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RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES INCLUDING BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY, INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE. VOLUME I

Walter Reed Army Institute of Research
Washington, D. C.

30 June 1972

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 1971 - 30 June 1972

VOLUME I

WALTER REED ARMY INSTITUTE OF RESEARCH

WALTER REED ARMY MEDICAL CENTER

WASHINGTON, D.C. 20012

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 1971 - 30 June 1972

Volume I

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

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 Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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.

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 OB 6464	72 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
71 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	099
b. CONTRIBUTING	62703D	ARPA AO 0798 9M10		
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code): (U) Further Studies of the Hazards of Microwave Irradiation as Indicated by CNS Neurotransmitters (21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 012900 Physiology; 016200 Stress Physiology; 01400 Radio and Radiation Chemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD
69 06	71 09	DA	DD	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: 27,810
e. KIND OF AWARD: Ext f. CUM. AMT. 57,175

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	0.5	25
	CURRENT 72	0.5	25

19a. RESPONSIBLE DOD ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
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NAME: Meyerhoff, James L., MAJ 1

21. GENERAL USE
Foreign Intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U)Microwave Hazards; (U) Nonionizing Radiation Hazards; (U) Behavioral Effects; (U) Neurochemical Systems; (U) Military Medicine

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

23. (U) To investigate the nature and extent of hazards to the central nervous system (CNS) and its function due to microwave and radio frequency emanations from military devices and equipment such as radar and communications gear.

24. (U) Chemical determination of changes in CNS levels and turnover rates of important neurotransmitters involved in thermoregulation as well as levels of consciousness and behavioral excitation. Those transmitters are serotonin (5-hydroxy-tryptamine) and norepinephrine.

25. (U) 71 07 - 72 06 The turnover of brain serotonin and norepinephrine was examined. When rats were acutely exposed for one hour to microwave irradiation at 40 mW/cm^2 there was a significant acceleration of serotonin turnover. Since the body temperature of these animals was elevated by about 3°C, it is possible that this was an effect of "heat stress," a procedure which is known to increase the rate of serotonin turnover. The most striking results of the study were obtained when rats were exposed to low levels (10 mW/cm^2) microwave irradiation 8 hours a day for seven days and were sacraficed one day following the last day of microwave exposure. Under these conditions, at the time serotonin turnover was estimated, rats had a normal body temperature and showed no apparent behavioral abnormalities. Nonetheless there was a dramatic slowing of serotonin turnover to a rate of 1/4 that of control animals treated identically except for the absence of microwave exposure. This result suggests that the firing rate of serotonin neurons in the brain is markedly slowed following chronic low level microwave irradiation. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72; these research studies were continued and reported under Project 3A062110A824, Task 00, Work Unit 057.

*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 68 AND 1498-1, 1 MAR 69 (FOR ARMY USE) ARE OBSOLETE.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6472	72 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
71 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	109
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Hypersensitivity in the immunopathology of helminthic infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 11	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 72	3	0
FISCAL YEAR CURRENT 73	3	70

19. RESPONSIBLE DOD ORGANIZATION

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NAME: MOON, A. P. DA

NAME:

PII Redacted

21. GENERAL USE

Foreign Intelligence Not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Radioactive; (U) Antigen; (U) Antibody; (U) Immunoglobulin; (U) Hypersensitivity; (U) Parasite

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 extent of hypersensitivity produced by helminthiases of military importance and their role in the immunopathology. This knowledge may be applied to developing effective agents for the prevention, or amelioration of these diseases in military personnel.

24(U) After careful study of pertinent literature and consultations with immunochemists and pathologists, both classical and new methods will be used to obtain data from controlled experiments.

25(U) 71 07 - 72 06 A radioactive iodine labeled microprecipitin assay was developed for measuring the binding of antigen by antibody in trichinosis. Rabbit immune serum was absorbed with goat antirabbit IgE antiserum. When tested after absorption, the reactivity of the serum in the radioactive assay was significantly reduced and the reactivity in the passive cutaneous anaphylaxis test was eliminated. However, the titer in the soluble antigen fluorescent antibody test was not reduced. These studies indicate that the radioactive assay can reliably measure antibodies to T. spiralis belonging primarily to the immunoglobulin E class. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*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 100 Hypersensitivity in the immunopathology of helminthic infections

Investigators
Principal: E. H. Sadun, Sc.D., Lib. Doc.
Associate: R. W. Gore, J. S. Williams

1. Homocytotropic antibody response to parasitic infections.

Many parasitological articles are currently devoted to problems related to immunity. Although Erlich (1907) and the Sergent brothers (1918) had already established some of the roles of acquired immunity in trypanosomiasis and malaria, up to the second quarter of the century most parasitologists were reluctant to recognize that immunity plays an important role in parasitic infections. This reluctance was a result of the paucity of immunological knowledge in general and of the unique complexities of life cycles, size and metabolic requirements of animal parasites. Taliaferro (1929) in his classic publication on the immunology of parasitic infections had pointed out that the large size and accessibility of parasites provide a great advantage in immunological investigations. Because of their size, parasites can be observed in vivo in relation to the host reaction and are a convenient source of large quantities of antigens for analysis and purification. However, the size and complexity of parasites also confront immunologists with a baffling array of antigens, some of which are stage specific and others which are common to other parasites or to antigens of the host.

In the past decade there has been an explosion of information pertaining to immunology in general, and the study of parasitic diseases has felt its impact. Numerous publications which have appeared recently describe the development of immuno-diagnostic techniques, and evaluate them under field conditions. Some of these publications point out the complexities of parasitic antigens; others deal with identification and purification of parasitic antigens; and still others explore factors which on one hand may be important in host resistance and on the other in the production of diseases through allergic or hypersensitivity mechanisms.

A summary of information available on homocytotropic antibodies in parasitic infections is much more meaningful if viewed from a perspective of the potential achievements of these studies rather than on past accomplishments. Yet, this review is particularly appropriate at this time, since in recent years there has been a considerable increase in the number of investigators interested in homocytotropic antibodies in parasitic infections and new findings are being reported at a rapid pace.

Hypersensitivity is an acquired state which develops as a result of exposure to some external harmful antigenic agent. Based on the time-scales with which the reactions occur, hypersensitivity is divided into immediate-type and delayed-type. This actually reflects fundamental differences in mechanisms. The immediate-type reactions are those which are mediated by serum antibodies and whose first manifestations occur within minutes of the contact of antigen with antibody. Delayed-type hypersensitivity reactions appear to be independent of serum antibodies and dependent upon sensitized cells. They differ from immediate-type reactions in at least the following respects: 1) the intradermal injection of antigen into a previously exposed animal initiates a slowly evolving, indurated and erythematous reaction which reaches a peak within 48 hours and wanes slowly during the following weeks; 2) the serum of a sensitized subject does not confer passive sensitivity to a non-sensitized recipient; 3) lymphoid cells from a reactive donor confer passive sensitization to a normal subject.

In this discussion of immediate hypersensitivity only minimal consideration will be given to the diagnostic and protective aspects to be derived. This review is not intended to be all-inclusive and the list of individual studies published on immediate hypersensitivity in parasitic infections is somewhat incomplete. Although for reasons of greater familiarity undue emphasis may have been placed on investigations with which the author and his collaborators have been directly involved, this should by no means be construed as a suggestion that these studies are of greater significance than others which might have been omitted or mentioned only briefly. It is hoped that even though this report discloses some wide gaps in our present knowledge of the immediate hypersensitivity of parasitic infections, it will serve the purpose of stimulating further collaborative research between parasitologists and immunologists and will lead to the development of more fruitful approaches to these problems in terms of immunological concepts.

I. Reactions Mediated by Anaphylactic Antibodies

Antibodies to parasites and their products are easily demonstrated in the blood of infected humans or animals. This is not surprising when one considers the complex structure of the invading organisms, the various stages of this life cycle and their many secretions and metabolic products which may serve as antigens. Moreover, many parasites may harbor a diverse array in various stages of development and decay and may be contaiminated by the products of these cells. This mosaic of antigens in various parasites stimulates the production of a remarkable multitude of antibodies which are found in different immunoglobulin classes.

Nearly 80 years ago, when antibodies were demonstrated by toxin neutralization, agglutination, precipitation and by hemolysis, immunologists believed that the antibodies responsible for each manifestation were different from one to another. However, the idea was reversed by several findings which indicated that the same antibody molecules could be responsible for different immunological reactions.

The unitarian theory; i. e., one and the same antibody being responsible for different immunological manifestations, has been accepted for a long time despite the heterogeneity of antibodies with respect to physicochemical properties. In addition to 7S gamma globulin, 19S antibody was detected in 1939. Twenty years later, a third type of gamma globulin which is antigenically related but distinct for 7S and 19S gamma globulin was found by immunoelectrophoresis. It is now established that biological properties of antibodies differed depending on immunoglobulin classes and subclasses, and that the functions of the antibodies are based on certain structures in the Fc portion of the molecules. Five main classes of antibodies (IgG, IgA, IgE, IgM and IgD) have been described in man, and similar classes are gradually being characterized in other animals. Detailed descriptions of the immunoglobulins and their properties have been presented in several recent reviews.

It may be pertinent here to indicate that, a) the antibodies involved in the conventional serologic tests (Precipitin, fluorescent antibody, agglutination and complement fixation tests) belong mostly to the IgG and IgM classes of immunoglobulins, the latter being the most primitive immunoglobulin both ontogenetically and phylogenetically; b) IgA, although present in serum, predominates in many mucous secretions; and c) IgE, found in minute amounts, is produced primarily in tissues adjacent to mucous surfaces. The only identified function of the IgE immunoglobulins is in the mediation of immediate hypersensitivity reactions such as local and systemic anaphylaxis. One type of homocytotropic (reaginic) antibody resides in this fraction.

The term homocytotropic antibody was introduced by Becker and Austen to describe a unique function of an immunoglobulin, i. e. the capacity to attach to certain target cells of the same species so that subsequent contact with antigen leads to the noncytotoxic release of pharmacologic mediators of anaphylaxis from that cell. Tissue or cells with homocytotropic antibodies fixed to the surface are referred to as sensitized. Two types of homocytotropic antibodies have been described; one fixes to the skin for a relatively brief period (2 to 6 hours) after intradermal injection for optimal antigen-induced local increase in vascular permeability as measured by the extravasation of the dye. This antibody has an electrophoretic mobility of gamma-1, a sedimentation coefficient of 7S and the capacity to fix to tissue and bind antigen, and withstands heating at 56 C for 4 hours or reduction and alkylation. The second antibody, referred to as skin sensitizing or reaginic antibody, is associated with the IgE immunoglobulin class. It is capable of fixing to skin after intradermal injection for days or weeks and continues to elicit antigen-induced vascular permeability. This antibody has the electrophoretic mobility of a fast gamma globulin, a sedimentation coefficient of approximately 8S, is heat labile, and is susceptible to inactivation by reduction and alkylating agents. Human homocytotropic (reaginic) antibody is capable of sensitizing tissues of monkeys and chimpanzees, but not those of other animals. In humans the only homocytotropic antibodies described so far are reaginic and belong to the immunoglobulin class IgE. In lower animals (mouse, rat, guinea pig, rabbit) both reagin-like

homocytotropic antibodies and other nonreaginic (heat stable 7S) homocytotropic antibodies have been reported. The human reaginic antibody was found in the serum after development of hypersensitivity to various antigens and appeared to mediate immediate skin reactivity, the Prausnitz-Kustner (PK) reaction, certain systemic reactions, and in vitro antigen induced release of histamine from actively or passively sensitized leukocytes.

Sensitized tissues, following antigen challenge in vivo or in vitro release one or more of the following pharmacologically active substances (mediators): histamine, serotonin, kinins, slow reacting substance, lysosomal enzymes, and eosinophilic chemotactic factor. The sources of these substances (target cells or substrate) vary in different species, but have usually been attributed to neutrophilic leukocytes, mast cells, enterochromaffin cells, basophils, platelets, macrophages and eosinophils. The release of pharmacologically active substances produces a series of rapid, explosive and evanescent reactions which vary in different animals depending on the shock organs for that species. The role of these substances in the host-parasite relationship has not been defined.

Cutaneous anaphylaxis, a clinical manifestation of mediator release, can be elicited by the intradermal injection of antigen into a sensitized individual or animal or through intradermal injection of antibody into the skin of a normal animal with subsequent antigen challenge injected intravenously. The latter is referred to as passive cutaneous anaphylaxis (PCA). A blue dye (Evans or pontamine blue) which is injected intravenously prior to or at the time of antigen challenge binds to plasma proteins and is a convenient indication of the increased vascular permeability occurring 5 to 10 minutes after antigen interaction with homocytotropic antibody. Benaceraff, Ovary and their coworkers, White and coworkers, Mota, and Mota and Peixoto have shown that the various antibodies produced by a given species differ greatly in the kinds of anaphylactic reactions which they can mediate.

The term "cytophilic" has been used to describe immunoglobulins which have a special tendency to become attached to the surface of certain cells, not by virtue of their antibody combining sites, but of some other configurational property of the molecule. When these attach to cells of the same or closely related species (usually those of the class IgE) they are referred to as homocytotropic; when instead they attach to cells of unrelated species (those of other immunoglobulin classes) they are termed heterocytotropic.

II. Allergic Response of the Host

A. Protozoan Infections

Although delayed hypersensitivity has been demonstrated or postulated for a variety of protozoan infections such as leishmaniasis, trypanosomiasis, toxoplasmosis, and amebiasis, the antigens of parasitic protozoa are in general relatively ineffective in stimulating immediate hypersensitivity.

Suggestive evidence of homocytotropic (reaginic) antibodies being stimulated by a protozoan has been presented in Trichomonas foetus infections in cattle. The immune reaction to this parasite is localized in the uterus and according to Robertson, anaphylactic reactions may follow repeated sensitization. Since no precise information is available as to the immunoglobulin class to which this anaphylactic antibody belongs, further studies with modern immunochemical techniques are warranted.

Infection with African trypanosomiasis produces the liberation of short chain peptides with kinin-like activity in mice, rats, rabbits and cattle.

The release of kinin is associated with antigen reaction and has been responsible for changes in vascular permeability that lead to some of the abnormalities occurring in diseased animals. Seed and Gam postulated that host tissue antigens released by hypersensitivity-induced cell destruction are responsible for an autoimmune condition which plays an important role in the pathogenesis of trypanosomiasis. Monkeys and guinea pigs infected with T. cruzi, the agent of American trypanosomiasis, developed cytophilic antibodies. The antibodies detected in the skin of guinea pigs were 7S and those detected in monkeys were defined as "beta-2A." The latter were sensitive to mercaptoethanol and were tentatively designated as reaginic antibodies.

Two types of cytophilic antibodies in sera from patients infected with Leishmania braziliensis were detected. The antibodies fixed in the skin of guinea pigs were of the 7S type and those in the monkey were "beta-2A." He postulated that these antibodies are responsible for the symptomatology of this infection. However, the presence of homocytotropic antibodies has not been demonstrated in this leishmaniasis even though it has been stated by Goodwin: "It is likely that vascular damage and kinin release may be related to the allergic reactions that occur when antibodies are formed to successive antigenic variants of the parasite."

The anoxemia-induced tissue damage in malaria also has been attributed to the release of pharmacologically reactive substances. Furthermore, a pharmacologically active agent, presumably histamine, has been demonstrated in the blood of monkeys infected with malaria. It has been postulated that a similar factor may be responsible for plasma leakage and hemoconcentration in P. falciparum infections. However, mediator release can also occur by nonimmunological mechanisms.

Immediate and delayed hypersensitivity to Entameba histolytica was observed in patients hospitalized with severe amebiasis and in asymptomatic individuals. The authors could not detect homocytotropic antibodies in monkeys which received sera from these patients, although passive cutaneous anaphylactic reactions in guinea pigs were obtained. Immediate hypersensitivity was observed more frequently in invasive clinical amebiasis, whereas delayed hypersensitivity was more common in the asymptomatic group.

Alimentary hypersensitivity induced by Giardia lamblia infection has been reported. Biopsy of the duodenum showed marked eosinophilic infiltration of the lamina propria. Since the syndrome with Giardia infection was concomitant with ingestion of meat of mammalian origin, it was postulated that meat might provide the appropriate environment for maximal liberation of parasite excretory allergens of that parasite metabolic products may serve as haptens, with the allergy being a result of the degradation products of the mammalian muscle. A third possibility was that Giardia infection induces a tissue sensitization in which there is an allergy to one or more components of the diet.

Therefore, it is concluded that homocytotropic (reaginic) antibodies in protozoan infections have not been convincingly demonstrated as of the present time. However, one must consider the possibility that failure of detection might be due to the scarcity of proper antigens and not necessarily to the absence of these antibodies.

B. Helminthic Infections

Contrary to the findings with protozoan infections, there is no problem in demonstrating homocytotropic antibodies in the blood of humans or animals infected with helminths. In fact, most parasitic worm infections elicit an immediate type of hypersensitivity and the development of high titer reaginic antibodies. This may be aided by the uniquely complex helminth life cycles which permit intimate contact with the mucosae of the respiratory or gastrointestinal systems at some stage during the migration of the parasite within the mammalian host.

The nearly universal capability of helminthiases to stimulate the immediate type of hypersensitivity has been used widely in immunodiagnosis for more than half a century. Intradermal tests of the histamine type have been described for the presumptive diagnosis of such human infections as ascariasis, creeping eruption, dracontiasis, enterobiasis, filariasis, strongyloidiasis, hookworm infection, trichinosis, diphyllobothriasis, echinococcosis, taeniasis, paragonimiasis, clonorchiasis and schistosomiasis. These intradermal reactions, although very useful for diagnosing helminthic infections, do not necessarily provide precise information on the presence, time course development or role of reaginic antibodies in parasitic infections.

Many helminthic infections cause various manifestations of immediate hypersensitivity such as eosinophilia, edema, dermatitis, asthma, acute diarrheas and in some cases anaphylactic shock, but most of the efforts designed to detect, define immunochemically and classify homocytotropic antibodies are directed toward only a relatively few infections. In some of these efforts, reagin-like antibodies with characteristics similar or identical to those of human IgE immunoglobulin class have been defined, whereas in others the antibodies which induce the allergic response to parasites have not been clearly defined or have been ascribed to IgA or IgG immunoglobulin classes.

In addition to being important inducers of homocytotropic antibody, helminths have long been known for their unique ability to evoke eosinophilia appears characteristically in many instances of allergy. An increase in the number of eosinophils, either local or general, usually is associated with local hypersensitivity reactions. This enhancement in number is so commonly associated with hypersensitivty reactions of the immediate type, that according to Raffel abnormal numbers of eosinophils in any disease of unknown etiology should suggest the probability of an allergic component. More recently Samnter called attention to the fact that eosinophils participate conspicuously in allergic reactions, and he noted the particular effectiveness of a derivative of Ascaris worms in the induction of eosinophilia.

High levels of circulating eosinophils are usually observed in those helminthic infections in which the association of the parasite with the host tissues is the closest. Examples of this are seen in eosinophilic meningitis due to infection in man of Angiostrongylus cantonensis, a worm which normally inhabits the pulmonary arteries of rats. Loeffler's syndrome or pulmonary eosinophilia is another complication of helminthiases. The symptoms produced include spasmodic bronchitis and bronchial asthma accompanied by massive leukocytosis most of which is eosinophilic. This may be caused by a passage to the lungs of parasites of man such as A. lumbricoides or by nonhuman parasites such as Toxocara canis or T. cati. Tropical eosinophilia or eosinophilic lung is also a respiratory tract condition which is associated with elevated eosinophil numbers. The fact that the signs associated with this condition frequently subside after administration of antifilarial drugs, suggests that tropical eosinophilia might be a form of filariasis. Infection with larvae of Brugia malayi of monkey origin or B. pahangi of feline origin produced the classical symptoms of the disease which were terminated by treatment with antifilarial drugs. This led to the belief that the disease was due to infection with non-human filarial parasites. Lung biopsies performed on patients with tropical eosinophilia showed eosinophilic foci surrounding the generating microfilariae. Therefore, tropical eosinophilia or eosinophilic lung has been ascribed to filarial worms that become established in a normal host and produce an unusual degree of hypersensitivity. On that basis this disease has been defined as an immediate hypersensitivity reaction. As pointed out by Andrews however, it is important to remember that while eosinophilia is commonly associated with immediate hypersensitivity, this dyscrasia may also be a prominent sign in a number of other disorders which thus far have not been identified with certainty as having hypersensitivity components. In his presidential address to the American Society of Parasitologists, he lamented that "little is known about the actual incidence of allergic morbidity and mortality due to hypersensitivity to helminth parasites." Although information on these aspects is accumulating rapidly, major gaps in our knowledge still exist.

Ascaris and related nematodes.

Many descriptions of toxic effects following the administration of Ascaris extracts to animals have appeared in the literature. Symptoms resembling those of anaphylactic shock occur after intravenous injection of Ascaris tissues. Similarly, digestive disturbances and degenerative changes have been observed following oral administration of Ascaris body fluid. Ascaris extracts caused an increase in tonus and in the rate of contraction in the isolated intestine of several species of mammals and produced vomiting and diarrhea. An aqueous extract of Ascaris lumbricoides and Parascaris equorum was toxic to horses, guinea pigs, dogs and rabbits but not to mice and rats. Weinberg and Julien found that the body fluid of P. equorum when instilled into the eyes of horses caused acute conjunctivitis and edema of the eyelids. These results were probably manifestations of immediate hypersensitivity, although they might have been due to toxic products. However, evidence of hypersensitivity produced by tissues and fluids of Ascaris has been presented in infected individuals in laboratory workers who frequently handle worms and in animals experimentally sensitized. Even minute amounts of volatile substances from A. lumbricoides are sufficient to induce phenomena of immediate hypersensitivity. The mere odor of fluids from A. lumbricoides may be sufficient to bring about severe symptoms, and laboratory coats worm while handling this nematode may induce allergic rhinitis in a sensitive person. Sensitization to Ascaris protein was also observed after infection with other nematodes. The fact that antibodies are involved in the mechanism of Ascaris allergy was demonstrated by transferring sensitivity by means of the P-K reaction in humans and in dogs. Oliver-Gonzalez reported that guinea pigs injected intraperitoneally with the serum from rabbits immunized with whole worm material developed anaphylactic symptoms when challenged with Ascaris intestine, blood or body fluids but not when challenged with cuticle, muscle, egg or sperm homogenates of the worm. Sprent was able to demonstrate that homogenates of body fluid, intestine, cuticle, muscle and ovary of A. lumbricoides from the pig failed to produce symptoms of shock when injected intravenously into mice, rabbits and guinea pigs, provided that no previous sensitization had occurred. However, these substances were shown to cuase active anaphylactic sensitization of the guinea pig to the same antigen, skin sensitivity and precipitins in rabbits, and skin reactions in persons sensitized to Ascaris. Guinea pigs infected with the larvae of A. lumbricoides, P. equorum, Trichinella spiralis and Paraspidodera spp. were found to be hypersensitive to Ascaris body fluid. Guinea pigs infected with A. lumbricoides manifested anaphylactic shock when injected intravenously with extracts prepared from A. columnaris, Toxascaris leonina and Phytoloptera maillaris but not by extracts prepared from Trichinella spiralis nor by those from three cestodes and a trematode. The changes in the histamine content and the mechanisms of the shock induced by anaphylaxis-like reactions produced by Ascaris have been studied by Rocha and Grana.

The first indication that homocytotropic (reaginic) antibodies were involved in Ascaris hypersensitivity was provided by Weiszer et al. They demonstrated cutaneous reactivity by skin testing and passively

transferred it to the skin of monkeys. The antibody activity was lost after heating at 56 C and was found to reside in the same fraction of a Sephadex G-200 eluate as the human reaginic sera. Normal rhesus monkeys were also shown to produce reaginic antibodies when injected with Ascaris crude extracts or with an electrophoretically isolated Ascaris fraction. Ishizaka et al. showed that monkeys immunized with Ascaris extract possessed detectable amounts of a serum protein that cross reacted with antisera specific for human IgE. The physicochemical properties of this monkey protein such as electrophoretic mobility, molecular size and behavior on column chromatography also resembled those of human IgE. The sedimentation velocity of the monkey protein was likewise comparable with that of human IgE and significantly faster than that of IgG. They concluded that this monkey protein represents a distinct immunoglobulin class corresponding to human IgE and that IgE formation may be stimulated by parasitic infections. Strejan and Campbell sensitized rats with crude Ascaris extracts as well as with chromatographically isolated fractions. They found that when these fractions were administered with killed B. pertusis organisms as adjuvants, they elicited homocytotropic antibodies in relatively high titers. They called attention to the fact that Ascaris extracts are much more important inducers of homocytotropic antibodies than standard protein antigens such as ovalbumin or bovine serum albumin. The histamine releasing capacity of one fraction was not inhibited by specific rabbit anti-rhesus immunoglobulins (IgA, IgG, IgM). This suggested that the histamine release factor which they identified as an immunoglobulin may be an IgE-like antibody. Booth and his coworkers identified in dogs an antibody which can transfer sensitivity to Ascaris and which is analogous to human IgE immunoglobulin. Winsor observed that sensitization of guinea pigs to A. lumbricoides antigen can be elicited not only by infection and by parenteral injection of crude antigens from adult worms, but also by exposure to air passed over whole Ascaris. When tested by the P-K test, guinea pig sera reacted to an Ascaris fraction believed to be the allergen described by Hogarth-Scott. He observed that the airborne antigens from hemolymph reacted with sera from sensitized guinea pigs as well as with human reaginic sera, and concluded that the active allergen is present in the worm hemolymph and metabolites. A homocytotropic (reaginic) antibody which reaches high titers during Ascaris infections in guinea pigs was reported by Dobson, et al. This antibody was considered analogous to human IgE antibody and differs from other guinea pig anaphylactic antibodies by virtue of its greater molecular weight, its sensitivity to heat and mercaptoethanol, and by its ability to sensitize homologous skin sites for prolonged periods of time and to remain active at high dilutions. Further studies revealed that the reaginic activity was not absorbed by rabbit anti-guinea pig IgM and IgG, but was absorbed by rabbit anti-guinea pig IgE antisera. An 8S and 11S component were detected by sucrose sedimentation of guinea pig IgE. The 11S reaginic fraction had a greater skin sensitizing activity than the 8S component. They could not exclude the possibility that a distinct reaginic antibody other than 8S IgE is present in guinea pigs. The reaginic activity in a canine reaginic antiserum against Ascaris antigen was studied by Patterson et al. who concluded that because of similarities of the characteristics of the canine and human reagin, the

canine immunoglobulin with reaginic activity should be considered to the IgE equivalent. The IgG, IgA and IgM antibodies to A. lumbricoides antigen were measured in the sera of normal and allergic persons by Parish who suggested that reagins are not necessarily confined to a single class of immunoglobulins. Further work on the heterogenicity of IgE has confirmed these observations.

Homocytotropic antibody formation against dinitrophenylated Ascaris extracts was selectively suppressed in rats by the passive administration of homologous antibody against the same antigen. The suppression was preferentially directed to the homocytotropic antibody response while the hemagglutinating antibody formation was unaffected. The results indicate the presence of a "feed back" regulation in the homocytotropic antibody response. Whole body x-irradiation greatly suppressed the production of IgG antibody and resulted in a defective humoral feed back regulation. Homocytotropic antibody formation was greatly enhanced and prolonged by splenectomy and thymectomy in adult rats. However, in contrast to adult thymectomy, neonatal thymectomy caused a loss of the ability to produce homocytotropic antibodies to the dinitrophenylated Ascaris extracts.

Specific homocytotropic (reaginic) antibody measured by passive cutaneous anaphylactic tests in baboons and monkeys was detectable in the sera of patients with proven visceral larva migrans. These patients had 10 to 15 times the normal level of IgE as determined by the radio immunosorbent test. Johannson et al. found that whereas bacterial and viral infections are of minor importance in raising IgE levels, parasitic infections gave rise to high IgE concentrations in individuals without atopic manifestations. Very high IgE levels were found in an unselected group of Ethiopian children who had on the average about 20 times as high IgE levels as healthy Swedish children of the same age. When children were selected for Ascaris infection, a mean IgE level 28 times higher than that of the Swedish children was found.

Nippostrongylus braziliensis.

In 1964 Ogilvie reported that rats infected with N. braziliensis developed a skin sensitizing antibody in the third week after an initial infection. This antibody persisted for many months even in the absence of subsequent reinfection. However, after a second infection, there was an abrupt rise in antibody titer. This skin sensitizing antibody had many of the properties of human reagin. It was heat labile and persisted in the skin so as to produce a PCA reaction after a 72-hour latent period. It produced passive cutaneous anaphylaxis in the skin of the rat but did not sensitize the skin of other species. Rats vaccinated with fresh extracts of adult worms with Freund's complete adjuvant produced precipitating antibodies indistinguishable in gel diffusion tests from those produced by repeatedly infected rats. However, vaccinated animals did not produce reagins but contained blocking antibodies which when incubated with antigen in vitro prevented the PCA reaction. Reagin production following initial infection was delayed in neonatally thymectomized

rats and in immunologically immature newborn rats, and further there was a reduced anamnestic response after reinfection. Conversely, splenectomy in adult rats had no effect on the level of reaginic antibodies. Reagins could not be detected in young rats suckled by immune mothers, although they were found in the milk and the sera of the mothers.

Attempts were made to characterize physicochemically the homocytotropic antibody developing in the rat following infection with N. braziliensis and to define the antigen which combined with the rat homocytotropic antibody. The rat reaginic antibodies to N. braziliensis were shown to be intermediate in molecular size between 7S and 19S globulins and migrated with fast immunoglobulins but could not be related to either IgG or IgA rat immunoglobulins. The same serum fractions which gave homologous passive cutaneous anaphylaxis also produced systemic anaphylaxis. The antigenic material for both the homologous PCA and systemic anaphylaxis seem to be a protein with a molecular weight of approximately 12,000 to 17,000.

Washed peritoneal mast cells obtained from actively infected rats or normal mast cells passively sensitized in vitro with serum from infected rats released histamine upon contact with worm antigen. Degranulation of mast cells occurred at the site of PCA reactions. Both the PCA and mast cell disruption were maximal 5 minutes after antigen challenge in the rat reagin system. However, the skin reaction was not primarily dependent on or associated with mast cell disruption, since it was possible to induce skin reaction when the mast cells had been disrupted by other means and, conversely, skin reactions could be obtained without significant mast cell disruption. The appearance and course of sensitization of rat peritoneal mast cells and pleural cells was followed by measuring antigen induced release of histamine in vitro. Two peaks of sensitivity were detected. The first was observed approximately one month after initial infection and soon after a second infection, the second peak was seen after a fourth infection. These results were similar to those obtained in the investigation of systemic anaphylaxis in vivo with N. braziliensis by Keller.

Trichinella spiralis.

Infection with T. spiralis elicits an immediate type hypersensitivity demonstrable by in vitro and in vivo immunological techniques. Allergic manifestations such as muscle pain, fever, skin rashes, edema and eosinophilia usually accompany heavy infections with this parasite. Most of these characteristic signs reach their peak between the 12th and 20th day following infection and tend to subside at the time when the larvae achieve maximal growth. These symptoms could be related to irritation and mechanical damage due to migration of the larvae. The eosinophilia precedes the development of serological reactions or positive skin reactions. However, the presence of eosinophils in the initial invasion of striated muscle fibers and in the muscle walls of the arterioles often accompanied by inflammatory changes resembling those seen in periarteritis nodosa or serum sickness, suggest an allergic or immune reaction.

Allergic sensitization has been demonstrated in experimentally infected guinea pigs and mice. Intravenous or intracardial injection of antigens into T. spiralis infected animals produced generalized anaphylactic reactions and frequently death. Such sensitivity was demonstrable soon after infection and persisted for many months, possibly for the life of the host. Sensitization of individual tissues was also shown by: 1) in vitro exposure of intestinal tissues from infected guinea pigs to somatic and metabolic antigens of Trichinella, and in some cases heterologous worm antigens, which resulted in muscular contractions and 2) exposure of subcutaneous mast cells of mice to similar antigens which produced cell disruption with release of granular contents. However, mast cell disruption was not produced with antigens from other worms. Such sensitivity of intestinal and subcutaneous tissues could be passively transferred to normal animals by injections of serum containing anti-Trichinella antibodies from homologous hosts. Antiserum from rabbits only poorly sensitized mouse subcutaneous tissues. Although some of these studies are suggestive of reactions elicited by reaginic antibodies, it is not possible to determine with certainty whether gamma E antibodies are involved in them.

T. spiralis infection has recently been shown to elicit the formation of homocytotropic antibodies in man, mice, guinea pigs and rabbits. PCA activity in rabbits was detected as early as two weeks after feeding the infected larvae; it did not reach its peak until 18 weeks later and was still present 38 weeks after infection. The instability of mouse and rabbit PCA antibody after heating or reduction and alkylation contrasted with the failure of such treatment to alter the activity of fluorescent antibodies. Electrophoretically faster immunoglobulins contained PCA activity, but did not contain detectable fluorescent antibodies. Cross reacting reagin-like antibodies were detected between T. spiralis and Dirofilaria uniformis.

Mice produced two homocytotropic antibodies following infection with T. spiralis. A heat-resistant antibody responsible for PCA reactions induced after a short sensitization period (2-4 hours), and a heat labile antibody responsible for PCA reactions induced after 72 hours. Both antibodies appeared in the circulation 5 weeks after infection and reached the highest levels on the 9th week. Later, the antibody which induced PCA reactions with a latency of 72 hours disappeared from the serum in some animals whereas the antibody having a 4 hour latency remained. In animals subjected to repeated reinfections, the reagin-like antibody either decreased or disappeared from the serum. On the other hand, the antibody with a 2 hour latency increased. Absorption of mouse antisera with a highly specific rabbit anti-mouse 7S gamma-1, completely removed the 2-hour PCA activity of mouse antiserum without changing its ability to induce PCA reactions after 72 hours. The two homocytotropic antibodies detected in serum of mice following infection with T. spiralis could be distinguished by their biological and chromatographic behavior.

Immunization with larval extracts of T. spiralis led to the appearance of both (2 and 72 hours) antibodies in the serum, with similar properties to those obtained in the course of infection. Although a

second dose of antigen resulted in increases in the levels of both antibodies, further injections resulted in a high level of the antibody with a 2-hour latency and the disappearance of the reagin-like antibody. The two homocytotropic antibodies could also be separated by biological screening. Antisera collected 8 days after single antigenic stimulation demonstrate both homologous and rat heterologous PCA activity. Heating of these early antisera resulted in complete inactivation of heterologous PCA activity and almost complete inactivation of homologous PCA activity, thus suggesting that the rat heterologous antibody was due to mouse reaginic antibody. These results were strengthened by those following serum absorption. Absorption of these same sera with rabbit anti-mouse gamma-1, caused no change in homologous or heterologous PCA activity, indicating that the PCA activity of the very early antisera was due to mouse reaginic antibody. Early mouse antiserum was found to be very efficient in passive sensitization of rat mast cells and heating at 56 C resulted in the complete loss of this capability. The main reason for using a closely related heterologous system such as the rat is that, of the homocytotropic antibodies present in mice, only the reagin-like IgE and not the gamma-1 is detected. Moreover, a shorter latent period is observed.

Homocytotropic (reaginic) antibodies in guinea pigs infected with T. spiralis were demonstrated by Catty. He observed PCA activity in the 7S region at 17 hours after intradermal injection and reagin-like antibodies which were detectable after a latent period of 7 days. The reagin-like antibodies resided in an electrophoretically faster region, had a sedimentation rate greater than 7S, and were highly sensitive to heat. This long-term sensitizing antibody with biological and physicochemical properties analogous to the human reagin could be demonstrated in the serum of guinea pigs infected with T. spiralis but not in those immunized with a worm extract.

A radioactive iodine labeled microprecipitin (RAMP) assay was developed for measuring the binding of antigen by antibody. In both naturally infected humans and experimentally infected animals reaginic antibodies could be detected by PCA after a latent period of 72 hours. Heating or reduction and alkylation of the immune sera destroyed or markedly reduced the T. spiralis antibody reactions in the RAMP assay. The results indicate that the RAMP assay can reliably measure activity due to antibodies primarily of the immunoglobulin E class and can be used as a means of demonstrating binding of antigen by reaginic antibody in trichinosis.

Schistosoma spp.

Allergic manifestations in man occur as a result of infection with all three species of human schistosomes. In persons previously exposed to infection, cercarial penetration is frequently followed by urticaria, asthmatic attacks, subcutaneous edema, leucocytosis and eosinophilia. A second clinically recognizable phase beginning one month after exposure to infection and usually coincidental with the onset of egg laying of the

worms consists of anorexia, fever, headache and weight loss. This has been known in Japan as "Katayama fever." It has been generally acknowledged that these two clinical phases of schistosomiasis are due to immediate hypersensitivity phenomena, although until recently no definite proof has been obtained to that effect. When cercariae of nonhuman species penetrate the skin a dermatitis termed "swimmer's itch" often develops which consists primarily of pruritic papules. Evidence that swimmer's itch is a sensitization phenomenon was provided by McFarlane and Oliver. Whereas primary exposure to cercariae was followed in some subjects by no visible reaction, repeated exposures brought a reaction usually seen immediately after penetration and followed within several hours by the development of large papules accompanied by erythema, edema and pruritis. Secondary reactions revealed edema and massive round cell invasion of the dermis and epidermis. A similar condition has been observed in Sardinia following repeated exposures by man to cercariae of S. bovis. A form of swimmer's itch has long been recognized in Japan as "Kabure." This is also apparently produced by nonhuman schistosome cercariae. The wheal and flair type reaction involved in swimmer's itch has been ascribed to local anaphylaxis and can be relieved by the use of antihistamines.

The second phase of schistosomiasis "Katayama fever" is more systemic in character. In addition to fever and eosinophilia, there is usually splenomegaly, generalized lymphadenopathy, urticaria and often diarrhea or dysentery. Congested spleens with large numbers of histocytes and eosinophils were seen by Bogliolo in man and by Fairley in experimentally infected monkeys. The latter also observed swollen kidney glomeruli which had been infiltrated by neutrophils and eosinophils.

Homocytotropic reagin-like antibodies in schistosomiasis were reported recently in rats and monkeys, rabbits, human patients, chimpanzees and in rhesus monkeys infected repeatedly with irradiated cercariae of S. japonicum. These reactions are mediated by antibodies which differ from the conventional 7S immunoglobulins.

The immunological response of people, chimpanzees, rhesus and Cercopithecus monkeys, rabbits, guinea pigs and mice was recently studied by Sadun and Gore. Antibodies detected by fluorescent and flocculating techniques were heat stable, not sensitive to mercaptoethanol and were eluted from Sephadex G-200 in the IgG fraction. Antibodies detected by anaphylactic reactions were destroyed by heating, were inactivated by mercaptoethanol reduction and alkylation, had a faster electrophoretic mobility than IgG and were eluted from Sephadex G-200 after IgM and before IgG. There was a suggestive correlation between the natural resistance of these animals to infection with S. mansoni and the presence of reagin-like antibody in the blood of these animals. Rabbits and rats which are highly resistant to this infection developed high titers of PCA antibody. Conversely, mice which have little or no resistance to schistosomiasis, did not show reagin-like antibody even though they were capable of making this kind of antibody when challenged with T. spiralis. The results for humans, chimpanzees

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and rhesus monkeys were intermediate between these two extremes. This admittedly circumstantial evidence of a possible protective role of reaginic antibodies is further supported by the fact that rabbits which are highly susceptible to schistosomiasis japonica develop only relatively low titers of reaginic antibodies to this infection.

The observation by Zvaifler and his coworkers that a homocytotropic reaginic antibody is produced in rabbits infected with S. mansoni led to a study designed to determine if infection "sensitized" platelets and leukocytes for in vitro histamine release following the addition of S. mansoni antigen in the absence of rabbit plasma. Histamine release was detected from rabbits regardless of whether or not passive cutaneous anaphylactic antibodies were produced in the infected animals. A pure platelet suspension from sensitized rabbits did not release histamine when challenged with antigen. Histamine release did occur, however, in the presence of whole washed blood when similarly challenged. It is now believed that basophilic leukocytes, sensitized with homocytotropic antibody, release a factor (P.A.F.) which induces histamine release from platelets.

A radioactive microprecipitin (RAMP) assay using iodinated (^{125}I) extracts of S. mansoni cercariae as antigen was used in testing sera from schistosomiasis patients and from experimentally infected chimpanzees, monkeys, rabbits and mice. Like the PCA test with a 72 hour latent period, this test reacted in all species except in mice. Antibodies detected by this test were thermolabile and their reactivity could be prevented by mercaptoethanol reduction and alkylation. A striking similarity was observed with the time course development of homocytotropic antibodies and antibodies detected by the RAMP assay. Both reactions could be inhibited by absorption with goat anti-rabbit gamme E serum but not with anti-rabbit gamma G serum.

Filarial worms

The many different species of filariae which affect man produce a wide variety of clinical signs, a number of which appear to be attributable to immediate hypersensitivity. The widespread occurrence of tropical eosinophilia and its probable filarial etiology have been discussed previously. Profound allergic reactions associated with infection with a nematode related to filarial worms, Dracunculus medinensis, are well known. These are particularly evident at the time of the sudden release into the host's system of toxic by-products of the gravid female worms. In addition to local cutaneous lesions, there are pronounced systemic prodromes consisting of erythemia and urticarial rash with intense pruritis, nausea, vomiting, diarrhea, severe dyspnea and giddiness. Anaphylactic shock following the accidental breaking of the worms during surgical removal has also been reported. Some of the clinical manifestations of filariasis can be relieved by injecting Dirofilaria immitis antigen. Temporary swellings commonly observed in Loa loa infections following the migration of the worms in the subcutaneous tissue have been interpreted as being hypersensitivity reactions.

Allergic symptoms occurring in oncocerchiasis have also been described. The sera from a high proportion of American military personnel evacuated from the South Pacific with suspected Wuchereria infection gave positive P-K tests with filarial antigens. Serum from persons with other filarial infections such as Acanthocheilonema perstans, Loa loa and Onchocerca volvulus also produced positive P-K reactions. In general, all filarial infections are associated with eosinophilia.

Rabbits infected with Dirofilaria uniformis produced homocytotropic (reaginic) antibodies detectable by passive cutaneous anaphylactic reactions after a 60 to 72 hour latent period. This antibody was found to be different from the IgG class of rabbit immunoglobulin, had a faster electrophoretic mobility than gamma G globulin and was eluted in Sephadex G-200 after gamma M and before gamma G globulin. It was largely destroyed by heating or by reduction and alkylation. Serum fractions which contained the major amount of IgG immunoglobulin and all of the detectable fluorescent antibody activity did not react in the PCA test. Conversely, electrophoretically faster immunoglobulins which contained passive cutaneous anaphylactic activity showed no fluorescent antibody titer.

Other helminths

Dobson compared various immunoglobulin responses of rats to a nematode infection with Amplicaecum robertsi and demonstrated serum PCA activity after 6 days' infection. The P-K titers rapidly rose to a peak at the end of one month and then decreased to extinction before the 3rd month of infection. The antibodies responsible for these reactions were heat labile and disappeared after being heated at 60°C for one hour.

Skin reactions were provoked by a saline extract of Ostertagia in sheep. The ability to provoke a PCA reaction in the sera of sheep was greatest just after the "self cure" phenomenon had taken place. Several skin-tested animals showed signs of severe general anaphylaxis following administration of the allergen, manifested by prostration, copious salivation, cyanosis, dsypnea, defecation and urination. However, most of the animals recovered within half an hour. The author concluded that the homocytotropic antibody detected in sheep in response to Ostertagia infection has properties similar to the antibody which occurs in other mammalian species in contact with nematode allergens and is probably analogous to human IgE. However, he pointed out that the successful detection in vitro of this antibody will depend on the isolation of an ovine gamma E analogue, and that the present lack of information available on sheep myeloma proteins renders this possibility somewhat remote. Subsequent investigations revealed the presence of reaginic antibodies in the sera of infected sheep by means of Evans blue dye and a radiolabelled ^{131}I technique. The author concluded that this homocytotropic antibody is probably associated with the "self cure" reaction.

The sera of mice immune to Nematospiroides dubuis contain homocytotropic antibodies which can be detected by immunoelectrophoresis and by passive cutaneous anaphylaxis. Immune mice are subject to anaphylactic shock and the intestine of such mice participates strongly in the reaction. The author concluded that a state of immediate hypersensitivity is involved in the immunity of mice to N. dubius and he suggested that in immune mice a fresh intake of larvae initiates an anaphylactic reaction which prevents a large proportion of the larvae from becoming established.

Homocytotropic antibodies were detected in several other animals infected with various helminths. However, it is not clear whether or not these are true reaginic antibodies of the IgE immunoglobulin class. Some of these antibodies have not been immunochemically defined, and others, such as those resulting from Ostertagia and Trichostrongylus infections in sheep, have been shown to be related to the subclass $IgG1_A$. Homocytotropic antibodies were also semonstrated in the intestinal mucous of sheep infected with Oesophagostomum columbianum by means of the cutaneous anaphylactic test. Homologous PCA tests in guinea pigs revealed the production of skin sensitizing antibody by Metastrongylus spp. infection. However, it could not be determined whether or not two types of anaphylactic antibodies were present as suggested earlier. Injection of antigen into the skin of sensitized rats led to mast cell degranulation in those which were infected with Strongyloides ratti.

Hookworm infection in endemic areas is characterized frequently by a persistent asthmatic cough. Rabbits infected with Ancylostoma develop antibodies which produce a P-K reaction in uninfected recipients of the homologous species. As quoted by Winsor, Fall and Bartlett studied the time course development of antibodies detectable by the P-K tests with sera from hookworm patients. They reported that the P-K tests became positive within 4 weeks after infection, reached the maximum titer 3 months later and were no longer detectable after 1 year.

Potentiated reaginic responses in helminthiases

Recently it was shown that N. braziliensis infections may influence reagin-producing systems in rats and that this influence is not specific for reagins against N. braziliensis antigens, but can influence the existing production of reagins against other antigens. The potentiated response was found to occur only in those animals given antigen before the helminthic infection. The authors concluded that a factor may be present in the helminthic infections which stimulates the reagin-producing system and which under normal conditions results in a high titer of homocytotropic antibodies against the parasite. However, if the reagin system is already producing antibodies against the given antigen, the helminthic infection potentiates the reagin production against that antigen. Maximal PCA titers against egg albumin were obtained when rats were infected with N. braziliensis 10 days after immunization. Results from experiments with sensitized peritoneal mast cells from these animals

indicated that histamine release on challenge with egg albumin was maximal within 3 weeks, whereas challenge with worm extract released histamine maximally between 4 and 5 weeks. Homocytotropic antibodies against the antigen as demonstrated by PCA were not obtained when DNP-BGG was substituted for egg albumin in this system.

Similar mechanisms of potentiation may be involved in producing allergic manifestations to many allergens in individuals infected with helminths. A significant association, as yet unconfirmed, was observed by Tullis between bronchial asthma and intestinal parasites. He postulated that the acquisition of an allergy to an antigen may depend on exposure to the antigen at some stage of infection by one or more helminth parasites.

Summary and conclusions

It is obvious from this review that different types of antibodies are produced following parasitic infections. These can react with antigen within tissue spaces such as in the conventional antibodies which belong to the immunoglobulin classes IgG, IgA and IgM, or the antibodies may become fixed to tissues. The latter, known as reagins in humans, belong to the immunoglobulin group IgE and are thought to be responsible for the different manifestations of anaphylaxis. Since reagins adhere strongly to tissues, they are often called tissue sensitizing antibodies. Whereas conventional antibodies can be detected in the serum by standard in vitro procedures such as precipitation, agglutination, complement fixation and immunofluorescence, reagins can best be detected in vivo by passive or direct cutaneous anaphylaxis. In lower animals antibodies other than reagins can produce anaphylaxis in other homologous and heterologous species and can be detected by passive cutaneous anaphylaxis, but only after a very short latent period. Those antibodies which produce anaphylaxis in the homologous species are also referred to as homocytotropic antibodies. They can easily be differentiated from reagins by the fact that in addition to their short latent period they are heat stable, cannot be destroyed by mercaptoethanol reduction and alkylation, and have a different electrophoretic mobility than that of IgE. In vitro tests for reaginic activity are now in the process of being developed.

Although homocytotropic antibodies and cutaneous anaphylaxis have been reported in many protozoan and helminthic infections, reaginic antibodies have been demonstrated only in those parasitic infections which are due to helminths. Anaphylactic phenomena are caused by the antigen-antibody reaction activating a series of enzymes leading to the release of certain pharmacological agents such as histamine, serotonin, slow reacting substance, kinins and eosinophilic chemotactic factor which produce local or systemic anaphylaxis. Cutaneous urticaria and the formation of wheal and flare lesions in the skin are anaphylactic phenomena usually ascribed to homocytotropic antibodies. However, non-immune reactions such as trauma or cold, can also release pharmacological agents. Therefore, the numerous observations of kinin release, urticaria,

wheal and flare lesions in the skin in the course of protozoan infections are not necessarily immunological phenomena and may be mistakenly interpreted as demonstrating the presence of reaginic antibodies.

The eosinophiles are closely associated with helminthic infections and anaphylactic reactions. However, their exact role is still controversial. As reported by Vaughn an association between eosinophiles and allergic states has been known since 1898 and the attraction of eosinophiles to sites of parasite infections has been reported since the beginning of the century. There is no doubt that infiltration of the tissues with eosinophiles is a common denominator of many anaphylactic reactions and that most helminthic infections are generally associated with eosinophilia. Eosinophiles are attracted by antigen-antibody complexes and *have* been shown to phagocytose these complexes. However, eosinophiles may also be found in the skin in urticaria reactions regardless of whether these reactions have an immunological or pharmacological basis. This would suggest that the eosinophiles are attracted by concentrations of mediators, and that they are potentially capable of causing *histamine* release from mast cells. Although release of histamine and other mediators, as well as hyper-secretion of mucous are phenomena associated with allergic response, the role which eosinophiles may play in accentuating allergic reactions or in protecting against allergy has not been clarified. The intradermal injection of histamine into young horses has been shown to cause an accumulation of eosinophiles at the site of the developing wheal. Moreover, equine eosinophiles have been shown to inactivate histamine and serotonin both in vivo and in vitro.

Since the earliest observation of a virtual disappearance of eosinophiles from the blood of persons following injection of ACTH, dramatic variations in the level of eosinophiles have been reported following injections of cortisone or cortisone and atropine. Further experiments showed that the pituitary and adrenal glands were necessary for maintenance of a normal level of eosinophils in the small intestine. Hypophysectomy or adrenalectomy resulted in the increased eosinophile levels in the gut which could be counteracted by cortisone injections. In experimental trichinosis it has been shown that the release of histamine and not serotonin is associated with eosinophilia. The association between histamine and eosinophiles is still not clear even though it is known that mast cells contain most of the histamine of the body and that basophiles contain most of the histamine in the human blood. The accumulation of eosinophiles *in* the peripheral blood can be inhibited by antihistaminic drugs.

In general, eosinophilia could be considered as the earliest evidence of antibody induced sensitivity in helminth infections. Experimentally, eosinophilia is frequently followed by sensitivity of the mediator releasing tissues and by demonstrable anaphylactic reactions to worm extracts. Eventually, circulating homocytotropic reaginic antibodies can be detected by PCA in a percentage of the infected animals. In vitro tests such as antigen induced histamine release and

radioactive microprecipitin assays appear to be considerably more sensitive than PCA for detecting reaginic antibodies. This sequence of events which can be postulated primarily on the basis of separate experiments conducted in laboratory animals may parallel the succession of occurrences observed in man and lower animals following natural infections with helminth parasites. In these, one often observes eosinophilia, followed by changes in the numbers of mast cells and platelets, increases in serum levels of pharmacologically active substances and cutaneous anaphylaxis.

What is the reason for the development of high titer homocytotropic (reaginic) antibodies frequently resulting from helminthic infections? The explanation cannot be found in the continuous release of antigen or in the specific location of the parasite. All evidence accumulated so far indicates that production of large amounts of reaginic antibodies are obtained only when an actual infection takes place. Conversely, immunization with helminth extracts frequently results in failure of eliciting reagins or at best only produces reagins at an extremely low titer. It has also been demonstrated in vitro that the antigens involved in stimulation of reaginic antibodies are released by the living helminths. This contrasts with the production of high levels of homocytotropic (non-reaginic) antibodies by artificial immunization with a variety of parasite antigens.

The reason helminthic infections stimulate reagins and eosinophils so efficiently still remains a matter of speculation. Recently, however, evidence has been provided that at least one helminth species possesses some kind of adjuvant effect on the production of reagin containing immunoglobulins. These results, which were obtained in rats infected with N. braziliensis, must be extended to other helminthiases and other hosts before suggesting that helminths stimulate nonspecifically the proliferation of reagin producing cells. Further studies have indicated that the reaginic adjuvant effect of helminthic infections is not observable if DNP-BGG has been used as sensitizing antigen. Therefore, the observed potentiation may not necessarily be a general phenomenon, but may be restricted to specific antigens and, possibly, to only some host parasite relationships.

Only suggestive evidence has been obtained thus far on the possible relationship between homocytotropic (reaginic) antibodies and the protective mechanisms of the host. Some aspects of this subject will be reviewed by Murray.

2. Antigen-antibody interaction in trichinosis assayed by radioactive iodinated antigen.

Indirect evidence for the existence of a "mast cell sensitizing" antibody in mice infected with Trichinella spiralis was supported by the finding of anaphylactic)reaginic) antibodies in a patient infected with T. spiralis. Subsequent studies provided detailed biological and physicochemical information on homocytotropic reagin-like antibodies

in mice and rabbits experimentally infected with T. spiralis. These skin sensitizing antibodies can be detected by passive cutaneous anaphylaxis (PCA) in the homologous species, have many of the properties of human reagin and are remarkably similar to those belonging to the IgE class of human immunoglobulin described by Ishizaka et al.

Antibodies in the blood of human sera or animal sera infected with T. spiralis can be demonstrated by a variety of serological procedures, most of which detect antibodies of the IgG and IgM classes. Among these procedures is a sensitive and specific fluorescent antibody test for trichinosis developed by Sadun et al. and subsequently adapted to the use of soluble antigens fixed onto a matrix of cellulose acetate filter paper. This soluble antigen fluorescent antibody (SAFA) test was evaluated with sera from infected humans and with sera from experimentally infected mice, rats and rabbits.

SAFA and PCA activities of immune rabbit serum fractionated by *DEAE* chromatography were located in different fractions. Whereas most of the detectable fluorescent antibody activity and none of the PCA activity was found in the electrophoretically slow gamma globulin (IgG), electrophoretically fast immunoglobulins contained PCA activity but no detectable fluorescent antibodies. Moreover, the instability of mouse and rabbit PCA antibody upon heating or reduction and alkylation contrasted with the failure of such treatment to alter the activity of antibodies detectable by the SAFA technic.

A radioactive antigen microprecipitin (RAMP) assay was developed recently for measuring the binding of antigen by antibody in schistosomiasis. Results were similar with RAMP assays and PCA tests of sera from human patients and experimentally infected animals, although antigen binding activity in the RAMP assay appeared earlier and persisted longer than PCA activity. It was also found that essentially all the RAMP and skin sensitizing activity in the serum specimens was removed by absorbing with anti-IgE but not by absorption with other monospecific antiglobulins, thus indicating that the RAMP assay measures primarily antibodies of the IgE immunoglobulin class.

The present investigations were designed to develop a radioactive antigen microprecipitin assay for trichinosis, to characterize the antibodies responsible for this reaction and to compare the results with those of the SAFA and PCA tests.

Antigens

A lipid-free somatic extract of T. spiralis larvae was prepared at 4 C in Tris buffered saline (TBS), lyophilized in one ml aliquots and stored at 4 C. This antigen was used in the SAFA and PCA tests as described. For the RAMP assay the lipid-free preparations were placed on a 2.5 cm X 100 cm Sephadex G-200 column and eluted with 0.14 M, pH 7.2, phosphate buffered saline as described by Williams et al. The first fraction containing 23 percent of the protein was labeled with radioactive iodine (^{125}I) according to the technique of McConahay and Dixon. The iodinated antigen was divided into aliquots and stored at -70 C until used. Titrations were done with each new lot of iodinated antigen to determine the desired dilution for optimal reactivity.

Sera

Human sera obtained from 18 patients in whom an infection with trichinosis had been established by clinical and serological evidence were tested. To determine the specificity and sensitivity of the RAMP test, 98 sera from individuals with other parasitic infections or with viral, bacterial, rickettsial or degenerative diseases were studied. In addition, 71 sera obtained from h althy individuals were used. All sera were either lyophilized or stored at -20 C until tested.

Sera from Wistar rats (150-200 gm) and New Zealand albino rabbits (2500-3000 gms) were collected before and during these experiments. The rats were infected with approximately 10 Trichinella larvae per gram of body weight and the rabbits were exposed by gavage to 50,000 larvae each.

Antibody Determinations

The soluble antigen fluorescent antibody (SAFA) test for trichinosis was conducted as described previously. The titers were expressed as the reciprocal of the highest dilution giving a positive reading.

Passive cutaneous anaphylaxis (PCA) tests of human sera were conducted in monkeys following the technic described previously. PCA tests in rabbits and rats were conducted as reported by Zvaifler and Becker and Mota. Reactions were induced in these tests 72 hours after sensitization and the results were recorded 30 minutes after injecting antigen. A positive reaction was recorded when the area of cutaneous bluing was greater than 5 mm in diameter.

The radioactive antigen microprecipitin assay (*RAMP*) was performed with close adherence to the technic described for schistosomiasis. Serum samples which precipitated more than 15 percent of the radioactive T. spiralis antigen were considered reactive.

Sera were fractionated, reduced and alkylated, heated and absorbed as described earlier.

RAMP Assay in Trichinosis

Preliminary studies indicated that the RAMP assay using the iodinated antigen could differentiate between serum specimens obtained from infected animals and normal controls. Optimal results were obtained when test sera were initially *diluted* 1:10 in a 1:100 normal serum-TBS diluent. To assess the sensitivity and specificity of this assay, sera from rats and rabbits experimentally infected with T. spiralis were tested along with normal serum controls at a constant dilution of 1:10 (Table I). Except for the serum of one rat, which precipitated less than 15 percent of the antigen, positive results were obtained in all specimens from infected animals. None of the 75 specimens from uninfected rabbits and none of the 29 specimens from uninfected rats reacted in this assay. Nearly seven times as much antigen was precipitated by the sera from infected rabbits compared to those of the uninfected controls. In rats, the ratio was more than 4 to 1.

Serum samples from patients with trichinosis, with a variety of infections other than trichinosis and from patients with lupus erythematosus were tested for their ability to react in the RAMP assay. As shown in Table II, positive reactions were obtained in 15 of the 18 serum samples from trichinosis patients. Conversely, no reactions were observed in any of the 71 sera from healthy persons or in any of the 98 sera from individuals with various parasitic, bacterial, rickettsial, viral or degenerative diseases. The ratio of antigen precipitated by the sera from trichinosis patients and that precipitated by the sera from healthy controls was more than 5 to 1. The sera from persons with infections or diseases other than trichinosis precipitated between 1.5 to 1.9 times as much antigen as those from healthy controls. The three sera from trichinosis patients which were classified as nonreactive precipitated 3 times as much antigen as the sera from the healthy controls.

In order to obtain some information on the reproducibility of results, serum pools from infected and uninfected rabbits and rats were divided into aliquots and tested at different times with different lots of iodinated antigen. As indicated in Table III,

TABLE I

TABLE I

Sensitivity of RAMP Assay Using Trichinella spiralis *Antigen and Sera of Experimentally Infected Rats and Rabbits*

Serum Source	No. specimens	No. reactive	Mean % Ag. ppt.	Ratio[a]
Uninfected rabbits	75	0	5.3	7.0
Infected rabbits	41	41	37.0	
Uninfected rats	29	0	7.9	4.0
Infected rats	34	33	32.0	

[a] Ratio = % antigen precipitated by sera from infected animals / % antigen precipitated by sera from uninfected animals

TABLE II

TABLE II

Sensitivity and Specificity of RAMP Assay Using Trichinella spiralis *Antigen with Human Sera*

Diagnosis	Number	Reactive[a]			Nonreactive		
		No.	Mean % ppt.	Ratio[b]	No.	Mean % ppt.	Ratio[c]
Healthy	71	0	—	—	71	4.1	—
Trichinosis	18	15	22.7	5.5	3	12.3	3.0
Other parasitic infections	39	0	—	—	39	7.8	1.9
Viral	26	0	—	—	26	6.1	1.5
Rickettsial	13	0	—	—	13	6.4	1.6
Bacterial	10	0	—	—	10	6.1	1.5
Lupus erythematosus	10	0	—	—	10	6.3	1.5

[a] Sera producing more than 15% antigen precipitate are defined as reactive.

