamma A globulin. Family studies revealed one sister, who had the same dysgammaglobulinemia.

c. A survey was carried out using agar gel electrophoresis to determine the incidence of monoclonal gamopathy in an 18 to 25 year age group. Sera from three thousand donors were examined and in only one was a monoclonal gamopathy found. This incidence is considerably less than reported from an older age population, implying an age dependent increase. In the serum of one other of these same donors, hypogammaglobulinemia was detected.

d. Clinical evaluation of a married couple revealed husband and wife to be affected simultaneously with multiple myeloma. Such a coincidence of a relatively rare disorder is some support of the theory citing an environmental basis for this disease. Both patients had a serum monoclonal gamma G globulin, but the light chains of the two proteins were different.

e. A low molecular weight protein, B_2-microglobulin, is a trace constituent of normal human serum and urine. Studies in rats indicated that B_2-microglobulin is a protein which is metabolized in large part by the kidney (1).

The Coagulation Laboratory has continuing activity in three areas: clinical research stemming from major clinical support in all areas of coagulation procedures for Walter Reed General Hospital and other military medical facilities; evaluating conformance of coagulation reagents submitted on bid for government purchase; and basic research. Reported observations are as follows:

a. A 48 year old Caucasian woman spontaneously developed antibodies in high titre to her own factor VIII, which led to several episodes of life-threatening hemorrhage. As is the usual experience, many attempted modes of therapy were to no avail until - borrowing from organ transplantation experience - two short courses of massive doses of an alkalating agent (cytoxan) intravenously were temporally associated with decline and eventual disappearance of the antibody. The antibody producing cells were "primed" by intravenous administration of concentrated factor VIII 36 hours before the time of the first dose of Cytoxan in each course. Since such antibodies may disappear spontaneously, as they come, cause and effect with this treatment were not established. Nevertheless, the encouraging experience may prove beneficial in other such cases.

b. A 10 year old Negro female with severe factor X deficiency has been studied, a new therapeutic agent utilized and observations concerning distribution, half life, etc., of factor X reported (2).

c. An 8 month old Caucasian male with combined deficiencies of factor VIII and IX was studied, and later his family also. The data support previous speculation that factor IX production may be controlled by a gene distinct from that involved in classical factor IX deficiency.

d. Disseminated intravascular coagulation (DIC) associated with pneumococcal sepsis was observed in an elderly Caucasian woman. Pneumococcal sepsis is not common and has in almost all reported cases been associated with asplenia. Thus, this case was of additional interest because at autopsy a 35 gram spleen was found and the atrophy was apparently due to Thorotrast, which had been administered years earlier. This is the first reported clinical corollary to the established experimental model in which Thorotrast is injected into animals to block the reticuloendothelial system and facilitate the development of DIC.

e. Hemorrhage is a prominent manifestation of scurvy. The basis for this hemorrhage was sought in scorbutic guinea pigs. Evidence for abnormal blood coagulation in addition to increased capillary fragility was obtained (3).

f. Little attention has been directed to the potential procoagulant and anticoagulant capabilities of heavy metals which are related to calcium on the periodic table. Studies conducted thus far indicate that strontium, magnesium, cobalt and manganese are capable of substituting for calcium and causing coagulation of citrated plasma. Higher concentrations of these metals inhibit coagulation, as do those of calcium. A variety of other heavy metals also inhibit coagulation entirely, often at low concentrations. Such information is potentially useful in standardization of clinical laboratory techniques and may have clinical implications.

g. Companion studies of the role of the chloride ion in coagulation were performed. The chloride ion is in fact a weak inhibitor of coagulation in the prothrombin time, partial thromboplastin time, and whole blood clotting time tests. Studies are planned to establish whether chloride ions have significant effects in more physiologic settings routinely used in the coagulation laboratory.

h. Animal studies intended to aid in the elucidation of the hemorrhage and thrombosis noted in children with congenital heart disease and severe erythrocytosis have been completed. Rhesus monkeys made erythremic in hypobaric chambers developed coagulation abnormalities thought due to increased blood viscosity (4).

1. Disseminated intravascular coagulation (DIC) has been demonstrated as a pathogenic factor in many diseases. The well known hemorrhagic manifestations of yellow fever therefore prompted investigation in animals. Monkeys infected with yellow fever virus developed coagulation changes compatible with DIC. Heparin ameliorated the coagulation abnormalities but did not prolong life (5).

Study of platelet kinetics in various diseases and evaluation of the platelet preservation and transfusion program have been undertaken during the past year. Reproducibility and validity of the methods used have been established in eight normal studies. A study of a group of patients with splenomegaly, before and after splenectomy, is nearing completion. Platelet counts in these individuals have been normal or low. When the count is normal, the data indicate that its maintenance is by dint of 2 to 3 times normal platelet production. Life span of the platelets is normal. The main defect is platelet pooling in the enlarged spleen. Removal of the spleen has been followed by return to normal of platelet production rate, when elevated.

Conclusions.

1. Further study is supporting the hypothesis that reduced negative charge is an important factor in recognition and destruction in vivo of diseased and aged red blood cells.

2. Study of three Caucasian soldiers with G6PD deficiency lends some support to the prevalent belief that red cell destruction after an oxidant stress in deficient Caucasians is more severe than in Negroes. However, in these individuals the effect was also self limited and successive doses of oxidant drug caused less and less hemolysis.

3. In the dog disease Tropical Canine Pancytopenia, evidence is accumulating that the low cell counts are due to peripheral destruction rather than bone marrow failure per se. There seems to be no disseminated intravascular coagulation.

4. Clinical immunoglublin and coagulation studies have led to several observations which appear to have significant research and clinical implications in terms of both the etiology and treatment of a number of diseases.

5. Study of platelet kinetics in a group of patients with splenomegaly indicates that a major reason for demonstrated increased production rates of platelets and normal or lowered circulating platelet counts is splenic pooling. Many or all of these sequestered platelets are normal and in equilibrium with the circulating pool.

As reported in the past a method for "in vitro" cultivation of malaria parasites throughout their intraerythrocytic life cycle was developed here. Progress on the application of this method of investigation of the biochemistry of the parasite and the effect of antimalarial drugs has also been reported in its developmental stages. The culmination in published form of the latest work of the primary investigator before he took another appointment is listed in the bibliography.

Recommendations.

Further studies of the mechanism of hemolysis of malarious red blood cells are required because patients without concommitant hemolysis are now ill. The importance of methemoglobinemia induced by antimalarial chemoprophylactic drugs has not been investigated. The demonstration of a genetic abnormality in a significant proportion of the population which predisposes them to methemoglobinemia has been documented. The significance of this disorder in performance requires further study. The etiology of bleeding in war dogs with a disease commonplace among animals in Vietnam requires additional investigation to establish its etiology and definition of its pathophysiology.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 086, Military Hematology

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION: DA OA 6452	2. DATE OF SUMMARY: 70 07 01	REPORT CONTROL SYMBOL: DD-DR&E(AR)636
3. DATE PREV SUM'RY: 69 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. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A061102B71R	02	087
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

10. LEVEL OF SUM: A. WORK UNIT

11. TITLE (Procede with security Classification Code): (U) GASTROINTESTINAL DISEASE (09)

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

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: NA EXPIRATION:
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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: 70	8	80
FISCAL YEAR — CURRENT: 71	9	100

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21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Diarrheal Disease; (U) Intestinal Blood Flow; (U) Intestinal Reactivity; (U) Intestinal Absorption; (U) Intestinal Enzymes

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

23. (U) The incidence of diarrheal disease in combat troops has been shown to detrimentally effect field operations. This department's mission relates to the pathophysiology of diarrheal disease in order to better approach treatment and control of these conditions in the field.

24. (U) A team is studying the etiology of the prevalent diarrheal diseases in Vietnam. Animal models are being used to assess the effect of diarrheal disease on intestinal neuromuscular mechanisms, absorption and secretion, blood flow and enzyme activity.

25. (U) 69 07 - 70 06. A large part of the eighty percent of diarrheas of unknown etiology in Vietnam appear to be due to pathogenic E. coli and a hybrid E. coli/Shigella organism. Diarrheal disease is associated with a loss of inhibitory and an increase of excitatory nerve/muscle mechanisms in the intestine. The fluid lost in these diseases results from a reversal of water and salt absorption to secretion; this condition is benefited by providing the gut with a solution containing increased glucose. A new study of intestinal blood flow has been inaugerated to evaluate the effect of enteric disease on the interaction of blood flow with motility and absorption. Disaccharidase enzyme activity is reduced in several malabsorption diarrheas. These animal studies have provided specific information concerning absorption and motility changes in diarrhea; a corresponding study of diarrhea in the human is currently being organized. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1969 - 30 June 1970.

* A valid No. or contractor name or initials or approval.

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 087, Gastrointestinal disease

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Description.

The research activity in the Department of Gastroenterology remains oriented on the pathophysiology of diarrheal disease. The multidisciplined approach employed within the department has been expanded to include an etiological study of diarrheal disease in Vietnam, a study of mesenteric blood flow, and a study of mitochondrial metabolism. The results of these studies are to be correlated with the previously established departmental studies on intestinal salt and water transport, neuromuscular interactions, mucosal disaccharidase activity and microvascular architecture in order to further evaluate the effect of diarrheal disease on intestinal function.

The study of the pathogenesis of gastric stress ulcer that was started last year has continued. The emphasis of the study, at this time, has been to describe specific changes in blood chemistry and correlate them with the applied stress and ulcer formation.

Progress and Results.

1. Pathophysiology of Diarrheal Disease.

a. Intestinal Transport in Diarrheal Disease. Intestinal transport studies have been continued on both normal and Salmonella typhimurium infected rats by using an in vivo perfusion method. These studies are being done in an attempt to elucidate the normal mechanism of water and salt transport and reveal the manner in which it is altered during diarrhea. A study of net intestinal water and salt flux in experimental Salmonella infection has been accepted for publication. In a separate study, it has been shown that despite glucose being capable of stimulating enough sodium and water absorption in diarrheic animals to offset their ileal secretion, there is interference with glucose absorption in all of the animals. This observation suggests that oral therapy of such diarrheal disease states in man may not be as efficacious as it is in cholera. However, this possible route for therapy is being investigated extensively. The relationship between glucose and Na transport has been studied by measurements of the intestinal

reflection coefficient. The results suggest that sodium transport in the jejunum may not be the passive solvent drag phenomenom that it has been thought to be. These latter studies are being prepared for publication. A radioisotope study is being done to determine unidirectional sodium and chloride fluxes in order to more accurately describe the movement of electrolytes in the pertinent animal model.

Intestinal transport has been studied in animals treated with aminophylline. This drug, when delivered intravenously, causes ileal secretion that is identical to that occurring in the Salmonella model. Work is continuing on this observation to determine if the aminopylline is causing the effect through changes in blood pressure or flow, or by an action directly on mucosal cyclic AMP activity.

b. Intestinal Neuromuscular Interactions: The study of the normal interactions of the longitudinal and circular intestinal smooth muscle in coordination of the peristaltic reflex is being continued. The reflex is being studied by an *in vitro* approach in which coaxial electrical and pharmacological stimuli are employed to elicit the reflex. The results suggest that the muscle layers are activated and inhibited reciprocally through separate neural mechanisms of the myenteric plexus. The chemical mediator of longitudinal contraction appears to be cholinergic in nature whereas contraction of the circular muscle appears to be a noncholinergic mechanism. Inhibition of the circular muscle results from activation of neuronal elements that are not blocked by adrenergic antagonists. The exact nature of the circular muscle excitatory and inhibitory transmitters is unknown.

The previously reported absence of intestinal smooth muscle alpha adrenergic blocking properties of WR-2823 is being studied in collaboration with the Department of Pharmacology, Division of Medicinal Chemistry. The current study is a thorough evaluation of the action of this drug on the intestinal musculature in comparison with standard alpha adrenergic blocking agents. The original observations have been submitted for publication in an article with personnel of the Department of Pharmacology.

c. Intestinal Disaccharidase Enzyme Activity. Many malabsorption syndromes are accompanied by reduced mucosal disaccharidase activities. On the basis that fecal enzyme activity might reflect mucosal enzyme activity, the relationship between these activity levels is being studied. If a high correlation is ultimately shown, the possibility of employing fecal enzyme analysis as a screening test for malabsorption will be tested. At the present time the data suggests a reasonable correlation between mucosal and fecal activities in germ-free mice. Fecal enzyme activity from conventional animals is extremely high and does not correlate well with mucosal activity. Work is continuing on this relationship in conventional mice.

The distribution of mucosal disaccharidase activity has been studied in human fetuses and a paper submitted for publication. By four months of fetal age, maltase and sucrase reached adult levels of activity. Lactase activity remained very low throughout the course of fetal development.

The effect of protein deficiency on disaccharidase activity was studied in collaboration with the Department of Experimental Pathology. This study showed a drastic loss of mucosal weight paralleling total body weight loss and a total loss of enzyme activity with the normal ratios of lactase, maltase and sucrase being maintained. This study has been published.[1]

d. Microvascular Architecture. The silicone rubber injection technique was used to elucidate the normal microvascular architecture of the human colonic mucosa and compare it to the vascular changes seen in ulcerative colitis. The arterial supply is distributed to the base of the colonic mucosa and runs into "feeding" capillaries that rise in the lamina propria. These vessels in turn supply capillary rings that surround the luminal openings of the colonic crypts. Venous drainage of the mucosal surface capillaries return through the lamina propria to the submucosa. In ulcerative colitis the "feeding" vessels appear dilated and the vascular geometry is disrupted at the margin of the ulcer. The vascular patterns seen support the concept of mucosal ischemia in ulcerative colitis. These observations were presented at a symposium on "Vascular Disorders of the Intestinal Tract" held by the Albert Einstein School of Medicine and Montefiore Medical Center.

e. Mitochondrial Metabolism. Several bacterial toxins have been shown to have specific deleterious effects on isolated mitochondrial preparations. The effect of purified staphylococcal enterotoxin B on isolated mitochondria has been studied. The results indicate that this toxin does not alter metochondrial respiration, it does not alter the mitochondrial membrane permeability, and it does not alter the rate of oxidation of $NADH_2$, succinate, or ascorbate. In collaboration with Dr. E. J. Davis, Indiana University School of Medicine and the Division of Biochemistry, WRAIR, the possibility of a malate-aspartate cycle being involved in transporting reducing equivalents across the mitochondrial membrane was investigated. The results of this study have been published.[2, 3]

f. Mesenteric Blood Flow. A preliminary study of the adrenergic mechanisms of the splanchnic vascular bed using electromagnetic flowmeter techniques has been completed. The results show both epinephrine and norepinephrine to be primary vasoconstrictors that operate through alpha adrenergic receptors. Epinephrine, when studied in does of 10^{-9} to 10^{-6} Gm/Kg, failed to elicite beta adrenergic action. Only following alpha blockade does epinephrine yield vasodilation through beta receptor stimulation. The mesenteric vascular bed is unique in that the classically pure alpha and beta agonists yield "reversal" type

responses following appropriate alpha or beta blockade. The results of this study have been accepted for presentation at the 1970 Fall meeting of the American Physiological Society. This study will now be extended to investigate possible changes of the mesenteric vascular bed in diarrheal disease. Further studies are in progress to evaluate the action of the alpha adrenergic blocking drug WR-2823 in this vascular bed.

g. Etiology of Diarrheal Disease in Vietnam. As a follow-up to the epidemologic survey of infectious diarrhea and enteritis conducted in Vietnam last year, a field study of the etiology of these conditions is currently being carried out. In the previous field study, 80% of the diarrheal disease encountered was classified as being of unknown etiology. The current study is designed to more completely define the causative agents involved in the large population of diarrheas of unknown etiology. Preliminary results from the present study indicate the involvement of pathogenic strains of E. coli and an overgrowth of small intestinal flora in these diarrheas. In approximately 20% of the group of patients with disease of unknown etiology, there appears to be a "hybrid" or variant strain of E. coli/shigella organisms involved. The possibility of viral agents being responsible for part of the unknown etiology population is being investigated. However, the data are in the early stages of analysis and thus do not indicate the degree to which viruses might be involved.

2. Pathogenesis of Gastric Stress Ulcer.

The study of gastric stress ulcer that was initiated last year is being continued in collaboration with the Division of Biochemistry. The rotational device used to subject small animals to a tumbling stress has been perfected and a descriptive article is being prepared for publication. It is possible to induce a highly predictable form of gastric ulceration by subjecting an animal to a predetermined period of rotational stress. During this year, emphasis has been placed on describing the changes in blood corticosterone and catecholamine levels in response to stress. At the onset of rotational stress, there is a sharp rise in both blood glucocorticoid and catecholamine content. As stress continues over a period of time, the corticosterone response becomes biphasic. That is, the sudden sharp rise is followed by a fall in blood steroids which in turn is followed by a prolonged or chronic increase. At the time of stress, the animals have been fasted and are therefore hypoglycemic. Stress further reduces blood sugar content. The persistent hypoglycemic condition of the animal during the period of stress seems to be causatively related to the development of gastric stress ulcers. These studies will be extended to consider other metabolic responses to an imposed stress condition.

Conclusions and Recommendations.

The study of experimental diarrheal disease in laboratory animal models has yielded sufficient information to enter a pathophysiological investigation of the clinical disease in the human. This department has been invited to collaborate with the Department of Infectious Disease, University of Maryland Medical School, on a study of intestinal transport mechanisms during experimental diarrheal disease in human volunteers. Efforts are presently directed toward establishing a mutually agreeable protocol to investigate the relationship between intestinal length and the disease state. Following this relatively simple initial study, a definitive investigation of water and electrolyte transport will be undertaken in the same volunteer population. All phases of animal experimentation will continue during the clinical investigation to further define intestinal changes associated with diarrheal disease.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 087, Gastrointestinal disease

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6453	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISD'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	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	61102A	3A061102B71R	02	088
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11. TITLE (Precede with Security Classification Code)

(U) Military Nursing (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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58 03	CONT	DA	C. In-house

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	1	70
	CURRENT 71	4	250

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Nursing; (U) COMPSY; (U) Wound Healing; (U) Diabetes; (U) Operative Care

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

23 (U) Develop rationale underlying military nursing through the study of the effect of moist heat on tissue as this applies to wound healing; the use of computers in military psychiatry (COMPSY); evaluation of the health teaching of the military diabetic patient; and the effect of pre-operative preparation of war wounded on postoperative recovery.

24 (U) Polarographic assays of tissue respiration in guinea pigs in response to various temperatures, durations, and solutions; collection of three forms of comparable nursing reports; identification of programmed instruction booklets for diabetic patients, while appropriate tests of patient attitudes and knowledge learned are being formulated; development of observational tools has been started.

25 (U) 69 07 - 70 06 The report of the completed assays of tissue respiration in guinea pigs is being published in the 1970 ANA Sixth Nursing Research Conference volume. Samples of three forms of daily nursing reports are still being collected: a verbal one given by a nurse to the psychiatric team, the standard written note, and the mark-sense form of automated nursing note. (See COMPSY Report WRGH Project No. MEDEC-GP, 68-4-7.) A programmed instruction booklet has been identified and ordered; opinion scale and pre- and post-tests of content have been developed. Patients are being observed for pre-operative preparation and post-operative response. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1969-30 June 1970.

*Available in controlled area upon originator's approval

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Project No 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 088, Military Nursing

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Objective:

Research in the clinical aspects of military nursing is concerned with identifying and testing principles underlying nursing care. A few studies are involved with basic sciences; however, the majority are investigations in the clinical situation. The seven studies being reported are concerned with: effect on tissue respiration in guinea pigs after application of hot moist soaks; a survey of moist soak procedures; validity of blood pressure readings; comparison of three methods of blood pressure measurement; description of the leukapheresis procedure; comparison of three methods of diabetic teaching; and computer support in military psychiatry.

Tissue Respiration in Guinea Pigs:

The disagreement among practitioners as to the best or proper method of application of moist soaks is well illustrated in the results of a recent survey (see Survey of Moist Soak Procedures below) into the procedures actually employed in the treatment of body wounds. The nurse who wishes to make her practice most beneficial and efficient must attempt to select those conditions which will best meet the intended purpose of the soak, i.e., that temperature, duration, solution, etc., that best serves to promote wound healing. Unfortunately, recourse to the literature for this information discloses a void regarding the effects of these procedural variables on body tissues. It was in view of the need for such basic data for the improvement of nursing practice that this study was conceived.

Wound healing is an energy requiring process, involving an energy requirement above and beyond that needed for normal tissue and cell maintenance. The source of energy to the cell is cellular metabolism or cellular respiration. An increase in the rate of cellular respiration will normally result in increased production of energy, some portion of which can be directed to support healing. Such interdependence of the two processes provides a reasonable basis for the selection of tissue respiration as a quantitative estimator of the goodness of any treatment in the promotion of wound healing. The work of previous experimenters showed that the rate of tissue respiration is considerably increased in the healing tissue of skin wounds, (1) and in granulation tissue. (2)

Using a standard laboratory animal, the guinea pig, the measurement of specific respiratory activity of skin to which a moist soak has been applied at different levels of each of the three procedural variables forms the experimental basis of the study. Allowing maximum metabolic activity to indicate optimal procedural variation, the data generated in this study are able to serve as a factual basis for the formulation of technical guidelines in the effective use of moist soaks in the promotion of tissue healing.

Adult male guinea pigs, weighing 250-300 g., of an inbred Walter Reed strain were chosen for use in this study primarily because of the continual, mosaic nature of it hair shedding (like man). The importance of this consideration lies in the fact in those animals (e.g. rat, mouse, and rabbit) where shedding is a seasonal, cyclic process, the metabolic activity of the skin varies greatly with the stage of shedding. (3) The use of the guinea pig eliminates this source of variability.

In preparation for the application of the moist soak, an area approximately 5x7 cm on both flank areas was shaved of hair. In those experiments evaluating temperature and duration, a single thickness, 4x4 gauze sponge, moistened in distilled water at the same temperature as that to be maintained, was applied to these areas in binder fashion. The temperature of interest was then held constant for the period of time desired by surrounding the moist application with an Aquamatic K-pad^{R} fed by a pre-set, water circulating temperature control unit, Model No. K-1-3.* The temperature

*Product of Gorman-Rupp Industries, Inc., Belleville, Ohio.

of the skin of each flank area under the soak was checked at regular intervals by inserting a standard laboratory thermometer between the skin and the soak. Under these conditions it was found that the temperature deviated no more than ±0.5°C from that desired.

In those experiments testing the effects of solution cotton elastic roller gauze, saturated in the test solution, was applied to the flank areas by encircling the body with 5-6 thickness. Plastic sheeting was used to cover this in order to prevent concentration of the test solution through evaporation. An external heat source was not used in these experiments since not all those solutions tested are used therapeutically at elevated temperatures. All were carried out for a period of one hour. The temperature of the skin, under these conditions, was found to remain at 37.5 ±0.5°C.

At the end of the time interval of the treatment, the moist application was removed, and the animal was sacrificed by decapitation, allowing as little time as possible to intervene (10 seconds or less). Skin was then rapidly dissected from both flank areas simultaneously, freed of adhering subcutaneous tissues, weighed, and placed in an ice cold buffered medium. The skin sample in all cases consisted of complete epidermal and dermal elements.

In all experiments reported, tissues were homogenized in a 1% solution (w/v) of glucose in 0.1 M tris--HCl buffer, pH 7.4, in the proportions of 1 g. (wet weight) of tissue to 5 ml. of this medium. A motor driven rotary blade homogenizer was used. The respiratory activity of this homogenate was then measured without further addition.

The oxygen consumption of skin homogenates was measured at an oxygen cathode, using the Yellow Springs Instrument Co. Biological Oxygen Monitor, Model 53 in conjunction with a potentiometric recorder. All oxygen assays were carried out at a temperature of 37°C. The respiratory activity of a sample was made specific on the basis of tissue dry weight as a Q_{O_2} value defined as $\mu l\ O_2 \times hour^{-1} \times mg$ (dry weight) $tissue^{-1}$ at 37°C.

In summary (4) and on the basis of these findings with the guinea pig, using the measure of specific respiratory activity as the estimator of physiologic response, the following recommendations can be made in the use of moist soaks for the promotion of tissue healing: The use of a soak without an external source of heat is not to be

desired, since this lowers the skin temperature and decreases significantly its rate of metabolism. Application of a soak which only maintains normal skin temperature produces no effect on the rate of skin metabolism and is, therefore, without effect on the healing process. Desirable conditions for the soak do include maintenance at a temperature higher than normal skin temperature for a period of time not less than 30 minutes and preferably longer. A temperature greater by 5°C (=9°F) is to be considered optimum since it evokes the maximum metabolic response, temperatures both higher and lower evoking a lesser response.

The evaluation of five moistening solutions--distilled water, normal saline, saturated solution of boric acid, 0.5% neomycin sulfate, and 1% silver nitrate--showed the first four of these to be without significant effect on the specific respiratory activity of tissues exposed to them for one hour without the use of external heat. Alternately, under the same conditions, silver nitrate produced nearly a three-fold acceleration of respiratory activity.

Survey of Moist Soak Procedure:

This study was the outgrowth of an effort to find out just how great a diversity of techniques exists in the use of moist soaks, and which persons on the health-care team are responsible for the selection of the technique employed. Information was gathered by questionnaire from nurses employed in eight hospitals, each of which was representative of a particular type of health care facility. Over a period of 16 months, 1,147 questionnaires were completed and returned providing information concerned with three broad categories: first, personal data relative to the age, sex, and military or civilian status of the patient being surveyed; second, data pertinent to the wound or injury such as etiology, site, integrity of skin at wound site, presence or absence of infection, causative organism if known, and antibiotic therapy, if any; third, data regarding the actual procedure employed in the application of the soaks, which included the verbatim order as written, method, duration, frequency of application, type of solution used, temperature of the soak, and the individual routinely responsible for carrying out the procedure. The findings of this study were presented in tabular form in last year's progress report and show that in the vast majority of cases reported, the

choice among procedural variables in the application of moist soaks rests with nursing personnel. Considerable procedural variation is encountered in the application of moist soaks. This lack of consistency or agreement in procedure attests to the need for evaluative study of the variables involved--i.e., temperature, duration, and moistening solution of the soak--if the choice among them is to be that which provides the maximum benefits desired. Considering the great extent to which nursing personnel are involved in this choice, these evaluative studies become germaine to the field of clinical nursing research.

Validity of Blood Pressure Measurements:

During the presentation of a report concerning the reliability of blood pressure measurements (1967), a question was posed concerning the validity of the readings. Accordingly, an attempt was made to determine the accuracy by which registered nurses read the blood pressure manometer.

Three filmed sequences of actual blood pressure measurements were incorporated into a convention exhibit. Each sequence showed the excursion of mercury in a manometer with electronically synchronized sounds. One sequence was a hypertensive reading; one was normotensive; one was low normotensive. The films were presented on a rearview projector.* In front of the screen was placed a plaster model of an arm with attached sphygmomanometer cuff and stethoscope through which sounds were transmitted to the reader; this simulation allowed the viewer to read the blood pressure on the film in much the same manner as in a real life situation. Each viewer read two pressures (systolic and diastolic measurements were considered one pressure); the first for practice, the second for analysis. Eight hundred and sixty-two registered nurses took part in the study with approximately one-third of the total reading each sequence. All participants provided applicable background data related to their age, sex, functional area of practice, educational level, and area of clinical practice. Each participant was asked whether he had any visual or hearing impairment.

Although this study was conceived of as an appropriate means of determining validity, the actual data collection method imposed crippling limitations. The area in the

*Fairchild Projector, Model Mark IV.

exhibit hall where data collection was undertaken, was continuously noisy. Many participants did not believe that the study was real and thus gave haphazard responses. In addition, no method was incorporated into the protocol to check the accuracy of participant responses, especially in regard to visual or hearing impairment. To summarize the findings, there were no statistically significant relationships of blood pressure reading accuracy related to age, sex, functional area, educational level, area of clinical practice, or visual acuity. For those who claimed hearing impairment (78 out of 862), there was a marked increase in non-valid readings. Since only five of the 78 persons who said they had hearing loss wore any type of corrective device, it is not known whether the remaining seventy-two had real loss or psychic insecurity in measuring blood pressures. Therefore, the findings are suspect and inconclusive and no further detail will be presented.

Comparison of Three Indirect Methods of Determining Blood Pressure:

Alternate indirect methods of determining blood pressure are needed for the nurse practitioner. This is true, especially in the military setting, where emergencies are routine in the field and where frequently there is a high level of ambient noise.

The three methods chosen for comparison were the standard ausculatory, palpitory (pulse), and mercury fluctuation indirect methods. Accordingly, a system was devised whereby the blood pressure of subjects (young, healthy, normotensive males) could be measured simultaneously by three observers.

All of the observers were experienced with the ausculatory method but had varying familiarity with the other two methods. Each observer measured blood pressure by each of the three methods. All readings were made independent of the other readings by means of walls between observers and individual manometers.

Analysis showed a very close relationship between the systolic readings for the mercury fluctuation and palpitory methods. Ausculatory systolic readings were consistently and statistically higher by a seven point average increase. For the diastolic readings, the mercury fluctuation method was consistently lower than the other two methods while the other two methods were within one point of each other (average difference). The average difference between the

mercury fluctuation method (lowest readings) and the auscultatory method (highest readings) was only four points. Considering the reading range acceptable to the American Heart Association, plus or minus eight points, the findings of this study show all three methods to be well within this acceptable range. It may be hypothesized that if the observers were experienced in all three methods, a lesser discrepancy would have been found!

The investigator primarily interested in exploring blood pressure measurements has been transferred. Therefore, no further explorations are anticipated.

Description of the Leukapheresis Procedure:

The leukapheresis procedure, being recently introduced to this medical center, was a completely unknown experience for the nursing personnel involved with donor care. In order to develop guidelines for making meaningful observations a descriptive study of donor response was undertaken. Twenty donations were observed; 16 male and one female normal donations and one male and two female leukemic donations (the latter two were the same donor).

Appropriate observations were determined during a pilot study of five other donors. Temperature showed no patterns of response and no marked fluctuations. Pulse rates formed three distinct patterns: 1) Initially elevated pulse followed by a decrease and stabilization; 2) The same as the first except for a temporary peaking after two to three hours of the procedure; 3) A stable pulse rate throughout the procedure. These three patterns were found without regard to smoking, sensations of cold, or apparent anxiety.

Respiratory rate showed no patterns of response. Symptomatology was not remarkable except for activity. Random movements of the head, arching the back, crossing the legs, etc. were consistently observed and probably were due to mild discomfort caused by the restrictions of movement placed on donors. Emotional response was probably related to the purpose of donation; that is, helping a family member, obtaining temporary relief of symptoms (in leukemic donors), or giving a routine donation on request.

Laboratory response showed an increase in white blood cells in normal donors and a decrease in leukemic donors. The

hematocrit decreased in all. The platelet response was inconsistent. No bleeding difficulties were seen during or after the procedure.

Guidelines for nursing attendants have been formulated based on the above findings. In addition, the resulting manuscript has described the development of the cell-separator machinery and discussed its present and potential uses.

Comparison of Three Methods of Diabetic Teaching:

US Public Health Service reports, "There are far more diabetic patients dying, having amputations, and related illnesses than is necessary. If they followed the regimen prescribed by their physicians, it is believed these numbers could be reduced. There are few diseases that make so many demands on the patient. Perhaps if better teaching materials were available to supplement the educational efforts of physicians, these diabetics could have better health records." (5)

Active duty Army personnel with diabetes can be retained on duty if under proper control. Perhaps another form of instruction or a combination of several educational methods can provide a more effective means of health teaching and keep more soldiers on active duty. According to the Patient Administration Division of Walter Reed General Hospital, an average of 450 to 480 diabetics are admitted to the hospital each year. These patients receive instruction from the Preventive Medicine Classes or from their physician or nurse. DeWitt Army Hospital, Ft. Belvoir, Virginia, admitted 190 diabetics to their hospital last year. Consequently, there is a need in the Washington, D.C. area to find a thorough, efficient, and economical method for diabetic instruction. There is reason to believe that this situation is replicated at other military installations throughout the country.

Therefore, a study has been formulated to test the following three types of instruction:

Group I, the Didactic-Demonstration Method Group, will serve as the control group.

Group II, Programmed Instruction Text Group, will serve as one experimental group.

Group III, combination of Didactic-Demonstration and Programmed Instruction Texts Groups, will serve as the mixed teaching experimental group.

One hundred fifty newly diagnosed adult diabetic patients on an inpatient or outpatient basis will be assigned to the above three groups of 50 each. General background information will be obtained. Each member of each group will be given: Reading level test (to be compared with their comprehension); Opinionnaire toward diabetes (to evaluate motivation for learning); Pre-test (to determine level of knowledge before teaching); Post-test (given immediately after the method of instruction and 2, 4, and 16 weeks later to determine retention). Improvement in scores will be determined statistically and attributed to the method of instruction received by the individual. Interviews with each patient at intervals of approximately 30 days for 4 months will record such health data as weight, days of illness, special visits to the physician and/or Army Health Nurse, days in the hospital, and relationship of present illness (if any) to diabetes.

Other than new diabetic patients, as well as that family member who prepares meals in the household, will be placed randomly in similar groups to those described above for separate analysis.

The pilot study and the definitive study is being conducted at Walter Reed General Hospital or at a station hospital at a nearby troop concentration.

Computer Support in Military Psychiatry (COMPSY):

This study is a joint project between the Department of Nursing, Walter Reed Army Institute of Research and the hospital's Department of Psychiatry and Neurology. It is being reported under Project No. 6215601A-3A025601A823, Task No. 00-048.

Summary:

The seven studies reported above are independent, yet interrelated in that each has a bearing on the overall practice of nursing. These and other nursing investigations seem to build a foundation for the practice of nursing based on scientifically tested principles rather than empirical directions.

Project No 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 088, Military Nursing

Literature Cited

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3. Ghadially, F.N. "Effect of Trauma on the Growth of Hair." Nature, 181: 993, 1958.

4. The complete study is to be published in the 6th Annual ANA Research Conference volume.

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1. Glor, Beverly A.K., et al. "Reproducibility of Blood Pressure Measurements: A Replication." Nursing Research, March/April 1970, p. 170-172.

2. Estes, Zane E. "Auto-Oxidation of Thioridazine HCl and Trifluoperazine Dihydrochloride: Implications for Clinical Practice." Military Medicine, April 1970, p. 296-299.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6464	70 07 01	DD-DR&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 07 01	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	61102A	3A061102B71R	02	089
b. CONTRIBUTING				
[redacted]	CDOG 1412A(2)			

11. TITLE (Procede with security Classification Code)

(U) BODY FLUID AND SOLUTE AND RENAL HOMEOSTASIS (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012 900 Physiology 003500 Clinical Medicine 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	CONT	DA	C. In-House

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a. DATES/EFFECTIVE: NA EXPIRATION:

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18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	2	50
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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (... Security Classification Code)

(U) Heat Stress; (U) Renal Failure; (U) Dialysis; (UA) Acid-Base; (U) Electron Microscopy
(U) Kidney Function; (U) Renal Hemodynamics; (U) Fluid & Solute Homeostasis; (U) Shock

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

23. (U) - To investigate mechanisms for maintaining fluid, electrolyte and hemodynamic homeostasis in response to disease, injury and environmental stresses of military significance, such as shock, heat stress, infectious disease, gastrointestinal disorders, and renal failure in order to provide rational basis for prevention and treatment.

24. (U) - Clearance methods, externally monitored isotope methods, isotope dilution, experimental models, in vivo renal micropuncture, in vitro renal microperfusion, membrane transport, light and electron microscopy.