[b] Ratio between percent antigen precipitate in sera from trichinosis patients and healthy controls

[c] Ratio between percent antigen precipitate in sera from other groups and healthy controls

TABLE III

TABLE III

Reproducibility of Results of RAMP Assay Using Aliquots of Pooled Sera Obtained From Experimental Animals Infected with Trichinella spiralis

Serum source	No. times tested	Reactive	Percent Antigen Precipitated	
			Mean	Range
Infected rabbits (Pool I)	11	11	42.1	39-49
Infected rabbits (Pool II)	11	11	38.5	34-43
Uninfected rabbits (Pool I)	11	0	5.4	5-6
Uninfected rabbits (Pool II)	11	0	5.6	4-7
Infected rats (Pool I	5	5	31.4	27-36
Infected rats (Pool II	5	5	31.0	25-38
Uninfected rats (Pool I	5	0	4.6	4-6
Uninfected rats (Pool II	5	0	4.4	4-5

the results were highly reproducible. All of the specimens from infected animals and none of the specimens from normal controls gave positive reactions. The iodinated antigen appeared to be stable for at least 8 months when stored at -70 C.

Correlation of RAMP and SAFA Activities

The 18 serum specimens from trichinosis patients were also tested for SAFA activity. All of the specimens were reactive in the SAFA test with titers of 1:8 to 1:512. The results of the RAMP assay and SAFA tests were compared by scatter diagram (Fig. 1) and by the use of the Spearman rank correlation coefficient. No obvious correlation was observed between the level of reactivity in the two tests ($r = 0.399$, corrected for tied ranks).

Time course development of antibodies detected by the RAMP assay and by the SAFA test was determined by testing 6 rabbits before and at regular intervals after infection with T. spiralis (Fig. 2). Fluorescent antibody reactivity was observed in all the animals within 2 weeks after they were fed Trichinella larvae, reached a peak in 5 weeks and remained elevated throughout the study. Antibodies were detected by the RAMP assay in all the animals within 4 weeks after infection, reached a peak after approximately 7 weeks and were still elevated 12 weeks later.

Characterization of Antibodies

Investigations were conducted to define some of the properties of the antibodies which reacted in this assay and to compare the results with the antibodies reacting in the PCA and SAFA tests.

The effect of heat and reduction and alkylation was studied with specimens from infected rabbits. Two pools of sera were selected for this study, one which was reactive in both the RAMP and the PCA tests and another which was PCA negative with a marked reactivity in the RAMP assay. The results (Table IV) show that heat and treatment of serum with 0.1M 2-mercaptoethanol followed by alkylation with 0.02M iodoacetamide destroyed the reaginic antibodies as detected by PCA and markedly reduced the reactivity with the RAMP assay. Conversely, similar treatment failed to decrease fluorescent antibody reactivity. An additional ten human serum specimens which had reacted with conventional serological tests were retested after heating. All specimens were still SAFA positive after heating, whereas none of them gave positive results in the RAMP assay.

In order to separate some of the antibodies present in the serum of infected rats, the sera were fractionated by molecular sieving and ion exchange chromatography. The pooled serum was separated into 3 fractions by Sephadex G-200. Fraction III

Figure 1

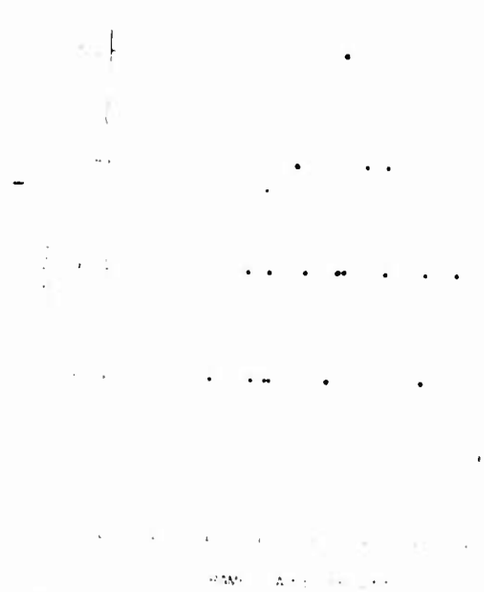


FIG. 1. Scatter diagram of results of RAMP and SAFA tests in trichinosis patients.

Figure 2

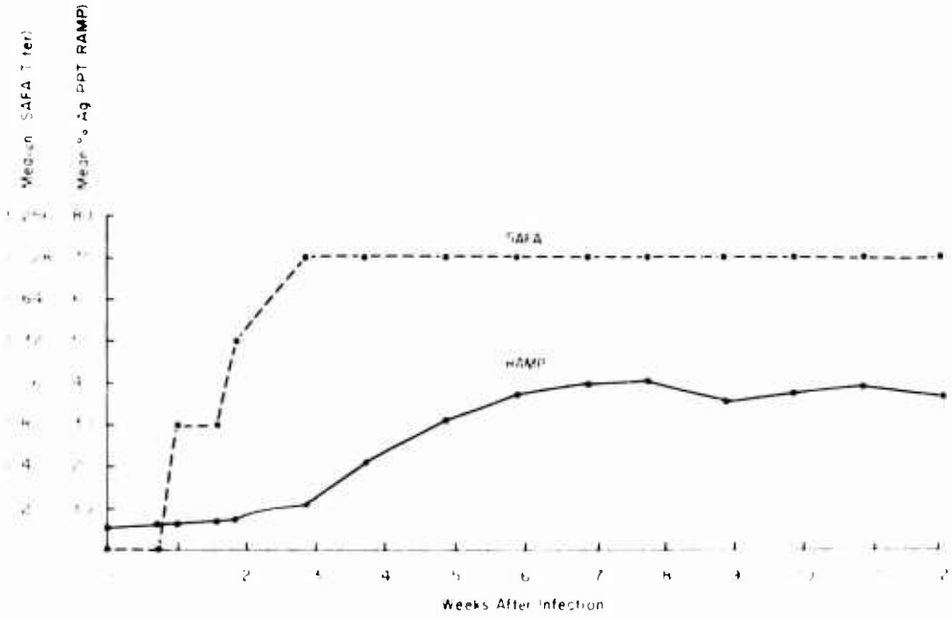


FIG. 2. Time course development of antibodies in rabbits infected with *Trichinella spiralis*.

TABLE IV

Effect of Heat and Reduction and Alkylation of Sera in the RAMP, PCA, and SAFA Tests

Serum Treatment	Rabbit Pool #1			Rabbit Pool #2		
	RAMP[a]	PCA[b]	SAFA[c]	RAMP	PCA	SAFA
Untreated	33.3	500	1,024	37.2	Neg	1,024
56 C 4 hr	14.1	10	1,024	15.0	Neg	1,024
2-Mercaptoethanol Iodoacetamide	14.1	0	1,024	14.5	Neg	1,024
Iodoacetamide	25.2	50	1,024	37.8	Neg	1,024
PBS dialysis	25.4	50	1,024	37.9	Neg	1,024

[a] Percent antigen precipitated in the assay. [b] Highest dilution of serum giving the minimum positive reaction. [c] Highest dilution of serum giving the minimum positive reaction.

contained most of the albumin and was not tested for antibody activity. Fraction II was separated into 3 fractions by DEAE A-25 chromatography and designated as Fractions II_1, II_2 and II_3 (Fig. 3). In the serum collected before infection all fractions gave negative results with all three tests. In the immune serum all SAFA activity was found in Fractions I, II_1 and II_2. Most of the PCA and RAMP activities resided in Fraction II_3 (Table V, Fig. 3).

Immune rabbit serum was absorbed with goat antirabbit IgE antiserum. When retested after absorption, the reactivity of the serum in the RAMP assay was reduced from 37.0 to 13.0 percent and the reactivity in the PCA test was eliminated. However, the titer in the SAFA test was not significantly reduced.

These data indicate that the radioactive antigen microprecipitin assay described for Schistosoma mansoni infections can be successfully applied to the detection of antibodies elicited by infections with T. spiralis.

Heating or reduction and alkylation of the immune sera lowered considerably their reactivity in the RAMP assay, indicating that the fractions precipitating the iodinated antigen are labile. This contrasts with fluorescent antibody reactivity which was not destroyed by heat or mercaptoethanol treatment. Fractions of sera from experimental animals gave similar results in both the RAMP assay and the passive cutaneous anaphylactic tests. Conversely, no correlation was observed between the results obtained with the RAMP assay and the SAFA tests in human sera. Absorption of immune rabbit serum with antirabbit IgE antiserum completely removed PCA activity and markedly reduced the antigen binding activity of the RAMP assay but failed to decrease the SAFA activity.

On the basis of this evidence, the RAMP assay as used in these studies measures primarily antibodies in the IgE immunoglobulin class.

3. Immunological reactions in chimpanzees experimentally infected with Schistosoma japonicum.

Helminthic infections elicit an immediate type of hypersensitivity reaction as evidenced by intradermal reactions and by the development of high titer reaginic antibodies. Although the ability of helminthiases to stimulate immediate hypersensitivity has been used in immunodiagnosis for more than half a century, the responsible antibodies have been demonstrated in the blood of humans or animals infected with helminths only recently.

TABLE V

TABLE V

Results of RAMP, PCA and SAFA Tests on Various Fractions of Pooled Sera Obtained From Rats Infected with Trichinella spiralis

Fraction	Normal Sera			Immune Sera		
	RAMP[a]	PCA[b]	SAFA[c]	RAMP	PCA	SAFA
I	1	Neg	Neg	1.2	Neg	64
II_1	1	Neg	Neg	1.4	Neg	256
II_2	1	Neg	Neg	1.2	Neg	256
II_3	1	Neg	Neg	4.2	4+	Neg

[a] Ratio = Percent antigen precipitated by sera from infected animals/Percent antigen precipitated by sera from uninfected animals

[b] Neg to 4+.

[c] Reciprocal of titer.

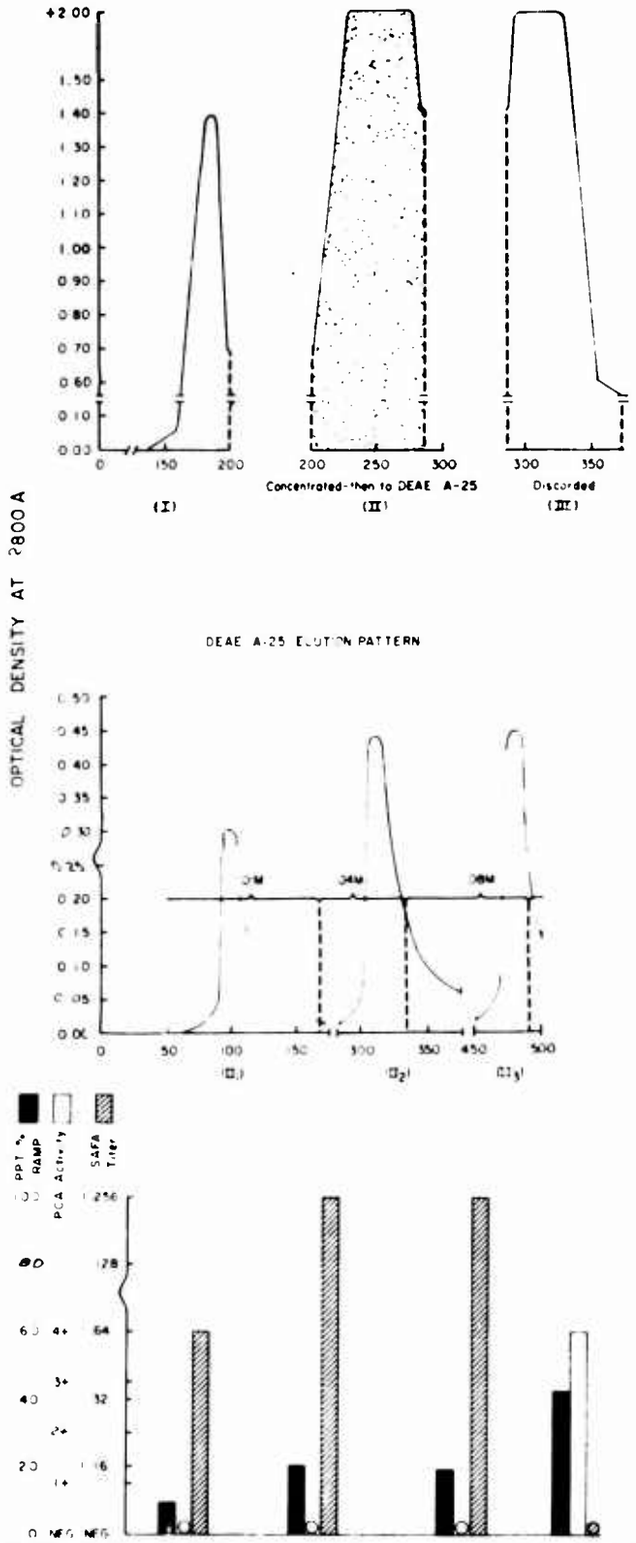


FIG. 3. RAMP, SAFA and PCA activity of fractions of rabbit anti-*Trichinella spiralis* serum. Serum was fractionated on G-200 Sephadex. Three fractions were collected and labeled I, II and III. Fraction II was concentrated and refractionated on DEAE A-25. These fractions (II_1, II_2, and II_3) and Fraction I were analyzed for RAMP, SAFA and PCA activity.

Schistosomiasis may in a sense be an immunological disease. In persons previously exposed to infection, cercarial penetration is frequently followed by urticaria, subcutaneous edema and massive round cell invasion of the dermis and epidermis (cercarial dermatitis). A second distinct clinical syndrome usually coincidental with the onset of egg laying consists of anorexia, fever, headache and weight loss (toxaemic phase, Katayama fever). These first two clinical phases may result from immediate hypersensitivity phenomena. The third stage of the disease (hepato-splenomegaly) may be caused by granulomatous host reaction to schistosome eggs which is regarded as a phenomenon of delayed hypersensitivity.

Homocytotropic reagin-like antibodies in schistosomiasis were reported in people, in rats and monkeys, rabbits, chimpanzees and in rhesus monkeys infected repeatedly with irradiated cercariae of Schistosoma japonicum. These reagin-like antibodies were detected by passive cutaneous anaphylaxis (PCA) in the homologous species in vivo. In vitro histamine release from platelets and leukocytes of rabbits was determined after the addition of S. mansoni antigen. Another in vitro test to detect reagin-like antibodies is the radioactive microprecipitin (RAMP) assay using iodinated extracts of S. mansoni cercariae as antigen. This assay was used in testing sera from schistosomiasis patients and experimentally infected chimpanzees, monkeys, rabbits and mice. The time course of development and immunochemical characteristics of homocytotropic antibodies detected by PCA and antibodies detected by the RAMP assay were strikingly similar. These reactions could be inhibited by absorption with goat antirabbit IgE serum but not with antirabbit IgG serum.

Although reagin-like antibodies were found in people, chimpanzees, rabbits, guinea pigs, rhesus and cercopithecus monkeys infected with schistosomes, mice did not show these antibodies to S. mansoni when tested either by PCA or by the RAMP assay. A correlation between the natural resistance of various animals to infection with S. mansoni and the presence and titers of reagin-like antibodies suggested that these antibodies may play a protective role in infection.

Parasitologic, serologic, pathologic, biochemical and clinical studies conducted in 15 chimpanzees exposed to varying numbers of S. japonicum cercariae indicated that this combination is a particularly valuable model for studies on the pathogenesis of S. japonicum because the infection in chimpanzees closely parallels various manifestations of this disease in man.

Immunologically mediated injuries such as serum sickness frequently result in glomerulonephritis. Kidney glomerular lesions also occurred in most of the S. japonicum infected chimpanzees. The association of glomerulonephritis and hepatosplenic schistosomiasis had been first observed in man. These findings along with our observations in chimpanzees emphasized the significance of schistosomal glomerulopathy. The possibility that pathogenic mechanisms of antigen-antibody reactions lead to injury or disease has been postulated frequently. Soluble immune complexes can cause a local increase in vascular permeability, generalized anaphylaxis, acute vasculitis and glomerulonephritis. Heavy deposits of host gamma and beta 1C globulins have been found along the glomerular capillary basement membranes in renal biopsies of Nigerian children with malarial nephrosis and in monkeys experimentally infected with Plasmodium cynomolgi. A decline in the complement activity of sera from humans, monkeys, hamsters and chickens infected with malaria has been reported. The changes in complement are apparently due to the immune fixation of the complement components to antigen-antibody complexes with consequent depletion from the serum.

A series of investigations was begun to determine the presence and extent of hypersensitivity reactions in chimpanzees experimentally infected with S. japonicum. Moreover, the effects of S. japonicum infection on serum complement activity were studied in the hope of finding some explanation for the glomerulonephritis encountered in our previous experiments.

Twelve young chimpanzees from West Africa were experimentally infected with a single dose of 50 cercariae per kilogram of body weight. Two animals were used as uninfected controls. The animals weighed 11 - 24 kilograms at the time of exposure. Splenectomy was performed on all of the chimpanzees for use in unrelated experiments with Plasmodium falciparum. These animals were utilized after termination of the malaria infection by chemotherapy and were healthy when selected for this study. At autopsy, accessory spleens had developed in some of the splenectomized chimpanzees. Previous studies indicated that splenectomy had no demonstrable effect on the host response of primates to schistosome infections except for a reduction in the rate of self cure in Macaca mulatta.

Cercariae of the Japanese strain of S. japonicum were obtained from pools of 50 to 100 infected Oncomelania hupensis nosophora snails which had been infected 2 to 4 months earlier with miracidia obtained by hatching eggs from livers of infected albino mice. The chimpanzees were exposed under light anesthesia as described earlier. Feces of each chimpanzee were examined weekly for schistosome eggs as evidence of infection.

Between 3 and 4 months after exposure to infection, 6 of the 12 infected chimpanzees were treated with a nitrovinylfuran derivative (SQ 18,506) from Squibb Laboratories. Previous investigations had revealed that administration of this drug to rhesus monkeys infected with either S. mansoni or S. japonicum was markedly therapeutic and resulted in no apparent host toxicity. The drug was administered by gavage under light anesthesia twice a day for 10 days. The course of treatment was repeated each time eggs reappeared in the stools.

Blood was collected from each chimpanzee at month intervals for serological and hematological studies. Lipid-free S. mansoni and S. japonicum cercarial extracts were employed as antigen throughout these studies. Cross reactions were observed between these two antigens. Serum specimens from chimpanzees were tested for reagin-like antibodies in the skin of rhesus monkeys weighing 2 to 3 kilograms each. The PCA tests were carried out with strict adherence to the published method and performed at 3 different times. Soluble antigen fluorescent antibody (SAFA) tests and RAMP assays were done according to the described method at the end of the experiment.

The amounts of serum IgE immunoglobulin at various intervals following infection were done by a radioimmunoassay (Ishizaka and Ishizaka, 1967).

For the blastogenesis studies, 10 ml of heparinized blood was obtained aseptically from each chimpanzee. One ml of 6% dextran in saline (70,000 molecular weight) was added to each sample of heparinized blood. The dextran blood suspension was mixed thoroughly and allowed to settle for 45 to 60 minutes. The leukocyte-enriched plasma layer was removed and placed in a 50 ml conical bottom graduated polyethylene centrifuge tube, the volume was brought up to 40 ml with 25% Eagle's minimum essential media (MEM) in Hank's balanced salt solution (HBS) and the mixture was centrifuged at 55 g for 8 minutes. The supernatant fluid was drawn off to 2.5 ml and the leukocyte button was resuspended very gently. The cell suspension was washed by removing the supernatant fluid to 5 ml and then adding 35 ml of 25% MEM in HBS. The suspension was centrifuged at 500 RPM for 5 minutes and the procedure was repeated. After washing three times, an aliquot was used to determine the number of leukocytes present in the suspension in a hemocytometer. The cell suspension was then diluted with MEM so that 1 ml would contain 1 X 10^6 cells per ml of suspension. The following cultures were set up in triplicate to contain 2 X 10^6 cells in 2 ml of medium: (a) cells and MEM only; (b) cells, MEM and phytohemagglutinin (PHA); (c) cells, MEM and antigen. The optimal amount of PHA was 0.1 ml of 1 to 60 dilution. Antigen was used at a concentration

of 1:8, 1:16 and 1:32. A dilution of 1:16 usually gave optimal results. All cultures were incubated at 37 C in an atmosphere of 5% carbon dioxide in air for 72 hours. Following incubation, 2 microcurie of tritiated thymidine was added to each culture and incubated at 37 C in an atmosphere of 5% carbon dioxide for 18 hours. After incubation with radioactive thymidine, the samples were centrifuged at 2,000 RPM for 10 minutes and the supernatant fluid was removed by aspiration. The cultures were then washed 3 times to remove the excess nonincorporated radioactive thymidine by adding 10 ml of 0.9% saline to each culture, mixing with a Vortex mixer and centrifuging at 2,000 RPM for 10 minutes. After the final wash the supernatant fluid was removed as close to the sediment as possible and 2 ml of NCS solubilizer was added to each tube, mixed well and allowed to stand overnight at room temperature. Cultures were prepared for liquid scintillation counting using 15 ml of PPO-POPOP toluene cocktail per vial. In tabulating the data, the scintillation counts per minute obtained from the antigen-stimulated cultures were divided by the counts per minute obtained from the nonantigen-stimulated cultures and then divided by the percent of lymphocytes in the culture. After infection higher stimulation was more often obtained with antigen than with PHA. The same lot of cercarial antigen containing 10 mg protein/ml was used in all of the blastogenesis experiments.

The complement levels were determined with sheep red cells which had been stored no longer than 3 weeks in modified Alsever's solution. They were washed and spectrophotometrically standardized to contain 5 X 10^8 cells per ml. Sensitized cells were then prepared by combining the standardized cells with an equal volume of an optimal dilution of rabbit hemolysin. A precise spectrophotometric method for complement assay was used and complement titers ($C'H_{50}$/ml) were expressed as the reciprocal of the amount of test serum required to lyse 50% of the optimally sensitized erythrocytes in a reaction volume of 1.5 ml after 30 minutes incubation at 37 C. Triethanolamine buffered saline (TBS) containing optimal concentrations of Ca^{++} and Mg^{++} was used as the reagent diluent.

Biometric analysis of the data was conducted only when the significance of the results was not obvious. Comparisons of observations in different groups were based on the Wilcoxon 2-sample Rank test.

No schistosome eggs were found in the stools of the chimpanzees before experimental infection and for at least 5 weeks afterwards, but all began excreting eggs in the feces between 5 and 6 weeks after exposure. Fecal egg counts in untreated

chimpanzees remained elevated throughout the course of this study although there was an irregular decline until necropsy. In contrast, the passage of eggs in the feces of treated animals was markedly suppressed. No eggs were seen in the feces 2 to 3 weeks after treatment. When they reappeared in the feces of some chimpanzees, these animals were treated again.

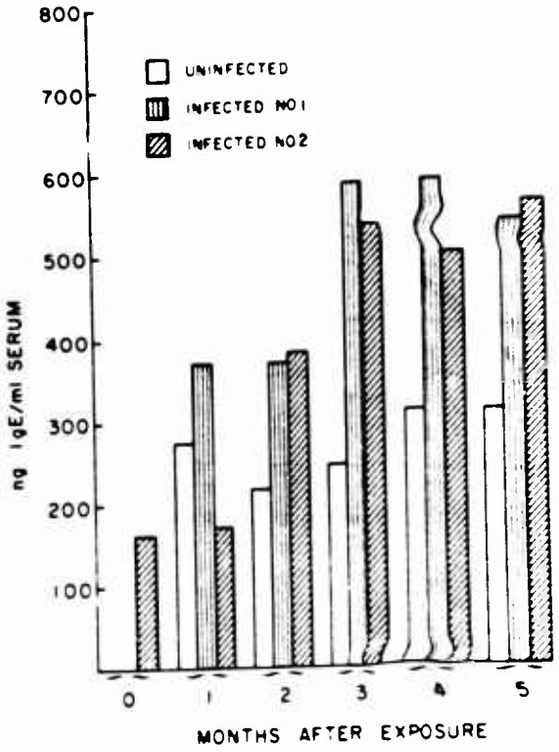
Four of 12 infected animals produced reaginic antibodies detectable by passive transfer to recipient rhesus monkey skin. PCA antibodies appeared 2 months after exposure to infection in 4 of 12 infected chimpanzees and persisted for only one or two months. In 8 infected and 2 uninfected controls no reaginic activity was demonstrated by PCA. No relationship was observed between the presence of dermal sensitivity to antigen and the intensity of infection as determined by egg counts.

All animals were tested with S. mansoni and S. japonicum cercarial antigen in the eyelid for immediate and delayed hypersensitivity. These tests were conducted shortly before necropsy in order to insure that introduction of antigen would not affect the serological determinations. A positive dermal reaction was observed in all of the infected animals (treated and untreated) within 3 hours after the antigens were inoculated intradermally. Conversely, no reaction was observed in either of the 2 uninfected controls. No delayed hypersensitivity reactions were observed in any of the chimpanzees with either of the two antigens used, 24 and 48 hours following introduction of the antigen.

The level of gamma E immunoglobulin in the serum of 3 chimpanzees (2 infected and 1 uninfected control) before exposure and at monthly intervals up to 5 months after infection showed considerable increases following exposure to S. japonicum cercariae (Fig. 4). Two of 4 PCA positive chimpanzees were included in this group. This increase parallels the increase in gamma globulins in S. japonicum infected chimpanzees.

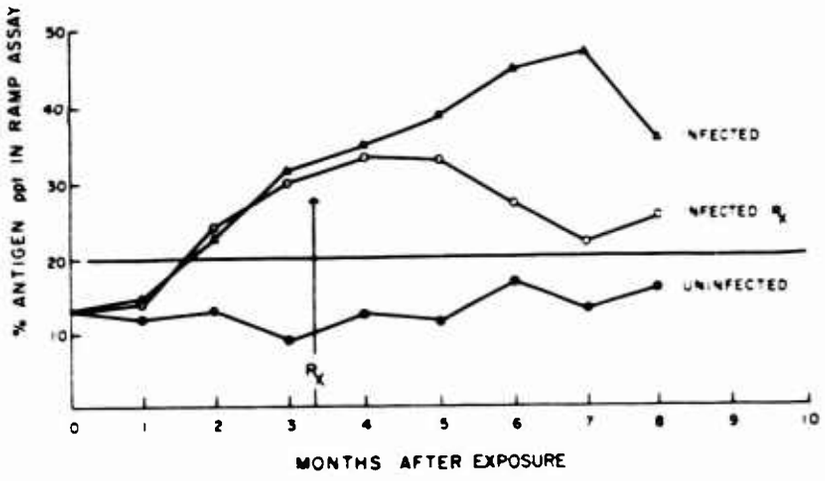
Previous studies had indicated that the RAMP assay can reliably measure activity due to antibodies primarily of the IgE class and can be used as a means of demonstrating binding of antigen by reaginic antibody in schistosomiasis. Therefore, all the sera from the chimpanzees were assayed monthly for RAMP activity. As shown in Fig. 5 all animals gave negative results before infection and one month after infection. However, beginning with the second month, the sera of infected animals precipitated increasing amounts of radioactive antigen, reaching a maximum 7 months after infection. A marked decrease in RAMP activity was observed in the sera of the infected animals following treatment. There was a significant difference between treated

Figure 4



Serum IgE levels in chimpanzees at various intervals following exposure to 50 cercariae/kg of body weight.

Figure 5



Average percent antigen precipitated by sera of chimpanzees at various intervals following exposure to 50 cercariae/kg of body weight.

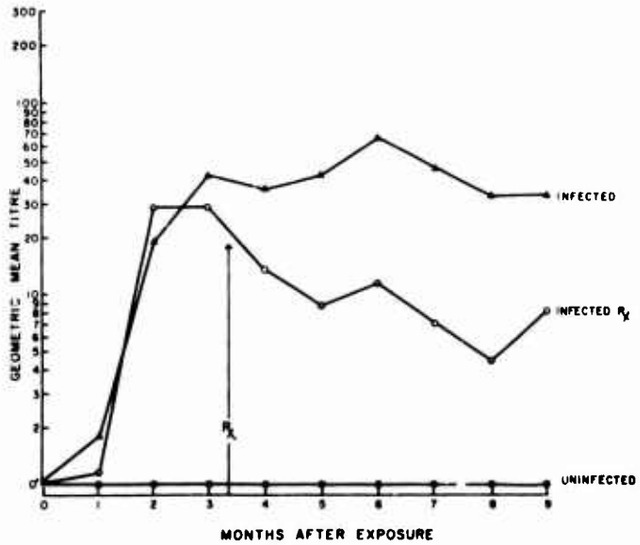
and untreated groups 6 and 7 months after infection. At the end of the experiment (8 months after exposure) the values obtained from treated and untreated animals were not significantly different from those of the uninfected controls.

Antibody production as measured by the SAFA test followed a different time course of development. Fluorescent antibodies were detected at low titers in the sera of all the animals one month after exposure to infection. The titers increased rapidly and remained elevated for the duration of the experiment (Fig. 6), although values for the treated animals were consistently lower than those for the untreated group. However, none of these differences were significant at the 95% confidence level.

The most reproducible and quantitative technic for measuring the response of lymphocytes to an antigenic stimulus is an assay of the total tritiated thymidine uptake by sensitized lymphocytes. This quantitative measure of the total DNA synthesis by the lymphocytes is a sensitive indicator of their transformation. Blast transformation was studied with lymphocytes from the peripheral blood of chimpanzees at monthly intervals following exposure to infection. Hematological observations indicated that the number and percentage of lymphocytes in the infected animals markedly decreased beginning with the second month following exposure to infection and remained depressed throughout the course of the infection. Whereas in uninfected chimpanzees approximately two-thirds of the white blood cells were lymphocytes, shortly after exposure to infection the percentage of neutrophils far exceeded that of lymphocytes. Therefore, in estimating the amount of thymidine incorporation in the leukocyte cultures of the chimpanzees at various intervals, the findings were corrected to take into account the percentage of lymphocytes in the culture. The rate of DNA synthesis as measured by thymidine incorporation is shown in Fig. 7. The thymidine incorporation in the lymphocytes obtained from noninfected chimpanzees remained low and constant throughout the experiment. Increased DNA synthesis in the infected animals became apparent as the infection progressed and reached a peak 4 months after exposure. The difference in values between infected and noninfected chimpanzees was highly significant beginning with the second month of infection except for the seventh month. Although consistent differences were apparent between the treated and untreated groups, these differences were not statistically significant.

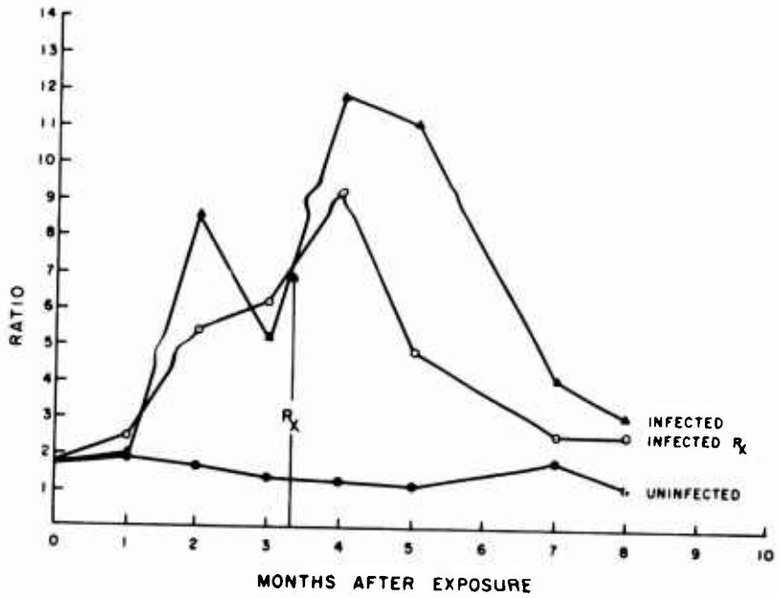
A small decrease in complement level occurred in the infected chimpanzees 2 and 3 months after exposure to infection. However, this decrease as well as changes which occurred later, were not statistically significant.

Figure 6



Geometric mean of soluble antigen fluorescent antibody titers in serum of chimpanzees at various intervals following exposure to 50 cercariae/kg of body weight.

Figure 7



Average ratio of tritriated thymidine incorporation between antigen stimulated and nonstimulated lymphocytes in blood cultures of chimpanzees at various intervals following exposure to 50 cercariae/kg of body weight.

The results of these investigations indicate that chimpanzees infected with S. japonicum develop homocytotropic reaginic antibodies and suggest that allergic manifestations may be an important component of schistosomal infections. Reagin-like antibodies could be detected in vivo by intradermal tests and by passive cutaneous anaphylaxis in the skin of rhesus monkeys and in vitro by the radioactive microprecipitin test. These results confirm and extend our earlier reports.

The results obtained in measuring the serum immunoglobulin E levels in infected chimpanzees are in agreement with the observations that parasitic infections give rise to high IgE concentrations even in individuals without atopic manifestations. Thus, elevated IgE levels have been observed in schistosomiasis japonica in chimpanzees as well as in people with ascariasis, visceral larva migrans, capillariasis and trichinosis.

The time course of the development of PCA antibody activity and of in vitro precipitation of antigen indicates that the RAMP activity was present in more animals, was detected earlier and persisted longer than PCA antibody activity in the serum of infected animals. This incomplete correlation suggests either that more than one antibody may be responsible for the two phenomena or that the RAMP assay is a much more sensitive method of measuring immediate hypersensitivity.

Negative results were obtained in attempts to detect delayed hypersensitivity by skin testing the animals before death. Since the reaction may be stimulated by eggs and may be stage specific, the negative results may be attributed to the failure of cercarial antigen to detect anti-egg antibodies. However, this seems unlikely since detailed cross absorption and double diffusion studies have shown that cercariae and eggs share most of their antigens and have more in common with each other than either has in common with the adult excretory antigens. Moreover, occasional skin reactions of the delayed hypersensitivity type were observed with cercarial S. mansoni antigen in Sardinian women frequently exposed to S. bovis. Another possibility is that delayed hypersensitivity would have been detected by this method at an earlier stage of the infection, since the tests reported in this study were carried out considerably after the peak of blastogenesis, as noted in Fig. 4. In fact, no significant differences in blastogenesis were observed in our animals toward the end of the experiment when intradermal reactions were performed in these chimpanzees. The consistent and significant increases in values obtained by stimulation of lymphocytes to antigen in infected animals seems to suggest that a delayed hypersensitivity phenomenon does indeed occur in schistosomiasis japonica. The apparent inconsistency between the results of

blastogenesis and those of skin tests might be due to the fact that the in vitro response might antecede the appearance of positive skin tests and that it can be detected occasionally in association with antibody production by cells in the apparent absence of positive skin tests. This suggests that lymphocyte transformation may be more sensitive than skin testing for detecting delayed hypersensitivity. However, since stimulation occurred at approximately the time of the earliest appearance of antibodies detected by the RAMP assay, blastogenesis might have been stimulated by the production of antibodies such as reaginic antibodies. Perhaps a better correlation between an in vitro system and delayed hypersensitivity could have been obtained by using a macrophage migration inhibition tests. We conducted such tests on our chimpanzees but due to technical difficulties inconclusive results were obtained.

The immunological responses of the treated animals which were somewhat intermediate between the infected animals and their uninfected controls may have resulted from an undescribed immunosuppressive or anti-inflammatory activity of the drug or from an interference with in vitro binding of gamma E antibody. Although no consistent and significant differences were obtained when the mean values of the two infected groups were compared, such possibility cannot be excluded since the number of animals was small and the assay technic might not be sensitive enough to measure decreases which may have been present.

Although no significant difference was noted in the mean values of infected and uninfected groups, there was a decline in complement activity in the more heavily infected animals 2 and 3 months after infection. This may have resulted from in vivo immune fixation. The time at which this decline was observed coincides with the massive release of eggs near the beginning of patency, suggesting the possibility of fixation of the complement components to antigen-antibody complexes with consequent depletion from the serum.

The schistosome egg plays an important role in the pathogenesis of schistosomiasis. The granulomatous reaction to the eggs trapped in the tissues appears to be a form of delayed hypersensitivity, which is specific, can be transferred by spleen or lymph node cells and can be inhibited by immunosuppressive drugs, antilymphocyte serum and by Hodgkin's like lesions. It has also been suggested that some of the lesions of schistosomiasis may result from immune complexes. Specific circulating antigen has been observed in heavily infected mice and in hamsters. This circulating antigen, reacting with specific antibody and coupled to complement, could lodge on some organs and cause considerable damage. The reports of a high coincidence of glomerulonephritis,

the presence of electron dense deposits in glomerular basement membranes in humans infected with S. mansoni and the general occurrence of glomerulitis and necrotizing arteritis in chimpanzees experimentally infected with S. japonicum further suggest that in addition to delayed hypersensitivity humoral immunologic mechanisms may play a role in the pathogenesis of schistosomiasis. Although these mechanisms could not be more precisely defined in the present investigations, detailed immunofluorescent and ultrastructural investigations in chimpanzees infected with S. japonicum are underway to determine the role of circulating antibodies or antigen-antibody complexes in the production of glomerular lesions and in the development of pipestem fibrosis.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 100 Hypersensitivity in the immunopathology of helminthic infections

Literature Cited.

Publications:

1. Aikawa, M., Schoenbechler, M. J., Barbaro, J. F. and Sadun, E. H.: Interaction of rabbit platelets and leukocytes in the release of histamine. Am. J. Pathology 63:85-91, 1971.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6473	72 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 / A. WORK UNIT
71 07 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	101
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Provide PIN Security Classification Code)

(U) Immunoprophylaxis of protozoan infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002:00 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 11	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	PRECEDING/CURRENT	a. PROFESSIONAL MAN YRS	b. FUNDS ($ Thousands)
FISCAL YEAR	72	3	0
	73	3	70

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Provide EACH with Security Classification Code)

(U) Immunize; (U) Irradiate; (U) Parasite; (U) Trypanosome

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

23(U) To develop means for inducing acquired immunity to protozoan infections of military importance. The results may be eventually applied to the prevention or suppression of these infections in military personnel.

24(U) Conventional and experimental serological methods will be used to evaluate host reactions to infection following treatment with gamma irradiated parasites. Data will be used to devise effective means of diagnosis, treatment, and/or immunoprophylaxis of infection.

25(U) 71 07 - 72 06 A recently isolated human strain of Trypanosoma rhodesiense (EATRO #1886) from Uganda was infective for mice and rhesus monkeys. Mice receiving two or three immunizing inoculations of gamma-irradiated trypanosomes were markedly protected from a challenge infection of 1,000 unirradiated organisms one week after immunization. Complete protection from a challenge infection was induced in 5 or 6 monkeys given six immunizing inoculations with irradiated trypanosomes. Conversely, the five unimmunized controls became patent 5 to 8 days after challenge with 10,000 unirradiated organisms, showed signs of progressive severe illness and died between 12 and 49 days after challenge. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

* 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 101 Immunoprophylaxis of protozoan infections

Investigators
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1. Quantitation of Trypanosoma rhodesiense by electronic particle counting.

Much effort is expended in the laboratory in the standardization of suspensions of protozoa and helminths for a variety of experimental studies. Microscopic examination of wet blood films or of stained thick blood films have long been used for estimating parasitemia levels in trypanosome infections. Counts based on a measured volume of blood, such as those made by Neubauer hemacytometer, have permitted further standardization in trypanosome quantitation. However, all these procedures are tedious and are subject to substantial variability, due primarily to a limitation on size of the samples examined.

Recently, a relatively simple method was described for isolating trypanosomes from infected blood by adsorbing particulate blood components onto DEAE cellulose columns and eluting the trypanosomes. The specific adsorption-elution characteristics of both trypanosomes and erythrocytes are interpreted in relative terms of the surface negative charge. With this technique trypanosomes can be obtained readily in a clear suspension.

The present studies were undertaken to determine whether an electronic particle counting technique could be used to estimate accurately the numbers of trypanosomes in such clear suspensions, with accuracy and with a saving of time and effort.

The technique of Lanham and Godfrey for the recovery of Trypanosoma rhodesiense from rat blood was used. Heparinized blood from Wrm:WRC (WI) BR albino rats infected with T. rhodesiense was diluted 1:4 in a phosphate buffered saline-glucose (PSG) solution (pH 8.0). Two milliliters of the diluted blood was put into a 1.5 x 30 cm column containing DEAE (DE52) cellulose packed to a height of 14 cm. A volume of 80 ml eluate was collected by passing PSG through the column. A small subsample was diluted for trypanosome counts on the electronic particle (Coulter) counter.

For some studies a large (3 x 50 cm) column was used to quantitate trypanosomes processed from relatively large volumes of blood. This column was packed to a height of 22 cm. An original blood sample of 10-15 ml was used in a 1:4 dilution. Collections of 20-, 40- or 80-ml

aliquots were made. For studies of total yield, volumes of up to 2 liters were collected.

Hemacytometer counts were made both on the blood and on the eluate for comparison with the Coulter counts. Comparisons were made to estimate the percentage yield of trypanosomes in standard volumes (40 or 80 ml) of eluate, and from exhaustive elutions (total trypanosomes in elutions up to 500 ml). The tests were repeated to provide reliable estimates of percentage yield from the column filtration process.

Based on numerous daily operations with the filtration-counting techniques, a standard threshold (lower 5, upper 50) was established for use of the Coulter counter in our laboratory (Model B, aperture 70μ), based on criteria of maximal counts with acceptably low background. Dilutions of the suspensions of trypanosomes were made according to expected magnitude of counts, and appropriate factors were applied when count comparisons were made on a standard volume basis.

Counts were standardized as follows: Replicate (usually 10) counts were made on each diluted test sample, and on a control solution of PSG buffer that had passed through the column before the blood was introduced. The counts were estimated by using the formula:

$$\text{Count} = \frac{2V_2}{DV_1}\ \bar{x} + C - \bar{x}_b$$ in which $\bar{x}$ represents the mean count of trypanosomes, $\bar{x}_b$ represents background buffer particle-count: V_1 the volume (ml) blood introduced into columns (1/2 ml for the small column); V_2 the volume (ml) of eluate from which counts were made; D the dilution factor for subsample used in counts; 2 the volume factor; since 1/2 ml volume is processed for each count in the Coulter apparatus; and C the coincidence factor (adjustment for simultaneous passing of more than one particle through the aperture) = $.357\ (\bar{x}/1000)$.[2]

At least 4 (usually 10) background counts were made from column-filtered buffer alone, and these values were used in adjusting the gross trypanosome counts, unless the background and coincidence factors combined were low enough to have only a negligible effect, in which cases the adjustments were deleted. This buffer (i.e. initial elution before blood was introduced into the column) generally gave lower particle counts than did buffer which had passed through a paper filter.

Uninfected rat blood was also tested in the filtration system, and counts from the resulting column eluates were not greater than the buffer-only background control.

In all of the experiments performed, the recovery of trypanosomes from the DEAE cellulose column appeared to be very satisfactory. Active trypanosomes passed through the column in numbers which generally were very close to expected values based on counts from whole blood. Lysis or agglutination of trypanosomes occurred after several hours at room

temperature. This lysis apparently occurred in whole blood as well as in the buffered saline-glucose solution. However, if counts were made within two hours after column elution, there was no preceptible loss of trypanosomes. Moreover, refrigeration appeared to prolong the survival of the trypanosomes.

Several attempts with the Coulter apparatus were made to count trypanosomes in diluted, unfiltered blood before and after lysis of erythrocytes with saponin (3 drops of 1% saponin in 10 ml of 1:500 blood dilution). In none of these tests on unfiltered blood was it possible to make satisfactory counts, apparently due to (a) an overlapping of thresholds (because of a similarity in particle sizes) for erythrocytes and trypanosomes and (b) a lytic effect of saponin on the trypanosomes.

Relative efficiency of recovery. Several experiments were undertaken to compare mean Coulter counts with hemacytometer counts. When a 40-ml volume of filtrate was collected from the small column, there was consistently an almost total recovery of trypanosomes, as evidenced by comparison of Coulter counts from the filtrate, versus hemacytometer counts from whole blood, after dilution factors were applied to adjust to the units of trypansomes/ml blood.

The ration (R) of trypanosome counts (per ml blood) was calculated. This ratio consisted of the Coulter count (filtrate) estimate divided by the hemacytometer count (unfiltered blood) estimate. The R values from 11 separate experiments were: 1.05, 0.87, 1.02, 0.70, 0.74, 1.35, 1.09, 0.71, 1.19, 1.06, 0.70. A similar comparison was made between Coulter count (filtrate) estimates and those made by hemacytometer counts on the same filtrates: The R values were 0.97, 1.02, 0.75, 0.96. The reproducibility of Coulter count estimates is indicated from duplicate column runs of 40-ml aliquots from the same blood samples (Table 1). The R values for comparison to hemacytometer counts were 0.99, 0.70 respectively.

Relative variability. The variability of counts from replicate determinations was assessed for each of the methods employed. The mean standard error and the coefficient of variation (V) = $\frac{\text{Standard deviation}}{\text{mean}}$ were calculated. In all cases the coefficient of variation (V) was smaller for the Coulter counts on filtered trypanosomes. Counts made by hemacytometer had lower values when made on whole blood than when made on the column filtrate (Table 2).

Relative sensitivity. The sensitivity of counts was assessed on blood with relatively light trypanosome densities, by both the Coulter and hemacytometer techniques. When the concentration of trypanosomes was so low that it could be infrequently detected by hemacytometer counts on whole blood, an estimate was then made of the requisite further dilution to cause the Coulter count to become negative also. A count of 1000 or less above background was arbitrarily considered to represent reversion to negativity.

Table 1

Counts of Trypanosome rhodesiense made by Coulter counter on column filtrates from duplicate column runs with different specimens of infected blood. Mean ($\bar{X}$) and coefficient of variation ($V = \frac{\text{Standard deviation}}{\text{Mean}}$) reflects the magnitude and reproducibility of counts in the filtration-particle-count procedures.

Blood Specimen	Method	Number Counts	$\bar{X}$	V
A	Coulter-Filtrate*			
	Run No. 1			
	1st 20-ml eluate	10	1,754,100	0.06
	2nd 20-ml eluate	10	81,900	0.10
	Run No. 2			
	1st 20-ml eluate	10	1,410,700	0.03
	2nd 20-ml eluate	10	439,200	0.05
	Hemacytometer-Blood			
	Technician A	6	248	0.92
	Technician B	8	308	1.25
B	Coulter-Filtrate*			
	Run No. 1			
	1st 20-ml eluate	10	3,338,800	0.03
	2nd 20-ml eluate	10	372,800	0.06
	Run No. 2			
	1st 20-ml eluate	10	5,240,100	0.03
	2nd 20-ml eluate	10	419,600	0.07
	Hemacytometer-Blood	4	448	0.32

*Trypanosome densities in the filtrate required a 1:100 dilution for Coulter counting, and the counts were multiplied by a factor of 100.

Table 2

Counts of Trypanosoma rhodesiense obtained by Coulter counter and Hemacytometer methods. Mean ($\bar{X}$) (see previous table).

Experiment	Method	Number Counts	$\bar{X}$	V
A	Coulter-Filtrate*	10	809,000	0.04
	Hemacytometer-Blood	8	214	0.07
	Hemacytometer-Filtrate	8	3	0.09
B	Coulter-Filtrate*	10	2,120,930	0.06
	Hemacytometer-Blood	8	560	0.10
	Hemacytometer-Filtrate	8	11	0.37

*Trypanosome densities in the filtrate required a 1:100 dilution for Coulter counting, and the counts were multiplied by a factor of 100.

In one experiment a mean value of 2 trypanosomes from 4 hemacytometer counts (i.e., just above borderline positivity) corresponded to a mean Coulter count of 8500 trypanosomes in the first 20-ml elution; which is equivalent to an estimated 700,000 trypanosomes/ml blood. In a lighter infection effected by mixing of the infected rat blood with uninfected rat blood, so that each of 4 hemacytometer counts was zero, the Coulter count was 4000 trypanosomes, which is equivalent to an estimated 320,000/ml blood, in the first 20-ml elution. In the second 20-ml eluate there was a count of 1800 trypanosomes, equivalent to an estimated 144,000/ml blood. Under these experimental conditions the Coulter count technique remained positive even if the light infection which could not be detected routinely by hemacytometer, were as much as three times lighter.

In a second experiment blood with a mean hemacytometer count of 4 trypanosomes remained positive by the Coulter counter technique even if diluted up to 12 times (with a 20-ml collection), or 8 times (with a total 40-ml collection). A third test indicated, similarly, that a 5-fold lighter than borderline-positive infection was detectable by the Coulter count method.

Elution pattern of trypanosomes. Serial collections of small (e.g. 10 ml) volumes of eluate were made, and trypanosomes were counted in each volume. A majority of the parasites were recovered in the first 10 to 20-ml elutions, and almost total recovery was obtained in the first 40 ml, although a few trypanosomes were eluted after 100-200 ml were collected. From several experiments the estimated proportion of trypanosomes recovered in the first 20 ml (of a 40-ml collection were as follows: 0.955, 0.763, 0.900, 0.926, 0.713, 0.743,

0.827, 0.673. As stated previously, almost all trypanosomes were recovered from each blood sample, when as much as 40 ml was collected from the column.

In the large column a similarly high proportion of trypanosomes was recovered in the early eluates, and a vast majority of the parasites was found in the first 100 ml. There were some parasites collected in elutions well beyond the precipitous decline in parasite recovery, and counts were still positive after 600 ml had been collected.

Tandem filtrations of blood specimens. In all of the experiments described above, each filtration was made with a freshly packed cellulose column. In one experiment a second blood sample from a blood specimen was run through the same column as the first, without repacking. For these two runs very similar trypanosome counts were recorded by the Coulter counter:

	First 20 ml collected	Second 20 ml collected
Run No. 1	340,000	27,800
Run No. 2	340,000	32,500

The results of our investigations indicate that the electronic particle counts of T. rhodesiense after column separation from infected blood is simple, highly reproducible, and the technique is less variable and more sensitive than the hemacytometer method. In very light infections when the parasite number was too low to be detected by hemacytometer, the parasites could be found and counted by the Coulter counter. The increased sensitivity is due to the relatively large volume of liquid on which particle counts are based (0.5 ml) as compared to the hemacytometer counts (0.000002 ml). In infections detectable by hemacytometer there was a remarkably close correlation between the hemacytometer counts and those made by the particle counting procedure. The ratios of mean counts by each method ranged from 0.7 to 1.4 in eleven separate experiments, with most ratios being very close to 1.0. These results justify the application of the particle counting procedure for preparation of inocula for experimental animals as well as for monitoring the courses of infection. In such cases relatively small elutions can be used, since most of the trypanosomes recovered are in the initial eluates.

The relatively high reproducibility of Coulter counts of filtered trypanosomes is due in part to a reduction of random error, because of the increased magnitude of each count in the relatively great volume of parasite suspension counted.

Both the particle count and the hemacytometer methods involved certain errors. A potential source of error in the particle count technique may arise from the presence of cellular debris which may be counted as trypanosomes by the counting apparatus. Although in our studies there was no contamination from cellular blood components, it is advisable that occasional microscopic observations of drops of column

eluates be performed to obviate this possible error, and to verify the presence of trypanosomes. A major source of variation in hemacytometer counts resides in the individual counting errors among various technicians. Human error can be precluded by the use of automatic equipment such as the Coulter counter, except for variations in the dilution procedures.

In addition to the greater sensitivity and reproducibility of the procedure, the particle count technique can be performed with a considerable saving of time in experiments involving clear suspensions of trypanosomes. Once the system is standardized, 5 background and 10 trypanosome counts should be made within 5 minutes by the use of the Coulter counter, whereas with the hemacytometer an initial 20 minute staining and settling period is required in addition to the time spent at the microsocope to perform the counts. This, for an experienced technician, amounts to 5 to 15 minutes for each count depending on the number of trypanosomes present.

The particle-count technique may have a variety of applications in laboratories in which a Coulter counter is available. Once the procedure is standardized for a laboratory there may be a number of uses in screening specimens from humans (blood donors in endemic areas) or for surveys of potential animal reservoirs. The efficiency of such procedures would depend on the numbers of specimens to be examined, the adaptability of the filtration process to simultaneous mass screening, and on specific confirmation of positives by microscopic examination of concentrated filtrates. In laboratory experiments in which clear suspensions are to be used, the Coulter particle-count technique appears to be both a convenient and reliable method of trypanosome quantitation.

2. Immunization of mice and monkeys with a gamma-irradiated, recently isolated human strain of Trypanosoma rhodesiense.

Immunity can be induced in rodents against African trypanosomiasis by inoculations with live parasites attenuated by ionizing radiation. Mice inoculated with unirradiated Trypanosoma rhodesiense (Wellcome strain) developed progressive infections and died in an average of 3.6 days after inoculation. Conversely, those inoculated with irradiated trypanosomes resisted challenging infections with unirradiated trypanosomes. All mice survived challenge when they received two or more immunizing inoculations of trypanosomes irradiated at doses of more than 20 Kr. Similar results were obtained in rats. A sterile immunity developed in the animals that survived challenge as evidence by subinoculations of blood and of splenic and hepatic tissues into susceptible animals.

Further investigations were conducted to determine: (1) whether the marked protection obtained with the laboratory-adapted Wellcome strain of T. rhodesiense could also be obtained with a newly isolated human strain of this parasite; and (2) whether these experiments could be extended to include immunizations of monkeys.

The mice used in these studies were albino males, Walter Reed (ICR) BR strain, weighing 20-25 g. and the monkeys were Macaca mulatta of both sexes, weighing 7-12 lbs. at the beginning of the experiments. The strain of T. rhodesiense (EATRO #1886) was isolated from a human patient in Uganda and passaged once in a rat before it was frozen and shipped to our laboratory. In the second experiment with monkeys the trypanosomes were separated from rat blood by using a phosphate buffered saline-glucose (PSG) solution in a DEAE cellulose column. The parasites recovered from the column were washed twice by centrifugation in PSG solution and the numbers of trypanosomes were determined by electronic particle counts. In all the other experiments trypanosomes for immunization and challenge were collected from heavily parasitized donor mice and monkeys, counted with a hemacytometer and inoculated in whole blood.

The parasites were exposed to a 60 Kr dose in a Gammacell cobalt 60 irradiator. The immunizing and challenge inocula were given intraperitoneally to the mice and intravenously to the monkeys. During the immunization period and after challenge, wet blood smears from mice and monkeys were examined microscopically for trypanosomes. The blood of the experimental animals was subinoculated into susceptible mice in an effort to detect trypanosomes not seen on direct examination. The mice surviving challenge were observed for at least 30 days before they were considered to be free of infection; the monkeys were observed for 90 days after challenge.

The susceptibility of mice to the newly isolated T. rhodesiense strain (EATRO #1886) was determined by inoculating them with graded doses of 1×10^3 to 1×10^6 trypanosomes. High levels of parasitemia (up to 5×10^8 trypanosomes per ml of blood) were produced after 3 to 7 days. The parasites could no longer be detected in the peripheral blood of some of these animals in 8 to 10 days, after which parasitemia recurred. Unlike the Wellcome strain trypanosomes which invariably established a fulminating infection in mice resulting in 100% mortality within a week, those of the EATRO #1886 strain sometimes disappeared temporarily from the peripheral blood and did not produce fatal infections in all of the animals. However, parasites were seen intermittently in the blood of the survivors for 30 days or more after inoculation.

On the basis of these preliminary observations, 40 mice were given 3 inoculations at weekly intervals with either 2×10^6 or 2×10^7 irradiated T. rhodesiense (EATRO #1886) and challenged with unirradiated trypanosomes of the same strain. As indicated in Table 1, 30 mice received 3 immunizing inoculations (Group I), 10 mice received 2 immunizing inoculations (Group II) and 10 received no inoculations and were kept as untreated controls (Group III). All animals of the 3 groups were challenged one week after the last immunization. The results (Table 3) indicate that a marked protection to a challenging infection was induced in the immunized mice. Greater protection was observed in the animals receiving the higher total (4×10^7) immunizing dose even though it was given in 2 instead of 3 inoculations. The differences

eluates be performed to obviate this possible error, and to verify the presence of trypanosomes. A major source of variation in hemacytometer counts resides in the individual counting errors among various technicians. Human error can be precluded by the use of automatic equipment such as the Coulter counter, except for variations in the dilution procedures.

In addition to the greater sensitivity and reproducibility of the procedure, the particle count technique can be performed with a considerable saving of time in experiments involving clear suspensions of trypanosomes. Once the system is standardized, 5 background and 10 trypanosome counts should be made within 5 minutes by the use of the Coulter counter, whereas with the hemacytometer an initial 20 minute staining and settling period is required in addition to the time spent at the microsocope to perform the counts. This, for an experienced technician, amounts to 5 to 15 minutes for each count depending on the number of trypanosomes present.

The particle-count technique may have a variety of applications in laboratories in which a Coulter counter is available. Once the procedure is standardized for a laboratory there may be a number of uses in screening specimens from humans (blood donors in endemic areas) or for surveys of potential animal reservoirs. The efficiency of such procedures would depend on the numbers of specimens to be examined, the adaptability of the filtration process to simultaneous mass screening, and on specific confirmation of positives by microscopic examination of concentrated filtrates. In laboratory experiments in which clear suspensions are to be used, the Coulter particle-count technique appears to be both a convenient and reliable method of trypanosome quantitation.

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Further investigations were conducted to determine: (1) whether the marked protection obtained with the laboratory-adapted Wellcome strain of T. rhodesiense could also be obtained with a newly isolated human strain of this parasite; and (2) whether these experiments could be extended to include immunizations of monkeys.

between the percent of mice which remained parasite-free in the various groups are significant ($P < 0.05$).

Table 3

Development of Immunity in Mice Inoculated with Irradiated (60 Kr) Trypanosoma rhodesiense (EATRO #1886) and Challenged with 1×10^3 Unirradiated Organisms One Week After Immunization

Group No.	No. of Mice	Immunizing Inoculations: No. Doses	Immunizing Inoculations: No. Trypanosomes	Percent Parasite-free After Challenge*
I	30	3	2×10^6	40
II	10	2	2×10^7	90
III	10	0	0	0

*Differences between Groups I and II, I and III, and II and III significant at 95% confidence level (Chi-square test).

In view of these results obtained in mice immunized with a human strain of T. rhodesiense, the studies were extended to include subhuman primates. In a preliminary experiment to determine the susceptibility of monkeys to this parasite, the infection was passed 3 times (inoculating dose 1×10^6 trypanosomes) in a total of 11 rhesus monkeys. This newly isolated strain produced infections which invariably killed these animals. The one monkey in the first passage developed a peak parasitemia on the 24th day and died on the 47th day after inoculation. However, the infection became more acute in the next two passages and there was a decrease in the average survival time of the remaining 10 monkeys.

Two experiments were designed to determine whether subhuman primates develop an acquired immunity following inoculation with irradiated T. rhodesiense. In the first experiment, five rhesus monkeys were divided into two groups. Three of the animals (Nos. 611, 664 and 670) received six twice-weekly immunizations with inocula varying from 1×10^8 to 1×10^9 irradiated trypanosomes and the other two (Nos. 628 and 723) were inoculated with irradiated, unparasitized monkey blood in the same volume as used for immunization (Table 2). Three weeks after the last immunizing dose, all five monkeys were challenged with 1×10^4 unirradiated parasites. The two unimmunized controls which received irradiated, normal blood became patent five days later and died as a result of the infection 31 and 44 days after challenge. Two of the three immunized monkeys remained parasite-free after challenge for the period of observation (90 days). The third animal (No. 611) became patent in 13 days and died 75 days after challenge.