25. (U) - 69 07 70 06 - Renal cell sodium localization studies were extended to show alterations induced by injury, and transport inhibitors. The role of physical composition of blood was found to be as or more important than the volume status of the animal in regulating renal salt excretion. A method for extracorporeal support of the kidney has been developed for studies of isolated kidney function and response to discrete manipulations. Our method for external measurement of renal hemodynamics on-line was extended to clinical use for study during development and treatment of shock. Membrane dialysance characteristics were studied to support rational development of more efficient clinical dialysis. Heat stress studies assessed basic physiologic alterations quantitated degree of tissue breakdown and developed indications for hemodialysis and a pathogenetic schema showing potential preventive measures. Combat zone studies of nonoliguric renal failure, traumatic renal failure, and renal failure in malaria were completed. New methods for study of isolated kidney tubules and isolated perfused whole kidneys were developed to study stress on kidney function apart from extraneous influences. Evidence for active transport of chloride has been found. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

* Available to contractors upon originator's approval.

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 089, Body fluid and solute and renal homeostasis

Investigators:
Principal: LTC William J. Cirksena, MC; MAJ James H. Knepshield, MC
Associates: MAJ Jordan J. Cohen, MC; MAJ Andrew Saladino, MC; MAJ Charles Wallas, MC; CPT Vincent Dennis, MC; John A. Gagnon, Natalie Lawson, James McNeil, Amy Lee, Martha Huddleston, James Roberson, and Doug Grove

Description: Studies are directed at investigation of mechanisms for maintaining body fluid, electrolyte and hemodynamic homeostasis or their correction in response to disease, injury and environmental stress of military significance, including shock, heat stress, infectious disease, gastrointestinal disorders, and renal failure. A variety of methods have been developed and utilized, including externally monitored isotope techniques, isotope dilution, experimental models of acute renal failure and shock, in vivo renal micropuncture, in vitro microperfusion, membrane transport, dialysis systems, and light and electron microscopy. The role of adaptive homeostatic mechanisms, including renal and extrarenal mechanisms, whereby body fluid and solute balance is achieved and maintained in the face of stress has been emphasized in order to provide a rational basis and develop improved methods for prevention and treatment of altered fluid, electrolyte and hemodynamic states and acute and chronic renal failure induced by these stresses.

Progress:

1. Renal Solute and Water Handling:

a. Studies in intact, conscious dogs were undertaken to assess the quantitative relationship between increases in extracellular fluid volume (positive sodium balance) and augmentation of sodium excretion during acute volume expansion. Each dog was studied with four different infusion rates at weekly intervals. A consistent pattern of change in extracellular fluid volume versus rate of sodium excretion was observed for each animal but there was considerable variability among animals. The factors responsible for this variable pattern will require further investigation.

b. The relationship of volume expansion per se to alterations in renal sodium excretion and proximal tubule sodium rejection was studied in dogs using micropuncture methods. Volume expansion was produced by using normal saline, homologous plasma or homologous blood. Renal perfusion pressure was kept constant so that only alterations in blood hematocrit and/or plasma concentration in association with volume

were studied. Results indicate that proximal tubule sodium reabsorption is decreased when volume expansion is associated with a decrease in hematocrit or a decrease in hematocrit and plasma protein concentration. When these changes are avoided by expansion with homologous whole blood, no change in proximal sodium reabsorption was observed early after infusion.[1,2] A late effect of volume expansion per se, consistent with formation and release of a circulating humoral substance affecting late proximal tubules could not be ruled out; current studies will compare early and late effects of blood expansion without change in physical composition of the blood. Further studies will investigate the effects of volume contraction without alteration in renal perfusion pressure on proximal sodium reabsorption.

c. The concentrating ability related to renal morphology of three species of Macaque monkeys was evaluated in order to determine the structural requirements vital to formation of a concentrated urine. Results indicate that in these species, unlike man, a direct relationship between concentrating ability and length of the renal medulla does not exist and that factors other than overall length of the loop of Henle must be important in the attainment of a concentrated urine in these animals.[3]

d. A study in dogs has been completed directed at defining the mechanism(s) responsible for the pharmacologic effect of propranolol on sodium metabolism noted in human subjects. Findings suggest that effects of propranolol on renal hemodynamics and sodium excretion are not attributable to an intrarenal effect, but are likely mediated by effects of the drug on cardiac output and total peripheral resistance.[4]

2. Acute Renal Failure:

a. Studies directed at pathogenesis and prevention of acute renal failure in an experimental animal model have allowed postulation of certain mechanisms for its development. Clearance and micropuncture studies in the methemoglobin-ferrocyanide model of acute renal failure in the rat have yielded evidence favoring an early renal vascular event consisting of afferent arteriolar constriction and relative efferent arteriolar dilatation as the central alteration leading to oliguria and azotemia. Studies using β_2 microglobulin have supported such interpretation and further suggest an absence of any significant obstructive element early in the course of development of the lesion. Studies in which lissamine green dye is injected in tubule lumina offer strong evidence against an increase in passive back diffusion. Micropuncture studies utilizing intratubule pressure measurements and intravenous lissamine green dye infusion suggest the transepithelial movement of fluid (and solute) in a direction from interstitium toward lumen early in the course of the lesion. Such vascular alterations and pressure relationships would explain the consistent finding of tubule dilatation in this lesion in the presence of documented oliguria, azotemia, and low intratubular pressure. Further studies will require new and improved methods in order to 1) discern the critical stimulus for the initial vascular alteration 2) confirm the suspected pressure gradient relationship betwen lumen and interstitium.

Further evaluation of the possible role of renin release in initiating the vascular response will be possible only through contract with the laboratory of Dr. K. Thurau (University of Munich) which has been proposed as a joint protocol study under DA contract. Such studies would allow assessment of individual nephron renin release and vascular response in shock and acute renal failure, and are strongly urged as the single most important avenue of scientific exploration from the standpoint of military significance in this area of hemodynamic homeostasic mechanisms.

New methods are being developed for pressure measurements which should allow confirmation or denial of proposed intrarenal pressure relationships which may be intimately tied to pathogenesis and prevention of this lesion. Results of these studies to date have been presented to an international symposium on acute renal failure[5] and publication in book form is in press.

b. Extensive light and electron microscopic studies of biopsy material obtained from Vietnam war casualties with severe wounding and acute renal failure have been completed. Findings in these studies in which preparation artifact has been scrupulously minimized are strikingly similar to those previously noted in studies of the experimental model of acute renal failure. Minor tubule epithelial changes comprised the only histologic evidence of damage and there was a total lack of widespread lethal alteration in tubule cells. These findings are in contrast with previous studies by others in which preparation artifact associated with histologic examination may have been prominent. Marked tubule dilatation noted in present studies is in concert with our evolving notions regarding pathogenesis.

c. Studies performed in Vietnam in the evaluation of patients with acute non-oliguric renal failure have been prepared for presentation and publication.[6] Two studies of heat stress, dealing with hypercatabolism in heat stress nephropathy,[7] and pathophysiologic mechanisms involved in heat stress[8] have been completed. A joint service protocol for evaluation of these mechanisms and prevention of heat stress under combat conditions in a hot environment has been prepared for evaluation of recruits in training. Studies of acute renal failure in Vietnam have been presented[9] and prepared for publication.

3. Acid-Base Homeostasis:

a. Studies have been undertaken in rats to determine the influence of extreme hyponatremia on the ability of the kidneys to maintain normal acid-base equilibrium. Animals have been rendered hyponatremic by the chronic, daily administration of pitressin and forced water-loading. After a new steady-state has been achieved, acid-base parameters in arterial blood are assessed. Preliminary observations

suggest that normal acid-base equilibrium is maintained despite marked disruption of normal extracellular electrolyte composition. This finding implies that bicarbonate reabsorption by the kidney is regulated by factors which are independent of and which may override the factors regulating sodium and chloride reabsorption. Further studies of this homeostatic mechanism are in progress.

b. Clinical observations have suggested that severe potassium depletion may result in the development of a variety of metabolic alkalosis that is resistant to the provision of chloride ("chloride wasting nephropathy"). Attempts to reproduce this lesion in dogs by selective potassium depletion have been unsucessful; total potassium depletion can be produced without any change in acid-base equilibrium. Similar studies have been undertaken in rats and have indicated that selective potassium depletion does produce a chloride-resistant metabolic alkalosis in this species. The prevalence of this species difference and the mechanisms responsible for it are being explored by extending these observations to other animals.

c. Studies investigating the relationship between urine flow rate and renal acid-excretion have been completed and published.[10]

4. Renal Hemodynamic Studies:

a. Previously completed studies developing a new isotopic method for the instantaneous measurement of effective renal blood flow in the dog have been published.[11] Extension of these studies to human subjects is in progress. Overestimation of renal blood flow limits the usefulness of the method for precise quantitation, but may allow its use as a relative measure of blood flow during rapidly altering clinical situations such as shock and developing renal failure. External monitoring methods remain reliable for estimating GFR in man, and are being employed in appropriate clinical studies.

b. A study demonstrating inhibition of tubular secretion of ^{131}I iodohippurate by p-amino hippurate has been submitted for publication.[12]

c. Extensive simultaneous use of ^{125}I iothalamate and inulin in both man and dog during development of external monitoring methods for measuring GFR have afforded the opportunity for careful assessment of the validity of using renal clearance of iothalamate as a measure of GFR. Results from over 200 simultaneous clearance periods indicate that in man, there is close correlation between the clearance of ^{125}I iothalamate and the clearance of inulin (mean ratio iothalamate: inulin = 1.01). In the dog, however, GFR is consistently underestimated using iothalamate compared with inulin (mean ratio = 0.89). Dialysis studies suggest the discrepancy is not due to the presence of free ^{125}I; simultaneous infusion of cold iothalamate ruled out a

micromolar reabsorption phenomenon. A possible direct effect of ADH on tubule permeability for iothalamate has been explored in antidiuretic dogs and dogs undergoing water diuresis and found not to be responsible for this discrepancy. The quantitative inaccuracy of iothalamate as an estimate of GFR in the dog thus appears to be due to some mechanisms for iothalamate reabsorption. Results are being prepared for publication.

d. Studies of renal function in the chimpanzee performed by Mr. Gagnon and gleaned from the literature have been collected into a review chapter and published in book form.[13] A histologic study of the morphology of the chimpanzee kidney compared with human kidney has been published as a book chapter.[14]

e. A clearance study in dogs showing absence of evidence of a role for the renin-angiotensin system in renal autoregulation of filtration rate is in press.[15] Further exploration in this area of controversy will require single nephron studies of renin secretion and hemodynamics. These studies have been proposed for support by DA contract with Professor K. Thurau (University of Munich) because of lack of facilities for such measurements elsewhere in the world. Investigation of the mechanisms by which the kidney maintains hemodynamic competence during shock is considered to be of the highest military significance, and support for this study has been strongly urged.

f. In order to study renal hemodynamic alterations independent of extra-renal influences, development of an isolated perfused kidney preparation has been begun. A pump-oxygenator system and the necessary surgical techniques for removal and extracorporeal support of the kidney have been developed. Preliminary findings suggest that cellular integrity of the kidney can be maintained for at least 507 hours in a normothermic state; fractional reabsorption of sodium is in excess of 99%; glycosuria does not occur; urine flow rate is high, and resistant to pitressin; renal blood flow increases and GFR decreases with time despite maintenance of perfusion pressure at 110 mm Hg. The relation of substrate depletion to deterioration of renal hemodynamics is being studied. The role of plasma potassium, suggested to be of significance in initial studies in the maintenance of normal fractional sodium reabsorption, is being investigated currently. Substrate and pharmacologic manipulation will continue in an effort to obviate the decline in functional status of the isolated kidney. The preparation will be used, then, to study the isolated effects of changes in perfusion pressure, perfusate composition, and various nephrotoxic substances and to clarify the role of various humoral agents in renal fluid and solute handling.

5. Cellular Transport:

a. Studies have been performed on the metabolic activity as measured by lactate production of RBC's from patients with advanced renal disease. RBC's from 20-30% of these patients have an increased metabolic activity which appears to be due to an acceleration of the proximal portion of glycolysis. Preliminary measurements of hexokinase and phosphofructokinase, the two rate limiting enzymes of this portion of glycolysis show a two-fold increase in activity in RBC's which are hypermetabolic. This RBC abnormality does not appear to bear any relationship to dialysis of the patient and may reflect the fact that the RBC's of some uremic patients represent a young population due to a hemolytic process. In addition, it appears that all patients with renal insufficiency studied to date have a decreased susceptibility to osmotic environments which is also not affected by patient dialysis. Studies will be extended to malarial red cells which have been shown to have a defect in active sodium transport.[16]

b. An alveolar macrophage isolated cell system has been devised to study the simulated injurious effects of decreased vascular perfusion or blood stasis from the standpoint of cellular ultrastructural and functional correlation. Initial studies indicate that following attainment of a 1:1 Na:K cytoplasmic ratio, cell recovery does not occur after such injury. The participation of the cell membrane in these ionic regulatory mechanisms will be studied in the next phase.

c. Preliminary studies of ion localization and movement and ultrastructural capillary alterations in P. berghei-infected hamsters have been made. In this infection, the disposition of altered cellular elements suggests that the cerebral lesion may involve capillary endothelial membranes or cell junctions resulting in leakage of fluid and extravasation of red blood cells. Furthermore, discrete analysis by electron microscopic techniques is in progress. Cellular localization of major ions, and their movements with injury and infection are planned for study using electron probe techniques.

d. A study of muscle biopsy material has been begun to investigate the macromolecular and functional alterations in muscle mitochondria in patients with malignant hyperthermia secondary to halothane anesthesia.

e. Studies reporting new methods for localizing sodium deposits in cells by electron microscopic histochemistry have been published.[17] Discrete localization of sodium, confirmed by electron microscopic autoradiography, has been noted along the plasma membrane intracellularly under normal conditions in rat renal tubules perfused and fixed *in vivo* using micropuncture techniques. Perfusion with ouabain produced widespread disruption of this discrete pattern of localization, with increased and scattered sodium deposits over the

entire cell. Studies in which various inhibitors of sodium transport (DNP, furosemide, ethacrynic acid and actinomycin) have been perfused directly into peritubular capillaries before histochemical perfusion and fixation, have been completed. Analysis of electron microscopic material is in progress. The differential effects of these inhibitors on intracellular sodium localization should do much to advance understanding of the basic cellular mechanisms by which sodium ion movement occurs.

f. These latter studies have been extended to study the effects of nephrotoxic injury. Injury to rat renal tubules was produced by injection of potassium dichroacetate before intratubule perfusion of potassium pyroantinonate for sodium localization and fixation. Results showing marked disruption of the normal discrete linear deposition of sodium, with increased uptake of sodium by mitochondria and other cellular organelles, have been presented and prepared for publication.[18]

g. Studies in isolated perfused rabbit tubules have been aimed at developing a reproducible model for the study of renal tubule solute and water handling, and the effects of drugs, other chemical and physical stresses apart from complicating physiologic conditions. The method for dissection, mounting, and perfusion of single segments of tubules has been perfected and initial studies completed. Evidence obtained from simultaneous study of chloride and ^{131}I tagged albumin indicate evidence for the active transport of chloride ion against an electrochemical gradient. Possible elution of protein on transfer pipettes is being investigated and further studies using ^{14}C inulin will be conducted. The availability of this method will allow study of localization and movement of sodium by histochemical electron microscopic techniques and to explore the isolated effects of ion transport inhibitors, trauma, and chemical and physical agents on sodium movement.

6. Dialysis Systems:

Studies have been undertaken to enhance the efficiency of extracorporeal dialysis in various clinical settings. Considerable progress has been made with the experimental evaluation of lipid dialysis for intoxication with pentobarbital. Various commercially available oils were tested for lipid:water partition coefficients for pentobarbital. Results showing corn oil to have a slight advantage over other oils for use as a lipid dialysate have been published in abstract form.[19] A dialyzer has been contructed and found to be effective and efficient for use as a lipid dialyzer. Reservoir studies have demonstrated, however, that blood protein binding of the drug (approximately 20%) is one factor limiting dialysance. Dialysis of dogs given pentobarbital will be studied in the next phase, leading ultimately to extension of the method to human subjects.

Project 3A061102B71R IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 02, Internal Medicine

Work Unit 089, Body fluid and solute and renal homeostasis

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 089, Body fluid and solute and renal homeostasis

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PROJECT 3A061102B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 03
Psychiatry

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6454	70 07 01	DD-DR&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 07 01	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	61102A	3A061102B71R	03	025
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code): (U) Analysis and Mamagement of Behavior and Stress (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS:
013400 Psychology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	11	300
FISCAL YEAR CURRENT 71	6.5	185

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Animal and Operant Behavior: (U) Avoidance; (U) Motivation; (U) Conditioning; (U) Performance Decrement; (U) Reinforcement Learning

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

23.(U) Complex behavioral models are developed which simulate conditions likely to lead to ineffective performance or psychiatric decompensation in the military environment. A wide range of physiological and psychological variables relevant to the environment-individual interaction are studied (e.g., the behavioral toxicity of therapeutic and nontherapeutic drugs, the effects of stress and fatigue upon alertness and performance.

24.(U) The techniques of modern experimental psychology, particularly operant conditioning, are combined with the techniques of neurophysiology, neuroanatomy, neuroendocrinology and pharmacology are to precisely define the nature of an organism's response to stress.

25.(U) 69 07-70 06 Animal studies are now in progress to evaluate the effects of synthetic marihuana and anti-malarial drugs on sensory capacities, vigilance, decision making, aggression and responsiveness to stress. Preliminary findings indicate that marihuana exerts its main effects on the subject's motivation to perform effectively rather than its ability to detect and recognize stimuli. Preliminary data also suggest that large doses of marihuana may increase aggressiveness. A study of hormonal factors in adaptation to behavioral stress indicate that low testosterone levels may prevent the taking of long-range action to avoid hazardous events. Preliminary data are being collected on the behavioral effects of the rapid transportation of humans across time zones. These data are relevant not only to the effects of geographical relocation of patients, but to the performance of medical personnel as well. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 03, Psychiatry

Work Unit 025, Analysis and management of behavior and stress

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This report covers the following problem areas: (1) Stress: laboratory techniques for the production and investigation of stress; (2) Central nervous system: its role in the adaptation of organisms to stress; (3) Pharmacological agents: the search for behavioral toxicity of drugs of military importance and the development of new methods for the evaluation of drug effects on behaviors of particular relevance to military psychiatry.

A group of monkeys is being evaluated in a study designed to confirm and extend earlier findings of concomitant blood pressure and heart rate changes associated with alterations in operant performance produced by both appetitive and aversive Pavlovian conditioning procedures superimposed on both appetitively and aversively maintained on-going operant performance. The present study, composed of three separate experiments, is directed at mechanisms underlying psychosomatic disorders resulting from enforced exposure to psychological stress. The experiments have been designed to systematically explore a number of instrumental and respondent interactions for the purpose of developing experimental models of psychosomatic disease to permit evaluation of potentially useful treatment procedures.

Adult chimpanzees are being maintained in individual cages which permit controlled access to a larger social chamber. Initial development of behavioral procedures for delivering daily rations of food and water and for arranging access to the social chamber have now been completed. The second phase of the study is presently under way. In this phase, the effects of social interaction will be assessed against a complex behavioral baseline. Following completion of this phase, the animals will be instrumented with transducers for telemetering physiological data related to social interaction and stress. These telemetered data will be compared with similar data obtained from humans functioning in the field. The chimp studies are intended to serve as a bridge between more controlled laboratory studies and field studies of stress in man. The complexity of both their individual behavior and collective social repertoires makes this organism ideally suited for this purpose.

Monkeys have been trained to perform in an avoidance procedure in which the monkeys can avoid a shock immediately prior to its delivery or they may avoid it at a point in time considerably earlier. The data indicate that the monkeys avoid 50% of the time in the stimulus condition remotely removed in time from the shock and 50% of the time in the stimulus condition temporally close to the shock. After castration the monkeys completed most avoidance responses in the stimulus closest to the shock. This suggests that the diminished testosterone levels have brought the animals more under the control of the immediate consequences of their behavior and less under the control of the remote consequences. This effect is related to the responsiveness of humans to the long-range versus short-range effects of their behavior.

Exogenous injections of 5, 125, 25, and 50 mg of testosterone propionate were systematically given to both monkeys. Increased amounts of the hormone increased the number of avoidance completions in the remote stimulus condition and decreased the number in the stimulus closest to the shock. At 50 mg levels of testosterone, performance of both monkeys was similar to pre-castration performance. These data indicate that the effect is reversible by the administration of exogenous testosterone. These hormone studies provide an insight into the interaction of the physiological substrate of emotion and the behavior of primates under stressful conditions. Further, the primate data provide a model to help understand the behavior of personnel under the stressful conditions that may be experienced in combat.

Following completion of a study that demonstrated changes in the morphology of the autonomic nervous system of rats as a result of exposure to an acute stress procedure, an attempt is being made to identify similar changes in non-human primates. Rhesus monkeys are being exposed to 72 hr. avoidance and sacrificed following administration of radioactively labelled thymidine. The data on the first two groups of animals indicates that acute stress does modify the structure of the autonomic nervous system and that the effect is most pronounced in those animals required to emit responses to avoid electric shocks. Electric shock alone, with no avoidance requirement, produces a less pronounced effect. Preliminary analysis of urine and serum endocrine measures suggest that the morphological response may be mediated by hormonal changes resulting from exposure to the stress procedure. These data bear directly on the problem of identifying the physiological consequences of exposure to stress and to the development of a model for interpreting the failure of individuals to successfully adapt to the acute stresses encountered by military personnel.

The identification of morphological changes in the autonomic nervous system of non-human primates exposed to acute psychological stress raised questions as to the effect of prolonged chronic stress on the structure and function of the nervous system. Rhesus monkeys are now being exposed to repeated 72 hr. avoidance sessions. Following ten weeks of stress

exposure, the morphology of their nervous systems will be examined using autoradiographic techniques. Data resulting from these studies may contribute substantially to our understanding of the anatomical and physiological mechanisms underlying the behavioral breakdown of military personnel exposed to chronic psychological stress.

A preliminary study is underway to confirm reports that air deployment of personnel across long distances in an East-West direction produces psychological and physiological changes, known as transmeridian desynchonization. These changes may result in temporary performance decrements in tasks requiring sustained attention. Pilot data are now being collected from a human subject following a direct flight from Washington, D.C. to Bangkok, Thailand and return. Initial plans call for selected WRAIR personnel, already required to travel long distances to complete assigned missions, to also serve as volunteer subjec-s in the study. Portable laboratory equipment has been developed for this purpose. Although the study has relevance to the performance of all military personnel after geographical relocation, assessment of performance decrements in medical personnel (e.g. surgeons) is of special interest. The potential relevance of these data to the geographical relocation of patients is also being evaluated.

The technique of making discrete lesions or neural structures known to be involved in mediating the response of an organism to stress is being used with a number of widely differing stress procedures in an attempt to better characterize the variables that contribute to an organism's adaptive response to both acute and chronic stress. Identification of stress-related variables materially contributes to the development of improved methods for predicting responses to stress as well as to the development of more effective techniques for dealing with stress-induced psychiatric and psychosomatic problems in military patients. A study has been completed demonstrating that subjects with lesions of the septal area of the limbic system are superior to intact subjects of their performance on Sidman avoidance procedures. Subjects with this lesion emit fewer, better spaced responses and their performance can be characterized as more efficient than that of intact animals. Subjects with lesions of the cingulate gyrus are being tested on Sidman avoidance. Preliminary data suggest complete loss of the ability to perform in this stress procedure following surgery. Subjects with septal lesions are stressed using a punishment procedure in which responding for food was punished with electric shocks. Unlike their performance on Sidman avoidance, the septal animals' performance on this procedure was not superior to that of intact subjects. Analysis of the data of this study is not yet complete. Subjects with septal lesions are also being stressed using punishment of Sidman avoidance performance. Analysis of the data of this study is not yet complete. Preliminary indications are that both intact and operated subjects responded equally to the punishment of aversively maintained performance.

Another study is being concluded using subjects with septal lesions in a behavioral testing procedure that compared the same performance under appetitive and aversive control. Differences between normal and lesioned subjects appear in the appetitive procedure, but not in the analogous stress-avoidance procedure. A study has also been completed in which animals with septal lesions have been exposed to concurrent appetitive and aversive schedules. Preliminary analysis of the data indicate that the lesioned animals do not respond to concurrent contingencies as intact animals do. The lesioned animals responded solely to the appetitive procedure without evidencing performance changes attributable to the concurrent stress contingency.

Recent clinical experiments indicate that low level electrical stimulation of peripheral nerves may act as an analgesic for patients suffering from causalgia. In this pilot experiment the analgesic properties of low level electrical stimulation were investigated in normal human subjects. The index finger of each subject was heated by a thermal stimulator (dolorimeter) until the subject reported feeling a "burning pain." A tolerance threshold for thermal stimulation was determined for all three subjects. This value was between 5-7 seconds at a standard heat intensity. After two minutes of low level electrical stimulation of the peripheral supply innervating the index finger, all subjects showed an increase in their tolerance threshold; i.e. they allowed the stimulus to stay on for 8-10 seconds before reporting "burning pain." The results of this pilot study suggest the need for further experiments. These additional studies will determine pain tolerance both before and after low level electrical stimulation of the peripheral supply innervating the index finger using psychophysical scaling methods. Such techniques will give an accurate estimate of pain tolerance. The results of these experiments will provide an insight into the mechanisms both psychological and neurophysiological that may produce causalgia. In addition these experiments may provide new information and techniques to relieve the causalgia symptoms experienced by wounded troops and veterans.

Clinical reports have often implicated the frontal lobes in emotional behavior. Some investigators have proposed that this area of the brain is primarily involved in the inhibitory control of emotional behavior. Recent experimental work however has revealed that the frontal lobe is not a unitary structure. In particular, the dorsolateral areas appear to serve different functions than the orbital areas. The orbital frontal cortex, with numerous connections to the limbic system, would seem more likely to play a role in emotional behavior. Therefore, a group of monkeys is being trained on two tasks, one stressful and one innocuous. It is anticipated that orbital cortex lesions will have a more powerful effect on conditioned emotional responses and their extinction, while dorsolateral control lesions will mainly affect the non-stressful control task.

A series of experiments on the phylogenetic development of the auditory and visual systems is now in a terminal phase. This project has combined the techniques of discrimination learning, psychophysics, neuroanatomy and neurophysiology in order to shed light on the evolutionary development of the brain in general and the auditory and visual systems in particular. The purpose of this research has been to determine the degree to which the data of central nervous system investigations of animals can be applied to humans and to indicate which organisms may most adequately represent humans in such investigations. The subjects of this current series of studies have been pigeons. These animals have been shown to resemble primates in the anatomy and physiology of their auditory and visual systems far more closely than had been previously recognized.

In pigeons, the auditory projection passes from cochlear nuclei to the nucleus mesencephalicus lateralis, pars dorsalis (comparable to the inferior colliculus of mammals) to nucleus ovoidalis thalami (comparable to the medial geniculate nucleus of mammals) to the telencephalic Field L of Rose (comparable to auditory cortex of mammals). Lesions of the various cell groups of this system have resulted in deficits in audition quite similar to those reported after comparable lesions in mammals.

Two ascending visual pathways to the telecephalon have been found in pigeons. These seem comparable in many ways to the dual ascending visual pathways recently discovered in mammals. The thalamofugal pathway passes from retina to the principal optic nucleus of the thalamus (comparable to the lateral geniculate nucleus of mammals) to the nucleus intercalatus hyperstriati accessorii (comparable in many ways to the striate cortex of mammals). The tecto-fugal pathway passes from retina to optic tectum (comparable to the superior colliculus of mammals) to nucleus rotundus thalami (comparable to the nucleus lateralis posterior of mammals) to the ectostriatum of the telencephalon (comparable to the circumstriate neocortex of mammals). Lesions in the tectofugal pathway have resulted in severe visual losses that eventually recover after extensive retraining. Lesions of the thalamofugal pathway have resulted in more modest losses that do not improve, even after extensive retraining. Combined lesions of the two pathways result in extremely severe losses that subside to the level of the thalamofugal lesions after extensive retraining. These data suggest that these pathways are not carrying information per se, but are serving to modulate information that is carried by one of the other outputs of the optic tectum or by one of the other cell masses receiving terminations of the optic tract such as the pretectal complex.

A series of experiments is being carried out on the behavioral effects Δ 9 tetrohydrocanabinol (THC), which is the principal active compound in marihuana.

Monkeys performing in an auditory discrimination task (click frequency) are given THC orally by injecting the drug into an orange slice which is then given to the animal. Doses will be given over a broad range, and the effects of chronic administration of relatively high doses will be studied. Preliminary results indicate that the minimum effective dose is about 1 mg/kg, and that tolerance to the drug develops rapidly. In addition, it appears that the rate at which the animals perform the problems can be affected with no noticable change in the accuracy of the discriminations. Urine samples from these animals are being collected for use in the Department of Biochemistry, WRAIR, which is studying the metabolism of THC.

A pigeon was trained to discriminate between a white noise of 65 db and one of 90 db. After learning this discrimination to a criterion of 90% or better for three consecutive sessions, the bird was required to make successive discriminations between 65 db and intensities ranging from 90 to 68 db. From the data obtained, the difference threshold for intensity discrimination was calculated. After the difference threshold had stabilized, THC was administered orally. Preliminary data indicate that doses as large as 800 μg/kg, caused periods of inactivity, but when responding, the pigeons' threshold performance was unaffected. The results of this experiment and the one preceding suggest that THC does not affect auditory mechanisms, but may alter the motivational state of the subject.

Rhesus monkeys held in restraining chairs in light tight booths were trained to respond within 10 seconds in the presence of a dim light to avoid a brief electrical shock. Once avoidance was well trained, a response in the presence of the light avoided the shock and decreased the intensity of the light programmed for the next trial by .20 log unit. A failure to respond in the presence of the light resulted in a brief electrical shock and increased the stimulus programmed for the subsequent trial by .20 log unit. Using this method the monkey's visual detection threshold was tracked over long periods of time. After stable baseline performance was established, THC was administered to the monkey orally. Preliminary data suggest that at dose levels of less than 1.5 mg/kg (in ethanol) the visual detection threshold is unaffected when compared to placebo days. At 1.5 mg/kg, the detection threshold is elevated. However, this dose is approximately 15 times the quantity of THC in one marihuana cigarette.

Fighting can be readily and reliably elicited from pairs of albino rats by electric shock delivered to the feet of both through a grid floor. Tests at three different current levels have showed a monotonic, nearly linear, increase in the probability of fighting as shock intensity is increased. THC in doses of 64 and 200 micrograms/kilogram produced no change in the probability of fighting, but dosages of 400 and 800 μg/kg appear to produce higher levels of fighting than placebo injections.

This increase may be secondary to an increase in activity or a change in shock sensitivity as a result of the drug. These possibilities will be investigated as well as the effects of higher doses of THC.

Recent pharmacalogical studies indicate that anti-malarial compounds routinely given to troops may cause met-hemoglobinemia in some individuals. This condition is accompanied by symptoms of muscle cramps and reports of fatigue. In this study, the visual detection threshold of a Rhesus monkey was determined. Once a stable baseline was established, the monkey was given prophylactic doses of primaquin (15 mg once a week). Preliminary data indicate this dose of primaquin did not produce met-hemoglobinemia in the Rhesus monkey studied. In addition, no alteration in the vigilance behavior was observed. We are currently studying the effects of therapeutic doses of primaquin (3 mg daily) upon vigilance performance. These studies may provide a model to determine whether anti-malarial compounds have any deletarious effects upon motivation and vigilance.

A baseline procedure has been adapted for use in monkeys to permit assessment of effects of administration of both therapeutic and non-therapeutic drugs on complex behavior that can be characterized as "decision making." This baseline measures the animal's ability to maintain optimal performance during changing work requirements. The baseline procedure will now be employed to evaluate the effects of THC and anti-malarial agents on decision processes.

Some of the interrelations between procedural variables and discriminative functions are being investigated with monkeys. In the initial part of the study, a "yes-no" psychophysical procedure is in effect in which the animals are reinforced in the presence of one brightness level for responding on one lever, and in the presence of a second brightness level for responding on a second lever. Fixed and variable ratio schedules of reinforcement of this discrimination problem will be compared. A later comparison will involve the use of "two-alternative-forced-choice" procedure. The primary purpose of this experiment is to investigate the effects of procedural variables in the study of sensory function in non-human animals, as a means of interpreting the effects of drugs on discriminative performance.

Rats have been studied using a "yes-no" psychophysical procedure. The animals are trained to discriminate between flashing lights of different rates. Variables being manipulated are differences between flash rates, probability of reinforcement of correct responses on either lever, and a priori probability of presentation of the stimuli. Data are being collected on the behavior during transition from one experimental condition to the next. In addition, this procedure is in effect 24 hours daily, and data are being gathered on the work cycle of the animals. It is planned to use this procedure as a baseline for the study of THC and anti-malarial drugs.

A study is being carried out to establish a behavioral baseline involving a temporal discrimination which can be used for a study of THC effects. In a "yes-no" procedure, monkeys are reinforced on one lever following a stimulus of 60, 80 or 90 sec duration, and on a second lever following a 100 sec stimulus. An index of detectability is being determined for each animal. After the data stablize, THC will be administered. This will provide a laboratory test of the clinical observations regarding the effects of marihuana on time discrimination.

An effort is being made to develop an animal model for the social phenomena of cooperation and the subordination of one's own interests to the needs of other group members. If this model is successful, it could serve as an useful means of evaluating the influence of drugs, hormones and stress on overall group effectiveness. Two monkeys are currently being trained in a situation which requires them to choose between two ratio schedules of reinforcement. One ratio is fixed at 120, while the other is a progressive ratio which starts at one and increases in steps of ten responses after each reinforcement. Choice of the fixed ratio 120 resets the progressive ratio to one. Performance on this schedule should be stable very soon, at which point a new contingency will be added: Each lever press during the fixed ratio 120 schedule will occassionally produce electric shocks for the other monkey of the pair. If the cooperative interaction is effective, the monkeys will choose the contingency that results in more work for themselves in order to avoid delivering shocks to their partners. These social effects will be assessed under the influence of THC and anti-malarial agents.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 03, Psychiatry

Work Unit 025, Analysis and management of behavior and stress

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA OA 6455	70 06 30	DD-DR&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 07 01	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	61102A	3A061102B71R	03	026
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code) (U) Analysis of Behavior and of Mediating Mechanisms - Psychophysical and Electrophysical Data Correlation (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
012900 Physiology 013400 Psychology - Indiv

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	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	5	110
FISCAL YEAR CURRENT 70	4	125

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21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Perception; (U) Sensory Neurophysiology: (U) Psychophysiological Correlates; (U) ERG: (U) EEG; (U) Visual Perception; (U) EMG

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

23. (U) This research is concerned with the perceptual determinants of behavior as these are reflected in the relationship between sensory input and neurophysiological function. Emphasis upon the visual system as a model for such psychophysiological interactions provides the basis for analyzing central and peripheral mechanisms involved in differential sensitivity to the environment and attentional factors in perceptual performance.

24. (U) Psychophysical methods and electrophysiological recording techniques are utilized to correlate sensory input from the environment with behavioral and neurophysiological output in the analysis of mediating mechanisms in perceptual performance. Microelectrode and gross electrode electroencephalography, electroretinography, and behavior control procedures are integrated through the application of computer programming to data analysis.

25. (U) 69 07 - 70 06 Objectives of the work unit have been consolidated with those of 3A062110A823 00 031 which is reported under DA OA 6456.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

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PROJECT 3A061102B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 03, Psychiatry

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

Investigators.
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Description.

This research is concerned with the perceptual determinants of behavior as these are reflected in the relationship between sensory input and neurophysiological function. Emphasis upon the visual system as a model for such psychophysiological interactions provides the basis for analyzing central and peripheral mechanisms involved in differential sensitivity to the environment and attentional factors in perceptual performance.

Progress.

1. Validation of electroretinogram for assessment of visual receptor function.

The electroretinogram (ERG) is widely used as a clinical tool for objective assessment of ocular visual function. It is especially useful because it may be recorded from the intact eye and therefore does not require risky surgical procedures. Since the ERG is a mass response from the entire eye, questions have been raised as to its relation to the activity of specific receptors within the retina. Since this question cannot be tested directly on human eyes, it has been pursued experimentally using an animal model. One of the ways of characterizing visual receptor activity is by their spectral sensitivity; in the human eye there are a number of receptor classes, each with a particular spectral sensitivity. Consequently, we have experimentally obtained data permitting the derivation of spectral sensitivity functions in an animal model, the Limulus dorsal ocellus. The Limulus model not only has the advantage of permitting both mass and single cell recording, but also has two distinct receptor classes whose spectral sensitivities are well separated. Furthermore, its neural organization is considerably simpler than that of the multi-layered human retina, thereby aiding analysis of fundamental characteristics of receptor activity. Recording ERG's from the

corneal surface of the dorsal ocellus in Limulus Polyphemus has shown that the dorsal ocellus contains two spectral mechanisms, one in the near ultraviolet region (360 nm maximum) and another in the visible (530-535 nm maximum) region of the spectrum. This information was derived originally from ERG data and subsequently tested directly by recording from single receptor cells within the eye. It was hypothesized that the two spectral mechanisms were due to the presence of two visual pigments housed in two kinds of visual cells. The slow receptor potentials elicited by illumination were recorded intracellularly with micro-electrodes in the visual cells and the spectral sensitivity of these cells determined. Two classes of visual cells were encountered. Sixty percent of the receptor cells responded only to the near-ultraviolet part of the spectrum, while another group responded to the visible part of the spectrum. In the second group, 5% of the cells responded both to the near ultraviolet and the visible part of the spectrum, but the sensitivity in the near ultraviolet was lower than that in the visible. On an average the overall sensitivity of near ultraviolet cells was about 1.5 - 2.0 log units more than that of the visible cells. Thus, our earlier findings of two spectral mechanisms based on the ERG in Limulus dorsal ocellus can now be attributed primarily to these two classes of visual cells. The ERG, a mass response, is presumably a weighted average response of all the visual cells in the ocellus. When the average spectral sensitivity curves of the two classes of receptor cells are put together, they closely approximate the spectral sensitivity curve obtained from ERG's with respect both to the two wavelength maxima (360 and 530 nm) and to the respective heights of the sensitivity of the two maxima. From the ERG data certain waveform differences were found to be associated with the presumed spectral mechanisms. These hypotheses are being pursued at the single cell level. The research thus far completed supports the use of the ERG as a way of assessing visual receptor function.

2. Physiological and behavioral spectral mechanisms in a vertebrate.

An important parameter of visual performance is the wavelength sensitivity of the neural-behavioral perceptual mechanisms. In order to pursue this problem into the central nervous system it is necessary to utilize an animal preparation from which microelectrode recordings may be obtained and on which experimental brain lesions may be performed for subsequent behavioral analysis. Rana have been used for this purpose since a great deal of prior information on Rana is available, including much of the fundamental visual pigment work. Using carefully controlled light stimuli (wavelength, intensity, duration, stimulation rate) a large number of recordings have been made from single neural cells

in various parts of the diencephalon. Some of the data have been gathered to assess the sites from which special short-wavelength sensitive activity may be obtained. From these sites data have been gathered to indicate the spectral response functions of individual cells. In addition, more extensive series have now been run which permit the establishment of spectral sensitivity functions. These are especially useful since they permit quantitative comparisons with similar data from other kinds of experiments, e.g., behavioral, ERG, visual pigment, eye cells, etc. The data have been recorded on magnetic tape so that detailed analysis may be made with the aid of computer techniques. In order to assist this procedure a special window discriminator has been developed and is now being fabricated after successfully being tested in the breadboard mode. Experimental lesions in these areas have been given a preliminary test in preparation for more extensive behavioral testing which is designed to evaluate the actual functional significance of the areas being investigated.

3. Opthalmologic hypoxia studies.

A study was done to further elucidate the effects of hypoxia on the absolute visual threshold, dark adaptation, and the critical flicker frequency.

The subjects were made hypoxic by breathing a tank mixture of 90% nitrogen and 10% oxygen.

A modified Goldmann-Weekers adaptometer was used to make the measurements.

Hypoxia raised the absolute threshold of both cone and rod vision. The effect was greater on peripheral rods (45° eccentricity) than on either central rods or cones (5° eccentricity). Cone thresholds were elevated to a greater degree than rod thresholds in the same retinal area (5° eccentricity).

The first 4 minutes of both cone and rod dark-adaptation were not affected by hypoxia. The later cone and rod segments of the dark-adaption curve, however, were elevated.

The effect of hypoxia on critical flicker frequencies at scotopic and at photopic light intensities were studied. Both the rod and the cone segments were decreased but there was not a significant difference in the magnitude of the decrease in the two functions.

4. Electrophysiological studies of brachial muscle function.

A series of experiments to determine the time relationship between electrical activity and mechanical output of human brachial biceps muscle has been completed. Subjects were asked to sinusiodally

vary isometric tension in the muscle between 0 and a known level by following a visually displayed target. Three maximum tension levels, 1.5, 3.0, and 7.5 Kg. were used. The frequencies of the tension output were 0.05, 0.1, 0.2, 0.5, 0.75, 1.00, 1.5, 2.0 Hz. The phase relation between the electrical activity of a few motor units (as recorded by bifilar fine-wire electrodes previously described) and gross tension output was measured. Results are as follows:

a. Electrical activity leads mechanical activity by a phase angle varying from 0° at .05 Hz to about 90° at 2.0 Hz under 0 to 1.5 Kg load.

b. Phase angle varies from 0° at .05 Hz to about 60° at 2.0 Hz under 0 to 7.5 Kg load.

c. Phase shift is roughly linear with log frequency.

These results suggest that this muscle moves significantly even under external isometric load, and that this motion is heavily damped. This viscous damping is reduced as more motor units are recruited. Isometric tension that varies at a rate exceeding one cycle in 10 seconds is thus not isometric, as a steady state is never reached.

Summary and Conclusions:

During the six-month period before this work unit was merged with Work Unit 031, several significant studies were completed. The correlative investigation of electroretinography and single cell recordings in Limulus Polyphemus has closed a gap in understanding relationships between electroretinograms and activity at the single cell level. The ERG is interpreted as amass response which is a weighted average of all the visual cells in the Limulus Polyphemus ocellus. The ERG can therefore be considered as a measurement of visual receptor function. The work on study of spectral response functions of individual cells in the diencephalon of Rana has also been of significance in pursuing relationships among the various levels of visual system organization and their relationships to visual functioning. Hypoxia effects on the visual system were found to include increments in the absolute threshold, both for cone and rod vision, but not rate of dark adaptation. Critical flicker-fusion thresholds were reduced, both in the rod and cone ranges of illumination. The electrophysiological studies of brachial muscle function have revealed that electrical activity precedes mechanical activity by a phase angle which is increased as a function of increasing rate of sinusoidal contraction and relaxation. This phase shift appears to be roughly linear with log frequency. The studies accomplished under Work Unit 026 have had value not only in relation to their scientific results, but also in their methodological contributions which are now receiving utilization in study of more clinically-oriented problems.

PROJECT 3A061102B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 03, Psychiatry

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

Literature Cited.

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PROJECT 3A061102B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01
Biochemistry

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6430	70 07 01	DD-DR&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 07 01	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	61102A	3A061102B71P	01	070
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Biochemical Variations during Disease and Treatment (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012900 Physiology 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 06	CONT	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 70	9	140
FISCAL YEAR CURRENT 71	13	195

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Protein Synthesis; (U) Enzymes; (U) Genetic Interrelationships; (U) Gene Isolation

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

23. (U) The technical objective of this work unit is to provide a program of differentiating the biochemical response between the host and infecting organism of those diseases of military medical importance. Particular emphasis is paid to the relationships of the nucleic acids and to enzymatic responses associated with infectious diseases.

24. (U) Macromolecular separations and characterizations, sequential analysis of nucleotide components, isotopic precursor incorporation and degradation will be used to study host infecting organism interrelationships. Bacterial and viral model test systems will be employed.

25. (U) 69 07 - 70 06 Change in emphasis has been introduced to expand the interdisciplinary nature of the effort. DNA synthesis and replication, RNA synthesis and function and protein synthesis and regulation have been collated, established and defined in a number of bacterial test systems. tRNA and its specific characterization along with comparative studies of different types of tRNA have been undertaken. Isolation and characterization of the tRNA gene is imminent. The transcription of the gene function is being initiated. The nature, frequency, and configuration of binding sites have been investigated. Particular emphasis has been given to drug interaction at binding sites in order to establish structure function interrelationships. The activities within this work unit are to be consolidated into a single work unit, 070. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 070, Biochemical activity in health and disease

Investigators.
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Associate: D. J. Brenner, Ph.D.; B. P. Doctor, Ph.D.; G. R. Fanning, M.S.; A. A. Faulkner, B.S.; CPT M. J. Fournier, Jr., MSC; M. A. Sodd, B.S.

Description.

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

1. The mechanism of protein synthesis

2. The role of nucleic acids in protein synthesis, the mechanism of transcription, translation and transfer of the genetic information.

3. The homology and heterology of nucleotide sequences in various species of DNA and its correlation to the pathogenesis of these species. This in turn will provide basic knowledge about the host-parasite relationship at the genetic level.

4. The regulatory function of various macromolecules under normal and abnormal conditions of cellular growth.

5. Interaction of antimetabolites with the macromolecules such as proteins, nucleic acids and the enzymes.

6. Isolation and *in vitro* characterization of structural and functional genes.

Progress.

The outstanding contribution from this work unit during this past year has been the isolation and characterization of a group of genes responsible for the synthesis of tRNA in *E. coli*. This landmark accomplishment will permit us to understand for the first time exactly how the DNA

functions as a storehouse of genetic information. It will further facilitate experimentation that will lead to understanding of basic concepts of *in vitro* synthesis of RNA and also furnish the answers to many questions which have baffled the scientists who are concerned with the understanding of the mechanism of protein synthesis.

Attempts are being made at the present time to synthesize *in vitro* nascent tRNA using these well characterized genes as a template.

1. Relationships.

We have established (Kingsbury, Fanning, Johnson and Brenner, 1969) that the pathogenic species of *Neisseria* are very closely related, probably due to the severe selection pressure of the environment on the divergence of obligately parasitic organisms. Included in this study were the causative organisms of meningitis and gonorrhoea. The pathogens were distantly related to the nonpathogenic *Neisseria* strains tested and the nonpathogens showed wide divergence among themselves. These studies were carried out in collaboration with D. Kingsbury of the Naval Research Laboratory.

Our main effort in this area is a continuing investigation of nucleic acid relationships within the *Enterobacteriaceae* or enterobacteria. The enterobacteria contain an extremely diverse group of organisms. One group contains plant pathogens and all of the other genera are pathogenic to man and animals either routinely or at least occasionally. Many of these organisms are also carried as normal intestinal, nasopharyngeal or skin flora. Included are causative agents of bacterial pneumonia, some meningitis, enteric fever, bacterial dysentery, food poisoning, bacteremias, diarrheal and enteric disorders.

Thus far our results are based on the relation of enterobacteria to three strains of *Escherichia coli*. By varying the stringency of our deoxyribonucleic acid (DNA) reassociation conditions, we have been able to assay both closely and distantly related nucleotide base sequences. In addition we have been able to assay the amount of divergence in related nucleotide sequences by determining their stability relative to the stability of homologous base sequences. Our results show that the *Escherichia coli* strains tested, both by DNA reassociation and by computer taxonomy form a more diverse group than previously suspected for a species. It appears that organisms with as little as 75% closely related DNA fall into the same species. The Alkalescens-Dispar group, in our hands, is virtually indistinguishable from *E. coli* strains. Strains of *Shigella* species appear to form another species group, closely related to but distinct from *E. coli*. The other groups in the enterobacteria fall into two categories based on polynucleotide sequence relationship with *E. coli* DNA. These organisms contain usually 7-12% of sequences

closely related to E. coli. This group includes the Citrobacter species, Aerobacter, Klebsiella, Salmonella, Bethesda group and some Erwinia species. The second category contains organisms showing 25% or less distantly related sequences and less than 5% closely related sequences. These organisms include the Proteus and Serratia organisms and some of Aerobacter, and Klebsiella species. Also in this group are most of the Erwinia species tested.

It is not surprising that a group with such a diverse habitat and so many free living or inobligately parasitic forms shows such a high degree of divergence. It is interesting that the Erwinia (plant pathogens) group retains in some cases, as much relationship to E. coli as does Aerobacter. This may again reflect a common denominator in pathogenic organisms. It appears that the individual genera within the enterobacteria have diverged only slightly. This seems especially true for the largely pathogenic genus Shigella and organisms normally found in intestinal flora. These groups must all be investigated further (see Brenner, Fanning, Johnson, Citarella and Falkow 1969, Brenner and Fanning, 1969). These studies were, in part, a collaborative effort with S. Falkow of Georgetown University.

2. Host-parasite interaction.

The problem of virus-host interaction has been attacked in a bacterium-bacterial virus (phage) system. This system was chosen as it is the best studied host-parasite system and the system most amenable to meaningful study at the molecular level. It is hoped that the results obtained from this system will serve as a prototype for studies with animal viruses and other intracellular parasites. It was previously established (see Brenner and Cowie, 1967; Cowie and Brenner, 1968) that temperate coliphages had several striking characteristics in common: they are heterogeneous in DNA base composition; their DNA molecules show a significant degree of interspecies reassociation, but they do not react with DNA from virulent coliphages; they show at least 20% relatedness to E. coli. Experiments recently carried out (Yehle and Brenner, 1970) indicate that temperate subtilis phage SPO2 DNA is not heterogeneous in base composition and is not extensively related to its host, Bacillus subtilis. A small amount of relatedness would not have been detected but it is clear that the high extent of reassociation seen in enterophage-E. coli DNA reactions does not occur in SPO2-B. subtilis reactions. SPO2 was not related to either of the virulent subtilis phages tested.

The amount of DNA held in common between temperate enterophages and E. coli is not contained in a contiguous region of the phage genome and does not appear to be necessary for virus integration into the bacterial genome. It is possible that relatedness between these enterophages and their hosts is due to some severe selection pressure that prevents divergence or that the conservation of this DNA results

from a continuing interchange of DNA between the host and phage. In any case, neither of these mechanisms operates between B. subtilis and SP02. If these organisms were once related they are "old" enough to have diverged almost completely. The data obtained with SP02 DNA indicate that neither heterogeneity in DNA base composition nor extensive polynucleotide sequence relatedness is a universal requirement for intracellular parasitism between a virus and its host cell.

3. Methodological advances in the speed, reproducibility and sensitivity of the hydroxyapatite assay system for separating double- and single-stranded nucleic acid molecules.

We have instituted several changes in methodology designed to increase our output, reproducibility and sensitivity in separating double- and single-stranded nucleic acids on hydroxyapatite. The first of these was to develop a batch technique (Brenner, Fanning, Rake and Johnson, 1969) that allows us to process five times as much material as we could using column separations. The increase in output is achieved without significant loss in sensitivity or reproducibility.

Hydroxyapatite is a modified form of calcium phosphate. It deteriorates with time and becomes reconverted to what is known as brushite. There is a marked decrease in specificity for nucleic acids when deterioration occurs. The nonspecificity can be decreased by boiling before use. It has been shown that the hydroxyapatite can be regenerated by reconverting it from the brushite stage in its preparation. It is more convenient to use sodium lauryl sulfate to overcome the specificity problem. In addition we have been able to bind transfer ribonucleic acid to hydroxyapatite in relatively high salt. This had not been achievable earlier and it paves the way for the isolation of the DNA cistrons that specify transfer ribonucleic acid (tRNA). We have devoted a significant portion of our time during the past year to these methodological improvements.

4. Isolation purification and characterization of tRNA genes.

Many questions regarding the mechanism of protein synthesis need to be answered. Some of them are:

a. What factors control the rate and extent of nucleic acid and protein synthesis?

b. Regarding tRNA, how are these tRNA cistrons arranged in the genome?

c. What is the nature of initiation and termination sites?

d. What is the function of modified bases in tRNA, and

e. Is the nascent tRNA (gene product) active biologically?

One of the best ways to answer these questions is to isolate tRNA genes and study these parameters in vitro.

This goal was accomplished and the details are as follows:

^{32}P labeled DNA was isolated and sheared by pressure to an average size of 1.25×10^5 Daltons. The sheared DNA was incubated at high temperature with large excess of highly purified E. coli tRNA and allowed to reassociate with its cistrons. This is done under controlled conditions that were ideal for the complete hybridization of tRNA and DNA. The DNA/DNA duplex and DNA/tRNA hybrid were separated from single stranded DNA and tRNA by column chromatography at 60° on hydroxyapatite. By repeated (3-5) chromatography on hydroxyapatite 0.04 to 0.05% DNA was isolated which was hybridized to tRNA. From the hybridization of tRNA with fixed (filter) DNA the value of 0.04 to 0.05% hybridization value has been reported. Thus the isolated cistrons obtained by our procedure is pure. This further implies the tRNA genes are contiguous. The isolated DNA/tRNA hybrids were treated with RNase T1 to remove tRNA. The tRNA cistrons were then reacted with unlabeled DNA isolated from E. coli and from P. mirabilis. More than 80% of the label reassociated with E. coli DNA. The reassociation with P. mirabillis DNA was approximately 70% relative to the E. coli. The stability of the proteus reaction indicated that most of the isolated DNA is specific as the reaction between bulk E. coli and P. mirabillis is less than 10% and is markedly unstable. In addition these results show that tRNA cistrons have been preferentially conserved with respect to the bulk enterobacterial genome. The reassocation of E. coli tRNA cistrons with rat liver tRNA was less than 3% and that of E. coli ribosomal RNA was less than 7%. This shows that the rat liver tRNA and E. coli ribosomal RNA cistrons are not closely related to the E. coli tRNA cistrons.

Experiments are in progress to further characterize these genes, determine their biological activity, structure and physical properties, and finally to synthesize tRNA and tRNA genes in vitro.

5. X-ray diffraction studies on tRNA crystals.

E. coli $tRNA_f^{met}$ has been crystalized by (a) ammonium sulfate, (b) dioxane, (c) 2-methyl-2,4-pentanediol methods and their cell dimensions and other X-ray diffraction studies are being pursued in collaboration with Dr. D. R. Davies of the National Institutes of Health. Yeast $tRNA^{Phe}$ has also been crystallized by 2-methyl-2,4-pentanediol and X-ray diffraction. Studies are in progress. In order to pursue these studies E. coli $tRNA^{val}$, $tRNA_f^{met}$, and $tRNA^{tyr}$ are purified in large quantities. Yeast $tRNA^{val}$, $tRNA^{tyr}$, $tRNA^{Phe}$ are also obtained in purified form and in large quantities.

6. Mechanism of protein synthesis.

Whether there are two, three or four sites involved in ribosomal function in protein synthesis was conclusively determined. This work was done in collaboration with Drs. D. J. Roufa and Philip Leder of National Institutes of Health, Bethesda, Maryland.

A number of useful models for protein biosynthesis specify that two tRNA's are bound to the ribosome prior to the translocation reaction. One of these tRNA's is thought to be the most recently recognized aminoacyl-tRNA and the other, the nascent peptidyl-tRNA. Reasonable modifications of the two site models have been suggested in which additional sites are required either for initiation or sequential alignment of oncoming aminoacyl-tRNA's. In order to distinguish between these multisite models we have used mRNA derived from the bacteriophage f_2 and have taken advantage of two degenerate serine codewords which, by analogy to the related virus R17, occur in the third and second positions of the coat and RNA polymerase cistrons, respectively. Further, we have employed a purified E. coli protein synthetic system which differs from others in that translocation is completely inhibited by the addition of small amounts of anti-G factor antibody. Under these conditions only the dipeptides initiating f_2 coat, maturation and RNA polymerase proteins, Fmet-ala, Fmet-arg and Fmet-ser, respectively, are synthesized.

Since the initial sequence of f_2 coat protein synthesized in vitro is Fmet-ala-ser-aspn. . . . and synthesis is limited to the initial dipeptide, the binding properties of the first pre-translocation intermediate permit us to distinguish between two or three (or greater) site models for ribosomal function. We have previously reported that unfractionated Fmet-, ala, and ser-tRNA's are bound to ribosomes in the presence of f_2 mRNA, whereas aspn-tRNA is not. A fourth binding site is thus eliminated, and the question arises as to whether the ser-tRNA binding is in response to the third codon of the coat cistron (UCU) or the second codon (UCG) of the polymerase cistron of the coat cistron (UCU) or the second codon (UCG) of the polymerase cistron.

We were able to distinguish between recognition of these degenerate serine codons using two countercurrent fractions of ser-tRNA. One fraction responds only to the codon UCU (ser-$tRNA_{UCU}$); the other to UCG and UCA as well as to UCU (ser-$tRNA_{UCG,A,U}$). Incorporation of serine from these tRNA's into initial f_2 coat and polymerase peptides is consistent with the nucleotide sequence data obtained from the related bacteriophage, R17, by Steitz. Further, of the two ser-tRNA's only ser-$tRNA_{UCG,A,U}$ is bound to the ribosome prior to translocation. Its binding can only be in response to the second polymerase codon, UCG, since the third coat codon, UCU, is not available for the binding of ser-$tRNA_{UCU}$. The result is consistent with the binding of no more than two tRNA's to the ribosome prior to translocation.

7. Interaction of antimetabolities with macromolecules.

For the aminoacylation of tRNA by aminoacyl tRNA synthetase, Mg^{++} is known to be an absolute requirement. Mg^{++} also stabilizes the conformation of tRNA. It is established the tRNA-synthetase interaction is dependent on the conformation of tRNA. Thus systematic studies of Mg^{++} requirement will throw some light as to the nature of interaction between proteins and nucleic acids.

Polyamines, or organic amines such as chloroquine, also affect the conformation of nucleic acids. Thus it is possible that the polyamines may be able to replace Mg^{++} requirement in the above mentioned reaction. In the E. coli system, spermine or spermidine can replace Mg^{++} requirement for the amino acylation of $tRNA_f^{met}$ and $tRNA^{val}$. However, this is not the general phenomenon, since in the case of yeast $tRNA^{tyr}$ or $tRNA^{Phe}$ amines have no effect on replacement of Mg^{++} requirement.

In the case of yeast $tRNA^{Phe}$ it was previously shown that using E. coli enzyme it is not possible to charge this tRNA under normal conditions. However, in the presence of physiological amount of amines it is possible to charge yeast $tRNA^{Phe}$ with E. coli synthetase. This clearly shows that amines alters the conformation of tRNA, thereby making it possible to interact with heterologus enzyme.

The role of amines and other antimetabolites in aminoacylation of tRNA is pursued at the present time.

8. Structural studies with macromolecules.

a. As a supporting unit for Army wide activities, approximately 15 determinations for serum proteins were carried out. These include sedimentation velocity runs with Model E ultra centrifuge. The main studies dealt with the quantitation of serum lipoproteins.

In addition, density gradiant centrifugation, using succrose, cesium chloride, cesium sulfate and alkaline succrose were carried out for the analysis of tRNA, ribosomal RNA, native and denatured DNA. The Model E runs on these samples were also performed to estimate the S values.

b. Regulatory function of macromolecules under normal and abnormal cellular growth conditions. Changes in tRNA patterns detected by column chromatography have been seen in various metabolic situations. Thus far, detection of new tRNA species resulting from amino acid starvation has been limited to methionine. In this case, the new subspecies are methyl-deficient. We wish to report the production of new tRNA's due to leu deprivation of E. coli (RC^{rel}). Leu-tRNA from leu-starved cells has an additional species (5-20% of total leu-tRNA) not present in tRNA of

non-starved cells. Formation of the new species requires RNA synthesis since its appearance is prevented by leu starvation of stringent control cells or uracil starvation of RC^{rel} cells. While the new species has chromatographic properties similar to a methyl-deficient species of leu-tRNA, the inability of leu-starved tRNA to accept methyl groups *in vitro* argues that it is not methyl-deficient. Leu starvation also produces new arginine (arg) and histidine (his) tRNA's, while deprivation for arg or his doesn't result in the formation of new species of tRNA for leu, his or arg. The change in patterns of arg, his and leu-tRNA induced by leu starvation suggests that leu has a specific metabolic effect on species of tRNA whose codons begin with C. The absence of detectable chromatographic changes in other tRNA's examined supports this hypothesis.

Summary.

Relationships among pathogenic bacteria and between pathogens and non-pathogens was studied. The enterobacteria fall into two groups; one contains organisms showing 30-50% distantly related sequences which have diverged some 15-20% from *E. coli* DNA and contain usually 7-12% of sequences closely related to *E. coli*. The organisms in this group include *Citrobacter* species, *Aerobacter*, *Klebsiella*, *Salmonella*, Bethesda group and some *Erwinia* species. They have 25% or less distantly related and 5% or less closely related sequences.

The genes responsible for the transcription (synthesis) of tRNA from *E. coli* were isolated, purified and characterized using DNA/tRNA hybridization and purification of hybrids by hydroxyapatite column chromatography. $tRNA_f^{met}$ from *E. coli* and tRNA from yeast have been crystallized and their three dimensional structure is being determined using x-ray diffraction techniques.

Positive evidence of the two site model for the ribosomal function in protein synthesis have been elucidated.

It is shown that spermine or spermidine can replace Mg^{++} for the aminoacylation of $tRNA_f^{met}$ and $tRNA^{val}$ in *E. coli*. However, these amines have no effect on the aminoacylation of $tRNA^{tyr}$ or $tRNA^{phe}$ from yeast. The change in patterns of arginine histidine- and leucine-tRNA induced by leucine starvation suggests that leucine has a specific metabolic effect on species of tRNA whose codons begin with C.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 070, Biochemical activity in health and disease

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6460	70 06 30	DD-DR&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 07 01	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	61102A	3A061102B71P	01	071
b. CONTRIBUTING				
[redacted]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Biochemical Variations in Abnormal Health States (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 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	4	70
FISCAL YEAR CURRENT 70	4	70

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Carbohydrates; (U) Lipids; (U) Proteins; (U) Enzymes; (U) Vitamins and Hormones

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

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

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

25. (U) 69 07 - 70 06 Application of developed methodology has been extended to a variety of clinical abnormalities to include hepatitis, cardiovascular disease and the problems of fat embolism. A profile of analyses has been assembled that provides information on the extent of the injury produced and the expected recovery. Studies have been initiated to examine a number of clinically abnormal profiles and attempts are being made to establish changes that are indicative of the beginning of the abnormality. Genetically monitored abnormalities are critically considered. Separations by ultracentrifugation has been compared to separation by electrophoresis on paper as well as agar gel. For routine use, the methods of electrophoretic separation on agar gel appear to be of greater applicability. Techniques developed by the group have been applied to the newborn and a compendium of these results are being prepared. Activities within this work unit are to be consolidated into a single work unit, 070. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval

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Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 071, Biochemical variations in abnormal health states

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Description.

The technical objective of this work unit is to examine the variations in biochemical entities as a result of abnormal health states. Activities with the work unit include the following sub-units:

1. Methodology development.

The examination of physiological fluids requires a broad program of methodology screening and development. Each method is studied for its reproducibility, reliability and adaptability to automatic analysis.

2. Protein, lipid and isoenzyme analysis.

Agarose gel electrophoretic analysis coupled with the estimation of lipids, cholesterol, lactic dehydrogenase and creatine phospho kinase are applied to a variety of clinically defined diseases.

3. Microanalysis and application to the newborn.

Biochemical tests are adapted to microlevels and applied to the comparative biochemistry of the newborn.

4. Role of inorganic ions in the physiological chemistry of man.

A variety of inorganic ions particularly those associated with sub-cellular components and physiological fluids are evaluated by either atomic absorption spectrometry or by the use of the ion specific electrode in order to understand their mode of action in the physiological environment.

Progress.

1. Methodology development.

During the reporting period, a variety of analytical procedures have been established, evaluated and inter-compared with other similar procedures. Serum lipid analysis continues to occupy a prominent role in analytical biochemistry with respect to heart disease and arteriosclerosis. A comparative study between agarose gel electrophoresis and acrylamide gel electrophoresis in the separation of lipoproteins has been completed. The results of this study indicate that both methods are of equal efficiency in separating the alpha lipoproteins but agarose gel electrophoresis gives more information on the total lipid fractionation. A micro procedure for cholesterol that eliminates the interference of high serum bilirubin has been established.

Methods for plasma histamine, catecholamine and cortisol have been refined and applied to pharmacological studies with bee venom and endotoxin in the presence of WR 2823, an antishock drug. Detail of these efforts will be reported by the Division of Medicinal Chemistry. These methods are also being applied to a variety of studies being performed in the Department of Gastroenterology, Division of Medicine and will be reported by that Division.

Procedures for the measurement of ATP have been established and standardized. These analytical tools are to be applied to a study of muscle metabolism in the biochemical definition of malignant hyperpryexia. Hydroxy proline analyses are being carried out for studies involving malaria patients.

2. Protein, lipid and isoenzyme analyses.

Lipid profiling and integration with serum protein electrophoresis, immunoelectrophoresis and discrete enzymic tests has led to a screening procedure that promises to be of value in those diseases of the cardiovascular system where the lipoproteins are implicated. Use of this system in coordination with the Cardiovascular Service, Walter Reed General Hospital, has proven its value as a diagnostic tool.

Lactic acid dehydrogenase isoenzyme activity has been shown to be associated with heat stress. The system defined above has been applied to studies of this condition in recruits. The results of these studies can be expected to provide valuable information leading to the biochemical consequences of this problem. The isoenzyme system is being extended to other types of shock models.

Characteristic lipoprotein patterns are evident in various forms of human liver disease. Differences in the type of disease can be made

by careful resolution and definition of these lipoprotein patterns. Combination of this type of biochemical separation with one or more immunological test may strengthen diagnosis.

3. Microanalysis and application to the newborn.

A series of biochemical tests have been adapted to the microchemical level. The procedures included uric acid, acid and alkaline phosphatases, total protein, albumin, bilirubin, blood urea nitrogen, glucose, calcium, magnesium and phosphorus. Each procedure was standardized and evaluated for reliability and reproducibility.

The methods have been applied to a series of normal newborn infants during the first four days of life. Comparisons within the experimental sample could be made with respect to sex and race, as well as between individual days after birth.

The results of this study have pointed out differences hitherto unknown. The only variable that showed no statistically significant difference was magnesium. The remaining differences are summarized in Table 1.

Table 1.

Comparative Differences in Biochemical Variables in Newborn Infants

Variable	Race	Sex	Differences Between Days
Total Protein		X	X
Albuminum		X	X
Glucose		X	X
BUN			X
Bilirubin			X
Calcium	X		X
Alkaline Phos.	X		X
Uric Acid	X		X
Inorganic Phos.		X	X

These results are being assembled for publication.

4. Role of inorganic ions in the physiological chemistry and biochemistry of man.

a. The measurement of serum ionized calcium in normal and disease states, and during therapy. It is well known that calcium homeostasis is one of the most closely regulated of parameters in living organisms because of the multiple and vital functions that this element serves. The determination of total serum calcium concentration has served as the mainstay in the assessment of normal and abnormal states. However, calcium in serum exists in equilibrium in three forms: bound to proteins, complexed with organic and inorganic acids and as the free ion. The percentage of the ionized form, while constant under normal physiologic conditions, changes drastically when the protein concentration, the anion concentration and the pH of the blood are altered, either singly or jointly. Hence measurement of the total serum calcium is not an accurate means for determining the concentration of ionized calcium. Since the ionized form is the species that is active physiologically and biochemically and is the moiety that is in equilibrium with calcium stores in tissues, the availability of a method for rapid, direct and accurate determination of serum ionized calcium is highly desirable. In this regard, we have been able to operate successfully, a recently-developed, flow through calcium-ion specific electrode system to measure serum ionized calcium concentrations in a variety of conditions.

1) Using this technique more than 300 sera from volunteers and from blood donors from the Blood Donor Clinic of Walter Reed General Hospital have been examined. The normal ranges of $[Ca^{++}]$ in serum is 1.20 ± 0.10 mM (mean $\pm$ 2 S.D.). Hyper- and hypoparathyroid states have been readily identified. In hyperparathyroidism, ionized calcium concentration was consistently and significantly elevated even when total serum calcium values were only equivocally abnormal. We are currently also examining the usefulness of this method in the diagnosis and management of patients with hypercaluria, and other disease states.

2) The method of blood collection (e.g. vacutainer vs. syringe), storage, and the effect of tourniquet ligature and stasis upon ionized calcium concentration have also been examined in order to define the acceptable limits in sample collection and processing.

3) Banked blood using ACD anticoagulant contains virtually no ionized calcium--all of the calcium is complexed with the large excess of citrate ions. Hence the effect of rapid and multiple transfusions upon serum ionized calcium was studied. The first condition examined was exchange transfusions in newborns. The use of ACD blood lowered serum ionized calcium drastically. Calcium administration in the form of $CaCl_2$ or calcium gluconate in the doses currently recommended did not significantly raise serum Ca^{++}, although total serum Ca increased. In contrast, when heparinized blood was used in exchange, serum Ca^{++}

remained unaltered throughout the procedure. These studies emphasize the marked and acute alteration of calcium metabolism during transfusions with ACD blood and the need to monitor this parameter during rapid and massive transfusions of plasma and whole blood. Further studies are in progress.

b. The role of inorganic ions in the structure, composition and function of erythrocyte membranes. The structure and composition of cellular membranes is currently a subject of intensive investigation. In our studies of the human erythrocyte membrane, we have found that, in addition to proteins and lipids which are the major components of the membranes, there are present also significant quantities of firmly bound metal ions. Erythrocyte ghosts prepared by hypotonic lysis showed the presence of 100-200 ug Zn, 500-600 ug Ca, 90-150 ug Mg, and 25-40 ug Cr per gram dry weight of membranes. These data suggest that metal ions may play a role in the structural integrity of the membranes. While Ca has been shown to be critical for the structural integrity of erythrocyte membranes in some species, a similar role for other metal ions has not been demonstrated thus far. In this regard, erythrocyte membranes are also known to bind Ca^{++} when it is added exogenously. Recently published and our own data indicate that this interaction of calcium and other inorganic cations with membranes is important in the binding of small organic molecules to the membrane. By means of a fluorescence probe (toluidinyl-naphthalenesulfonate) we have shown that in the absence of an electrolytic solution, erythrocyte membranes do not bind the dye, and that the nature of the cation profoundly influences the binding. Thus divalent cations such as Ca, Mg, Zn are much more effective than monovalent cations in promoting dye binding and monovalent cations such as Al and La are even more effective. The interaction of the cations presumably neutralizes the negative charges of the membrane, thereby facilitating hydrophobic interactions. Such studies are pertinent to the study of the binding of drugs, hormones, and other organic molecules to membranes and their transport across cellular membranes.

Summary.

The work unit represents a broad, diverse work area that encompasses methodology development, lipid, protein and isoenzyme assay, microanalysis and the biochemical action of metal ions. The methodology development aspect represents the continued effort to review, standardize and employ the best of biochemical technology.

Lipid analysis and protein assay procedures have been established and applied to a variety of medical problem areas. Microanalysis of a number of biochemical variables have been studied in newborn infants. Efforts have been initiated to study calcium under a variety of conditions. Metal ion concentrations in membranes have been evaluated.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 071, Biochemical variations in abnormal health states

Literature Cited.

1. Papadopoulos, N.M. and Kintzios, J.A.: Determination of human serum lipoproteins by agarose gel electrophoresis. Anal. Biochem. 30:421, 1969.

2. Papadopoulos, N.M., Bloor, C. and Standefer, J.: Effects of exercise and training on plasma lipics and lipoproteins in the rat. J. Applied. Physiol. 26:760, 1969.

3. Jordan, W.J. Jr. and Knoblock, E.C.: A micromethod for total serum cholesterol that eliminates interference by high bilirubin concentrations. Clin. Chem. 16:19, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6437	70 06 30	DD-DR&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	9a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	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	61102A	3A061102B71P	01	072
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c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Biochemical Laboratory Automation Systems (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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17. CONTRACT/GRANT: NA
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
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e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	4	100
FISCAL YEAR CURRENT 70	6	100

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Biochemistry; (U) Automation; (U) Clinical Chemistry

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

23. (U) The technical objective of this work unit is to provide a modular system for the sequential analysis of physiological fluids and tissues over a wide range of environments. Particular attention is paid to reliability, precision, and the reduction of output data to useable format.