In the second experiment six monkeys were divided into two groups. Three of the animals (Nos. 506, 523 and 833) received six twice-weekly

immunizations with total inocula containing approximately the same number of trypanosomes used in the previous experiment, and the other three (Nos. 403, 484 and 505) were kept as unimmunized controls. All six monkeys were challenged with 1 x 10^4 unirradiated parasites three weeks after the last immunizing dose. The three unimmunized controls became patent in 5 to 8 days and all three died between 12 and 49 days after challenge. Conversely, all three immunized monkeys remained parasite free, as determined by blood examination and subinoculation into mice, for the duration of the experiment.

Table 4

Development of Immunity in Rhesus Monkeys Inoculated with Irradiated (60 Kr) Trypanosoma rhodesiense (EATRO #1886) and Challenged Three Weeks Later with 1 x 10^4 Unirradiated Trypanosomes

Exp. No.	Monkey No.	Immunizing Inoculations: No. Doses	Immunizing Inoculations: No. Trypanosomes	Prepatent Period (Days)	Survival Time (Days)
1	628	0	0	5.0	44
	723	0	0	5.0	31
	611	6	$1x10^8$ to $1.8x10^9$	13.0	75
	664	6	$1x10^8$ to $1.8x10^9$	*	>90
	670	6	$1x10^8$ to $1.8x10^9$	*	>90
2	403	0	0	8.0	12
	484	0	0	6.0	47
	505	0	0	5.0	49
	506	6	$2x10^8$ to $1.3x10^9$	*	>90
	523	6	$2x10^8$ to $1.3x10^9$	*	>90
	833	6	$2x10^8$ to $1.3x10^9$	*	>90

*No parasites were recovered from the blood at any time after challenge.

Detailed clinical, hematological and pathological observations which will be published elsewhere indicated that the immunized and challenged monkeys, with the single exception of No. 611 which became infected, had values similar to those of normal monkeys. Conversely, the unimmunized and challenged monkeys rapidly developed progressive anemia, thrombocytopenia, reticulocytosis and signs of progressive severe illness.

A marked acquired resistance to infection with a recently isolated strain of T. rhodesiense was produced in mice and monkeys by vaccination with irradiated trypanosomes of the same strain.

Until recently, successful immunization against African trypanosomiasis in domestic animals and man appeared to be an unlikely goal unless antigens which stimulate protective antibodies were isolated, purified, and characterized. Even in this improbable event, the well known characteristics of trypanosomes to develop antigenic variants with each relapse made it unreasonable to contemplate the possibility of active artificial immunization. Soon after the host responds to one antigenic variant, the parasite population usually evades the response by changing antigenic type. It is not yet clear whether the new variant arises from a mutant which is selected because its multiplication is not affected by the antibodies present, or whether individual trypanosomes throw off surface antigens and synthesize new antigens. Whatever the mechanisms for producing antigenic variation, it seems clearly established that the phenomenon allows the parasite to partially evade the host's immune response and initiate a chronic infection.

The possibility was considered that the strong acquired resistance to infection with T. rhodesiense produced in mice and rats by vaccination with irradiated trypanosomes of the Wellcome strain might have resulted from the inability of this strain to produce relapse variants after continuous blood passage in laboratory animals for many years. Therefore, another strain newly isolated from a human patient was used in the present experiments. The results indicate that protection from a challenging infection was also induced in mice with this strain, though to a lesser degree than was observed with the Wellcome strain. The encouraging results obtained in inducing a protective immune response in different experimental models by these two unrelated strains made it appear well worthwhile to continue this study in primates. The results reported here suggest that a marked protection can also be induced in monkeys by inoculating them with irradiated trypanosomes. Moreover, detailed clinical and laboratory observations indicated no signs of disease in the immunized animals resulting from either the immunization or challenge.

The mechanisms underlying variation and the potential number and chemical nature of variant antigens are unknown. It is possible, however, that exposing the host to a given variant may condition it for a rapid immune response to many other variants. This, in essence, would parallel the situation observed in malaria reflecting a quantitative relationship between the titer of protective antibodies and antigenic variants rather than a quantitative all or none reaction. This suggestion seems to be substantiated by the observation that the acquired resistance to infection developed after immunization with irradiated parasites is directly related to the number and size of immunizing inoculations. The results reported here are in agreement and extend those previously reported for trypanosomiasis and for malaria.

Since the animals used in our studies have all been challenged with homologous strains, the question of whether cross protection can also be obtained is unanswered. Studies designed to resolve this question are now underway.

3. Immunization of mice and cattle with gamma-irradiated Trypanosoma congolense.

Trypanosoma congolense is undoubtedly the most important trypanosome affecting ungulates in Africa. In West Africa trypanosomiasis in cattle is generally regarded as being a relatively mild chronic infection, but in East Africa T. congolense infection usually results in an acute, subacute or chronic infection leading to the death of the infected cattle. Virulent strains also produce high and sustained parasitemias in dogs, which may die within one to four weeks.

Killed vaccines against T. congolense in laboratory animals gave some protection against the homologous strain. Apart from the early experiments by Schilling, who was moderately successful in immunizing calves with killed trypanosomes, the only known attempts to vaccinate cattle are those recently made by Neal who used T. congolense antigens with adjuvants, but without great success. Soltys found that cattle repeatedly treated and maintained in areas endemic for T. congolense acquired a resistance to reinfection. Treated animals which were repeatedly challenged with virulent T. congolense were marginally more resistant than animals which received only the drug. However, since the drug alone in the absence of challenge had some lasting protective effect, it was difficult to interpret to what extent the resistance which developed was in fact due to an acquired immunity.

Following the observations that immunity is produced in rodents, cattle and monkeys against T. rhodesiense by immunization with gamma-irradiated blood forms, investigations were conducted to determine whether or not a similar technique would be effective in immunizing mice, dogs and cattle against T. congolense.

The Trans-mara I strain of T. congolense was used in all of these experiments. The parasites used for immunizing inoculations were exposed to 60 Kr doses in a Gammacell with a cobalt 60 source. The immunizing inoculations were given weekly to the mice and cattle and biweekly to the dogs. The number of trypanosomes inoculated was determined by counts made with a hemacytometer.

Mice. The mice used in these studies were albino males, Walter Reed (ICR) BR strain, weighing 20-25 g at the start of each experiment. Trypanosomes for irradiation were collected in blood from the axillary vessels of heavily infected donor mice. The whole blood containing the trypanosomes was irradiated, then diluted in Locke's solution to prepare a 0.2 ml inoculum of the desired number of parasites. Immunizing and challenge inoculations were made intraperitoneally. In addition to uninoculated mice used as challenge controls in all the experiments, a

separate group of 10 mice in Experiment 1 was inoculated with 0.2 ml irradiated, uninfected blood at the time the immunizations were given. When it was noted that the results of challenge of these mice were essentially the same as with the uninoculated groups, this additional control was not included in subsequent experiments.

Dogs. The dogs used in these studies were Beagles of both sexes, weighing between 8.5 and 11 kg. The trypanosomes were obtained from donor dogs and inoculated intravenously in whole blood.

Cattle. Herefords of both sexes, weighing from 400 to 900 lbs., were used in the first experiment which was conducted in Kenya. The trypanosomes were obtained from donor cattle and inoculated intravenously in whole blood.

Mixed local breed cattle of both sexes, 400 to 900 lbs., were used in the second experiment, also done in Kenya. The same procedures were used as in the first experiment except that the trypanosomes were obtained both from a donor steer and from rats. The rat blood was washed twice by centrifugation at 2000 rpm for 15 minutes to separate the trypanosomes from the blood. A mixture of phosphate buffered saline with 5% glucose and 10% calf serum at pH 7.8 was used in centrifuging and resuspending the parasites.

Holstein bull calves which weighed between 450 and 600 lbs. were used in two other experiments, conducted in Maryland, USA. The trypanosomes were collected from mice and inoculated in whole blood for the first two immunizations. Since inoculating rodent blood cells into the cattle produced anaphylaxis in some of the animals, it became necessary to administer diphenhydramine as a preventive measure. For the subsequent immunizations the trypanosomes were separated from the blood by centrifugation. Inoculations were given intravenously and intraperitoneally, the latter into the right paralumbar fossa. At the same time the immunizing inoculations were given, the calves subsequently used as challenge controls were inoculated with irradiated, uninfected blood in the same volume as the immunizing doses.

Fresh preparations of blood were examined microscopically for trypanosomes during the immunization period and after challenge. The blood of the experimental animals was also subinoculated into susceptible mice in an effort to detect any trypanosomes not seen on direct examination. The animals surviving challenge were observed for at least 30 days before they were considered to be free of infection.

Preliminary experiments were conducted to determine the effect of the Trans-mara I strain of T. congolense in mice. A single intraperitoneal dose of 1000 trypanosomes produced consistently high levels of parasitemia after a prepatent period of 3 to 6 days with a mean of 4.6 days. The parasitemia persisted in these animals for at least a 30 - 40 day observation period. Unlike the infections with T. rhodesiense (Wellcome strain) which invariably produced fulminating infections in

mice resulting in 100% mortality, infection with this strain of T. congolense did not kill all the mice and some of the animals were still alive 30 days after inoculation.

The first series of experiments was designed to determine whether mice inoculated with irradiated T. congolense would exhibit a demonstrable resistance to a challenge infection with unirradiated trypanosomes. As indicated in Table 5, 185 mice were used in 5 experiments and divided into 19 groups. All animals were challenged with 1000 unirradiated T. congolense 7 days after the last immunization except those of Group XIX which were challenged 72 days after immunization.

In the first experiment, 24 mice were divided into 3 groups. Four of 7 mice receiving four doses of irradiated T. congolense (Group III) remained parasite free after challenge. The mean prepatency in those that became infected was significantly longer than that of the unimmunized controls. Conversely, all of the mice which received irradiated parasite-free blood (Group II) and all of the uninoculated controls (Group I) became infected approximately 4 days after challenge. In a second experiment in which 40 mice were separated into 4 groups, greater numbers of trypanosomes were used for the immunizing inoculations (Groups V, VI, VII). Most of the inoculated mice remained parasite free after challenge, and the prepatency of those that became infected was significantly longer than that of the unimmunized controls (Group IV). The third and fourth experiments were essentially similar to the previous ones except that six immunizing doses were used in some groups (IX, X, XI, XVI, XVII). Between 50 and 100 per cent of the mice were free of parasites during a 30-day observation period after challenge. The unimmunized control animals invariably developed a parasitemia and more than half of them eventually died.

Since in all of the previous experiments, challenge was given 7 days after the last immunizing inoculations, a fifth experiment was designed to determine whether a greater interval between immunization and challenge might influence the degree of immunization. All 10 animals which received 4 immunizing inoculations and were challenged 73 days after the last immunization remained parasite free (Group XIX). Conversely, the 10 unimmunized controls became infected in an average prepatent time of 4.3 days and all but one died as a result of the infection.

Another experiment was designed to determine the effects of repeated challenges in mice which had been vaccinated with irradiated trypanosomes and challenged one week after the last immunizing inoculation. Some of the mice which had survived challenge with 1000 trypanosomes in the previous experiments were rechallenged 9 weeks after immunization and again 26 weeks after immunization. Forty-one mice were inoculated with unirradiated trypanosomes and used as infectivity controls. As summarized in Table 6, all of the animals which had not been immunized previously developed parasitemias. Conversely, most of the animals which had been immunized previously remained parasite free when challenged 1, 9 and 26 weeks after the last immunization.

Table 5

Development of Immunity in Mice Inoculated Weekly with Irradiated Trypanosoma congolense

Exp. No.	Group No.	No. Mice	Immunizing Inoculations: No. Doses	Immunizing Inoculations: No. Trypanosomes	Percent Parasitemia-free	Mean Prepatency (Days)	Percent Survival
1	I	7	0	0	0	4.3	43
	II	10	6	0	0	3.7	10
	III	7	4	2 x 10^6	53	7.3	71
2	IV	10	0	0	0	4.5	30
	V	10	4	2 x 10^6	70	8.7	100
	VI	10	4	1 x 10^7	80	6.5	100
	VII	10	4	2 x 10^7	80	8.0	100
3	VIII	10	0	0	0	3.7	30
	IX	10	6	2 x 10^6	50	6.6	60
	X	10	6	1 x 10^7	60	7.3	70
	XI	9	6	2 x 10^7	55	9.3	56
4	XII	10	0	0	0	4.3	10
	XIII	9	4	2 x 10^6	89	9.0	89
	XIV	10	4	1 x 10^7	90	9.0	100
	XV	10	4	2 x 10^7	100	-	100
	XVI	10	6	1 x 10^7	90	10.0	100
	XVII	10	6	2 x 10^7	78	10.0	100
5	XVIII	10	0	0	0	4.3	90
	XIX	13	4	1 x 10^7	100	-	100

Table 6

Rechallenge of Mice Immunized with Irradiated Trypanosoma congolense

No. Mice	No. Immunizing Inoculations	Time of Challenge (wks)	Time of Challenge (wks)	Time of Challenge (wks)	Percent Parasite-free	Mean Prepatency	Percent Survival
10	0	1	-	-	0	4.3	10
29	4	1	-	-	93	9.0	97
22	0	-	9	-	0	5.0	0
27	4	1	9	-	60	7.5	70
10	0	-	-	26	0	5.0	20
10	4	1	9	26	70	7.0	80

An experiment essentially similar to those conducted in mice was undertaken to determine whether dogs developed an acquired resistance by inoculation with irradiated T. congolense. Preliminary studies had shown that a single dose of 10,000 trypanosomes produced fulminating infections in Beagle dogs after a prepatent period of 4 to 6 days. All inoculated dogs died usually within 12 days. As indicated in Table 7, all four unimmunized controls developed parasitemias 5 days after a challenge with 8.5×10^4 unirradiated trypanosomes and died one week later. The immunized dogs (Group I) had a more prolonged prepatent period but eventually became infected and died at approximately the same time as the unimmunized controls.

Table 7

Development of Immunity in Dogs Inoculated Biweekly with Irradiated Trypanosoma congolense and Challenged with 8.5×10^4 Unirradiated T. congolense

Group No.	No. Dogs	Immunizing Inoculations: No. Doses	Immunizing Inoculations: No. Trypanosomes	Mean Prepatent Period (Days)	Mean Survival Time (Days)
I	4	5	1.15×10^{10}	8.0	12.2
II	4	0	0	5.0	12.0

Further investigations were conducted to determine whether or not a similar technique could be applied to the immunization of cattle against T. congolense. A series of 4 experiments was conducted. The first two experiments were done in Kabete, Kenya and two more were conducted in Maryland, U.S.A. As indicated in Table 8, only partial protection could be induced by inoculation with irradiated trypanosomes in T. congolense infections. This partial protection was manifested by delayed prepatency in all of the immunized animals. Eventually, however, all of the challenged cattle became infected and the levels of parasitemia in the immunized animals were comparable to those observed in the controls.

Although the possibility of producing effective immunization against trypanosomes has occupied the attention of investigators for more than fifty years, only limited success has been achieved in protecting experimental animals by inoculating them with killed trypanosomes and various adjuvants. Control of trypanosomiasis by immunoprophylaxis in domestic stock will continue to demand the application of recently developed immunological techniques. Immunization with live vaccines may be the most successful and practical method since this procedure requires relatively small numbers of trypanosomes without major changes in their nature. Attenuated live vaccines were used by Schilling in calves with partial success.

Table 8

Development of Immunity in Cattle Inoculated Weekly with Irradiated *Trypanosoma congolense* and Challenged with Unirradiated Trypanosomes Seven Days After Immunization

Exp. No.	Group No.	No. of Cattle	Immunizing Inoculations: No. Doses	Immunizing Inoculations: No. Trypanosomes	Challenge (No. trypanosomes)	Prepatency (Days)
1	I	3	0	0	1×10^{5}	5.7
	II	3	4	1×10^{6}	1×10^{5}	7.0
2	III	3	0	0	1×10^{4}	5.3
	IV	3	7	1×10^{9}	1×10^{4}	7.0
3	V	2	0	0	5×10^{4}	5.0
	VI	6	4	1×10^{9}	5×10^{4}	7.3
4	VII	1	0	0	5×10^{4}	6.0
	VIII	2	5	1×10^{9}	5×10^{4}	9.5

In view of these considerations and of the promising results obtained in rodents, cattle and monkeys by previous inoculations with irradiated *T. rhodesiense*, the possibility of producing effective immunization against *T. congolense* by this method was investigated. Although many of our mice were immunized successfully, the inability to produce effective immunization of dogs and cattle against *T. congolense* was disappointing. However, an increase in the prepatency was observed in all of the immunized animals. The degree of resistance conferred on animals by natural or artificial immunization is assessed by the outcome of the infection following a challenge with a known infective dose of trypanosomes. The results are usually determined in terms of the prolongation of the prepatent period, the reduction or delay of the height of parasitemia and the time of survival of the immunized animals as compared to the unimmunized controls. A severe challenge may overcome a weak immunity which would be sufficient to enable the test animal to withstand a milder challenging dose. Therefore, it is essential to relate the results of immunization in terms of the load of the challenging infection.

It seems likely that in our experiments involving infections of extended duration, antigenically distinct variants appeared in succession, each corresponding to a particular wave of parasitemia. In other words, the serum of immunized animals might have contained antibodies which were effective against the antigens of the immunizing inoculations, but ineffective against those yet to materialize. It is also possible that

the differences in resistance between immunized and unimmunized dogs and cattle in our experiments would have been much more apparent if we had used a smaller challenge dose. This possibility, as well as the potential role of cellular processes in the protection of the host against infection, is now under investigation.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-house Laboratory Independent Research

Work Unit 101 Immunoprophylaxis of protozoan infections

Literature Cited.

Publications:

1. Duxbury, R. E., Sadun, E. H., Wellde, B. T., Anderson, J. S. and Muriithi, I. E.: Immunization of cattle with X-irradiated African trypanosomes. Trans. Royal Soc. Trop. Med. & Hyg. 66:349-350, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6474	72 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. SPECIFI CONTRACT-	DATA-ACCESS	9. LEVEL OF SUM
71 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	102
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Enzyme Activity Measurements by Centrifugal Chemistry

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 11	72 06	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 71	10	250
FISCAL YEAR — CURRENT 72	10	250

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Enzyme Measurements; (U) Intermediary Metabolism

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

23. (U) The technical objective of this work unit is to employ centrifugal chemistry as a technology in the measurements of enzymes and their kinetics. The enzymes selected are specifically linked to problem areas of military medical importance.

24. (U) Utilizing a centrifugal chemistry unit, develop methodologies and apply the standardized methodologies to enzyme levels in physiological fluids.

25. (U) 71 07 - 72 06 During the reporting period, the centrifugal chemistry unit as supplied by the American Instruments Company has been evaluated extensively for its operational characteristics. A reported precision of + 0.001 absorbance units has been unattainable consistently in a variety of standard enzyme measurements. Sample handling problems tend to extend the time of analysis. The instrument is capable of handling any number of enzymatic tests and will find its greatest application in the research laboratory as opposed to the clinical laboratory. The system will be utilized in the definition of the effects of chemotherapeutic agents on enzyme rates of reaction in mechanism of action studies. These efforts will be consolidated under Biochemical Methodology and Laboratory Automation. For technical report, see the Walter Reed Army Institute of Research Annual Progress Report, 1 July 1971 - 30 June 1972.

*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 102 Enzyme Activity Measurements by Centrifugal Chemistry

Investigators:
Principal: COL Charles R. Angel, MSC
Associate: LTC Douglas J. Beach, MSC, Billy G. Bass, M.S.:
CPT Eric S. Lichtenstein, MC

Description.

The work unit is designed to evaluate enzyme methodologies utilizing centrifugal chemistry.

Progress.

The centrifugal chemistry machine as manufactured by American Instruments Company, Silver Spring, Maryland was originally intended to sustain a precision of plus or minus 0.001 absorbance units with a time averaging device. After detailed evaluation of common enzyme tests such as alkaline and acid phosphatase, glucose oxidase and lactic acid dehydrogenase, lack of consistency was established. Integration of a small mini-computer with the instrument failed to markedly improve the consistency of results. A detailed study of specific chemistries on a quantitative basis has been initiated in an attempt to bring the instrumental results in line with expected performance. The first test system studied was lactic acid dehydrogenase. Under ideal conditions with careful sample handling, a precision of plus or minus 2% was obtained.

It has become evident that centrifugal chemistry must be accomplished under the most rigid conditions. At the present time, integration of this instrumentation into the routine clinical laboratory is impossible and results would be totally discouraging.

Summary and Conclusions.

A centrifugal chemistry unit has been evaluated and studied for routine enzymatic determinations. The results have lacked the desired level of accuracy.

PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6475	72 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. ORD'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	103
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procode with Security Classification Code)

...ents in the Field Interfaced with Mini-Computers

12. ...TECHNOLOGICAL AREAS

002300 Biochemistry 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
70 11	72 06	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: 71	10	288
FISCAL YEAR CURRENT: 72	10	288

19. RESPONSIBLE DOD ORGANIZATION

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

RESPONSIBLE INDIVIDUAL
NAME: Buescher, COL E. L.
TELEPHONE: 202-576-3551

21. GENERAL USE
Foreign Intelligence Not Considered

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Biochemistry
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)
NAME: Angel, COL C. R.
TELEPHONE: 202-576-2211
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

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

22. KEYWORDS (Procode EACH with Security Classification Code)

(U) Biochemistry; (U) Clinical Chemistry; (U) Computer Interfacing; (U) Field Teams

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

23. (U) The technical objective of this work unit is to interface surplus DOD computers with automated biochemical systems to produce more timely information for the military physician.

24. (U) Mini-computers that have become surplus will be interfaced with standardized laboratory instruments such as wet chemical analysis equipment, gas chromatographs, UV and other laboratory analytical systems. Particular emphasis is placed upon the utilization of the developed systems for field use.

25. (U) 71 07 - 72 06 During the current reporting period, the applicability of two field mini-computer systems to analytical chemistry systems has been demonstrated as feasible. One, the Field Artillery Digital Automatic Computer (FADAC) after thorough documentation and interfacing can be utilized effectively with gas liquid chromatographs, amino acid analyzers and other slow response time instrumentation. Because of its size and configuration, its use would be limited to centralized laboratories although a telephone input system has been developed for use with the system. The other system, the D17 (Minute Man Missile Guidance System) has been interfaced and programmed to operate with wet chemical analysis equipment such as the AutoAnalyzer(R). This system may be applicable to small medical facilities not ordinarily afforded data processing capabilities. The developed systems will be used in the study of drug abuse methodology. The efforts under this work unit will be consolidated under the work unit entitled Biochemical Methddology and Laboratory Automation. For technical report, see the Walter Reed Army Institute of Research Annual Progress Report, 1 July 1971 - 30 June 1972.

*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 103 Biochemical Measurements in the Field Interfaced with Mini-Computers

Investigators.
Principal: COL Charles R. Angel, MSC
Associate: Billy G. Bass, M.S.; John I. Davis, B.S.; Helen Sing, M.S.

Description.

The work unit is designed to examine the use of the mini-computers as an aid to data reduction in either the analytical or clinical laboratory. The subject of the on line processor versus time sharing and their applications to the chemical laboratory has been critically examined. Necessary software to accomplish tasks is developed within the scope of the work unit.

Progress.

Specific tasks that have been addressed during the reporting period are listed below:

1. Commercial mini-computer linked systems.
2. Modification and adaptation of excess DOD computers.
3. The laboratory use of time shared systems.
4. Software development.

1. Commercial Mini-Computer Linked Systems.

The dedication of central processor facilities to analytical chemistry instrumentation has led to the development of the mini-computer for actual data reduction within the laboratory. During the current year, two systems have been tested and examined. The first is the centrifugal chemistry unit and the second is the Hewlett-Packard automated gas chromatograph. The two mini-computers have effectively reduced the data workload when used with existing software. Change in the system requires extensive effort and development of extensive algorithms. This can be above and beyond the technical capacity of usual laboratory personnel.

2. Modification and Adaptation of Excess DOD Computers.

Two excess DOD computers have been under study to determine the feasibility of utilizing these systems under field conditions. The field artillery digital automatic computer (FADAC) has been used principally to process gas liquid chromatographic data. In order to accomplish this application, it was necessary to develop an extensive algorithm. The system is rather large and cumbersome and can best function at a fixed location. In addition, the system has a relatively slow responsive time thus limiting the use to slow analytical instrumentation like the gas chromatograph. The other system, the D17 (minuteman guidance system) has been adapted to the wet chemical analysis as typified by the Technicon AutoAnalyzer™. The analytical system has been successfully adapted to the D17 and this application has been demonstrated. Extension of this system to routine clinical laboratory operations will require extensive software development.

3. The Laboratory Use of Time Shared Systems.

The use of time shared systems offers the best solution to most research institute applications. All systems that have been tested to date have available extensive libraries for general statistical use. Turn around time is gratifying and complex problems can be solved economically with minimal wait. The division has made extensive use of the Air Force system at Rome AFB, New York. Problems have arisen in unexplained termination of telephonic linkages and the loss of files that have been placed in disk storage. Considerable effort has been expended in using the teletype terminal linked to the CDC 3300 RESPOND system. Successful program runs have been minimal but it is anticipated that these difficulties will be overcome. The G.E. time shared system in BASIC language has been utilized to introduce incoming WRGH interns to the use of the computer in the biomedical field.

4. Software Development.

Software development constitutes the major problem area in extending the computer directly into the laboratory. During the reporting period, the major part of the effort has been spent in the development of software for every aspect of the program. Successful accomplishments in the area include: 1) development of the programmable calculator as a research tool; 2) development of computational methods for standard curve generation in both the FRAT and fluorescence assay technologies in the drug abuse methodology program; 3) statistical testing of inference data from varied experimental data.

Summary and Conclusions.

During the reporting period, mini computers have been evaluated, two excess DOD computers have been adapted to laboratory instruments, several time shared systems have been utilized and extensive software development has been undertaken.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA 0B 6476	72 07 01	DD-DR&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
71 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	104
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code): (U) Management of Primary Hypertension and Autonomic *Dysfunction* Using Operant Conditioning Techniques.

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 013400

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 11	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 72	02	60
FISCAL YEAR CURRENT 73	02	60

19. RESPONSIBLE DOD ORGANIZATION
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TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION
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NAME: Hegge, F.W., Ph.D.
TELEPHONE: 202-576-5257
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Robinson, MAJ M.G.
NAME: Sodetz, CPT F.J. DA

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with Security Classification Code): (U)Blood Pressure;(U)Operant Conditioning;(U)Human Volunteer; (U)Biofeedback;(U)Autonomic Dysfunction;(U)Psychosomatic Disease;(U)Primary Hypertension.

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

23. (U) Development of behavioral techniques for the outpatient management of primary hypertension in military personnel through appropriate application of existing principles of operant and respondent conditioning. The systematic exploration of the role of these principles in the pathogenesis of hypertension, autonomic dysfunction, and psychosomatic disease under conditions of military performance related stress.

24. (U) Existing knowledge of operant principles is applied to both normal and hypertensive individuals to effect reductions in blood pressure of sufficient duration to warrant development and standardization of a optimal procedure for management of primary hypertension in outpatients. Techniques are extended to include the modification of esophageal, gastric, and colonic motility for the management of psychosomatically based gastrointestinal disorders. Concurrently, studies in non-human primates are conducted to update operant technology, to explore potentially productive methods for treatment of patients, and to facilitate development of required bioinstrumentation.

25. (U) 71 07 - 72 06 The continued evaluation of pressure cuff based monitoring of blood pressure has demonstrated the unsuitability of this approach. Work is proceeding on the application of tetrapolar impedance techniques to the monitoring of peripheral vascular resistance. The continuous feedback afforded by this technique will be used in conjunction with periodic cuff pressure measurements to provide an adequate measurement system. Preliminary evaluation of solid state motility probes is under way. Significant progress has been made in the study of blood pressure control in non-human primates using chronically implanted catheters. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 71 - 30 JUN 72.

* 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 104 Management of primary hypertension and autonomic dysfunction using operant conditioning techniques.

Investigators.
Principal: Frederick W. Hegge, Ph.D.
Associate: MAJ Malcolm G. Robinson, MC; CPT Frank J. Sodetz, MSC

Description.

A rapidly expanding body of literature (cf Barber, et. al., 1971) demonstrates the feasibility of applying operant conditioning technologies to the management of primary hypertension, autonomic dysfunction, and psychosomatic disease. The basic research strategy of this work unit involves: a) the development of appropriate non-invasive techniques for monitoring physiological variables of interest; b) the establishment of conditioning protocols that will effect changes in physiological variables of sufficient magnitude to produce meaningful therapeutic affect; and c) the translation of laboratory procedures to techniques useful for clinical outpatient managment. Specific areas of current research include: 1) the application of tetrapolar impedance measurement to the continuous monitory of peripheral blood volume and flow; 2) the development of procedures based on the continuous modulation of sensory feedback as a programmed function of physiological output; and 3) the acquisition and assessment of a multitransducer, solid state motility probe suitable for the operant modification of esophageal and colonic motility.

Progress

1. Previous reported work dealt with attempts to design and construct an automated pressure cuff blood pressure monitoring system. This phase of the research program has been discontinued. Successful operant conditioning requires that there be close temporal contiguity between the response-to-be-conditioned and programmed reinforcing consequences. Cuff-based measurement systems cannot provide the continuous output necessary to meet this requirement. In addition, operation in the systolic mode produces unacceptable levels of discomfort in experimental subjects.

Recently, a self-balancing tetrapolar bioimpedance measuring system was acquired. Overcoming most of the past objections to impedance plethysmography systems, this unit permits continuous measurements of blood volumes with tissue volumes as large as a leg. The system is non-invasive, safe, and it produces no discomfort during long sessions. Preliminary evaluation has been completed.

2. New experimental procedures have been designed to maximize the effect of reinforcers on physiological behaviors. In the usual biofeedback experiment, a reinforcer acquires strength through the use of instructions, an unreliable procedure at best. The new procedure links the conditioned reinforcer for desired physiological change to a well-maintained overt operant behavior. In addition, the transfer function relating physiological output to the strength of the conditioned reinforcer has been made variable and non-linear to permit the shaping of particular levels of physiological output. The procedure is currently being implemented in hardware and initial experiments will be conducted early in FY 1973.

3. A multitransducer solid state motility probe has been acquired for the purpose of developing techniques for operantly modifying colonic motility. This unit has undergone preliminary evaluation and early thermal instability problems have been eliminated. The procedures described above for blood pressure conditioning will be modified to permit their application to the study of spastic colitis during FY 1973.

4. Concomitant modifications of cardiovascular variables and behavior. Several experiments are in progress directed at the effect of environmental manipulations, known to produce perturbations in on-going behavior, on cardiovascular dynamics. Primates have been instrumented for continuous long-term monitoring of arterial blood pressure and heart rate. Pavlovian conditioning procedures have been used to elicit cardiovascular changes. Because Pavlovian conditioning has been superimposed upon on-going operant performance it has been possible to simultaneously identify both disruptions in behavior and perturbations in cardiovascular dynamics. Reliable changes in arterial blood pressure up to 40 mm Hg as well as 20-70 BPM changes in heart rate have been produced by conditioning procedures alone. By manipulating the character of the on-going behavior, that is, the behavioral context in which the cardiovascular changes are elicited, it has been possible to modify the outcome of the cardiovascular conditioning. It has been possible to obtain increases and decreases in arterial blood pressure, both with and without changes in heart rate, simply by changing the on-going behavioral context. These data point up the complexity of the visceral changes that accompany overt behavior and represent an initial step toward an objective analysis of the interaction between these two broad classes of events.

5. Cardiovascular responses to acute behavioral stress and drug challenge following chronic elevation of arterial blood pressure maintained under behavioral control. A study has been undertaken in which four rhesus monkeys with arterial catheters are being run in a procedure requiring them to maintain a predetermined level of diastolic blood pressure in order to obtain food and water reward. The purpose of this study is to examine the response of the cardiovascular system to both acute behavioral stress, in this case a 72 hour avoidance session,

and to pharmacologic challenge by a number of drugs with specific actions on the cardiovascular system. Once the baseline response to stress and to pharmacologic challenge has been established the animals will be required to maintain successively higher levels of diastolic pressure in order to obtain food and water reward. At the end of a six-month period, during which elevated diastolic pressure will have been maintained for 12 hours each day, these animals will again be challenged by both drugs and acute behavioral stress. By comparing the response to these challenges, with the responses obtained before blood pressure conditioning was instituted, it may be possible to begin to define some of the changes in cardiovascular function which take place early in the etiology of hypertension.

Project 3AØ61101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task ØØ In-House Laboratory Independent Research

Work Unit 104 Management of primary hypertension and autonomic dysfunction using operant conditioning techniques

Literature Cited.

References:

1. Barber, Theodore, DiCara, Leo V., Kamiya, Joe, Miller, Neal E., Shapiro, David, Stoyva, Johann.: Biofeedback and Self-Control: Aldine Atherton, Chicago, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6477	72 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. DISB'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	62110A	3A061101A91C	00	107
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code): (U) Treatment of pain states arising from combat related peripheral nerve injuries by transcutaneous stimulation techniques (TST). (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 012900 Physiology 016200 Stress Physiology 013400 Psychology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 07	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 72	1.5	25
	CURRENT 73	1.5	25

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

PII Redacted

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
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ADDRESS: Washington, D.C. 20012
PRINCIPAL INVESTIGATOR
NAME: Jennings, CPT J.R.
TELEPHONE: 202-576-5257
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]
ASSOCIATE INVESTIGATORS
NAME: Hegge, F.W., Ph.D.
NAME: Hall, Stanley DA

21. GENERAL USE: Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Pain; (U) Peripheral Nerve Injury; (U) Psychophysiology; (U) Psychophysics; (U) Analgesics; (U) Human Volunteer (U) Combat

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS

23. (U) Peripheral nerve injury, frequently resulting from a high velocity missile, may result in causalgia - a state of local hypersensitivity causing chronic pain. Transcutaneous stimulation techniques may relieve this pain and other forms of currently intractable pain. The validity of this analgesic method and the nature of its physiological mechanism are being identified.

24. (U) Using pnychophysical and psychophysiological techniques, the amount of pain an individual experiences is measured before and after low level electrical transcutaneous stimulation. The individual's verbal reports of pain and his peripheral physiological reactions are both analyzed. Methods are adjusted to study both clinical (nerve injury) and normal volunteers. Methods are designed to insure that the volunteer experiences the minimum possible amount of pain and discomfort.

25. (U) 71 07 - 72 06 A study of 24 normal volunteers demonstrated the effectiveness of transcutaneous stimulation in reducing pain reports. Pain reports were significantly less in treated subjects than those with placebo treatments. Autonomic reactions were concomitantly reduced but not at a statistically significant level. The results further indicated that TST was bilaterally or centrally effective rather than ipsilaterally effective. Certain puzzling temporal effects emerged which are currently under stud For technical reports, see Walter Peed Army Institute of Research Annual Progress Report, 1 JUL 71 - 30 JUN 72.

*Available to contractors upon originator's approval.

DD FORM 1498 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

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 107 Treatment of pain states arising from combat related peripheral nerve injuries by transcutaneous stimulation techniques (TST)

Investigators.
Principal: CPT J. R. Jennings, MSC
Associate: Frederick W. Hegge, Ph.D.; Stanley Hall, M.A.

Description

The optimum clinical application of peripheral stimulation techniques for the treatment of pain depends on an understanding of the biological and psychological effects of the technique. In particular, information on the reliability over time and people, generality over pain states, and specificity to pain sensation is important. The initial successful application of the techniques was to causalgia patients. These patients reported up to four hours of pain relief following three minutes of stimulation (Meyer & Fields, in press). Current efforts are directed at modifying laboratory induced pain under conditions allowing an assessment of the specificity and reliability of the pain reduction. In other terms, a primary goal was to determine whether the pain reduction was due to the stimulation per se or due to a psychological placebo effect. Over time and a variety of clinical application, a stimulation exerting influence through psychological suggestion would prove *unreliable*.

Progress

The effectiveness of TST in reducing judgements of laboratory-induced pain and autonomic reactivity were demonstrated in an initial experiment (Krasnegor, Jennings, & Fields, 1972). After three minutes of stimulation, pain judgements in response to high but not low level thermal stimuli decreased. In other words the TST influenced reactions only to intense pain. A parallel decrease in galvanic skin response to the pain stimuli was found after TST. In short, this initial experiment demonstrated the feasibility of the laboratory investigation of TST.

A second experiment was designed to provide control over placebo effects, the influence of the order of collecting baseline and post-stimulation judgements, and finally to test the effect on a relatively large sample of normal, experimentally-naive, persons. This experiment has recently been completed. Two control-conditions were employed, first TST applied to the arm opposite the one used for induced pain, and second, a true placebo i.e. TST was applied without any electrical current applied. The first control serves as a placebo, if and only if,

the TST effect is confined ipsilaterally with the induced site. The theories of Melzack and Wall (1965) and the data of Higgins, Tursky, and Schwartz (1971) suggested such an ipsilateral effect. If TST was effective contralaterally a central or bilateral effect would be suggested.

Following TST, persons who are treated on either arm demonstrated less reported pain than those who received placebo TST. These results then suggested a central or bilateral locus of the effect. Training day judgements of persons in the treated group, however, tended to be lower than those of the control subjects. This fact prevents a clear interpretation of the drop in pain judgments. Skin conductance (galvanic skin response) results showed a parallel drop in reactivity due to TST, but this drop was not statistically significant. The skin conductance results showed the influence of order of stimulation and baseline conditions. These temporal effects are currently being investigated. To summarize, TST seems to be effective in reducing pain and seems to do so by a central or bilateral mechanism. Certain aspects of the influence to TST such as temporal influences are not yet well understood.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 107 Treatment of pain states arising from combat related peripheral nerve injuries by transcutaneous stimulation techniques (TST)

Literature Cited.

References:

1. Higgins, J.D., Tursky, B., & Schwartz, G.E.: Shock-elicited pain and its reduction by concurrent tactile stimulation. Science 172: 866-867, 1971.

2. Melzack, R., & Wall, P.D.: Pain mechanisms: A new theory. Science 150: 971-979, 1965.

Publications:

1. Krasnegor, N.A., Jennings, J.R., Orr, W.C., & Fields, H.L.: The after effects of peripheral nerve stimulation: Change in psychophysical judgements and autonomic reactions during the scaling of cutaneous pain. Psychonomic Science 26: 74-76, 1972.

2. Meyer, G.A., & Fields, H.L.: Causalgia treated by selected large fiber stimulation of peripheral nerves. Brain Pt. 1, Vol 95, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6456	72 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
71 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	109
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Nuclear-Cytoplasmic Transplantation

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: 71 08 EXPIRATION: 72 07
b. NUMBER: DADA-17-69-[illegible]-9182
c. TYPE: S.CT d. AMOUNT: 0
e. KIND OF AWARD: CONT f. CUM. AMT. 23,974

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 72	0.1	0
	CURRENT 73	0.1	0

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Noyes, H. E. PhD 1
NAME:

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 observations on nuclear transplantation of mammalian cells and to apply the technique to immunologic studies of diseases of military importance.

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) 71 07 - 72 06 It was determined that cytochalasin B (CB) inhibited chemotaxis of polymorphonuclear leukocytes at doses of 1-2 mcg/ml but enhanced chemotaxis irreversibly at 0.1 mcg/ml. Further studies on transport of small molecules showed that CB is a competitive inhibition of glucose, glucoseamine, thymidine, uridine, and adenosine transport but not of choline transport. Studies were initiated on the action of CB on cell membrane. Current emphasis is on determining the effects of drug on reaggregation of tumor cells as well as on the cell itself. This contract is expected to terminate on 31 July 1972. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1971-30 June 1972.

* 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: Howard E. Noyes, Ph.D.

Preliminary work at WRAIR confirmed observations by Carter (1967) that the drug cytochalasin B (CB) had two dramatic effects. First, at doses of 1 ug/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 ug/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. Three areas have been studied: 1) the mechanism of cytokinesis through the use of low doses of CB; 2) nuclear transfer through the use of CB in high doses; and 3) effect of CB on human polymorphonuclear leukocyte phagocytosis.

1. During this period the effects of Cytochalasin B (CB) on biological systems have been further extended to the directed movement of polymorphonuclear leukocytes (chemotaxis). CB inhibited chemotaxis at doses of 1-2 ug/ml but enhanced it irreversibly at doses of 0.1 ug/ml. In light of the known effects of the drug on microfilament structure, no easy explanation exists for this action, since at the higher doses microfilaments may be altered. However, the enhancement of chemotaxis is unlikely to be caused by a similar mechanism.

2. Further studies on transport of small molecules have shown that CB is a competitive inhibition of glucose, glucoseamine, thymidine, uridine, and adenosine transport but not of choline transport. This inhibition of glucose transport was the most effective known with a Ki of about 1 uM (glucose Km 1-2 mM). No intracellular inhibition of processes of phosphorylation, formation of UDP sugars on incorporation of precursors into macromolecules has been detected. Again the action of the drug on these processes has been shown to be rapidly reversible (within 2-4 minutes), and action at the surface only has been implied.

3. Further studies are now being directed at the action of the drug at the cell membrane. The effects of the drug on reaggregation of tumor cells as well as its effects on the cell itself are being studied. Preliminary data indicate that CB can produce isolated cell fragments

enclosed by plasma membrane (minicells). While such phenomenon are interesting in themselves such isolated fragments may be useful in the study of cytoplasmic events.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 109 Nuclear-cytoplasmic transplantation

Literature Cited.

References:

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

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

Publications:

1. Estensen, R. D.: Cytochalasin B I: Effects on cytokinesis of Novikoff hepatoma cells. Proc. Soc. Exp. Biol. & Med. 136: 1256, 1971.

2. Hamer, M. G., Sheridan, J. D., and Estensen, R. D.: Cytochalasin B II: Selective inhibition of cytokinesis in Xenopus laevis eggs. Proc. Soc. Exp. Biol. & Med. 136: 1158, 1971.

3. Davis, A. T., Estensen, R. D., and Quie, P. G.: Cytochalasin B III: Inhibition of human polymorphonuclear leukocyte phagocytosis. Proc. Soc. Exp. Biol. & Med. 137: 161, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6495	72 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
71 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	113
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

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

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 003500 Clinical Medicine 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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 72	2	65
FISCAL YEAR CURRENT 73	2	65

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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20. PERFORMING ORGANIZATION

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Washington, D. C. 20012

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Buescher, COL E. L. DA

NAME:

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

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

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

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

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

25. (U) 71 07 - 72 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 two manuscripts have been completed in draft form and a third manuscript is in preparation. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

*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 113 Effects 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.

Two manuscripts of the findings in this study have been prepared, one dealing with the changes in thyroid hormones, the other with changes in corticosteroids and catecholamines, both before and during respiratory illness. A third manuscript is in preparation, dealing with an abnormal tendency for extremely high or extremely low adrenal, thyroid, and gonadal hormone levels to occur during the pre-illness week in subjects destined to develop respiratory illness.

Summary and Conclusions.

Adrenal cortical, adrenal medullary, thyroid, and gonadal hormonal abnormalities were noted during the pre-illness week in recruits who subsequently developed acute Adenovirus IV respiratory illnesses. The causes and possible pathogenetic significance of these pre-illness hormonal changes remains to be evaluated in future studies.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6486	72 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
	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	126
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Development of Capillary Blood Flow Sensors (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
71 09	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 72	2.25	100
	CURRENT 73	2.25	100

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Khouri, E. M. DA

NAME: Bass, B. G.

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide EACH with Security Classification Code)

(U) Blood Flow; (U) Shock; (U) Trauma; (U) Capillary; (U) Washout

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 equipment for the measurement, separately and simultaneously, of capillary blood flow during chronic experiments in various organs, e.g., liver, kidney, heart, skeletal muscle, in conscious unanesthetized animals. Data derived from this study should be directly applicable to military patients undergoing surgery.

24. (U) Beta radiation detectors encapsulated in special configurations are implanted surgically in trained animals and are used to measure the rate of washout of radioactive gasses, thus providing an estimate of capillary blood flow rate. Diodes which are at least 10 times more sensitive than those used in previous studies have recently become commercially available.

25. (U) 71 09 - 72 06 Such a device has been implanted on the surface of the heart in one dog and performed satisfactorily for a period of 21 days, yielding low noise clearance curves in agreement with a precordial counter. In order to increase the longevity of the unit, a study has been made of temperature, mechanical stress, humidity, etc., on the diodes. The compatibility of various types of polymers such as epoxy resins, silastic rubber, polyvinyl chloride has also been determined. A prototype unit applying the latest information thus obtained is now under construction and evaluation. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*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 126, Development of capillary blood flow sensors

Investigators.
Principal: COL Ray A. Olsson, MC
Associate: Edward M. Khouri; Billy G. Bass

Description.

Development of equipment for the measurement, separately and simultaneously, of capillary blood flow during chronic experiments in various organs, e.g., liver, kidney, heart, skeletal muscle, in conscious unanesthetized animals.

Progress and Results.

Techniques have been developed for the encapsulation of the avalanche diode semiconductor beta ray detector, for use in the study of capillary blood flow by tissue clearance of isotope indicators, in chronic experiments. Such a device has been implanted on the surface of the heart in one dog and performed satisfactorily for a period of 21 days yielding low noise clearance curves in agreement with a precordial counter. In order to increase the longevity of the unit, a study has been made of the effects on the diodes of mechanical and environmental stresses and of various types of polymers. A prototype unit applying the latest information thus obtained is now under construction and evaluation.

Conclusions.

Experiments are underway to increase the longevity of a semiconductor beta ray detector.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6532	72 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
71 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

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

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 72	10	185
FISCAL YEAR CURRENT 73	10	185

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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TELEPHONE: 202-576-2211
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Beach, LTC D. J.
NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Toxicology; (U) Drugs of Abuse
(U) Automation; (U) Electron Spin Resonance (ESR); (U) Atomic Absorption;

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 continuously review and develop biochemical methodology for application to mass screening of military specimens.

24. (U) Automated, analytical instruments, data reduction equipment, ESR systems, and semi-automated manual techniques will be utilized to identify and quantitate various compounds for both developmental and research uses. Emphasis will be placed on fast analytical systems, on identification of a variety of compounds, and on feasibility of systems for general laboratory use. Efforts will be concentrated on compounds of interest in red cell metabolism, on enzyme analysis, on the fast identification of drugs of abuse, and utilization of automated analytical systems for data collection.

25. (U) 71 07 - 72 06 Analytical chemistry development as applied to drugs and other biochemical tests has continued. Major progress within this work unit has been centered around ESR, gas liquid chromatography and gas liquid chromatography-mass spectrometry. The effect of temperature and pH on ESR signals in the region of the FRAT test has been categorized. Variation in ambient temperature of +3°C can cause a reduction in signal peak height of 3%/°C. This effect could allow weakly positive urines to go undetected. The spin label on morphine is pH dependent being completely destroyed at pH 4.0 and below. Stability is achieved at neutral and alkaline pH's. A statistical program utilizing a programmable calculator has been completed, this program allows for the establishment of absolute sensitivities of any FRAT type assay. The screening tests for sickle cell hemoglobin have been modified to produce more rapid and less expensive tests. Electrophoretic separation of lipids and proteins with application to cardiovascular disease continues. A quality control program for drugs of abuse has been established. For technical report, see Walter Reed Army Institute of Research Annual Progress Report [illegible].

DD FORM 1 MAR 68 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 INHOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 170 Biochemical Methodology and Laboratory Automation

Investigators.
Principal: COL Charles R. Angel, MSC
Associate: LTC Douglas J. Beach, MSC; MAJ James B. Powell, MC; MAJ Charles H. Tripp, Jr., MSC; Edmund S. Copeland, PhD; Robert T. Lofberg, PhD; Jean Matusik, B.S.; John Davis, B.S.; Clarence R. Emery, B.S.; John Kintzios, B.S.

Description.

This work unit is primarily dedicated to biochemical methodology and the application of these methods to clinical chemistry. With the advent of drug abuse, comparative methodology is continuously evaluated to provide the best technology for drug detection in biological fluids. Automation of manual procedures are carried out whenever appropriate. Activities within the work unit for the reporting period are collected into clinical biochemical methodology and drug abuse methodology.

Progress.

1. Clinical Biochemical Methodology.

a. Automated clinical chemistry analyses.

The AutoAnalyzer™ system continues to be utilized throughout the institute on an as required basis with the requesting divisions providing technicians to carry out the desired procedures. Mobile cart modifications have been completed and extensive modification of semi-micro to micro procedures have been accomplished. Technical advice and co2trol of AutoAnalyzer™ procedures continues for the metabolic service on Ward 30, Walter Reed General Hospital.

b. Comparative methodology evaluations.

Accurate measurement of glucose in metabolic disorders continues to occupy a prominent place in clinical chemistry. During the reporting period, glucose oxidase and ortho-toluidine methods for estimating glucose have been compared with conventional methods. Results indicate the glucose oxidase and ortho-toluidine best estimate true glucose. Where the patient is uremic, glucose

oxidase has been found to be the only valid method for measuring glucose.

The measurement of 2,3-diphosphoglyceric acid is an important variable in a number of clinical disorders. A variety of methods exist for its estimation. Comparing these methods, it has been found that the most satisfactory and reliable method for measuring 2,3 DPG in erythrocytes is the estimation of DPG phosphatase activity of phosphoglycerate mutase. This procedure has been automated and operates efficiently.

The T-4 test has replaced the PbI for measuring thyroid function in many laboratories for reasons of greater specificity. The T-4 test entails the use of resin columns which have been prepacked and are commercially available. A study has been conducted to examine the use of laboratory packed columns versus commercially available ones. The results of this study indicate that laboratory columns have less variability from regeneration cycle to regeneration cycle and can, with careful handling, be used for extended periods of time. It is also evident that a 25% cost saving can be realized using laboratory packed columns.

c. Sickle cell anemia screening methods

Mass screening of the Negro population for sickle cell trait and the presence of sickle cell anemia has been a prominent effort during 1971-1972. Activities within the division during the year have been concerned with the modification of Itano's solubility test, modification of the sickledex™ test and the automation of a screening procedure for sickling hemoglobins. The results have been published or accepted for publication. Training in these technologies has also been given to interested groups within the D.C. area.

d. Sialic acid studies in meningococcal vaccines (collaboration with DCD&I)

In the development of meningococcal vaccines, it is evident that a sialic acid polymer is present as a part of the active material. Extensive evaluation of methodology to measure sialic acid has been carried out with the development of a satisfactory method that measures the desired constitutent. The procedure has been automated and is currently employed in following sialic acid content during various stages of vaccine purification.

e. Electrophoretic studies

The system developed within the division for measurement of proteins, lipids and isoenzymes by agarose gel electrophoresis continues to be employed in conjunction with Walter Reed General Hospital in the evaluation of lipidemias, cardiovascular disease and liver disease. A simple chromatographic method utilizing two dimensional separation has been developed for measuring neutral lipids in plasma and tissues.

f. Automated systems

The division has acquired a high pressure liquid chromatographic system for differential separation and an automated ion exchange chromatography system for measuring amino acids. Both systems have been installed and standardized. They are currently dedicated to the study of the influence of drugs of abuse upon major metabolic pathways.

2. Drug Abuse Methodology

a. Free radical assay technology

The efforts carried out during the reporting period have centralized themselves on the systematic examination of various factors that influence drug screening and confirmation. With the procurement of a free radical assay system and an E-9 electron spin resonance spectrometer, it has been possible to examine the free radical assay system in great detail.

(1) Effect of Temperature on ESR signal response

The effect of temperature during ESR observation was studied over the temperature range from 0-45°C under two conditions (1) slight antigen excess and (2) where 95% of the spin labeled morphine was in the free form. Three simultaneous temperature effects were found. In the 20-30°C range, free spin labeled morphine showed a peak height increase of 1%/°C. In the same temperature range with bound spin labeled morphine, the peak height increased 3%/°C. Higher temperatures result in the release of antibody bound morphine. The third effect of temperature deals with the rotational correlation time. In the 20-30°C temperature range, rotational correlation time did not decrease sufficiently to narrow the broad tightly bound spectrum into the magnetic field region of the FRAT line. If ambient temperature varies more than ±3°C, serious inaccuracies can arise particularly if the temperature were significantly lower when unknowns were run than

when standards were analyzed. These inaccuracies could lead to failures in detection near established threshold levels.

(2) Effect of pH on the stability of spin labeled morphine

At pH <4.0, spin labeled morphine is extremely unstable and the free radical is completely destroyed in 30 minutes. At pH 5.5-6.5, nitroxide radical concentration decreased about 5% in two hours. At neutral and alkaline pH's, no decay of spin label was observed after two hours.

b. Study of substances producing FRAT signal response

The mass screening of urine from a population allows little time in which to examine substances that produce a false positive response in the FRAT system. The following compounds have been evaluated:

(1) Codeine Phosphate

Codeine phosphate in prescription cough medicine (40 mg Codeine) was taken over a 12 hour period. The FRAT system responded at 12 hours and 24 hours giving 12.5 µg/ml and 5.0 µg/ml morphine equivalent. Thirty-six (36) hours after taking the last dose no evidence of response was found. Saliva samples were analyzed and presented no response.

(2) Valium

Valium at a dosage of 5 mg per day was taken and urine collected by aliquot over twenty-six hours. Twelve hours after taking the 5 mg dose a positive response equivalent of 0.8 µg/ml morphine was observed. This response is being further evaluated. This preliminary study suggests an age dependence since similar doses in young people do not produce the response.

(3) Chloroquine and Primaquine

The FRAT system does not give false positive responses to urine following the usual prophylactic dosage of chloroquine or primaquine.

(4) Lomotil

Twenty-five normal individuals given Lomotil over a period of three days gave no response in the FRAT system. This preparation is recommended over paregoric preparations for the treatment of diarrhea.

c. Stability of Drugs in Urine

Development of Quality Control for evaluating laboratory performance necessitates the addition of the compounds of interest to urine. Many questions arise as to the length of time that urine can stand without preservative before it is no longer valid as to the original concentration of the drug.

During the reporting period, extensive study has been made of the effect of storage on both physiological and spiked urine specimens. The results of these studies have indicated that urine samples can be maintained for periods of time up to six weeks without preservative. Within this time frame, there have been no failures to detect and confirm the presence of any drug added. The drugs that were used in these studies included morphine, codeine, amphetamine, metamphetamine, amobarbital, secobarbital and phenobarbital. It should be pointed out here that normal urine begins to age shortly after it has been delivered. Many mistakes are made in identifying materials that are products of normal urinary aging.

Summary and Conclusions

The work unit has as a fundamental objective the continuous extension and development of biochemical methodology for any problem of military medical interest. During the reporting period, emphasis has been placed upon the extension of drug abuse methodology and the critical examination of the many factors that tend to confuse results. Among the progress that has been accomplished are the following:

a. A series of comparative studies of methodology has been accomplished.

b. Methodology for the study of sickle cell anemia has been standardized and training has been given to interested agencies.

c. A detailed study of factors involved in the FRAT system has been carried out.

d. Stability studies on urine that are being used for quality control indicate that samples are stable for all classes of drugs for periods of time up to six weeks.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 070, Biochemical Methodology and Laboratory Automation

Literature Cited.

Publications.

1. Palmer, D. C., Kintzios, J. A., and Papadopoulos, N. M.: A simple separation of neutral lipid classes with *two*-two dimensional thin layer chromatography. J. Chrom. Sci. 10:107, 1972.

2. Powell, J. B., Djuh, Y.-Y.: A comparison of automated methods for glucose analysis in patients with uremia before and after dialysis. Am. J. Clin. Path. 56:1, 1971.

3. Powell, J. B., and Beach, D. J.: A modification of the "Sickledex" test for hemoglobin S. Clin. Chem. 17:1055, 1971.

4. Matusik, J. E., Powell, J. B. and Gregory, D. M.: Rapid solubility test for detection of hemoglobin S. Clin. Chem. 17: 1081, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6539	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. ORD'R NUMBER	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 07 01	D. Change	U	U	NA	NA	[X] 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 (Procede with Security Classification Code)

(U) Migratory Animal Pathological Survey

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

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

17. CONTRACT GRANT

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 72	3	29
FISCAL YEAR CURRENT 73	1	10

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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

ASSOCIATE INVESTIGATORS
NAME: DA
NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

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

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

23. (U) Study ectoparasites, hematozoa, and serum antibodies of migratory animals to assess the disease patterns of geographic areas of potential military importance not accessible for direct epidemiological observations.

24. (U) Major effort 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) 71 07 - 72 06 This has been an eight-year intensive study of the migration of birds of eastern Asia and of their ectoparasites and hematozoa. Collected data on file at the Applied Scientific Research Corporation of Thailand include band records for 1,200,000 birds representing 1200 species. More than 500 species of ectoparasites were collected, and 100,000 blood films were examined. At this time most of the analyses are completed, and the next year will be used to prepare not less than 5 publications based on these studies. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

* 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 172 Migratory animal pathological survey

Investigators.
Principal: H. ElliotMcClure, Ph.D.

This has been an eight year intensive study of the migration of birds in eastern Asia and of their ectoparasites and hematozoa. Collected data on file at the Applied Scientific Research Corporation of Thailand (ASRCT) include band-records for 1,200,000 birds representing 1200 species. Some 50,000 of these were captured more than once, and 5,000 have been recovered and reported by other than MAPS personnel. More than 500 species of ectoparasites were collected, and 100,000 blood films were examined from 200,000 individuals. By July 1972 most of the analyses and data collection will be completed. During the next year Dr. McClure plans to complete analyses of data as a basis for publication of the following:

a. The ectoparasites and their distribution of birds of eastern Asia.

b. Migration and survival among Asian birds.

c. Haematozoa and their distribution among Asian birds.

d. A monograph of the House Swallow in Asia.

e. Contribution to the bionomics of Asian birds (a symposium by the team leaders).

Starting 1 July 1972 this study will be funded by the USAMRDC from Project 3A061102B71P and is expected to terminate not later than 30 June 1973.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6440	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. USER'S INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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

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 NA

a. DATES/EFFECTIVE — EXPIRATION

b. NUMBER

c. TYPE — d. AMOUNT

e. KIND OF AWARD — f. CUR. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 72	3	60
CURRENT FISCAL YEAR 73	3	65

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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Washington, DC 20012

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TELEPHONE: 202-576-3758

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) 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. The goal is to develop vaccines against each of the antigenic types of meningococcus producing disease in military populations.

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) - 71 07 - 72 06 Continuing experience with group C meningococcal vaccine in Army training centers has shown that group C disease has decreased markedly and there has been no sharp outbreak of disease due to another serogroup. Over 300,000 recruits have been immunized since 1968. An Escherichia coli polysaccharide has been extracted and shown to be chemically and immunologically similar to group B meningococcal polysaccharide. The E. coli antigen was immunogenic in rabbits but not in eight human volunteers. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

* Available to contractors upon originator's approval

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 181 Development of a meningococcal immunizing agent

Investigators.
Principal: Malcolm S. Artenstein, M.D.
Associate: MAJ Dennis L. Kasper, MC; Brenda L. Brandt; MAJ Edmund C. Tramont, MC; CPT Richard L. Cohen, MSC; CPT Wendell D. Zollinger, MSC; SP5 Dennis O. Broud; SFC Adam D. Druzd; and SP5 Charles L. Pennington.

Description.

Meningococcal polysaccharide vaccines have been under investigation since their development by this laboratory in 1968. Group C vaccine has been proven safe and effective by large scale field tests. The continuing experience with production, serologic testing and clinical use of this vaccine will be described in this report. New data on response of children to varying doses of group C vaccine have been accrued. Studies with group B polysaccharide in man have been essentially negative in terms of immunogenicity. Experiments were, therefore, carried out to evaluate as a meningococcal vaccine an Escherichia coli polysaccharide which is similar immunologically to group B meningococcal polysaccharide.

Progress.

1. Continuing experience with group C meningococcal vaccine in basic training centers.

Immunization of Army recruits with group C vaccine was carried out in a cautious, stepwise fashion over a 20 month period in order to evaluate the possible emergence of other meningococcal serogroups as causes of epidemic disease. Vaccine was administered as a single pulse to an entire population of recruits (8-10,000 men) or as a single pulse followed by several weeks or months of vaccination of newly arrived recruits. These procedures appeared to interrupt outbreaks of group C disease without inducing the emergence of other serogroups as significant disease problems. Beginning in October 1971 group C vaccine was administered to all new recruits at all BCT posts. The routine use of group C vaccine in Army training centers during the past "season" has been accompanied by a very low incidence of meningococcal disease.