24. (U) Automatic wet chemical and analytical modules are developed on mobile carts for specific assignments. Each system is critically examined for quality control and the absolute limits of reliability and precision. Design characteristics are such that maximum flexibility is obtained.

25. (U) 69 06 - 70 06 The institution of a system analysis approach has gone far in development of the system. Each project assigned is considered on the basis of the required chemistry and the means to accomplish it. Methods are developed to fit the mission with the highest level of quality control. Profiles or multiple analytical tests are an important part of the effort to attain maximum flexibility. Analytical systems designed include tests for specific drugs such as diaminodephenyl sulfone. A lipid profile system to include triglycerides, free fatty acids, phospholipids and cholesterol is under study. Enzyme measurement for methemoglobin reductase is nearing completion. The use of ion specific electrodes particularly for calcium measurements has been established. Toxicological examination of fluids and tissue through a developed system employing ultraviolet spectrometry, gas liquid chromatography and mass spectrometry continue to provide information on various foreign materials. These data are being assembled into an information bank that will be made available to Army Medical Department laboratories on an as-required basis. Activities within this unit are consolidated and reported under 3A061101A91C 00 170. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

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Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 072, Biochemical laboratory automation systems

Investigators.
Principal: LTC C. R. Angel, MSC
Associate: B. G. Bass, B.S.; J. I. Davis, B.S.;
L. Kayzak, B.S.; R. T. Lofberg, Ph.D.;
J. E. Matusik, B.S.; R. C. Permisohn, B.S.;
MAJ J. B. Powell, MC

Description.

The automation of analytical procedures in order to provide chemical data on large numbers of samples continues to occupy a prominent role in analytical chemistry. The ultimate objective of this work unit is to provide a modulized unit of maximum capability coupled with the highest level of flexibility. In addition to the analytical train, interfacing of the equipment to data reduction and processing equipment forms a subjective part of the work unit. Applied research efforts under this work unit include:

1. Analytical toxicology.

Gas liquid chromatography, ultraviolet and infrared spectroscopy and the mass spectrometer are interfaced and multiplexed to data reduction equipment that provides the input to a central processing unit. Programs are available to process the data in terms of retention time and retention index.

2. Wet analytical chemistry.

The Technicon Autoanalyzer forms the basis for development of multiple analytical systems that provide analyses for the divisions of the WRAIR on an as-required basis. Quality control systems have been developed for each procedure in terms of reliability and precision.

Progress.

1. Analytical toxicology. (Mr. Kayzak, Mr. Permisohn)

The activity of the analytical toxicology group during the reporting period is divided into the measurement of antimalarial drugs in human body fluids and the development of methodology for the automated system leading to compilation of characteristic drug data.

Eleven hundred urine specimens have been examined for their chloroquine levels as a part of the program at the Oklahoma State Penitentiary in the study of diformyl dapsone (DFD) distribution. This effort is in support of the Department of Pharmacology, Division of Medicinal Chemistry. Another aspect of this distribution study was to examine the urine for dapsone (DDS), alleged to be the principal urinary excretion product of DFD. In the urines examined to date, no evidence of the presence of DDS has been demonstrated. Continuing efforts are being made to define the urinary excretion patterns after oral administration of DDS and DFD.

The analysis of chloroquine in the plasma of individuals infected with strains of Plasmodium falciparum is being carried out in support of the British Medical Command in Malaysia. The samples of both the cells and the plasma are being examined by mass spectrometry for presence of intermediary metabolites of chloroquine. This work is somewhat hampered by the low levels of chloroquine found in the plasma.

With the completed development of an automated gas liquid chromatography system coupled with ultraviolet spectroscopy and mass spectrometry, the need for a dependable standardized data reference system to replace the retention time has been recognized. A study has been initiated to adapt the retention index (Kovats index) to a variety of compounds of toxicological interest. Using retention indices for three different columns as a basis, a computer identification program has been developed that will identify compounds of toxicological interest. The data bank accumulated to date consists of 163 types of compounds representing alkaloids, tranquilizers, narcotics, insecticides, antihistaminics, barbiturates and amines.

Another activity of the analytical toxicology group that is worthy of mention is the specific identification of toxic materials that have been accidentally or purposefully administered to soldiers. Each urine, plasma or other physiological tissue becomes an unknown that requires a screening for grouping followed by a specific identification within group. Results of such studies from whatever the source form a basis for medico-legal proceedings. Falling within this category are those compounds that form the group known as illicit drugs. Procedures under development are for specific identification for marijuana either as the parent compound or its metabolites.

2. Wet analytical chemistry. (MAJ Powell, Mr. Davis, Mr. Bass, Dr. Lofberg)

The activities of this group are logically subdivided into a service support function for the research divisions of the WRAIR and the developmental efforts necessary to maintain the service support function.

The service support activity is divided into two parts, one located in close proximity to Ward 30, WRGH, and the other in the main section of the Institute. The system centers about a modulized Technicon Autoanalyzer system coupled with a digitizer system designed to produce peak height analysis. The basic difference between this system and that of the standard clinical laboratory is its modularization that permits the units to be assembled and disassembled consonant with investigator need. The use of movable carts designed to contain up to four modules increases flexibility in permitting units to be moved to areas where the experimental requirements are located. During the reporting period, a total of 104,364 separate tests have been performed involving 34 different procedures. Each procedure has its own quality control system involving aqueous standards and natural or artificially fabricated plasma or serum controls. Statistical analysis of control data provides the usual population variables of mean, variance, standard deviation, standard error, and confidence interval. Each procedure is compared with its manual counterpart utilizing linear regression and correlation. Recovery studies are performed to determine process control and determine potential interferences.

Development and adaptation of biochemical test procedures to the Autoanalyzer is dependent of investigator need and requirement for large numbers of samples to complete research protocols. Each developed procedure is standardized and compared against the manual method, coupled to the digitizing system and a quality control program instituted. Particular emphasis is placed on the volume of materials required to adequately perform the test. As the majority of tests developed require one or more enzymes for endpoint completion, this attention to concentration is in the best interests of economy. During the reporting period, procedures for methemoglobin, methemoglobin reductase, diamino diphenyl sulfone, hydroxyproline, 2, 3 diphosphoglyceric acid and the triglycerides have been processed.

A series of experimental protocols have been designed to determine the most appropriate means of processing data generated by the wet chemistry group. Time sharing as typified by utilization of a terminal linked to a large volume central processor is one such experiment. The feasibility of central processor dedication is also in progress and it is expected that a unit of sufficient capacity will be available to cover the needs of analytical toxicology.

Summary and Conclusions.

The work unit has been a composite of service support function and methodology development in both analytical toxicology and wet chemistry.

The service support functions of the two working groups could best be funded out of Institute overhead as they serve the Institute as a

whole. Developmental aspects of the program should be expanded to include in addition to methodology development, informational processing, new instrumental approaches and extended biomedical engineering.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 072, Biochemical laboratory automation systems

Literature Cited.

Kayzak, L. and Permisohn, R. C.: Retention indices for compound identification by gas chromatography. Am. J. Forensic Sci. in press, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

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a. PRIMARY	61102A	3A061102B71P	01	073
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11. TITLE (Precede with Security Classification Code)

(U) Molecular Pharmacology of Chemotherapeutic Drugs (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 07	CONT	DA	C. In-House

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Molecular Pharmacology; (U) Antimalarials; (U) Quinine; (U) Berberine; (U) Naphthoquinones; (U) Distamycin; (U) Mechanisms of Drug Action

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

23. (U) This work is directly related to safeguarding the health of the Army against communicable diseases. Its objective is explanation of mechanisms of action of clinical or investigational drugs for the cure or prevention of communicable diseases of military importance, for example, malaria. From this: explanation of relationships between chemical structure and medicinal activity of drugs, formulation of principles for the design of new or improved drugs for which there is a military need, and ways to overcome natural insensitivity or acquired resistance to drugs in disease-producing microorganisms.

24. (U) Experimental studies at 3 levels of biological organization: 1. Biophysical studies on the interaction of drug molecules with their biopolymer sites of action. 2. Biochemical studies of the functional consequences resulting from the above interactions. 3. In vivo studies of the manifestations of the above events on the physiology and population dynamics of intact microbial cells.

25. (U) 69 07 - 70 06 The antibiotic, distamycin, forms a complex with DNA as shown by: 1. DNA-induced alterations in distamycin's absorption spectrum; 2. A DNA-induced Cotton Effect in the spectrum of distamycin; 3. Flow-dichroism of the DNA-distamycin complex; 4. Displacement of methyl green from DNA by distamycin; 5. Shift of the thermal strand separation profile of DNA to higher temperatures by distamycin. - A typical antimalarial naphthoquinone blocks uptake of uracil into susceptible Bacillus megaterium; this is one manifestation of a general effect of rendering susceptible organisms impermeable. - Quinine and berberine inhibit Pneumococcus; quinine is bacteriostatic while berberine is bactericidal. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 Jun 70.

* A WORD OR CONTRACTOR'S GRANT OR GRANTEE'S APPROVAL

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Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 073, Molecular pharmacology of chemotherapeutic drugs

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Description.

Experimental research studies in depth on the molecular pharmacology, biochemistry, biophysics, microbial physiology, and biochemical genetics of the actions of antimicrobial drugs, especially antimalarials, with a view to elucidating principles of modes and mechanisms of drug action at the primary level, explaining phenomena of acquired drug resistance and offering conceptual guidance to both improved methods of chemotherapy of infections with existing drugs as well as rational development of novel chemotherapeutic substances.

Progress and Results.

1. Mode of Action of Chloroquine. As the result of our published studies and those of others, it is now generally accepted that chloroquine forms a molecular complex with DNA and acts as a DNA template poison, inhibiting foremost the replication of DNA and, to a lesser extent, the transcription of RNA from DNA in susceptible micro-organisms, including plasmodia. The nature of the DNA-chloroquine complex has been further studied by measuring the transfer of energy in this complex. The effective distance over which the drug inhibits in DNA the formation of pyrimidine dimers by irradiation with uv of 254 mμ is 8 base pairs in double-helical DNA. Radiant energy absorbed by DNA is transferred to intercalated chloroquine as measured by the drug's fluorescent or phosphorescent emission. The phosphorescence measurements indicate the occurrence of triplet-triplet energy transfer which is predicated upon physical proximity of purine/pyrimidine energy donors, and the chloroquine acceptor; this furnishes independent proof of the validity of the intercalation theory of the drug's binding to DNA. Since the binding of chloroquine to DNA involves prominently the attraction of protonated centers of the drug molecule to negatively charged phosphates of the double helix, proton magnetic resonance studies have been carried out on chloroquine solutions with the view to determining which of the possible ionic species of the drug molecule interact with DNA. The analysis and pH dependence of the PMR spectrum of chloroquine

indicates that at pH 7.0 the heterocyclic ring exists as a pyridinium-like cation with delocalization of the charge-deficiency to the carbocyclic and heterocyclic rings as well as to the 4-amino nitrogen. This necessarily requires coplanarity of the 4-amino group and the quinoline ring. The large region of positive charge at the quinoline ring and the positively charged diethylamino group means that chloroquine exists as an asymmetric dipolar cation in aqueous solutions. Because of the repulsive forces between the positively charged centers of the chloroquine dication, the molecule would be expected to adopt a molecular conformation with the maximum separation possible between the charge centers.

2. Mode of Action of One Antimalarial Quinoline Methanol. Quinine, which we reported to form a complex with double helical DNA, is a natural quinoline methanol. Structure-activity rules suggest that synthetic quinoline methanol drugs will also bind to DNA. This was studied and proved for α-piperidyl-6,8-dichloro-2-phenyl-4-quinoline methanol. Spectrophotometric observations indicated that the synthetic drug forms a complex with DNA and spectrophotometric titration showed that, on the average, 10 times more of the synthetic drug is bound than of quinine. The quinoline methanol shifted the thermal strand separation profile of DNA to higher temperatures, i.e. it stabilizes duplex DNA; this should result in inhibition of DNA replication which requires separation of the component strands of the double helix. The binding of the synthetic drug to DNA resembles that of quinine and the greater antimalarial potency of the drug is probably the result of a larger number of molecules becoming bound to DNA. The drug, at low concentrations, also precipitates DNA which renders further biophysical studies difficult.

3. Mode of Action of One Antimalarial Naphthoquinone. Studies on the mode of action of 2-hydroxy-3-cyclohexylpropyl-1,4-naphthoquinone are almost complete. Results of previous studies had indicated that the drug exerts its bactericidal effect on Bacillus megaterium by selectively inhibiting RNA and protein biosynthesis. When deoxyadenosine promotes the extensive entry of exogenous thymidine into DNA, the addition of naphthoquinone also causes immediate and demonstrably complete inhibition of DNA biosynthesis. Naphthoquinone also inhibits cell wall biosynthesis, as evidenced by the failure of ^{3}H-diaminopimelic acid to become incorporated into cell wall mucopeptide. These results demonstrate that there is no gross preferential inhibition of macromolecular biosynthesis in B. megaterium.

The concurrent inhibition of protein, nucleic acid, and cell wall biosynthesis might be explained by an effect of the naphthoquinone upon energy generation or upon transport phenomena. Since permease-mediated transport and the generation of energy through oxidative phosphorylation are membrane-associated functions, the inhibitory site of naphthoquinone might be the cell membrane. Hence, experiments were conducted on the uptake and localization of

tritiated naphthoquinone in B. megaterium. The addition of tritiated naphthoquinone to exponentially growing cultures resulted in an immediate and saturating uptake of radioactive label. Titration studies on resting cell suspensions using ^{3}H-labeled naphthoquinone revealed an uptake curve of sigmoid character suggesting that the drug binds tightly and specifically to the inhibitory site. When growing cells were incubated with ^{3}H-naphthoquinone and treated with lysozyme, 65 per cent of the radioactivity resided in the protoplasts. The membrane fraction, obtained by osmotic lysis of the labeled protoplast preparation, contained all the radioactivity. Furthermore, ^{3}H-naphthoquinone remains bound to hot trichloroacetic acid precipitated material suggesting that naphthoquinone is covalently bound to the membrane.

The observed inhibitory effects of naphthoquinone might result from interference with transport of added precursors. To test the possibility that naphthoquinone was inhibiting transport into the cell, the total uptake of ^{14}C-uracil, as well as its conversion into acid-insoluble material, was measured at short time intervals. At bactericidal concentrations of drug, the bacterial uptake of labeled uracil was immediately and completely arrested.

In addition, the chasing of radioactive uracil from the cells by excess nonradioactive uracil was also prevented.

4. Mode of Action of Nitroakridin 3582. Studies on the mode of action of Nitroakridin 3582 (NA) are nearing completion. NA is a potent antibacterial and antirickettsial drug resembling quinacrine in its structure (1-Diethylamino-3-[(2,3-dimethoxy-6-nitro-9-acridinyl) amino]propanol). NA inhibits, strongly, multiplication of both gram-negative and gram-positive bacteria, induces lysis of two gram-positive species, Bacillus licheniformis, and Micrococcus lysodeikticus, and induces formation of filaments of gram-negative organisms. NA induction of filament formation was traced by isotope incorporation studies to the ability of NA to inhibit DNA synthesis more effectively than it inhibited either RNA or protein synthesis. A bacteriostatic condition was induced by the inhibition of DNA synthesis at low concentrations of NA. At higher concentrations ($>10^{-4}M$), the bacteria lost viability exponentially; this death was accompanied by cessation of RNA and protein synthesis as well as of DNA synthesis.

In vitro observations of the effect of NA on synthesis of DNA, RNA, and protein were consistent with those of cell studies. NA inhibited cell-free synthesis of DNA by DNA polymerase more effectively than it inhibited synthesis of RNA by RNA polymerase. The ED_{50}s for inhibition of both cell-free and cellular DNA synthesis were lower than the ED_{50}s for inhibition of RNA and protein synthesis, both cell-free and cellular. In addition, adsorption isotherms showed NA to bind very strongly to DNA consistent with intercalation.

NA was also found to inhibit synthesis of polyphenylalanine, that is, to inhibit in vitro model protein synthesis. Such inhibition was consistent with the finding that NA, similar to other acridines, binds to all three classes of RNA engaged in protein synthesis. Adsorption isotherms showed NA to bind strongly to transfer RNA. NA also inhibited the function of tRNA through suppression of charging of tRNA by amino acids and ATP, and by inhibition of binding of aminoacyl-tRNA to messenger-encoded ribosomes. Single-stranded polyribonucleotides used as messenger RNA in synthetic amino acid incorporation systems altered the absorption spectrum of NA, indicating that NA binds to these compounds. NA also binds to ribosomes, as evidenced by previous observations that NA reduces the thermal stability of ribosomes. Thus, NA inhibition of polyphenylalanine synthesis can potentially occur on any or all RNAs engaged in protein synthesis.

Despite the ability of NA to inhibit both RNA and protein synthesis in vivo and in vitro, suppression of cell multiplication correlates most closely with inhibition of DNA synthesis, and the mode of action of NA is attributable to its binding to DNA, regardless of other physiological and biochemical effects.

5. Mode of Action of Distamycin A. The antibiotic distamycin is known to inhibit the induction phase of inducible enzyme synthesis in bacteria but not the subsequent synthesis of enzymes after induction. Distamycin also has antiviral and antitumor properties, and has been found to inhibit the DNA polymerase reaction in vitro. This suggested that the antibiotic might form a complex with DNA. This hypothesis was verified by the following results: (1) DNA produces changes in distamycin's absorption spectrum which suggest that individual chromophores bind to DNA. (2) Distamycin stabilizes DNA to thermal strand separation. (3) Distamycin displaces externally bound methyl green from its complex with DNA by a second order reaction. (4) Flow dichroism of the distamycin-DNA complex indicates that the N-methylpyrrole chromophores of distamycin are not intercalated into DNA but oriented parallel to the long axis of the double helix. (5) Double-stranded as well as single-stranded DNA induces multiple Cotton Effects in the ORD spectrum of distamycin.

6. Mode of Action of Berberine. We have shown previously that berberine forms a complex with DNA by intercalation. Further experiments have characterized the DNA-berberine complex: (1) Instability of the complex in high concentration of urea and low concentration of salt suggests that the interaction of berberine with DNA involves both the formation of urea-sensitive bonds and electrostatic attractions between ionized groups. (2) Spectrophotometric and spectrophotofluorometric titrations of berberine with DNA show that berberine binds to more than one class of sites in DNA. For the stronger binding process, an apparent association constant between 5 and 20 x 10^5 M^{-1} and a stoichiometry of 1 berberine molecule per 10 DNA bases was obtained. Weaker binding process involves the

maximum number of binding sites for a planar ligand binding to DNA by intercalation, i.e. one berberine molecule per two DNA base pairs with an apparent association constant of $1 \times 10^5\ M^{-1}$. (3) Enhancement of the intrinsic viscosity of DNA by berberine also shows that berberine is intercalated into DNA. (4) Changes produced in berberine's absorption spectrum in the presence of single-stranded DNA and tRNA suggest that berberine also binds to these polynucleotides. In vivo studies are in progress to elucidate the mode of action of berberine on micro-organisms.

The test strain, Diplococcus pneumoniae, ATCC #9163, was inhibited by a concentration of 2.5×10^{-4}M. When the organisms were exposed to a growth inhibitory concentration, the drug exerted a strong bactericidal effect. The number of colony-forming bacteria decreased by at least three orders of magnitude during one generation time.

Radioisotope studies showed that there was no incorporation of phenylalanine ^{14}C and thymidine ^{14}C into the cells in the presence of berberine, thus indicating that there was complete cessation of DNA and protein synthesis. However, when the medium contained uridine ^{14}C, there was approximately 10 per cent incorporation of the label into the cells in the presence of the drug. Apparently the cells synthesized a small amount of RNA.

When E. coli were grown in the presence of subinhibitory concentrations of berberine for a prolonged period of time, a phenomenon was exhibited that we have previously reported for quinacrine. The bacteria showed various stages of filament formation and pleomorphism.

7. Mode of Action of Quinine. In vivo studies on the mode of action of quinine advanced slowly because of the long search for a suitably sensitive test organism. Initial studies were carried out with Bacillus megaterium which was moderately sensitive to the drug. Exposure of the bacteria to growth inhibitory concentrations of quinine was bactericidal. Quinine caused complete inhibition of protein and DNA synthesis. RNA biosynthesis was also inhibited and there was a slight loss of RNA from the cells. The effect resembled that produced by chloroquine in B. megaterium. An attempt to study the bacteriostatic instead of the bactericidal effects was unsuccessful because of too many day to day variations in the narrow concentration range at which quinine was bacteriostatic. The strain of Diplococcus pneumoniae, ATCC #9163, is susceptible to quinine at a concentration of 3.1×10^{-5}M.

When a growth-inhibitory concentration of quinine was added to this strain in exponential growth, bacteriostasis resulted. Effects on protein synthesis, DNA synthesis, and RNA synthesis were investigated by measuring the incorporation of carbon-14 labeled

phenylalanine, thymidine and uridine, respectively, into the polymers of quinine-treated bacteria. Following the addition of quinine, all three biosyntheses were reduced to the same extent, that is, by approximately 85 per cent.

Bacteria which had been exposed to quinine for a generation time were centrifuged, washed three times, and resuspended in fresh medium. These organisms resumed growth immediately.

Conclusions.

The intercalation model of the chloroquine-DNA complex has been further substantiated by measurements of energy transfer in the complex. A representative antimalarial quinoline methanol, like quinine, forms a complex with DNA and stabilizes the double helix. A representative antimalarial naphthoquinone leads to cessation of all macromolecular biosyntheses in a test bacterium. The drug becomes covalently bound to the bacterial membrane and impairs active transport across this membrane. The broad-spectrum synthetic antimicrobial drug, Nitroakridin 3582, forms complexes with nucleic acids and inhibits DNA synthesis strongly, and RNA and protein biosynthesis appreciably. At bacteriostatic concentrations it produces filament formation. At bactericidal concentrations, all tested macromolecular biosyntheses are blocked. The antiviral antibiotic, Distamycin A, forms a molecular complex with DNA by binding to the periphery of the double helix. Berberine is bactericidal for pneumococcus and inhibits DNA and protein biosynthesis completely while permitting some RNA biosynthesis. Quinine is bacteriostatic for pneumococcus and inhibits DNA, RNA, and protein biosynthesis to the same large extent.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 073, Molecular pharmacology of chemotherapeutic drugs

Literature Cited.

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10. Fean, C.L., and Hahn, F.E.: Spectrophotometric studies of the interaction of an antimalarial quinoline methanol with DNA. Abstr. 9th Intersci. Conf. Antimicrob. Agents & Chemotherapy 57, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE 6453	70 07 01	DD-DR&E(AR)436

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. OMB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	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	61102A	3A061102B71P	01	074
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Molecular Basis of Biological Regulation (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
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FISCAL YEAR — CURRENT 71	2	50

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code) (U) Chloramphenicol; (U) Dapsone; (U) Blood dyscrasias; (U) Episomes and R-factors; (U) Antimutagens; (U) Drug Resistance

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

23. (U) Selected studies with the common objective of rendering the drug treatment of communicable diseases of military importance either (1) safer, or (2) effective in those instances in which multiple drug resistance is encountered or emergence of resistance to individual drugs is predictable.

24. (U) Experimental studies on (1) influence of chemotherapeutic drugs on the development, differentiation, and maturation of human blood cells in culture, (2) elimination of circular DNA episomes from bacteria by DNA-complexing compounds and determination of the fate of episomal DNA, (3) nature of complexes of biologically active compounds, for example, antimalarials, with circular DNAs, (4) molecular genetics of the action of quinacrine and other antimutagens and of their apparent ability to block microbial mutations to drug resistance in vitro.

25. (U) 69 07 - 70 06 Quinacrine, ethidium, acridine orange, chloroquine, and marginally, miracil D and quinine eliminate the F lac episome from E. coli at sub-bacteriostatic concentrations. Episomal DNA does not disappear entirely but is found to be reduced in quantity when estimated by its content of radioactive thymine. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 Jun 70.

*Available to contractors upon originator's approval

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Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 074, Molecular basis of biological regulation

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Description.

A new work unit comprised of experimental research studies in molecular biology concerned with the physiology, biochemistry, biophysics and molecular genetics of the title processes with a view to elucidating mechanisms underlying biological events of special importance to military medicine, for example, currently, the biological role of supercoiled DNA in micro-organisms, the influence of chemicals on DNA's function, the toxicity of chemotherapeutic drugs, or the mechanism underlying the formation of polyglutamate in bacilli.

Progress and Results.

1. The Relationship of Colchicine to DNA. The literature contains one report of a synergistic curative effect of colchicine and quinine in the treatment of falciparum malaria; another paper reported a mutual influence of DNA and colchicine upon each others specific optical rotation. Colchicine is a well-known mitotic poison whose structure is remindful of that of the antibiotic, anthramycin, which is a DNA complexing drug. In our hands, double- or single-stranded DNA failed to alter the absorption spectrum of colchicine or to induce a Cotton Effect in the optical rotatory dispersion spectrum of the alkaloid. Colchicine did not stabilize DNA to strand separation by heat. While we did reproduce the observation of nonadditivity of the specific rotations of colchicine and DNA, these observations alone are not sufficient to conclude that the cytological effects of colchicine are produced in general by reaction with DNA

2. Biosynthesis of Polyglutamic Acid by Bacilli. A D-polyglutamate capsule is the determinant of the pathogenicity of Bacillus anthracis. Bacillus lichinoformis synthesizes the same polymer but excretes it into the liquid medium. It has been previously reported from this laboratory that this biosynthesis is inhibited by one of the stereoisomers of chloramphenicol but not by the natural antibiotic; this discovery has been considered a clue to the mechanism of action of chloramphenicol. Others have subsequently developed a crude cell-free system from B. lichinoformis which produces polyglutamate in vitro. Studies are in progress to resolve the in vitro reaction sequence of polyglutamate formation. The most interesting result so

far is the discovery that this biosynthesis of a homopolymer resembles protein biosynthesis in that it is mediated by bacterial ribosomes.

3. Probes of DNA Structure and Interactions. The formation of cyclobutyl dimers, foremost thymine dimers, induced in DNA by light of 254 mμ is not only mutagenic or lethal but also affords a structural probe for the interaction of DNA with substances which interfere with photodimerization. Additionally, DNA-sensitized fluorescence of substances binding to this nucleic acid is another physical method of probing the structure of DNA vs. that of substances interacting with the polymer. The intercalating compound, ethidium bromide, and the nonintercalating dye, methyl green, both inhibit the formation of pyrimidine dimers in DNA by ultraviolet radiation. The critical interval over which this effect extends is 20 base pairs for methyl green and 17 base pairs for ethidium. Exterior binding of Cu^{++} to DNA increases photodimerization but interior binding of Cu^{++} to amino groups of DNA bases, following local strand separation, decreases dimer yield by holding the DNA strands apart. Probing the copper-DNA complex by physical methods is important because of the multiple mutagenic effect of Cu^{++} on many cells.

The sensitization spectrum for ethidium fluorescence (at 580 mμ) in the DNA complex resembles the absorption spectrum of DNA. Each dye molecule receives energy absorbed by about four base pairs. The significance of these DNA probing studies lies in the fact that the theoretical principles and experimental approaches which have been developed can be used to investigate the interaction between DNA and substances which regulate DNA's function, such as chemotherapeutic drugs which form DNA complexes.

Conclusions.

Substances which form complexes with DNA inhibit the photodimerization of thymine in the double helix. This effect is produced by intercalative substances as well as by those which bind to the periphery of DNA. A figure β can be determined for each complexing compound which gives the effective length of double helical DNA over which energy transfer, as indicated by photodimerization, is interrupted. It is possible that the figures for β are related to the extent to which DNA complexing molecules appear to exclude each other's occupancy of the double helix. Colchicine, proposed by others to interact with DNA, gave no such indication in a variety of experimental tests. D-glutamyl-γ-polypeptide is polymerized by a cell-free system of Bacillus lichinoformis which requires the ribosomes of this organism.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 074, Molecular basis of biological regulation

Literature Cited.

1. Sutherland, B.M., and Sutherland, J.C.: Inhibition of pyrimidine dimer formation in DNA by cationic molecules: role of energy transfer. Biophysical J. 9:1045, 1969.

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PROJECT 3A061102B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03
Entomology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	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 A	9. LEVEL OF SUM
69 07 01	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	61102A	3A061102B71P	03	035
b. CONTRIBUTING				
c. [redacted]	CDOG1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Ecology and Control of Disease Vectors and Reservoirs (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 005900 Environmental Bio 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	CONT	DA	C. In-House

17. CONTRACT/GRANT Not Applicable

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 70	3	60
CURRENT FISCAL YEAR 71	1	20

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Arbovirus; (U) Trypanosomes; (U) Ecology; (U) Mosquitoes; (U) Vectors (&) Tsetse flies; (U) Control; (U)

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

23. (U) Studies emphasize control of vectors of arbovirus and parasitic diseases of military significance. Objectives are incrimination of vectors and understanding of host-parasite relationships initially, understanding of vector biology and disease transmission mechanisms ultimately in order to develop more effective control procedures.

24. (U) Invertebrate vectors and vertebrate reservoirs and hosts are collected in areas of known disease activity. Infection rates are determined, as are flight ranges, blood meal sources, breeding habits, and other biological characteristics. Other biological processes, such as pathogen transmission, flight physiology, and diapause are studied in the laboratory.

25. (U) 69 07 - 70 06. Over 300,000 mosquitoes collected in an area in Maryland endemic for arbovirus diseases. 19 isolations of EEE and WEE viruses obtained all from Culiseta melanura. Larval breeding habits and seasonal distribution determined for dominant mosquito species of area. Factors responsible for infection of 2 species of tsetse flies with trypanosomes have been partially determined. Blood meal source of 200 engorged mosquitoes of 2 species determined. Five species of mosquitoes tested for flight ability on mechanical flight mill. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

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Project 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03, Entomology

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

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Description

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

Progress

1. Eastern Maryland Arbovirus Study

a. Introduction. A program to investigate the ecology of arthropod-borne virus diseases in swamp habitats of eastern Maryland has been underway for several years by investigators at WRAIR. Until this year, the emphasis has been on isolation of virus agents from invertebrate and vertebrate animals. This year, intensive entomological studies were begun, with an emphasis on population dynamics and feeding, oviposition, and flight habits of the most abundant species occurring in the swamp, and especially those species yielding virus isolates. Strictly virological aspects of this program are reported under Project 3A061102B71Q, Communicable Disease and Immunology, Work Unit 166, Viral Infections of Man. For background of the overall study, and a detailed description of the study site, see WRAIR annual reports for previous years.

b. Objectives. The entomological aspects of the study sought to study the possible role of species other than Culiseta melanura Coquillett as vectors of mosquito-borne virus diseases in the Pocomoke Cypress Swamp. In addition to evidence of present involvement as vectors, evidence was sought pertaining to factors which relate to potential involvement. These factors include: flight range, seasonal occurrence, susceptibility to virus infection, host preference, daily activity patterns, and larval breeding habitats. Until these factors can be fully evaluated for the most abundant species occurring in endemic areas,

the likelyhood of involvement of other vertebrates, including man, in virus transmission under altered ecological or climatic conditions cannot be assessed.

c. Methods. Sites were chosen for study in and around a fresh water swamp adjacent to the Pocomoke River a few miles downstream from Pocomoke City, Worcester County, Maryland (Fig. 1). Seven major habitat types (macrohabitats) occurring in the area were identified and chosen for location of collection sites. The microhabitats chosen were: closed swamp (floating root mat), open swamp, upland forest, marsh, farmland, upland forest-marsh transition area, and upland forest-closed swamp transition. In most instances there are no clear-cut boundries between the macrohabitats - they tend to merge gradually into one another. There is no sharp line of demarcation, for example, between the closed swamp and the open swamp, although each is clearly identifiable in its characteristic form. The topographic features which determine the type of swamp are not known, but the open swamp is the wetter of the two. The marsh, farmland, and upland forest habitats are clearly definable and have reasonably sharp interfaces. Between the upland forest and the marsh, on the one hand, and between the upland forest and the closed swamp on the other, the interface is vague, and the areas have been designated by us as transitional. The former transitional area is subject to occasional flooding by brackish water due to tidal action, the latter by fresh water from precipitation.

The macrohabitats were sampled weekly for mosquito larval breeding and also for presence of adult mosquitoes. Collection techniques used included light traps baited with dry ice, Shannon traps, human biting collections and sweeping collections. Engorged females were identified and frozen for virus isolation after the abdomens had been cut off and saved for precipitin test identification of the blood meal. Unengorged females were identified and frozen without dissection. Collection records were coded on a standard machine data punching format (WRAMC Form 1200) and all data were stored on magnetic tape for machine data processing.

d. Results

(1) Mosquito population studies

(a) Larval surveys

During the period February 1969 to January 1970, 589 collections of mosquito larvae were made. Table 1 lists the species collected in the 7 macrohabitats. The relative frequency and density of a particular species of mosquito is only indirectly dependent upon the primary features of the macrohabitats chosen for study. These macrohabitats vary chiefly in topography which determines drainage and thus vegetation. The distribution of mosquito larvae is dependent upon the relative abundance of particular types of microhabitats. These microhabitats often occur in several or even all of the microhabitats

but their abundance varies greatly. Table 2 lists the collections of dominant mosquito species according to macrohabitat and microhabitat. The choice of categories of microhabitat was somewhat arbitrary. A brief description of each follows:

Temporary open pool. - Any accumulation of water that did not develop emergent vegetation. Some of these pools held water for up to 2-3 months, replenished by rain.

Crypt pool.- Usually formed at the base of a fallen tree, surface is at least partially covered by tree roots stumps, soil, or other natural debris. Water usually present year-around, but no emergent vegetation.

Semipermanent pools.- Like temporary pools, but with characteristic emergent vegetation. Water present most of year.

Open hole.- An artificial opening made for purposes of collection and dug into the root mass of the closed swamp habitat. About 2 ft. in diameter. Usually dry in mid-summer.

Covered hole.- An open hole covered by a piece of plywood.

Tree hole.- Naturally occurring cavity in a living tree.

Rot hole.- A cavity resulting from the rotting away of the stump and roots of a dead tree. Found at ground level.

Artificial container.- Any man-made object that will hold rain water.

Semipermanent ditch.- Same as semipermanent pool, but man-made.

Culiseta melanura larvae were most abundant in the closed swamp and in upland forest rot holes. They were scarce in the transitional areas and in the open swamp, were collected but once in the pasture, and never in farmland. Within the closed swamp, they were always found in the covered holes, nearly always in the crypt pools, frequently in the open holes, and infrequently in the temporary open pools. When found in open pools, they were found among dead leaves at the margin, and the pool was well shaded. During the cooler portions of the year, C. melanura larvae were usually associated with larvae of Aedes canadensis. In mid- and late summer, C. melanura larvae were commonly associated with larvae of the genus Culex.

Aedes canadensis larvae were found everywhere during the winter and spring months. They became scarce during the warm months until heavy rains occurred in mid-summer. Their numbers decreased again in late summer, then increased in the late fall.

The narrowest ecological amplitude observed was that of Aedes thibaulti. This species occurred only in rot holes in the upland forest habitat.

Culex territans were present from early May through December. They were collected in 6 of the 7 macrohabitats, but were most abundant in the closed swamp. Far fewer adults were captured than expected from the large numbers of larvae collected.

Culex salinarius also occurred from May to December. It was collected in both types of swamp, and in the upland forest-marsh transition area, which was subjected to some brackish water tidal flooding, but not in the upland forest-open swamp transition area, which was not subject to tidal flooding. It was also found in the farmland type of habitat.