Reported (provisional) cases of meningococcal disease from eight BCT posts for FY 1972 (through 7 April) were 22 including three deaths. Of 15 strains of meningococci from active duty personnel received at WRAIR during the period, four were serogroup C and 11 were serogroup Y (Bo). Although the total number of group Y infections was not

greater than that seen in previous years, the increased proportion of Y cases will bear watching. There was no report of group C disease in an individual who had received the vaccine.

Group C vaccine has been administered to more than 300,000 young adult Army recruits since 1968 with no evidence of toxicity. Occasional reports of reactions to the vaccine have not been substantiated. Even though the sharp decrease in all meningococcal disease in the past year cannot be attributed to vaccination alone, routine year-round use of the group C vaccine has been instituted at all Army BCT posts. Although emergence of other meningococcal serogroups as causes of epidemic disease has not occurred, rapid case reporting and identification of the causative serogroup will remain a necessary requisite for epidemiological assessment of vaccine effectiveness.

2. Human immunogenicity of new lots of group C polysaccharide vaccines.

(These studies were performed in collaboration with COL John Einarson, MC, Preventive Medicine Activity, Fort Ord, CA and LTC Creed Smith, MSC, Microbiology Department, Sixth USA Medical Laboratory, Fort Baker, CA.)

During the reporting period Merck Institute for Therapeutic Research, under contract with USAMR&D Command, produced eight lots of vaccine totalling 413,000 human doses. Immunogenicity was determined in recruits at Fort Ord, CA using 50 microgram subcutaneous injections with serologic tests (passive hemagglutination) performed on sera obtained prior to and two weeks post-vaccination. Results are shown in Table 1.

These data show that all new lots of group C vaccine appear to be equally immunogenic. Further analysis of two older lots (Squibb) and three new lots (Merck) using the more quantitative radioactive antigen binding assay (C-ABA) is shown in Table 2. Because of the large variance in individual titers, the geometric mean titer differences are not considered to be significant. Many of these lots have already been used in routine recruit vaccination with apparent success.

3. Responses of children to meningococcal polysaccharide vaccines.

(Study performed in collaboration with Dr. Arnold Monto, University of Michigan School of Public Health, Ann Arbor, MI.)

Preliminary studies had suggested that the antibody response of children was quantitatively different from that of recruits who had received the same dose. Therefore, a dose response titration of group C vaccine was carried out in 102 children, age two-six years.

Table 1. Immunogenicity of group C meningococcal polysaccharide vaccines in man.

Test*	Vaccine preparation	Geometric mean serum hemagglutination titer (log 2) at indicated week		Mean titer change	No. with 4-fold or greater rise of no. tested
		0	2		
1.	C-9 Squibb	2.03	6.94	4.91	34/35
	Merck 419 (C-A258)	1.54	7.16	5.62	36/37
	Merck 420 (C-A259)	1.66	7.38	5.72	32/32
	Merck 422 (C-A261)	1.36	6.47	5.11	34/36
2.	C-9 Squibb	0.33	4.22	3.89	42/45
	Merck 421 (C-A260)	0.30	4.70	4.40	43/43
	Merck 423 (C-A262)	0.32	4.30	3.98	38/43
	Merck 424 (C-A263)	0.79	4.41	3.62	45/46
	Merck 426 (C-A265)	0.34	4.49	4.15	42/47
	Merck 438 (C-A406)	0.26	4.77	4.51	42/44

* Test 1 performed at WRAIR; Test 2 performed at Sixth USAML.

Table 2. Radioactive antigen binding assay results following group C polysaccharide vaccination.

Vaccine preparation	No. men tested	Geometric mean C-ABA (nanograms bound)		
		0 week	2 week	6 week
Squibb C-7 (Fort Lewis)	25	2.86	302.9	
Squibb C-9	25	8.57	451.6	374.6
Merck 419	25	3.14	260.2	151.1
Merck 420	25	4.87	417.1	302.6
Merck 422	25	3.84	339.6	259.8

Informed consent was obtained from parents of these children who resided in state homes for mentally retarded children. Dosages of 5, 25, 50, 100 and 250 micrograms of group C vaccine were administered to groups of children. Antibody response to each dose level appeared to be directly related to age; 100 and 250 mcg gave higher serum antibody responses than the lower doses, but even these high dose responses in children were significantly lower than the geometric mean antibody (GMT) response of recruits given 50 mcg doses. The 5, 25 and 100 mcg dose groups received booster inoculations of 50 mcg eight months after the primary. In each group GMT following booster inoculation was significantly greater than the primary. All children responded to group A vaccine (50 mcg) given at the same time as the group C booster. Figure 1 shows in graphic form the antibody responses to varying doses and booster of group C vaccine.

During these studies comparison of hemagglutination (HA) tests with radioactive antigen binding assay (RABA) showed the latter test to be much more sensitive and quantitative than the HA and thus the RABA is the serologic test best suited for studies in children.

4. Studies of group B polysaccharide vaccines.

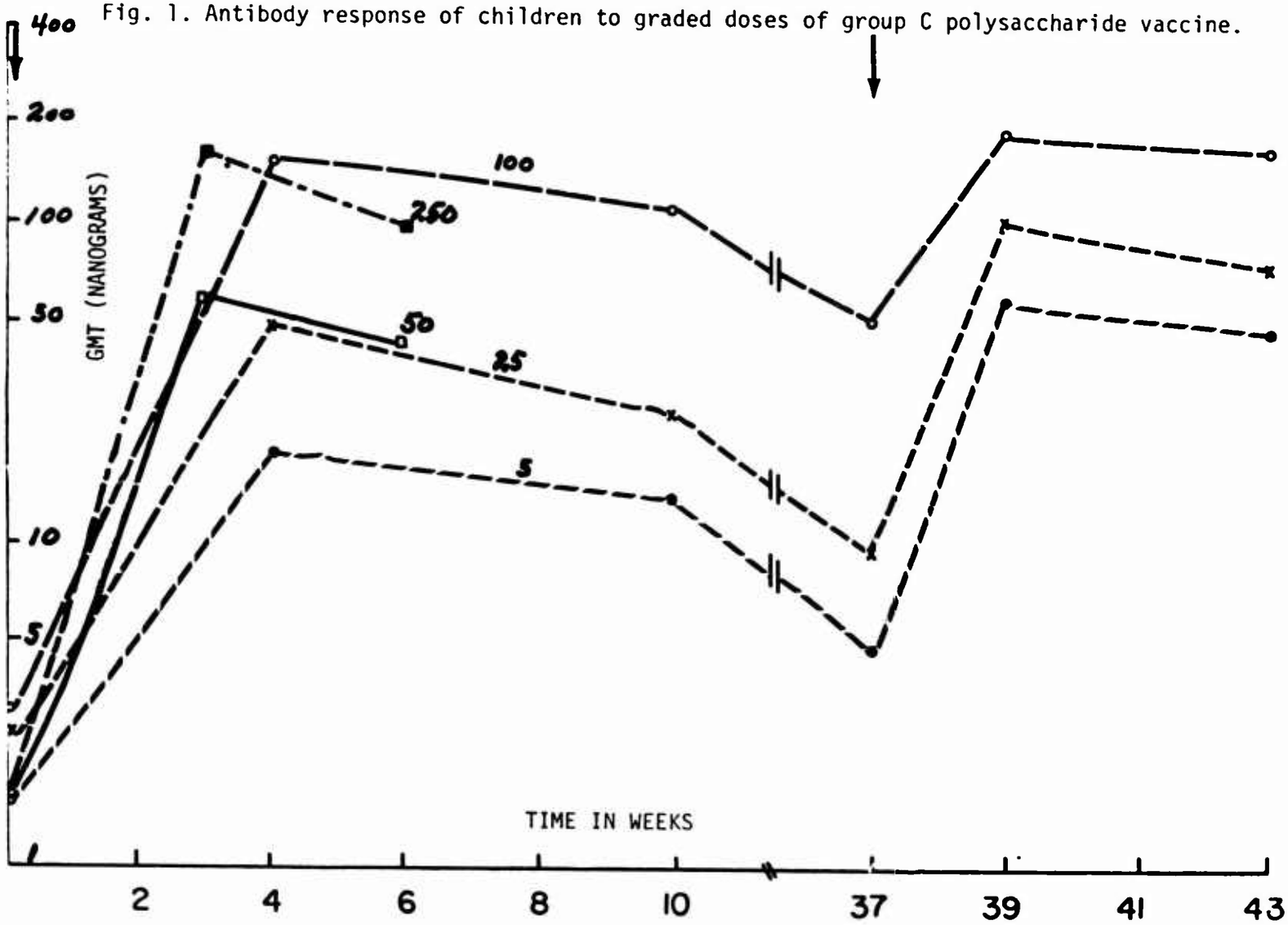
Polysaccharide vaccines prepared from group B meningococci were tested in 620 human volunteers. Antibody responses were tested by five different assays. Titration of one vaccine (10, 50, 100 and 250 microgram doses) showed only one confirmed antipolysaccharide antibody rise in 51 men tested. Another 50 volunteers who had received vaccines of different molecular sizes failed to develop significant antibody responses. Eight volunteers who received the largest molecular size polysaccharide also showed no antibody response; three of these men also failed to respond to a booster injection. Only one individual of four who received a combined influenza virus - meningococcal group B vaccine developed significant increase in serum antipolysaccharide titer. Failure of the group B polysaccharide to stimulate antibody in all but a few individuals may be an intrinsic deficiency of the vaccines or may represent an example of immunological tolerance.

These results plus in vitro studies of bactericidal activity of purified antipolysaccharide antibodies (which killed less than 50% of group B strains tested) have led to the exploration of alternative approaches to a group B vaccine.

5. Studies of an Escherichia coli polysaccharide which is immunologically similar to group B meningococcal polysaccharide.

Attempts to prepare a group B meningococcal vaccine from purified polysaccharides of this organism have failed to produce an immunogenic preparation. As an alternative approach a polysaccharide was

Fig. 1. Antibody response of children to graded doses of group C polysaccharide vaccine.



prepared from E. coli O7:K1(L)NM because of the known cross reaction of this strain with the group B meningococcus.

Tests at WRAIR have confirmed that the O7:K1(L)NM strain of E. coli does indeed cross react and the relevant antigen is the polysaccharide K antigen (Table 3). When extracted by the same procedures used for preparation of meningococcal polysaccharides the E. coli K antigen is recovered in highly purified high molecular weight form and is characterized by a high sialic acid content like the group B meningococcal antigen.

The K polysaccharide is more active immunologically, however, in that it induces antibodies in rabbits, a property not characteristic of the meningococcal polysaccharides. Seven of seven rabbits injected intravenously developed titers of hemagglutination ranging from 1:8 to 1:64. In addition, these anti-K antibodies have been shown to have bactericidal activity against the prototype group B meningococcal strain. One lot of vaccine (EC-1) was prepared for human use. After AIDRB approval, four laboratory volunteers were immunized with a 50 microgram dose administered subcutaneously. No local or systemic reactions were observed. Antibody studies were carried out on sera collected at intervals following the injection. Results are seen in Table 4.

Because of these negative antibody responses with the new lot EC-1, yet with previous positive (although weak) rabbit responses with the original purified E. coli antigen, the latter material was removed from frozen storage and processed into a vaccine for human use. No physico-chemical differences were detected between the two lots on the basis of Sepharose 4B chromatography.

EC-1 and EC-2 were then inoculated into rabbits by the following schedule: 50 micrograms IV three times weekly for two weeks, then tested one week later for antibody.

Results of antibody tests are shown in Table 5.

Three of four rabbits which received EC-2 developed anti-meningococcal group B HA antibody. Only one of four rabbits given EC-1 showed antibody increase and this titer was only 1:8 in B-HA test. These results suggested that EC-2 may be more immunogenic than EC-1.

Thus, Lot EC-2 was given to six volunteers subcutaneously in a 50 µg dose. Two of these volunteers had already received EC-1.(WZ,KR). None of this group responded to EC-2 by HA, RABA or RBT assays as seen in Table 6.

Table 3. Serological characteristics of E. coli K polysaccharide.

A. Serogrouping

Whole organisms	Meningococcal serogrouping antisera				
	A	B	C	Y	29E
E. coli 07:K1(L)NM	-	+	-	-	-
group B meningococcus	-	+	-	-	-

B. Hemagglutination inhibition

Purified antigen	No. tubes inhibition (Log 2) of meningococcal antiserum	
	B	C
E. coli K antigen	9	0
Meningococcus B_2 (100γ)	6	0
Meningococcus C pool (100γ)	0	≥11

Table 4. Antibody response following E. coli polysaccharide (EC-1) vaccination.

Volunteer	Week	B-ABA*	B-Cidal**
DK	0	3.8	14.8
	1/2	5.5	
	1	3.3	
	2	4.9	13.9
	4	4.3	13.6
MSA	0	6.4	24.8
	1/2	6.1	
	1	7.3	
	2	5.4	21.4
	4	5.5	25.8
KR	0	4.3	0
	1	4.2	
	2	5.8	0
	4	5.5	0
WZ	0	7.6	27.5
	1	6.8	
	2	9.7	28.1
	4	10.1	32.9

* Radioactive antigen binding assay using meningococcal B antigen. Results expressed as nanograms antigen bound by undiluted serum.

** Radioactive bactericidal test using ^{14}C labelled group B meningococci. Results expressed as net percent release of radioactivity over control values.

Table 5. Antibody tests on rabbits immunized with lots EC-1 and EC-2 polysaccharides.

Vaccine	Rabbit No.	B-ABA*		B-HA**	
		Pre	Post	Pre	Post
EC-1	542	3.9	4.9		
	546	2.8	3.6		
	97			<2	<2
	104			<2	8
EC-2	95			2	32
	88			2	2
	93			<4	16
	102			<4	16

* Nanograms radioactive antigen bound.

** Reciprocal of serum dilution.

Table 6. Antibody response to EC-2 vaccine.

Volunteer	HA*			RABA-B**			RBT-B***		
	Pre	2 wk	4 wk	Pre	2 wk	4 wk	Pre	2 wk	4 wk
WZ	32	32	16	1.2	2.1	3.6	12.5	14.3	16.2
KR	8	16	16	3.0	0.8	2.4	0	0	0
DB	32	32	16	1.6	1.9	2.6	14.1	17.7	18.3
JW	16	32	16	1.3	0.3	1.9	0	0	0
WB	8	16	16	0.9	1.1	1.6	0	0	0
RB	16	32	16	2.2	2.1	3.2	0	0	0

* Antigen EC-1 used to sensitize erythrocytes; titer expressed as reciprocal of serum dilution.

** Nanograms B polysaccharide bound.

*** Net % release over control values.

Thus, despite its immunogenicity in rabbits the E. coli polysaccharide gave no antibody response in human volunteers.

These results suggest that the lack of immunogenicity of the meningococcal B polysaccharide may be a complex immunological phenomenon related to immunological tolerance in man. An alternative explanation, however, would be that these polysaccharides are being broken down in the subcutaneous tissues to a smaller inactive form.

The search for a group B meningococcal vaccine has been influenced by these failures to develop an immunogenic polysaccharide. However, other studies in this laboratory have shown that anti group B polysaccharide antibodies have a very limited spectrum of bactericidal activity in that less than 50% of B strains tested were killed. Therefore, anti-B polysaccharide antibodies even if they could be induced might fail to provide satisfactory protection. New studies have been initiated to examine protein antigens of the meningococcus as vaccine candidates.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 181 Development of a meningococcal immunizing agent

Literature Cited.

Publications:

1. Artenstein, M. S., Brandt, B. L., Tramont, E. C., Branche, W. C., Jr., Fleet, H. D., and Cohen, R. L.: Serologic studies of meningococcal infection and polysaccharide vaccination. J. Infect. Dis. 124:277-288, 1971.

2. Artenstein, M. S.: Polysaccharide vaccines against meningococcal infections. In Proceedings, International Conference on the Application of Vaccines against Viral, Rickettsial, and Bacterial Diseases of Man, 14-18 Dec 1970. Scientific Publication No. 226. Pan American Health Organization, Wash., D. C., 1971, pgs. 350-352.

3. Artenstein, M. S.: The prevention of meningococcal infections: vaccine. In Changing Patterns of Bacterial Infections and Antibiotic Therapy. H. C. Neu, editor. Excerpta Medica 1971, pgs. 77-81.

4. Artenstein, M. S., Schneider, H., and Tingley, M. D.: Meningococcal infections. 1. Prevalence of serogroups causing disease in U. S. Army personnel in 1964-1970. Bull. Wld. Hlth. Org. 45:275-278, 1971.

5. Gold, R. and Artenstein, M. S.: Meningococcal infections. 2. Field trial of group C meningococcal polysaccharide vaccine in 1969-70. Bull. Wld. Hlth. Org. 45:279-282, 1971.

6. Artenstein, M. S., Branche, W. C., Jr., Zimmerly, J. G., Cohen, R. L., Tramont, E. C., Kasper, D. L., and Harkins, C.: Meningococcal infections. 3. Studies of group A polysaccharide vaccines. Bull. Wld. Hlth. Org. 45:283-386, 1971.

7. Artenstein, M. S.: Meningococcal infections. 4. Stability of group A and group C polysaccharide vaccines. Bull. Wld. Hlth. Org. 45:287-290, 1971.

8. Artenstein, M. S.: Meningococcal infections. 5. Duration of polysaccharide vaccine induced antibody. Bull. Wld. Hlth. Org. 45:291-293, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA OB 6443	72 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. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	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	71 08	DA	B. Contract

17. CONTRACT GRANT
a. DATES/EFFECTIVE 70 07 EXPIRATION 71 08
b. NUMBER DADA17-69-C-9014
c. TYPE S.CT d. AMOUNT: 8,000
e. KIND OF AWARD EXT f. CUM. AMT. 141,016

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	0.1	15
	CURRENT 72	0.3	8

19. RESPONSIBLE DOD ORGANIZATION
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ASSOCIATE INVESTIGATORS Rose, R. M., MD
NAME:
NAME: 1

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 infuence on success of task performance and aggressive behavior. The relationship between hormone secretions and performance success, breakdown, and interactions between groups will be determined.

25. (U) 71 07 - 71 08 Effective 1 August 1971 this contract was transferred from 91C, In-House Laboratory Independent Research to 71R, Research in Biomedical Sciences.

*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

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6444	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. GROUP MBR'S	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	183
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Provide 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 72	6	120
	CURRENT 73	6	140

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Binn, Dr. L. N. OA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered.

22. KEYWORDS (Provide EACH with Security Classification Code) (U) Military Dogs; (U) Tropical Canine Diseases; (U) Tropical Canine Pancytopenia; (U) Ehrlichia canis; (U) Babesia Trypanosoma

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

23. (U) To define, study, diagnose and control known and potential infectious diseases of military dogs in Southeast Asia and other areas of potential military significance. 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 medical problems in military dogs.

24. (U) Conventional methods are employed for epidemiological, pathological and microbiological examinations, and new procedures are developed as needed.

25. (U) 71 07 - 72 06 TCP, a hemorrhagic disease caused by Ehrlichia canis has been responsible for the death of approximately 300 US military dogs in Southeast Asia. Studies on Ehrlichia canis have continued. The red fox, Vulpes fulva, and the gray fox, Urocyon cinereoargenteus, were successfully infected with Ehrlichia canis. Transtadial transmission of Ehrlichia canis from an infected fox to a laboratory dog by the tick, Rhipicephalus sanguineus, was demonstrated. The fate of DF32 P-labeled platelets was examined in normal and thrombocytopenic Ehrlichia canis infected dogs. It was found that platelet survival was shortened during the febrile phase of the infection. An indirect fluorescent antibody test for detection and titration of antibody to Ehrlichia canis was developed. Studies have been initiated to determine the value of tetracycline treatment in dogs during the chronic phase of TCP. Neorickettsia helminthoeca, the causative agent of Salmon poisoning, has been serially passed in monocyte cultures. Electron microscopic studies of Neorickettsia helminthoeca are currently in progress. As of 1 Jul 72 these studies will be conducted in support of the Biological Sensor System Program. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

(Available to contractors 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 183 Diseases of military animals in Southeast Asia

Investigators.

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Description.

To define, study, diagnose and control known and potential infectious diseases of military dogs in Southeast Asia and other areas of potential military significance. 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 Southeast Asia. Studies are also being conducted on the epidemiology, treatment and control of Babesia and other parasitic infections which are medical problems in military dogs.

During the reporting period research activities have been concerned with: (1) the development of an indirect fluorescent antibody test for the serologic diagnosis of TCP; (2) pathogenicity of Ehrlichia canis in red and gray foxes; (3) platelet survival in TCP infected dogs; (4) therapeutic value of tetracycline in dogs with chronic tropical canine pancytopenia; and (5) comparison of Ehrlichia canis and Neorickettsia helminthoeca. Some of the investigations reported here have been done jointly with Dr. Miodrag Ristic and other investigators at the University of Illinois.

Progress.

1. Development of an Indirect Fluorescent Antibody Test for Tropical Canine Pancytopenia.

Tropical canine pancytopenia (TCP) is a tick-borne rickettsial disease of dogs caused by Ehrlichia canis (1,2). The organism was first recovered from dogs in Algeria in 1935. Infections with E. canis are now known to occur in various parts of the world, including the United States (3,4,5). The disease has been responsible for the death of approximately 300 U. S. military dogs in Southeast, Asia. The acute form of the disease is characterized by pancytopenia, particularly thrombocytopenia, anorexia, emaciation, dehydration, and fever. Epistaxis is a terminal manifestation of the disease. In acutely infected animals the organism can be detected in the cytoplasm of circulating monocytes as an inclusion body referred to as a morula. The dogs which recover remain carriers of the infection but the organism usually cannot be demonstrated in films of peripheral blood. These animals may serve as a source of the infection for susceptible dogs in areas in which a suitable arthropod vector exists. The need for a serologic test which can be used for detection of E. canis antibody has been emphasized by various investigators. However, the lack of a method for production of ehrlichial antigen has hampered development of such a test.

Recently Nyindo et al. (6) developed a method for propagation of E. canis in monocyte cell cultures and demonstrated an antibody to the organism in blood serum of infected dogs. The method provided a means whereby the antigen of this organism can be produced in quantitites needed for development of serodiagnostic procedures for TCP. This has permitted the development of an indirect fluorescent antibody (IFA) test for detection and titration of antibodies to E. canis. The test is specific and applicable to diagnosis experimentally and naturally induced TCP.

The organism used in this study was recovered in 1969 from blood of a German Shepherd dog with signs of TCP in Florida (4). Infected monocyte cell cultures grown in 3 oz. plastic flasks for 10 to 14 days according to the method of Nyindo et al. (6) were used as the source of antigen. Approximately 25 to 40% of the cells in these cultures were infected with E. canis. The fluid portion of the culture was removed and to each flask 2 ml. of 0.02% ethylene diaminetetraacetate (EDTA, tetrasodium salt) in Ca++ and Mg++ free phosphate buffered saline, pH 7.2, was added. The flask was agitated on an electric rotator (Fisher Scientific Co.) at 170 oscillations per minute at 37°C for 40 minutes. The content was poured into a 5 ml. conical glass tube and centrifuged at 206 x g

for 5 minutes. The supernatant fluid was replaced with distilled water containing 1.75% bovine serum albumin (BSA) fraction V (Armour Pharmaceutical Co.), cells gently brought into suspension, and the tube centrifuged as above. The washing procedure was repeated, 0.25 ml. of 1.75% BSA solution added, and final cell suspension (antigen) prepared. The suspension was placed on the slide in 6 spots of 0.025 ml. volume arranged in 2 rows. The antigen spots were dried at 37°C for 1 hour, the slides separated by porous paper, wrapped in aluminum foil, and stored at -65°C.

Antiserum was produced in 2 healthy rabbits inoculated with normal dog gamma globulin prepared by precipitation with ammonium sulfate in accordance with the method described by Goldman (7).

The gamma globulin was extracted from the immune rabbit serum by precipitation with 15% (w/v) sodium sulfate solution. The precipitate was dissolved in a volume of 0.15 M sodium chloride solution equal to 20% of the original serum volume and dialyzed overnight against 0.175 M sodium phosphate buffer (pH 6.3) at 3°C. The dialyzed protein was separated in diethylaminoethyl (DEAE) cellulose equilibrated in 0.0175 M phosphate buffer (pH 6.3). The protein concentration was adjusted to 10 mg./1 ml. and labeled with fluorescein isothiocyanate (0.033 mg./mg. of protein) dissolved in 0.5 M carbonate buffer solution (pH 9.5). The mixture was stirred at 22°C for 2 hours and then passed through a Sephadex G-25 column (Pharmacia Fine Chemicals) to remove free fluorescein isothiocyanate. The conjugate was then absorbed for 30 minutes at 22°C with lyophilized bovine spleen powder in a ratio of 1 ml. of conjugate to 10.0 mg. of the powder. The conjugate was stored in aliquots of 0.25 ml. at -65°C.

Test sera consisted of: (a) Time sequence sera collected from experimentally infected dogs beginning with the day of infection and during periods from 3 to 18 months thereafter. (b) Single serum samples collected from 61 U. S. military dogs in Southeast Asia where clinical TCP was diagnosed; from 6 military dogs at Lackland Air Force Base, Texas; from 21 dogs experimentally infected at Division of Veterinary Medicine, Walter Reed Army Institute of Research; from 13 experimentally infected and 16 non-infected dogs at the College of Veterinary Medicine, University of Illinois. Isolates from 4 diverse geographic regions (Puerto Rico, Virgin Islands, Florida, and Southeast Asia) were used to infect individual dogs from which the above sera were obtained. (c) Sera from dogs infected with Leptospira canicola, Brucella canis, canine herpes virus, para-influenza virus SV 5, distemper virus and hepatitis virus, and Neorickettsia helminthoeca. (d) Guinea pig sera specific to 8 species of rickettsia which included: Rickettsia tsutsugamushi (Gilliam strain), R. Rickettsii, R. canada,

R. burnettii, R. mooseri, R. akari, psittacosis agent, and Tatlock agent, a rickettsia-like organism isolated from, and pathogenic for, the guinea pig. For use in the test, all sera were diluted 1:10 with phosphate buffered saline pH 7.2 (PBS). Serial two-fold serum dilutions were prepared when titer was to be established.

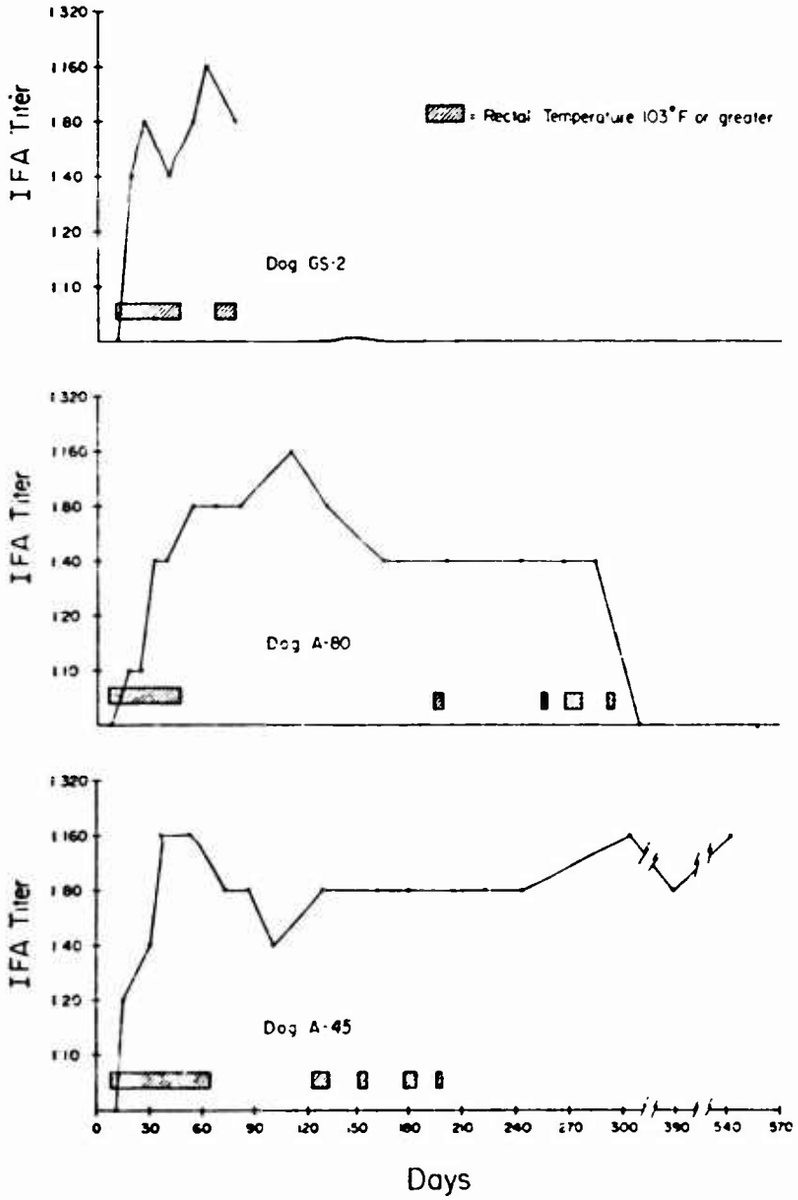
Antigen slides were removed from the freezer and placed in a desiccator jar over calcium chloride. The jar was evacuated and slides maintained in it for 1 hour at 22° C. The slides were then fixed in absolute acetone at 22° C for 5 minutes, allowed to dry for 15 minutes and a circled area (1 cm. in diameter) flooded with test serum. Appropriate positive and negative control sera were included with each run. The slides were placed in a humidified chamber and incubated at 37° C for 30 minutes; they were rinsed twice for 5 minutes each time in PBS, 5 minutes in distilled water, air-dried, and then the fluorescein-conjugated anti-canine globulin applied. Incubation and rinse procedures were repeated as above. Mounting fluid containing 9 parts of glycerin and 1 part PBS was placed on each slide and all 6 antigen spots covered with a 22 x 40 mm. coverslip. The slides were examined on a microscope equipped with an ultraviolet light source.

A correlation between the presence and/or absence of indirect immunofluorescence reaction and actual infectivity status of the dog being examined was attempted in a few instances by: (a) inoculating whole blood (5-20 ml.) from test subjects into susceptible recipients and manifestation of TCP in the latter, (b) determination of specific microscopic tissue abnormalities in reactor dogs. The presence of plasmacytosis in the meninges and kidneys was considered to be a consistent manifestation of TCP.

The appearance of the fluorescence reaction in infected cells of specimens treated with serum from a dog with TCP varied. The antigens detected in the cytoplasm of these cells were those of mixed populations of elementary and initial bodies, singly occurring morula, and multiple morulae. In each microscopic field there were also cells containing apparently soluble antigen diffusely distributed throughout the cytoplasm. No fluorescence of any kind was noted in or outside the cells when sera from E. canis free dogs were used in the test.

The titer of sera collected from 10 experimentally infected dogs during periods from 3 to 18 months after exposure ranged from 1:10 to 1:640. The earliest reactions were demonstrated 11 to 28 days after inoculation. Three examples, indicating a relation between the antibody titer and an increase of body temperature are given in Figure 1. In all three instances febrile reactions preceded the rise of antibody titer. Transitory febrile episodes were noted later in the infection. German Shepherd dog (GS-2) died

Figure 1. The relation between antibody titer detected by the indirect fluorescent antibody test and the febrile response in 3 dogs experimentally infected with *E. canis*: GS-2, German Shepherd dog, died 90 days after exposure; A-80, Beagle dog, killed when moribund approximately 1 year after infection; A-45, Beagle dog, a chronic carrier at the time testing discontinued approximately 18 months after infection.



with typical signs of TCP 90 days after exposure; Beagle dog (A-80) was killed when moribund approximately 1 year following infection, and Beagle dog (A-45) was a chronic carrier when testing was discontinued approximately 18 months after infection. At the time of death, GS-2 had an antibody titer of 1:80, whereas the titer of A-80 fell below detectable level.

The results of the test on 3 groups of dogs using 1:10 serum dilutions are given in Table 1. In the experimentally induced disease group all 34 animals reacted in the test. The blood from 27 of these animals was inoculated into susceptible dogs. All recipients developed the infection. Of 61 dogs from regions of Southeast Asia, where naturally occurring disease was reported, 24 reacted in the test. The organism was recovered from 2 reactor dogs but not from 2 non-reactors. One reactor was detected among 6 dogs from Lackland Air Force Base, Texas. (This case is discussed below). The dog which received the blood from this reactor developed TCP 10 days after inoculation. None of the 26 dogs from Georgia and Illinois, where the disease is not known to occur naturally, reacted in the test. Attempts to recover the organism from 7 negative dogs failed.

Eight of 9 serologically positive dogs showed plasmacytosis of the meninges and kidneys at the time of their death.

None of the specific sera against common dog pathogens and sera against 8 rickettsial species reacted in the test (Table 2).

The results, in general, indicate that the IFA test is applicable to both experimental laboratory and field epidemiologic investigation of TCP. In experimentally infected dogs the period prior to detection of antibodies varied from 11 to 28 days. The analysis of the inoculation data indicates that this variation is apparently due to individual animal responses rather than to the volume of inoculum used. From 10 experimentally infected animals, monthly blood subinoculations were made into susceptible dogs. The latter animals consistently developed clinical infections demonstrating the existence of active carrier state in the donor animal. A further indication that the positive test may be indicative of an active infection was provided by transmission of the infection from 3 naturally occurring reactor dogs into susceptible dogs.

The test antigen (Florida isolate) depicted antibodies in dogs infected with isolates from Puerto Rico, Virgin Islands, and Southeast Asia, indicating serologic similarity among E. canis organisms from different geographic areas. In most dogs the antibody titer persisted during the entire observation period. However, in the terminal case, Beagle A-80 (Fig. 1), the titer sharply subsided

Table 1. Relation between Positiveness of the Indirect Fluorescent Antibody (IFA) Test for TCP and Actual Recovery of the Organism Accomplished by Blood Subinoculation into Susceptible Dogs.

		IFA	Recovery of the Organism	
			**IFA+	IFA-
TCP	Location	Positive/Tested	Accomplished/Attempted	Accomplished/Attempted
Experimentally Induced	Walter Reed	21/21*	19/19	0/0
	U. of Ill.	13/13	8/8	0/0
Naturally Occurring	Southeast Asia	24/61	2/2	0/2
	Texas	1/6	1/1	0/0
Not Known to Occur	Atlanta, Ga.	0/10	0/0	0/4
	Urbana, Ill.	0/16	0/0	0/3

*Number of dogs
**(IFA+ and IFA-) positive and negative indirect fluorescent antibody test, respectively

Table 2. Specificity of the Indirect Fluorescent Antibody (IFA) Test for Tropical Canine Pancytopenia (TCP)

Agent	Titer	Number of Sera Tested	IFA for TCP 1:10 Serum Dilution
Leptospira canicola	1:200 agglutination lysis	10	Negative
Brucella canis	1:400 tube agglutination	4	Negative
Canine Herpes virus	1:32 serum neutralization	6	Negative
Parainfluenza virus SV_5	1:16 serum neutralization	15	Negative
Neorickettsia helminthoeca	No test - animal known positive	2	Negative
Distemper virus and Hepatitis virus	No test - immunized with live attenuated viruses	45	Negative
Rickettsia tsutsugamushi	1:20,000 IFA test	1	Negative
Rickettsia rickettsii	1:20,000 IFA test	1	Negative
Rickettsia canada	1:20,000 IFA test	1	Negative
Rickettsia burnettii	1:20,000 IFA test	1	Negative
Rickettsia mooseri	1:20,000 IFA test	1	Negative
Rickettsia akari	1:20,000 IFA test	1	Negative
Psittacosis agent	1:20,000 IFA test	1	Negative
Tatlock agent	1:20,000 IFA test	1	Negative

below 1:10 during the last week. This could have been due to reduced immunologic responsiveness apparently caused by a total exhaustion of the bone marrow as revealed by histopathologic examination of terminal TCP cases (8). The role of the immune response in the pathogenesis of TCP may be further investigated by careful correlative study of clinical pathological manifestations and variations in IFA titer. Plasmacytosis of the meninges and kidneys appears to be a characteristic pathologic manifestation associated with TCP.

A relatively high incidence of reactors found among the military dogs in Southeast Asia where the disease was diagnosed and serious losses occurred, indicates applicability of the test to epidemiologic investigation of TCP. Thus, the test may be used to determine the incidence of the disease in the U.S., particularly in areas where clinical cases have been described. Because of striking clinical resemblance between canine distemper and TCP, it would be of interest to examine by the IFA test suspected distemper cases, especially those occurring in tick-infested regions.

The test revealed no cross serologic relation between E. canis and any of the common canine pathogens. Also, none of the high titer specific sera produced against 8 rickettsial species reacted in the test. Apparent lack of serologic relationship between E. canis and the above described rickettsieae renders further support that the agents of the genus Ehrlichia might be taxonomically a unique rickettsial group.

2. Antibody Response In Dogs Treated With Tetracycline During The Acute Phase of TCP.

The development of the IFA test has permitted an examination of the antibody response in dogs treated with tetracycline during the acute phase of TCP. A study in which tetracycline was evaluated as a therapeutic and prophylactic agent in dogs infected with the agent of TCP was described in detail in a previous annual report (15). Eight German Shepherd dogs were used as part of this study. The eight dogs were divided into a treated group of 5 dogs and an untreated group of 3 dogs. All dogs were inoculated intravenously with 5 ml. of whole blood collected in EDTA from an acutely ill dog. Treatment was initiated after all dogs had a rectal temperature of 104 F. (40 C.) or greater and had altered hemograms. This was 13 days post inoculation. Each treated dog was given 30 mg./lb. (0.45 kg.) of body weight of tetracycline HCl daily for 14 days. The drug was administered orally in a divided dose, half in the morning and half in the evening. On the 30th day after the last day of treatment, 20 ml. of blood was collected in EDTA from each dog. The blood was inoculated intravenously into susceptible laboratory Beagles to determine the infectivity of each dog. All treated dogs freed of the infection were reinoculated with the

homologous strain of E. canis to determine susceptibility to reinfection. Serum was collected weekly from all dogs for serological studies. All dogs developed signs of disease within 2 weeks after inoculation. The signs included pyrexia, anorexia, conjunctivitis, increased erythorocyte sedimentation rate, and decreased red blood cell, white blood cell, and thrombocyte counts. Morulae of E. canis were demonstrated in monocytes in capillary blood smears. Twenty-four hours after initiation of tetracycline therapy, the rectal temperature of the treated dogs had returned to normal. During the ensuing 2 weeks, the hemograms of the treated dogs returned to normal, whereas in the untreated dogs, the hematologic signs remained.

After the acute stage of the disease had passed in 1 untreated dog, the red and white blood counts returned to normal. At approximately the 75th day after inoculation, a relapse characterized by a reappearance of earlier signs was noticed in this dog. The dog died with signs of hemorrhage at the 88th day after inoculation. Throughout the infection, the thrombocyte count remained extremely low and at the time of death, thrombocytes could not be detected in the blood. The red and white blood cell counts of a 2nd, untreated dog gradually returned to near normal over a 60-day period; however, during this time, febrile episodes were common and the thrombocyte counts remained depressed. The treated dogs did not have any evidence of relapse after treatment. The organism was not recovered from the blood of the treated dogs.

All dogs freed of the infection responded to reinoculation with the homologous strain of E. canis. The 2nd infection was equally as severe as the 1st, and intracytoplasmic inclusions of E. canis were easily demonstrated in capillary blood smears of all reinfected dogs.

The sera collected from the dogs were examined in the Ehrlichia canis IFA test as described above. The results are summarized in Table 3. Antibody was demonstrable in the sera of all dogs, and there was no apparent difference in antibody titer between the treated dogs and untreated dogs. It is also significant that all dogs responded to reinoculation with the homologous strain of E. canis in spite of an antibody titer.

3. TCP In a Recruit Military Dog at Lackland AFB, Texas.

In October 1971 this laboratory received from Lackland AFB a serum specimen from a military dog (Sampson, 5S65) which had been acquired for military use in June 1971. The dog had a history of having been whelped in England. After 18 months in England, it was known to have spent one month in Tennessee, one year in Wyoming

Table 3. Antibody Response in Dogs Treated with Tetracycline During the Acute Phase of *Ehrlichia canis* Infection*

	Reciprocols of IFA Antibody Titers								
	Days Post Inoculation								
	0	14	20	32	39	62	78	91	104
Dog No.									
Controls									
32	Neg	Neg	20	ND**	40	40	160	80	40
35	Neg	40	80	80	80	ND	80	Dead	Dead
36	Neg	Neg	Neg	ND	80	80	40	80	80
Treated									
27	Neg	Neg	20	80	80	40	10	20	40
33	Neg	Neg	Neg	40	80	20	40	40	ND
37	Neg	Neg	40	80	80	80	80	80	160
38	Neg	20	80	80	80	80	80	40	160
39	Neg	20	40	80	80	80	80	80	80

*Administration of tetracycline was initiated at the 13th day after inoculation and was continued for 14 days. The dog was reinoculated with E. canis on day 86.

**Not done

and five months in Colorado before it was acquired for military use. After arrival at Lackland AFB the dog showed signs of hemorrhage in the sclera and gums, loss of weight and an abnormal hemogram, particularly a low white blood cell count. Such signs are compatible with tropical canine pancytopenia. Treatment included extensive administration of chloramphenicol and steroids. In October 1971 LTC Paul W. Husted, Chief, Military Dog Service, Lackland AFB, requested the assistance of this laboratory in determining if this dog did in fact have tropical canine pancytopenia. A serum specimen was submitted from the dog and examined for antibody to Ehrlichia canis using the newly developed indirect fluorescent antibody test. An antibody titer of 1:80 was demonstrated. A whole blood specimen from this dog was then requested. The blood was received on 11 November 1971 and immediately inoculated into a laboratory German Shepherd dog. The inoculated dog developed typical signs of E. canis infection and morulae of E. canis were demonstrated in the cytoplasm of monocytes in blood smears. Fortunately collaborative studies with Lackland AFB have resulted in a system whereby all dogs arriving at Lackland are bled upon arrival and the serum is sent to the Division of Veterinary Medicine, WRAIR. A specimen collected from Sampson, 5S65, on 21 June 1971 was examined and found to have an antibody titer of 1:640. A follow-up serum specimen collected on 12 January 1972 from the same dog had a titer of 1:320. From this series of examinations the following conclusions are drawn: (a) the dog was already infected at time of arrival in June 1971; (b) the time when the dog became infected cannot be determined; (c) the administration of chloramphenicol plus steroids did not clear the dog of infection.

4. Pathogenicity of Ehrlichia canis in Red and Gray Foxes.

While basic pathogenicity and chemotherapy studies on TCP have been done, the epidemiology of the disease remains for the most part, speculative. The host range and natural cycles of transmission have not been well established. Unsuccessful attempts have been made to infect mice, rats, guinea pigs, hamsters, rabbits, sheep, cattle and cats (3,9). Neitz and Thomas (10) have suggested that the wild dog, Lycaon pictus, served as a reservoir for Ehrlichia in the Kruger National Park in Africa, and reported that the jackal can be infected. The only evidence for a susceptible host, other than the dog, in the United States was supplied by Ewing and co-workers (11) when they experimentally infected a coyote, Canis latrans. It is apparent from these reports that the host range may be limited to canines.

The most numerous and widespread wild canines in the United States are the red fox, Vulpes fulva, and the gray fox, Urocyon cinereoargenteus. Both are common in the southern United States,

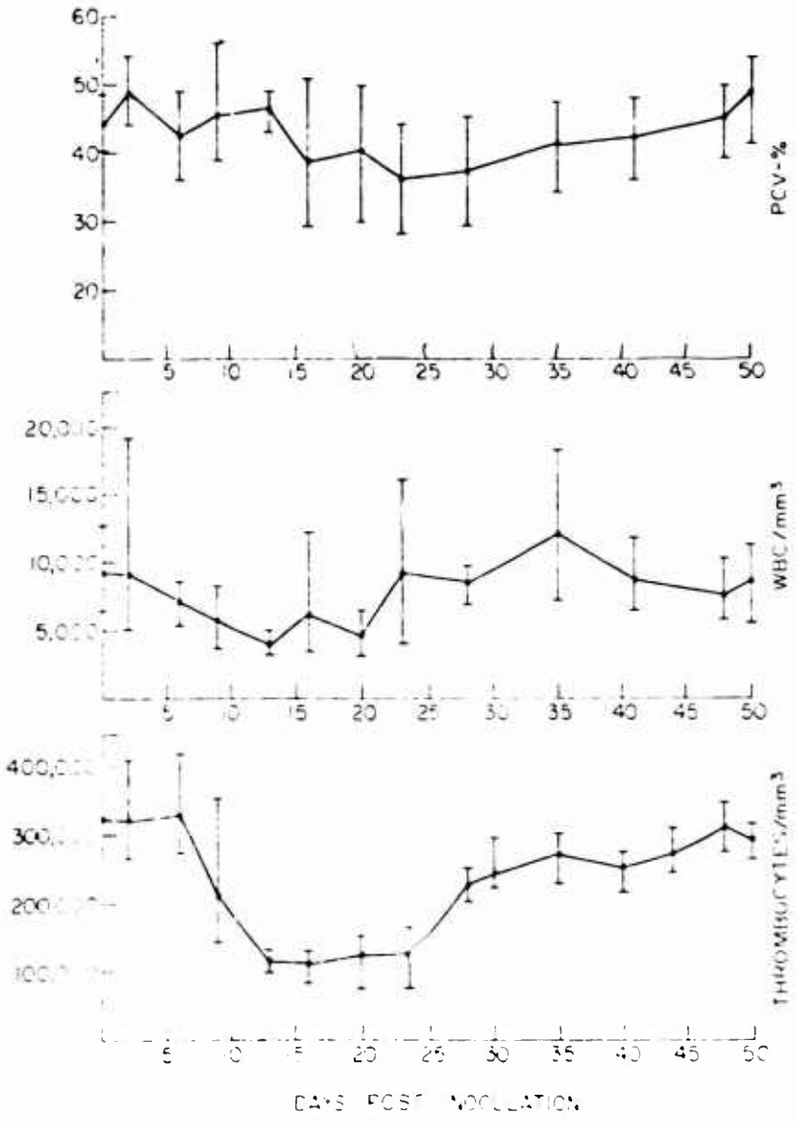
especially the gray fox in Florida and Texas. In these areas Rhipicephalus sanguineus, the natural arthropod vector of Ehrlichia, can overwinter out of doors and would be more likely to attack wildlife. This is also the area where Ehrlichia is endemic. The present report describes experimental infections with Ehrlichia canis in red and gray foxes, and transmission of the agent from foxes to dogs via the natural arthropod vector.

The isolate of Ehrlichia canis used in this study was recovered from a German Shepherd dog. The severity of the experimental disease produced by this strain in dogs has been previously described (12,4,13). Six young adult foxes, 4 red and 2 gray, were used in the experiment. One red and 1 gray fox were held as uninoculated controls. The others were inoculated intravenously with 5 ml of blood collected in EDTA from a laboratory Beagle in the acute stage of infection with Ehrlichia canis. Each fox was examined daily thereafter for clinical signs of ehrlichiosis. Following inoculation and two weeks prior to inoculation, 3 ml of blood were collected twice a week in sealed vacuum tubes containing EDTA. These samples were used for determination of packed cell volume, erythrocyte sedimentation rate and erythrocyte, leukocyte, and thrombocyte counts. A standard microhematocrit centrifuge was used for all packed cell volume determinations. The Wintrobe tube was used for determination of erythrocyte sedimentation rates. Leukocyte and erythrocyte counts were determined with an electronic cell counter. Thrombocyte counts were made with a phase contrast microscope. Blood films were made twice per week, stained by the Giemsa method, and examined for morulae of Ehrlichia canis.

One month following experimental infection, 5 ml of blood were collected in EDTA from the infected gray fox and inoculated into the control gray fox. When morulae of Ehrlichia canis were demonstrated in the peripheral blood during the acute stage of infection, approximately 6,000 larvae of Rhipicephalus sanguineus were fed on the fox. The larvae were from a colony of ticks which had been maintained in the laboratory over 3 generations by feeding on normal healthy dogs and had never transmitted disease. The engorged larvae were recovered, allowed to molt, and the resultant nymphal ticks were fed on a normal laboratory Beagle. The Beagle was then examined and specimens collected as described for the foxes.

The experimentally infected foxes showed none of the typical clinical signs of ehrlichiosis in dogs. Nasal or ocular discharges, anorexia, weight loss, or hemorrhage were not observed. Clinical laboratory tests demonstrated a mild anemia, thrombocytopenia, and leukopenia in the acute stages of the disease, along with a rise in erythrocyte sedimentation rate which varied greatly with the individual animal. Figure 2 summarizes the findings in the first two months following inoculation. Typical morulae of Ehrlichia

Figure 2. Mean and range of packed cell volume, white blood cell count, and thrombocyte count of foxes following intravenous inoculation with Ehrlichia canis.



canis were easily demonstrated in Giemsa stained venous blood smears from the second week through the fifth week post inoculation. Three months following inoculation blood was collected from the foxes and inoculated into laboratory dogs. The dogs developed signs of disease, and the organism was recovered. This confirms persistence of the infection in the fox as in the dog.

Rhipicephalus sanguineus ticks fed on the infected gray fox as larvae transmitted Ehrlichia canis to the Beagle as nymphs. Eight days after the ticks were fed, the Beagle began to show the typical signs of ehrlichiosis, and 11 days after feeding, morulae of Ehrlichia canis were demonstrated in the blood.

These studies confirm the susceptibility of foxes to infection with Ehrlichia canis. The mild disease observed in the fox is in contrast to the severe, often fatal, disease described in the German Shepherd dog and referred to as tropical canine pancytopenia (2,13). This study provides further evidence that disease manifestations of infection with Ehrlichia canis are host dependent. The strain of Ehrlichia canis used in this study has been used in pathogenicity studies of Ehrlichia canis in German Shepherd and Beagle dogs (4,12,13). The strain produced severe diseases with high mortality in German Shepherd dogs, clinical disease with no mortality in Beagle dogs and mild disease with no clinical signs in foxes.

Transmission of the disease from infected foxes to dogs by the tick, Rhipicephalus sanguineus is important epidemiologically. Rhipicephalus sanguineus is a three-host tick which feeds in all stages on canines (14). In the southern part of the United States where foxes are numerous, Rhipicephalus sanguineus can overwinter out of doors. Tick sharing by foxes and dogs may occur where dog and fox populations intermingle in rural and suburban areas.

In areas where the disease occurs in domestic dogs, wild foxes and other wild canines may serve as reservoir hosts for Ehrlichia canis.

5. Survival of P-Labeled Platelets in Normal and TCP Infected Dogs.

Marked thrombocytopenia is known to be the cause of the mucosal and serosal hemorrhages characteristic of TCP (2). The cause of the thrombocytopenia, however, is not known. The perivascular infiltration by plasma cells in the lungs, meninges, kidney, and spleen of dogs with TCP, and certain serum protein changes, prompted the suggestion that E. canis infection initiates the development of a hypersensitive or autoimmune reaction (16). This would account for the pancytopenia observed during the post-febrile phases of infection, but not for the development of pancytopenia early in the febrile phase of the disease.

An alternate explanation is that E. canis organisms somehow alter the production of platelets in the bone marrow. This is consistent with the finding of bone marrow hypoplasia in E. canis infected dogs (2,17).

Since the circulating platelet level is the net result of the rate of platelet production versus destruction, a comparison of the platelet survival times in normal and E. canis infected dogs would indicate the mechanism by which the thrombocytic series are affected in TCP.

A total of 11 dogs of mixed breed (9-12 months old) weighing from 7-32 kg, were included in 3 platelet survival trials. Three of the dogs served as uninfected controls, although one of them exhibited moderate pancytopenia and was later diagnosed as having a reticulum cell sarcoma. The other 8 dogs were studied in one or more phases of E. canis infection. Two of these dogs had been splenectomized one month prior to infection.

Dogs were infected by the intravenous injection of 5 ml of blood from an E. canis carrier dog. A Florida isolate of the parasite was used (4).

The course of the infection was monitored by pre- and post-infection determinations of rectal temperature, packed cell volume (PCV), and total leukocyte and platelet counts.

Each dog received from 60-240 uCi (0.15-0.60 mg) of $DF^{32}P$ intravenously. The label was diluted to 5 ml with saline immediately prior to injection via the cephalic vein over a 3 minute period.

Platelets were recovered from 4.5-13.5 ml of blood collected in plastic syringes containing 1 part of 1.5% Na_2EDTA in phosphate-buffered saline (PBS) pH 7.2, for 9 parts of blood. Each sample was transferred to a siliconized glass tube and centrifuged for 10 minutes at 206-600 g. Three-quarters of the platelet-rich plasma (PRP) were transferred to a second siliconized tube and the remaining cells and plasma resuspended in 0.5% Na_2EDTA, recentrifuged, and the supernatant added to the PRP.

Following removal of an aliquot of platelet suspension for enumeration of platelets with Unopette diluters, the suspension was centrifuged at 1000 g for 30 minutes. The volume of the supernatant was measured and the platelet button resuspended in 1% ammonium oxalate in distilled water, centrifuged, and the supernatant discarded. Following a second wash, the platelet button was lysed with 0.5 N sodium hydroxide and transferred to polyethylene scintillation vials. The vials were stored at -20°C until counting.

Platelet recovery was calculated by multiplying the volume of the platelet suspension by the average of 2 platelet counts on it. This method assumed that negligible numbers or a constant percentage of platelets were lost in washing.

Immediately prior to radioactive counting, distilled water was added to the scintillation vials to a final volume of 20 ml. The vials were placed in a low-temperature Packard Tri-Carb 3000 series liquid scintillation counter. Each sample was counted twice for 50 minutes and corrections for decay during counting were included. Background was 7-10 counts per minute.

Percent platelet survival was plotted by considering the highest counts per minute (CPM) per billion platelets as the 100% value and expressing all others as a percentage of it.

The criterion used to compare platelet survival among the dogs was the time (in days) at which 50% of the labeled platelets had disappeared. This is not to be confused with half-life, which may or may not be applicable to platelet survival curves.

The course of TCP in 6 experimentally infected dogs over a 10-week period is presented in Figure 3 (A,B,C,D). Immediately following infection, rectal temperature rose while PCV, total leukocyte and platelet counts decreased. The maximal febrile response (14 days) coincided with maximal depression of blood cell levels. The febrile phase ended at approximately 3 to 3½ weeks. It was followed by a chronic phase characterized by a slow return toward preinfection erythrocyte (PCV) and leukocyte levels. The platelet corresponds to the subclinical phase observed by other workers (18,20). Terminal epistaxis never occurred with any of the dogs.

Figure 4 (A,B,C,D) presents the results of platelet survival studies in 2 uninfected (AcC) and 2 febrile dogs (B,D). One of the uninfected dogs (A) was moderately thrombocytopenic (160,000/ul) and was later found to have a reticulum cell sarcoma. Marked splenoand hepatomegaly were present.

The 50% survival time in the uninfected dogs was approximately 4 days. It was reduced to 2.5 and 0.7 days in the 2 acute phase dogs (B and D, respectively). Note also the suggestion of a platelet release phenomenon in the sarcoma dog (A) and one of the febrile phase dogs (B).

A second trial was performed comparing platelet survival in 1 uninfected, 1 acute phase, and 2 chronic phase dogs. Results are shown in Figure 5 (A,B,C,D, respectively). The 50% survival time

Figure 3. Clinical data on 6 dogs infected with Ehrlichia canis.

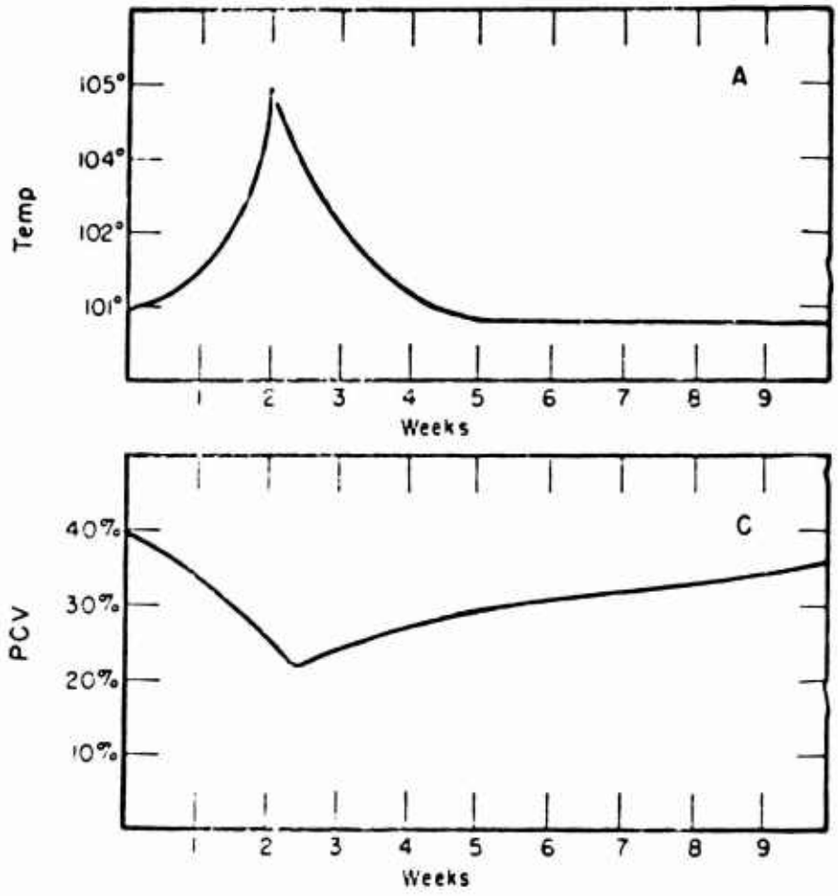
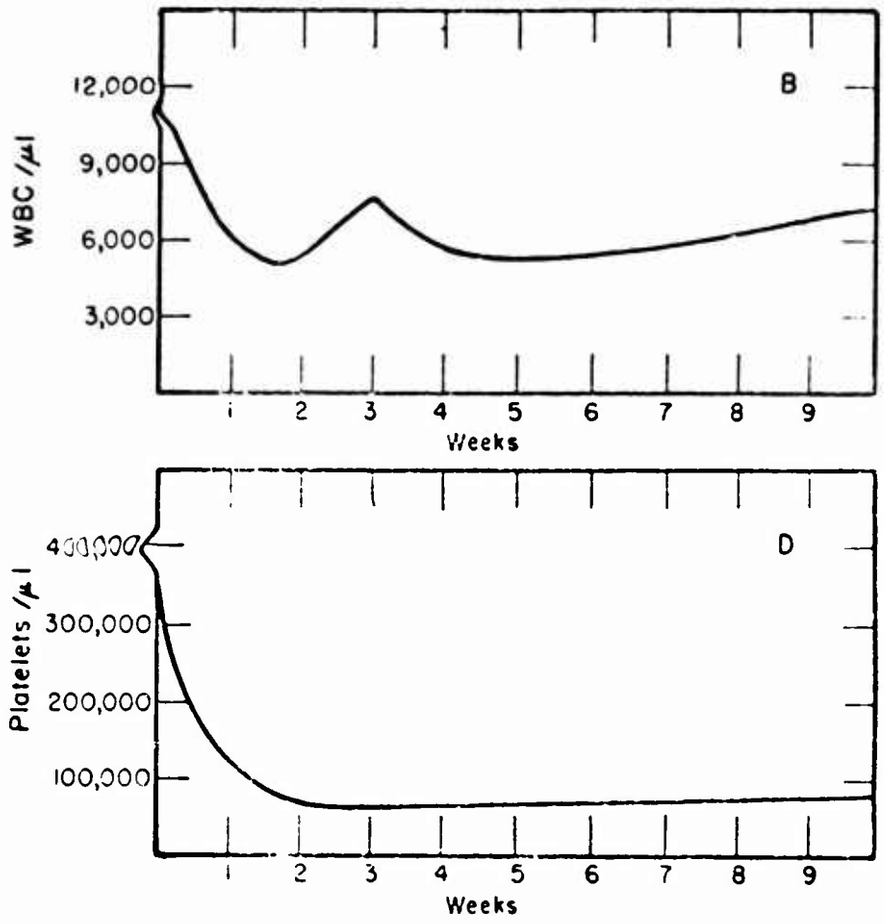


Figure 3. Clinical data on 6 dogs infected with Ehrlichia canis.



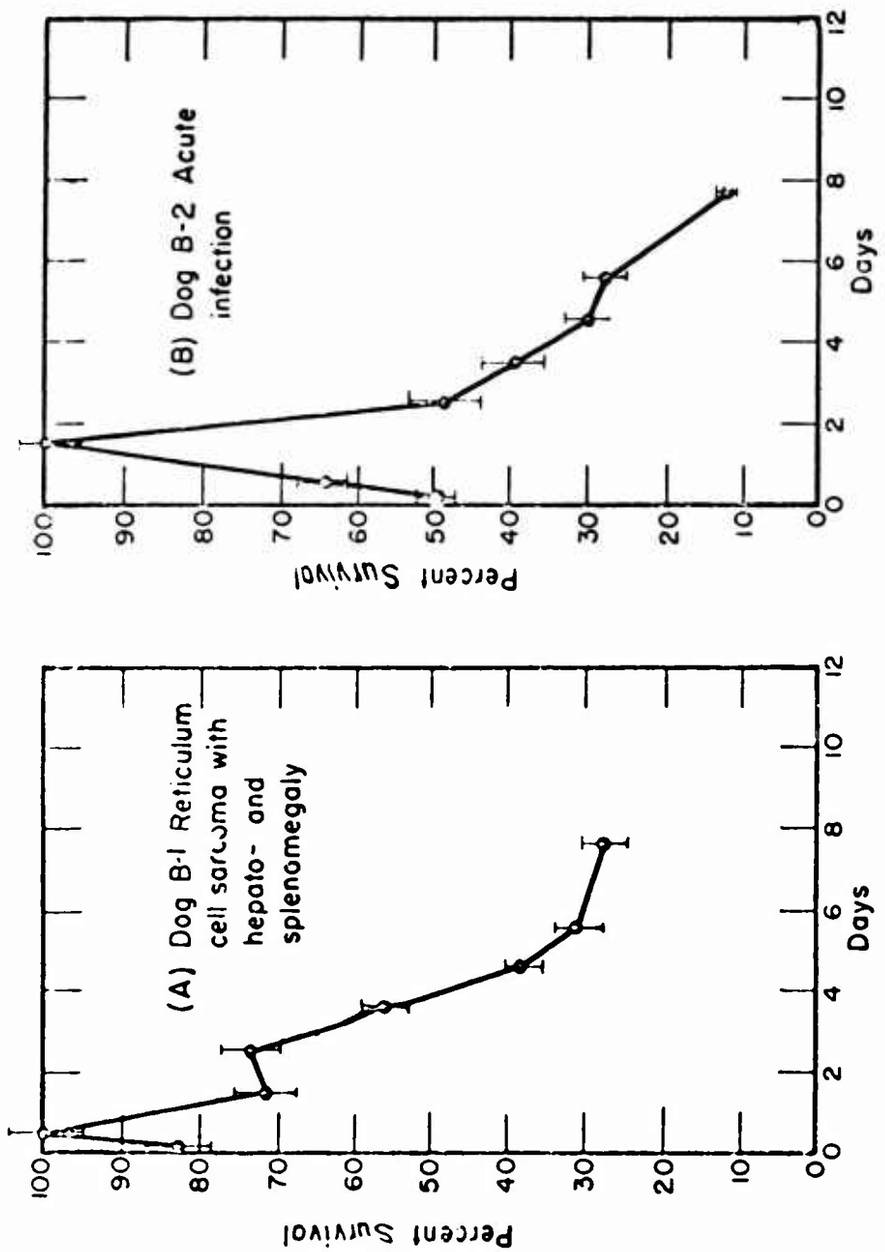
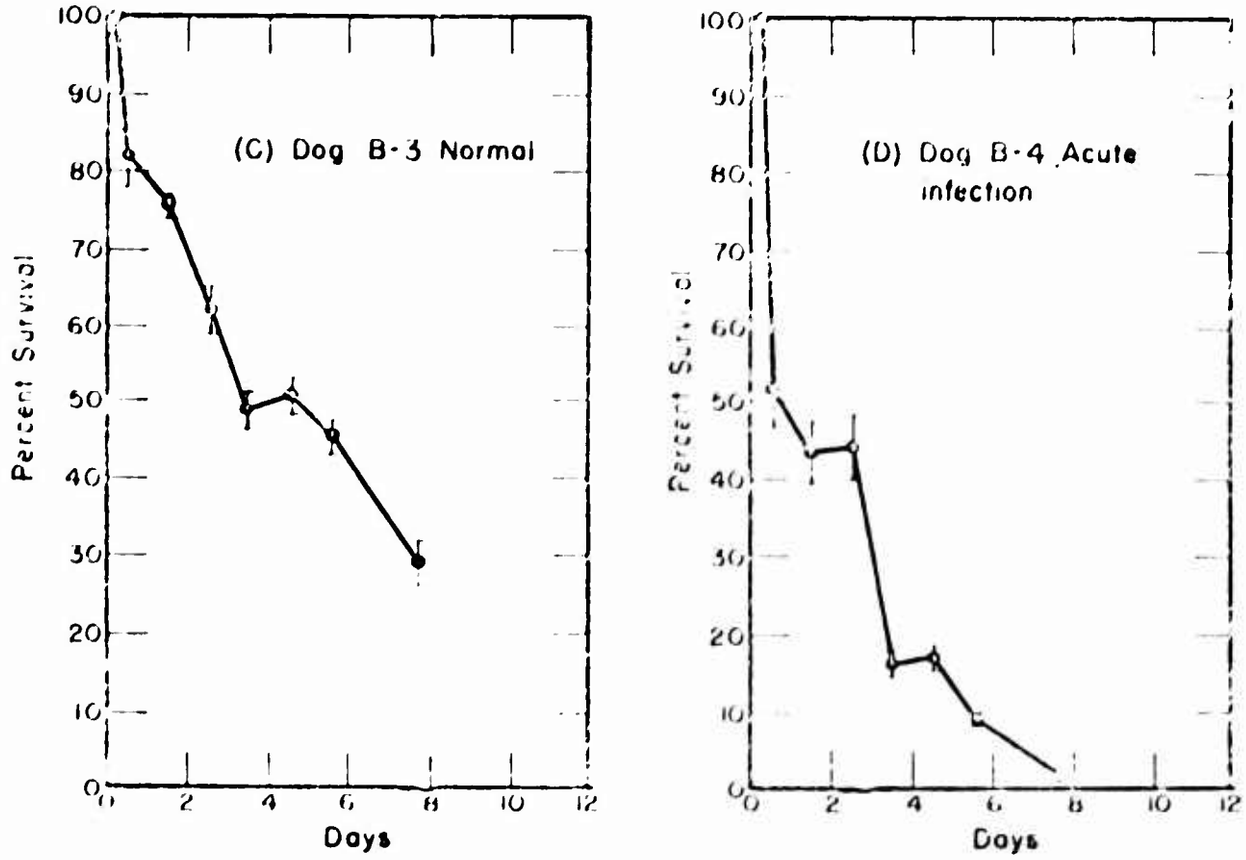


Figure 4. Percent survival of P-32 labeled platelets: Trial 1

Figure 4. Percent survival of P-32 labeled platelets: Trial 1.



for the uninfected dog (A) was 4.5 days compared with 1.6 days for the acute phase dog (D). Surprisingly, the 50% survival time was essentially normal in the chronic phase dogs at 3.6 and 4.2 days (B,C, respectively). It is also interesting that dog B-2 which exhibited a marked release of labeled platelets from sequestration during the febrile phase (Figure 4,B) failed to do so at 2 months post-infection (Figure 5,B). The release phenomenon is apparent, however, in dogs N-2 and N-1 (Figure 5 C,D) at 1 month and 6 days post-infection, respectively.

A third trial included 1 uninfected, 2 febrile phase, and 2 chronic phase dogs. One febrile phase and 1 chronic phase dog had been splenectomized 1 month prior to infection with E. canis.

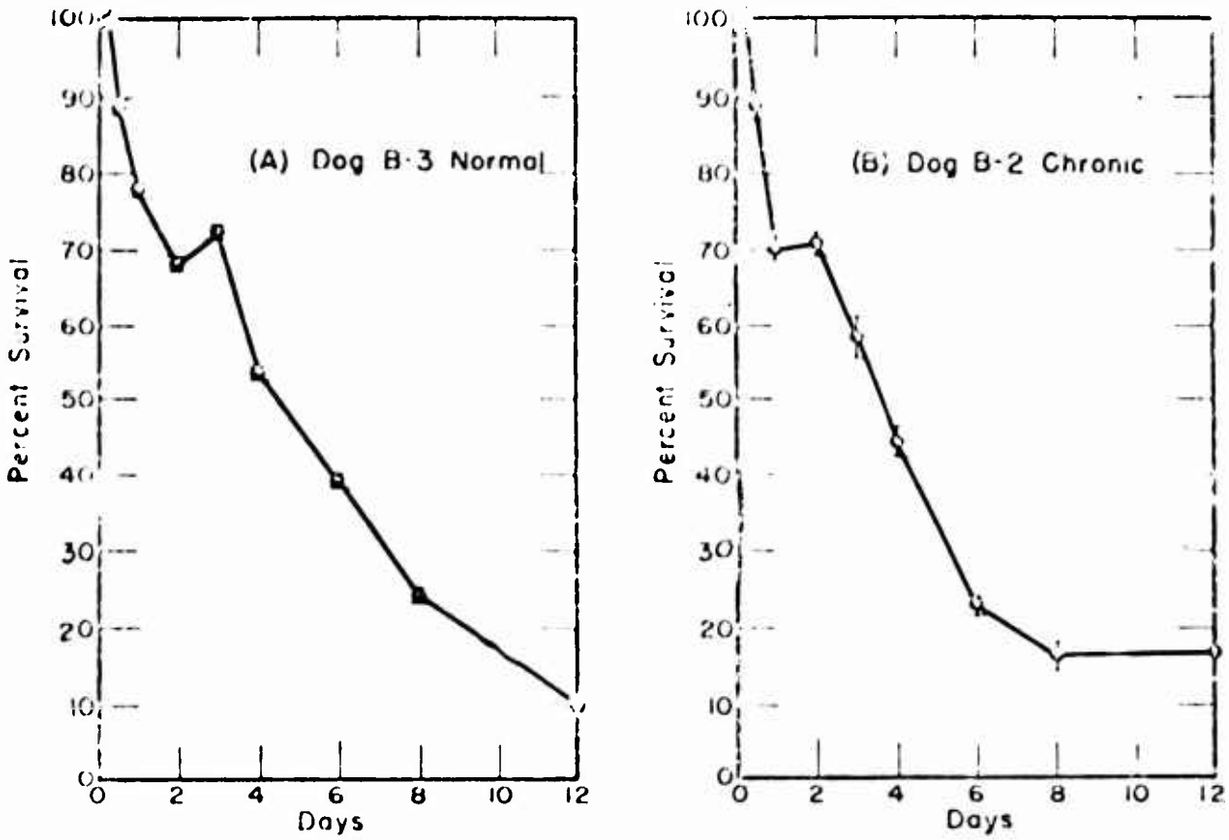
There was essentially no difference between the platelet survival curves of the splenectomized dogs and their intact counterparts. The 50% survival times followed the same pattern observed in the first 2 trials, i.e., markedly reduced during the acute phase and essentially normal during the chronic phase. Splenectomy prior to infection did not alter the 50% platelet survival time. The results of all trials are summarized in Table 4.

The nature of platelet survival curves in normal animals is still unresolved. In man, opinion is divided between random destruction and removal of platelets due to senescence (19). The resulting platelet survival curves would be curvilinear (exponential) in the former and retilinear (arithmetic) in the latter case. Studies in dogs have favored an exponential curve (20,21). In the present study, the platelet survival curves in normal and infected dogs were compared by noting the point at which 50% of labeled platelets were removed. This criterion avoids the issue of the shape of normal canine platelet survival curves.

Platelet sequestration and release have been observed in normal canine (21,22) and human (19) subjects. It has been attributed to reversible and/or irreversible damage to platelets incurred during in vitro labeling with $DF^{32}P$ or ^{51}Cr and in vivo labeling with $DF^{32}P$. The spleen and liver have been implicated in the removal of such altered platelets. If these tissues are altered during the febrile phase of TCP, a change in the rate of removal of labeled platelets could be anticipated.

During infection, morulae (inclusion bodies of E. canis) are found within mononuclear cells in the liver, spleen, lungs, and kidney, as well as in circulating monocytes (2). It is probable that a state of non-specific macrophage activation accompanies this form of parasitism, as has been demonstrated to occur with other intracellular parasites (25). An increased rate of pinocytosis has been

Figure 5. Percent survival of P-32 labeled platelets: Trial 2.



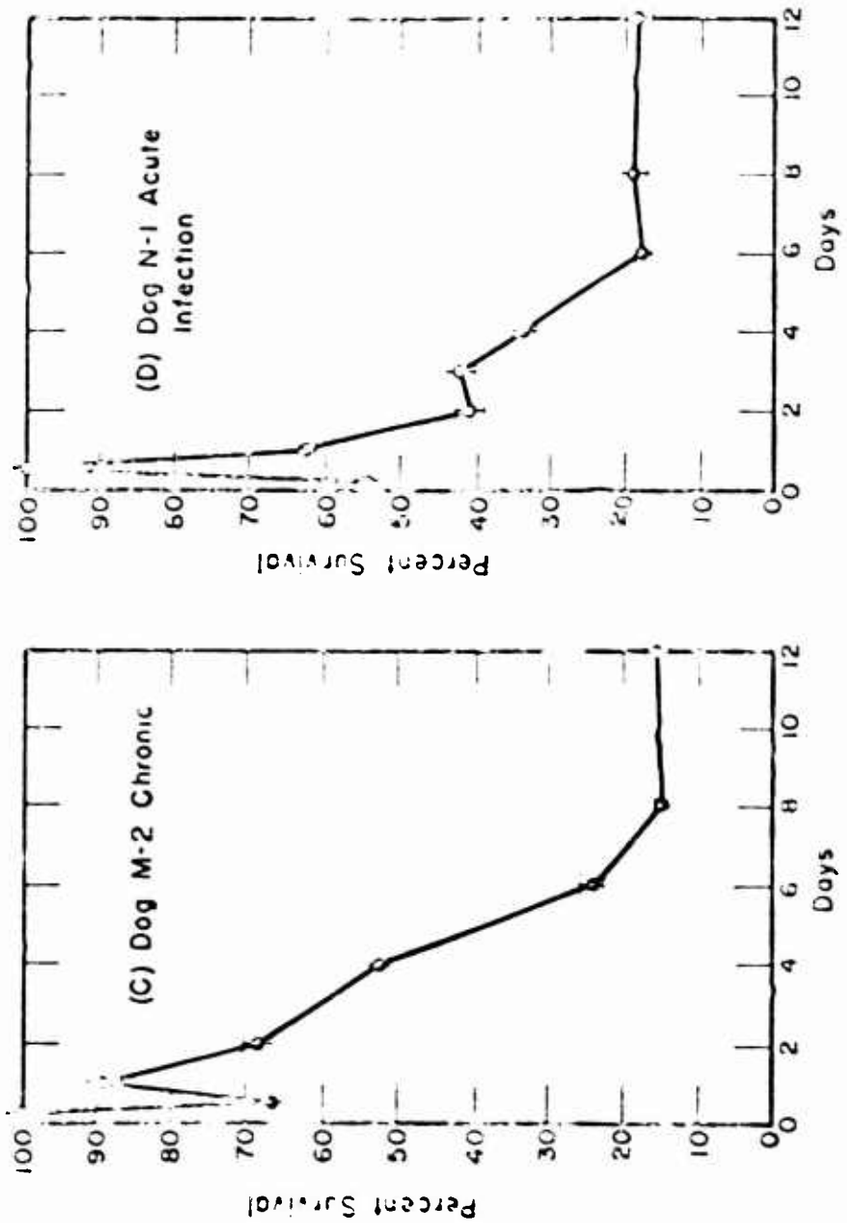


Figure 5. Percent survival of P-32 labeled platelets: Trial 2.

Table 4. 50% Platelet Survival Time in Ehrlichia canis-Infected Dogs and Controls

Dog		50% Platelet Survival (in days)		
		Uninfected	Febrile Phase	Chronic Phase
B-1[a]	thrombocytopenic (175,000/ul)	3.9 ± .2*		
B-3	normal (384,000/ul)	4.1 ± .2		
B-3	normal (274,000/ul)	4.5 ± .1		
44	normal	[illegible]		
B-2	[illegible] (46,000 ul)		2.5 ± .1	
B-4	thrombocytopenic (50,000/ul)		.7 ± .5	
M-1	thrombocytopenic (117,000/ul)		1.6 ± .1	
49[b]	thrombocytopenic (52,000/ul)		2.3 ± .1	
50	thrombocytopenic (61,000/ul)		2.6 ± .1	
B-2	thrombocytopenic (58,000/ul)			3.6 ± .2
M-2	thrombocytopenic (57,000/ul)			4.2 ± .1
40[b]	normal (403,000/ul)			3.6 ± .2
47	thrombocytopenic (137,000/ul)			3.7 ± .1

* expressed as the mean ± 1 standard deviation.

[a] dog afflicted with a reticulum cell sarcoma, marked hepato- and splenomegaly.

[b] splenectomized 1 month prior to infection.

noted in such non-specifically activated macrophages. It is conceivable that these cells show a decreased selectivity toward foreign substances and altered host cells. This may occasion a heightened reaction toward ^{32}P-labeled platelets resulting in greater sequestration and/or destruction of labeled platelets. Some release occurs, but labeled platelet survival time is shortened. Following the febrile phase, these tissues may return to normal function despite persistent infection.

Splenectomy prior to infection with E. canis failed to alter the febrile phase platelet survival curves. If cells of the reticuloendothelial system are involved in the sequestration and release of labeled platelets, we would not expect splenectomy alone to prevent it. Splenectomy has been helpful, however, in cases of human idiopathic thrombocytopenic purpura where autoradiography has shown the spleen to be the principal organ removing ^{51}Cr-labeled platelets (24).

Thus, the phenomenon of the sequestration and release of labeled platelets, as well as the shortened platelet survival time in febrile dogs, may be only a reflection of the selective removal of ^{32}P-labeled platelets by activated macrophages. Unlabeled platelets are not affected. The fact that platelet survival is essentially normal during the chronic phase of the disease indicates that decreased production of platelets is responsible for the persistence of thrombocytopenia.

Although idiopathic thrombocytopenic purpura of "autoimmune" etiology has been reported in dogs (26), the clinical syndromes described are characteristic of TCP. As platelet survival studies were not carried out, the cause of thrombocytopenia may not be ascertained. One may speculate, however, that some of the suspected cases of idiopathic thrombocytopenia purpura may have been due to E. canis.

6. Therapeutic Value of Tetracycline in Dogs with Chronic Tropical Canine Pancytopenia.

At the present time the control of TCP in military working dogs involves prevention of tick infestation and the treatment of dogs with antibiotics. The therapeutic value of tetracycline during the acute phase of Ehrlichia canis infection has been reported (12,15). The present study was undertaken to evaluate the efficacy of tetracycline in the treatment of dogs chronically ill with TCP. Additionally, the study was designed to gather further information regarding the pathogenesis of E. canis infection and the serologic response in dogs treated during the late stages of TCP.

Ehrlichia canis was isolated from a naturally infected dog in Southeast Asia and maintained by passage in laboratory dogs. An acutely infected German Shepherd dog served as the source of inoculum for this study.

Fourteen German Shepherd dogs of both sexes ranging from 10 months to 3½ years of age, were obtained and baseline data recorded for 60 days. Dogs were examined and rectal temperature recorded daily. Three times weekly blood was collected in vacuum tubes containing ethylene diaminetetra-acetic acid (EDTA) for clinical laboratory studies. White and red blood cell, and thrombocyte counts were performed on electronic cell counters. Hematocrit was determined by the micro-hematocrit technique, and hemoglobin by the cyanmet-hemoglobin method. Erythrocyte sedimentation rate (ESR) was determined using the Wintrobe tube and was not corrected for hematocrit. Blood smears were stained using Wright's, Wright-Leishman, May-Grunwald Giemsa, or new methylene blue stains. Once weekly serum was collected from each dog and stored at -70C.

Tetracycline HCl in tablet form and administered orally, was used throughout the study.

Bone marrow aspirations were made under thiamylal sodium anesthesia from 6 dogs randomly selected prior to inoculation with E. canis. Aspirations were made every other week from the iliac crest, using a 16 gauge silverman type needle. Preliminary results of bone marrow studies are discussed in a separate section of this report.

Fourteen dogs were inoculated intravenously with 5 ml of whole blood from an acutely ill dog. The donor dog was then included in the study. Clinical signs and hemograms were followed and at the first sign of external hemorrhage, peripheral edema, or a rapidly dropping PCV, tetracycline therapy was initiated. The dosage was 30 mg/lb. of body weight given daily, in 2 divided doses, for 14 days.

Thirty and 90 days after the last day of treatment 5 ml of whole blood was collected from each surviving, treated dog and inoculated intravenously into normal Beagles to determine the infectivity of the treated dogs.

Results to date are preliminary. All infected dogs developed physical signs of disease within 10 days following infection with E. canis. Principal clinical signs included pyrexia, lethargy, anorexia, and conjunctivitis. Thrombocytopenia was noted in all dogs by the 6th day post inoculation, and decreases in hematocrit and white blood cell counts had occurred by the 10th day in most dogs. ESR was elevated in most dogs during the acute febrile period.

Recovery from the acute phase occurred in all dogs 20 to 30 days post inoculation and was characterized by a variable decrease in the rectal temperature and the disappearance of clinical signs. However hematologic values during this period remained abnormal. At this point, infected dogs were observed to follow one of 2 clinical courses. Those dogs referred to as "relapsing" showed a worsening of condition with severe thrombocytopenia (less than 15,000 platelets/cmm), leukopenia (less than 5,000 wbc/cmm), episodic pyrexia, elevated ESR, emaciation, and anorexia. In contrast, "non-relapsing" dogs showed clinical improvement with a gradual return of hematologic values to near normal levels. However, non-relapsing dogs were observed to have a mild thrombocytopenia (100-200,000 platelets/cmm), mild leukopenia (5-7,500 wbc/cmm), and persisting episodic pyrexia. Nine of the 15 experimentally infected dogs were classified as relapsing and 6 as non-relapsing. Tetracycline therapy was initiated in 7 of the 9 dogs when signs of hemorrhage, or peripheral edema were noted. Treatment was initiated on an impirical basis in the remaining 2 relapsing dogs which did not show signs of hemorrhage after 105 days post inoculation.

One of these dogs had platelet counts of less than 12,000/cmm and leukocyte counts of less than 4,000/cmm for 25 days, and the other dog had platelet counts of less than 35,000/cmm and leukocyte counts of less than 6,000/cmm for 30 and 18 days respectively, before treatment was initiated.

Five of the 9 dogs responded to treatment with a noticeable and rapid improvement in activity and appetite. Hematocrit, thrombocyte counts, and leukocyte counts began to increase either during or following the treatment period, but hematologic changes occurred gradually in most cases. The slowest response to therapy was noted in the thrombocyte counts which sometimes increased only 20 to 30,000/cmm over a 30 day period. The remaining 4 "relapsing" dogs died either during or after the period of therapy.

The data collected to date indicate that the response to tetracycline of dogs relapsing with TCP differs greatly from the response of acutely ill dogs. The latter show dramatic improvements both clinically and hematologically to therapy (4), whereas response in the chronically ill, relapsing dog is very undramatic and slow. Indeed, in 4 dogs in the present study therapy was unsuccessful in preventing death. Further conclusions must await completion of the study.

7. Evaluation of Bone Marrow Cytology in TCP.

Investigations into the changes occurring in the bone marrow of dogs acutely and chronically ill with TCP have been initiated. Pathogenicity studies have indicated previously that bone marrow is unaffected or even hyperplastic during acute TCP, but that severe depression of hematopoeisis occurs if the disease becomes chronic.

Bone marrow samples were aspirated twice monthly from the iliac crest of 6 German Shepherd Dogs included in the chronic treatment study. Stained smears were examined and a bone marrow differential count performed. Preliminary results indicate that minor bone marrow changes occur the first 30 days following infection. These changes include an increased myeloid-erythroid ratio, a decreased percentage of erythroid precursor cells, an increase! percentage of lymphocytes, and an increase in reticuloendothelial cells. From 30 to 60 days following infection the bone marrow becomes more hypoplastic, with decreased numbers of megakaryocytes, an elevated myeloid-erythroid ratio, and a decreased percentage of erythroid cells.

Inclusion bodies typical of E. canis were observed in bone marrow cells during the first 30 days of illness. Most inclusions were observed intracytoplasmically in lymphocytes. None were observed in any red or white blood cell precursor.

Further study will involve the cytology of bone marrow in Beagles infected with TCP, fluorescent antibody technique applications to positively identify E. canis antigen in bone marrow cells, and bone marrow kinetic studies to elucidate the complex changes occurring in TCP.

8. Comparative Study of Ehrlichia canis and Neorickettsia helminthoeca.

The only other rickettsia commonly recognized as a pathogen for the dog is Neorickettsia helminthoeca, the causative agent of salmon poisoning disease (SPD). Because of its apparent similarities to E. canis, the SPD agent was acquired for comparative purposes.

For many years salmon poisoning disease has been recognized in the Pacific Northwest as an acute, usually fatal disease of dogs, foxes, and coyotes. The etiological agent was first described by Cordy and Gorham in 1950 when intracytoplasmic bodies were observed in reticuloendothelial cells of infected dogs and foxes (27).

They suggested that the organism belonged in the order Rickettsiales, and Philip, et al. proposed the name Neorickettsia helminthoeca (28). A trematode, Nanophyetus salmincola (29,30), serves as a vector for the SPD agent. Natural transmission occurs when animals ingest raw salmon or trout containing the infected metacercariae of the trematode.

In the dog the SPD agent produces characteristic clinical signs which include fever, depression, lymph node enlargement and severe dehydration (31,32). The histologic changes, which are confined primarily to lymphoid tissues, have been described (27,33).

The association of the SPD agent with the life cycle of the trematode vector has been extensively studied (32,34,35,36), however, the inability to propagate the agent in an in vitro system has hampered research related specifically to the etiological agent N. helminthoeca (37). Recently Nyindo and co-workers (6) reported the successful propagation of another canine rickettsial agent, Ehrlichia canis, in monocyte cell cultures derived from acutely infected dogs. The purpose of the present study was to determine if N. helminthoeca could be cultured by a similar in vitro technique.

Six small trout furnished by Dr. Doyle Frank, Washington State University, Pullman, Washington, were triturated and fed in equal amounts to each of two Beagles. A rise in temperature was detected in both dogs within 5 days and reached a peak in 7 days. Other signs associated with SPD were noted, including lymph node enlargement, anorexia, diarrhea, fluke ova in feces, and dehydration. Death occurred in all untreated dogs 12 and 15 days post-inoculation. The isolate was passed serially in laboratory dogs by intravenous inoculation of 5 ml of blood collected in heparin from an acutely affected dog. Necropsy findings included

lymph node enlargement, especially the mesenteric nodes. Impressions of lymphatic tissues were stained by the Giemsa method and microscopic examination revealed organisms within the cytoplasm of mononuclear cells.

The mesenteric lymph nodes and spleen were removed from a dog acutely infected with SPD. A 20% tissue suspension was prepared by homogenization of the lymph nodes and spleen in Snyder I solution containing 1% bovine serum albumin. The suspension was rapidly frozen in a dry ice-alcohol bath and stored at -80°C. When this suspension was thawed and inoculated intravenously into laboratory Beagles typical signs of SPD developed.

Infected monocyte cultures were prepared from acutely infected dogs using the culture technique described by Nyindo et al. However, propagation of normal monocyte cultures required slight modification of the above technique. To increase the erythrocyte sedimentation rate of blood collected from healthy dogs, two parts of blood were mixed with one part of a solution containing 3% dextran in 0.85% NaCl. The blood-dextran mixture was allowed to stand 30 minutes at 37°C. The overlying supernatant consisting of plasma, leukocytes, and platelets was transferred to Leighton tubes, or culture flasks, and incubated at 37°C. In 4 to 6 hours the supernatant was discarded and the developing monolayer was rinsed with Hank's balanced salt solution. The cell culture was maintained with Eagle's minimum essential medium containing 20% canine serum and 0.1 mM L-glutamine per ml. In vitro passage of the organism was achieved by inoculation of normal monocyte cultures with fluids from infected cultures.

Monocyte cultures prepared from dogs infected with E. canis were used for comparative studies. The procedures used for their preparation and maintenance was identical to that described for cultivation of cultures infected with N. helminthoeca.

Two dogs with typical signs of SPD were treated 8 days post-inoculation by intravenous administration of fluids and of chloramphenicol at the rate of 10 mg/lb body weight twice daily. Both dogs recovered in 5 days. Ten days after recovery the dogs were challenged by intravenous inoculation. Serum was collected 3 weeks following recovery.

Laboratory dogs chronically infected with a Puerto Rico isolate of E. canis were used as the source of antisera. At the time of serum collection they had been infected for approximately 5 months. The globulin fraction was conjugated according to the method described by Nyindo et al. (6), and stored at -80°C. Prior to use the conjugate was diluted 1:3 with PBS, pH 7.2.

Leighton tube coverslip cultures were routinely stained by the May-Grunwald Giemsa (MGG) method and examined microscopically at 24 hour intervals for evidence of multiplication of N. helminthoeca. For immunofluorescence studies monolayers on coverslips were stained by the direct fluorescent antibody method (6).

Monocyte cell cultures were composed of a heterogeneous population of cells which were similar in morphology to both monocytes and macrophages. Many cells of the macrophage type were multinucleated. Conclusive evidence of multiplication of N. helminthoeca was apparent after 72 hours when minute, darkly stained coccoid bodies were readily identified within the cytoplasm of a few cells. Initial development was usually in an area adjacent to the nucleus. The mode of multiplication was apparently by binary fission and in 5 to 7 days numerous organisms were present in cells throughout the monolayer. The organisms were usually dispersed loosely throughout the cytoplasm; however, in some cells the organisms appeared in aggregates or colonies within the cytoplasm. In many cells multiplication continued until the cell became packed with organisms. Their approximate size ranged from 0.3 to 1 u.

In unstained cultures microscopic evidence of cytopathic effects (CPE) was minimal for approximately 7 days. Extensive CPE then appeared suddenly and within 24 hours the monolayer was destroyed.

Normal monocyte cell cultures readily supported growth of N. helminthoeca. The organism was identified within the cytoplasm of an occasional cell 24 hours post-inoculation. Both development of the organism and cytopathogenicity appeared identical to that observed in monocytes prepared directly from infected dogs. The rate of multiplication and appearance of CPE was related to the amount of inoculum. Definitive titrations were not attempted. The organism was maintained in vitro over a period of 4 months by 23 serial passages in monocyte cell cultures. It was possible to preserve the organism propagated in vitro by freezing infected cell culture suspensions at -80°C. After 3 months the frozen suspensions contained viable organisms which were capable of multiplication when inoculated into normal monocyte cell cultures.

A laboratory Beagle which was infected with organisms propagated in cell culture prepared directly from infected dogs developed typical clinical signs of SPD. A fever was detected 5 days post-inoculation and death occurred in 13 days. Another Beagle which was infected with organisms propagated after 6 serial passages in monocyte cell culture developed similar clinical signs and died in 18 days. In both dogs the lesions at necropsy were compatible with those described for SPD. During the acute stages of infection N. helminthoeca was reisolated using the monocyte cell culture technique.

Specific immunofluorescence permitted identification of N. helminthoeca in monocyte cells stained with anti-SPD fluorescent antibody. The organisms appeared as brightly fluorescing, coccoid bodies similar to those observed in cells stained by the May-Grunwald Giemsa method.

When coverslip cultures singly infected with either N. helminthoeca or E. canis were stained in parallel with fluorescent antibody specific for each organism respectively, immunofluorescence was present only when the organisms were stained with their homologous antibody. Therefore, based upon the presence or absence of immunofluorescence there was no antigenic cross-reactivity between N. helminthoeca and E. canis.

A technique for the in vitro propagation of N. helminthoeca has not been previously described. In this study the monocyte cell culture technique proved to be a reliable method of propagation in which conventional aseptic techniques were employed without the use of antibiotics. The technique provides a practical means for growing the organism in quantities sufficient for more detailed studies of its physical and chemical characteristics.

Serological studies of the antigenic properties of N. helminthoeca have been limited and there has been no antigenic comparison of strains. Elokomin fluke fever has been described as a second antigenically distinct rickettsia-like disease transmitted by the intestinal fluke and associated with SPD (38). The precise relationship of Elokomin fluke fever to N. helminthoeca is not clear. The monocyte cell culture technique will permit isolation and antigenic characterization between the SPD agents or agent. Since dogs which recover from the acute infection are apparently immune to reinfection (39) it may also be feasible to develop a vaccine consisting of an avirulent strain of N. helminthoeca or a purified antigenic extract.

Preliminary observations by light microscopy indicate there is a difference in the manner of in vitro development by N. helminthoeca and E. canis. Development of N. helminthoeca is more typical of that described for the classical human rickettsial agents (40). It characteristically develops in loosely dispersed colonies, multiplies abundantly and demonstrates a high degree of cytopathogenicity. In contrast, E. canis characteristically develops in densely structured morulae and demonstrates a low degree of cytopathogenicity (6). A comparative study of the ultra structure of N. helminthoeca and E. canis will provide a better understanding of the true nature of the organisms and a more firm basis for resolving their proper taxonomic classification.

Two canine rickettsial agents have now been propagated in vitro by utilization of the monocyte cell culture technique. It is anticipated that the technique has broader application and can be used for the isolation and propagation of other rickettsial agents of animals and man. The technique offers several inherent advantages. This is the only known means for in vitro propagation of at least two rickettsiae. Blood collected during the acute febrile stage provides an uncontaminated source of viable organisms in a medium suitable for preparation of cell culture. The technique may provide a means whereby the diagnosis of a suspected rickettsial disease can be confirmed prior to development of the specific antibodies. Utilization of a similar technique to propagate the equine infectious anemia virus of horses (41) indicates some viruses could also be isolated in this manner.

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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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. Agency accession	DA 036445
2. Date of summary	72 07 01
Report control symbol	DD-DR&E(AR)636
3. Date prev. sum'ry	71 07 01
4. Kind of summary	D. Change
5. Summary scty	U
6. Work security	U
7. Regrading	NA
8. Dissem instr'n	NL
8a. 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	184
b. Contributing				
c. Contributing				

11. Title: (U) Diseases of Recruit Military Animals (09)

12. Scientific and technological areas: 010100 Microbiology

13. Start date: 68 07

14. Estimated completion date: CONT

15. Funding agency: DA

16. Performance method: 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	Fiscal year	a. Professional man yrs	b. Funds (in thousands)
Preceding	72	1.2	21
Current	73	2.1	30

19. Responsible DoD organization

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21. General use: Foreign intelligence not considered.

22. Keywords: (U) Military Dogs; (U) Recruit Dog Diseases; (U) Canine Parainfluenza; (U) Canine Rhinovirus; (U) Canine Coronavirus; (U) Canine Cell Culture

23. (U) To determine and characterize causative agents of infectious diseases occurring in military dogs during their induction and training; to determine how the infections are spread; and to develop effective control measures. Studies on agents associated with acute respiratory disease and diarrheal diseases are specifically emphasized. The potential 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) 71 07 - 72 06 Epizootics of respiratory disease occurring at induction and training centers from 1966 to 1968 affected approximately one-fourth of the dogs. The disease episodes disrupted training and shipment of the dogs to operational areas. Parainfluenza SV 5 virus was primarily related to the outbreaks. In the past year, more than 75 percent of newly procured dogs and all dogs from the Biosensor Research Facility at Edgewood Arsenal were susceptible to SV 5 infection on basis of serological test findings. The rate of SV 5 infections at the procurement center appears to have markedly declined from previous years. The transmissible agent recovered from sentry dogs involved in an epizootic of diarrheal disease appears to be a member of the coronavirus groups. Further studies on the pathogenicity, immunology, and other properties of the canine rhino-like virus and canine corona-like isolates have been carried out. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*Available to contractors upon originator's approval.

DD FORM 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 184 Diseases of recruit military animals

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Description.

Objectives are first to determine the etiology and epizootiology of respiratory disease in military dogs and to develop methods of control and prevention and second to isolate and characterize viruses of military dogs and laboratory animals which may be potential zoonotic agents or interfere with the utilization of the animals. Further studies were done on the epizootiology of parainfluenza SV5 in recruit military dogs, characterization of a viral isolate obtained from military dogs having a gastrointestinal disease, and on the characteristics of corona and rhino-like viruses recovered from dogs.

Progress.

1. Respiratory Disease in Military Dogs.

In the spring of 1966, an epizootic of respiratory disease occurred at the military dog induction and training centers. The disease episode disrupted the shipment and training of the dogs. In the next 2 years epizootics of respiratory disease continued to occur at the induction and training centers. In these epizootics approximately 25% of the dogs developed signs of upper respiratory disease. Parainfluenza SV5 was recovered from the initial outbreak [1,2] and the virus was found to be highly communicable. The SV5 infections occurred soon after recruitment and at the time of the respiratory disease cases.[3] In subsequent years, SV5 infections have continued at the recruitment and training centers affecting more than 80% of the dogs. This report summarizes further observations on SV5 infections in military dogs at the induction and training centers.

As observed in previous years, most newly procured dogs in 1971-72 were serotest susceptible to parainfluenza SV5 infection (Table 1). Seventy-nine percent or 137 of 174 dogs arriving at the Lackland reception center were devoid of SV5 antibody. This is a slight decrease from previous years in which the number of serotest susceptibles was nearly 90%. The dogs from California had the highest rate of positive reactors as 12 of 20 or 60% had SV5 antibody. The remaining serotest positive dogs came from 12 states representing all areas of the United

States. To date, 1004 of 1144 or 88% of newly procured dogs were found to be susceptible to SV5 infection.

Additional serum samples were obtained from 69 of the new dogs prior to shipment from the reception center. Approximately half of the dogs were at the reception center for 60 or more days. Only 3 converted to positive antibody status. In previous years antibody conversions to SV5 were demonstrated in approximately 50% of the dogs at the reception center. This observation suggests a decrease in the rate of SV5 infections from previous years. These findings are consistent with the few cases of respiratory disease observed at the reception center.

Table 1. SV5 Antibody Status of Newly Procured Military Dogs at Lackland, AFB

Study Period	Antibody Status		Tot.	Percent Sero-Suscept.
	No. Sero-Pos.	No Sero-Neg.		
1969 - 1970	67	618	685	90
1970 - 1971	36	249	285	87
1971 - 1972	37	137	174	79
TOTAL	140	1004	1144	88

Serological tests were also done on Ft. Benning dogs. Twenty-seven dogs shipped to Ft. Benning from the Bio-Sensor Unit at Edgewood Arsenal were found to be sero-susceptible to SV5 infection at the time of their arrival. Serum specimens were received from 91 dogs on departure from Ft. Benning. Eighty-one or 89% were serotest positive for SV5 antibody. These dogs originated from Lackland and were at Ft. Benning for at least one year. The time of these SV5 infections is unknown.

2. Studies of 1-71 Virus Recovered from Military Dogs with Gastrointestional Disease.

The isolation of a transmissible agent from the fecal specimen of a dog with diarrheal disease was described in the previous annual report (1 July 70-30 June 71). The agent, designated 1-71, produced giant cells in primary dog kidney cell cultures (PDKC) similar to those produced by canine corona-like virus L198R (Ann. Rept. - 1 July 69-30 June 70). During the past year further studies on the host range, growth, and properties of the 1-71 isolate were carried out.

In addition to PDKC, 1-71 produced cytopathic effects (CPE) in diploid German shepherd embryo (GSE) cell cultures (Flow Laboratories). In GSE cells, slight CPE were observed in 24 hours post infection and increased markedly at 36 to 48 hours post infection. The infected cells were larger in size and contained many nuclei. Inclusion bodies were not

seen in infected cells. Examination of the growth curve revealed maximal titer at 24 hrs when CPE were beginning. The initial increase in infectivity was detected 8-to-12 hours post infection. The 1-71 isolate did not produce CPE in primary bovine embryonic kidney, swine kidney, feline kidney cells, or in diploid feline lung, or continuous swine PK15 cells. The virus was not pathogenic for suckling or weaning mice by the intraperitoneal and intracranial routes.

Table 2 summarizes studies on the chemical and physical properties of the virus. Chloroform treatment markedly reduced the titer of virus. The virus was stable at pH 3.0 which might be anticipated for a virus recovered in the feces. A 100-fold reduction in virus titer occurred when the virus was filtered thru a 100nM millipore filter and the virus did not pass thru a 50nM filter. These findings indicate the virus is enveloped and slightly smaller than 100nM in size.

Table 2. Chemical and Physical Properties of 1-71 Virus

Test Virus (strain)	Treatment	Virus Titer (Log)		
		Treatment Before	After	Change (Before-After)
ICH (Cornell)	Chloroform	5.8	6.3	+0.5
Canine herpes (D004)	"	4.3	<1.0	>3.3
1-71	"	5.0	2.0	3.0
ICH (Cornell)	pH 3.0	5.3	5.4	+0.1
Canine Herpes (D004)	"	4.3	<2.0	>2.3
1-71	"	5.8	5.3	0.5
1-71	Filtration	6.5	6.3	0.2
	450nM*		5.5	1.0
	220nM		4.5	2.0
	100nM		<1.0	>5.5
	50nM			

*Millipore membrane filter of nanometer (nM) porosity.

To obtain additional information on the classification of the isolate ultra-thin sections of infected cells were examined by the electron microscope. These experiments were conducted in conjunction with Dr. A. Strano of the AFIP. Forty-eight hours post infection GSE cell cultures were fixed with gluteraldehyde and processed. Examination of the sections revealed enveloped virus particles in vesicles of the cytoplasm. The particles were round or oval varied in size from 50 to 90 nM in diameter and had an inner core of 35 to 45nM. From several particles projections could be discerned. The virus appeared to be forming from

the vesicle walls in the cytoplasm. These observations are consistent with the chemical and physical properties described above. The structure observed by electron microscopy would place the isolate in the coronavirus group.

Further studies on the properties and antigenic relationships of 1-71 and other corona-viruses are in progress.

3. Studies on the Infectivity of Canine Coronavirus Isolate L198R in Neonatal Dogs and Swine.

The recovery and characterization of a coronavirus, L198R, from a laboratory dog with fatal respiratory disease was described in the two previous annual reports. The L198R isolate was neutralized by transmissible gastroenteritis (TGE) virus antiserum at one-sixty-fourth of the homologous titer. Initial attempts to infect sero-test negative swine with the L198R isolate was unsuccessful. However, as TGE virus is most virulent in neonatal swine additional experiments were carried out. The pathogenicity of the L198R isolate for neonatal dogs was also determined.

For these experiments a TGE and L198R serotest negative pregnant sow and a TGE and L198R serotest negative pregnant dog were obtained. Three-to-four days after birth, the baby pigs and dogs were given approximately 1 million tissue culture infected dose$_{50}$ ($TCID_{50}$) of virus by the oral or intracranial route. Uninoculated litter mates were given a placebo preparation and kept with inoculated animals. With the exception of one puppy, no signs of disease occurred in either the baby pigs or dogs. On the third day after this puppy had been fed the virus, minimal signs of upper respiratory disease were evident. Sneezing and a serous nasal discharge persisted to the fourteenth day. L198R virus was recovered from rectal swabs of each puppy (inoculated as well as contacts) for 1 to 6 days. The virus was also recovered from the throat of the bitch on day 8. After infection each puppy and the bitch had low levels, i.e. 1:2 - 1:8 of L198R neutralizing antibody and all but 1 puppy developed similar TGE antibody titers.

In contrast to the puppies, the baby pigs did not shed virus or develop neutralizing antibody to either TGE or L198R viruses. These findings suggest that puppies can be infected with L198R virus but that baby pigs are refractory to infection and provide further evidence that TGE and L198R viruses are antigenically related. However, the low neutralizing antibody levels produced after infection require further studies to assure that undetected antibody is not preventing or modifying the infection.

Table 3. Experimental Infection of Newborn Dogs and Swine with Coronavirus L198R

Animal	Route of Infection	Virus Recovery	Neutralizing Antibody Response (Day) L198R (14)	(28)	(42)	(56)	TGE (14)	(28)	(42)	(56)
Bitch B15	contact	Day 8 throat	2	2	2	2	2	2	2	2
pup 1	"	Day 6 stool	-	0*	0	2	-	0	0	0
2	"	Day 11,13,17 stool	-	2	2	2	-	2	2	2
3**	per os	Day 7 stool	-	2	2	2	-	2	8	2
4	" "	Day 3,4,5,6,7&8 stool	-	0	0	2	-	2	2	0
5	" "	Day 4,6,7 stool	-	2	2	8	-	2	2	2
Sow 78	contact	0	0	0	0	0	0	0	0	0
piglet# 1	per os	0	0	0	0	0	0	0	0	0
2	" "	0	0	0	0	0	0	0	0	0
3	" "	0	0	0	0	0	0	0	0	0
4	" "	0	0	0	0	0	0	0	0	0
5	contact	0	0	0	0	0	0	0	0	0
6	contact	0	0	0	0	0	0	0	0	0
7	intra-cranial	0	0	0	0	0	0	0	0	0
8	"	0	0	0	0	0	0	0	0	0

* Less than 1:2

** Signs of upper respiratory disease occurred on day 3 thru day 14. The signs included sneezing and serous nasal discharge.

4. Further Studies on the Properties of Canine Rhino-like Virus (CRV) Isolates.

The recovery and characterization of 4 antigenically distinct CRV isolates were previously reported (1 July 69 - 30 June 1970 and 1 July 70 - 30 June 1971). The recovery of these agents was made possible by use of a canine cell line developed in this laboratory.[4] The CRV isolates differed from human rhinoviruses in being stable at pH 5.0 and were not heat stable in the presence of molar magnesium chloride. The CRV resembled the feline rhinoviruses (FRV) and the vesicular exanthema virus of swine (VEVS).[5] This report summarizes further studies on the physical properties of 2 representative CRV isolates. In addition, initial studies on the infectivity of the 3-68 isolate for puppies are reported.

To determine the buoyant density and size by electron microscopy approximately one-half liter pools of high titered virus were prepared. The virus pools were concentrated and purified by differential contrifugation. The virions were sedimented at 27,000 rpm for 3 hrs in the 30 head of a Beckman preparative ultracentrifuge. The concentrated virus preparation was either layered on or mixed with cesium chloride solution (refractive index 1·3800). The mixture was centrifuged at 33,000 rpm for approximately 20 hrs in the SW39 head of the Beckman ultracentrifuge. Following centrifugation, fractions were collected for infectivity and density determinations. For control purposes, poliovirus type 1 was centrifuged at the same time as the test virus. Polio type 1 virus had the expected peak titer at 1.333 g/ml. The test virus, L198T, had a density of 1.375 and 1.385 in the first and 1.385 and 1.390 in the second test. A similar value of 1.385 was obtained with the 3-68 isolate.

Following determination of the buoyant density, the fractions containing highest virus titers were pooled diluted with distilled water and centrifuged for 3 hr at 27,000 rpm in the 30 head of the Beckman ultracentrifuge. The pellet was resuspended in 0.05 ml of distilled water for examination in the electron microscope. Electron microscopic examination was done by Mr. Walter Engler of the AFIP in a Siemens electron microscope at 40K. The virus preparations were examined after negative staining with 2 per cent phosphotungstic acid (PTA). For size determinations a reference calibration grid was employed in each series of micrographs. The 3-68 virus was naked and both "empty" and complete virions were observed. The complete virions averaged 42 nM and the empty 43 nM in diameter. The L198T isolate was similar in appearance as 3-68 and averaged 36 nM in diameter.

Both the size and buoyant density of L198T and 3-68 more closely resemble the FRV and VEVS than the human rhinovirus. The FRV and VEVS have buoyant densities of 1.38 and measures 35-40 nM in diameter whereas the human rhinoviruses are 30 nM and have a buoyant density of 1.40[5].

To determine the pathogenicity of 3-68 virus for dogs a serotest negative pregnant dog was obtained. One month after birth of 3 puppies the bitch and two puppies were given approximately 100,000 $TCID_{50}$ of the virus by the oral and nasal routes. The remaining puppy received control tissue culture fluids by the same routes. Elevated rectal body temperature or other signs of disease did not develop and the virus was not recovered from throat or rectal specimens from any of the puppies or the bitch. The bitch and the 2 inoculated puppies developed neutralizing antibody on day 7 but the uninoculated puppy did not develop antibody. The neutralizing antibody titers ranged from 1:8 to 1:128. On day 35 the inoculated dogs were boosted and the neutralizing antibody titers increased to $\geq$512. Further studies on the pathogenicity and immunology of the CRV isolates are in progress.

5. Canine Viral Antibodies in Mammalian Wildlife.

During the course of a survey of Maryland wildlife mammals for selected zoonoses, infectious canine hepatitis (ICH) virus was recovered from 2 skunks.[6] Moreover, significant neutralizing antibodies for ICH were demonstrated in 63% of 94 skunks. These serological findings prompted additional examinations for canine adenovirus antibodies in sera from racoons, opossums and woodchucks which were trapped from the same area and during the same period the skunks were collected. In addition, the wildlife sera were tested for presence of antibodies to other viruses, viz, canine distemper (CD), canine herpes and parainfluenza SV5, which are commonly found in dogs.[7]

Neutralizing antibody to ICH and CD viruses were found in the serum specimens of skunks and racoons but not in opossums and woodchucks (Table 4). The CD neutralizing antibody was a common occurrence in racoons; 84% were sero-test positive. Two positive racoon serum specimens randomly selected for end titer determination each had titers of 1:256 or greater. Two of 25 skunk sera had CD neutralization antibody. Each had titers of 1:64. Both skunks and racoons have been reported susceptible to CD.[8] The finding of ICH neutralizing antibody in racoon extends the observations of Parker and his co-workers.[10] They found ICH complement fixing antibody in 1 of 9 racoons. As the complement fixation test may measure a group adenovirus reactions, the demonstration of neutralizing antibody indicates that ICH or a closely related virus was infecting the racoons. A positive racoon serum neutralized the skunk ICH isolate[6] and the reference ICH virus to the same titer (1:64).

Neutralizing antibody for canine herpes and SV5 virus were not found in sera for 4 species of wildlife.

Experimental infection and further field observations are required to evaluate the significance of skunks and racoons in the epizootiology of CD and ICH. The wide host range of these 2 viruses[8,9] make them ideal agents to study epizootiology of viruses in domestic and wild animals.

Table 4. Neutralizing Antibody to Canine Viruses in Mammalian *Wild* Animals

Animal	No. (%) Positive / Total Tested Against Virus			
	Infectious Canine Hepatitis	Canine Distemper	Canine Herpes	Parainfluenza SV5
Skunks	59/94 (63)	2/25 (8)	0/25	0/25
Racoons	6/50 (12)	21/25 (84)	0/25	0/25
Opossums	0/25	0/25	0/25	0/25
Woodchucks	0/9	0/9	0/9	0/9

Summary and Conclusions.

1. Respiratory Disease in Military Dogs.

Nearly 80% of newly procured dogs in 1971-1972 were serotest susceptible to parainfluenza SV5 infection. The rate of SV5 infections in dogs at the procurement center appears to have declined in the past year. Almost 90% of the dogs completing scout dog training at Ft. Benning, Ga., were serotest positive to SV5 virus. The dogs provided by the Biosensor Research team at Edgewood Arsenal, Maryland continue to be serotest susceptible to SV5 infection. Parainfluenza SV5 vaccines for use in dogs are currently undergoing commerical development. The high degree of susceptibility of dogs at procurement and at the Bio-Sensor Unit may warrant the trial of this product to prevent SV5 caused respiratory disease in military dogs. Experimental vaccine trials in German shepherds is recommended to evaluate the safety and potency of the produce for this breed. Further observations on the epidemiology of respiratory disease in recruit military dogs will be continued.

2. Studies of 1-71 Virus from Military dogs with Gastrointestional Disease.

Studies on the growth of 1-71 virus in German shepherd embryo cell cultures were carried out. Maximal infectivity titers occurred 24 hours post infection when cytopathic effects were beginning. Following electron microscopic observation, the virus was found in vesicles in the cytoplasm. The virion resembled coronaviruses in size and morphology. The 1-71 isolate did not produce cytopathic effect in bovine, swine, or feline cell cultures. Further studies on the pathogenicity and antigenic relationships to other coronaviruses are recommended.

3. Studies on the Infectivity of Canine Coronovirus Isolate L198R in neonatal Dogs and Swine.

L198R virus was fed to 3-to 4-day old dogs and pigs. The virus was

recovered from only the dogs and minimal signs of respiratory disease occurred in 1 puppy. A low level of L198R and transmissible gastroenteritis (TGE) neutralizing antibody developed in the dogs but not in the baby pigs. Further studies on the relationship of L198R and TGE are in progress.

4. Further Studies on the Properties of Canine Rhino-like Virus (CRV) Isolates.

Two CRV isolates had buoyant densities of approximately 1.38 and measured 36 to 42 nM in diameter. The first CRV isolate 3-68 was inoculated into 1-month-old-puppies by the oral-nasal routes. Signs of disease were not detected, nor was the virus recovered. The inoculated dogs developed neutralizing antibody. Further studies on the pathogenicity and immunological relations of the CRV isolates are in progress.

5. Canine Viral Antibodies in Mammalian Wildlife.

Sera of skunks, raccoons, opossums, and woodchucks collected in Maryland were examined for neutralizing antibodies to infectious canine hepatitis (ICH), canine distemper (CD), canine herpes, and parainfluenza SV5 viruses. Neutralizing antibody ICH and CD were found in the skunk and racoon serum specimens, but not in the sera of opossums or woodchucks. No neutralizing antibody to canine herpes or SV5 were found in any of the sera tested.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 184, Diseases of recruit military animals

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6481	72 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. DISB'N INSTR'N	8a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	3A061102A91C	00	183
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)
(U) Preparation of Tubercular Antigens (24)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
64 03	72 02	DA	B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 72 03 EXPIRATION: 72 02

b. NUMBER: DADA 17-64-C-9108

c. TYPE: S. CT d. AMOUNT: FS 9,347

e. KIND OF AWARD: EXT. f. CUM. AMT.: FS36,104

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING	72	0.3	7
CURRENT	72	0.3	4

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Resrch

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E.L.

TELEPHONE: 202-576-3551

21. GENERAL USE

Foreign Intelligence Not Considered

20. PERFORMING ORGANIZATION

NAME: George Washington University

ADDRESS: Washington, DC 20006

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

NAME: Affronti, Dr. L.F.

TELEPHONE: 202-331-6531

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Fife, E. H. Jr. DA

NAME:

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. Precede text of each with Security Classification Code.)

23. (U) Continued 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, for monitoring tuberculosis in the WRAIR non-human primate colony, and for current studies on the serodiagnosis of human tuberculosis in a military hospital.

24. (U) By procedures developed by the principal investigator and published in the scientific literature.

25. (U) 71 07 - 72 06. Regular deliveries of A-protein, C-protein and polysaccharide antigen fractions from M. tuberculosis have been made according to the agreement in the contract. Additionally, C-protein fractions were prepared from the atypical mycobacteria M. kansasii, M. battey, M. gause, and M. avium. Each antigen fraction currently being characterized by chemical, ultraviolet absorption and polyacrylamide gel electrophoresis analyses. Also the efficacy of the various antigens for the differential diagnosis of typical and atypical tuberculosis is being evaluated in the SAFA procedure. Hopefully, this will avoid the confusion that prevails because of inherent cross reactivity in the hypersensitivity tests. From 1 Mar 64 thru 29 Feb 72 this contract was funded from 910 funds. Starting 1 Mar 72 funding was from 71P-05-Microbiology funds, and will be reported under accession No. DAOB 7340. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*Available to contractors upon originator's approval.

DD FORM 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.

PII Redacted

Project 3A161101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 189, Preparation of tubercular antigens

Investigators
Principal: Lewis P. Affronti, Ph.D.
Associate: Earl H. Fife, Jr., M.S.

Description.

This Work Unit consists of a contract to prepare specific tuberculin antigen fractions and to supply the Department of Serology, WRAIR, with specified amounts of each antigen. Fractionation procedures developed by the principal investigator are used to isolate A-protein antigens from filtrates of M. tuberculosis cultures, C-protein antigen from the bacterial cells, and polysaccharide antigen from the cell walls of the tubercle bacillis. These antigens are used by investigators in the Department of Serology in studies on the serodiagnosis of simian and human tuberculosis, and for screening for tuberculosis in the animals of the nonhuman primate colony, WRAIR.

Progress.

In accordance with the contract, the following amounts of antigen fractions from typical and atypical Mycobacterium sp. were supplied to the Department of Serology during the present reporting period:

Antigen	mg	Organism
A-protein	3.0	M. tuberculosis
C-protein	7.5	M. tuberculosis
C-protein	2.0	M. kansasii
C-protein	2.0	M. battey
C-protein	2.0	M. gause
C-protein	3.0	M. avium

The antigens from both the typical M. tuberculosis and the atypical mycobacteria were prepared in the indicated manner by the procedures developed by the principal investigator[1]. The tubercle bacilli were cultured as surface pellicles on Proskauer-Beck medium for 6-8 weeks and then separated from the culture fluid by membrane filtration.

1. Preparation of A-protein antigen.

The culture filtrates served as the source of the A-protein antigens. The filtrates first were concentrated by pervaporation

to one-tenth of their original volume and then dialyzed vs distilled water to remove the dialyzable medium components and the salts that were concentrated during the pervaporation. The A-protein antigen fraction finally was precipitated from the dialyzed filtrate by treatment with 70% ethanol at pH 4.0.

2. Preparation of C-protein antigen.

The C-protein antigens were isolated from the organisms that had been separated from the culture medium by membrane filtration. The harvested mycobacteria first were suspended in isotonic saline and solubilized in a Branson Model 575 Sonifier. The extraneous debris then was removed from the sonicate by centrifugation in the cold (4°C) at 144,000 rcf for 100 min. The C-protein antigen finally was isolated from the supernate by isoelectric precipitation at pH 4.0.

Summary and Conclusions.

During the present reporting period, C-protein antigen fractions were prepared from four atypical Mycobacterium species as well as from the typical M. tuberculosis. The chemical and serological nature of each antigen currently is being characterized by chemical, ultraviolet absorption and polyacrylamide gel electrophoresis analyses. Additionally, the efficacy of the various antigens for the differential diagnosis of typical and atypical tuberculosis is being evaluated in the Soluble Antigen Fluorescent Antibody (SAFA) procedure. These latter studies are of more than academic interest since it has long been recognized that PPD tuberculin antigens prepared from various mycobacterial species exhibit considerable cross reactivity when tested in individuals presumably infected with the so-called atypical mycobacteria [4,5]. Furthermore, use of the SAFA test on humans and monkeys with naturally acquired atypical tuberculosis has provided indirect evidence suggesting that antibodies produced in response to the atypical organisms do not react well with antigens derived from typical M. tuberculosis. The present availability of antigens from both typical and atypical organisms will provide an opportunity to determine whether species or strain specificity can be demonstrated in the SAFA test and thus provide for differential diagnosis, minimizing the problems of cross reactivity that prevail in the hypersensitivity tests.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 189, Preparation of tubercular antigens

Literature Cited.

References:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 0454	72 06 30	CSRD-R&E AR-659

3a. TYPE PROG. SUMMARY	3b. KIND OF SUMMARY	3c. SUMMARY SCTY	3d. WORK SECURITY	4. RESEARCH	5a. DISEM RESTR	5b. SPECIFIC DATA CONTRACTOR ACCESS	6. LEVEL OF SUM
[illegible]	K. Completion	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. MO CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	190
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Include any Security Classification Code)

(U) Tropical Disease Bulletin Information Retrieval System

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

(U) 2600 Biology; (U) 0942 Computers

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
68 07	CONT	DA	B. Contract

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE

PROFESSIONAL MAN YRS	FUNDS ($ thousands)
PREVIOUS FISCAL YEAR	
CURRENT FISCAL YEAR	

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Inst of Research

ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: CompuMath, Inc.

ADDRESS: Silver Spring, MD 20910

PRINCIPAL INVESTIGATOR (Provide name, title, and telephone number)

NAME: Voccola, H. W.