(b) Adult collections

During the period March thru September 1969, 334,121 adult mosquitoes were collected and identified. 251,515 of these were females and were placed in pools of 25, frozen, and tested for virus isolation. Table 3 lists the numbers of each species collected in alphabetical order by month.

Collections from CDC light traps produced nearly 85% of the total. Vacuum sweeps accounted for 4.23%, Shannon traps, 3.82%, human baited collections 3.68%, resting boxes 2.34%; and miscellaneous collections 0.84%. Some minor species were captured less frequently in light traps than by the other methods. Among these were Anopheles quadrimaculatus and Culex erraticus, captured most often in resting boxes; and Culex territans, captured most often in vacuum sweeps. Vacuum sweeps provided essentially the same information on major species as did light traps (see Figs. 2 and 3), but tended to capture proportionately more specimens of those species not particularly attracted to light or CO_2. Vacuum sweep collections contained a higher percentage of engorged specimens than did light trap collections, but a greater proportion of specimens collected by vacuum sweeping were damaged than those collected by other means.

Resting box collections consisted largely of Culiseta melanura; followed by Culex territans, Anopheles quadrimaculatus and Aedes canadensis. These collections were made from 1 to 4 times a week, usually around 1200 hrs. Figure 4 gives the monthly average collection per resting box. Note that significant numbers of species other than C. melanura did not occur until after midsummer. This increase was due largely to An. quadrimaculatus and C. territans, two relatively minor species (see Table 3).

Early in the season, larger numbers of C. melanura per collection were obtained from resting boxes than from light traps, possibly related to inhibition of nighttime activity by cool evening temperatures at that time.

Figures for Shannon trap and human bait collections are not shown, as they consisted almost entirely of Aedes canadensis, and the curves did not differ greatly from those for Figures 2 and 3.

The great increase of mosquitoes occurring in August was largely a result of very heavy rains in late July and early August (see Fig. 2). These rains caused the water level in all parts of the swamp to return to, and in some cases exceed, that of early spring. This flooding allowed hatching of large numbers of Aedes eggs that otherwise might have lain domant until the following spring.

Table 4 compares trap-night captures of the 4 major species from the 7 macrohabits mentioned in the larval section. Largest numbers of Aedes canadensis were found in areas having abundant water after rains, especially if the water was shaded and the substrate was relatively firm. This included most of the closed swamp, the upland forest, and the upland forest-closed swamp transition areas. The upland forest-marsh transition and open swamp areas usually had water, and at least a fair amount of shade, but the substrate was usually mucky to some depth. No breeding activity was detected in the marsh, this area undergoing daily tidal inundations. Breeding appeared limited in the cultivated area primarily by a lack of shaded water.

Culiseta melanura was even more dependent on shade, as well as on a relatively permanent source of sheltered water. Areas having the lowest capture rate of C. melanura were characterized by a paucity of suitable breeding sites. One anomaly is the capture rate in the marsh, where no breeding was known to occur. Small numbers of C. melanura larvae were collected within a few hundred yards of the marsh traps. The marsh and farmland areas produced by far the largest numbers of Culex salinarius, in spite of an apparently lack of suitable breeding sites. In other areas, C. salinarius appeared more tolerant of pools with a mucky substrate and a lack of shade than did either A. canadensis or C. melanura.

Aedes cantator adults appeared fairly evenly distributed throughout the swamp, lower numbers occurring in the uplands and transitional areas, and fewer still in the marsh and farmland. Their breeding site requirements seemed similar to those for A. canadensis, the larvae of the 2 species often collected together in early spring. A. cantator did appear more tolerant of soft, mucky substrates than did A. canadensis.

Adults of most other species were collected in all the habitats, but some minor species were quite restricted in distribution. For example, Aedes aurifer and Uranotaenia sapphirina were seldom found outside the open swamp area.

In addition to the collections listed in Table 4, a few experimental light traps were run during daylight hours. In these collections, Aedes atlanticus, A. infirmatus and Psorophora ferox were proportionately

more abundant than in the night time collecting, while Culiseta melanura was seldom collected.

To date, 19 virus isolations have been made from Culiseta melanura collected during 1969. They include 12 confirmed WEE, 1 suspected WEE, 3 confirmed EEE and 3 suspected EEE isolates. Eighteen of the isolations have been from specimens from light trap collections, with a single isolate from a resting box collection. The ratio of light trap-collected to resting box-collected C. melanura was about 11:1, so this preponderance of isolates from light trap material is not too unusual. The first WEE isolate was from material collected 15 July, and all succeeding isolates were WEE until 12 August when C. melanura yielding EEE were first collected.

The initial isolation of each virus was made from C. melanura collected in the marsh, and succeeding ones from all macrohabits studied, with the closed swamp providing the largest number of isolates.

(2) Mosquito blood meal identification

Abdomens of engorged female mosquitoes were tested to determine the source of the blood meals. The testing technique used followed that of Tempelis and Lofy in which a precipitin test by the capillary tube method is performed with diluted blood from an engorged mosquito as the antigen source being tested against screening and specific antisera. Preliminary work consisted of collecting serum samples from the available hosts present in the swamp and subsequent preparation of screening and specific antisera. The screening antisera were prepared in rabbits and specific antisera in roosters. Acceptable antiserum titer was considered to be 1:10,000 with homologous antigen and no crossing with heterologous antigens at 1:1,000. Cross reaction between deer and bovine antisera has been eliminated by dilution, and hopefully deer-goat crossing can also be eliminated in a similar fashion. Table 5 shows the mammal-specific antisera prepared during the year.

A few engorged mosquitoes have been screened. The results of the blood-meal identification tests performed are shown in Table 6.

2. Basic Biology Studies

a. Studies of mosquito flight. During the year 4 flight mills were constructed by the Instrumentation Division, WRAIR, according to plans published by Rowley, et al. The objectives of the flight studies were:

(1) To determine whether gross differences in flight ability could be observed among different vector mosquito species. If specific differences exist, an attempt will be made to correlate such differences with differences observed in nature using mark-release techniques. The ultimate purpose of the correlation is to

determine the relationship between flying ability in terms of distance and natural flight range. The latter is of great epidemiological significance.

(2) To determine whether performance on a flight-mill can be used as a measure of quality of insectary rearing procedures.

(3) To determine the effect of pathogen infection (both mosquito pathogens and vertebrate pathogens) on flight mill performance.

(4) To determine whether flight-mill performance can be used as a quantitative assessment of the diapause state of the adult mosquito.

Initial work with the flight mills has been limited to perfecting orientation and attachment of mosquitoes to the mill arm. The species of mosquitoes which have been flown and the maximum distances flown are shown in Table 7.

b. Floodwater Mosquito Studies. The most abundant species of mosquito (Aedes canadensis) in the Pocomoke Cypress Swamp is apparently not involved in the transmission of eastern or western encephalitis virus. Because of the possible involvement in the transmission of Bunyamwera and California group agents by this and other floodwater Aedes, ecological studies of this group of mosquitoes was begun. In order to determine the types of ecological habitats preferred by females for oviposition, 18 soil samples (40-50 sq. in.) were taken at the Pocomoke Cypress Swamp at 3-foot intervals along a transect of a depression from which larvae of Aedes canadensis and other floodwater mosquitoes had been collected when the depression was flooded. The soil samples were comminuted in a Waring blendor and washed through a series of sieves (4.76, 2.38, 1.19, 0.35 and 0.15 mm openings). The material retained by the 0.15 mm sieve was further fractionated by differential flotation in saturated salt solution and in water. The material that sinks in fresh water was examined microscopically for mosquito eggs. Calibration trials with known samples indicate that this procedure recovers approximately 50% of the eggs present in the soil sample. In the transect described, samples 3, 10-13 and 16-18 were positive for floodwater mosquito eggs with a maximum density of 0.50 eggs/in^2 observed in sample no. 16. Most samples were divided in half and processed in two separate runs. It frequently occurred that one of the two half-samples contained eggs while the other did not, indicating that the eggs have an extremely localized and patchy distribution, presumably due to the oviposition behavior of the female mosquito. The eggs recovered have been preserved for identification.

The transect from which the samples were taken represented a gradual topographic transition from an upland habitat which became flooded only after heavy and persistent rains to ground which is normally flooded during part of the year after moderately heavy rains.

Figure 5 illustrates the location of the sampling sites in relation to the habitat type. These preliminary results indicate that along the transect study, maximum deposition of eggs occurred on soil which flooded only after particular heavy and persistent rainfall, minimally on soil which is annually flooded by spring melting of snow to form a woodland pool.

c. Photoperiod Studies. In an attempt to shed light on virus overwintering mechanisms, several species of mosquitoes which occur in the Pocomoke Cypress Swamp are being studied in regard to diapause physiology. Because strong evidence indicates that transovarian transmission of arbovirus by mosquitoes does not occur, the adult stage of the mosquito is the only possible vehicle for overwintering of virus if the virus of diseases such as WEE and EEE do indeed overwinter in the home of infected hibernating mosquitoes. Further, for an individual adult mosquito to be a likely carrier of virus from one season overwinter to the next, it would have to become infected during the warm summer months when the virus is active, and then remain alive during the fall, over the cold winter months, and into the following spring. It is unlikely that a species of mosquito would be adapted to this type of overwintering unless it was capable of undergoing a physiological diapause.

Preliminary studies have been made with Culex salinarius a common eastern Maryland mosquito. Parallel studies were run with Culex tritaeniorhynchus, a species known to undergo diapause in response to short daily photoperiod. Initial experiments involved study of the effect of photoperiod on blood feeding frequency and on subsequent ovarian development. Mosquitoes were reared under standard insectary rearing procedures (27°C, 16 hours daily photoperiod) and separated, upon pupation, into 2 experimental groups, and placed into low temperature incubators. One group was subjected to 20°C, 8 hours photoperiod, the other to 20°C, 16 hours photoperiod. Results of feeding trials and ovarian development rates for the 2 species tested are shown in Tables 7 and 8.

The differences in blood-feeding between the groups of Culex tritaeniorhynchus females are significant and demonstrate the well-known response of this species to photoperiod. The differences in blood feeding between the 2 groups of Culex salinarius are not significant ($X^2 = 2.62$, 1 df). Lack of ovarian development (genotrophic dissociation) was not observed in either group.

From these preliminary results, it would appear that Culex salinarius does not respond to short photoperiod, and probably does not undergo a physiological diapause. This conclusion is tentative, but is consistent with its generally southern range, although it does extend into Maine and Nova Scotia during the summer months.

d. Colonization attempts. In order to be assured of a uniform supply of live mosquito material for experimental pathogen transmission

and for studies of vector physiology, it is necessary to maintain colonized strains in the laboratory. Where species of interest have been colonized by other laboratories and published procedures are available or where species are being currently maintained by other laboratories, maintaining a reliable colony does not pose a difficult problem. The species of mosquitoes of greatest interest to us in connection with the eastern Maryland arbovirus study, however, either have never been colonized or have been so only recently. The species which we attempted to colonize during this year and the status of these attempts are as follows:

(1) Culex salinarius Coquillett. Successfully colonized from larvae collected at the Pocomoke Cypress Swamp, Maryland during the spring and summer of 1969. Colony is vigorous and supplies ample material for experimentation.

(2) Culiseta melanura (Coquillett) A colony has been maintained by artificial insemination at WRAIR in the past. Attempts this year concentrated on establishment of a naturally mating colony. These attempts were not successful, and will be continued during the coming year.

(3) Culex restuans Theobald. This mosquito has never been colonized. Attempts to establish a naturally mating colony this year failed. Absence of mating by adults while in captivity is apparently the stumbling block to colonization, and attempts this coming year will be to establish a colony maintained by artificial insemination.

(4) Aedes canadensis (Theobald). This species has never been colonized. In nature, the egg stage of this species undergoes a diapause, presumably facultative in response to environmental factors, which must be avoided if successful colonization is to be obtained. By manipulation of temperature and photoperiod, and by artificial insemination techniques, material collected in the larval stage has been reared through a single generation: larvae - pupae - adults - eggs - larvae. The nature of the diapause response will be studied when more material is available.

3. Glossina studies

A small colony of tsetse flies is maintained at WRAIR for studying the host-parasite relations of African trypanosomes in their insect host and as a source of material for attempted in vitro cultivation of tsetse tissues. Two species, Glossina austeni and G. morsitans, were reared in the insectary through December 1969. After that period only G. morsitans was maintained as it appeared more amenable to the laboratory environment than G. austeni.

Rearing procedures were modified so that all stages of the flies were maintained in low temperature incubators at 22 - 25°C with 70 - 80% RH. Adult flies were kept in Geigy-10 cages while pupae were placed in

Barraud cages over a layer of sand for emergence. Flies were offered blood meals daily with guinea pigs serving as donor hosts. Strains of Trypanosoma brucei have been maintained by cyclical transmission in guinea pigs and white mice.

During FY 1969 it was observed that the infection rate of G. austeni exposed to the Lugala I strain of Trypanosoma brucei was considerably lower (8.5%) than that previously observed (26.7%) at the London School of Hygiene and Tropical Medicine under similar experimental conditions. One possible explanation was that while the parasite had undergone several serial blood transfers during the year in the absence of cyclical transmission, the parasite had changed in its ability to infect the insect host. A new parasite strain (S-42) was acquired and the susceptibility of day-old tsetse flies to this and the old Lugala I strain were compared during FY 1970. The new strain proved to be more infective to both G. austeni and G. morsitans (Table 10) and is the one currently used.

Earlier observations indicated that only rarely did flies older than 24 hours after emergence from pupae develop a trypanosome infection when fed on parasitemic hosts (infection rate of 2-3 day old Glossina = 0.6%). During the past year it was observed that some groups of older flies which had been inadvertently offered an infective blood became infected at a low level. An experiment was made to test the susceptibility of groups of 3 week old flies, which had been exclusively fed on uninfected guinea pigs, as compared to the susceptibility of day-old flies. On 17 April 1970 G. morsitans of both age groups were fed on a guinea pig with the S-42 strain and flies were sacrificed 2-3 weeks later to assay the degree of trypanosome infection. 7/18 or 38.9% of day-old flies were infected while 5/63 or 7.9% of the older flies were infected. This difference was significant and indicated that barriers to fly infection which may be present in 2-3 day old flies are not as effective in older flies. Future studies will be directed towards an analysis of this change in tsetse susceptibility.

Conclusions and Recommendations

1. Culiseta melanura (Coquillett) is apparently the only species of any ecological consequence is the summer transmission of eastern and western equine encephalitis among birds in fresh water swamp habitats of eastern Maryland during years of normal rainfall.

2. Other species are probably involved in occasional transmission of these viruses outside the swamp habitat to vertebrates other than passerine birds and may be involved in overwintering of the virus in swamps. In this latter process, Culex salinarius is unlikely to have any part.

3. Future studies should be carried out to discover possible roles of other species as overwinter virus carriers. Culex restuans is a prime candidate for study.

4. Studies should be done on rates of laboratory infection and transmission of WEE and EEE in the 4 or 5 most abundant species occurring in the swamp.

5. Studies should be carried out on the flight range and ecological amplitude of Culiseta melanura.

6. Study of mosquito flight using mechanical flight mills is a feasible means of obtaining ecological and physiological data having epidemiological significance.

7. Floodwater mosquitoes of the genus Aedes are involved in the transmission of virus agents in the Bunyamwera and California groups of viruses. Additional studies on the ecology of these vector mosquitoes should be carried out.

8. Culex salinarius apparently does not undergo physiological diapause in response to environmental factors. Additional studies are needed, however, on other species which overwinter as adults, such as Culex restuans.

9. Colonies of two species of tsetse flies, Glossina austeni and G. morsitans, have been maintained by the Department of Entomology. Current studies are directed towards analyzing the factors responsible for the establishment of Trypanosoma brucei infections in these hosts. Work in this area can be accelerated by the establishment of a small colony of lopeared rabbits to provide a better host animal for normal uninfected tsetse fly maintenance.

TABLE 1

Mosquito larvae collected 1969, Pocomoke Cypress Swamp, Maryland. Treehole and artificial container collections excluded

Species	No. of collections in which species occurred: Alone	With other species	Total	% of total * collection
Aedes canadensis	126	121	247	47.05%
Culiseta melanura	49	122	171	32.57
Culex territans	22	108	130	24.57
Culex salinarius	9	90	99	18.85
Anopheles spp.	32	18	50	9.52
Culex restuans	7	41	48	9.14
Aedes vexans	3	29	32	6.09
Psorophora ferox	0	22	22	4.19
Aedes atlanticus	1	15	16	3.05
Aedes cantator	1	7	8	1.52
Culex pipiens	2	5	7	1.33
Aedes informatus	0	5	5	0.95
Psorophora confinnis	0	5	5	0.95

No other species exceeded 0.9% of total collections

* Based on 525 collections

TABLE 2

Dominant species of mosquitoes in macro- and microhabitats, Pocomoke Cypress Swamp, Maryland, 1969.
Species listed in order of dominance

Macrohabitats	Temp. pools	Crypt pools	Semi-perm. pools	Open Holes	Covered holes	Tree holes	Semi-perm. ditches	Rot holes	Artif. cont.
Closed swamp	1,4,3,2	2,1,3,4	-	1,2,4,3	2,1	11,12, 13	-	-	11,5
Open swamp	-	-	4,3,5,1	-	-	-	-	-	5
Upland forest	1,3,9,8	2	-	-	-	11,12, 13	-	1,2,14, 5	-
Forest-swamp tr.	1,2,7,8,	2,4	-	-	-	-	-	-	-
Forest-marsh tr.	1,3,8,9	-	-	-	-	-	3,4,5	-	-
Marsh	-	-	-	-	-	-	-	-	-
Farmland	1,7,5, 6,8	1,2,7,8	1,6,3, 7,8	-	-	12,11,13	6,1,3,4		5,10,13, 4,11

Species indicated by numbers:

1. Aedes canadensis
2. Culiseta melanura
3. Culex territans
4. Culex salinarius
5. Culex restuans
6. Anopheles spp.
7. Aedes vexans
8. Psorophora spp.
9. Aedes atlanticus
10. Culex pipiens
11. Aedes triseriatus
12. Orthopodomyia sp.
13. Toxorhynchites sp.
14. Aedes thibaulti

TABLE 3

Adult mosquitoes, captured in Pocomoke Swamp, Md., 1969

SPECIES	APRIL	MAY	JUNE	JULY	AUGUST	SEPT	TOTAL
Aedes atlanticus	0	0	8	142	4,359	3,496	8,005
Ae. aurifer	0	58	97	0	0	0	155
Ae. canadensis	1,739	56,933	20,258	2,450	62,228	4,191	147,799
Ae. cantator	7,650	6,628	1,003	134	1,042	28	16,485
Ae. cinereus	0	0	0	0	1	0	1
Ae. fulvus-pallens	0	0	1	0	0	0	1
Ae. grossbecki	9	14	13	2	0	0	38
Ae. infirmatus	0	0	16	106	1,115	470	1,707
Ae. sollicitans	198	324	319	403	352	118	1,714
Ae. taeniorhynchus	0	0	112	194	840	203	1,309
Ae. thibaulti	0	2	2	1	0	0	5
Ae. triseriatus	0	6	39	102	373	105	625
Ae. trivittatus	2	0	0	1	0	0	3
Ae. vexans	7	25	186	2,469	673	216	3,576
An. bradleyi-crucians complex	281	101	49	280	1,900	1,715	4,326
An. perplexans	1	0	0	2	1	2	6
An. punctipennis	18	16	8	13	31	43	129
An. quadrimaculatus	0	0	2	15	59	123	199
Culex erraticus	0	0	0	1	0	5	6

TABLE 3 (Cont'd.)

SPECIES	APRIL	MAY	JUNE	JULY	AUGUST	SEPT	TOTAL
Culex pipiens	20*	0	12	14	93	30	169
Cx. vestuans	58	122	45	114	115	163	617
Cx. salinarius	140	212	293	4,273	24,979	14,021	43,918
Cx. territans	8**	3	26	27	130	201	395
Culiseta inornata	1	0	0	0	0	1	2
Cu. melanura	1,040	4,860	17,595	19,691	35,678	11,775	90,639
Mansonia perturbans	0	18	2,180	981	36	65	3,280
Orthopodomyia sifnitera	0	5	3	1	2	4	15
Psorophora ciliata	0	0	0	1	1	0	2
P. confinnis	0	0	2	9	9	37	57
P. ferox	0	0	58	240	2,192	956	3,446
P. howardii	0	0	0	48	14	6	68
P. varipes	0	0	0	0	1	0	1
Uranotaenia sapphirina	2	1	2	8	69	123	205
Damaged specimens Unidentifiable to species	124	736	396	723	2,867	372	5,218
TOTALS	11,298	70,664	42,725	32,405	139,160	38,469	334,121

* Doubtful I.D.
** Includes 6♀♀ from late March

TABLE 4

Adult mosquitoes captured in CO_2-baited light traps in Pocomoke Swamp, Md., 1969

Macrohabitat	No. trap nights	Numbers per trap night: Aedes canadensis	Culiseta melanura	Culex salinarius	Aedes cantator	Total All species
Open Swamp	52	84.81	179.04	30.19	49.31	384.27
Closed Swamp	173	209.15	211.03	53.95	45.17	589.08
Upland Forest	47	240.70	44.74	16.13	30.51	381.26
Upland Forest-Closed Swamp Transition	26	168.62	80.27	31.54	33.58	342.96
Upland Forest-Marsh Transition II	29	223.52	201.93	30.03	37.86	601.48
Marsh/Farmland	44	52.45	44.43	275.18	19.48	452.98
Averages	417	160.19	153.15	101.78	37.43	516.31

TABLE 5

Specificity of Rooster Anti-Mammal Sera

Antiserum	No. lots prepared	Antigen												
		Bovine	Cat	Deer	Dog	Goat	Horse	Human	Opossum	Pig	Rabbit	Raccoon	Rat	Squirrel
Bovine	4	+	-	+	-	-	-	-	-	-	-	-	-	-
Cat	4	-	+	-	-	-	-	-	-	-	-	-	-	-
Deer	4	+	-	+	-	-	-	-	-	-	-	-	-	-
Dog	4	-	-	-	+	-	-	-	-	-	-	-	-	-
Goat	4	-	-	+	-	+	-	-	-	-	-	-	-	-
Horse	4	-	-	-	-	-	+	-	-	-	-	-	-	-
Human	4	-	-	-	-	-	-	+	-	-	-	-	-	-
Opossum	4	-	-	-	-	-	-	-	+	-	-	-	-	-
Pig	3	-	-	-	-	-	-	-	-	+	-	-	-	-
Rabbit	4	-	-	-	-	-	-	-	-	-	+	-	-	-
Raccoon	4	-	-	-	-	-	-	-	-	-	-	+	-	-
Rat	3	-	-	-	-	-	-	-	-	-	-	-	+	-
Squirrel	2	-	-	-	-	-	-	-	-	-	-	-	-	+

TABLE 6

Results of Preliminary Screening Tests of Engorged Mosquitoes

Mosquito Species	Total	Anti-Mammal	Anti-Bird	Anti-Reptile	Negative	Double Feedings
Culex salinarius	9	9	0	0	0	-
Aedes canadensis	219	162	24	44	3	15

TABLE 7

Maximum Distances Flown by Various Mosquito Species on a Mechanical Flight Mill

Species	Distance Flown (meters)
Aedes polynesiensis	208
Anopheles balabacensis	3008
Culex salinarius	4298
Anopheles stephensi	5543
Aedes aegypti	7114

TABLE 8

Blood Feeding of Culex tritaeniorhynchus and Culex salinarius after Exposure to 2 Different Photoperiods

	Photoperiod			
	8 hours		16 hours	
Culex tritaeniorhynchus	2/47	(4%)	20/30	(67%)
Culex salinarius	72/150	(48%)	86/150	(57%)

TABLE 9

Number of Female Culex tritaeniorhynchus and Culex salinarius showing full ovarian development after exposure to 2 different photoperiods

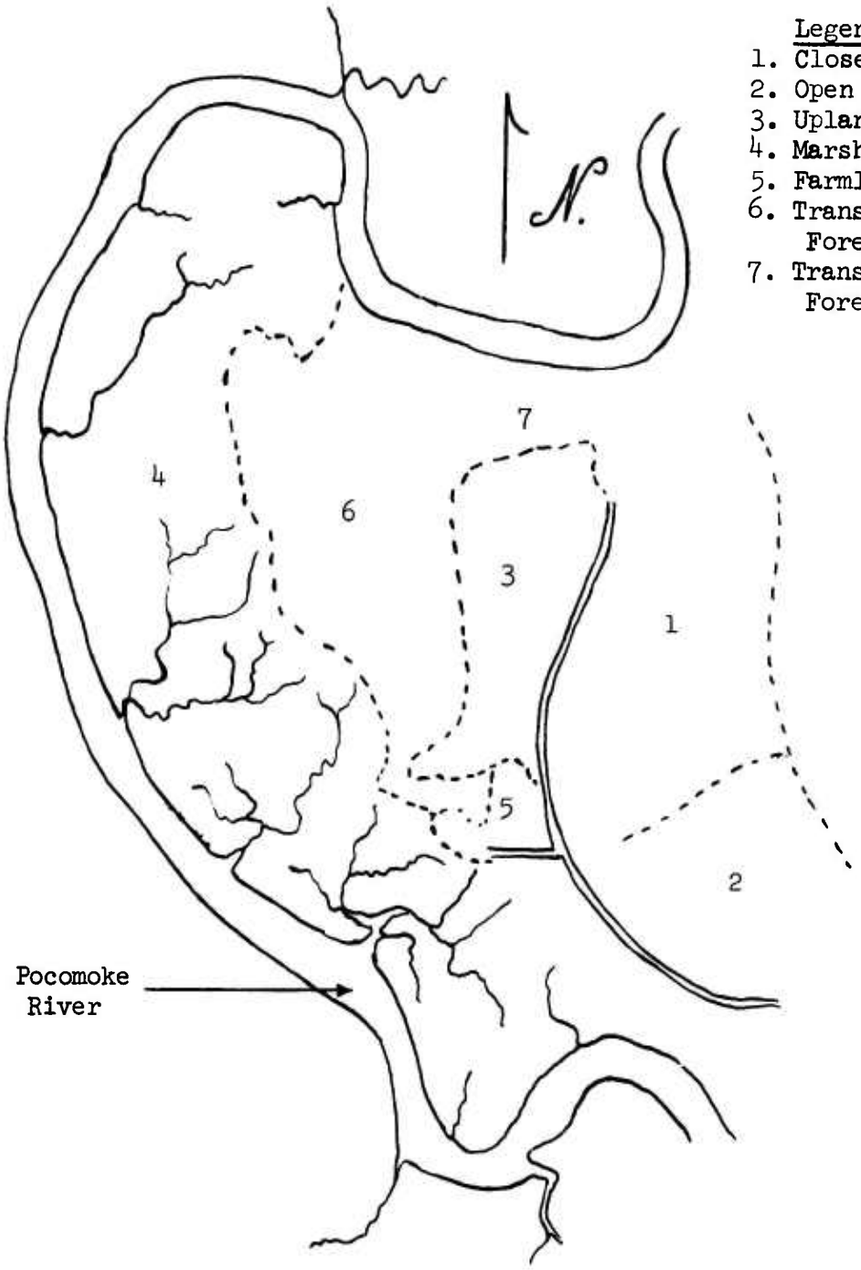
	Photoperiod	
	8 hours	16 hours
Culex tritaeniorhynchus	2/2	13*/20
Culex salinarius	72/72	85/86

* 7 mosquitoes died after feeding

TABLE 10

Infection of tsetse flies with *Trypanosoma brucei*

Parasite strain	Tsetse species	Location	No. infected/ No. dissected	% infected
Lugala I	*Glossina austeni*	London	26/97	26.9
Lugala I	*Glossina austeni*	Washington	11/145	7.6
S-42	*Glossina austeni*	Washington	3/19	15.8
S-42	*Glossina morsitans*	Washington	28/130	21.5



Legend
1. Closed swamp
2. Open Swamp
3. Upland Forest
4. Marsh
5. Farmland
6. Transitional (Upland Forest-Open Swamp)
7. Transitional (Upland Forest-Marsh)

FIG. 1 Pocomoke Cypress Swamp and vicinity, Worcester County, Maryland

FIG. 2
Light Trap Collections Pocomoke Swamp, Md. 1969

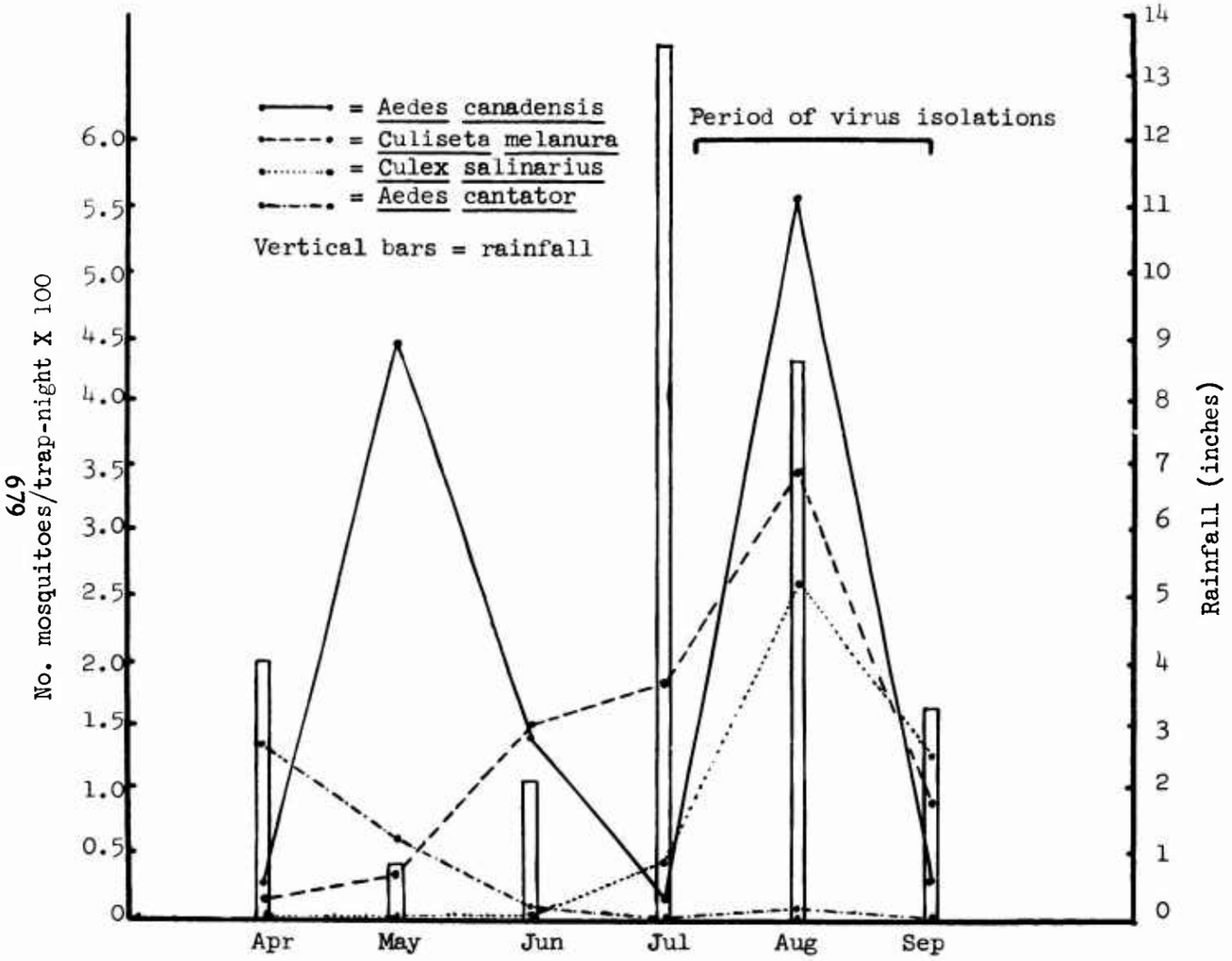


FIG. 3

Vacuum Sweep Collections-Pocomoke Swamp Maryland 1969

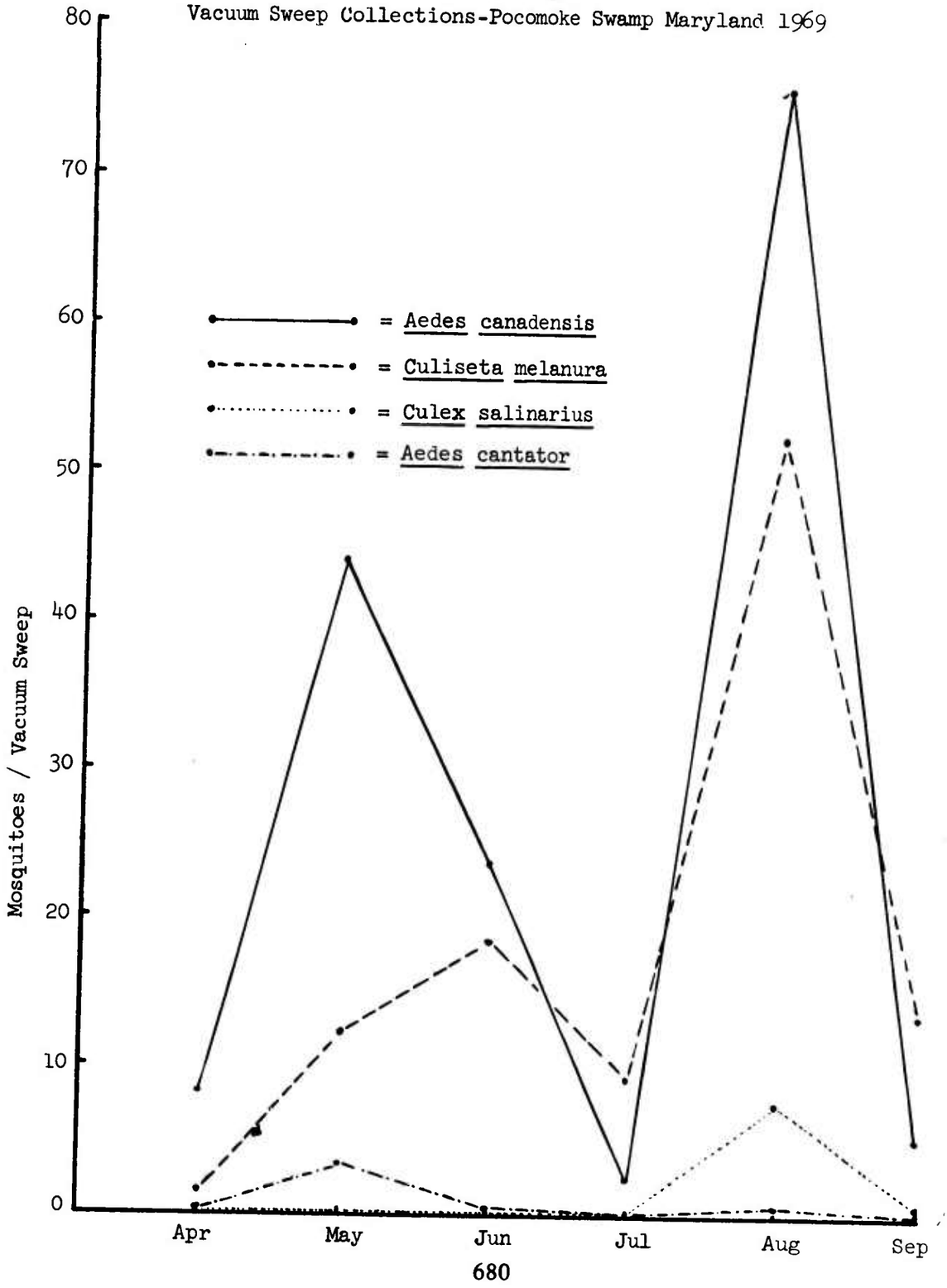


FIG. 4

Resting Box Collections - Pocomoke Swamp Maryland 1969

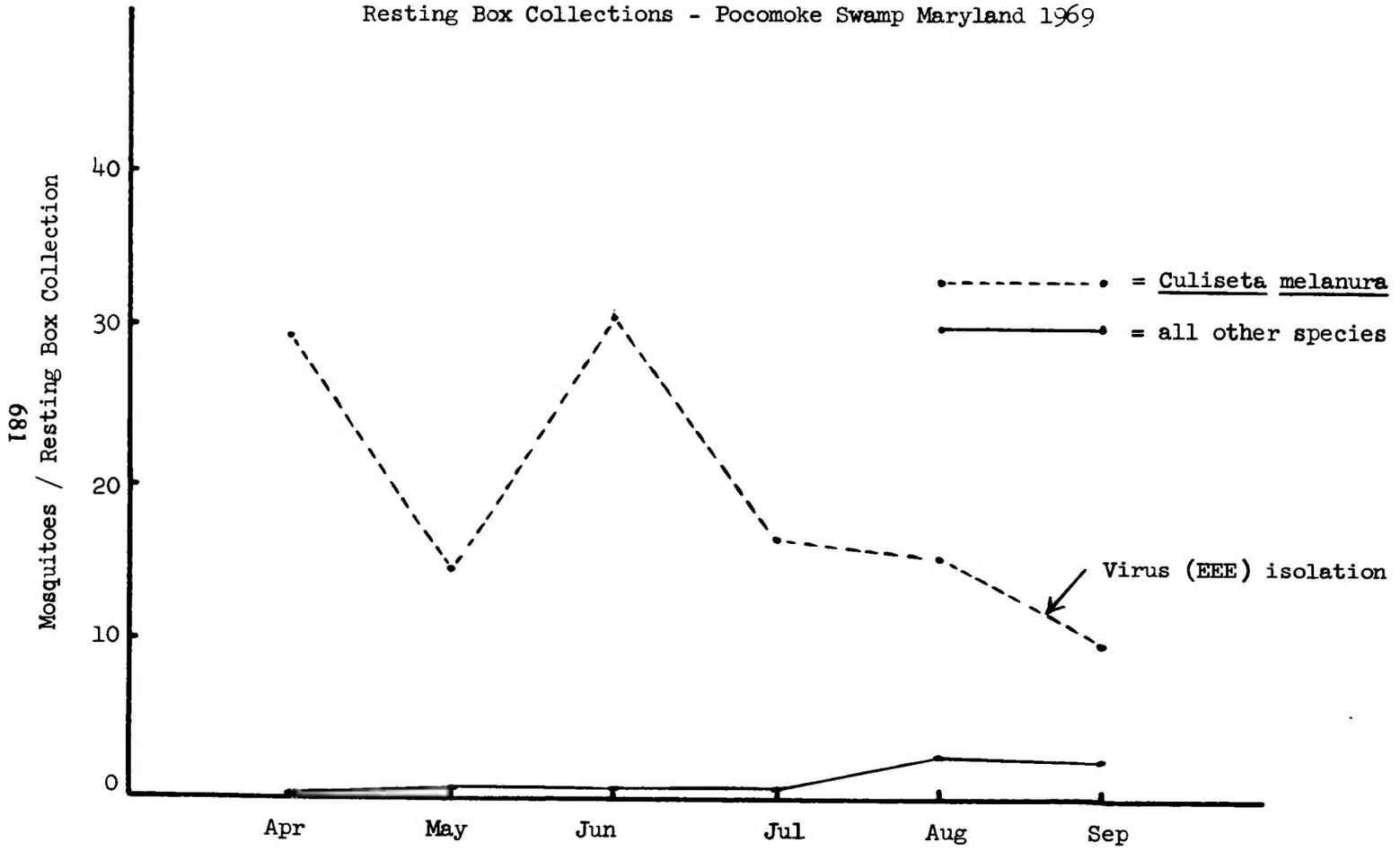
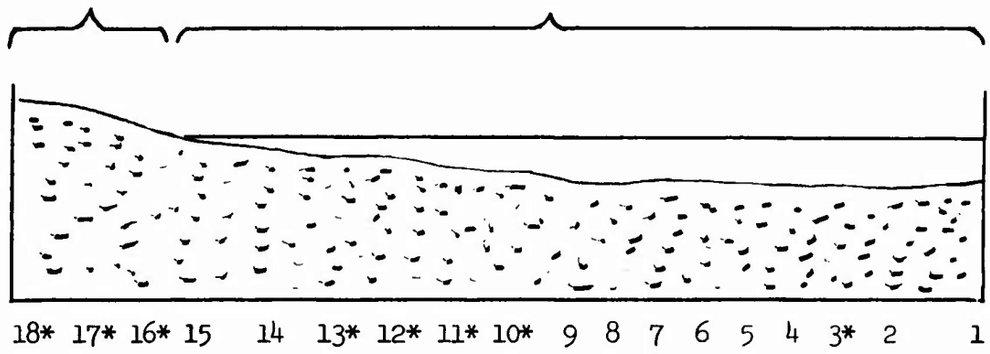


FIGURE 5 Diagrammiatic representation of Pocomoke Cypress Swamp transect showing location of sites sampled for presence of floodwater mosquito eggs. Site numbers positive for eggs indicated by asterisk.



Project 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03, Entomology

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

Literature Cited

1. References

(1) Tempelis, C.H. and Lofy, M.F. A Modified Precipitin Method for Identification of Mosquito Blood-meals. Amer. J. Trop. Med. Hyg., 12: 285, 1963.

(2) Rowley, W.A., Graham, C.L. and Williams, R.E. A Flight Mill System for the Laboratory Study of Mosquito Flight. Ann. Entomol. Soc. Amer. 61:1507, 1968.

2. Publications

1. Ward, R.A. Tsetse Fly Colonization (Diptera, Muscidae, Glossina spp.) Bull. Entomol. Soc. Amer. 16:111, 1970.

2. Ward, R.A., and West, H.J. In vitro Cultivation of Trypanosoma brucei. Trans. Roy. Soc. Trop. Med. Hyg. 64:159, 1970.

PROJECT 3A061102B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04
Immunology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6432	70 07 01	DD-DR&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 07 01	C. 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	61102A	3A0611028B71P	04	015
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)
(U) Antigen-Antibody Reactions In Vivo and In Vitro (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 09	CONT	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: 70	7	160
FISCAL YEAR CURRENT: 71	7	160

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

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

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Div of CD and I
Washington, D C 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Barbaro, J.F.
TELEPHONE: 202-576-3665
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Allergy, (U) Enzymes, (U) Immunology,(U)Antigen, (U) Antibody,(U)Hypersensitivity

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

23 (U) Work under this work unit involves the study of the basic mechanisms of the immediate type allergies from the viewpoint of the enzyme systems involves. This looks to the ultimate control of such allergies by a specific inhibition of these enzymes. The development of methods for the isolation and characterization of the enzymes involved in hypersensitivity reactions.

24 (U) Phosphonate inhibitors are being synthesized and tested in vitro for selective action against various enzymes. The distribution of blood group antibody between fluid phase and human erythrocyte is being investigated under various conditions of temperature and concentration of cells and antibody. The nature of the lymphocyte-dependent histamine release from rabbit platelets is being studies.

25 (U) 69 07 - 70 06. Data has been obtained that demonstrate that viable leukocytes are required for activation by antigen in the leukocyte-dependent histamine release for rabbit platelets. Once the activation process is completed the destruction of the cells does not affect their capacity to interact with platelets. The leukocyte-dependent histamine release reaction was used with rabbits infected with schistosomaisis and trichinosis to determine the degree of cross reactivity with various heterologous helminth antigens. Although the specificity was not perfect, the frequency of positive reactions was far less than with standard serological tests. The nature of the antibody involved in opsonization of sheep red blood cells was studied.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

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

Investigators.

Principal: John F. Barbaro, Ph.D.
Associate: Elmer L. Becker, M.D.,Ph.D.; MAJ R. Wistar, MC; MAJ E.J. Colwell, MC; D.T.O. Wong, PH.D.; M.B. Gibbs, PH.D.; P. Ward, MD; L. Hafner, M.S.; J. Fellman, M.S.; J. Ortaldo, M.S.; J.P. Bingham; M. McCormick; M.J. Schoenbechler; MAJ R. Evans, MC

Description:

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

Progress:

1. Histamine release from rabbit platelets

a. The previous annual report established that the leukocyte dependent histamine release from rabbits infected with S. *mansoni* could be divided experimentally into two distinct steps. The first involves the reaction of sensitized leukocytes with antigen; the second is concerned with the interaction of these activated cells with platelets. The results reported indicated that the activation of sensitized leukocytes by antigen is complex and probably enzymatic in nature. Work has continued in an attempt to determine the exact nature of the activation process.

b. The effect of divalent cations on the activation of leukocytes was investigated. Pre-incubation of leukocytes with 0.01 M EDTA does not affect their ability to be activated by antigen once divalent cations are restored. However, activation in the absence of divalent cations or in Tyrode's solution where cations are chelated with EDTA results in an irreversible decrease in activation. The results obtained also indicate that calcium may be the only cation required for maximal activation and that magnesium plays little or no part in the activation process.

c. Attempts at demonstrating the production of a soluble factor by pre-incubation of leukocytes with antigen which would be active towards platelets in the absence of intact cells were unsuccessful. Attention was directed towards determining if the histamine releasing activity could be extracted from activated cells by freeze-thawing. It was found that histamine releasing activity could be demonstrated with the supernatant as well as with the cells and debris from the freeze-thawed mixtures. These findings indicate that platelet reactive material can retain its activity once removed from the intact cell, but what relationship this has to a soluble factor is not known at present.

d. Data has been obtained that demonstrate that viable cells are needed for activation by antigen. However, once the activation process is completed the destruction of the cell does not affect its capacity to interact with platelets.

e. The deleterious effect of cold on the capacity of leukocytes to be activated by antigen suggests the possible involvement of the sodium-potassium pump in the activation process. Various parameters knwon to impair or restore the sodium-potassium pump are being investigated to determine what effects these parameters have on the antigen induced activation process. The cold impaired sodium-potassium pump can be restored by preincubation at elevated temperatures which results in a reaccumulation of potassium ions. Pre-incubation at 37°C of leukocytes stored in the cold results in a restoration of the capacity of these cells to be activated. However, these restored cells were only capable of 75% of the original activity. The time necessary for maximal restoration was 20 minutes and pre-incubation for as long as 4 hours gave no change in the results.

2. In vivo passive sensitization of rabbit leukocytes

a. Recent evidence indicates that leukocytes possess specific receptor sites which can bind homocytotropic (reaginic) antibody which in turn is responsible for the immune release of histamine from human leukocytes obtained from ragweed sensitive individuals. The fixation of homocytotropic antibody to human leukocytes was demonstrated by in vitro passive sensitization of normal leukocytes with sera from ragweed sensitive individuals. Since rabbits infected with helminths usually produce homocytotropic antibody and their antigen activated leukocytes are capable of interacting with platelets to cause histamine release from the platelets, it was felt that, similar to the human ragweed system, the binding of homocytotropic antibody to leukocytes was involved in this system.

b. Extensive in vitro attempts to passively sensitize normal rabbit leukocytes with homocytotropic antibody from Schistosoma mansoni infected rabbits were unsuccessful. The in vivo passive sensitization of normal rabbit leukocytes with homocytotropic antibody from rabbits immunized with egg albumin was undertaken. Antisera from immunized rabbits were tested for homocytotropic antibody by passive cutaneous anaphylaxis (PCA) concentrated by ultrafiltration and injected intravenously into normal rabbits. Passive sensitization of these leukocytes with homocytotropic antibody was determined by antigen-induced histamine release from normal rabbit platelets.

c. The results obtained desmonstrated that those rabbits sensitized with negative or low titered PCA sera (less than 1/50) were unable to release significant amounts of histamine. The range of histamine release was from 0 to 4.8 percent. In contrast, the leukocytes obtained from rabbits passively sensitized with high titered PCA positive serum (greater than 1/50) released significant amounts of histamine. The average release obtained was 40.3 percent with a range from 9.8 to 80.0 percent.

d. The wide range of histamine release obtained from these rabbits passively sensitized with high titered PCA positive serum is due largely to individual variation in the ability of recipient leukocytes to absorb the homocytotropic antibody. This was established by using the same aliquot of serum to passively sensitize different rabbits.

e. The time course of passive sensitization was examined to determine the optimal time for maximal sensitization. Significant histamine release was detected at 1.5 days and maximal release was obtained when leukocytes were collected 3 days after intravenous injection of PCA positive serum. Leukocytes tested at 5 to 7 days showed decreased sensitization and by 10 days significant histamine release was not demonstrable. The circulating PCA titer in the recipient rabbits showed an inverse correlation with the degree of sensitization. The PCA titer decreased rapidly and was virtually gone on the third day when the leukocytes were maximally sensitized.

3. Leukocyte-mediated histamine release with helminth antigens

a. This study was undertaken to examine the sensitivity and specificity of leukocyte-mediated histamine release with various helminth antigens in rabbits infected with schistosomiasis and trichinosis. The results obtained were compared with the passive

cutaneous anaphylaxis reaction as well as standard serologic reactions such as complement fixation.

b. All rabbits infected with S. mansoni or T. spiralis demonstrated significant histamine release when tested with the homologous antigens. The sensitivity of the leukocyte-dependent histamine release was demonstrated by the finding that antigen concentrations as low as 3×10^{-4} mg protein/ml were capable of significant histamine release.

c. Ten rabbits infected with S. mansoni were tested for leukocyte-dependent histamine release with antigen extracts of S. mansoni, S. japonicum, T. spiralis, D. immitis, P. westermani and E. granulosus. Eight rabbits showed cross reactions with S. japonicum antigen and 2 of the 10 showed cross reactions with P. westermani antigen while no significant release was obtained with T. spiralis, D. immitis or E. granulosus. Although significant histamine release was demonstrable in all infected rabbits, only 5 of the 10 were able to elicit positive PCA reactions with the homologous antigen. No positive PCA reactions were observed with any of the heterologous antigens. Of the antigens tested all showed cross reactivity when tested by the standard complement fixation reaction.

d. The results obtained with 10 rabbits infected with T. spiralis and tested for leukocyte dependent histamine release with these helminth antigens showed that 3 of the 10 rabbits cross reacted with S. mansoni antigen, in contrast to schistosomiasis infected rabbits that showed no cross reaction with T. spiralis antigens. This finding is in agreement with the non-reciprocal cross reactivity demonstrated by serologic cross adsorption studies with these antigens. In addition, 3 rabbits also showed significant release with D. immitis and/or E. granulosus antigens. The PCA reaction, similar to the schistosomiasis infected rabbits, showed no cross reaction with the heterologous antigen and only half of the rabbits serum responded with the homologous antigen.

4. Characterization of mouse homocytotropic antibodies

a. The previous annual report described the differentiation of mouse immunoglobulins by the technique of biological screening. The results demonstrated that in vivo passage of mouse antisera in normal mice was capable of discerning two antibodies i.e., the heterologous rat heat labile (reaginic) anti-

body and the homologous mouse heat stable (7Sγ1) antibody, on the basis of their different tissue binding affinities. After in vivo passage in normal mice the antibody responsible for the heterologous rat PCA was completely removed, while the homologous mouse PCA activity was quantitatively recovered. The results obtained by the biological screening method clearly demonstrated that the heterologous 4 hour rat PCA reaction, mediated by heat labile (reaginic) antibody, and the homologous 72 hour mouse PCA reaction were completely removed by in vivo passage in normal mice. These results indicate that both the heterologous rat and the homologous mouse PCA reaction were probably mediated by the same heat labile antibody, which possessed both homocytotropic and heterocytotropic affinities.

c. Additional evidence that heat labile heterologous rat and homologous mouse PCA reactions were mediated by the same antibody was obtained by adsorption with specific antiserum. Mouse antisera with both heat stable (7Sγ1) and heat labile (reaginic) antibodies was separated by Pevikon electrophoresis. Those fractions containing homologous 72 hour mouse PCA reaction were pooled and used to immunize rabbits. The specific rabbit antiserum was capable of removing both the heterologous 4 hour rat and the homologous 72 hour mouse PCA activity to the same extent. These additional studies add to the accumulating evidences that the same heat labile antibody is responsible for both the rat 4 hour and the mouse 72 hour PCA reaction.

d. A comparative study of mouse heat labile (reaginic) and heat stable (7Sγ1) antibody was undertaken to determine the sequence and optimal time for the production of these antibodies. Mice were given a single injection of alum precipitated antigen and then bled daily starting with the sixth day after stimulation. The results demonstrate that the heat labile (reaginic) antibody is the first mouse PCA activity to appear in the sera, followed shortly thereafter by the production of heat stable (7Sγ1) antibody. Eight days after the initial antigenic stimulation 90-95% of the mouse PCA activity can be attributed to the heat labile (reaginic) antibody and the remainder to the heat stable (7Sγ1) antibody. By the 10th day, over 75% of the mouse PCA activity was due to the heat stable (7Sγ1) antibody. At present the reason for the abrupt change in the ratio of heat labile and heat stable antibody is not known. Presumably, the rapid production of heat stable antibody interferes with either the production or binding of the heat labile (reaginic) antibody.

5. Chemotaxis of rabbit polymorphonuclear leukocytes

a. Results have been obtained that are compatible with the idea that an enzyme analogous to the sodium-potassium ATPase of mammalian tissue is necessary for the chemotactic responsiveness of rabbit neutrophils. This conclusion is based on the inhibition of chemotaxis by ouabain, a reasonably specific inhibitor of sodium-potassium ATPase, and also on the ability of potassium to reverse this inhibition.

b. The exact action of the sodium-potassium ATPase or an analogous enzyme involved in the chemotactic response of rabbit neutrophils is not known at this time. Nevertheless, these findings indicate that neutrophils contain another enzyme system in addition to the two esterase systems defined in previous annual reports that is involved in the biochemical sequence of chemotaxis.

6. The synthesis of novel phosphonates

a. Fourteen new phosphonates were synthesized. Their elementary and physical properties are given in Table I.

7. Enzymatic mechanisms of phagocytosis

a. The previous annual reports described an already activated esterase in or on the polymorphonuclear leukocytes of guinea pigs and rabbits that was involved in phagocytosis. This enzyme has many similarities with the analogous activated esterase required for chemotaxis. The findings that phosphonofluoridates would inhibit during phagocytosis was suggestive of the involvement of an activatable esterase. Cyclohexyl butyl and cyclohexyl propyl phosphonofluoridate both gave significant inhibition of phagocytosis when incubated with polymorphonuclear phagocytes prior to addition of opsonized erythrocytes. Work is presently under way to test all nine of the phosphonofluoridates which have been synthesized. The results obtained thus far indicate that phosphonofluoridate acts early in phagocytosis before the engulfment phase is completed which suggests a means of distinguishing the already active esterase from the activatable esterase.

b. The phagocytic assay used previously in this study was described as "complement dependent erythrophagocytosis". Studies done in the past year on the nature of the opsonins and co-factors involved in this system have demonstrated that

complement is not required for phagocytosis of erythrocytes by guinea pig polymorphonuclear leukocytes. Maximal phagocytosis occurs with only opsonizing antibody on the erythrocyte. The same pattern of phosphonate inhibition was obtained whether the erythrocytes were opsonized with purified antibody alone or both antibody and complement.

C. A study of the nature of the antibody involved in opsonization of sheep erythrocytes was undertaken. It was found that purified rabbit IgG antibody to either Forssman antigen or whole sheep erythrocytes was capable of opsonizing erythrocytes; whereas, the corresponding purified IgM antibody was ineffective in preparing erythrocytes for phagocytosis in spite of high hemolytic activity.

d. Pepsin digested guinea pig gamma 2 or rabbit IgG anti sheep red blood cell antibodies were prepared and corresponding F (ab') 2 fragments isolated. Although these fragments retained the ability to combine with red blood cells, as shown by direct agglutination and agglutination enhancement with anti-F (ab') 2 antisera, they were unable to opsonize red blood cells. The maximum phagocytosis of F (ab') 2 coated cells was 8 percent, whereas about 60 to 75 percent of the IgG coated red cells were phagocytized. These findings indicate that the opsonizing activity of the IgG antibody is dependent on the presence of the Fc portion of the H chain of the immunoglobulin.

8. Studies on blood group antigens and antibodies

a. Investigations directed towards the recognition of the "dangerous" universal group 0 donor by characterization of the binding properties of natural and immune anti-A and anti-B isoagglutinins were continued. As summarized in the previous annual report, evidence has been obtained with the log-probit assay in support of the Wurmsers' conclusion that naturally occurring anti-B agglutinins differ in their binding affinities according to the ABO genotype of the individual. The study of the anti-B binding affinities of a woman whose most probable genotype is homozygous A_2A_2 has provided further evidence in support of the Wurmsers' hypothesis. The isoagglutinins of this homozygote were found to have weaker binding affinities for B sites of human erythrocytes than the anti-B isoagglutinins from three of her heterozygous A_2O children.

b. As stated in the previous annual report the observations of the Wurmsers' that the ratio of the maximum number of B red cells agglutinated by a natural anti-B serum at

4°C and at 37°C (designated N_4/N_{37} ratio) is less tedious and time consuming than the determination of the binding affinities of the anti-B isohemagglutinin, further studies were done utilizing this method. The problems encountered, particularly with the log-probit assay at 37°C, have been resolved and the validity of the Wurmsers' observation could be evaluated. The N_4/N_{37} ratios of young individuals (age 12-20 years) of known ABO genotypes was determined. The characteristic N_4/N_{37} ratios reported by the Wurmsers were confirmed for 11 A_1O, 7 A_2O and 6 OO sera. The N_4/N_{37} ratio of a homozygous A_2A_2 serum, not previously reported, was also determined. The N_4/N_{37} ratio of a homozygous A_1A_1 serum reported by the Wurmsers could not be confirmed due to the unavailability of an A_1A_1 serum for testing.

c. The usefulness of N_4/N_{37} ratio as a means of determining the homo- or heterozygosity of phenotype A_1 and A_2 individuals was investigated. The N_4/N_{37} ratios of 80 random sera obtained from A_1, A_2 and O civilian donors in the WRGH Blood Bank were determined. Of the 34 A_1 sera tested, 17 sera were too low titered to be assayed; 14 sera could be identified as heterologous A_1O; 2 sera gave results characteristic of the homozygous A_1A_1 genotype and 1 sera could not be classified. The results with 14 A_2 sera were as follows: 4 were too low titered to be assayed; 7 sera could be identified as heterozygous A_2O; 2 sera gave ratios which were in accord with a homozygous A_2A_2 and 1 serum could not be classified. Eleven of the 31 group O sera were too low titered to be assayed; 11 gave ratios reported by the Wurmsers as characteristic of group O sera. The remaining 9 sera gave N_4/N_{37} ratios considerably higher than expected. The results obtained with these 9 sera could be attributed to unusually high levels of cross reactive AB antibody in these sera. After removal of the cross reactive antibody by adsorption with A_1 erythrocytes the sera gave the characteristic N_4/N_{37} ratios. The possibility that high levels of cross-reactive AB isoantibody may be the cause of the "dangerous" universal group O donor is being considered.

d. The direct hemagglutination of dried blood stains for the identification of ABO blood type used in forensic laboratories has many difficulties. Since the direct agglutination reaction of dried and aged blood is very weak and often contaminated with debris, the interpretation of the results is hazardous. To circumvent these difficulties a hemagglutination inhibition reaction was developed whereby the A_1, A_2, B and O type of dried blood samples could be determined. The red blood cells in a sample are disrupted by sonication and then clarified

of extraneous debris by centrifugation. The supernatant containing the red cell antigen in solution is reacted with anti-A, anti-B and anti-H serum. To each, a weak suspension of appropriate indicator red cells is added and the tubes centrifuged immediately. The lack of agglutination reaction indicates the presence of the antigen in the sonicated supernatant and indicates the ABO blood type of the original blood sample. The applicability of this procedure for the identification of ABO, MNS and P blood types of blood stains stored for various times on different types of materials is currently being studied.

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

a. The following commercial vaccines were tested for the presence of blood group A and B activity: 26 plague and 2 culture media for Salmonella. No detectable A or B blood group substance was found in any of the plague vaccines. The culture media were found to be highly contaminated with blood group A substance.

Summary and Conclusions:

1. The activation of leukocytes by antigen in the leukocyte-dependent histamine release from rabbit platelets requires the presence of calcium for maximal activation.

2. Viable leukocytes are needed for activation by antigen. However, once the activation process is completed, the destruction of the leukocytes does not affect their capacity to interact with platelets.

3. A soluble factor can be extracted from activated leukocytes which retains its capacity to interact with platelets once removed from the leukocyte.

4. The deleterious effects of cold on sensitized leukocytes can be partially reversed by pre-incubation of leukocytes at 37°C before activation by antigen.

5. In vitro passive sensitization of normal rabbit leukocytes with homocytotrpic antibody from S. mansoni was unsuccessful.

6. Normal rabbit leukocytes can be passively sensitized in vivo with rabbit sera possessing homocytotropic antibody. The optimal time for maximal in vivo passive sensitization was three days after intravenous injection of antiserum.

7. The circulating homocytotropic antibody in recipient rabbits showed an inverse correlation with the degree of sensitization.

8. Although the specificity of the leukocyte-dependent histamine release is not perfect, the frequency of positive reactions with heterologous antigens is far less than with standard serological tests.

9. Evidence has been obtained that indicates that both the heterologous rat and the homologous mouse PCA reactions are probably mediated by the same heat labile antibody.

10. Over 90% of the antibody obtained 8 days after initial antigen stimulation is attributed to heat labile (reaginic) antibody. By the 10th day over 75% of the mouse PCA activity is due to heat stable (7Sγ1) antibody.

11. Evidence of the involvement of the sodium-potassium ATPase in the chemotactic response of rabbit neutrophils has been obtained.

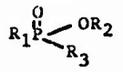
12. Fourteen new phosphonates were synthesized.

13. The opsonizing activity of IgG antibody is dependent on the presence of the Fc portion of the H chain of the immunoglobulin.

14. The Wurmsers' report that the ratio of the maximum number of B red cells agglutinated at 4°C to the maximum number of cells agglutinated at 37°C is characteristic of the ABO genotype has been confirmed with selected antisera; however, there were some discrepancies using random anti-B sera.

15. A hemagglutination-inhibition reaction was developed whereby the A_1, A_2, B and O type of dried blood samples could be determined.

TABLE I



	R_1	R_2	R_3	MO_{25}		C T	C F	H T	H F	P T	P F	F/Cl T	F/Cl F
$C_{16}H_{24}FO_2P$	$C_6H_5(CH_2)_4$	C_6H_{11}	F	1.4972	159/0.03	64.4	64.2	8.1	8.8	10.4	10.0	6.4	6.5
$C_{16}H_{24}ClO_2P$	"	"	Cl	1.5192	xylene[a]								
$C_{18}H_{23}O_3P$	"	C_6H_5	OC_2H_5	1.5302	167/0.02	67.9	67.8	7.3	7.3	9.7	9.7		
$C_{11}H_{17}O_3P$	C_3H_7	"	"	1.4898	73/0.05	57.9	57.6	7.5	7.6	13.6	13.3		
$C_{12}H_{19}O_3P$	C_4H_9	"	"	1.4850	87/0.05	59.5	59.3	7.9	8.0	12.8	12.6		
$C_{13}H_{21}O_3P$	C_5H_{11}	"	"	1.4850	105/0.05	60.9	60.3	8.3	8.6	12.1	12.2		
$C_{14}H_{23}O_3P$	C_6H_{13}	"	"	1.4830	107/0.05	62.2	62.0	8.6	8.8	11.5	11.2		
$C_{13}H_{20}ClO_3P$	$ClH_2CC_4H_9$	"	"	1.4998	toluene[a]	53.7	54.0	6.9	7.5	10.7	10.0	12.2	12.6
$C_{10}H_{23}O_3P$	C_4H_9	$CH(CH_3)_2$	$OCH(CH_3)_2$	1.4173	76/0.02								
$C_{12}H_{19}O_3P$	C_6H_5	"	"	1.4798	101/0.05								
$C_9H_{21}O_4P$	$OCH(CH_3)_2$	"	"	1.4031	129/WA	48.2	49.7	9.4	9.7	13.8	13.5		
$C_9H_{12}ClO_2P$	C_6H_5	Cl	"	1.5130	73/0.1								
$C_9H_{12}FO_2P$	"	F	"	1.4743	58/0.2	53.5	53.8	6.0	6.1	15.3	12.6	9.4	10.2
$C_{12}H_{27}O_3P$	$OCH_2CH(CH_3)_2$[b]			1.4225	123/WA	57.6	56.3	10.9	11.2	12.4	12.5		

(a) Heating medium for falling molecular still
(b) Compound is a phosphite

Project 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

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

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6433	70 07 01	DD-DR&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 07 01	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	61102A	3A061102B71P	04	016
b. CONTRIBUTING				
c. CONTRIBUTING	6D06 1412A(2)			

11. TITLE (Procede with Security Classification Code)

(U) Immunization Studies of Militarily Significant Viral Diseases (DD)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	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 70	2	90
FISCAL YEAR CURRENT 71	3	120

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Chikungunya; (U) Dengue; (U) Vaccine; (U) Immunology; (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) Chikungunya and dengue are mosquito-borne viral infections having widespread geographical distribution throughout populated areas of the world. Although mortality rates are low, the incapacitation effected by these viruses could have a serious impact on military timetables and troop movements. This investigation is concerned with the development, production and assay of vaccines suitable for use in man.

24. (U) Both, chikungunya and dengue are characterized by antigenically-related strains within their respective groups. Cognizance of these relationships enhanced the feasibility of producing vaccines capable of broad-spectrum protection against related as well as parent strains of the virus.

25. (U) 69 07 - 70 06 Of 20 volunteers receiving the Chik vaccine (Lot E-20), none experienced side effects and all developed significant levels of neutralizing antibody against the Chik virus, clearly indicating the safety and immunogenic potency of this vaccine. Fluorescent antibody studies on selected tissues of vaccinated and control monkeys challenged with the Chik virus revealed no invasiveness of the virus in vaccinated animals, whereas, 90% of the control animal tissues examined contained viral antigen. In the long term vaccine protection study, viremia levels are being determined in the first group of monkeys challenged 6 months after vaccination. Isolation and purification of antigenic subunits from specific types of the dengue virus is proceeding satisfactorily. It is anticipated that these preparations will measurably contribute to the production of a broad-spectrum protective vaccine against the dengue prototypes. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

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Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

Work Unit 016, Immunization studies of militarily significant viral diseases

Investigators.

Principal: V. R. Harrison, M.S.
Associate: K. H. Eckels, M.S.; C. Hampton, B.A.

Description.

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

Progress.

A formalin-inactivated chikungunya vaccine (Lot E-20) has been prepared in bank-frozen green monkey kidney tissue, certified-free from detectable adventitious agents. After the addition of 2% Human Serum Albumin (USP) as a stabilizer the vaccine was freeze-dried by the Department of Biologics Research. Upon the satisfactory completion of safety and potency tests the vaccine was delivered to the US Army Medical Investigation Unit, Ft. Detrick, Md. for immunologic studies in human volunteers.

Sixteen individuals received a 0.5 ml. subcutaneous injection of the experimental vaccine on days 0 and 28. No untoward reactions or side effects were observed in any of the vaccinees. At intervals of 14, 28 and 42 days after vaccination, the vaccinees were bled and their serologic response determined by complement-fixation, hemagglutination-inhibition and serum neutralization tests. These data are summarized in Table 1.

A plaque-inhibition test, wherein the zones of virus inhibition due to the neutralizing-antibody content of the serum are macroscopically visualized and measured, was also performed on these sera. The results are summarized in Table 2.

Thus, from the data presented, it is clearly evident that Lot E-20 chikungunya vaccine is safe and highly immunogenic when inoculated into human subjects.

To determine the long-term protective efficacy of the vaccine described above (Lot E-20), a parallel study is being conducted in

rhesus monkeys. Briefly, this study comprises 36 monkeys, 18 of which are vaccinated while the remaining 18 serve as non vaccinated controls. At intervals of 6, 12, 18 and 24 months after vaccination, 3 vaccinates and 3 control monkeys are challenged by the subcutaneous route with an highly infectious Asiatic strain of the chikungunya virus. The inoculated monkeys are bled on 7 consecutive days for the determination of viremia levels, and, at intervals of 30, 45, 60 and 90 days post-inoculation for serologic studies. Data pertaining to the 6 month post-vaccination group are summarized in Table 3.

TABLE 1

Serologic response of 16 human volunteers to formalin-inactivated chikungunya vaccine, Lot E-20

Subject	Complement-Fixation (1/CF)*				Hemagglutination-Inhibition (1/HI)**				Serum Neutralization (LNI)***			
	Pre	+14	+28	+42	Pre	+14	+28	+42	Pre	+14	+28	+42
BLW	-	-	-	-	-	-	-	20	-	0.7	1.3	2.3
CJM	-	-	-	-	-	10	-	20	-	2.0	1.7	2.3
CJW	-	-	-	16	-	10	10	320	-	1.0	1.7	3.0
CDD	-	-	-	4	-	40	20	20	-	2.6	2.3	3.3
DJ	8	8	4	8	-	20	20	20	-	2.0	1.3	2.3
DRR	-	-	4	8	-	160	40	160	-	2.7	3.0	>3.5
GRC	-	-	4	8	-	10	10	10	-	1.7	1.7	3.0
HSL	-	-	-	8	-	80	40	160	-	2.0	2.3	2.7
KCL	-	-	-	-	-	20	10	20	-	1.7	1.7	2.0
LJF	4	-	-	8	-	20	10	40	-	1.7	1.0	2.3
LDR	8	-	-	-	-	-	-	10	-	1.3	1.0	2.0
MJR	-	8	8	8	-	10	10	40	-	2.4	3.0	3.5
MDM	-	-	-	4	-	20	10	80	-	2.0	2.7	3.0
RRB	-	-	-	-	-	10	-	10	-	2.5	2.0	2.5
SWF	-	-	-	8	-	20	-	80	-	1.5	2.0	3.0

* Lowest dilution tested 1:4 (4 units antigen used in test).
** Lowest dilution tested 1:10 (8 units antigen used in test).
*** LNI, Log neutralization index. Average titer of Pre-vacc serum, $TCID_{50} = 10^{6.3}$.

TABLE 2

Plaque inhibition test on sera of 16 human volunteers receiving formalin-inactivated chikungunya vaccine, Lot E-20

Subject	Pre-bleed	Plaque Inhibition Test* +14	+28	+42
BLW	-	9	9	11
GRC	-	8	8	10
MJL	-	9	10	12
CJM	-	8	8	9
HSL	-	10	11	15
MJR	-	8	9	12
CJW	-	8p	9	18
KCL	-	8	9	12
RRB	-	9	9	12
CDD	-	9	9	11
LJF	-	9	9	12
SWF	-	8	8	12
DJ	-	8p	8	10
LDR	-	-	-	9
DRR	-	11	12	14
MDM	-	8	10	16

* Zone of plaque inhibition measured in mm.
p = partial zone of inhibition.

TABLE 3

Long-term Vaccine Study: Summary of data on monkeys challenged 6 months after vaccination (CHIK Vaccine Lot E-20)

MK #	Vaccine status	Serology - 30 days post challenge* CF	HI	PI**	Viremia
101	Yes	<4	20	10	No
999	Yes	<4	20	10	No
991	Yes	<4	<10	8	No
116	No	512	160	21	Yes
117	No	256	80	18	Yes
122	No	256	160	22	Yes

* Challenge consisted of approximately 1600 suckling mouse LD_{50}s.
** Plaque inhibition test, zone of virus neutralization measured in mm.

Work is continuing on the adaptation of chikungunya virus to the human diploid cell line WI-38. Several experimental lots of vaccine have been prepared in this substrate, however there is considerable variation in immunogenic potency between lots, indicating the need for further adaptation. Data on these experimental vaccines are summarized in Table 4.