TELEPHONE: 301-588-2531

SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS

NAME: Schafer, June A.

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Include any Security Classification Code)

(U) Information Retrieval; (U) Information Handling; (U) Computer Programming; (U) Text Processing

23. TECHNICAL OBJECTIVE; 24. APPROACH; 25. PROGRESS (Provide individually paragraphs identified by number. Proceed each with appropriate Security Classification Code)

23. (U) To develop computer techniques to create, maintain, index, and search large free text data bases of interest to the USAMRDC. Access to the information in large, non-indexed data bases such as The Tropical Disease Bulletin could be of great value to the Army, especially in the rapid development and support of research programs in new areas of interest. However, the cost of retroactive manual indexing is prohibitive and competent people trained in both subject area and indexing are not generally available. The purpose of this effort is to explore an alternate method that could be used to obtain this information.

24. (U) Rewrite most of the programs in FORTRAN so as to provide greater flexibility and enable us to use the WRAIR CAC 3300.

25. 71 07 - 71 06 The following were performed under this contract: (a) Completion of the Tropical Disease Bulletin (TDB) abstracts data base including editing to generate an [illegible] copy. (b) Title and author indexes for TDB volume 1-14 were generated and delivered to BIOSIS. (c) Subject indexes for 2 volumes were processed. (d) Consideration was given to the published works of several [illegible] in an attempt to derive a working (e) Binary Compression Table for representa[illegible] the TDB. A number of programs were written to generate and vary the [illegible] Master File; search this binary file; extract results from the full abstract data base and generate statistics. Completed programs and support documentation were submitted to the Division of Medicinal Chemistry, WRAIR, in July 1971; this consisted of master data files and tapes.

Availability to contractors upon originator's approval.

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, NOV 68 AND 498 MAR 69 FOR ARMY USE ARE OBSOLETE

PROJECT 3A061102B71Q
COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00
Communicable Diseases *and Im*munology

PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA
2. DATE OF SUMMARY: 81 07 01
REPORT CONTROL SYMBOL: CSD-DR&E AR 636

3. DATE PREV SUMMARY 4. KIND OF SUMMARY
5. SUMMARY SCTY 6. WORK SECURITY
7. REGRADING
8A. ORDER NUMBER
8B. SPECIFIC DATA CONTRACTOR ACCESS: [X] 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 | 3A161102BS10
b. CONTRIBUTING
c. CONTRIBUTING

11. TITLE (Precede with Security Classification Code)
Immunological Studies of Schistosomiasis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
Parasitology

13. START DATE: 7107
14. ESTIMATED COMPLETION DATE: 8306
15. FUNDING AGENCY: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE/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: 81: 2.0 | 30
FISCAL YEAR — CURRENT: 82: 2.0 | 30

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D.C. 20307
RESPONSIBLE INDIVIDUAL
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TELEPHONE: [redacted]

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army I[nstitute of Research]
Division of M&C
ADDRESS: Washington, D.C.
PRINCIPAL INVESTIGATOR (Furnish SSAN)
NAME: CADIEN, E. [redacted]
TELEPHONE: [redacted]
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]
ASSOCIATE INVESTIGATORS
NAME: MUN, A. S. DA
NAME

21. GENERAL USE
Foreign Intelligence Not Applicable

22. KEYWORDS (Precede EACH with Security Classification Code)
U; Parasitic; U; Schistosomiasis; U; Pathology; U; Primate; U; Chemotherapy

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

(U) The purpose of this research is to study various physiological, immunological and clinical aspects of parasitic diseases of military importance toward the goal of evaluating host and parasite factors of natural susceptibility, acquired resistance and the effectiveness of therapy with regard to the prevention, suppression and treatment of these infections.

(U) Through personal perusal of pertinent literature and discussion with other scientists both classified and now open is and used to set up controlled experiments.

(U) 81 10 - 82 06: Fifteen young chimpanzees were exposed to 50 to 100 cercariae of the Japanese strain of S. japonicum by single or multiple exposures. The animals were followed by clinical, parasitological, immunological and miscellaneous laboratory studies, and in some cases by endoscopies, serial biopsies and or X-ray examinations. After periods of 6 to 17 months, they were submitted to portal and hepatic pressure determination, and complete necropsies were performed including injection of portal collaterals, and determination of the worm and egg burdens. These studies showed that the chimpanzee is a suitable host for experimental studies on S. japonicum, is highly susceptible to infection, and develops pathological lesions similar to those seen in the toxaemic and hepatosplenic phases of human infection. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 71 - 30 Jun 72.

* Available to contractors upon originator's approval

DD FORM 1498 MAR 68 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A NOV 69 AND 498-1 MAR 68 FOR ARMY USE ARE OBSOLETE

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 001, Parasitic Diseases

Investigators
Principal: E. H. Sadun, Sc.D., Lib. Doc.
Associate: COL H. Worth Boyce, MC; A. W. Cheever; LTC D. G. Erickson, MSC; CPT A. J. Johnson, VC; F. von Lichtenberg

1. Immunity to parasitic infections.

Protozoal and metazoal infections frequently stimulate a vigorous immunological response which is partially cell-mediated and humoral. The specific antibodies produced have proved to be of both diagnostic value and of use in epidemiological surveys. Despite the established immunogenicity of parasites, their apparent capacity to evade the lethal effects of specific antibody is a fundamental feature of obligate parasitism. Parasitologists have long been impressed by this paradox and have coined terms confusing to immunologists, such as "premunition" and "tolerance," to describe the state of acquired, partial resistance associated with continuing low-grade parasitism.

There are several ways in which parasites might evade the host's specific immune defenses. Some, such as serological variation and anatomical location, concern the habits of the parasite; others, including the appearance of enhancing antibodies, the induction of tolerance, and the effects of antigenic competition, could involve the host's reaction. The discussions of this workshop have centered about these processes in relation to the survival of several parasite species within the immunized host.

I. Antigenic Variation and Parasite Immunity

A. Trypanosomiasis

During the usual course of an infection with trypanosomes in man and experimental animals, the initial high parasitemia is quickly brought under control by a host response involving lytic antibody. Subsequently, a growth-inhibiting antibody holds the parasitemia at a low level until a relapse occurs. In a typical infection several relapses occur, each successively brought under control by the host response and each less severe than the last. These waves of parasitemia are thought to correspond to the emergence on the parasite surface of new antigens; as soon as the host responds to one antigenic variant the parasite population evades the response by changing antigenic type. These antigens can be labeled with ferritin-conjugated antibodies and are located in a dense surface coat lying just outside the plasma membrane. Although originally thought to consist only of protein, the variant antigens are now known to contain carbohydrate as well. The dense surface coat contains other glycoproteins.

The variant antigens are continuously released by the trypanosome into the host's blood where they are called exoantigens.

Recently, various extensions of the surface have been detected on trypanosomes. These so-called plasmonemes are believed to be released from the parasite, and it has been suggested that they represent the sloughing off of an old membrane in preparation for the expression of a new one carrying a different antigenic variant. This speculation raises the question of the origin of new antigenic variants in the population of trypanosomes. Does the new variant arise from a mutant which is then selected because its multiplication is not affected by the antibodies present? Alternatively, can individual trypanosomes throw off surface antigens and synthesize new ones? This latter explanation implies that the genome of the parasite contains a number of genes for surface antigens, each of which can be turned off when an immune response is mounted against the protein it produces. At present, there is no evidence available which allows a choice to be made between these two possibilities. Whatever the mechanism for producing antigenic variation, it seems clearly established that the phenomenon allows the parasite to partially evade the hosts' immune response and set up a chronic infection.

B. Malaria

There is [illegible] evidence for synthesis of specific antibody during the pre-erythrocytic development of malaria parasites which follows mosquito infection. [illegible] inoculation of sporozoites taken from infected mosquitoes [illegible] with ultraviolet light or X-irradiation leads to immunity [illegible] sporozoite infection in bird, rat, and monkey malaria. The protective response is mediated partly by serum, since sporozoites heated with immune serum undergo morphological changes and suffer a considerable loss of infectivity; this effect is not complement dependent. Immune to sporozoites are fully susceptible to erythrocytic infections, demonstrating the specificity of the response in regard to developmental stages of the parasite.

Acquired immunity to natural malarial infection is directed mainly against the erythrocytic phase of the parasite although circulating gametocytes are apparently unaffected. Animals immune to the erythrocytic phase are still susceptible to pre-erythrocytic development, but do not manifest parasitemia. The role of serum antibody in protective immunity has been established by passive transfer in monkey, human, and rodent infections. Immune serum has also been shown to inhibit the cyclical proliferation of monkey parasites maintained in vitro. Immune serum does not affect the growth of intracellular parasites but inhibits red cell reinvasion after parasitic division. The action of immune serum is confined to IgG and IgM fractions, is not complement dependent, and requires at least two combining sites per antibody molecule. Claims that cell-mediated immunity plays a role in malarial resistance are, at present, based on inconclusive evidence.

Several plasmodial species are known to contain multiple strains which do not give cross protection as tested by superinfection tests. In addition, antigenic variants of single strains occur during the course of the asexual erythrocytic cycle in rodent, simian, and human malarias. These variants are recognizable on the basis of the schizont agglutination test which involves the interaction of IgG and IgM agglutinins with the surface of red blood cells containing mature parasites. During the course of chronic infections, each relapse is associated with the appearance of a distinct variant. Infection with a given variant appears to condition the host for a rapid immune response to many other variants. Such variants consequently give rise to low-grade infections.

These facts indicate that the specific immune response of the mammalian host can effectively eliminate given strains of the plasmodium but that chronic infections are maintained by continued antigenic variation during the course of somatic division. The mechanisms underlying variation and the potential number and chemical nature of variant antigens are not known.

2. Schistosomiasis

It is generally accepted that after infection with schistosomes man develops a resistance to reinfection. As early as 1916, Fujinami reported that people who lived in endemic districts in Japan were usually less seriously affected by schistosomiasis than were immigrants from other areas. Similarly, cattle brought to endemic areas from other parts of Japan became very ill and often died. However, those that recovered became much more resistant to reinfection. These initial observations were confirmed and extended by numerous epidemiologists and field investigators. Experimental laboratory animals develop acquired immunity to human species of schistosomes after infection.

Since schistosomiasis is of great medical, social and economic importance in vast areas of the world, many workers have studied the phenomenon of protective immunity and much interesting information has been obtained. An important reason for the failure to develop an effective vaccine is that, although we know that protective immunity occurs, we know very little of how it acts. Since the schistosomes are complex multicellular organisms with a complicated life cycle, involving specialized habitats in the host, it is very difficult to identify where acquired immunity acts. As pointed out by Humphrey, when a parasite goes through an elaborate life cycle with different surface antigens at each stage and when the right antibodies are likely to be important only when they come in contact with the right antigens in the right place and at the right time, one is faced with a monumental task in separating those which matter from the larger number of those which are irrelevant.

In the past decade there has been an explosion of information pertaining to immunology in general and the study of schistosomiasis has felt its impact. Several recent publications describe the complexities of schistosome antigens, others explore factors which may be important

in the production of disease through hypersensitive mechanisms and still others suggest that an antigen-antibody complex disease may result in glomerulonephritis. Until fully understood, these immunopathologic manifestations may be a further impediment to control by immunization in schistosomiasis. Moreover, it is well known that schistosomes can persist in chronically infected animals even after the animals are immune to further challenge infections. In this situation, which has been termed "concomitant immunity," the adult worm apparently stimulates an immune response which destroys the developing schistosomula but is not itself damaged by the immunity.

1. Vaccination Attempts

The degree of resistance acquired by vaccination in schistosomiasis varies greatly with the species of parasites studied, the host species, and the technique used in the process of vaccination.

(1) Non-living parasite material. Ozawa was able to protect dogs to a small degree of injections of saline suspensions of S. japonicum worms. Kawamura reported that animals which were given injections of worm extracts before being challenged developed a milder disease than the uninjected controls, and that the vaccinated animals harbored fewer and smaller worms which produced a significantly smaller number of eggs. Lin et al. succeeded in producing a barely detectable degree of protection by previous injections of saline suspensions of whole S. japonicum worms. Similar results were obtained by Sadun and Lin. However, several other authors were unsuccessful in immunizing various species of laboratory animals against S. japonicum by this method.

Most attempts to induce artificial immunization against S. mansoni have had even more limited success. Although mice injected with the cercarial antigens of S. mansoni were reported to be more resistant to infection than noninjected mice, Thompson was unable to obtain any noticeable degree of protection. Protection was induced in rats by vaccination with fresh homogerates of adult S. mansoni worms.

The inability of a dead vaccine to induce a high degree of resistance may be due to the difficulty of exposing the host to the immunizing agents over a long period of time. This, however, may be overcome in the future by the use of repository forms of antigen preparations. Another reason for the relative inefficacy of dead vaccines may lie in the difficulty of obtaining sufficient amounts of nondenatured "functional antigens." Since these antigens are probably present in only minute amounts at any given time, the logical approach to vaccination with dead antigen might be to cultivate the worms in vitro and to collect their excretions and secretions. However, suitable culture conditions for the extensive release of functional antigens have yet to be developed. Moreover, it is possible that if the functional antigens are very labile, they may be denatured by enzymes present in culture media.

(2) Passive transfer of serum. Except for early partial success obtained by Kawamura, nearly unanimous failure was reported in attempts to passively transfer resistance to either S. japonicum or S. mansoni from immunized to nonimmunized hosts. Failure to transfer immunity occurred even when three-quarters of the total blood volume was replaced with serum from immune animals or when antiserum was given over a period of seven weeks. Barely significant levels of protection were observed by Sadun and Lin by the injection of large amounts of immune serum in mice infected with S. japonicum.

(3) Living parasites. Numerous reports on the development of strong acquired immunity following infection with schistosomes have been reviewed by several authors.

(a) Exposure to the homologous parasite. Vogel and Minning reported that monkeys infected with male worms of S. japonicum were still protected one to three years after exposure to otherwise lethal challenging doses. Lin and his coworkers also found that mice infected with male worms developed a moderate resistance to superinfection. However, Hunter et al. found no marked immunological response to reinfection in mice, hamsters and rabbits with this parasite. Sadun observed in previously infected mice fewer worms from the challenging infection than in the controls. Furthermore, many of the worms from the immunized animals were considerably stunted.

As in artificial immunizations, attempts to induce resistance against S. mansoni following infection have been markedly less successful than with S. japonicum. Failure to demonstrate acquired resistance was reported by Olivier and Schneiderman after a single exposure of mice to male cercariae, by Stirewalt after a single mature bisexual infection of adult mice, and by Thompson after a single infection of adult mice, hamsters, guinea pigs and white rats. Hunter et al. reported no reduction in the numbers of worms maturing from a bisexual challenge. Meleney and Moore found that monkeys initially exposed to a single sex of cercariae and subsequently challenged by cercariae of the opposite sex gave no evidence of resistance. However, these authors reported that acquired resistance was observed when bisexual infection was induced. Resistance of rhesus monkeys exposed to S. mansoni re-infection was reported by numerous authors. Although no acquired resistance could be stimulated by adult worms injected intraperitoneally in monkeys, acquired resistance was induced by living adult S. mansoni worms transferred directly into the mesenteric veins of normal monkeys.

(b) Exposure to heterologous parasites. Evidence of protection conferred by infection with related schistosomes was reported simultaneously by different groups of investigators with different systems. The suggestion made on epidemiological grounds that exposure to cercariae of S. bovis might confer immunity on man to infection with S. mansoni has found considerable support from the experiments referred to above and other recent ones carried out in monkeys and rodents.

(4) Attenuated parasites. Irradiation of helminth larvae interferes with some of their physiological processes and frequently inhibits their normal development to adulthood. Standen and Fuller demonstrated that cercariae of S. mansoni failed to reach maturity in the mouse after exposure to ultraviolet radiation, but they were able to penetrate the skin and undergo the early stages of migration. Since then several groups of investigators have reported varying degrees of protection in experimental hosts induced by irradiated cercariae. However, detailed histopathological studies conducted on mice which were killed at regular intervals after having been exposed to irradiated cercariae showed that the death and disintegration of schistosomula was accompanied by local inflammation, particularly when large number of parasites died in the same organs. The host-tissue reactions to attenuated cercariae were strikingly similar to those observed following primary exposures of an abnormal host to nonattenuated cercariae and to those observed in the normal host which had become resistant to a secondary infection following a primary exposure to nonattenuated cercariae.

(5) Concomitant immunity. The transfer of adult S. mansoni directly into the mesenteric veins of rhesus monkeys protected the monkeys against a subsequent cercarial challenge. However, the adult schistosomes were able to evade the immune response which they themselves provoked. This phenomenon was termed concomitant immunity. The mechanisms by which it occurs may involve acquisition by the worms of a coating of molecules with antigenic determinants common to the host. The origin of the surface antigens of apparent host type is still unknown. They may be of host origin or may be synthesized by the parasites. Schistosomula grown in cultures containing human serum and erythrocytes acquire a human host antigen which is comon to A and B type red cells. Therefore, the insusceptibility of the adult worms to the immune response which they are known to provoke and their long persistance in the blood must be considered in any scheme contemplating control of schistosomiasis by immunization.

2. Immunopathology

(1) Immediate hypersensitivity. Allergic manifestations in man occur as a result of infection with all three species of human schistosomes. In persons previously exposed to infection, cercarial penetration is frequently followed by urticaria, asthmatic attacks, subcutaneous edema, leukocytosis and eosinophilia. A second recognizable clinical phase beginning between one and two months after exposure to infection and usually coincidental with the onset of egg laying of the worms consist of anorexia, fever, headache and weight loss. This has been known as the "toxemic" phase or "Katayama fever." It has been generally acknowledged that these clinical phases of schistosomiasis result from immediate hypersensitivity phenomena. Whereas primary exposure to cercariae is usually followed by no visible reaction, repeated exposures bring about a reaction usually seen immediately after penetration and followed within several hours by the development of large wheals accompanied by erythema, edema, and prutitus. Secondary reactions include edema and massive round-cell invasion of the dermis and epidermis. The wheal and flare type reaction involved in this dermatitis

could be relieved by the use of antihistamines and was ascribed to local anaphylaxis. Homocytotropic reagin-like antibodies in schistosomiasis were reported recently in rats and monkeys, rabbits, chimpanzees, human patients and in rhesus monkeys infected repeatedly with irradiated cercariae of S. japonicum. Homocytotropic antibodies could be detected in vivo by passive cutaneous anaphylaxis or in vitro by antigen-induced histamine release. A radioactive microprecipitin (RAMP) assay using iodinated extracts of S. mansoni cercariae as antigen also detected antibodies which were thermolabile and whose reactivity could be prevented by mercaptoethanol reduction and alkylation. A striking similarity was observed with the time course development of homocytotropic antibodies and antibodies detected by the RAMP assay. Both reactions could be inhibited by absorption with goat antirabbit gamma E serum but not with antirabbit gamma G serum.

(2) Delayed hypersensitivity. Although infectious granulomata were suspected to be a manifestation of delayed hypersensitivity, significant data supporting this concept have been gathered only in the last few years. Von Lichtenberg showed that in contrast to reactions against insoluble foreign body particles such as divinyl-benzene polymers, reaction of mice to purified viable schistosome eggs begins slowly in the unsensitized host. Although no cellular reaction was seen to occur before 24 hours after injection, the egg granulomata reached peak size at 15 days and thereafter receded slowly, disappearing as late as 5 to 6 months after intravenous injection. The cell population of the granulomata was mixed and included granulocytes, eosinophils, non-epithelioid macrophages as well as giant cells, epithelioid cells and fibroblasts. Lymphoid cells participated early and appeared usually in the periphery of the cell reaction. Soluble antigen initially diffused out of whole eggs and eventually both inside and outside the egg shell, mainly in the center portion of the granuloma where a substantial part of this material was sequestered and ultimately metabolized.

Warren and his colleagues have suggested that the development of granulomata around schistosome eggs is due to delayed hypersensitivity. Mice previously sensitized through schistosome eggs developed larger granulomata than unsensitized animals. This response was specific and sensitization was transferrable from infected to uninfected mice by lymph node and spleen cells, but not by serum. Granuloma formation was significantly diminished in mice which had been neonatally thymectomized, treated with antilymphocyte serum or with immunosuppressant drugs. Egg granulomata were also reduced in a "[illegible] skin-like" disease in mice in which cell mediated response is repressed. However, granuloma formation was unaffected by measures directed primarily against antibody-mediated reactions such as total body irradiation.

Soluble antigens isolated from S. mansoni eggs elicited granuloma formation and delayed type skin reactions in the absence of detectable circulating antibody. Specifically sensitized mice produced an enhanced granulomatous response around antigen-coated bentonite particles. Bentonite particles coated with pure S. mansoni egg lysophosphatase also produced granulomata which resembled those produced by purified schistosome eggs.

Lymphoblastogenesis was demonstrated in chimpanzees infected with S. japonicum by incorporation of tritiated thymidine into lymphocyte DNA in response to an antigenic stimulus. DNA synthesis increased progressively in all infected animals and reached a peak 4 months after exposure to infection.

(3) Cellular reactivity to antigens. There is histological evidence of a vigorous cellular reactivity in human and experimental animals infected with schistosomes. Since Fairley's early observations there has been speculation about the possible role of adult worm antigens in the marked granulomatous and exudative host response. Detailed investigations in chimpanzees infected with S. japonicum indicated the development of diffuse inflammatory and fibrotic lesions in large portal fields and in the bilharzomata, as well as severe vascular alterations in these sites. Portal vein lesions have been observed in S. mansoni infected persons and in S. japonicum infected rabbits, but those seen in the chimpanzee have been the most spectacular. Necrotizing arteritis has been reported in human transverse myelitis due to S. mansoni. These lesions were observed near sites of heavy egg deposition in our chimpanzee studies. Since arteritis is frequently associated with immunologically mediated injury such as serum sickness, this finding suggests that circulating antigen-antibody complement complexes may play a role in schistosomiasis.

The association of glomerulonephritis and hepatosplenic schistosomiasis was suggested in human patients. Glomerular lesions were observed in most of the chimpanzees infected with S. japonicum that had developed pipestem fibrosis of the liver. Our observations and similar ones conducted in humans emphasized the significance of glomerulopathy induced by schistosomiasis. Kidney sections from chimpanzees infected with S. mansoni and S. haematobium also showed minor focal glomerular changes comparable to the least diseased animals in the S. japonicum study. The finding of an association of renal lesions with cases of hepatorenal schistosomiasis raises interesting questions about the possibility that the immune responses to schistosome antigens may play a part in the pathogenesis of the renal lesions. Circulating antigen-antibody complexes can produce glomerular lesions with deposits containing antigen-antibody and complement which localize on the epithelial side of the glomerular basement membrane. Of interest are observations by electron microscopy of mesangial thickening and deposits of osmophilic material, similar to lesions which have recently been described in other types of glomerular disease. Present data are still too tenous to determine with certainty whether we are dealing with immune complex disease, hepatoglomerular sclerosis or autoimmune disease. Based on the description of circulating schistosomal antigen on the finding of prominent hyaline droplets in glomeruli, and on the admittedly minimal evidence obtained thus far on host glomerular globulin deposits, it seems likely that a schistosome glycoprotein antigen might be implicated. These recent reports have stirred up widespread interest and it is likely that the result of intensive studies currently being conducted in several laboratories may provide an answer to this question within the near future.

This synoptic and admittedly incomplete review indicates that immunization in schistosomiasis is indeed possible and that the degree of resistance induced varies greatly with the species of parasites, with the host species, and with the technique used in the process of vaccination. The basis of immunity in schistosomiasis is still obscure and it is not yet certain which stage in the life cycle of the parasite is primarily responsible for stimulating resistance. The main immunogenic stimulus appears to be related to excretory antigens released during the metabolic activity of the worms. Alternatively, an unstable somatic antigen may be present primarily in the schistosomes.

Studies conducted in the past few years suggest that we have the apparent paradox that humoral and cell-mediated specific immunity which may play a protective role may also be harmful to the host. Immediate and delayed hypersensitivity, local inflammatory responses and glomerulopathy may be the result of immune phenomena, whether mediated by humoral antibodies or by sensitized cells. It is important to be aware of these possibilities so as to avoid attempts at using vaccines for schistosomiasis control before the mechanisms of acquired immunity and immunopathology are well known. As yet, control of schistosomiasis by vaccination is not in sight. We do not even have means of isolating functional antigens or of reproducing in vitro the complete schistosome life cycle. However, there is no reason why this could not be accomplished if adequate research support were provided. Since no communicable disease has ever been eradicated until effective prophylactic immunization was developed there is a need for greatly expanded fundamental research conducted in close collaboration between parasitologists and immunologists.

Research in schistosomiasis has been hampered in the past by lack of experimental animals in which the disease as seen in human beings could be observed. In the past few years, however, we have been able to reproduce all the manifestations of human schistosomiasis in chimpanzees. These findings could help considerably in understanding the processes involved in immunity and immunopathology with either of the three species of schistosomes and might provide us with some of the basic information which will ultimately lead to the development of a safe and effective vaccine.

II. Anatomical Location and Parasitic Infection

While parasites appear to be protected from the immune response during intracellular phases of development, e.g., the malaria parasite in the red cell, it is not clear to what extent the specific anatomical location of other parasites, e.g., those within the gut, influences the afferent or efferent expression of the immune response.

There is definite evidence for specific immune rejection of helminth parasites by the host. This is obvious for parasites living in the lumen of the gut, but can be observed as well on those which have an intimate association with various other tissues. The immunity of the host is expressed either by massive expulsion of the worms, by inhibition of their

reproductive ability, or by a stunting of their larval form. These processes ultimately prevent or delay the formation or mature adult stages.

Because of the size of these parasites and the complexity of their life cycles, no clear cut evidence yet exists concerning the mechanisms involved in acquired immunity. Unlike protozoan infections, homocytotropic (γG_1 and γE-like) antibody can be clearly demonstrated. In fact, most parasite worm infections elicit an immediate type of hypersensitivity and the development of high-titer reaginic antibodies. This response may be related to the uniquely complex helminth life cycles which permit intimate contact with the mucosa of the respiratory gastrointestinal systems during migration of the parasites within the mammalian host.

All evidence accumulated so far indicates that production of large amounts of reaginic antibody takes place only when an actual infection occurs. Conversely, immunization with helminth extracts fails to elicit reagins or, at best, only produces these antibodies at a very low titer and at the same time stimulates little protective immunity. It has been postulated on this and other circumstantial evidence that reaginic antibodies are required for an adequate immune rejection of helminths.

III. Host Responses which Modify Immunity to Parasites

Experiments with rodent malaria have indicated that preliminary immunization with crude antigen extracts lead to increased mortality on challenge with the living organism. There is some evidence from passive transfer experiments that this effect may be due to enhancing antibody of the γG class. Similar experiments have been carried out with piroplasm infections, but enhancement is not yet a well-established phenomenon among protozoal infections.

Among helminth infections enhancement may occur with a filarial parasite. A first infection of rats with a hamster strain of the parasite results in rejection, but with subsequent challenges parasite growth is enhanced. Similar enhancement can be induced in rats by passive transfer of small amounts of homologous immune serum although larger transfers cause parasite rejection.

No convincing demonstration of tolerance in the strict immunological sense has yet been made in parasitic infections.

Antigenic competition has been observed in leishmanial infections in guinea pigs. Immunization of the host with antigens unrelated to the parasite, i. e., oil in water emulsions of Corynebacteria or Mycobacteria, caused diminished resistance to subsequent leishmanial infection. Resistance against this protozoan is considered to be primarily cell-mediated, and it is suggested that antigenic competition results in defective coordination between lymphocytes and macrophages involved in the induction and expression of cell-mediated immunity.

There have been a number of reports that malarial infections in mice may compete with and suppress the immune response to other non-malarial infections. In African children infected with malaria the response to tetanus toxoid was diminished as compared to children without the disease.

2. Experimental infections with Schistosoma japonicum in chimpanzees.

Investigations conducted with chimpanzees experimentally infected with Schistosoma mansoni and Schistosoma haematobium revealed that the course of infection, the location of worms, the egg distribution, the immunologic and biochemical findings and the development of lesions in various organs closely resemble those observed in man. Studies in macaque monkeys infected with a human strain of S. japonicum showed that lesions in these animals simulate those observed in human autopsy cases. However, recent histopathologic studies in four chimpanzees infected with S. japonicum demonstrated lesions which much more closely resemble those in man.

Since schistosomiasis japonica constitutes such a serious health menace and is the most severe of the three major schistosome infections of man, the need for a better understanding of the disease is great. Therefore, an attempt to determine the pathophysiologic sequence of schistosomiasis japonica was made by observing parasitologic, clinical, serologic, pathologic and radiologic changes in chimpanzees following single and multiple exposures to varying doses of S. japonicum cercariae. By using this unique animal model it has been possible to study some of the relations between the worm and egg distribution, the extent of pathologic changes and the time sequence of the development of lesions.

Fifteen young chimpanzees, eight males and seven females, (Nos. 9, 11, 13, 15, 16, 18. 59 192, 466, 467, 744, 782, 783, 361 and EA) originating in West Africa were experimentally infected. The 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 1.

TABLE 1

Exposure of chimpanzees to cercariae of S. japonicum

Chimpanzee No.	Sex	Weight (*kg*)		No. cercariae per exposure	No. of exposures	Total no. of cercariae	Cercariae per kg	Duration of infection (*months*)
		at exposure	at necropsy					
783	M	19.5	26.1	50	1	50	3	9
466	M	8.2	5.5	750	1	750	91	3
361	M	16.8	15.9	750	1	750	45	5
15	F	23.2	23.0	750	1	750	32	7
192	M	16.3	13.4	750	1	750	46	8
16	M	25.9	31.8	750	1	750	29	17
EA	M	36.5	34.0	2,000	1	2,000	55	2
13	F	23.6	19.5	2,000	1	2,000	85	8
18	F	20.9	23.2	2,000	1	2,000	96	17
9	F	21.8	22.7	50	5	250	11	17
11	F	22.2	27.7	50	6	300	14	17
744	F	11.1	7.3	750	3	2,250	203	5
467	M	7.9	7.3	750	3	2,250	285	6
782	F	9.0	8.1	750	3	2,250	250	6
59	M	19.5	19.9	2,000	2	4,000	205	17

Five additional animals (Nos. 1, 2, 3, 4 and 7) were used as uninfected controls. Splenectomy was performed on most of the chimpanzees for use in an unrelated experiment with Plasmodium falciparum. These animals were utilized after termination of the malaria infection by therapy, and were healthy when selected for the present work. At necropsy it was found that accessory spleens had developed in some splenectomized animals. Previous studies indicated that splenectomy had no demonstrable effect on the host response of primates to schistosome infections except for a reduction in the rate of self cure in Macaca mulatta.

Cercariae of the Japanese strain of S. japonicum were obtained from pools of 50 to 100 infected Oncomelania hupensis nosophora snails which had been exposed 2 to 4 months earlier to miracidia obtained by hatching eggs from livers of infected albino mice. The chimpanzees were exposed under light anesthesia with phencyclidine hydrochloride after the abdominal hair had been removed with clippers and the skin had been washed with dechlorinated water. The snails were crushed between glass slides and the cercariae were rinsed into a beaker of dechlorinated water. By means of a 4 x 5 mm monofilament nylon loop, cercariae were counted by microscopic observation and were placed on the damp abdominal skin of the chimpanzees. Mice were similarly exposed to 30-40 cercariae from the same suspension and used as infection controls. At autopsy, 6-10 weeks after exposure the percent recovery of adult worms from these controls varied from 60 to 75%.

Feces of each chimpanzee were examined weekly for schistosome eggs. One gram samples were concentrated by the formalin-ether-buffered alcohol technique and the entire sediment was examined microscopically in a Sedgwick-Rafter chamber. Egg viability was determined by miracidial hatching. Protoscopies were performed 1, 2, 3, 5, 8 and 16 months after exposure to infection. These included collection of mucosal biopsies for histopathologic studies and scrapings for parasitologic examinations. Esophagoscopies were performed on four chimpanzees one month before necropsy.

Blood was collected from these chimpanzees at regular intervals for leucocyte differential counts, hematocrit and prothrombin time determinations. Serum specimens were obtained before exposure and at monthly intervals thereafter for biochemical and serologic studies. Biochemical determinations included fasting serum glucose, blood urea nitrogen, total serum protein and serum paper electrophoresis. Blood ammonia was also determined.

Passive cutaneous anaphylaxis (PCA) with cercarial extracts as antigen was studied as described previously. Lipid-free extracts of S. mansoni cercariae were used in the PCA tests since insufficient amounts of S. japonicum cercariae were available for antigen extraction. Previous investigations had indicated that cross reactivity exists between these two species in the above tests.

Several of the chimpanzees were subjected to laparotomy at 7 and 11 months after infection. At this time the abdominal viscera were inspected, portal and intrahepatic pressures were measured and a wedge biopsy of the liver was removed for histologic examination. The portal pressure was measured at laparotomy and before necropsy using a Sanborn 267A pressure transducer and a model 301 Sanborn Amplifier-Recorder. A 19-guage needle was inserted into a branch of the inferior mesenteric vein and connected to the transducer by tubing filled with heparinized saline. Intrahepatic pressure was measured through a 15-gauge needle. A 19-gauge needle was used as a trocar within the 15-gauge needle during insertion to a depth of 3 to 5 cm into the liver. The larger needle was then filled with heparinized saline during trocar withdrawal, and was connected to the transducer. After 1 to 2 minutes, when the pressure had stabilized, a reading was taken. The needle was then partially withdrawn and the pressure was again recorded after 1 to 2 minutes. The level of the inferior vena cava was used as the zero reference point.

Cardiac catheterization was performed on two animals about one month before necropsy. The animals were anesthetized with phencyclidine hydrochloride (1mg/kg) and nembutal (26mg/kg) and a catheter was introduced into the right ventricle via the internal jugular vein. In three chimpanzees the right ventricular and pulmonary artery pressures were measured just prior to necropsy. After opening the abdomen, a #14 needle containing a polyethylene catheter was inserted into the right ventricle through the diaphragm. After removal of the needle, right ventricular pressure was measured; the catheter was then advanced into the pulmonary artery.

Hepatic blood flow and blood volume were estimated at 60 days after exposure to infection and again at 30 days before necropsy using microaggregated ^{125}I labeled human serum albumin. On each occasion, 9 infected and 5 uninfected animals were used.

An attempt to quantitate portasystemic collateral circulation was made by injecting 0.3 to 1.0 x 10^6 polystyrene beads 50 to 80 microns in diameter into a branch of the inferior mesenteric vein 5 to 10 minutes before death. For this purpose S-X8 Bio-Beads were suspended in water and washed onto a monofilament nylon cloth (Nytex) with a 73 micron aperture. Beads passing through the cloth were discarded. One lung and samples of the liver were removed prior to perfusion and digested with 4% KOH for determination of numbers of beads in these organs.

Lymphangiograms: Pontamine sky blue was used to observe the lymphatic drainage of the liver. Ten mgs of the dye in 0.2 ml saline were injected into the liver parenchyma near the middle of the anterior margin of the liver. Subcapsular and hilar lymphatics were observed continuosuly for the next five minutes and at intervals thereafter for 10 or 15 minutes, and were inspected again when each chimpanzee was killed about 30 minutes after dye injection.

During necropsy examinations the mesenteric-portal circulation was perfused separately from the intrahepatic circulation by ligation of the portal vein 1 to 2 cm from the liver. The abdominal viscera were removed, and 0.85% saline containing 325 mg of pentobarbital per liter was pumped into the inferior mesenteric, superior mesenteric and celiac arteries by placing a cannula into each artery in turn. As worms flushed from an opening in the portal vein, they were collected on a stainless steel screen in a capsule as described previously. The pulmonary vessels of one lung were also perfused. Recovered worms were counted by sex and measured unfixed. After perfusion of the mesenteric-portal system, colloidal latex was injected into the distal segment of the portal vein which had been cut at the hepatic hilus. Latex filled porta-systemic collaterals were identified during the course of the dissection.

The number of eggs in various tissues was determined after digestion in 4% potassium hydroxide at 37 C for 12 to 18 hours. About 90% of the intestine and mesenteries were digested. Smaller proportions of the liver and lungs were used. Mature eggs in the tissues were counted in samples of the liver, colon and small intestine digested in a 0.3% solution of pepsin in acidified saline for 6 to 12 hours. These counts were made less than 24 hours after the autopsy was begun. Viable, but immature, embryos were seldom differentiated by stage and were not categorized in counting. The number of mature eggs in the tissues was calculated by multiplying the proportion of mature eggs by the number of eggs in the tissues, as calculated from KOH digests. Methods for pathologic and histologic studies have been described previously.

Parasitologic Observations.

No schistosome eggs were found in the stools of the chimpanzees before experimental infection and for a number of weeks afterward. The onset of patency as determined by detection of eggs in the feces varied from 5 to 9 weeks (Table 2). The number of eggs in the feces increased

TABLE 2

Egg excretion and worm recovery in chimpanzees infected with S. japonicum

Chimpanzee No.	No. cercariae	First eggs detected (*week*)	Peak egg count (*week*)	NEPGF* Mean	NEPGF* Max	Duration of infection (*months*)	No. worms recovered M	F	Total	Percent recovery	Size of worms (*um*) M	F
783	50	8	31	20	73	9	2	3	5	10.0	16	20
466	750	6	9	670	1,830	3	186	175	361	48.1	18	20
361	750	5	13	129	240	5	69	70	139	18.5	18	20
15	750	8	27	9	41	7	4	4	8	1.0	ND	ND
192	750	6	16	291	740	8	25	20	45	6.0†	16	21
16	750	7	31	30	81	17	12	12	24	3.3	16	23
EA	2,000	6	8	92	190	2	352	382	734	36.7	15	23
13	2,000	6	19	93	240	8	6	6	12	0.6†	14	24
18	2,000	8	56	104	300	17	17	18	35	1.7	12	18
9	250	6	23	37	130	17	10	9	19	8.0	16	27
11	300	8	31	44	110	17	26	28	54	18.0	14	25
744	2,250	6	14	526	2,357	5	289	315	604	26.8	9	14
467	2,250	6	13	607	1,426	6	177	176	353	15.7	15	18
782	2,250	6	24	452	2,987	6	90	108	198	8.8	10	17
59	4,000	8	52	178	975	17	110	120	230	5.7	12	20

* Number of eggs per gram of feces.
† Perfused about 3 hours after death.

rapidly and reached a peak shortly after patency. Numbers of adult worms recovered by perfusion are listed in Table 2. The recovery rate of adult worms varied from 0.6 to 48.1%. The relatively low worm recoveries from 4 animals (15, 16, 18 and 59) probably occurred because insufficient water was placed on the skin with the cercarial suspension to allow complete cercarial penetration before the inoculum had dried. Since perfusion of chimpanzees Nos. 13 and 192 was performed some hours after death and was incomplete due to extensive clotting, it is probable that the number of worms recovered was considerably less than the number actually present. No consistent difference was observed in the percent recovery and location of the worms in the animals which were exposed to a single dose of cercariae and those which were exposed to multiple doses. Adult worms recovered by perfusion were active and well developed, and both sexes were present in comparable numbers. Approximately 3% of the worms recovered were found in the pulmonary arteries in four (466, 744, 467, EA) of the five animals infected with more than 10 worm pairs per kg body weight (Table 3). All females found in the lungs were immature. A single male worm was also found in one lightly infected chimpanzee (No. 15).

Most of the eggs recovered from tissue digests were in the liver and large intestine (Table 3). However, in three animals (Nos. 466, 744 and 467) approximately one-fourth of the eggs were in the small intestine. These were the only animals harboring more than 20 worm pairs per kg of body weight. Eggs were found in the lungs of most of the animals, but in only three of them (Nos. 192, 744 and 467) did this organ yield more than 10% of all eggs recovered by digestion from the various organs. The number of eggs in the tissues was generally related to the number of female worms recovered and the duration of infection.

TABLE 3

Number and distribution of eggs in various organs

Chimpanzee No.	Worm pairs per kg B.W.	Eggs per female†	Mean no. eggs per gram of tissue (1,000's)					Percent distribution in various organs				
			Liver	Small intestine	Large intestine	Lung	Mesentery	Liver	Small intestine	Large intestine	Lung	Others
783	0.1	155	0.4	0.1	0.1	0	0	60	9	29	0	2
466	31.8	79	19.9	18.4	14.8	7.5	0.5	42	28	24	5	1
961	4.4	72	2.5	0.2	2.7	1.5	1.2	44	1	47	5	3
15	0.2	119	0.5	<0.1	0.1	0	0	81	0	19	0	0
192*	1.5	473	7.1	1.0	4.4	7.1	0.7	38	3	28	28	3
16	0.4	157	1.4	0	0.6	0	0.1	72	0	26	0	2
EA	10.9	39	2.7	<0.1	7.4	0.1	0.2	32	0	67	0	1
13*	0.3	936	4.3	0.2	3.0	0.3	0.1	61	1	36	1	1
18	0.8	220	2.6	<0.1	1.3	0.5	0.1	59	0	37	2	2
9	0.4	115	0.9	<0.1	0.3	0	0	78	1	21	0	0
11	1.0	220	2.5	<0.1	0.5	0.1	0.2	78	1	18	0	3
744	34.4	80	9.2	14.8	21.9	14.4	0.9	18	25	45	11	1
467	24.1	85	20.9	14.4	9.1	10.7	1.8	48	22	19	10	1
782	12.6	71	5.7	<0.1	8.6	6.8	0.7	31	0	58	8	3
59	6.0	140	5.1	0.8	7.3	2.1	0.7	51	3	40	2	4

* These chimpanzees died several hours before perfusion; worm recovery was probably incomplete.

† These values indicate thousands of eggs per female worm found in the tissue digest.

The number of eggs recovered from the tissues was about 40,000 per worm pair 2 months after infection and ranged from 110,000 to 220,000 in those animals infected for 17 months. The number of eggs per gram of liver generally correlated well with the number of worm pairs per kg of body weight (Fig. 1). The number of eggs per mature female worm as determined from tissue digests usually increased with the average duration of infection (Fig. 2).

In one chimpanzee (No. 9) a mature S. mansoni female was recovered at perfusion. In another (No. 16) three mature S. mansoni worms (2 males and 1 female) were recovered. Although no S. mansoni eggs were detected in the stools of these or any other animals during the course of the experiment, KOH digestion demonstrated that an estimated 25,000 and 60,000 eggs, respectively, of this species were present in the tissues of these two chimpanzees at necropsy.

Clinical Observations.

All animals gained weight early in the experiment. This trend was later reversed, particularly in the heavily infected animals. After exposure, small papules with erythema appeared at the site of cercarial penetration. These became more prominent up to 48 hours after exposure, and were no longer visible after three or four days. No signs of illness were observed during the first six weeks after exposure. However, in the animals exposed to heavy cercarial doses, blood was observed in the stools intermittently after 8 weeks of infection. At the same time the biochemical tests indicated liver decompensation had occurred, some animals lost their apetites, showed rapid weight loss and became lethargic and gaunt. Five

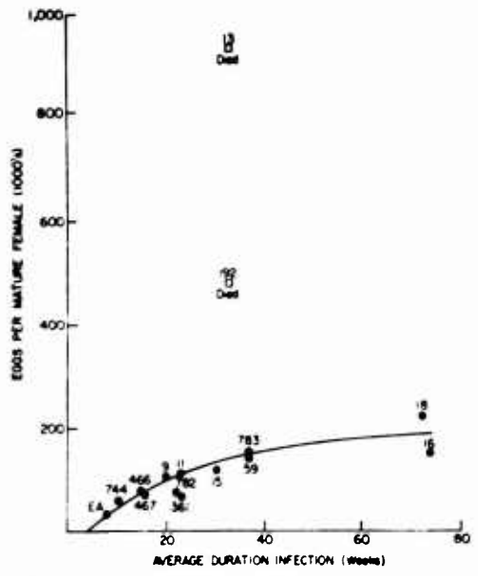


FIGURE 2. The number of *S. japonicum* eggs recovered from tissue digests per mature female worm related to the average duration of infection in weeks.

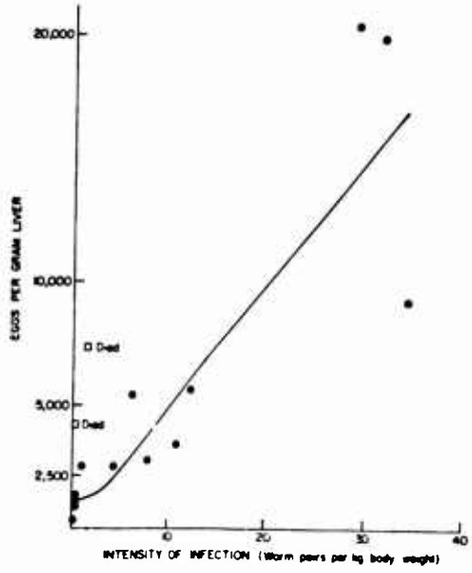


FIGURE 1. Regression graph relating the number of *S. japonicum* eggs per gram of liver to the intensity of infection (worm pairs per kg of body weight)

chimpanzees either died as a result of infection or were moribund when taken for necropsy (Nos. 466, 744, 467, 192, 13). Hematologic determinations indicated that anemia had developed in all infected chimpanzees. A marked increase in prothrombin time was observed in most of the animals with severe or prolonged infections (Table 4).

Proctoscopic examinations revealed occasional raised, discrete, 1-3 mm erythematous mucosal areas in some animals. Pale, raised granular areas of mucosa were also seen in one chimpanzee (No. 59) three months after exposure and thereafter. Mild to moderate lymphoid hyperplasia was seen frequently. Diffuse proctitis was present in some animals, and gross white exudate was also seen on occasion. Mucosal scrapings fixed in Schaudinn's solution and stained with trichrome revealed pinworms and their eggs, Balantidium coli and Troglodytella abrassarti in most animals. S. japonicum eggs were only occasionally seen in mucosal scrapings and press preparations of biopsies. No other parasites were found. On two occasions stained scrapings of mucosa covered with white pasty material resembling a purulent exudate contained masses of the ciliate, Troglodytella. Histopathologic examinations of biopsies showed chronic mild, nonulcerative inflammation of the lamina propria.

Biochemical Observations.

An increase in blood urea nitrogen (BUN) was observed in four chimpanzees (No.s 13, 18, 59, 192) in the latter part of the infection, ranging from 16 to 189 mg % as compared to 5 to 9 mg % in the controls. These animals had shown great clinical abnormalities resulting from infection. In one of them (No. 13) extremely high values were observed on two occasions (64 and 189 mg %). A striking increase in blood ammonia was observed in two chimpanzees (Nos. 13 and 192). Values of 700 and 800 mg/100 ml, respectively were reached in these two animals just before death. This contrasts with values of between 100 and 200 obtained in the uninfected controls. A varying degree of hypoglycemia was observed in most of the heavily infected chimpanzees toward the latter part of this study. Fasting blood glucose values as low as 46 and 37 mg % of glucose were obtained in two chimpanzees (Nos. 11 and 59) in the terminal stages of the disease. In addition, in chimpanzee No. 13 a value of 10 mg % glucose was obtained 10 minutes after death. For 2 days before death this animal had been comatose and responded dramatically to an intravenous solution of 5% glucose in saline.

Total serum proteins increased ... most animals as the infection progressed (Table 5). The largest increases occurred in the chimpanzees with infections of long duration and particularly in those showing the greatest clinical deterioration (Nos. 59, 18, 16, 11 and 13). The average total protein for the infected animals was 7.6 gm % at the time of exposure to infection and 12 gm % at the end of the experiment. A decrease in the albumin (Table 6) was observed in the severely infected chimpanzees as the infection progressed. Alpha-1, alpha-2 and beta-globulin levels in the infected animals remained essentially unaltered. However, there was a striking increase in gamma globulin which accounted for the increases in total serum proteins (Table 7). The greatest increases in

TABLE 4

Prothrombin time (%) in chimpanzees at monthly intervals after infection with S. japonicum

Chimpanzee No.	Values (%) at given months after first exposure																
	0	1	2	3	4	5	6	7	8	9	10	11	12	14	15	16	17
783	ND*	ND	99	75	63	68	ND	77	82	76	—	—	—	—	—	—	—
466	ND	ND	81	23	—	—	—	—	—	—	—	—	—	—	—	—	—
361	ND	74	40	19	15	14	—	—	—	—	—	—	—	—	—	—	—
15	79	100	ND	82	—	—	—	—	—	—	—	—	—	—	—	—	—
192	ND	ND	77	42	40	ND	57	53	19	—	—	—	—	—	—	—	—
16	93	100	ND	98	ND	ND	ND	ND	ND	ND	75	66	56	68	77	82	69
EA	ND	92	90	ND	—	—	—	—	—	—	—	—	—	—	—	—	—
13	ND	ND	89	59	53	40	53	65	44	—	—	—	—	—	—	—	—
18	68	100	ND	80	—	—	—	—	—	—	39	27	25	22	20	14	24
9	93	100	ND	86	ND	ND	ND	ND	ND	ND	99	67	77	67	63	65	62
11	93	100	ND	86	ND	ND	ND	ND	ND	ND	84	ND	65	56	63	59	61
744	ND	ND	64	75	30	—	—	—	—	—	—	—	—	—	—	—	—
467	ND	ND	94	43	40	40	—	—	—	—	—	—	—	—	—	—	—
782	99	89	52	ND	ND	53	63	52	—	—	—	—	—	—	—	—	—
59	100	93	ND	95	ND	ND	ND	ND	ND	ND	23	19	19	14	19	19	21

* Not done.

TABLE 5

Total serum proteins in chimpanzees at monthly intervals after infection with S. japonicum

Chimpanzee No.	Values (*g per 100 ml*) at given months after first exposure																
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
783	8.9	9.4	8.1	8.0	7.3	8.1	8.9	8.2	9.2	—	—	—	—	—	—	—	—	—
466	8.1	7.3	7.1	5.7	—	—	—	—	—	—	—	—	—	—	—	—	—	—
361	ND*	ND	6.3	ND	8.1	9.7	—	—	—	—	—	—	—	—	—	—	—	—
15	7.1	7.5	7.6	8.1	7.6	8.5	7.1	7.5	—	—	—	—	—	—	—	—	—	—
192	7.7	7.9	8.5	9.4	ND	9.1	10.5	10.4	—	—	—	—	—	—	—	—	—	—
16	7.0	7.6	7.9	8.6	8.9	9.1	8.3	9.3	9.3	9.4	9.9	9.3	9.2	ND	9.5	9.8	10.7	11.3
EA	ND	7.4	8.2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
13	9.8	9.5	9.4	9.5	10.4	ND	10.6	10.9	12.6	—	—	—	—	—	—	—	—	—
18	6.9	7.3	8.2	8.4	9.5	9.5	9.3	9.0	10.2	10.2	12.4	11.6	10.2	10.4	ND	11.7	11.8	12.0
9	6.5	7.0	7.3	7.8	7.8	7.0	7.0	7.0	7.0	7.2	6.0	7.6	8.7	9.6	10.2	10.0	10.9	—
11	6.6	6.9	7.3	7.3	6.8	7.4	6.7	7.3	7.3	7.3	7.6	7.8	8.3	9.8	10.2	12.6	11.8	—
744	7.9	6.9	7.4	7.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
467	8.3	6.5	6.4	8.0	6.8	ND	7.7	—	—	—	—	—	—	—	—	—	—	—
782	7.1	7.5	7.5	ND	9.1	10.9	9.3	9.1	—	—	—	—	—	—	—	—	—	—
59	6.5	7.6	8.6	9.0	8.6	8.6	10.5	10.6	10.6	11.2	12.0	12.4	11.2	ND	13.4	13.1	13.5	12.6
Mean	7.6	7.6	7.7	8.1	8.3	8.8	8.7	8.9	9.4	9.1	9.6	9.7	9.5	9.9	10.8	11.4	11.8	12.0

* Not done.

TABLE 6

Serum albumin in chimpanzees at monthly intervals after infection with S. japonicum

Chimpanzee No.	Values (*g per 100 ml*) at given months after first exposure																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
783	2.3	2.6	2.7	2.7	2.4	3.0	2.4	2.5	2.8	—	—	—	—	—	—	—	—	—
466	2.2	2.6	1.8	1.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
361	ND*	ND	1.5	1.3	1.3	1.1	—	—	—	—	—	—	—	—	—	—	—	—
15	3.5	3.4	3.2	3.7	3.2	3.0	3.0	3.1	—	—	—	—	—	—	—	—	—	—
192	2.8	1.9	1.6	1.7	ND	1.8	1.8	1.8	—	—	—	—	—	—	—	—	—	—
16	3.3	3.5	3.3	3.2	3.4	3.4	2.9	2.7	2.4	2.4	2.5	2.5	2.3	ND	2.1	2.1	2.1	2.3
EA	ND	2.7	2.4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
13	2.7	3.1	2.8	2.0	1.7	ND	1.5	1.5	—	—	—	—	—	—	—	—	—	—
18	3.3	3.0	2.1	2.3	2.6	2.3	2.2	1.9	2.2	1.9	1.9	2.1	1.9	ND	2.1	1.9	2.0	2.3
9	2.9	2.8	2.9	3.1	3.2	3.3	3.5	2.9	2.6	2.6	2.7	2.3	2.4	1.9	2.0	2.2	2.5	—
11	2.9	3.0	3.1	3.2	2.9	3.1	3.4	3.1	2.1	2.2	2.5	2.4	2.7	2.2	2.2	2.4	2.4	—
744	2.5	2.3	1.5	1.3	—	—	—	—	—	—	—	—	—	—	—	—	—	—
467	3.1	2.6	1.8	1.7	1.0	ND	1.2	—	—	—	—	—	—	—	—	—	—	—
782	3.1	2.7	1.9	ND	2.0	1.9	1.4	1.7	—	—	—	—	—	—	—	—	—	—
59	3.4	3.6	3.0	3.2	2.8	2.8	3.0	2.6	2.4	2.2	1.3	1.0	1.0	ND	1.0	1.1	1.2	1.2
Mean	2.9	2.8	2.4	2.3	2.4	2.6	2.4	2.4	2.4	2.3	2.2	2.1	2.1	2.0	1.9	1.9	2.0	1.9

* Not done.

TABLE 7

Serum gamma globulin in chimpanzees at monthly intervals after infection with S. japonicum

Chimpanzee No.	Values (*g per 100 ml*) at given months after first exposure																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
783	2.8	2.6	2.2	2.5	2.3	2.4	2.8	2.7	3.0	—	—	—	—	—	—	—	—	—
466	2.4	2.0	2.3	2.6	—	—	—	—	—	—	—	—	—	—	—	—	—	—
361	ND*	ND	2.2	ND	3.3	4.4	—	—	—	—	—	—	—	—	—	—	—	—
15	1.3	1.4	1.7	1.8	1.7	2.0	1.7	1.8	—	—	—	—	—	—	—	—	—	—
192	2.4	2.6	3.8	4.6	ND	4.5	5.4	5.2	—	—	—	—	—	—	—	—	—	—
16	1.5	1.4	1.8	2.0	2.1	2.2	2.4	2.5	3.5	3.5	3.8	3.5	3.7	ND	4.0	4.3	5.2	4.4
EA	ND	1.2	3.2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
13	2.3	2.4	3.1	3.9	5.1	ND	6.1	5.6	6.6	—	—	—	—	—	—	—	—	—
18	1.1	1.4	2.3	2.8	3.5	3.8	3.9	4.1	4.7	5.5	8.4	4.4	4.4	ND	5.9	6.0	5.7	6.2
9	1.8	1.8	2.0	2.0	1.7	1.3	1.3	1.4	1.6	1.6	1.2	1.9	2.7	3.9	4.3	4.2	4.7	—
11	1.4	1.4	1.3	1.3	1.3	1.2	1.1	1.5	1.8	1.8	2.2	2.4	2.8	3.9	4.8	4.6	5.0	—
744	2.2	1.4	3.1	3.2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
467	1.3	1.5	1.5	3.2	3.3	ND	4.0	—	—	—	—	—	—	—	—	—	—	—
782	1.7	1.8	2.8	ND	4.1	5.3	4.6	3.3	—	—	—	—	—	—	—	—	—	—
59	1.4	1.4	2.4	2.4	2.6	2.6	3.0	4.5	4.5	6.2	7.6	7.9	7.2	ND	10.1	8.8	7.1	7.2
Mean	1.8	1.7	2.4	2.8	2.8	3.0	3.3	2.8	3.8	3.7	4.7	4.0	4.1	3.9	5.8	5.6	5.6	5.9

* Not done.

gamma globulin levels occurred in the animals showing the greatest degree of hepatic dysfunction (Nos. 59, 192, 18, 13). Values as high as 10.1 gm % of a gamma globulin were observed in one animal (No. 59). A typical electrophoretic pattern of the serum of this animal is shown in Figure 3. Although some correlation was observed between worm burdens and the time at which significant increases in serum protein and gamma globulin levels occurred, no consistent differences were observed between the chimpanzees infected by a single exposure and those given multiple exposures.

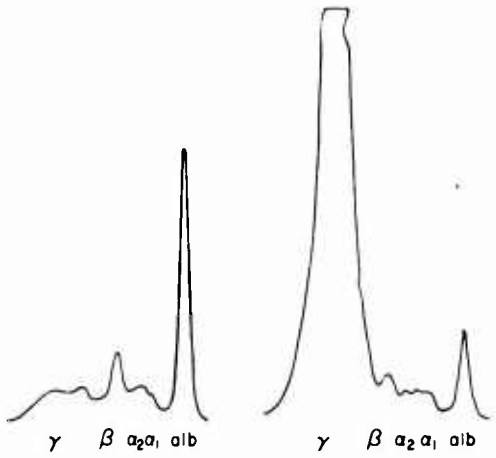


FIGURE 3. A typical electrophoretic pattern of serum from chimpanzee No. 59 before exposure (pattern on left) and after 14 months of infection with *S. japonicum*.

Passive Cutaneous Anaphylaxis (PCA).

Cutaneous reactivity was successfully transferred from 5 of the 15 infected chimpanzees to recipient tuberculin-negative Rhesus monkeys. The time course development of reaginic antibodies varied considerably and these antibodies persisted for only a relatively brief time. In 10 animals no reaginic activity was demonstrated at any time.

Blood Flow and Collateral Circulation.

Obstructed intrahepatic portal branches were seen macroscopically and microscopically. In chimpanzees Nos. 192 and 467 the obstruction was complete, since saline pumped into the hepatic veins during perfusion could not reach the portal vein. Porta-systemic collateral venous circulation was well developed. Submucosal esophageal varices were seen during esophagoscopy or following injection of latex into the portal vein

during necropsy. Collateral veins were also identified on the serosal surface of the esophagus, on the abdominal wall and joining the splenic vein to that of the left adrenal. Direct portacaval connections were also present. Individual collaterals were usually less than 0.3 cm in diameter, but there was evidently very effective decompression of the portal system, since the portal pressure was only slightlt elevated, even in animals with complete intrahepatic portal obstruction (Table 8). The intrahepatic pressure paralleled the portal pressure.

The estimated hepatic blood flow in infected chimpanzees was slightly decreased as compared to that in uninfected animals, but the degree of change showed no relationship to the extent of portal fibrosis or portal obstruction. Two of the animals with very severe hepatic lesions (Nos. 18 and 59) had normal hepatic blood flow. The blood volume was moderately decreased.

The size and number of porta-systemic collateral veins visualized by latex injection in this study correlated poorly with other indicators of the degree of shunting. One animal with complete portal obstruction showed only moderate collaterals, although 43% of polystyrene beads injected into the mesenteric circulation (and 100% of those recovered in tissue digests) were in the lungs. In another animal (No. 744), 25% of injected beads were recovered from the lungs, but only minor collaterals were identified macroscopically. No injected beads were recovered from the liver or lungs of 8 chimpanzees.

The right ventricular and pulmonary artery pressures were normal in the few animals thus studied (Nos. 9, 16, 59 and 782). Only one of these animals (No. 782) had large numbers of eggs in the lungs.

TABLE 8

Physiological measurements in chimpanzees after infection with S. japonicum

Chimpanzee No.	Body weight (*kg*)	Blood volume (*ml/kg*)	Hepatic blood flow (*ml/min/kg*)	Pressure (*cm saline solution*)	
				Portal	Intrahepatic
783	26.1	81	16	16	14
466	5.5	ND*	ND	15	8
361	15.9	81	16	21	21
15	23.0	ND	ND	ND	ND
192	13.4	ND	ND	24	22
16	31.8	92	15	15	ND
EA	34.0	ND	ND	16	15
13	19.5	88	12	17	19
18	23.1	85	21	18	21
9	21.5	79	14	13	13
11	27.7	72	14	15	15
744	7.3	ND	ND	10	11
467	7.3	ND	ND	15	8
782	8.1	102	14	13	13
59	19.9	112	19	25	23
Mean (experimental)		88	15.7 ± 0.8	16.6	15.6
Mean (control)		104	19.1 ± 1.1	16.0	ND

* Not done.

The hilar lymphatics were visible within two minutes after injection of pontamine dye into the liver parenchyma of two chimpanzees (Nos. 59 and 361) and advanced portal fibrosis. In these animals the lymphatics were dilated, and the thoracic duct of No. 59 was dilated and totuous. In the other animals in which lymph flow was studied, the findings were unremarkable.

Gross Pathologic Observations.

The gross pathologic findings varied in degree and distribution, and with a few exceptions, were directly related to the number of female worms recovered and the number of eggs found in the tissues (Table 9). Alterations were found in the liver, intestines, lungs, abdominal lymph nodes and soft tissues, spleen and kidneys.

Liver.

Hepatomegaly, with liver borders palpable well below the costal margin, was present in some animals. In others, liver size was within normal limits. Capsular and parenchymal granulomas were visible grossly in all infected animals. These varied in size from barely visible to 4 mm in diameter, and in color from whitish-gray to yellow. The liver surfaces were usually smooth, but were finely granular or bosselated in a few animals with advanced lesions (Fig. 4). The liver capsule was thickened in the more chronic infections. The degree of portal fibrosis could be appreciated only after sectioning. Only mild, focal granulomatous lesions were seen in chimpanzee Nos. 783 and 15. One chimpanzee (No. EA), in addition to massive granulomatous involvement, exhibited a delicate, translucent thickening of many of the finer portal branches, with an occational prominent enlarged triad (Fig. 5). All other animals showed variable degrees of pipe-stem lesions of the portal fields (Table 10). The most striking lesions were seen in chimpanzee No. 59, which showed a broad, continuous tree-like broadening of the entire portal framework (Fig. 6). A similar pattern was seen in several other long-term severe infections (Fig. 7). A finely arborescent fibrotic portal pattern was seen in chimpanzee Nos. 9, 11, 16 (Fig. 8), 782 and 466. In several other animals (Table 10) marked focal variation of the portal fibrosis was apparent (Fig. 9). This varied from coarsely broadened triads to mildly enlarged ones, from translucent, to opaque portal fields. Organizing thrombi were occasionally observed in portal veins (Fig. 10), many of which appeared thickened. In some livers (Table 10), a delicate nodularity of the parenchyma and a fine greyish streaking were noted, especially in their subcapsular portions (Fig. 8). The liver parenchyma showed variable degress of dark brown pigmentation or mottling. In one chimpanzee (No. 361) there was green discoloration due to bile stasis.

Intestines.

All animals showed patchy colonic involvement except one which harbored only four female worms (No. 15). The patches were granular elevations of the mucosa. They varied in color from tannish-brown to yellow

TABLE 9

Necropsy findings in chimpanzees infected with S. japonicum

Chimpanzee No.	Infection (*months*)	NEGLT* (1,000's)	No. of females	Globulin (g%)	Prothrombin time (%)	Gross pathological abnormalities					
						Ascites	Liver 1 to 4	Large intestine	Small intestine	Lungs to + +	Esoph collaterals	Enlarged lymph nodes
783	9	0.4	3	3.0	76		0–1	Patches, cecum; some scattered lesions	-	ND†	ND†	Mesentery, retroperitoneum
466	3	19.9	175	2.6	23	20 ml	3–4	Ulcerated patches, sigmoid and transverse colon	Patches, proximal and distal ileum, jejunum	+ +	ND	Mesentery, transverse colon
361	5	2.5	70	4.4	14		4	Patches, sigmoid and hepatic flexure	-	+ +	+	Liver hilus, retrogenital mass, pararectal mass
15	7	0.5	4	1.8	82		0	No focal lesions	-	Rare 0.1 nodules	ND	Mesentery
192	8	7.1	20‡	5.2	19		4	Patches; sigmoid, hepatic flexure, and descending colon	-	+ + granulomas	ND	Retrorectal mass, retroperitoneal mass, periaortic masses
16	17	1.4	12	4.4	69	—	3	No focal lesions	-	-	+	Liver hilus
EA	2	2.4	373	3.2	90	220 ml	1	Lesions in 50% colon, patches in appendix	-	-	-	Liver hilus, mesocolon
13	8	4.3	6‡	6.6	44	—	4	No focal lesions	-	-	+	Left mesocolon, periaortic mass, peripancreatic mass
18	17	2.6	18	6.2	80	—	3–4	Patches, sigmoid, splenic flexure, and cecum	-	+ +	+	Liver hilus, mesocolon
9	17	0.9	9	4.7	62	—	2	Patches, splenic flexure and rectosigmoid	-	-	-	Mesentery, retroperitoneum
11	17	2.5	28	5.0	61	—	3	Patches, sigmoid and appendix	-	-	+	Mesocolon
744	4	9.2	251	3.2	30	—	4	Patches, low sigmoid and ascending colon	-	+ +	-	Liver hilus, mesentery, peripancreatic
467	5	20.9	176	4.0	40	1,525 ml	4	Patches in 50% mucosa of sigmoid and ascending colon	Patches, mid-jejunum	+ +	+	Retroperitoneal mass
782	6	5.7	102	3.3	52	—	3–4	Lesions, hepatic flexure, transverse colon, and rectosigmoid	-	-	+	Not recorded
59	17	5.1	120	7.2	21	—	4	Patches, sigmoid and splenic flexure	-	-	+	Hilar retroperitoneal mass, transverse mesocolon

* NEGLT = Number of eggs per gram of liver tissue

† Not done.

‡ Died, perfusion probably incomplete.

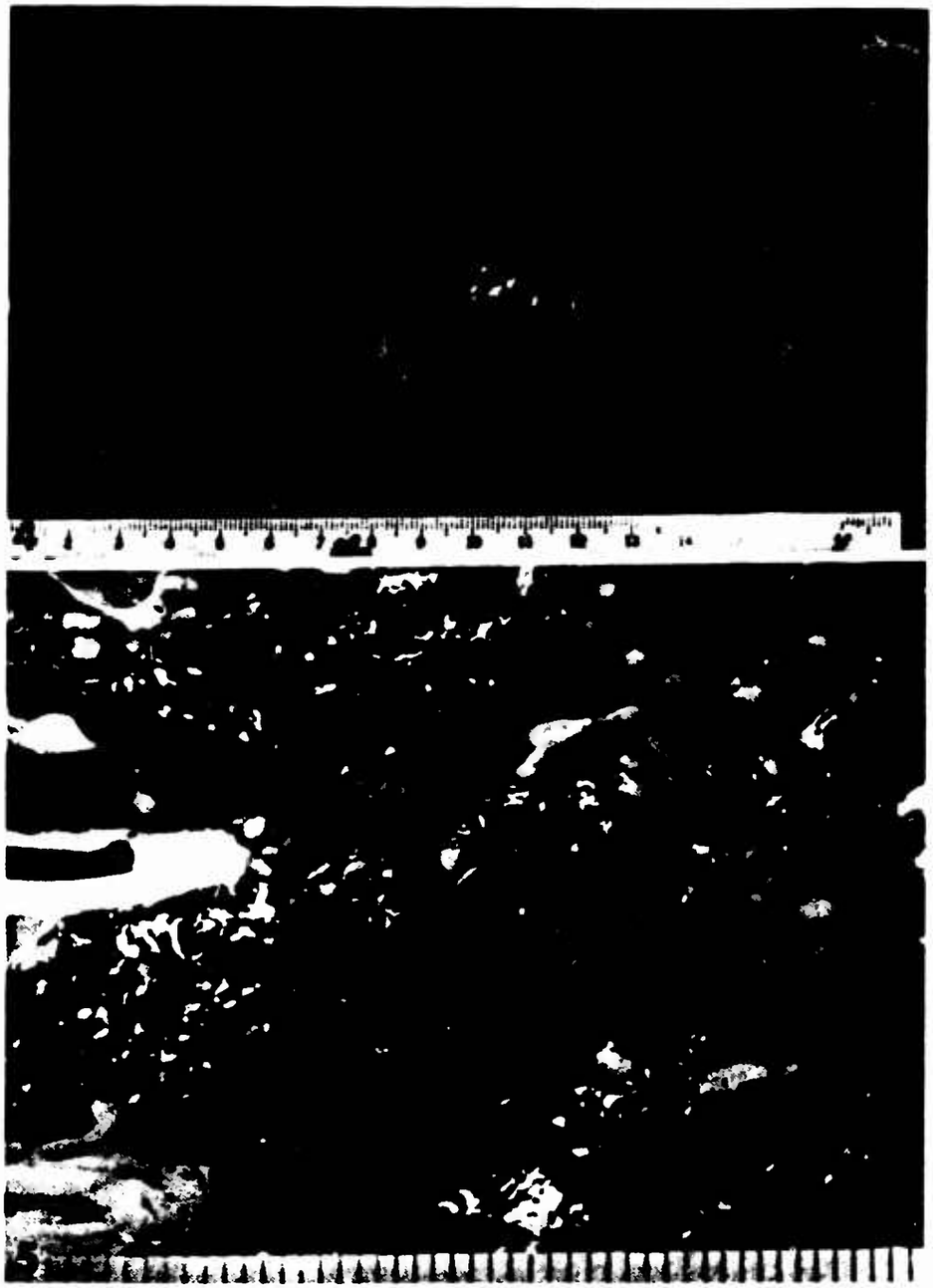
TABLE 10

Histopathological findings in chimpanzees infected with S. japonicum

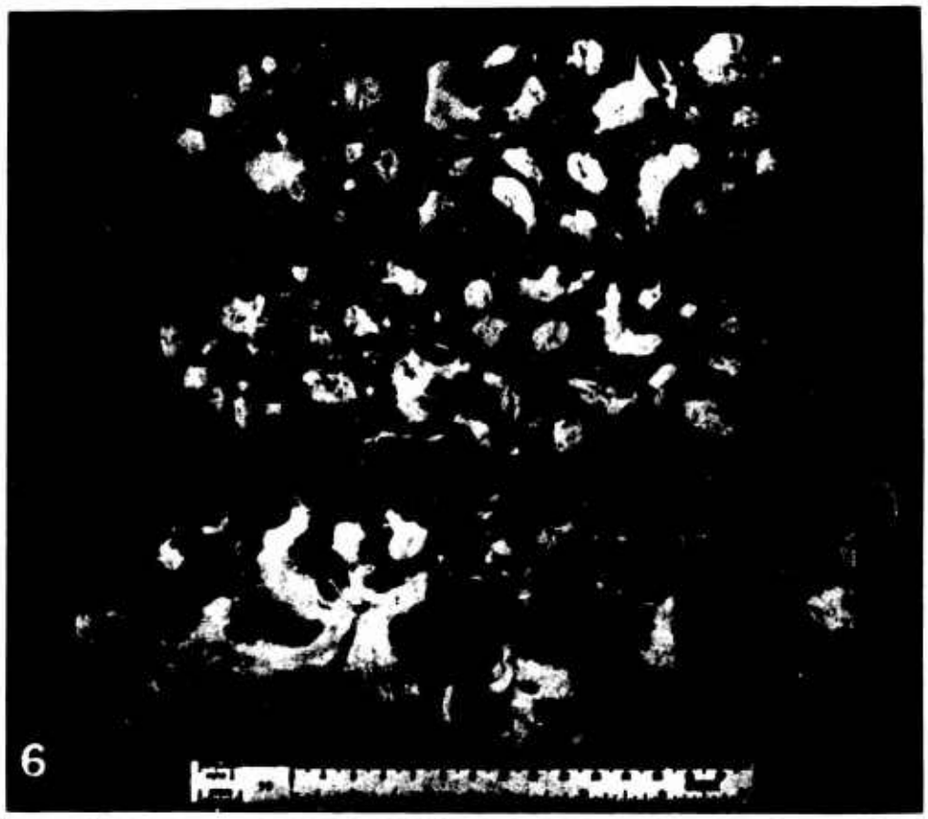
No.	Body weight (kg)	Eggs/g liver (1,000's)	Months	Index	Liver* 0	I	II	III	Portal fibrosis type†	Terminal triad fibrosis	Eggs/g lung	Granul.	Lungs: Diffuse lesion	Pulmonary arteritis	Kidneys: Glom. path.
783	26.1	0.4	9	4	0	13	1	0	1)			0			
15	23.0	0.5	7	5	0	12	2	0	1)			0			
EA	34.0	2.7	2	19	0	0	13	0	2)	S		0.1	+		+
9	22.7	0.9	17	26	0	1	4	8	2)	S		0			+
11	27.7	2.5	17	28	0	1	4	6	4)	S	+	0.1	+		+
16	31.8	1.4	17	32	0	0	2	9	4)	S		0			+
361	15.9	2.5	5	36	0	0	3	7	7)	V	+ + +	1.5	+ + +	+	+ +
18	23.1	2.6	17	37	0	0	1	6	8)	B	+ + +	0.5	+ +		+ + +
13	19.5	4.3	8	37	0	0	0	8	7)	B	+ + +	0.3	+		+ +
192	13.4	7.1	8	37	0	0	1	6	8)	V	+ +	7.1	+ + +	+	+	+ + +
782	8.1	5.7	6	37	0	0	3	2	10)	S	+ + +	6.8	+ + +		+ +	+ + +
744	7.3	9.2	4	37	0	0	2	4	9)	V	+ +	14.4	+ + + +	+	+ +	+ +
466	5.5	19.9	3	38	0	0	1	5	9)	S	+ +	7.5	+ + +	+	+	+ +
467	7.3	20.9	5	39	0	0	1	4	10)	V	+ +	1.7	+ + + +	+	+ +	+ +
59	19.9	5.1	17	42	0	0	0	6	10)	B	+ + + +	2.1	+ + +		+	+ + + +

* Number of portal tracts of 15 counted showing these characteristics. Normal (0). Diffuse inflammation (I). Fibrosis without egg deposition in the central part of the portal tract (II). Fibrosis with egg deposition in the central part of the portal tract (III).

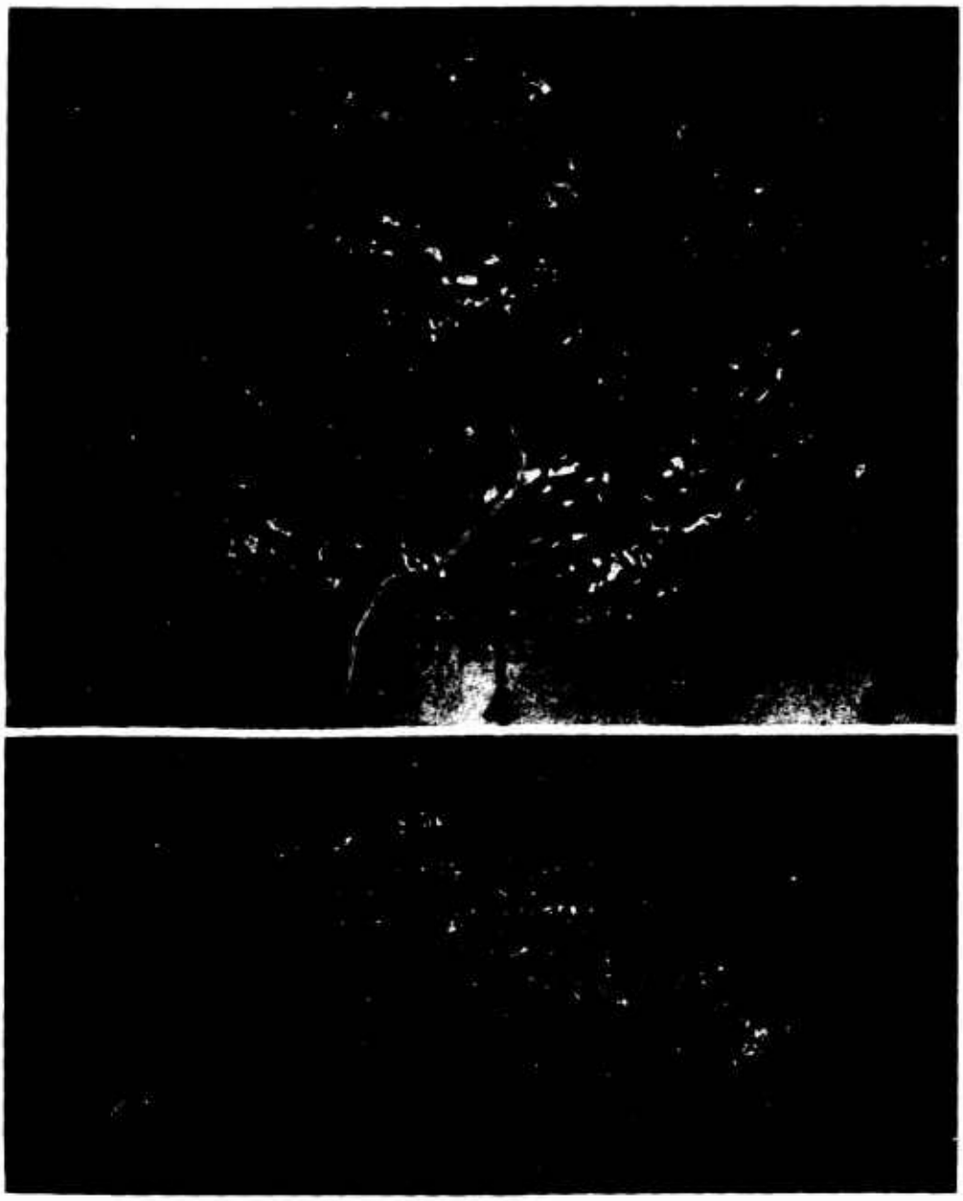
† S = Slender; V = Variable; B = Broad.



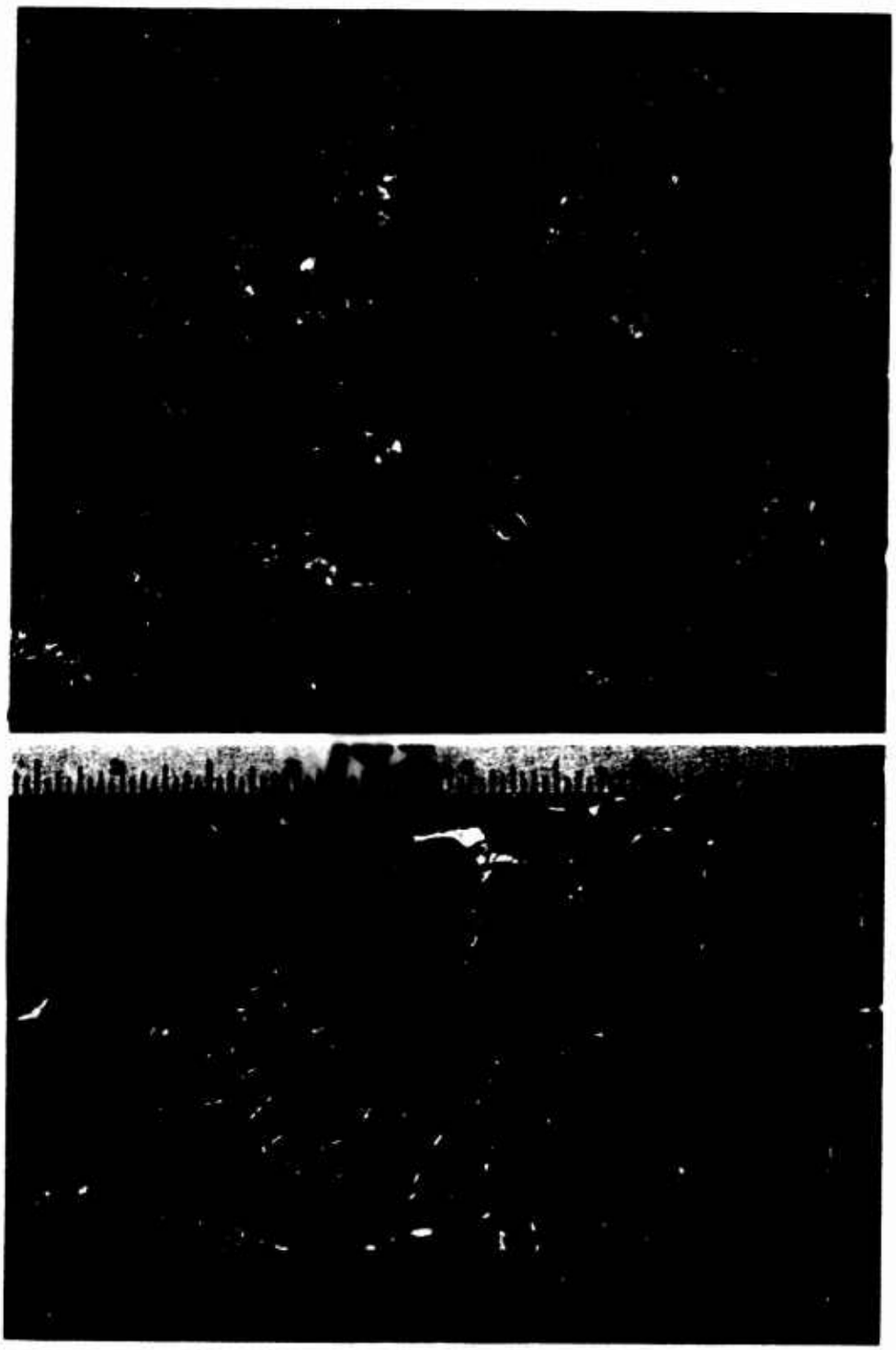
FIGURES 4 and 5. **4.** Chimpanzee No. 16. Liver surface showing bosselation and granulomas in the capsule (mm scale). **5.** Chimpanzee No. EA. Cross section of liver showing massive granulomatous involvement and delicate translucent thickening of many of the finer portal branches (mm scale).



FIGURES 6 and 7. **6** Chimpanzee No. 59. Cross sections of the liver showing advanced pipe-stem fibrosis with a continuous tree-like broadening of the entire portal framework and with narrowing or obliteration of vein lumina within the fibrous tracts (mm scale). **7** Chimpanzee No. 192. Cross section of liver showing broad fibrous portal enlargements. Virtual obliteration of vein lumina within fibrous tracts had occurred (mm scale).



FIGURES 8 and 9. **8**. Chimpanzee No. 16. Cross section of liver showing moderately advanced lesions with a finely arborescent fibrotic portal pattern. The delicate nodularity of the parenchyma and the very fine grayish streaking around portal areas, especially in subcapsular portions, is indicative of septal fibrosis (mm scale). **9** Chimpanzee No. 467. Cross section of liver showing marked focal variation of the portal fibrosis with coarsely broadened triads alternating with mildly enlarged ones (mm scale).



FIGURES 10 and 11 **10** Chimpanzee No. 18. Cross section of liver with organizing thrombi in the portal veins of the larger pipe-stem lesions. **11** Chimpanzee No. 192. Hepatic flexure of colon showing sharply demarcated patches involving the mucosa and submucosa (mm scale).

after perfusion, in size from 0.5 to several cm in diameter and were irregularly outlined and occasionally ulcerated (Fig. 11, 12). The larger patches produced slight to moderate thickening of the bowel wall. Several of the larger lesions were intensely fibrotic and extended through the muscular layers to involve the serosa. The localization of the patches varied. They were often concentrated in the lower sigmoid, usually at least 20 cm above the anus, where they would not have been visible during proctoscopy. Other frequently involved sites were: the splenic flexure, the hepatic flexure, the cecum, the transverse colon and the ascending colon. The appendix was involved in three cases. Although the distribution of eggs and of lesions was focal, there was a general correlation between the number of worms recovered, the concentration of eggs in the colon and the extent of colonic lesions. All 7 animals with more than 4,000 eggs/g color had severe mucosal lesions, while only 2 of 8 animals with less than 4,000 eggs/g colon showed marked lesions. The concentration of eggs in the lesions was considerably greater than the average for the colon; e.g. 49,000 eggs/g were found in one lesion digested separately from the remainder of the intestine.

In the small intestine gross lesions were found in only 2 animals. Although numerous eggs were found by digestion of the small intestine of chimpanzee No. 744, no gross lesions were seen. Lymphoid hyperplasia was occasionally observed.

Lungs.

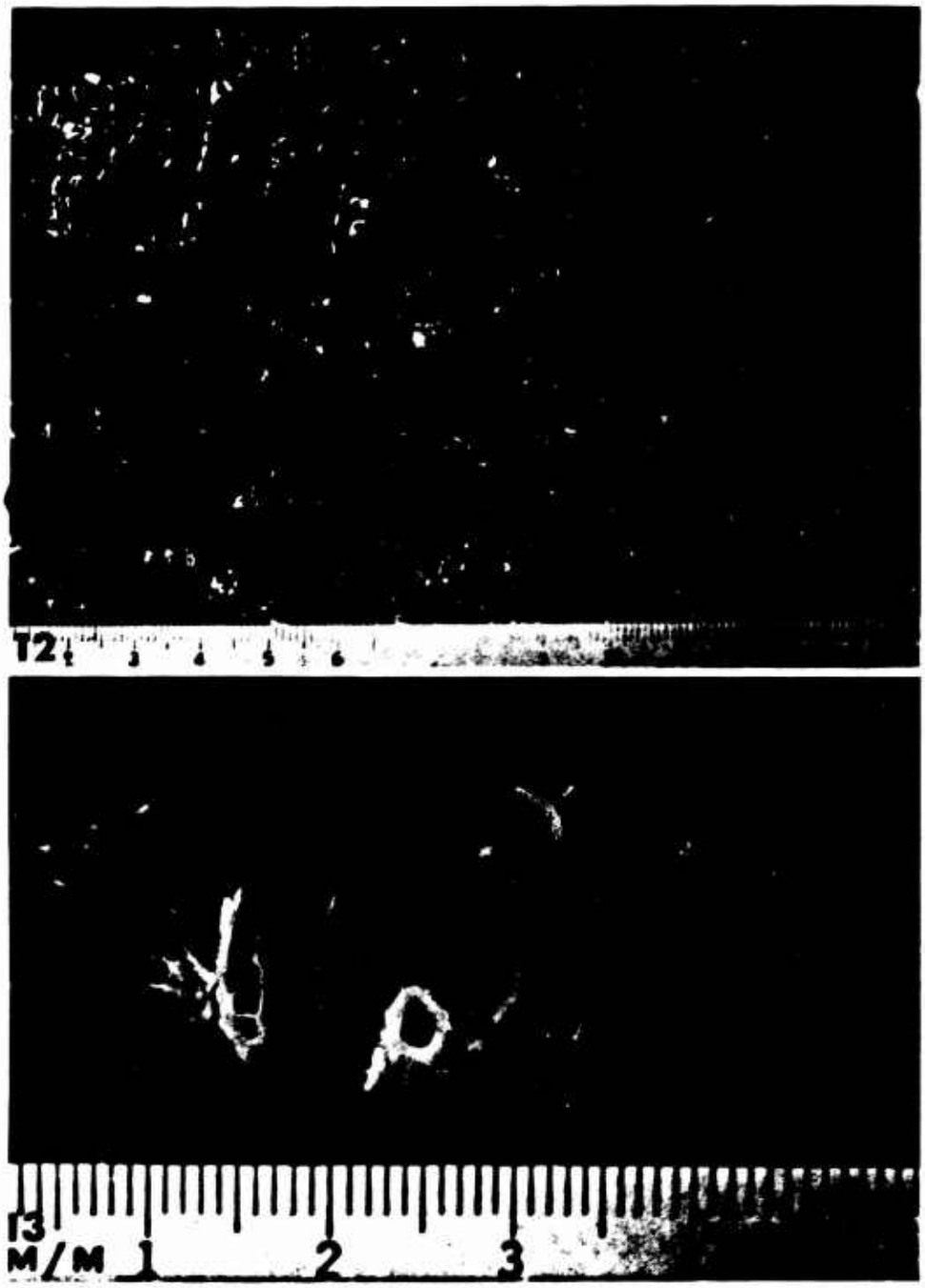
Seven chimpanzees showed subpleural and parenchymal granularity (Fig. 13) on visual inspection or palpation, in some instances accompanied by focal hemorrhages or areas of consolidation. All of these animals had portal fibrosis.

Lymph Nodes.

The mesenteric and other abdominal lymph nodes were enlarged. Some were soft, juicy, lacked focal lesions and were frequently seen at the liver hilus or along the colon; others showed areas of fibrosis, discoloration or induration, but appeared to be well encapsulated and discrete. Some lymph nodes were fused with the surrounding areolar or fibrous tissue, forming part of bilharziomata.

Bilharziomata.

Large retroperitoneal masses weighing 60 to 310 grams were present in four chimpanzees (Nos. 59, 192, 361 and 467). Pararectal fibrosis was present in all four animals, and in three, this was continuous with a retroperitoneal mass extending along the aorta. In chimpanzee No. 467, the mass virtually obliterated the pancreas and extended into the porta hepatis. In chimpanzee Nos. 59 and 361 the masses were broad and extended beneath one or both ureters. In three of the four animals, the bilharziomata were adherent to the colon on its mesenteric border at some point. When sectioned, the bilharziomata were found to be composed of an irregular



FIGURES 12 and 13. **12** Chimpanzee No. EA. Transverse colon showing irregular involvement of the mucosal surface (mm scale). **13**. Chimpanzee No. 466. Cross section of one lobe of the lung showing subpleural and parenchymal granular lesions (mm scale).

mass of fibrous tissue interspersed with fat and lymph nodes. Between 100 and 1000 eggs per gram of tissue were found in the bilharziomata.

Kidneys.

In animal No. 59 the kidneys were pale, granular, reduced in size and cortical width. Whitish streaking of the tubules was seen when the kidneys were sectioned. In another infected animal (No. 469) there was a small granulomatous mass in one kidney. No other changes were observed macroscopically.

Spleen.

Most of the chimpanzees had been splenectomized before infection. In those chimpanzees with intact or accessory spleens, splenic tissue was hyperplastic or normal in structure.

Brain and spinal cord.

The brain was examined in 9 animals and the spinal cord in 4. No gross abnormalities were seen. Concentrates of KOH digests of the brain showed 10 eggs/g in chimpanzee No. 466 and 2 eggs/g in No. 59. No eggs were found in the spinal cord.

Changes unrelated to schistosomiasis.

One animal (No. 192) had numerous acute superficial gastric stress ulcers. Chimpanzee No. 783 had serosal Oesophagostomum nodules (2-3 cm in diameter) along the transverse colon. One animal (No. EA) had a 1 cm submucosal nodule in the proximal jejunum and a dozen broadly stalked polypoid lesions in the distal ileum. Compression preparations revealed no eggs in these lesions.

Histopathologic findings.

Liver - The microscopic lesions were often more severe than had been suspected on gross examination. The following features will be described:

a) Focal egg lesions (exudative foci, granulomas, scars)
b) Disruption of small portal tracts
c) Diffuse inflammation and fibrosis of large portal tracts
d) Vascular lesions (veins, arteries, lymphatics)
e) Variable changes of hepatocytes, R.E. cells and bile ducts.
f) Variations of the liver architecture

a) Focal egg lesions: Classical pseudotubercles were present in variable numbers. Egg lesions tended to be large, exudative, and destructive. Residual scarring was more substantial than in S. mansoni infections. Most foci contained multiple eggs which were often peripherally scattered in early lesions, but were centrally clustered in their scars. The largest foci were related to mature eggs. Aging granulomas contained degenerated and calcified eggs.

Cell response was generally disproportionately intense; frequently no eggs were found in large lesions until multiple sections were made. Search for other particulate material in egg lesions revealed the presence of irregularly distributed P.A.S. positive hyaline droplets, some of which were clearly mast cell granules. No vitelline conglomerates were found.

Early granulomas often showed central massing of neutrophils and eosinophils; most lacked well-defined epithelioid cell borders. Some egg lesions, particularly those inside veins, consisted almost entirely of granulocytes (Fig. 14). Similarly, later foci consisted of sheet-like aggregates of eosinophils, neutrophils, plasma cells, macrophages, giant cells, fibroblasts and lymphocytes in variable proportions.

After two months of infection most egg foci were exudative, while by 17 months most were scarred. However, active foci persisted throughout all stages. There was great variation among the various chimpanzees. For example, two animals (Nos. EA and 744 showed many large foci with sporadic Hoeppli phenomena. By contrast, one of them showed small, acellular necrotic egg foci reminiscent of necrotic miliary tubercles. These were positive for fibrin and negative for amyloid on special staining. Healed egg foci took either a concentric (Fig. 15) or a stellate form (Fig. 16). These scar types varied in proportion in different animals without any evident explanation. The size of focal scars also varied greatly.

Early composite egg foci were bulky and tended to obscure the architectural landmarks of the liver by compressing or destroying adjacent normal structures. Some were concentrically surrounded by atrophic liver trabeculae invaded by leukocytes (Fig. 17), or encroached on the liver lobule end-plates bordering portal fields. Adjacent vessels or bile ducts were frequently inflamed.

When guided by the size of the arterial branches as markers of portal field location and magnitude, it could be seen that the majority of egg foci originated in small portal fields. With the onset of pipe-stem fibrosis in the larger tracts, granulomas were often seen near their borders. As the lesions evolved, egg foci became increasingly numerous deeply within pipe-stem lesions, especially in areas of endophlebitis and collateral formation (see below).

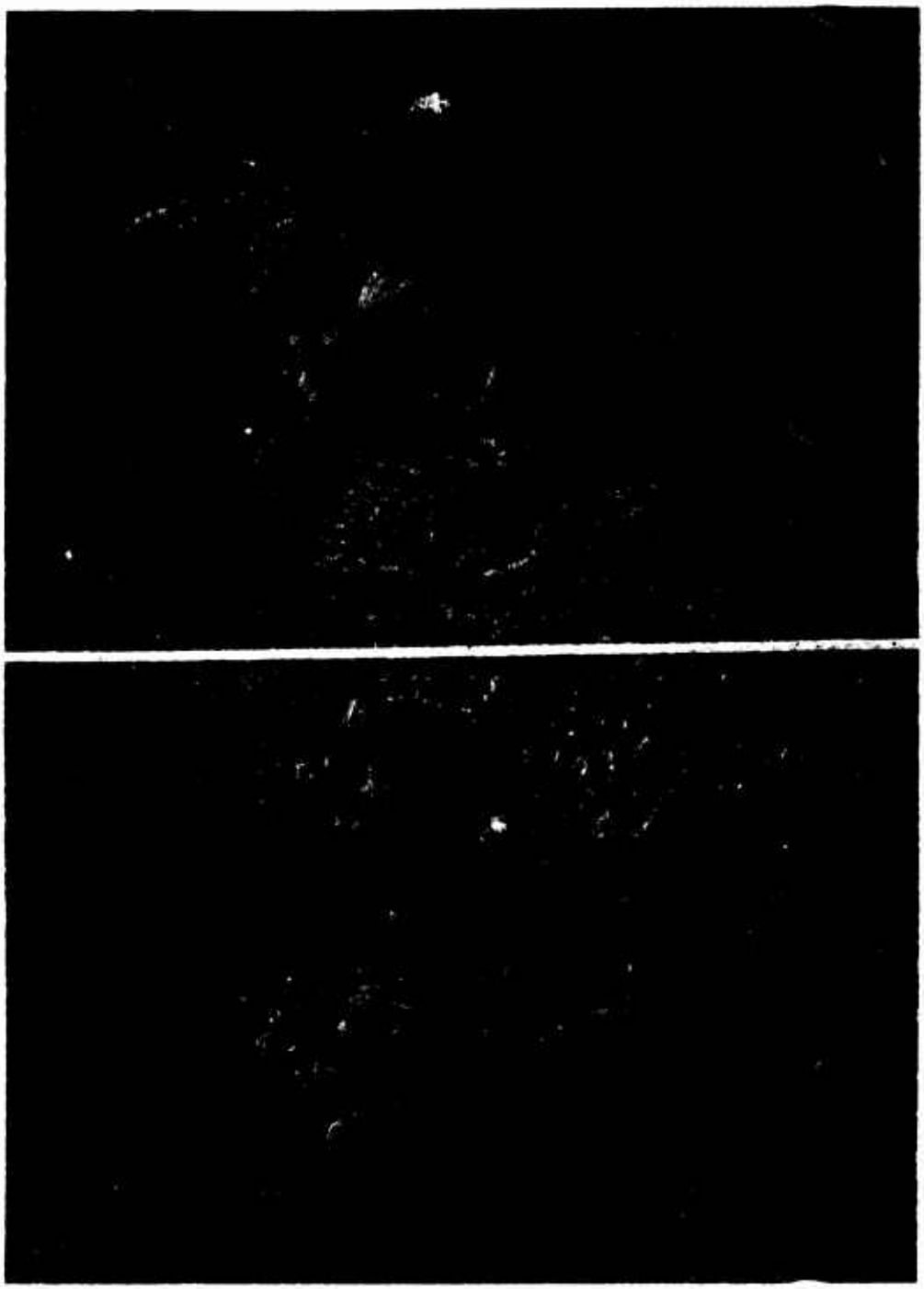
b) Disruption of small portal fields: This was clearly a widely distributed, but focal process. In early lesions small, thin-walled, severely dilated portal radicles were plugged by eggs and by granulocytic exudate diffusing through the wall and into the portal stroma (Fig. 18). Often there was segmental or total destruction of vein walls. In many small tracts the smooth muscle and elastic fibers were replaced by egg foci. Multiple thin-walled collateral venules were occasionally seen. The arteries and bile ducts were proliferated, thickened or dilated. All these structures were distorted or displaced by egg foci and connective tissue.

FIGURE 14. Liver, Chimpanzee No. 361 Two exudative egg lesions occupying an enlarged portal field, both consisting largely of neutrophils. On the right, there is one central egg; in the large, composite lesion on the left, five eggs (*arrows*) can be seen along the periphery, marked by a poorly defined epithelioid cell border. (H. and E., × 55.)

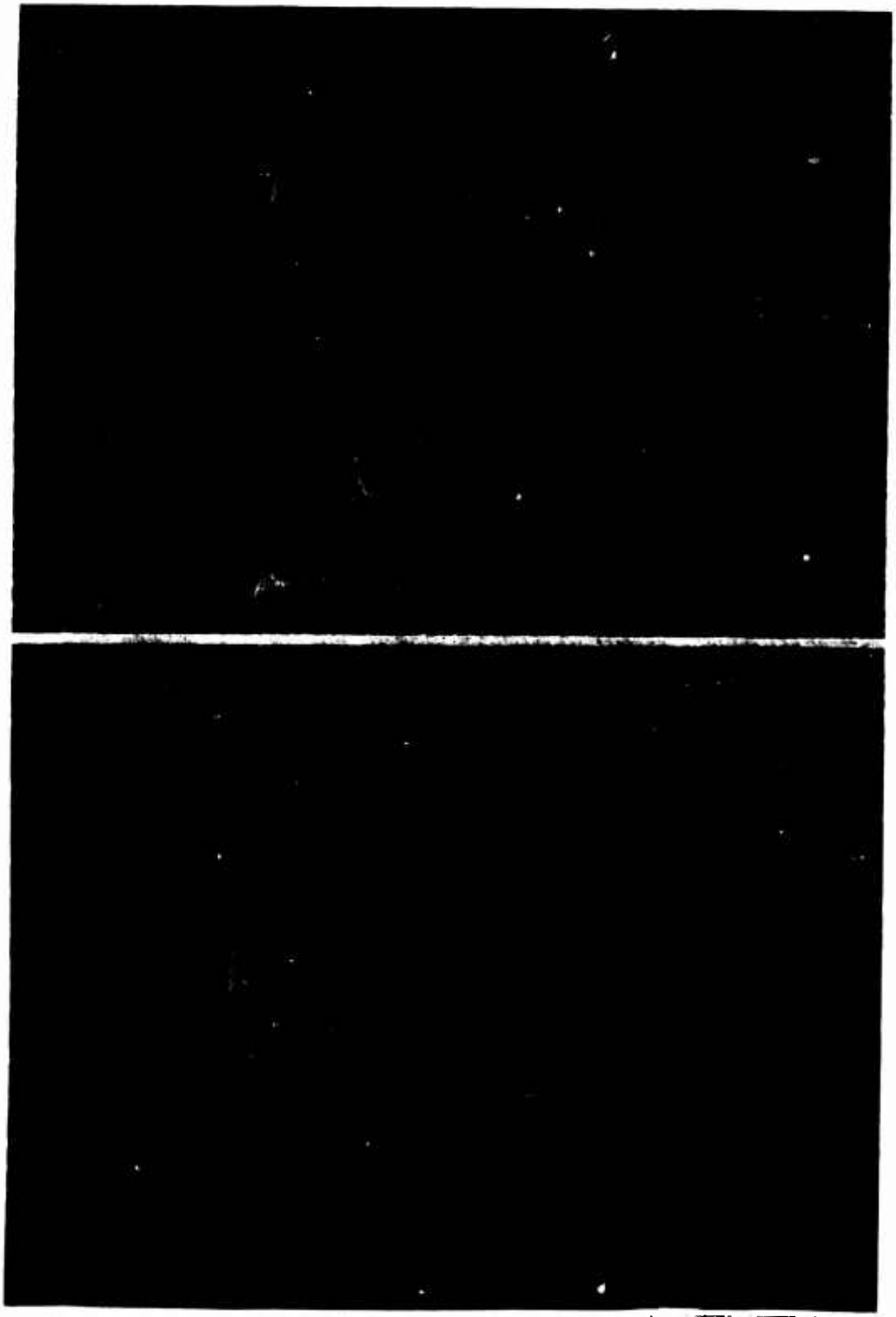
In severe "terminal triad fibrosis," fine septa divided nodular parenchyma units, especially near the liver capsule, in a pattern reminiscent of human post-necrotic or "post-hepatic" liver cirrhosis (Fig. 19).

c) Diffuse inflammation and fibrosis of large portal fields: The earliest diffuse lesions were seen after 2-4 months of infection and affected middle and large-sized portal tracts in areas where considerable focal disruption of the smaller triads had occurred. Few eggs and granulomas were seen in the larger triads at this time. The tracts were swollen by diffuse edema, inflammatory infiltration, and proliferation of plump fibroblasts. The infiltrate included numerous eosinophils, plasma cells, neutrophils and lymphocytes (Fig. 20). Occasionally, there was spotty fibrin deposition associated with vascular damage or accumulation of pink-staining, faintly granular proteinaceous material. The broad, wavy collagen bundles representing the normal endo-skeleton of these swollen triads were separated and disrupted by exudate and proliferating cells. Inflammatory cells, often extending from the portal tracts, disrupted the limiting plates of the liver lobules.

Subsequently, the inflammatory infiltrate shifted toward the lymphoid series, sometimes with numerous plasma cells, or with formation of follicular aggregates bearing germinal centers. Inflammatory infiltration



FIGURES 15 and 16. **15**. Liver, Chimpanzee No. 11. Healing granuloma of the concentric type showing seven eggs in its hyaline center. The surrounding portal space is chronically inflamed. (Masson's trichrome, × 68.) **16**. Liver, Chimpanzee No. 466. Healing granuloma of the stellate type, showing numerous eggs and persisting inflammatory infiltrate in its center. (Phosphotungstic acid-hematoxylin, × 55.)



FIGURES 17 and 18. 17. Liver, Chimpanzee No. 11. Typical, large composite granuloma with three eggs in plane of sectioning (*arrows*). There is early concentric fibrosis. Borders are indistinct with encroachment on the adjacent liver parenchyma. An adjacent small portal field is diffusely inflamed (*lower left corner*). (Masson's trichrome, × 68.) 18. Liver, Chimpanzee No. 744. Small portal field showing an immature egg occupying the vein lumen. The early granuloma obscures the vein wall and is contiguous with diffuse inflammatory infiltration. (H. and E., × 168.)

decreased and fibroblasts became less conspicuous, with the center of the triad usually clearing before its margins. Simultaneously, the infiltrate was replaced by a fine collagenous network (Fig. 21). In most animals, this evolution was followed by the appearance of new egg lesions and persistence of inflammatory activity, both focal and diffuse, particularly along the liver lobule end plates (Fig. 22). In others, the tracts progressed to a relatively acellular, hyaline condition (Fig. 23). Both in gross and microscopic appearance these changes varied between the portal tracts within the same liver, and they became increasingly uniform and universal in the most advanced cases. These architectural variations will be further discussed below:

d) Vascular lesions: The portal veins were severely affected. Besides the granulomatous replacement of small radicles, the larger branches also exhibited obliterative and destructive lesions which were focally distributed and varied in stage and morphology. These lesions appeared in conjunction with, but not prior to, diffuse portal tract inflammation and fibrosis. The most severe lesions were often associated with intravascular eggs. Overall, large vessel involvement was proportional to the degree of severity of pipe-stem fibrosis.

In many diffusely inflamed portal tracts, cellular infiltrate extended into the wall of the larger veins resulting in minor focal endothelial or intimal proliferation. A striking feature was an abrupt

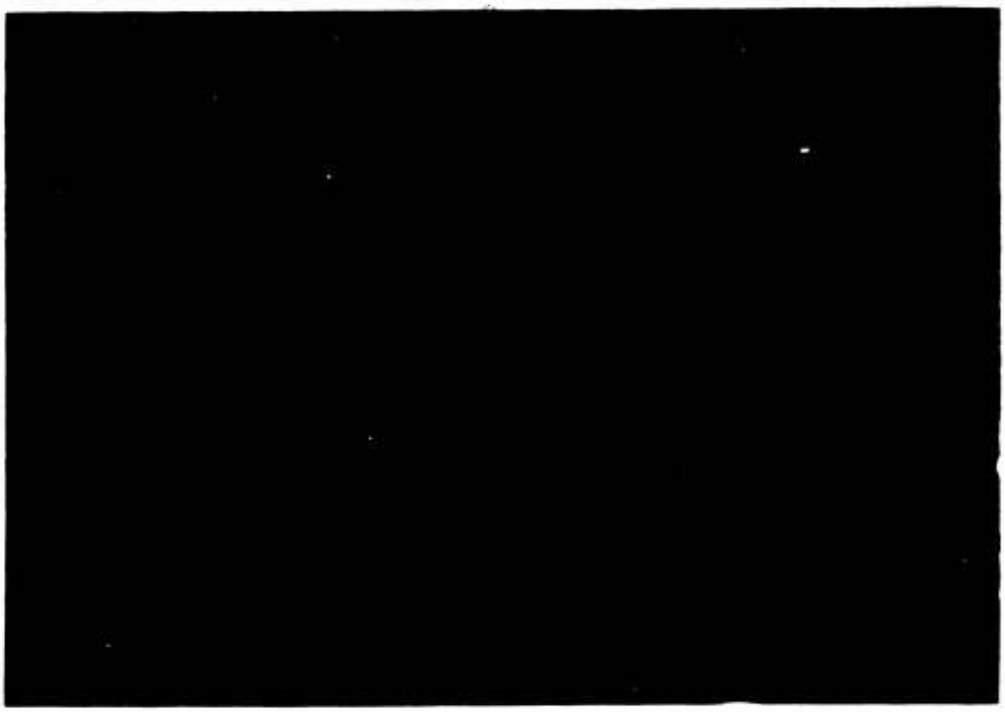
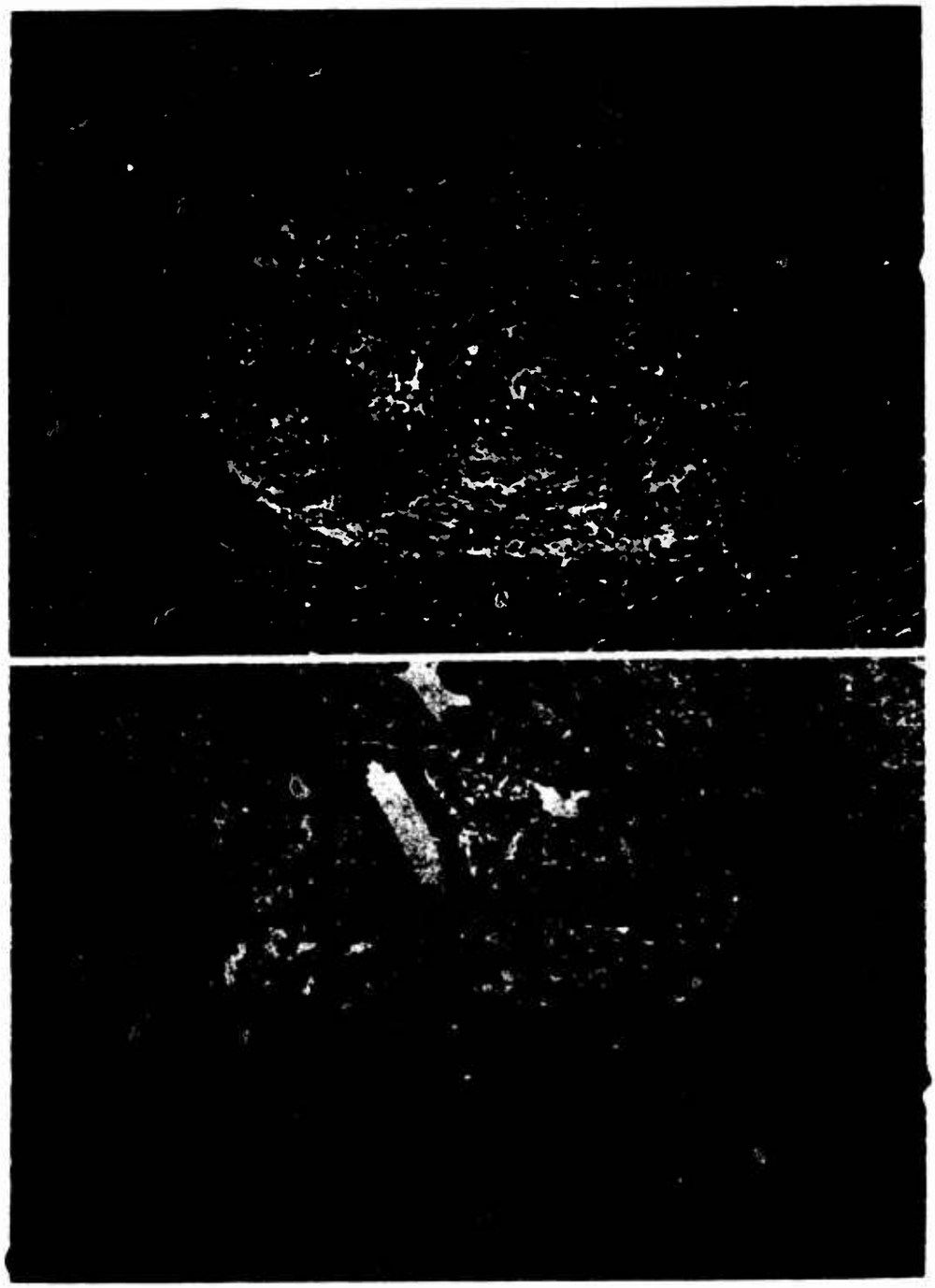
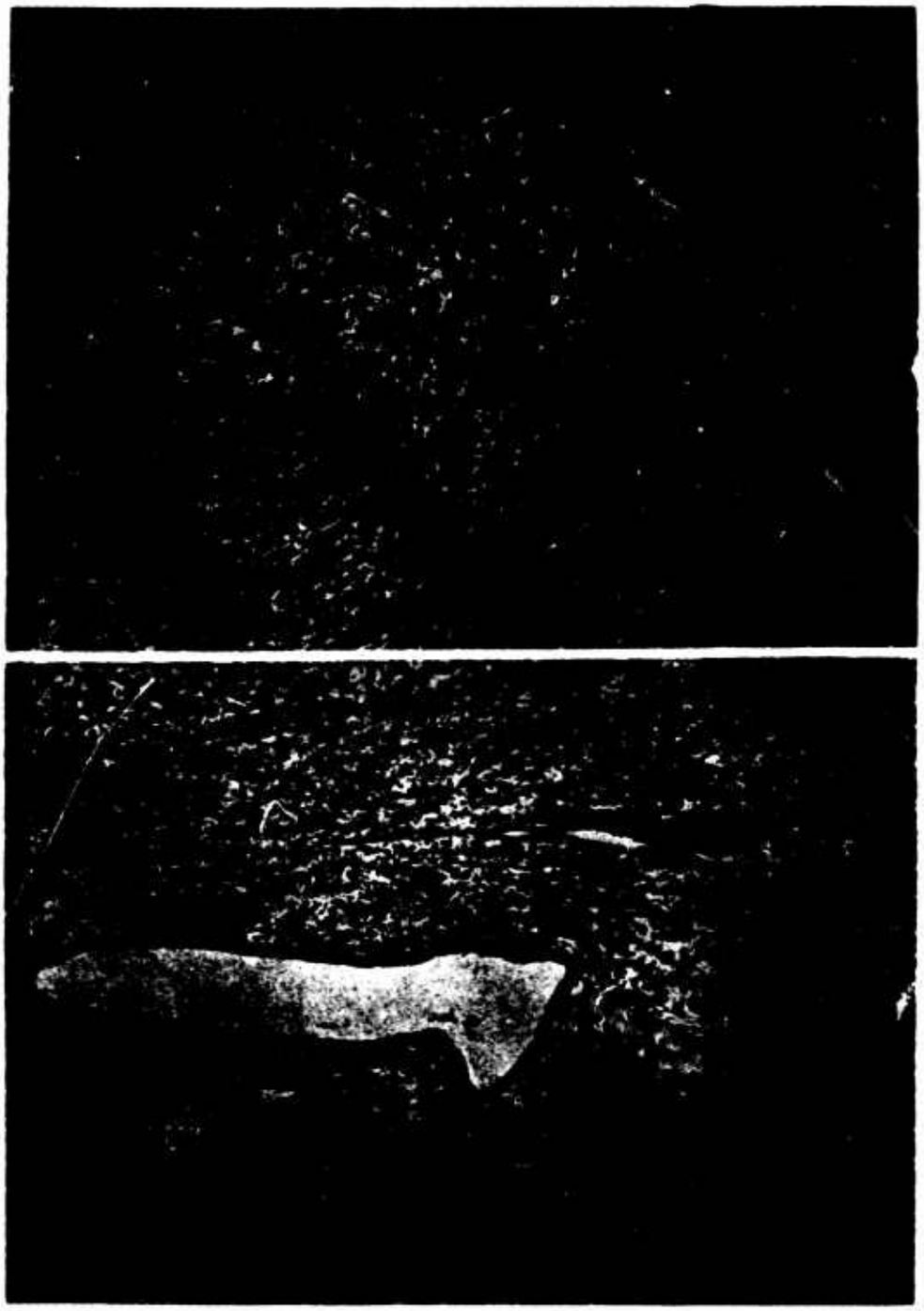


FIGURE 19. Liver, Chimpanzee No. 467. Advanced, terminal-triad fibrosis, with fibrous septa surrounding groups of parenchymal cells. Stellate scarring of granulomas is evident. (Masson's trichrome, × 55.)



FIGURES 20 and 21. **20.** Liver, Chimpanzee No. EA. Diffuse, portal inflammatory infiltration as seen 2 months after exposure. This marks the precursor stage of pipe-stem fibrosis. The portal vein lumen is replaced by inflammatory tissue. Faint strands of smooth muscle mark its former contour. Note the edematous state of the stroma. No eggs are present. (Masson's trichrome, × 62.) **21.** Liver, Chimpanzee No. 466. Large portal field with an established pipe-stem lesion. Note the partial obliteration of the portal vein lumen (*arrow*) and enlargement of the two arterial branches. The dark-staining, thick collagenous fibers represent the preexisting portal field, distorted and augmented by faintly staining new collagen. Inflammatory infiltration has become focal and predominantly mononuclear. An egg is present in a small satellite portal space (*right upper corner*) but none is seen in the area of maximal fibrosis. (Masson's trichrome, × 55.)

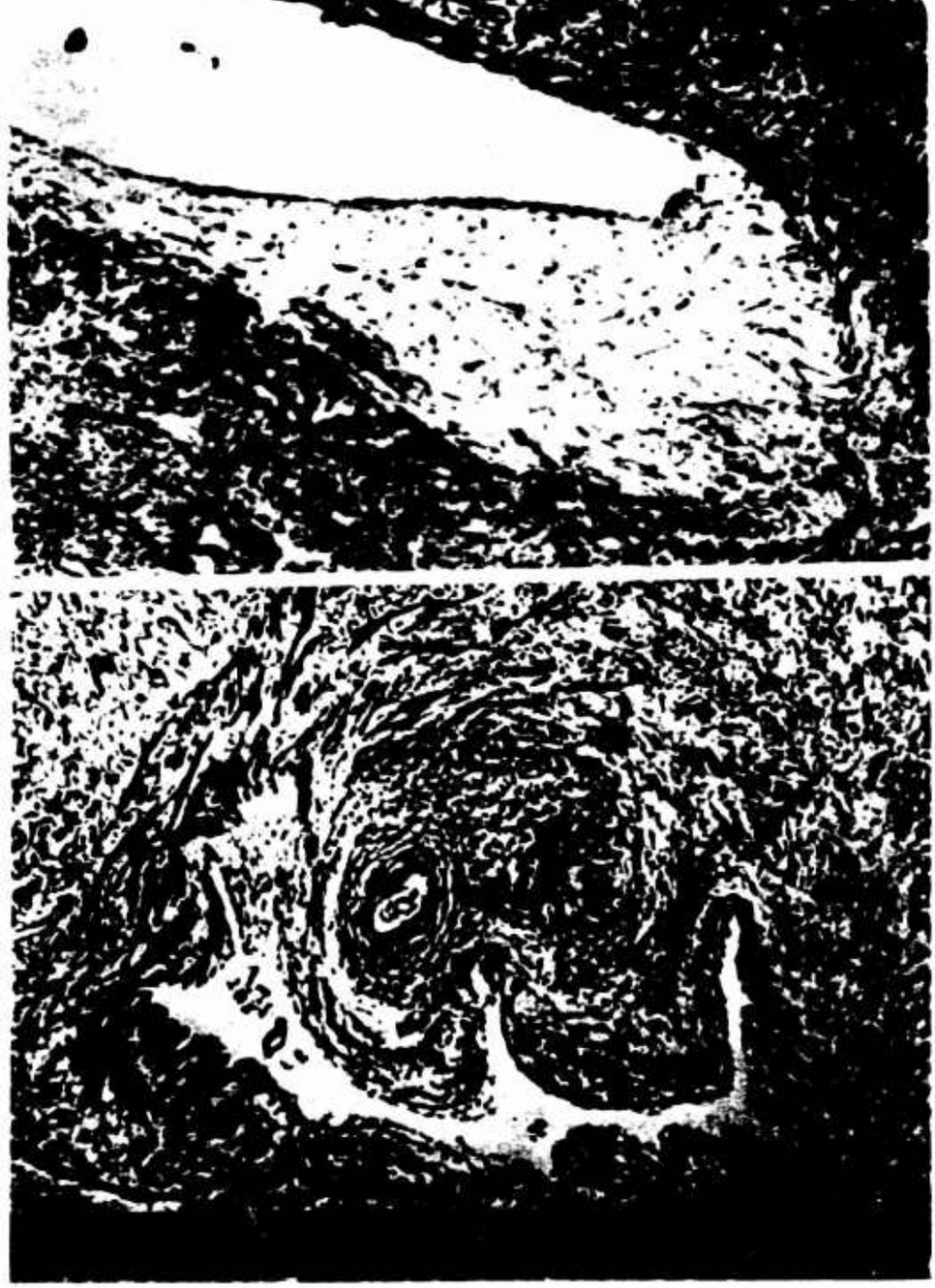


FIGURES 22 and 23. **22.** Liver, Chimpanzee No. 59. Pipe-stem fibrosis as seen 17 months after exposure. *The border* of this massively enlarged portal field shows dense, inflammatory-cell infiltration with irregularity of the liver *lobule end* plate. An active granuloma with an egg in its center protrudes into the liver tissue. Note the "onion skinning" around *the arteriole* and bileductule toward the left border. (Masson's *trichrome*. × 55.) **23.** Liver, Chimpanzee No. 13. Eight months after exposure, *the inflammatory* infiltration has largely subsided in this field, and the collagen has a hyaline appearance. In contrast to those in Figure 22, the liver lobule end plates are sharply delimited. The hepatic artery is markedly widened, contrasting with the faintly outlined portal vein, above and to its right, that shows two small luminal slits (*arrows*). No eggs are present. (Masson's trichrome, × 55.)