TABLE 4

Summary of data on chikungunya vaccines prepared in WI-38 cells

Vaccine Lot No.	Date Prepared	Mouse Serum 7 days post 2 doses CF	HI	ED_{50}/0.5 ml.
WI-38A	Feb. 69	4	10	0.12
WI-38B	Aug. 69	<4	<10	0.23
WI-38C	Sep. 69	<4	<10	0.44
WI-38D	Oct. 69	<4	<10	>0.50

Comparative assays to determine the relative stability of chikungunva vaccines prepared by formalin-inactivation and Tween-ether extraction are in progress. Both vaccines were found to be of comparable immunogenicity by mouse potency assays after 3 months storage at 4°C. It was of interest to note that the vaccines did not differ in immunogenic potency when either the 48 hr. or 96 hr. harvest fluids were used for vaccine preparation. Although the ratio of hemagglutinin to infectious virus was markedly different for the two harvest periods, the immunizing antigen concentration remained relatively constant as reflected by potency assay. Comparative data for the two vaccines are shown in Table 5.

TABLE 5

Tween-ether (TE) and Formalin (HCHO) Chik vaccines - Stability data

Vaccine* Type	Hemagg-Inhib		Comp-Fix		PI Test**		ED_{50}/0.5 ml.	
	0	90	0	90	0	90	0	90
HCHO, dry	40	20	8	8	12	11	.05	<0.1
HCHO, fluid	20	10	4	8	10	12	.03	.04
TE, dry	10	20	<4	<4	10	12	.13	.05
TE, fluid	20	10	<4	<4	10	9	.17	.04

* Vaccine given in 2 doses, 7 days apart (0.25 ml/dose), adult mice.
** Plaque inhibition test, values given as diameter (in mm) of zone of inhibition.

Preliminary studies have indicated the desirability of propagating viral antigens for vaccine production in tissue culture roller vessels rather than flat prescription flasks. Some advantages offered by the roller vessel culture method are conservation of incubator space, greater yields of virus per unit volume, a more rapid equilibration of gas exchange and a more efficient distribution of growth nutrients. Formalin-inactivated chikungunya vaccine was prepared in roller vessels having 670 cm^2 surface area. The ratio of inoculum to cells was approximately 1:1. A summary of the data pertaining to vaccine prepared in the roller vessels is presented in Table 6.

TABLE 6

Chikungunya vaccine prepared in roller vessel tissue culture

Lot No.	Inoculum (MOI)*	Harvest Fluids Titre	Hemaggl. 0.5 ml	Mouse Serum 14 da p. 2 dose CF	Mouse Serum 14 da p. 2 dose HI	ED_{50}/0.5 ml
E-23	$40x10^5$	6.8	32	8	20	0.16

* MOI, Multiplicity of infection.

This vaccine has been stored at 4°C in the fluid state for approximately 1 year with no detectable loss in potency! It is anticipated that the production of subunit antigens for several of the Group B arboviruses and particularly the dengue virus, may be significantly increased by the use of the roller vessel technique. Currently under investigation are several animal species cell types which will serve as a suitable substrate for the propagation of crude viral antigens in bulk.

Partially purified and concentrated subunit antigens of the dengue prototypes are being produced by column chromatography and Amicon cell concentration methods. In Table 7 are shown the yields for 8 partially purified and concentrated soluble CF antigens for dengue 2, dengue 4 and the Japanese encephalitis viruses.

TABLE 7

Soluble CF antigens extracted from Dengue and JE viruses

Virus	Prep. No.	Soluble CF Antigen Titre	Volume (ml.)
Dengue 2	1	1024	2.0
Dengue 2	2	2048	2.0
Dengue 2	3	2048	2.0
Dengue 4	4	64	1.6
Dengue 4	5	64	1.6
Dengue 2	6	512	15.0
Dengue 2	7	512	2.0
JEV	8	32	2.0

From the data presented it may be readily ascertained that current yields of the subunit antigens are extremely low. Adaptation

of crude antigen production to in vitro methods such as the roller vessel tissue culture may significantly augment the yield of these potential immunogens. Some preliminary data on crude antigen production in the roller vessel are presented in Table 8.

TABLE 8

Hemagglutinin produced by in vivo and in vitro methods for CHIK and Dengue

Virus	Method of Preparation	Infected Fluids (Hemagglutinin)	Conc. (10X) (Amicon Cell)
CHIK	Roller Vessel	1:10,240	N.D.*
Dengue 2	Suckling Mouse Brain	1:512	1:6400
Dengue 2	Suckling Mouse Brain	1:32	1:1024
CHIK	Roller Vessel	1:640	1:5120
CHIK	Roller Vessel	1:64	1:512

* Not done.

Summary and Conclusions.

A formalin-inactivated chikungunya vaccine has been prepared in bank-frozen green monkey tissue culture certified-free from detectable adventitious agents. This vaccine when injected into human volunteers caused no untoward reactions or side effects and elicited an excellent immunogenic response in the vaccines.

In a study to determine the long-term protective efficacy of this vaccine at 6, 12, 18 and 24 months after vaccination, rhesus monkeys were challenged with an highly infectious Asiatic strain of the chikungunya virus. Challenged 6 months after vaccination, 3 vaccinated monkeys developed no viremias and maintained low levels of CF and HI antibody, whereas 3 control (non-vaccinated monkeys) developed viremias and elicited high levels of CF and HI antibody. The protective capability of this vaccine will be further evaluated at 12, 18 and 24 months post-vaccination.

Work continues on the adaptation of the chikungunya virus to the human diploid cell line WI-38, predicated upon the potential use of this substrate for human vaccine production in the US.

Stability studies on formalin-inactivated and Tween-ether extracted chikungunya vaccines indicate that after 3 months storage at 4°C both vaccines are of comparable immunogenic potency. Further

storage stability studies will be performed at 6, 12 and 18 month intervals.

Purification and concentration of dengue subunit antigens are being accomplished by column chromatography and Amicon cell techniques. It is anticipated that application of the roller vessel tissue culture method to the production of crude antigen in bulk will significantly increase the yields of arbovirus subunit antigens for immunogenic analyses.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

Work Unit 016, Immunization studies of militarily significant viral diseases

Literature Cited.

1. Harrison, V.R.: Flow chart for safety testing monkey kidney cell suspensions used in vaccine production. Appl. Microbiol. 19:1, 1970.

2. Eckels, K.H., Harrison, V.R., and Hetrick, F.M.: Chikungunva virus vaccine prepared by Tween-ether extraction. Appl. Microbiol. 19:2, 1970.

PROJECT 3A061102B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 07
Pharmacology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6448	70 07 01	DD-DR&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
69 07 01	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	61102A	3A061102B71P	07	036
b. CONTRIBUTING				
c. XXXXXXX CDOG	1L12A(2)			

11. TITLE (Precede with Security Classification Code)
(U) Pharmacological Studies (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
68 07	CONT	DA	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 70	2	40
FISCAL YEAR CURRENT 71	2	40

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Pharmacology; (U) Medicinals; (U) Shock Therapy; (U) Drugs; (U) Stress

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

23 (U) Research is directed toward investigating the pharmacology of promising medicinal agents, drug interactions, developing and refining animal models for the study of hemorrhagic, septisemic, and traumatic injury shock as well as the exploitation of Army procured chemicals in the treatment and prevention of shock. Studies are directed toward determining the mechanisms of action of therapeutic agents as well as the nature and type of chemical which would be useful in shock therapy. The goal of this research is to develop a highly effective, non-toxic drug which would be useful in the treatment or prevention of trauma associated with battlefield injury.

24 (U) Drugs are tested in animal models for effectiveness in preventing or treating experimental shock resulting from hemorrhagic, endotoxin, traumatic injury, and anaphylactic stress.

25 (U) 69 07 - 70 06 Preclinical pharmacology and toxicology studies are being pursued in preparation for submitting an IND for compound WR 2823 AB for Phase I testing in man. The ultimate objective of this study is to test the efficacy of WR 2823 AB in the therapy of hemorrhagic shock in man. Compounds related to WR 2823 have shown efficacy in animal models for therapy of hemorrhagic, endotoxin and anaphylactic shock. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

* Available for release upon administrative approval

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Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 07, Pharmacology

Work Unit 036, Pharmacological studies

Investigators

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Description

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

Appropriate pharmacological, physiological, biochemical, and electrophysiological studies are conducted in vitro and in vivo. These studies encompass the acute responses to radioprotectant chemicals and their interaction with standard pharmacological and physiological agents. An outstanding feature of the capabilities of this department resides in the vast inventory of serially related and diverse chemicals which can be used in detailed and in screening studies of the nature of drug interactions with biological systems.

Progress

Experiments were continued in the development of isolated test systems for screening drug effects. Work continued in the definition of the mechanisms of action of aminoalkylaminoethyl phosphorothioate compounds to cause alpha adrenergic blockade. The use of snake and insect toxin as well as ionizing radiation as tools for the definition of drug receptors was continued. A test system has been developed using isolated rabbit aorta which is capable of screening for antihistaminic, antiserotonin, and antiadrenergic action simultaneously in four different tissues. Baseline evaluation of this test system is continuing. Studies have shown that the active form of WR 1729 and WR 2823 as alpha adrenergic blocking agents is that of WR 149,024.

Enzyme levels in tissues of normal, lead poisoned and iron shot loaded Mallard ducks were studied to assess the manifestation of lead poisoning in this species. The results suggest skeletal muscle damage from the result of lead poisoning. No further studies are contemplated.

The metabolism and excretion patterns of WR 40,070 and WR 109,342 are being studied to evaluate the possible pharmacokinetic mechanisms involved. Routine potency assays of Southeast Asia snake antivenoms intended for use in the field are in progress.

Summary and Conclusion

The surprising finding that WR 33,278 possesses efficacy against traumatic lethality but exhibits no adrenergic blocking properties leads us to investigate new pathophysiologic models for the pharmacological mechanisms involved in the chemoprophylaxis of traumatic injury. The correlations between traumatic, tourniquet, and burn injuries and hemorrhagic and endotoxin shock will be made in order to evaluate the similarities of mechanisms of these models in order to determine the pharmacophoric moiety responsible for such therapeutic effects.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 07, Pharmacology

Work Unit 036, Pharmacological studies

Publications

None.

PROJECT 3A061102B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08
Physiology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0A 6437	70 06 30	DD-DR&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 07 01	K.Completion	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3061102B71P	08	075
b. CONTRIBUTING				
c. CONTRIBUTING	C DOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)*

(U) Cell Growth and Regeneration (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

016200 Stress Physiology; 012900 Physiology; 002300 Biochemistry.

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
58 09	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:* NA

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	1.9	65

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Wound Healing; (U) Fibroblasts; (U) Collagen; (U) Hydroxyproline; (U) Oxygen Tension.

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

23(U) The definition of the cellular mechanisms controlling wound healing and tissue repair following combat injury such as mechanical, thermal, or chemical trauma, infection, ionizing radiation, and shock.

24(U) To maximize experimental control and analytical resolution, a well defined *in vitro* system is used to study fibroblastic cell populations under normal and simulated trauma conditions through the application of a wide spectrum of biological, physical and chemical procedures.

25(U) 69 07 - 69 12: It was found that, under the proper conditions, fibroblasts growing *in vitro* proceed from a phase characterized by a high rate of cell division and minimal collagen synthesis to a high density stationary phase characterized by minimal cell division and a high rate of collagen synthesis, thus paralleling the maturation of a wound in the body. In a further search for the determinants of this evolution it was found that, following renewal of the medium of the dense cultures, the oxygen tension of the cultures decreased progressively. To test whether decreased oxygen availability could act as inducer of the stationary phase by interfering with the energy metabolism of the cells but not with the oxygen requirement for the hydroxylation of proline, the cellular levels of hydroxyproline were compared with the kinetics of oxygen tension during the transition to the stationary phase. It was found that despite declining oxygen tensions, activation of hydroxyproline synthesis occurs in high density stationary cultures. Because of the considerable interest of these results for the control of wound healing, they have been communicated to clinical investigators to serve as guidelines in devising new methods for treating wounds.

For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08, Physiology

Work Unit 075, Cell growth and regeneration

Investigators.
Principal: André D. Glinos, M.D.
Associate: E.M. Bartos, Ph.D.; J.M. Vail, Ph.D.; R.J. Werrlein, M.S.

Problem and Background

While a great number of clinical studies in man and experimental studies in animals have revealed some of the factors influencing wound healing, the essential determinants of this process continue to elude us: we still do not know what makes fibroblasts proliferate in the early stages of a wound and what, at a later stage, causes these cells to stop dividing and to begin the synthesis of collagen responsible for the tensile strength of the wound. The reason for the failure of previous studies to reveal the nature of these determinants is the great complexity of the clinical situation in man and of the experimental preparation in the intact animal. To overcome this difficulty by simplifying the experimental situation, a well defined *in vitro* culture system has been developed in this department to study fibroblastic cell populations under normal and simulated trauma conditions. The major portion of the results obtained with this system have been described in detail in the annual progress reports of previous years and in the following publications: Glinos, A.D., Werrlein, R.J., and Papadopoulos, N.M., *Science,* 150: 350 (1965); Maxwell, R. E., and Glinos, A.D., *Federation Proc.,* 25: 297 (1966); Glinos, A.D.,, Vail, J.M., and Bartos, E.M., *Proc. of the 14th Oholo Ann. Biol. Conf.,* Israel Institute for Biological Research, Ness-Zinona, Israel, (1969); Glinos, A.D., in *Control of Cellular Growth in the Adult Organisms,* (edit. by Teir, H., and Rytomaa, T.), 41 (Academic Press, London, 1967). Accordingly, the final integration of the results relevant to oxygen tension and to hydroxyproline levels described in the present report represents the completion of this work unit.

Approach

The cells were grown in Eagle's spinner minimum essential medium (s MEM) supplemented with 10 percent horse serum. The cultures were incubated in the chamber shown in Fig. 1 maintained at 35°C. With a total volume of the incubation chamber of 2.75 L., a 200 ml cell suspension, and cell densities ranging from about 4 X 10^5 cells/ml at the beginning of the logarithmic growth phase to a maximum of 10^7 cells/ml

in the stationary phase, an atmosphere of 3 to 5 percent CO_2 in air was found sufficient to maintain the ph of the culture between 7.3 and 7.0 for a period of 24 hours following medium renewal. At the end of the 24-hour incubation period the medium of the culture was renewed by removing the culture from the chamber, centrifuging the cells at 225 X g for 25 minutes and resuspending the cells in fresh medium without dilution of the cell population. Cell counts were performed with a hemacytometer before and 2 hours after renewal of the medium in order to allow proper dispersion of the cells.

The paramagnetic oxygen analyser was calibrated using standard gas mixtures. The oxygen electrode was standardized daily by relating its response in the gas phase of the chamber to the readings of the paramagnetic oxygen analyser as follows: At the beginning of the 24-hour incubation period and with the culture in place, the chamber was gassed for 10' with 5 percent CO_2 in air and a positive pressure of 1/2 psi created by closing the outlet valve shortly before the inlet valve. After allowing 20' for thermal and water vapor equilibration the outlet valve was opened and three consecutive samples of the atmosphere of the incubation chamber were introduced into the analysis cell of the paramagnetic oxygen analyser after first passing through a $CaCl_2$ column to remove water vapor. With the interior of the analysis cell at atmospheric pressure, the oxygen content of the samples was determined and a mean value calculated. The incubation chamber was then also brought to atmospheric pressure, the outlet valve closed and the response of the oxygen electrode recorded. After incubating the culture for 24 hours in this closed system, the procedure was repeated with the response of the electrode obtained just before inflating the baloon in order to produce the positive pressure necessary for the introduction of the gas samples into the paramagnetic oxygen analyser. Finally, paired readings of the oxygen electrode and the paramagnetic oxygen analyser were obtained after removing the culture for medium renewal and reducing the oxygen content of the incubation chamber to 0-3 percent by gassing with nitrogen for a limited period of time. It was found that when polarization of the electrode was limited to 10' prior to each determination, its response to oxygen tensions varying from 140 mmHg to 0 was linear and relatively stable with deviations from the mean from day to day not exceeding ten percent. Accordingly, at desired time intervals after medium renewal, the electrode was polarized for 10', lowered into the liquid phase of the culture and its response recorded.

Results and Discussion

The daily kinetics of the oxygen tension of the medium during the progression of a culture from the logarithmic growth to the stationary phase is shown in Fig. 2. It can be seen that in the first day, as the density of the culture increased from 3.6×10^5 cells/ml to 7.94×10^5

cells/ml the oxygen tension of the medium declined from 141.4 mmHg after medium renewal to 98.5 mmHg at the end of the 24-hour incubation period. During the phase of near logarithmic growth of the culture, the rate of the daily decline of the oxygen tension of the medium increased progressively so that on the fifth day a pO_2 of 10 mmHg was reached within 12 hours after medium renewal. The rate of decline of the oxygen tension of the medium increased further during the retardation phase of the culture from the sixth to the tenth day, when medium pO_2 values below 10 mmHg were reached within 6 hours after medium renewal. From the eleventh to the fourteenth day when the culture was terminated, the cell density remained stationary at approximately 10^7 cells/ml. During this phase the oxygen tension of the medium fell rapidly after medium renewal, reaching values as low as 3 mmHg within the first 12 hours. In the second half of the incubation period the oxygen tension of the medium exhibited a slight tendency to increase, with pO_2 values as high as 12 mmHg recorded at 24 hours. Determination of the oxygen tension in the gas phase of the incubation chamber showed also a decrease, reflecting the utilization of oxygen by the cells. However, in no case did the pO_2 values recorded in the gas phase at the end of the 24-hour incubation period fall below 115 mmHg. This indicates that the striking decline of the oxygen tension in the culture medium is not due to the depletion of oxygen from the system as a whole but rather that as the cell density increases, the diffusion of oxygen from the gas phase into the medium of the culture lags behind its utilization by the cells. This suggests that decreased oxygen availability could act as an inducer of the stationary phase by interfering with the energy metabolism of the cells. To test this possibility and in view of the requirement for molecular oxygen for the synthesis of hydroxyproline, the amino acid characteristic for collagen, the cellular levels of this compound were determined during the progression of a culture to the high density stationary phase. Figure 3 shows that levels of cellular hydroxyproline remained low throughout the period of logarithmic growth and during the early stationary phase when the cell number fluctuated between 6 and 8 X 10^6 cells/ml. After stabilization of the density of the culture at 7.5 $\pm$ 0.3 X 10^6 cells/ml on day 15, cellular hydroxyproline increased rapidly and reached a new level exceeding by a factor of 3 the level characteristic for low density growing cultures.

Conclusions and Recommendations

It is concluded that in the model system used inhibition of fibroblastic growth and activation of hydroxyproline synthesis can occur in the presence of rapidly declining oxygen tensions. In regard to the latter it should be noted that the kinetics of the oxygen tension shown in Fig. 2 indicates that during the first hours after the renewal of the

medium of stationary cultures there was sufficient molecular oxygen present to satisfy the requirements of the hydroxylating enzyme, the Km of which for oxygen is 2.6 volumes percent or 19 mmHg. It is noteworthy that the pO_2 values observed during the second half of the incubation period are within the physiological range characterizing nonpoliferating tissues *in vivo*, if areas immediately adjacent to the capillaries are excluded. Also, it should be mentioned that the apparent association of relatively high oxygen tensions with fibroblastic growth and of low pO_2 with growth inhibition and activation of processes involved in collagen biosynthesis (Figures 2 and 3) has also been observed by Silver (in *Progress in Respiration Research*; edit. Herzog, H., 3, 124; Karger, Basel, 1969) during the growth and maturation of fibroblasts in rabbit ear chambers. The potential clinical significance of these results is readily apparent when they are considered in conjunction with the well known sequence in wound healing where the formation of new capillaries is associated with the proliferation of granulation tissue and their subsequent atrophy and involution with deposition of collagen fibers. Accordingly, these results have been communicated to clinical investigators and it is recommended that such contacts be maintained and increased. This is necessary in order for these findings to be properly utilized as guidelines for further research and development aiming at the rational treatment of wounds.

LEGEND TO FIGURES

Fig. 1 Diagram of the incubation chamber and the oxygen measuring devices operated as a closed system. A. Cell suspension. B. Magnetic stirring platform. C. Stainless steel culture chamber. D. Heating coil and connection to water bath. E. Pressure gauge. F. Gas inlet valve with 0.45 μ pore sterile filter. Gas outlet valve with connection to atmosphere or paramagnetic oxygen analyzer. H. Removable lid. I. Thermostat. J. Movable Clark-type oxygen electrode. K. Valve with attached inflatable balloon.

Fig. 2 Population density curve (A) and daily kinetics of medium pO_2 (B). A. Points in population density curve represent mean cell counts immediately before and 2 hours after daily medium renewal. B. The daily starting point of 140 mmHg represents the oxygen tension of new medium immediately after equilibration with 5% CO_2 in air. Subsequent points refer to a minimum of 3 determinations carried out at 2, 14 and 24 hours after medium renewal as on day 1 and a maximum of seven determinations at 2, 3, 4, 6, 7, 11 and 24 hours as on day 8.

Fig. 3 Cellular hydroxyproline levels during the transition from the logarithmic to the stationary phase. Points in cell number curve, to be read against the left ordinate, represent mean cell counts immediately before and 2 hours after medium renewal. Points in hydroxyproline curve, to be read against the right ordinate, represent the mean values obtained from duplicate samples of approximately 2×10^7 cells each, which were washed with buffered saline, hydrolysed for 16 hours with 6N HCL at 100°, neutralized and assayed with the method of Prockop and Udenfriend (*Anal. Bioch.*, 1: 228, 1960).

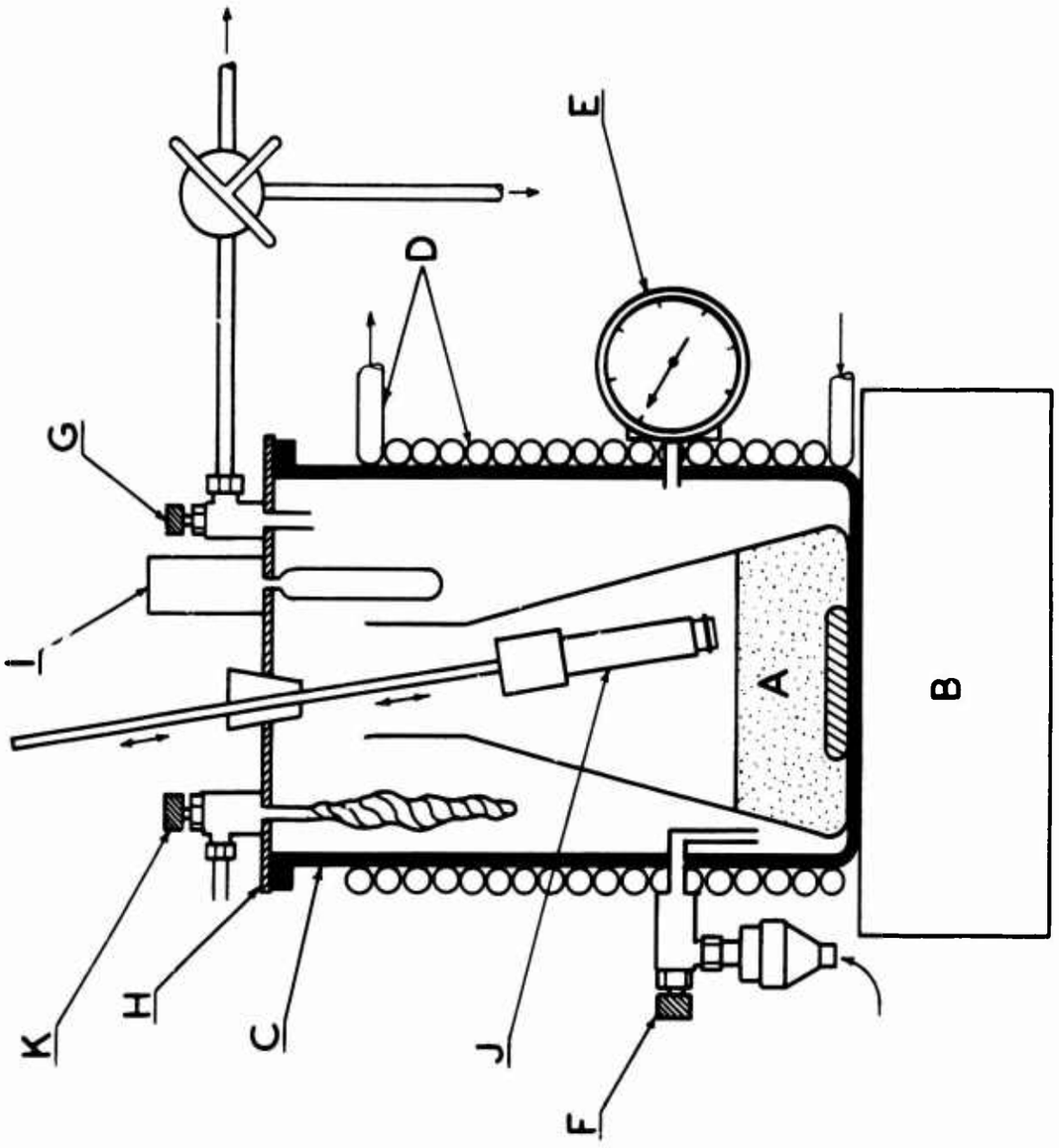

FIGURE 1

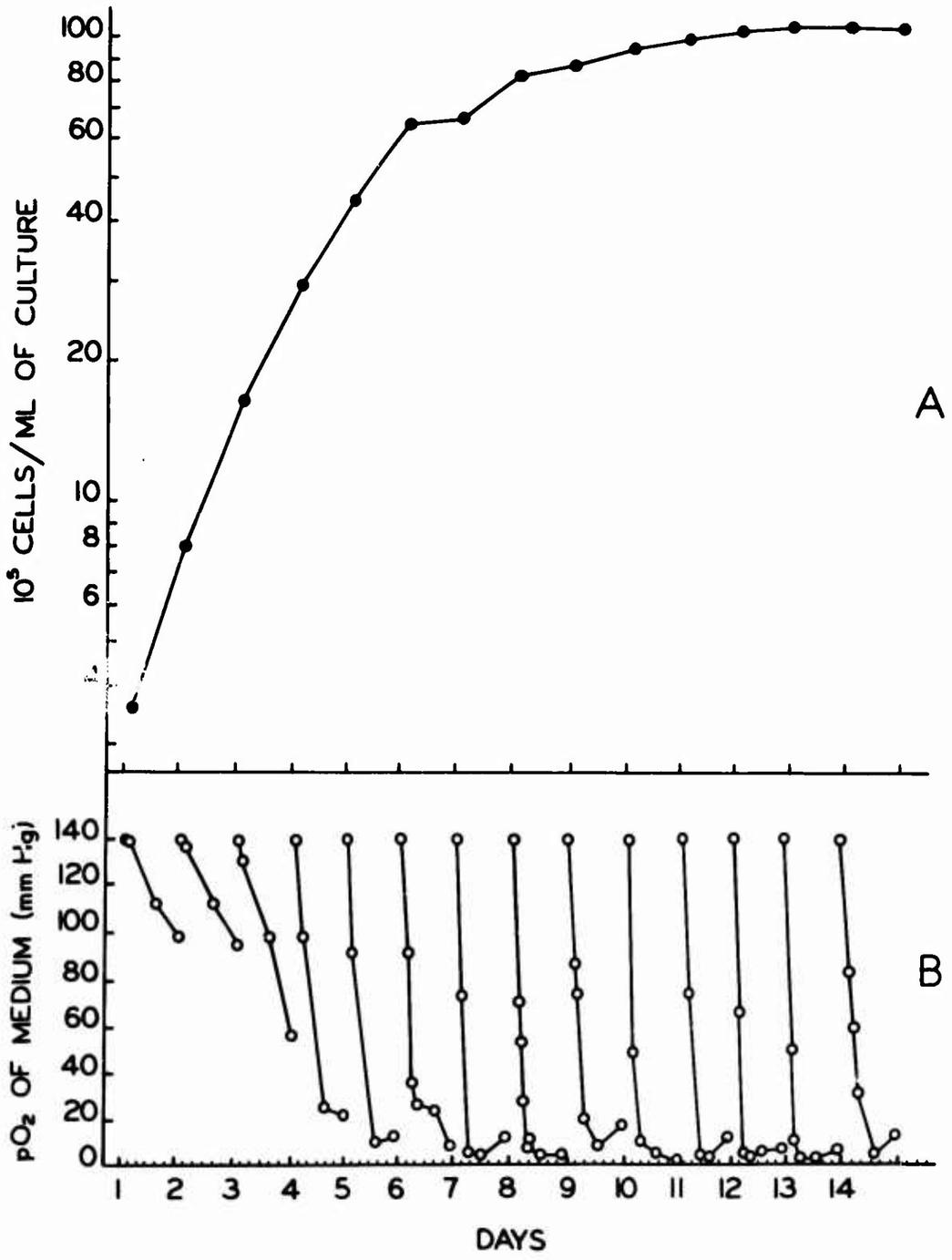
100
80
60
40
20
10
8
6
10^3 CELLS/ML OF CULTURE
A
140
120
100
80
60
40
20
0
pO_2 OF MEDIUM (mm Hg)
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAYS

FIGURE 2

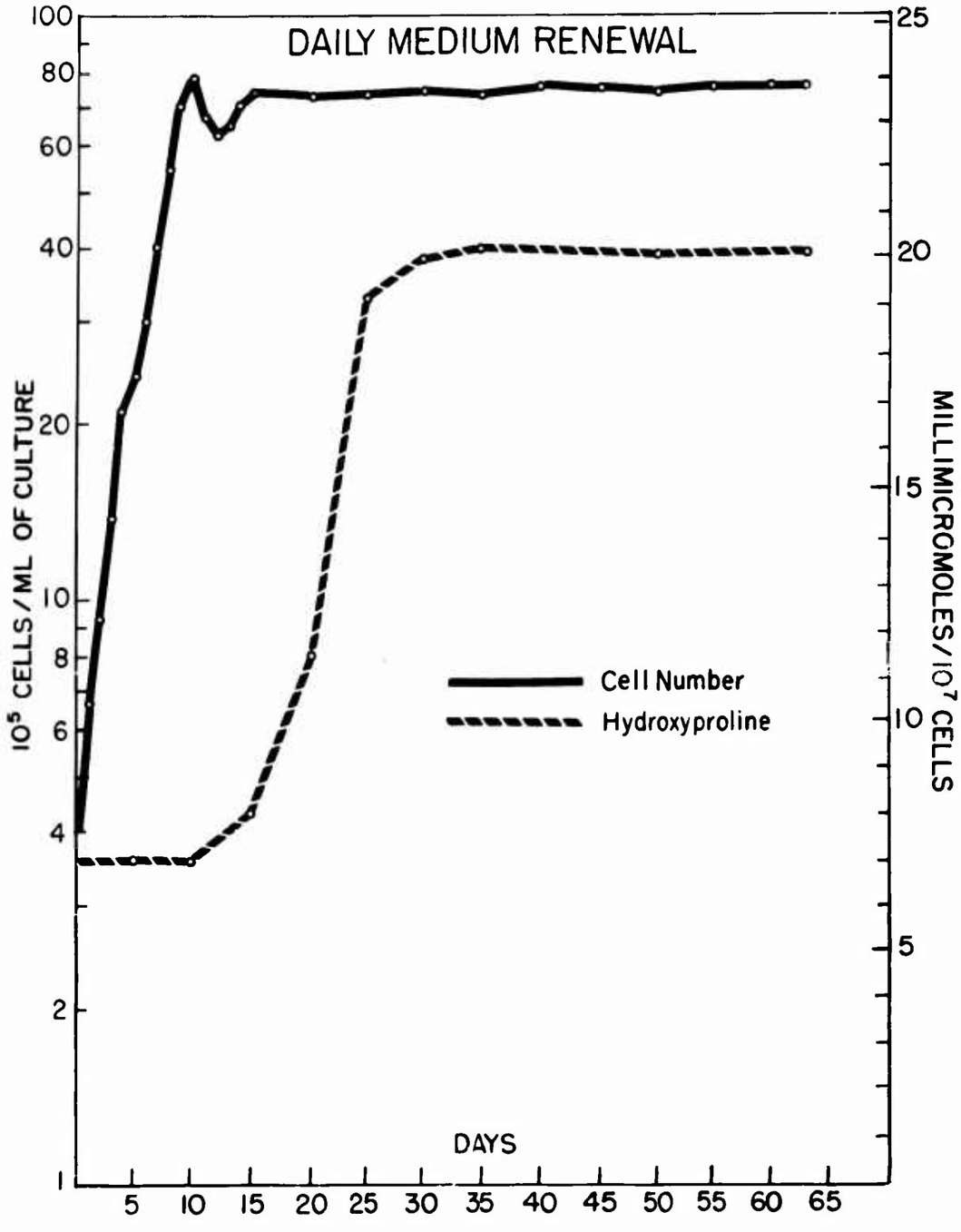
DAILY MEDIUM RENEWAL
100
80
60
40
20
10
8
6
4
2
1
10^5 CELLS/ML OF CULTURE
25
20
15
10
5
MILLIMICROMOLES/10^7 CELLS
Cell Number
Hydroxyproline
DAYS
5 10 15 20 25 30 35 40 45 50 55 60 65

FIGURE 3

3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08 Physiology

Work Unit 075 Cell growth and regeneration

Literature Cited

1. Reference:

Glinos, A.D., Vail, J.M. and Bartos, E.M.: High density suspension cultures of mammalian fibroblasts: Increased levels of hydroxyproline in the presence of rapidly declining oxygen tensions. To be submitted for publication.

2. Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6438	70 07 01	DD-DR&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 07 01	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	61102A	3A061102B71P	08	076
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code): (U) Maintenance of basic physiologic function and pain regulation in disease and stress -- Anatomic and Electrophysiological Factors (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 002400 Bioengineering 016200 Stress Physiology 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	cont	DA	C. Inhouse

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 70	15	280
FISCAL YEAR CURRENT 71	10.5	200

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NAME: Wylie, R., Ph.D. DA

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Neurophysiology; (U) Neuropsychiatry; (U) Anatomy; (U) Autonomic Nervous System; (U) Central Nervous System; (U) Biomedical Engineering

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

23. (U) The anatomic & physiologic basis of Central Nervous System control of blood pressure adjustments in medical & surgical (blood loss) shock. The effects of stress on these adjustments. An analysis of Transcutaneous Stimulation Technique (TST) for the control of pain in patients with high velocity missile injuries of peripheral nerves.

24. (U) Determination of Central Nervous System pathways & terminations involved in control of blood pressure adjustments by histological & Histochemical techniques. Recording and analysis utilizing computer techniques, of cardiovascular responses to controlled hemorrhagic shock in awake chronically implanted monkeys. Recording and analysis of single and multicell activity in the Central Nervous System and its relationship to physiological adjustment mechanisms. Stimulation of peripheral nerves in patients to relieve pain.

25. (U) 69 12-70 06 Autonomic centers in the spinal cord have been defined and structural changes in autonomic ganglia following stress are being detailed. Cardiovascular responses to controlled hemorrhagic shock are being quantitated. New centers of interaction have been discovered in the spinal cord between representations of internal organs and somatic structures. A new pathway carrying information from these centers to the brain is being detailed. Interactions between the vestibular system and other sensory inputs are under study. Hospital patients with peripheral nerve injuries have been treated with TST. The above constitutes a basis for favorably modifying life-threatening physiologic responses in stress and disease. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 69 - 30 June 70.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

PROJECT 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

TASK 08, PHYSIOLOGY

WORK UNIT 076, Maintenance of basic physiologic function and pain regulation in disease and stress: Anatomic and Electrophysiological factors

INVESTIGATORS.
PRINCIPAL: David L. Winter, M.D.

ASSOCIATES: MAJ. John C. Adkins, MC; MAJ. Robert D. Cardiff, MC; CPT. John J. Dropp, MSC; MAJ. Howard L. Fields, MC: CPT. Michael L. Katz, VC; MAJ. Glenn A. Meyer, MC; James M. Petras, Ph.D.; Andrew T. Pryzbylik, EE; Maurice E. T. Swinnen, EE; Richard Wylie, Ph.D.

DESCRIPTION:

The general object of this subtask is to study the neural mechanisms which underlie the physiologic responses to stress, disease and pain. Of particular interest are autonomic nervous system functions such as control of blood pressure, gastrointestinal motility, glandular secretion, heart rate, temperature control and bladder regulation. These autonomic functions comprise the basic components of homeostasis. An understanding of the mechanisms of action of the autonomic nervous system, its interaction with somato-motor systems and its response to physical and psychological stress establish a basis for favorably modifying life-threatening physiologic responses in disease.

Detailed information concerning the physiology and, especially the anatomy of the autonomic nervous system is not available. A major effort is being undertaken to obtain this detailed data as well as broader observations of the total system's responses to experimentally controlled and naturally occurring stress.

Progress

1. Neuroanatomic studies

A) Definition of autonomic nuclei in the spinal cord.

Using chromotolysis of neurons following sympathectomy, the normal anatomy of autonomic nuclei in the thoraco-lumbar spinal cord has been established. The results indicate that three separate nuclear groups send axons into the sympathetic chains.

B) Relationships between autonomic neurons and sensory inputs.

Having established the location of preganglionic neurons in the cord, dorsal root lesions were made at appropriate spinal cord levels following sympathectomy. Utilizing the Nauta and Fink-Heimer stains for degenerating terminal endings, the distribution of dorsal root fibers on spinal neurons was documented. It was found that sensory fibers did not terminate directly on autonomic preganglionics, thus establishing, for the first time, that the autonomic spinal reflex system must be polysynaptic.

C) Relationships between descending pathways and autonomic neurons.

A major project underway is to determine the origin of descending pathways to autonomic neurons. The initial studies are focusing on cortical and reticular formation sites as possible origins of pathways to autonomic nuclei.

D) Definition of Parasympathetic nuclei in the spinal cord.

Attempts are being made to selectively cut the parasympathetic input to the spinal cord in order to identify the cells of origin by retrograde chromatolysis techniques. At this time there are no clear cut studies in the literature which indicate where the parasympathetic cell bodies are located in the sacral cord. It is our hope to delineate these structures and to proceed with additional studies as we have done with the sympathetic neurons.

E) Studies on a new visceral afferent pathway.

A fiber degeneration study is underway to determine the course and termination of a newly discovered visceral-somatic afferent pathway (Fields & Winter, 1970, see below). Lesions have been made using dorsal and ventral approaches to the ventral funiculus of the spinal cord. Preliminary findings point toward degenerated ascending components in the spinal ventral funiculus which may correspond to the ventral pathway of Fields and Winter.

F) Changes in autonomic ganglia following behavioral Stress.

Last year's report indicated that cytological changes had been noted in autonomic ganglia following behavioral stress. This very important finding has been confirmed and a number of studies have been initiated to examine these changes in detail. In the rat, evidence has been gathered to indicate that neuroglial proliferation, neuronal hypertrophy and neuronal nuclear and nucleolar hypertrophy takes place.

These changes have been found in autonomic ganglia only and not elsewhere in the nervous system. The increase in 3H-Thymidine uptake is presumptive evidence of DNA synthesis leading to cell division. The glial response is perhaps a concomitant of increased metabolic (functional) demands of the autonomic neurons.

New studies have determined the time course of this response and tested modifications of the behavioral stress. Also in progress are studies in the monkey which are focusing on acute and chronic stress periods.

G) Mast cells in the CNS.

A survey of the distribution of mast cells in the CNS is being done in rodents. This is important since (1) there is increasing evidence that biogenic amines stored and released by mast cells have pharmacologic effects on neurons and neuroglia; and (2), mast cell secretions have recently been implicated in the genesis of histological brain damage and (3), there are very few reports of the occurrence of mast cells in the mammalian CNS.

H) Neurotropic Viruses.

In collaboration with other WRAIR laboratories the morphology of various antigens from dengue virus infected mouse brain has been studied. The purpose of the study was to determine the interrelationship between the virus and two non-infections dengue specific antigens SHA and SCF. As a result of the study of the ultrastructure of chemically degraded virion and SHA and SCF, it was concluded that neither was a result of viral breakdown.

2. Physiological Studies.

A) CNS control of blood Pressure.

A collaborative project with Dr. E. Hawthorne, Howard University, has suggested that electrical stimulation of diencephalic structures might selectively elicit cardiac contractibility and peripheral resistance changes. These are the two major factors controlling blood pressure and previously have not been thought to be represented separately in the CNS.

B) Autonomic responses in hemorrhagic shock.

The effects of slow hemorrhage and its progression towards shock are being studied in chronic, extensively instrumented monkeys. The basic aim of this study is to detail the mechanisms and sequences of breakdown of the normal homeostatic autonomic reflexes involved in the maintenance of blood pressure and blood flow. Blood

pressure, blood flows and derived values such as cardiac work and cardiac output have been documented for the slow hemorrhage model. The initial phase of this study has now been completed and Phase II is starting. This phase will consist of stepwise interruption of the nervous system, peripheral and then central in order to break up different feedback loops.

C) Spinal mechanisms in blood pressure control.

Increasing the spinal subarachnoid pressure in the spinal dog, causes an elevation of blood pressure to exceed the imposed spinal pressure. This model was used to study single preganglionic sympathetic neurons and their participation in the blood pressure increase. It was also possible to evoke Mayer waves, rhythmical blood pressure changes not related to respiration, and follow unit discharges through numerous cycles. A group of spinal cord autonomics was discovered which clearly followed the blood pressure fluctuations in a fixed-time relationship. This establishes the potential for spinal autonomics, independent of descending influences, to exert some degree of control of blood pressure.

D) Neurophysiologic studies of spinal cord integration.

Using microelectrodes, the electrical activity of single cells in the spinal cord has been studied. Of particular interest has been the question of how signals are integrated. Somato sensory and visceral sensory inputs have been tested to determine if such information was integrated at the spinal level. Indeed, a group of cells was discovered which did respond to both types of input. These cells had all of the features of cells generally found in the reticular formation of the brain stem. These findings indicate that considerably more integration takes place in the spinal cord than is generally recognized.

E) Identification of a new afferent pathway in the Spinal cord.

A new afferent sensory pathway was discovered in the ventral funiculus of the spinal cord. The fibers conduct at unusually fast speeds and carry both somatosensory and viscerosensory information. The location of the pathway is unusual in that no sensory tracts have previously been noted in this area. Its presence may explain residual pain following classical spinothalamic tractotomy since this new pathway would be spared.

F) Studies in the vestibular system.

A new major project area has been initiated in vestibular system physiology. This system provides information about the position of the head. Visual, somesthetic and visceral inputs provide additional sources of information about the position of the organism in the gravitational field. These diverse sensory inputs must be integrated with volitional and reflex activities of the nervous system in order to

maintain the organism in an appropriate position. The vestibular system has also been implicated in autonomic changes occurring in sleep and in homeostatic adjustments of vascular responses to position change. Vestibular-autonomic interactions are probably of great importance in problems of motion sickness and in problems of medical evacuation flights.

Initial studies in this system are focusing on the integration of somatosensory and labyrinthine information in the vestibular nuclei. Additional work is underway to determine the nature of synaptic transmission in the vestibular nuclei. These experiments all involve microelectrode techniques under stereotoxic placement. Due to the relatively small size of the vestibular nuclei and their location, this work represents a major achievement in technical development.

G) Modification of Pain perception.

In association with WRGH, studies on the modification of Pain perception have continued. Transcutaneous stimulation Technique (TST) has been more fully investigated with encouraging results. Patients with true causalgia have received marked relief of pain and have been able to start physical therapy earlier following TST therapy than untreated patients. As a result of these studies a project is being set up OCONUS, in Japan, to evaluate TST in patients treated earlier in the course of their pain syndrome. It is hoped that the pain syndrome will be favorably modified and rehabilitation started earlier in these patients as a result of TST. This OCONUS project will start in the fall of 1970.

H) EEG Studies in Uremia.

In conjunction with the Department of Medicine, WRAIR, a study has been completed in which an analysis of EEG activity during uremia was performed. A simple method of quantitation was developed which indicated that EEG monitoring has value in estimating the degree of uremia in experimental animals.

3. Technical Developments.

A) Experimental

The electron microscopy laboratory is still not completely functional. Difficulties with the electron microscope necessitated replacement by the vendor of a new scope. Minor problems still plague the scope but hopefully these will be corrected shortly. Installation of a chemical hood and proper plumbing still await completion.

B) Electronic

1) A special design 8-channel EEG preamplifier
2) Universal frequency counter
3) Luminance calibrator
4) Running time meter for the electron microscope
5) Voltage control variac
6) Temperature alarms for refrigerators
7) Dermohmmeter
8) Skin temperature indicator
9) Blood pressure display servo unit
10) Skin thermometer
11) AC spark suppressor systems
12) Portable laboratory for Psychological testing

SUMMARY AND CONCLUSION

The organism's response to disease and stress has been studied using a variety of experimental approaches. Particular emphasis has been given to the role of the autonomic nervous system in mediating these responses. Morphological studies have been directed towards determining the organization of this system and the interrelationships of successive levels of control. Physiological studies have focused on establishing functional properties of neuronal aggregates and their relationship to the regulation of homoeostatic processes. A new definition of the autonomic nervous system from both an anatomical and physiological viewpoint is clearly emerging from these studies. The role of this system in homeostatic reflexes and the ability of this system to perform discrete as well as general actions are new concepts. The potential ability to control pain perception by TST is an exciting and important development which has wide ranging implications. Several of the listed projects were accompanied and facilitated by new developments in technical instrumentation.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08, Physiology

Work Unit 076, Maintenance of basic physiologic function and pain regulation in disease and stress: Anatomic and Electrophysiological factors

Literature Cited.

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3. Cummings, J.F.,: Thoracolumbar preganglionic neurons and Adrenal innervation in the dog. Acta. Anat. 73:27, 1969.

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8. Winter, D.L. and Fields, H.L.: Rapidly Conducting Somatovisceral Pathway in the Spinal cord. Fed. Proc. 29:392, 1970.

9. Wylie, R.M. with Hunt, C.C.: Responses of snake muscle Spindles to stretch and Intrafusal Muscle Fiber Contraction. J. Neurophysiol. 33:1, 1970.

10. Wylie, R.M. with Wilson, V.J.: A short-latency Labyrinthine Input to the vestibular Nuclei in the Pigeon. Science. 168:124, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA 0A 6439	70 07 01	DD-DR&E(AR)636

3. DATE PRJV 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
69 07 01	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	61102A	3A061102B71P	08	077
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code) (U) Influence of Stress on Hormone Response, Performance and Emotional Breakdown in the Military (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
013400 Psychology /Indivi 016200 Stress Physiology 005900 Environmental Bio

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	8	240
FISCAL YEAR CURRENT 70	9	260

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21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Stress; (U) Emotions; (U) Homeostasis; (U) Psychophysiology; (U) Neuroendocrinology; (U) Psychoendocrinology

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

23. (U) Principal objective is to study the integrating influences of the central nervous system in controlling and coordinating the organs of the body and their metabolic functions under environmental and emotional stresses which are likely to produce casualties due to psychiatric or psychosomatic disease.

24. (U) This involves measurement of plasma and urinary hormone levels in monkeys and humans in a variety of acute and chronic stress situations, with emphasis on the concept developed by our earlier works that we must view changes in broad, overall hormonal patterns or balance, rather than in single endocrine systems as was previously customary in the stress field.

25. (U) 69 07 - 70 06 Emphasis during the past year has been given to neuroendocrine studies of physical stress, particularly to a study of muscular exertion in collaboration with ARIEM (Natick). In acute exercise, norepinephrine, epinephrine, growth hormone, and cortisol rise while insulin, testosterone, androsterone, etiocholanolone, estrone, and estradiol tend to decrease. A qualitative, as well as a quantitative, shift in hormonal balance was observed as a function of increasing workload. Preliminary findings also suggest a possible relationship between high insulin levels and physical endurance in exhaustion sessions. Additional observations showing distinctive patterns of multiple hormonal responses to cold, heat, and regional brain stimulation have been made. Clinical psychoendocrine studies of patients with obesity, colitis, and hypertension are providing leads concerning new approaches to the pathogenesis of these diseases. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 Jun 70.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08, Physiology

Work Unit 077, Influence of stress on hormone response, performance and emotional breakdown in the military

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Associate: MAJ Theodore A. Kotchen, MC; CPT Albert L. Ehle, MC; Edward H. Mougey, M.S.; Frances E. Wherry, A.B.; David R. Collins, B.S.; Percy T. Ricketts, B.S.; Lee L. Pennington, B.S.; Jennette Wade, B.S.; Norman Krasnegor, Ph.D.

Description.

This program is concerned with the role of the central nervous system in the co-ordination of endocrine regulation. Instead of the conventional study of single endocrine systems in isolation, multiple endocrine systems are studied concurrently so that the overall balance between the many interdependent hormones may be investigated. In recent years we have learned that various forms of psychological and physical "stress" elicit broadly organized patterns of hormonal response involving many hormones in addition to those of the adrenal systems. A major goal is to define conclusively these distinctive "overall" hormonal response patterns for various stressful stimuli, including psychological stimuli, cold, heat, hypoxia, fasting, exercise, hemorrhage, dehydration, trauma, infection, and various nutritional changes. Such basic knowledge of the integrative machinery is essential as a foundation for neuroendocrine approaches to the study of clinical and field problems concerned with such parameters as endurance, fatigue, host resistance and performance. Emphasis in our program during the past year has shifted to the study of neuroendocrine mechanisms which integrate bodily responses to physical stress and a very fruitful interdisciplinary collaboration has been established with investigators at the Army Institute for Research on Environmental Medicine (ARIEM) at Natick, Massachusetts. A substantial amount of work on the development of new or improved hormone assay procedures has also been continued in order to provide the necessary methodological foundation for this stress research program.

Progress.

1. Hormonal Balance in Physical Stress.

a. Exercise. A major portion of our effort during the past year has been directed to the study of the organization of multiple hormonal responses to muscular exertion in normal human subjects in collaboration with LTC LeeRoy Jones and LTC Howard Hartley at ARIEM. Hormonal

responses have been studied in relation to graded work loads (40%, 70%, and 100% of maximal oxygen uptake level of 5- to 10-minute duration and in relation to exhaustion in prolonged exercise sessions. In addition, neuroendocrine factors in relation to a vigorous physical conditioning program are under study. Results in acute, short duration exercise sessions so far indicate marked elevations in plasma growth hormone and norepinephrine levels, a moderate increase in plasma epinephrine level, but relatively little change in plasma 17-OHCS levels except at high work load levels. Plasma insulin levels fall during exercise. Urinary norepinephrine and epinephrine levels rise sharply, but urinary 17-OHCS levels do not change, even with 40% max. VO_2 exercise for three hours. Urinary testosterone and androgen levels appear to fall slightly. At various graded work load levels there is some qualitative difference in hormonal pattern which may be related to the shift in the muscular utilization of energy substrate (from glucose and FFA to glycogen) which occurs at higher work-load levels. Norepinephrine and epinephrine responses steadily increase with increasing work load up to the 100% max. VO_2 level, while growth hormone response peaks at the 70% level and rises less at the 100% level. The plasma 17-OHCS response is appreciable only at the 100% level. One interesting lead which has emerged in exhaustion studies is a possible correlation between high plasma insulin levels and endurance in prolonged exercise sessions. A second study is now under way to evaluate further this finding. Only preliminary results have been obtained so far on the physical conditioning studies.

Plasma renin activity was also measured in seven subjects during graded exercise. There were significant but transient elevations in renin levels immediately after exercise at the 70% and 100% max. VO_2 work-load levels, but not at the 40% level.

b. Cold. Several experiments have now been completed on the organization of multiple hormonal responses to cold in the monkey. Gradual lowering of temperature to 5°C elicits marked elevations in 17-OHCS, epinephrine and norepinephrine levels, as well as a delayed increase in plasma BEI (thyroxine) levels during six-week cold sessions. Plans are under way to do more detailed and complete studies of hormonal responses to cold in the human in collaboration with ARIEM.

c. Heat. Additional data have been obtained in the monkey indicating that heat actually suppresses 17-OHCS, epinephrine and norepinephrine levels if care is taken to minimize psychological reactions to the experimental situation. Monkeys kept at 85°F over a period of many weeks consistently show urinary 17-OHCS levels at least 50% lower than monkeys kept at 72°F. Experiments are currently under way to alternate four-week periods at 85°F versus four-week periods at 70°F in the same monkeys in order to establish the organization of

hormonal adjustments to heat more conclusively. Results so far do not show any individual hormonal responses which are common to both heat and cold, if psychological reactions are minimized.

2. Hormonal Balance in Emotional Stress.

In several subjects involved in exercise experiments, marked plasma growth hormone and 17-OHCS responses to venipuncture and venous catheterization were observed, presumably as a result of emotional reaction to these procedures. These experiments will provide us with our first opportunity to study systematically the overall pattern of hormonal response to acute emotional stimuli in the human in comparison with the pattern of psychoendocrine response which is now well established in the monkey.

In the monkey we have completed a small series of experiments on hormonal responses to avoidance following adrenalectomy. Marked growth hormone responses to avoidance persist following adrenalectomy, as does the usual suppression of testosterone and androsterone levels. Urinary 17-OHCS and epinephrine responses are abolished as expected, but some norepinephrine response persists. Thus, a number of hormonal responses to avoidance are not dependent upon the pituitary-adrenal system.

3. Hormonal Balance in Medical Illness.

a. Obesity. A psychoendocrine study of five obese patients in collaboration with Dr. Jules Hirsch of the Rockefeller University Medical Center has now been completed. Comparison of chronic mean "basal" hormonal profile in these patients with normal subjects shows a number of differences. Obese patients show higher levels of norepinephrine, TSH, estrogens, and insulin but lower levels of growth hormone than normal subjects. Another interesting difference suggested by our data is that testosterone and androgen levels tend to be lower than normal in the obese men and higher than normal in the obese women.

A second lead obtained was the occurrence of a peculiar pattern of acute psychoendocrine response in obese patients, in which insulin and testosterone levels increased along with the adrenal hormones. The possibility that this paradoxical rise of "anabolic" hormones during emotional stress may play a role in the pathogenesis of obesity may deserve further study.

These patients were also followed through a period of many weeks on a 500 calorie reducing diet and then on a weight maintenance diet at a considerably reduced weight level. On the weight reduction diet, there were marked decreases in 17-OHCS, norepinephrine, testosterone, androgen, estrogen, and insulin levels, and mild increases in growth

hormone and TSH levels. On the weight maintenance diet all these hormonal changes tended to return toward the initial profile except for norepinephrine levels which tended to remain lower than during the original high weight level period. These findings suggest that most of the hormonal abnormalities observed in the obese patient are not secondary to the presence of increased amounts of adipose tissue, but rather they appear to characterize the individual in a more enduring fashion even during periods of weight reduction.

b. Colitis. A pilot study of two colitis patients was initiated this year in collaboration with Dr. Arthur McMahon of the New England Medical Center in Boston. This study suggested that epinephrine levels are lower than normal and that testosterone, androgen, and insulin levels are higher than normal in colitis patients, a hormonal imbalance perhaps analogous to the postulated autonomic imbalance in these patients. Careful daily psychological evaluation of these patients was made and hormonal data, particularly 17-OHCS values, indicated that the confrontation of the patient with their longstanding problem of psychosexual immaturity and the suggestion of psychotherapy for this problem was repeatedly associated with marked disruption of physiological functions, with depressive affect, and with changes in bowel function, whereas other stressful experiences did not have similar effects.

c. Hypertension. MAJ Kotchen has initiated a study of hormonal profile and renin regulation in patients with essential hypertension. To date, four patients have been evaluated on Ward 30. All had normal or elevated renin activity on a measured high sodium diet, and three showed a renin elevation following dietary sodium depletion. Only preliminary hormonal and psychological data have been obtained so far on these patients.

Renal venous renin has been measured in ten patients suspected of having renovascular hypertension. There was no significant difference in renin levels between the right and left renal veins in eight of these patients. Two patients had elevated renin levels in the vein on the affected side and are being considered for surgery.

4. Hormonal Responses to Brain Stimulation.

CPT Ehle has now completed several experiments with chronically implanted electrodes in the chair-restrained monkey which indicate that electrical stimulation of the amygdaloid complex can increase the plasma levels of growth hormone as well as cortisol. Other hormonal measurements are currently being made and the effects of hippocampal stimulation are also being studied.

5. Hormone Assay Methodology.

An isotopic method for the measurement of testosterone secretion rate has been successfully worked out by Mr. Collins, with guidance from Dr. Rose. Mr. Collins has also set up a gas chromatographic procedure for measurement of pregnanediol, a major derivative of progesterone, which can be combined with our current urinary androgen assay. Mr. Mougey has continued work on the free thyroxine method, but finds the existing procedures very susceptible to error. Mrs. Wherry has set up a new radioimmunoassay procedure for the measurement of plasma estradiol levels which has been checked out chemically and is now being evaluated physiologically. Attempts are also being made currently by Mrs. Wherry and MAJ Kotchen to develop radioimmunoassays for the measurement of vasopressin and angiotensin.

Summary and Conclusions.

Our basic studies continue to show that multiple hormonal responses occur in various physical and psychological stress situations and that these multiple responses appear to be broadly organized in a way that is distinctive for the particular stimulus. We have reasonably extensive data so far on "overall" hormonal response patterns for acute psychological disturbances, fasting, muscular exercise, cold, heat, high carbohydrate intake, and hemorrhage, but need further studies of hypoxia, dehydration, barometric pressure, trauma, organic substrates, and several other factors which represent important natural inputs into the neuroendocrine integrative machinery.

Our clinical studies continue to turn up new leads and to provide information on the ways in which neuroendocrine approaches can be applied in the psychosomatic study of medical illnesses. In addition to the study of abnormal chronic hormonal profiles in medical patients, the study of disordered patterns of hormonal response to psychological or other acute stimuli may prove another important parameter and one which may provide a new approach to the study of disease pathogenesis.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08, Physiology

Work Unit 077, Influence of stress on hormone response, performance and emotional breakdown in the military

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PROJECT 3A061102B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 09
Radiobiology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6533	70 06 30	DD-DR&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 07 01	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	61102A	3A061102B71P	09	015
b. CONTRIBUTING				
XXXXXXXXXXXXXX	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Mechanisms (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

014100 Radiobiology 011500 Nuclear Weapons

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

17. CONTRACT/GRANT

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	11	180
	CURRENT 70	7	200

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS

NAME: Ginsberg, LTC D. M. DA

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Radiation Injury; (U) Radiobiology; (U) Combined Injury; (U) DNA; (U) Bacteria

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

23. (U) Quantitative measurements of the consequences of exposure to ionizing radiation in cellular, submammalian and mammalian systems.

24. (U) Effects of radiation and radiation modifiers on DNA and influence of alterations in cell metabolism on radiation survival are being investigated in bacteria. Electron spin resonance spectrometry is used to define free radical production and the time length of action of radicals within the biological medium. Changes in genetic material within mammalian cell lines as evidenced by chromosomal breaks and translocations are quantitated with biochemical cellular constituents after radiation. Influence of stresses such as trauma and burns combined with radiation are evaluated in relation to dose response.

25. (U) 69 07 - 70 06 Within the reporting period a spectrum of studies have been pursued at the cellular, organ and whole animal levels. Efforts at the cellular level have included the use of electron spin resonance spectrometry to study structure-function interrelationships on pertinent aminothiols. The study of radiation injury and cellular repair has been carried out utilizing E. coli infected with T4 bacteriophage. Results indicate that a single strand break is made after UV-irradiation for every dimer present. Organ level studies have been carried out on bone marrow and the gastrointestinal tract. Intermediary metabolic studies have shown that hydrogen equivalent transfer from the intra mitochondria space is based upon a substrate shuttle system. The work unit is being terminated and the effort consolidated within the Divisions of Biochemistry, Medicine, and Communicable Disease and Immunology. Project has been terminated and work unit is to be considered as terminated. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

* Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 09, Radiobiology

Work Unit 015, Mechanisms

Investigators.
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Associate: B. G. Bass, B.S.; A. R. Berman, B.S.; E. S. Copeland, Ph.D.; W. N. Crofford, M.S.; M. H. Davis, B.S.; MAJ E. C. Friedberg, MC; MAJ H. B. Gardner, MC; LTC D. M. Ginsberg, MSC; M. M. Hill, M.S.; LTC M. C. Johnson, MC; MAJ M. E. Liebowitz, MC; MAJ L. Lumeng, MC; LTC D. T. Mahin, MC; E. C. Richardson, M.S.; LTC H. M. Swartz, MC.

Description.

The work unit is being terminated and the personnel have been transferred to the Divisions of Biochemistry, Medicinal Chemistry, Experimental Pathology, Medicine, and Communicable Diseases and Immunology. The overall objective of this work unit was to define the biochemical and physiological mechanisms by which ionizing radiation affects living organisms at all levels. Research efforts reported here cover the period from 69 07 01 to 69 12 31.

1. Physico-chemical studies of radiation injury.

Electron spin resonance techniques are being used to detect, measure and quantitate free radical response as a consequence of radiation exposure.

2. Molecular and cellular biology of radiation injury.

This work entails the identification and measurement of specific radiation damage to bio-macromolecules. Repair processes at the cellular level are also considered.

3. Tissue, organ and whole organism studies.

Immediate and long term changes in the functional integrity of the whole organism after exposure to ionizing radiation are being investigated.

4. Radiation dosimetry.

The quality and quantity of radiation exposure are important considerations in making observations as to biological response. Projects within this work unit are designed and executed to accurately forecast and quantitate radiation exposure.

5. Human whole body radiation detection.

The Walter Reed Human Whole Body Counting Facility is used as a supporting tool for health physics and nuclear medicine activities at the Walter Reed Army Medical Center.

Progress.

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

The work within this section has been devoted to the study of the oxygen effect and the use of electron spin resonance spectrometry as an in vivo dosimeter.

Power saturation studies down to very low microwave power levels (5 microwatts) reveals that the oxygen freezing free radicals are few in number when compared to free radicals naturally occurring in bacteria. When bacteria are frozen in the presence of oxygen, the total number of radicals observed were significantly lower than when the bacteria were frozen in an anoxic atmosphere. These observations lend credence to the hypothesis that the toxic effect of oxygen upon freezing are due to free radical reaction.

A practical "in vivo" dosimeter for radiation exposure has been evaluated. Fingernails show a linear relationship between radiation exposure and ESR signal intensity. The use of this practical dosimeter could be of value in accidental exposure to ionizing radiation.

2. Molecular and cellular biology of radiation injury. (LTC Ginsberg, MAJ Friedberg)

The projects within this section have been principally devoted to the study of the repair of damaged DNA by T4-infected cells.

E. coli infected with coiled-type bacteriophage T4 contain an endonucleolytic activity that specifically degrades DNA exposed to ultraviolet-irradiation. A UV-sensitive mutant of T4V1 has been shown to be defective in this enzymatic activity.

This endonuclease has been purified to a state of physical homogeneity as most criteria of purity are concerned. The purified enzyme register in polyacrylamide gel as a single electrophoretic band shows no other demonstrable nucleolytic activities.

The properties of this enzyme have been studied. It is a relatively small protein with a relative S value of about 3.0. It has no cofactor requirement and unlike most endonucleases does not require metal activation for activity. The enzyme is absolutely specific for pyrimidine dimer or UV-irradiated DNA. Stoichiometric experiments indicate that a

single-strand break is made on UV-irradiation for every dimer present. These results lend new support to the postulate that pyrimidine dimers are the significant lethal lesion in UV-irradiated DNA. The purified enzyme is totally inactive on native unirradiated DNA. It is not confined to T4 DNA in its activity but also degrades E. coli UV-irradiated DNA.

The purified enzyme also degrades DNA alkylated with nitrogen mustard or methylmethane-sulfonate. Current experiments are attempting to demonstrate conclusively that this activity is indeed a function of the T4 UV-repair enzyme and not a contaminant. The observed degradation of alkylated DNA is of great interest since it has long been known that some UV-sensitive organisms are also sensitive to the effects of alkalating agents.

The purified enzyme does not cause excision of pyrimidine dimer from DNA, only endonucleolytic incision. However, the addition of crude extract of cells infected with the UV-sensitive mutant T4V1 to DNA preincubated with the endonuclease, does result in dimer excision. Current experiments are aimed at identifying and isolating these other activities required to complete dimer excision.

3. Tissue, organic and whole organism studies. (LTC Johnson, Mrs. Hill, MAJ Lumeng, MAJ Liebowitz)

Under this section of the work unit, a variety of studies have been accomplished during the reporting period. They may be subdivided into a. Bone marrow transplantation; b. Gastrointestinal studies; c. Lymphocyte culture; d. Intermediary metabolism and membrane transport.

a. Bone marrow transplantation. (LTC Johnson, Mrs. Hill)

The effect of different types of bone marrow preparations, as well as the influence of the time interval between radiation and wounding, on wound healing patterns, body weight and post-irradiation mortality were examined in inbred Fisher rats exposed to LD50/30 neutron irradiation. Within 6 hours of neutron exposure the rats were transfused with 100 million syngeneic bone marrow cells. Three sources of bone marrow cells were used: 1) from donor rats treated with rabbit anti-rat lymphocyte serum; 2) from donors treated with normal rabbit serum, and 3) from untreated donors. On the first, third or fourth post-irradiation day, a standardized open skin wound was made in the dorsal lumbar region. Antibiotic therapy was initiated on the day of exposure and continued for 14 days. Appropriate control groups for radiation lethality, wound injury and antibiotic effect were included in the study.

In previous studies it was shown that shielding of the bone marrow during, or transplantation of syngeneic marrow immediately after

$LD_{50/30}$ X-irradiation was associated with less mortality and more rapid wound closure than in non-transplanted, irradiated wounded controls. In striking and significant contrast, transplantation of the variously prepared marrows, in spite of being combined with antibiotic therapy, did not modify either post-irradiation wegith changes, mortality or wound closure patterns in neutron-irradiated rats.

Wound closure patterns and mortality were influenced by the time interval which elapsed between the two injuries. The effect was biphasic, with the most abnormal wound contracture patterns and the highest 30-day mortality, 91%, at the 3-day interval between radiation exposure and wounding. When either 1 or 4 days elapsed between the two injuries, mortality was just over half that at 3 days and wound closure was more rapid. Wound enlargement did not occur at these time intervals and healing (closure) commenced with little or no delay.

b. Gastrointestinal studies. (Mrs. Hill, Dr. Jervis, Division of Experimental Pathology)

The accepted result of exposure to high levels of radiation or of treatment with high doses of radiomimetic drugs is the denudation of the intestinal mucosa, i.e., the massive loss of epithelium, with accompanying diarrhea, contrary reports having been neglected. Our observations unequivocally estalish that no denudation of the villi occurs following neutron and X-ray irradiation and with nitrogen mustard and aminopterin treatment, except as a processing artifact. The mature epithelial cells present on the villi at the time of exposure or drug administration are normally shed from the villous tips and are replaced by a limited number of abnormal cells from the crypts. Even in case of severe villous atrophy the mucosa is covered by a continuous cell layer. Shedding of mature cells is completed in about 3 days in rats, and 4 days in mice at which time diarrhea begins. The abnormal epithelium lacks enzymatic activity and is presumably incapable of active transport. It serves, however, as a barrier and prevents indiscriminate outpouring of plasma constituents. Ulcerations of variable severity, usually accompanied by hemorrhages and cellular exudate, are mostly a later development.

c. Lymphocyte culture system. (LTC Johnson, Mrs. Davis, MAJ Lumeng)

The culture of lymphocytes continues to be improved with the separation of lymphocytes by gradient centrifugation. This procedure as modified permits isolation of lymphocytes from peripheral blood with 95% purity, 90% viability and 80% yield.

d. Intermediary metabolism and membrane transport. (MAJ Lumeng, MAJ Liebowitz)

Using an *in vitro* reconstructed system consisting of a suspension of rabbit liver mitochondria in a KCl medium supplemented with

NADH, glutamate, aspartate, aspartate aminotransferase and malate dehydrogenase, a substrate shuttle system involving malate dehydrogenase has been established to explain physiological transfer of hydrogen equivalents to and from the intramitochondria space. Continuing study of this hydrogen shuttle system has shown that high concentrations of inorganic phosphate ion inhibits the transfer.

The effect of purified Staphlococcal enterotoxin B on rabbit liver mitochondria, electron transfer particles from beef heart and purified cytochrome oxidase (beef heart) has been preliminarily examined and the following conclusions have been reached: purified Staphlococcal enterotoxin B at a level of 50 µg per mg of any of the above mentioned preparations does not affect impermeability of NADH across intact rabbit liver mitochondria; does not affect the oxidation of pyruvate plus malate or succinate by intact rabbit mitochondria; does not affect the oxidation of NADH or succinate by beef heart particles; and does not affect the oxidation of ascorbate plus cytochrome C by beef heart cytochrome oxidase.

A series of studies have been initiated to study whether lipid peroxide information mediates permeability change. Using synthetic pure lipid membrane in which peroxides had been induced by gamma radiation coupled with, appropriate addition of ascorbate, ferric ion and glutathione, the permeability of the system was measured by evaluating the flux of Na-24 and Na-22 across the membrane. Results shows that lipid peroxides do not produce permeability changes in the pure membrane.

4. Radiation dosimetry. (Mr. Bass, Mr. Crofford)

The activities of this area consists of reactor operations, dosimetry, electronic development, and instrument calibration.

The Walter Reed Research Reactor has been destined for dismantlement. Considerable effort has been expended in the preparation of a basic dismantlement plan that will successfully close the installation. During the period 1 July 1969 through 31 January 1970, reactor operations totalled 18,000 kilowatt-hours divided between activation analysis and radiopharmaceutical production. Since January these activities have been transferred to the Diamond Ordnance Radiation Facility (DORF) through a host-tenant agreement. Personnel assigned to reactor operations are being cross-trained in other areas of interest to the Division of Biochemistry and the WRAIR.

Dosimetric measurements have totalled more than 4,200 observations involving the X-ray machine, the Gamma Cell and the Small Animal Irradiator. Several research efforts have been under way to improve support in this area. A method utilizing sodium chloride as a means of quantifying ionizing radiation intensity in place of lithium fluoride has been

developed. Although one one-eighth as sensitive as lithium, the differential in cost of the two chemicals makes this method attractive. Measurements using sodium chloride are accurate within 5%. "In vivo" dosimetry has always been an attractive possibility in a number of biological situations. Miniature radiation detectors consisting of lithium drifted silicon wafers, 5 mm in diameter with a 2 mm thickness are imbedded in biologically inert epoxy resin and connected to a low noise signal conditioning circuit. These detectors are being implanted in dogs and monkeys for coronary blood flow studies in connection with coronary insufficiency.

The transplantation effort has been supported by the adaptation of a 3-probe renogram system to measure glomerular filtration rate in normal and hypertensive patients. It is expected that this system will aid in kidney transplantation.

5. Human whole body radiation detection.

The functions of the Whole Body Counter have been transferred to the Division of Medicine in support of the activities of the Department of Metabolism and the Department of Nuclear Medicine, WRGH. Efforts in this area primarily consist of an isotopic bone survey that has been developed into a useful clinical tool and a study of Calcium-47 kinetics. Estimation of total body potassium has been made available to the entire hospital staff. Use of these estimations have been limited to endrocrine patients (Ward 30) and renal patients (Ward 33).

Summary and Conclusions.

With the termination of this work unit, those parts of the unit that have application in Biochemistry, Medicinal Chemistry, Medicine, Communicable Disease and Immunology, and Experimental Pathology have been absorbed by the divisions cited above. The use of radiation as a tool continues to extend the interdisciplinary character of research within the WRAIR. More emphasis will be placed on systems development and extension of these systems to mission-oriented research.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 09, Radiobiology

Work Unit 015, Mechanisms

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