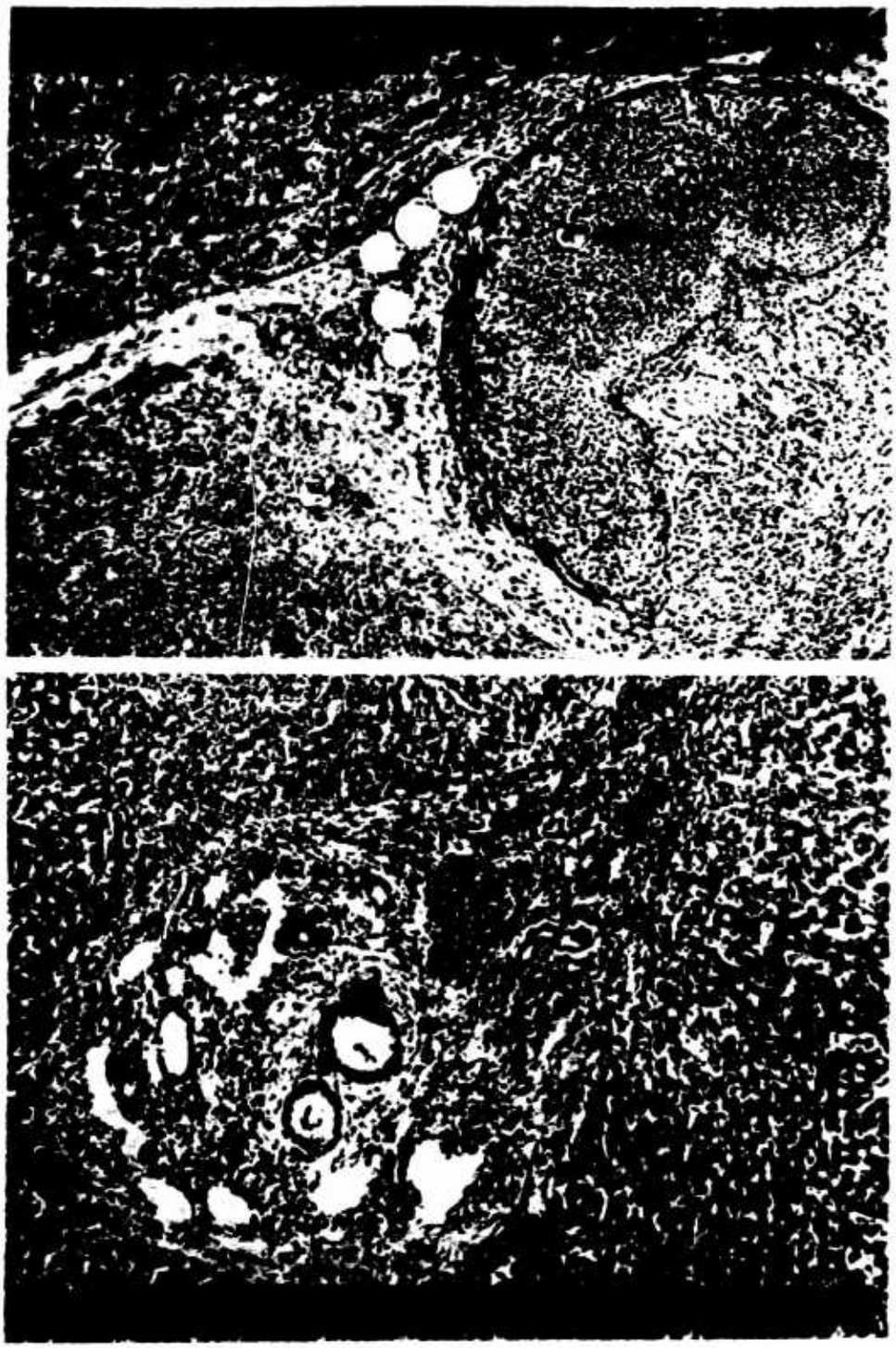
cushion-like engorgement of the intima with pale-staining proteinaceous material which usually contained only a few leukocytes (Fig. 24). At later stages these intimal swellings led to fibrous proliferation and organization with narrowing of the vein lumen. Many of these lesions were contiguous, or associated with severe, superimposed phlebitic lesions of three other types, namely: (1) organizing and recanalizing thrombophlebitis (Fig. 25); (2) granulomatous, or (3) exudative phlebitis. Fibrin deposits were occasionally found in all these forms, while egg lesions occurred mainly in the latter two and often resulted in focal smooth muscle and elastica destruction. In contrast to the total replacement commonly seen in terminal radicles, the healing residua of these larger vessels remained identifiable with trichrome or elastica staining (Fig. 25), but were difficult to recognize on hematoxylin and eosin stained slides.

Despite their polymorphism, all the occlusive and destructive variants of portal phlebitis seemed to be generically related. Exudative phlebitis, the most disruptive form, resembled bacterial pylephlebitis, except for its focal distribution and for the presence of eosinophils. Frequently, eggs embedded in aggregates of neutrophils and fibrin (Fig. 26), could be seen nesting on edematous intimal cushions near the major lesions. Thus, exudative phlebitis seemed to be the intravascular equivalent of the abscess-like portal egg lesions described earlier, and was probably due to the same etiological factors. Neovascularization of the portal connective tissue was among the most variable features of pipe-stem fibrosis. Some fields showed shrunken, organizing or recanalizing main portal branches together with numerous dilated thin-walled new channels around them which were suggestive of the "cuffed lesions" described in human portograms and post-mortem corrosion casts (Fig. 27). The new vascular channels promptly became the sites of new egg deposition and of recurrent endophlebitic activity.

A number of significant lesions occurred in hepatic artery branches. Arteries traversing areas of active inflammation often showed adventitial edema with peripheral "onion skinning" (Fig. 22). Focal necrotizing arteritis was seen in one chimpanzee (No. EA), both in the liver and in other organs. The lesions were confined to small arteries in portal fields with intense, diffuse inflammation. Two early infections showed focal "angiomatoid lesions" formed by irregularly proliferating smooth muscle cells separated by small clefts with endothelium (Fig. 28). Budding and branching of hepatic arteries with proliferation and thickening of the media also occurred. In most large portal fields, hepatic arteries were increased in size or number and, in some advanced cases, the larger arteries were dilated to the point where they sometimes exceeded the diameter of the corresponding portal branch (Fig. 23). This reversal of dimensions correlated well with the ante-mortem hemodynamic studies and with findings in human corrosion casts.



FIGURES 24 and 25. **24.** Liver, Chimpanzee No. 13. Large portal vein showing subendothelial edema. Note the disorganization of the smooth muscle layer and the newly formed capillary buds within the edematous intima. (Masson's trichrome, × 62.) **25.** Liver, Chimpanzee No. 18. Large portal vein showing recanalization. The original lumen is outlined by the dark-staining muscularis remnants; the recanalized lumen has its own, paler-staining muscular layer. An involuting granuloma is present (*arrow*). (Masson's trichrome, × 62.)



FIGURES 26 and 27. **26**. Liver, Chimpanzee No. 744. Large portal vein with diffuse subintimal edema. On the right, the lumen is almost obliterated by a large, exudative egg lesion (*arrow*). Next to it, five plastic beads injected immediately before death have become impacted. Note the fibrin strands overlying this abscess-like focus. (Masson's trichrome, × 55.) **27**. Liver, Chimpanzee No. 744. This middle-sized portal field shows two dilated arteries, but the portal lumen cannot be identified. There are many dilated venous collaterals, which appear empty after perfusion with saline solution. No eggs are present. (H. and E., × 159.)

Dilation of portal lymphatic vessels occurred irregularly both in early and late pipe-stem lesions. Dilated lymph vessels were often found peripheral to the areas of intense neovascularization and were associated with interstitial proteinaceous exudate.

Lesions were also found in the hepatic veins. In several heavily infected animals (Nos. 192, 744, 467, 59), there was focal endophlebitis of hepatic veins in sites adjacent to egg lesions or fibrous scars (Fig. 29). Some of these vessels showed thickening, obliteration or recanalization. Although similar to portal endophlebitis, these lesions were generally milder and less destructive than those found in portal branches.

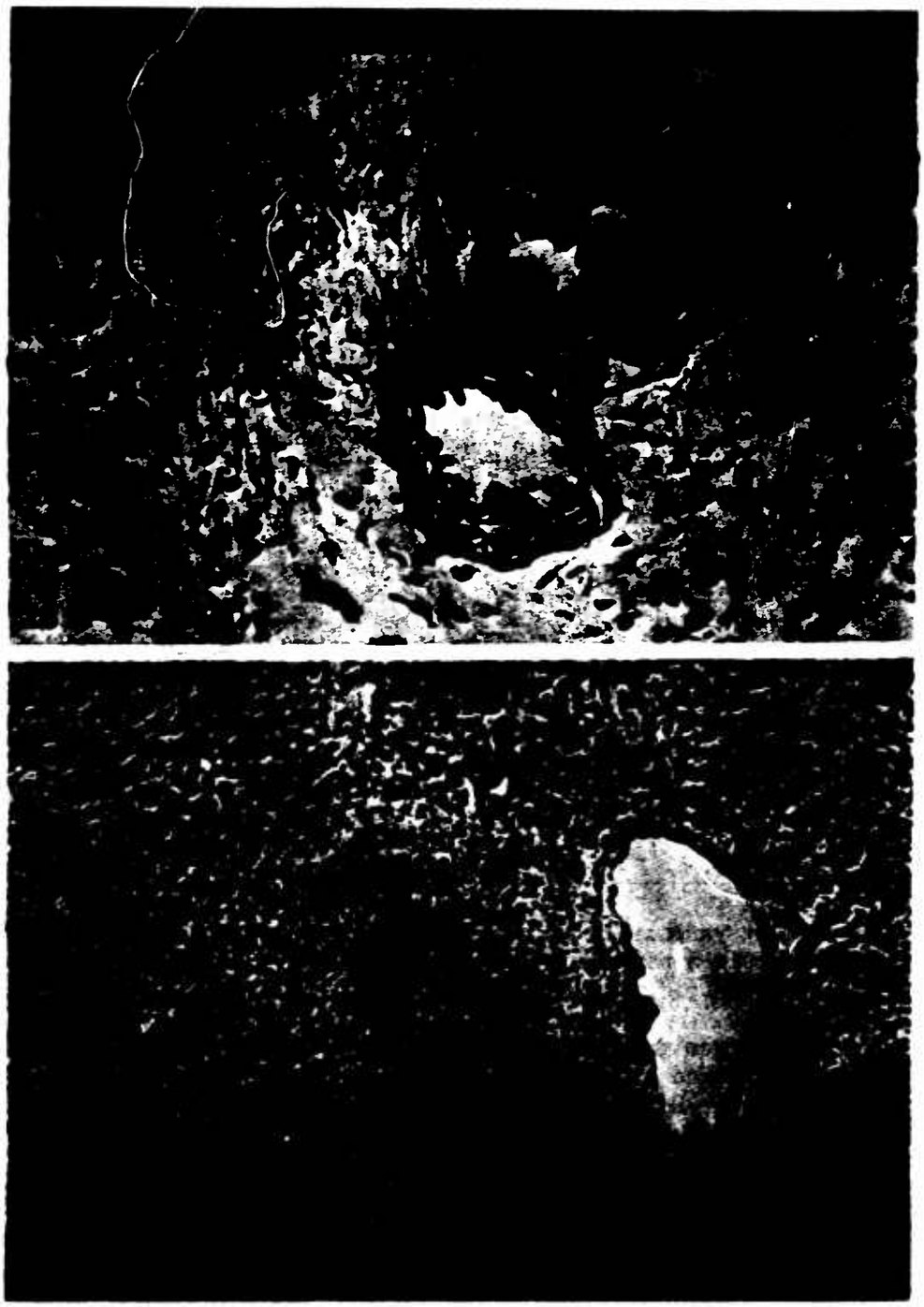
e) Variable and miscellaneous changes: Except in the areas of severe, subcapsular "terminal triad fibrosis," the lobular pattern of the liver parenchyma remained intact. Nodular regneration of liver cells was not seen. Hepatocellular damage was mostly confined to the periphery of early granulomas and to the margins of actively inflamed portal fields, as described earlier. However, in some cases with terminal disease and uremia, there was disseminated focal liver cell necrosis. Councilman bodies were present in one animal (No. 782), as well as occasional multinuclear or mitotic hepatocytes, and there was bile staining of centrolobular cells.

Ductular proliferation occurred along the enlarged portal field margins, especially in the most advanced cases (Nos. 59 and 192), and the interlobular bile ducts nearly always showed variable degrees of proliferation, sometimes progressing to miniature adenomatous patterns (Fig. 30). Chimpanzee No. 59, in addition to showing "piece-meal necrosis" along the liver end plates, exhibited active pericholangitis with marked neutorphilic infiltration of ductules and, to a lesser extent, of intertubular ducts. True cholangitis was not seen.

The reticuloendothelial cells were extensively loadened with birefringent, brown pigment and were prominent throughout. In some livers, sinusoids showed focal or diffuse inflammatory sinusoidal infiltration.

f) Variations of the liver architecture: While attempting to classify each infection, we noted that, in contrast to the orderly disease progression reported in S. mansoni infections, the S. japonicum-infected livers often had lesions of different stages existing side by side. This was most noticeable in animals which developed pipe-stem fibrosis within three to five months. Therefore, the four-stage classification proposed earlier remained applicable to individual portal fields, but could not be used to characterize the status of the entire liver.

As an alternative, 15 successive trichrome stained preparations of middle-sized and large portal fields of each chimpanzee were scanned under low power and were classified as: 0 - Normal, I - Diffusely inflamed, II - Fibrotic without central egg deposition, or III - Fibrotic



Figures 28 and 29. **28.** Liver, Chimpanzee No. EA. Hepatic artery showing angiomatoid lesion. Note widening of lumen, irregularity of elastica layer, and "onion skinning" of the adventitia. The angiomatoid excrescence consists of plump smooth muscle and endothelial cells lining small, irregular clefts. Some of these cells contain dark-staining hyaline droplets. (Periodic acid-Schiff, × 285.) **29.** Liver, Chimpanzee No. EA. This medium-sized central vein bordering a large, exudative egg lesion shows segmental endophlebitis with intimal fibrin deposition. This represents an early stage of involvement. (Masson's trichrome, × 62.)

with central egg deposition. The cumulative number in each category was multiplied by the respective denominator, and added together to yield an overall index of anatomical severity, as shown in Table 10. Thus, the scoring system used depended on subjective selection of the portal fields. Although there was a general correlation of this anatomical index with the number of eggs per gram of liver tissue throughout the series, this correlation was less evident in the infections of longer duration.

No two livers of the series showed identical patterns of architecture, either on gross examination or by panoramic microscopy. Nevertheless, two features could be graded subjectively: (1) comparative size of each large portal field (board, slender or variable), and (2) the estimated degree of terminal fibrosis (+ to ++++).

As shown in Table 10, the chimpanzees in our study could thus be divided into the following 4 groups: I) those with focal granulomatous involvement associated with mild infection (Nos. 783, 15); II) those with the precursor stage of pipe-stem fibrosis (No. EA); III) those with mild pipe-stem fibrosis (Nos. 9, 11, 16); and IV) those with severe pipe-stem fibrosis (all 9 other animals).

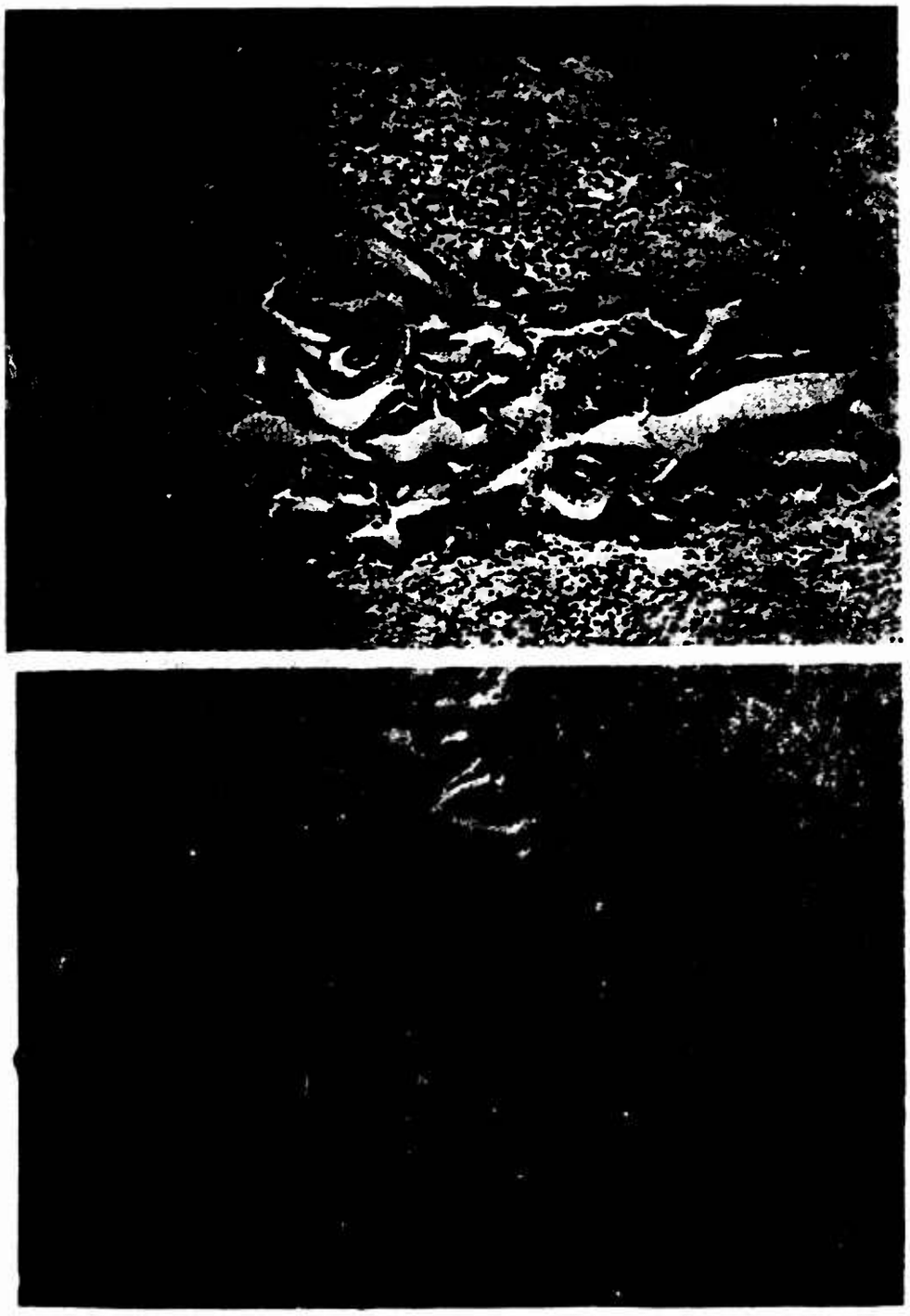
Enlargement of portal fields in the chimpanzees with the milder liver lesions of groups II and III were of the slender type; these were markedly arborescent on gross inspection. In group IV, the enlargement was either slender or variable in the severe infections of shorter duration and was uniformly and classically broad in type in severe prolonged infections. Although there was considerable individual variation, terminal triad fibrosis was most marked in severely infected animals.

Intestine.

The lesions at various levels of the intestine were similar to their gross and microscopic appearance, but were most severe in the colon. There was no evidence of mucosal atrophy of the small intestine.

Samples taken from grossly detectable lesions showed eggs of similar developmental stages clustered as "intravascular convoys" or in composite granulomas. Epithelial desquamation and crypt abscesses were commonly seen near the egg lesions. Focal hemorrhage and ulceration occurred in heavily involved areas. The lamina propria was infiltrated with eosinophiles and plasma cells, but was not fibrotic. In chronic patches, the mucosal crypts were proliferated and irregularly elongated with an increase in goblet cells, showing a near-adenomatous appearance.

In the smaller patches, eggs were mostly superficial, but in the larger ones, egg foci surrounded by diffusely inflamed or proliferating fibrous tissue often extended through the entire wall of the colon (Fig. 31) with partial disaggregation of the muscular layers. Some patches, primarily in the rectosigmoid, were continuous with pelvic or retroperitoneal "bilharziomata."



FIGURES 30 and 31. **30**. Liver, Chimpanzee No. 13. Large, interlobular bile duct showing marked epithelial proliferation and infolding adjacent to a follicular, lymphoid-cell aggregate. (H. and E., × 68.) **31**. Colon, Chimpanzee No. 16. Heavy egg deposition in the mucosa and submucosa with intravascular convoys and eggs in crypt lumina. The crypts are elongate and irregularly branched; occasional crypt abscesses are seen. The lamina propria is inflamed; the muscularis mucosa is effaced; the submucosa is thickened and shows large, exudative egg lesions extending toward the muscularis. (Masson's trichrome, × 32.)

The diffuse lesions were remarkably polymorphous and similar to those described in the large portal fields of the liver. Edema, granulation tissue, proteinaceous and fibrin deposits, proliferating fibroblasts and, ultimately, hyaline scarring were seen. Vascular lesions similar to those of the liver were also found in the colonic lesions including "edematous" and obliterative endophlebitis. In several instances, both the hepatic and the intestinal lesions seemed to illustrate the same stage and individuality. In one chimpanzee (No. EA) focal necrotizing arteritis occurred both in the liver and in the deep submucosa, muscularis, and subserosa of the colon, appendix and gallbladder (Fig. 32).

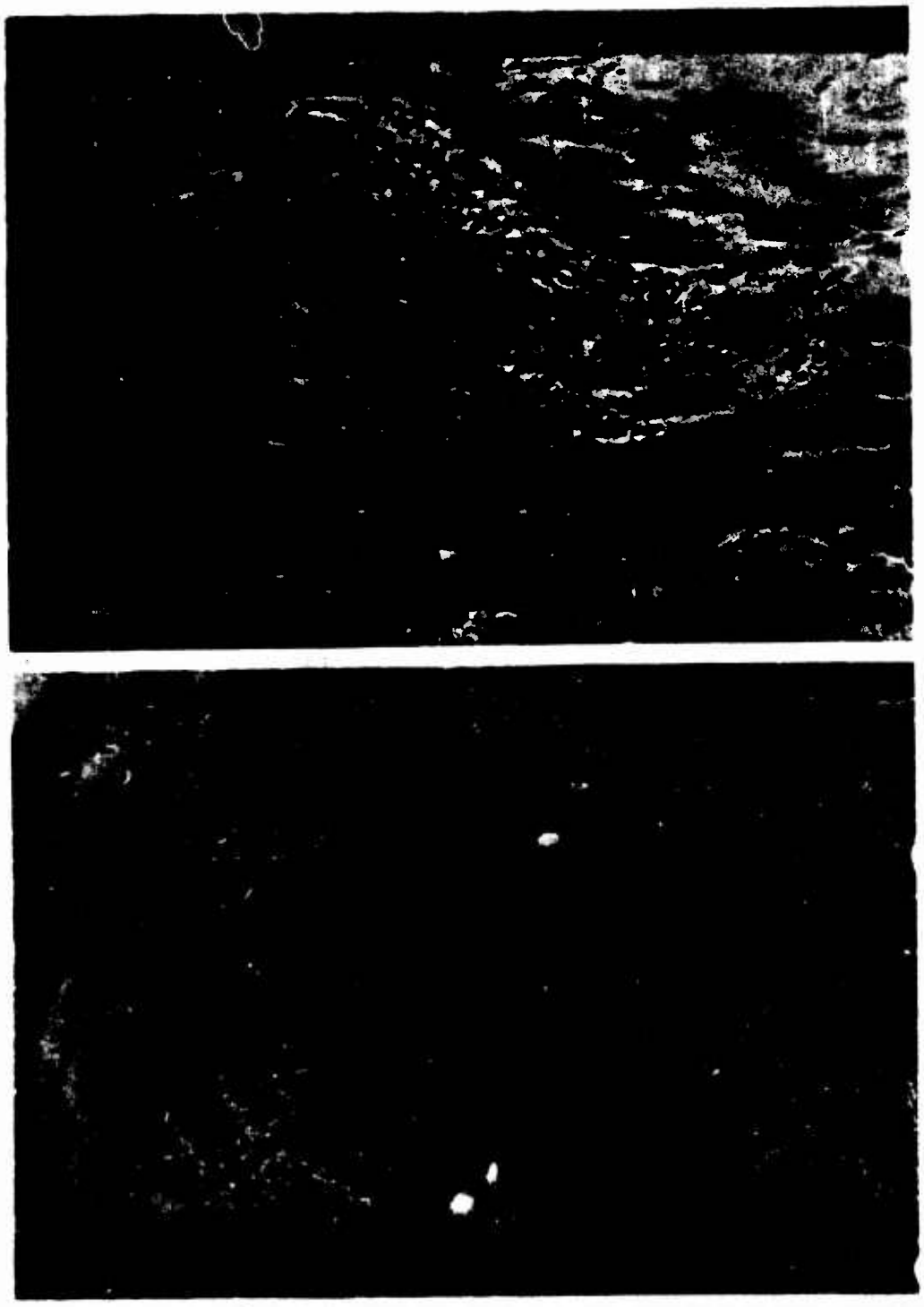
Lymph nodes and bilharziomata.

Some enlarged lymph nodes showed diffuse lymphadenitis and lymphoreticular hyperplasia with few or no egg lesions. The follicles were large with distinct germinal centers, and the sinuses were distended with numerous plasma cells and eosinophils forming sheet-like aggregates. Nodes with a gross spotted appearance showed egg foci reproducing the various lesions already described in the liver and gut (Fig. 33). Simultaneously, diffuse inflammation and fibrosis occurred in the surrounding areolar tissue which proceeded to fuse with the lymph node capsule. These inflammatory and fibrotic lesions led to disproportionate swelling and bilharziomatous transformation of the connective tissue and resembled the changes underlying large intestinal patches or found in the large portal triads of the liver.

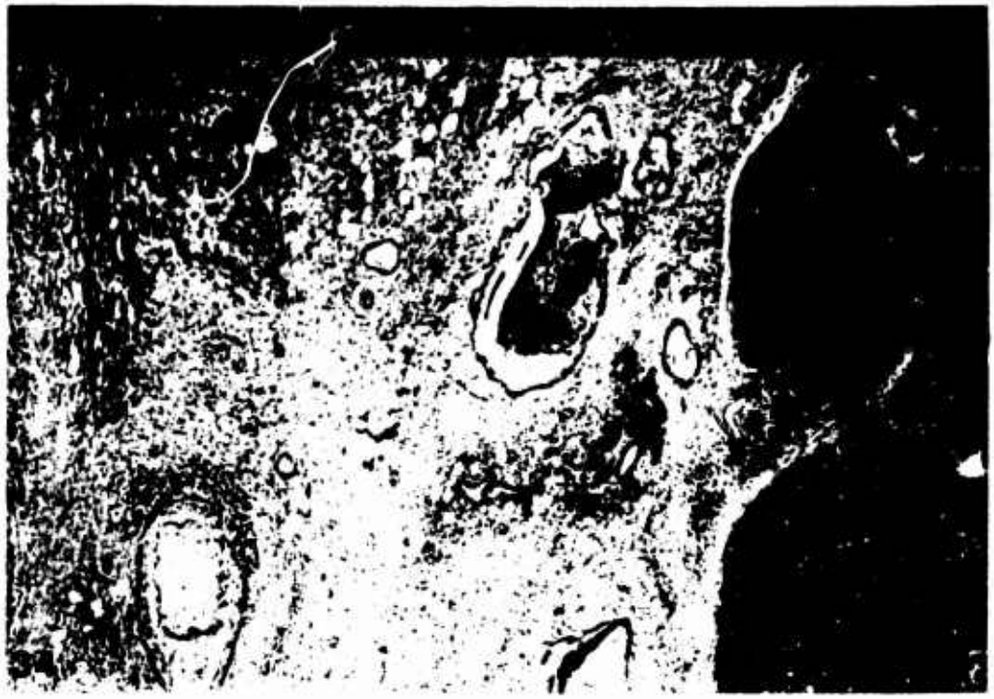
Although the fibrous tissue of retroperitoneal bilharziomata contained only sporadic schistosome eggs, these were commonly found in lymph nodes and in some of the veins. Early bilharziomata (i.e. No. EA) showed widespread infiltration of the connective tissue by proteinaceous edema, focal fibrin deposits and inflammatory cells (Fig. 34). In older lesions there was atrophy of areolar fat, fibroblastic proliferation, and increasing collagen deposition fusing all structures into a hyline mass. Vascular lesions here also resembled those of the liver and gut. Large vessel endophlebitis was less common than in the other two sites, and necrotizing arteritis was not seen. There was endolymphagitis with fibrin plugs and inflammatory cell aggregates inside dilated lymph vessels, particularly those afferent to a lymph node hilus (Fig. 34).

Lung.

Involvement was predominantly focal. Diffuse lesions and arteritis were present in heavy infections only. A pair of small schistosomes was seen in one heavily infected animal (No. 782). Egg foci was irregularly distributed in early infections (Nos. EA and 466), and the host response was predominantly exudative. In areas of massive egg deposition, the intervening lung tissue showed thickened alveolar septa infiltrated by leukocytes, with desquamation and fibrin deposition into alveoli. The overlying pleura was edematous and diffusely inflamed (Fig. 35). These lesions were rarer and less impressive than those seen in the abdominal viscera.



FIGURES 32 and 33. **32.** Appendix, Chimpanzee No. E4. An artery embedded in the outer muscular layer shows fibrinoid necrosis of the media and endothelial damage. It is surrounded by a loose, edematous inflammatory exudate including many dark-staining eosinophils. (H. and E., × 146.) **33.** Hepatic lymph node, Chimpanzee No. 13. The large, exudative egg lesion in the center of the field shows densely packed granulocytes bordered by an indistinct epithelioid-cell halo. Schistosome eggs are mostly peripheral (*arrows*) and are disproportionately few. The lymph node architecture around this lesion is preserved and shows reticular hyperplasia. (H. and E., × 42.)



FIGURES 34 and 35. **34.** Mesocolic lymph node, Chimpanzee No. 11. The lymph node tissue on the right is hyperplastic. The perilymphatic connective tissue on the left is diffusely inflamed and edematous, with denser infiltrates around vessels. Near the left upper border is a darkly stained sheet-like fibrin deposit. In the center a large, afferent lymphatic shows endolymphangitis. Near the left lower corner a relatively thick-walled vein branch shows subendothelial edema. (Masson's trichrome, × 32.) **35.** Lungs, Chimpanzee No. 467. Numerous, poorly defined composite granulomas contain many eggs. Note the thickening of alveolar walls and the desquamative pneumonitis of the lung tissue between the granulomas. The pleural subserosa is markedly thickened, edematous, and diffusely inflamed (compare with Fig. 22). (H. and E., × 32.)

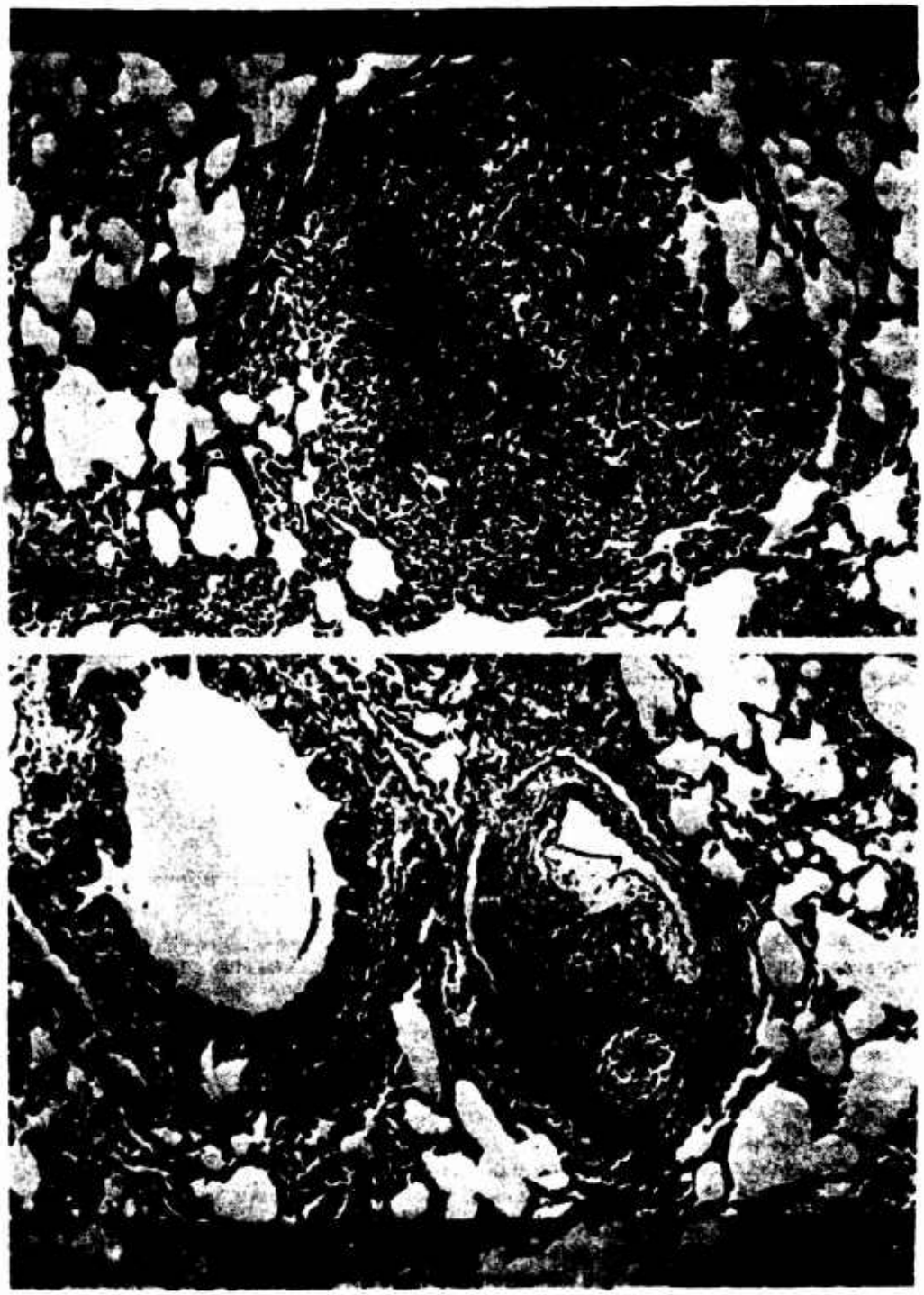
Arteritis was seen in seven animals (Table 10). In four of these, arteritis involved small arteries situated within active egg foci, and the lesions were few in number (Fig. 36). In three of the heaviest infections, occasional arger arteries were also affected. Edematous or thrombosing endarteritis was associated with egg lesions (Fig. 37). These lesions were similar to those in portal veins. Pulmonary arterial involvement was less than had been observed in a severe S. mansoni infection.

As shown in Table 10, there was a general correlation of the number of eggs/g of liver, versus the number of eggs/g of lung and the lung histopathologic findings. However, significant inconsistencies sometimes were found (*e.g.* Nos. 13, 744), indicating different degrees of egg shunting at comparable levels of infection. There was a close correlation between the number of eggs in the lung and the pathologic picture there.

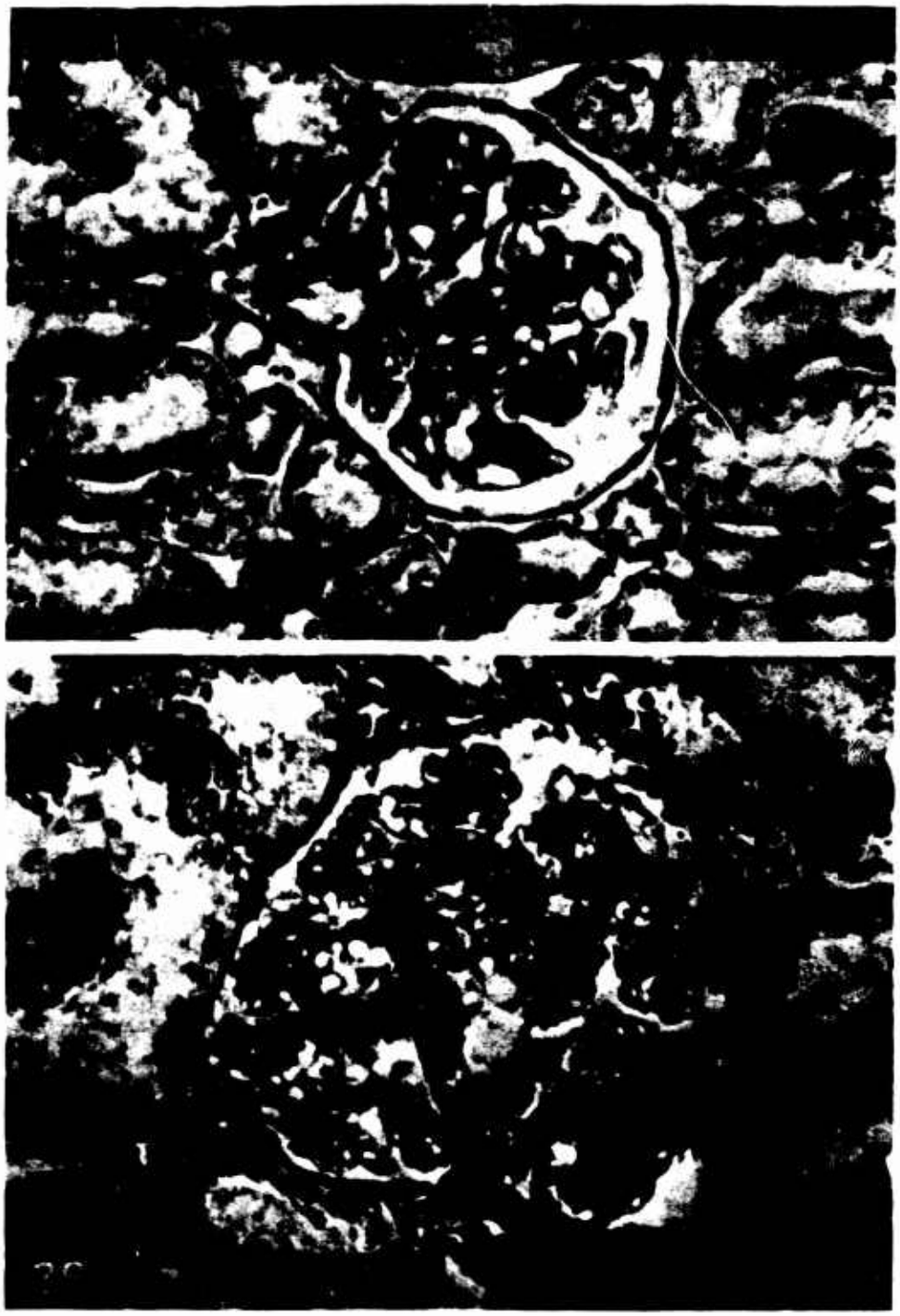
Kidney.

In a chimpanzee with one of the heaviest infections of the group (No. 467) a single subcapsular 2 mm-sized composite egg lesion was observed. This was the only such lesion found in the urogenital system.

Many animals (Table 10) showed a distinctive glomerulopathy similar to that reported in human hepatosplenic schistosomiasis. In its mildest form (No. EA) occasional glomeruli showed increased density and fuzziness of the glomerular stalks due to a minimal increase in mesangial substance and cells. Sporadic cells contained hyaline droplets. Moreover, there was albuminoid material in the Bowman's space. These changes could be appreciated only in PAS-stained slides and by comparison with normal controls (No. 38). In more advanced lesions, glomeruli appeared hypercellular and enlarged, especially in the inner cortex. Increase of mesangial matrix and proliferation of mesangial cells became more striking, and hyaline droplets and casts appeared in the proximal convolted tubules as well. With further development of the lesion, focal proliferation of the capsular epithelium occurred, and there was focal thickening of the capillary basement membrane in some of the loops. Some capillaries appeared ischemic or contained granulocytes. Finally, in the most severely affected animal of the series (No. 59) there were capsular adhesions, focal fibrous scars of the tuft, and occasional epithelial crescents. The interstitium and distal tubules remained remarkably free of change, but focal lymphoid cell and plasma cell aggregates were commonly seen. Some of these occurred around affected glomeruli, but others were between tubules. Similar foci were also seen in the uninfected controls. Altogether, these changes appeared to represent variations and successive stages in the development of glomerulonephritis (Figs. 38, 39). As shown in Table 10, glomerulopathy was absent in those animals which did not develop pipe-stem fibrosis, and could generally be correlated with the severity of schistosomal liver pathology. However, even in the most severe cases, renal lesions did not fully develop until about 6 months after exposure, and thus seemed



FIGURES 36 and 37. **36.** Lung, Chimpanzee No. 782. The small artery leading into *this composite* granuloma loses its elastica layer and its contour toward the center of the lesion. An intact artery is seen in the upper left corner. (Verhoeff-van Gieson, × 62.) **37.** Lung, Chimpanzee No. 744. A larger, juxtabronchial artery shows partial destruction of its wall by a granuloma. The overlying intima shows segmental subendothelial edema. The arterial lumen is narrowed. (H. and E., × 62.)



FIGURES 38 and 39 **38** Kidney. Chimpanzee No. 783. **Shows normal glomerular and tubular structure** Note delicate, well-defined mesangium (Periodic acid-Schiff, × 180) **39** Kidney. Chimpanzee No. 56. A representative glomerulus shows mesangial thickening from the stalk outward *with* increase in matrix and in mesangial cells. The matrix is fuzzy and ill-defined. There is slight capsular thickening *with an* adhesion of the tuft to the right of the vascular stalk. Near the stalk, a mesangial cell contains dark-staining hyaline droplets. The tubules are dilated and contain proteinaceous material. (Periodic acid-Schiff, × 180)

to lag behind the development of liver pathology. A more complete study of this lesion is currently being undertaken.

Spleen.

Splenic, or accessory splenic tissue was present in six chimpanzees. Sporadic egg lesions were found in the spleen of only one of these (No. 467). The Malpighian corpuscles were large, with well-defined germinal centers; in one animal (No. 466) pseudoamyloid was seen. The extent of congestion and thickening in the sinusoidal walls of the red pulp varied. Macrophages contained brown pigment and occasional haemosiderin.

Other schistosome lesions.

The patchy gallbladder involvement in animal No. EA was identical to that described in the intestinal lesions. Despite frequent involvement of the regional lymph nodes, pancreatic egg foci were present in two animals (Nos. 192, 467). The following organs, sampled extensively, were negative on histologic examination: brain, meninges, spinal cord, heart, skeletal muscle, and upper gastrointestinal tract.

Dilated, latex-injected veins were seen in the submucosa and outer two layers of the cardial esophagus of several animals. In two of them, the submucosal veins showed minimal phlebosclerosis. Lymphoid cell infiltration of the submucosa was noted twice.

Non-schistosomal pathology.

Many chimpanzees showed subepithelial lymphoid cell infiltration of the urinary bladder. This was most intense in chimpanzee No. 783. In the kidney, lymphoplasmocytic foci with a few eosinophils were found, mostly underneath the capsule or along blood vessels, with some of the smaller ones scattered throughout the cortex. Some glomeruli adjacent to these infiltrates were inflamed or desquamescent, and there was rare focal tubular atrophy and scarring. These changes were seen in all animals, including the controls, and were interpreted as evidence of a mild interstitial nephritis of unknown etiology.

The small ileal polyps described in animal No. EA were adenomatous, while the jejunal nodule was characteristic of a myoepithelial harmatoma. No schistosome eggs were seen in these lesions.

One chimpanzee (No. 15), which died from causes unrelated to schistosomiasis, had bronchopneumonia. Another (No. 192) which died spontaneously with terminal uremia, showed recent mucosal ulcerations of the stomach, and necrosis of superficial submucosal vessels. In No. 59, sporadic inflammatory foci possibly related to terminal sepsis, were seen in the cerebellum and diaphragm. No morphologic evidence of hepatic encephalopathy was found in sections of brain.

Non-schistosomal parasitic infection.

Pneumonyssus mites were seen in bronchioles of chimpanzee Nos. 9 and 744, and were probably present in others. Strongyloid larvae were seen in crypts of the small intestine (Nos. 744, 59) and low in the colon (No. 18). Oesophagostomum-like helminths occupied large, massively inflamed paracolonic cysts (No. 783). Adult filarid worms found in three animals were located in the submucosa of the small intestine (No. 466), lymphatics of the liver hilus (No. 16) and in a mesocolic lymph node (No. 13). The adult worms were surrounded by chronic inflammation with eosinophilia. No microfilariae were seen.

These studies indicate that the chimpanzee is well suited for investigations on the pathogenesis of Schistosoma japonicum infections.

Clinical and parasitologic course of the infection.

In all animals large numbers of viable eggs were passed in the feces after a prepatent period of 5 weeks or more. At necropsy, well developed adult schistosomes were found in the distal branches of the mesenteric veins. Most of the eggs in the tissues were found in the liver and large intestine, but numerous eggs were often present in the small intestines and in the lungs of the most heavily infected animals.

In the heavily infected chimpanzees blood was seen in the stools after 8 weeks of infection. This was followed by loss of appetite, decreased activity and responsiveness, and ultimately, a rapid loss of weight. These progressive symptoms were frequently accompanied by ascites, abnormal electrophoretic patterns and marked increase in the prothrombin time. Five chimpanzees either died as a result of infection or were moribund when taken for necropsy. As had been observed in chimpanzees infected with S. mansoni and S. haematobium, the infections with S. japonicum remained active and there was no evidence of a host self-cure phenomenon during the 4 weeks of observation.

Hepatic lesions and functions.

1) "Symmers" clay pipe-stem fibrosis of the liver

One of the most striking findings in our S. japonicum infections was the early appearance of portal fibrosis. Although the more advanced stages occurred with heavier infections of longer duration, one chimpanzee infected for only two months already had significant portal edema and early fibrosis at necropsy. High infection levels, (e.g. 70 or more female worms) resulted in pipe-stem fibrosis after 3 to 4 months, while lower infection levels progressed to the same stage in 7 to 17 months. Only the two animals which had light infections, or were killed shortly after exposure, failed to develop pipe-stem fibrosis. Thus, it appeared that the time of development of portal fibrosis was related to the intensity of infection.

In both S. mansoni and S. japonicum infections in chimpanzees, development of pipe-stem fibrosis was continuous and progressive and did not require particular sequence of exposure or any individual host predisposition. However, in S. japonicum infections this stage was particularly rapid in development and destructive in character. Re-infection was not necessary for the development of pipe-stem fibrosis, although it would, of course, increase the intensity of the infection. As was concluded in earlier chimpanzee experiments, there is no evidence that malnutrition was a factor in the progression of this disease, except as a terminal complication. Therefore, there is no indication that diet plays an important role in the development of pipe-stem fibrosis in infected chimpanzees as well as humans. No evidence of acquired immunity was obtained from our present investigations and, in contrast to the observations by Hsu and Hsu, no cross-immunity was apparent in two animals which had been naturally infected with S. mansoni before being exposed to S. japonicum.

2) Intrahepatic portal obstruction, portal pressure, collateral circulation and hepatic blood flow.

Marked obstruction of intrahepatic portal vein branches was evident in most infected chimpanzees, and in two of them the obstruction was complete. Despite this, the increase in portal pressure was modest and did not correlate well with the anatomical severity of hepatic lesions. Thus, five animals with marked pipe-stem fibrosis (No.s 13, 782, 466 and 467) had normal pressures (Table 8) although one of these had complete portal obstruction. As had been observed with the chimpanzees infected with S. mansoni, portasystemic collaterals were apparently adequate to compensate for the intrahepatic obstruction at this stage of the disease. The extent of collateral flow was evidently greatly underestimated by latex injection of the portal system, as only moderate collaterals were noted in animals 744 and 467, in which a minimum shunt of 25% and 43% of the portal flow was shown by injection of polystyrene beads. The failure to recover beads from either the liver or the lungs of the last group of eight injected animals might have been caused by clumping of the beads and shows that this technique apparently is inadequate to demonstrate the full extent of collateral venous channels present. Esophageal varices, though present, were not as highly developed as would be the case in man with comparable hepatic fibrosis, and bleeding from varices did not occur.

The nearly normal hepatic blood flow observed in animals having marked portal obstruction was presumably maintained by an increase in hepatic arterial flow, as reported in persons with pipe-stem fibrosis and in infected mice. The marked enlargement of hepatic artery branches seen histologically contrasted vividly with the narrowing and obstruction present in the portal channels. Although blood volume is increased in persons with pipe-stem fibrosis and in infected mice, it was below normal in the present series of S. japonicum infected chimpanzees, as well as in the S. mansoni infected chimpanzees.

3) Hepatic function.

The prothrombin time was abnormal in several of the more severely diseased animals and marked hypoalbuminemia was frequent. These findings were consistent with histologic evidence of hepatocellular damage in these animals.

The situation observed in those animals which became very lethargic or comatose appears to be quite complex and requires further study. Although blood ammonia levels were high, we have no evidence to relate this to the coma, and histopathologic examination of the brain failed to show the characteristic lesions seen in the human brain with hepatic coma. Hypoglycemia appeared to be an important factor in some of these animals, and uremia was of significance in one. The possible effects of dehydration and failure to eat at this stage of the disease must also be considered.

4) Hyperglobulinemia.

Significant increases in total serum proteins occurred in most animals as the infection progressed. The increase was due primarily to a dramatic increase in gamma globulin, which reached a level of over 10 g percent in one animal. This is in general agreement with observations reported in humans and rabbits with this parasite, although the increases in chimpanzees were much more striking. In fact, the increases in total protein and globulin concentration recorded in our chimpanzees with schistosomiasis japonica were considerably greater than those observed previously with schistosomiasis mansoni and schistosomiasis haematobia. Both hepatic involvement and the antigenic stimulation by the parasite are presumably significant factors here. The time-course development of antibodies detected by passive cutaneous anaphylaxis was unrelated to the degree or manner of exposure and followed an unpredictable course, as had been the case in schistosomiasis mansoni and hematobia.

Intestinal lesions.

The colon and small intestine of S. japonicum-infected chimpanzees often revealed marked lesions in some segments interspersed with large areas of nearly normal tissue. In contrast, the lesions in S. mansoni-infected chimpanzees were more uniformly distributed. Whereas submucosal colonic lesions produced by S. haematobium in the chimpanzee were focal, nodular and discreet, those produced by S. japonicum (Fig. 11) were broader, and flat with irregular borders. Marked serosal fibrosis was common only around the appendix in S. haematobium infected chimpanzees, while serosal fibrosis had no discernable pattern in S. japonicum infections. In persons infected with S. japonicum, segmental lesions of the intestine are apparently much more common than in persons infected with S. mansoni or S. haematobium. S. japonicum also produced focal lesions in the mouse intestine.

Bilharziomata.

Large fibrous tumors caused by schistosome infection (bilharziomata) have long aroused the interest of clinicians, partly because of the difficulty in differentiating the fibrous masses from neoplasms. Pathologists have likewise been intrigued by these lesions, particularly those large fibrous masses containing relatively few eggs. The masses in the intestinal serosa, in the mesentery and in the retroperitoneum appeared to be of a similar nature and are considered together here. These were similar to the fibrous masses occurring in the internal genitalia and the fibrous patches in the bladder and ureters in S. haematobium infections and in the intestinal submucosa in persons and mice infected with S. japonicum.

Retroperitoneal fibrosis in chimpanzees infected with S. mansoni and S. haematobium was usually associated with large numbers of eggs; for example, 7,000 and 52,000 eggs per gram of tissue were recovered from two retroperitoneal bilharziomata in two S. haematobium infections (unpublished data). In S. japonicum infected chimpanzees, however, only 100 to 1000 eggs per gram of tissue were found in the three retroperitoneal bilharziomata digested separately. Many more eggs were frequently found in other tissues which showed no significant fibrous response. Since the duration of the infections in two of the animals with bilharziomata was only five and six months, it seems unlikely that these lesions were caused by eggs which were destroyed in the tissue prior to the time of necropsy.

Development of the fibrotic lesions.

As was observed in schistosomiasis mansoni in the chimpanzee, fibrosis of the large portal fields of the liver often occurred in areas where few or no eggs had been deposited. Eggs and egg lesions were found mainly in the adjacent small portal triads. Similarly, in the diffuse fibrous proliferations seen in the intestinal serosa and in the bilharziomata, eggs were scarce in the foci of fibrosis and were found mainly in the neighboring intestinal mucosa, or in lymph nodes. On the other hand, fibrosis of the distal portal tracts and subcapsular septa in heavily infected animals appeared to be more closely related to the topography and number of eggs deposited locally. These observations suggest that, in addition to the direct local destructive effect of granulomas, diffuse fibrotic lesions may develop proximally to the site of extensive egg deposition.

Liver lesions in S. japonicum infections were more inflammatory and polymorphous than was the case with S. mansoni in the chimpanzee. The early lesions of the large portal fields were characterized by marked edema and inflammatory infiltration, rather than by granulomata. Focal deposits of fibrin or proteinaceous exudate were common. These lesions progressed from an inflammatory stage to exuberant fibrosis, and from a focal, to a generalized distribution which culminated in the classical pipestem picture. A similar sequence was seen in the subserosal lesions

of the gut and in the bilharziomata. In all three of these lesions, inflammation and fibrous proliferation were frequently accompanied by vascular inflammation. Endophlebitis of portal and mesenteric veins was the most prominent of these lesions, but the hepatic veins, arteries, and lymphatics were also affected, and there was capillary proliferation. These observations suggest that both intensive infection and an active host cellular response play a role in their pathogenesis in the chimpanzee. Bolicle pointed out that the reason for the marked variation in human tissue response to S. mansoni infection is still unknown. He also noted morphologic similarities between pipe-stem fibrosis and bilharziomata in man, such as were found in our experimental material.

Hyperactivity to schistosome antigens.

In addition to the humoral response, and the marked hypergammaglobulinemia observed, there was pathologic evidence of a vigorous cellular reactivity in chimpanzees infected with S. japonicum.

The frequency of large, exudative granulomas resembling abscesses in S. japonicum infection has been previously mentioned in the classical study by Faust and Meleney in dogs and man. This contrasts with observations made in other host species and indicates that the chimpanzees not only become heavily infected, but also are highly reactive to schistosome antigens. Since Fairley's time there has been speculation about the role of adult worm antigen as an explanation for this marked granulomatous and exudative host response. Faust and Meleney mentioned the possibility of uterine antigen released by female worms at the time of oviposition. Since the present observations are not sufficient to confirm or rule out this possibility, further immunologic and histochemical studies have been initiated.

Other features of the infected chimpanzees suggesting a strong immunologic response were the development of diffuse inflammatory and fibrotic lesions in the large portal fields and in the bilharziomata, as well as the severe vascular alterations in these sites. Since none of the vascular lesions constantly preceded or were present in the diffuse fibrotic lesions, these two features appear to be mutually related, but this is not necessarily a causal relationship.

Portal vein lesions have been observed in S. mansoni infected humans and in S. japonicum infected rabbits, but those seen in the chimpanzee have been the most spectacular. Unlike the portal phlebitis seen in the course of self-healing S. mansoni infections of macaque, the portal phlebitis in the S. japonicum-infected chimpanzee was associated with sustained heavy infections, occurred in the channels of egg embolization and was locally destructive with eventual permanent obliteration. In these animals, even the hepatic vein branches were sometimes involved.

Necrotizing arteritis has been mentioned by Maciel, Coelho and Abath in human transverse myelitis due to S. mansoni. We observed this lesion near sites of heavy egg deposition in the most recent and heaviest infection of the series. While this single observation is not conclusive, it is of interest, since arteritis prominently occurs in experimental serum sickness and in other conditions in which circulating antigen-antibody-complement complexes may play a role. The finding of antiomatoid lesions in hepatic arterioles was also noteworthy.

Glomerular lesions were observed in most of the chimpanzees which had developed pipe-stem fibrosis of the liver. Lima et al. recently reported the association of glomerulonephritis and hepatosplenic schistosomiasis. The renal lesions in these cases were described by Andrade et al., who proposed that this may represent a form of chronic glomerulonephritis which gradually develops in hepatosplenic schistosomiasis. Our observations provide experimental confirmation of their original studies which first noted and recognized the significance of schistosomal glomerulopathy. Spontaneous lesions of this type have not been reported for chimpanzees, although they do occur in other nonhuman primates, such as the bush baby.

Re-examination of kidney sections from chimpanzees infected with S. mansoni and S. haematobium prepared in our previous experiments, revealed that many were normal, but some showed minor focal glomerular changes comparable to the least diseased animals of the S. japonicum study. These alterations resembled those found in biopsies of S. mansoni-infected Brazilian patients by Caetano da Silva, et al. who on the basis of immunochemical electron microscopic studies believe they represent "hepatic glomerulosclerosis." However, the severe glomerular changes found in our S. japonicum study were clearly inflammatory and could be classified as "lobular," "mixed mesangial and membranous" or "membrano-proliferative" glomerulonephritis or "stalk proliferative" glomerulonephritis. This tentative classification would be consistent with the apparent long-drawn and relatively benign course of albuminuri, and with the virtual absence of significant tubular and interstitial lesions in most of these kidneys. A more detailed histopathologic study of these renal lesions is under way; in addition, immunofluorescent and ultrastructural investigations will be required to determine whether circu-schistosome antigen or antigen-antibody complexes may be involved.

The occurrence of glomerulitis and necrotizing arteritis in our animals, as well as the diffuse nature of some of the major inflammatory and fibrous lesions, suggest that, in addition to delayed hypersensitivity, humoral immunologic mechanisms may play a role in the pathogenesis of schistosomiasis japonica of the chimpanzee. With the present state of knowledge, these mechanisms cannot be further defined, but even the possibility of autoimmune phenomena cannot be excluded with certainlty. In a recent study, at least four groups of sera from hamsters infected with S. mansoni produced one or two precipitates against heat-denatured calf thymus DNA and Bacillus subtilis DNA. These lines showed reactions of identity when compared with S. mansoni DNA extracts. Our chimpanzee sera are being tested for similar activity.

chimpanzee is as highly reactive as is the heavily infected "toxemic" human patient, and it may not be coincidental that both develop pipestem fibrosis and bilharziomata. It is noteworthy that in the chimpanzee no individual predilection seems to be required for this "hyper-reactive" or "hyperergic" tissue response, although this has been postulated to be the case in man.

Much has been learned about the immunopathology of schistosomiasis from simple animal models, particularly from studies on experimental granuloma formation induced by intravenous injection of eggs in the mouse and guinea pig. However, our present studies show that in experimental schistosomiasis in the chimpanzee, as in severe chronic schistosomiasis in man, the immunologic status of the host and its role in pathogenesis is quite complex.

Our observations in this series of S. japonicum infected chimpanzees have once again demonstrated the relevance of this experimental model to the disease in man. While many problems of pathogenesis remain unsolved, leads for more definitive studies have been obtained and some are being pursued further.

Project 3A061102B71a, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 105 Parasitic Diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6441	72 07 01	DD-DR&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
71 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. [redacted]	CDOG 114(f)			

11. TITLE (Include with Security Classification Code)

(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
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: 72	13	420
FISCAL YEAR CURRENT: 73	13	420

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide Each with Security Classification Code)

(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 (Provide 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) 71 07 - 72 06 Hepatitis B Antigen (HB Ag) was purified from human plasma and used to prepare highly specific animal antisera. This antisera was used to detect HB Ag in human blood products by a variety of immune precipitin techniques and complement fixation. A study of detection techniques led to the selection and use of a commercial counterelectrophoresis test in U.S. Army blood banks by September 1971. Subsequently, a solid phase radioimmune assay was shown to be 2 to 3 times more sensitive for detecting hepatitis carriers among blood donors. Antigenic characteristics of HB Ag were compared by immunodiffusion: in addition to 3 previously recognized antigenic determinants (a,d,y), 2 new determinants (w,r) were identified. Three subtypes of HB Ag were found: ayw, adw and adr. The predominant subtype from donors in 3 countries was: U.S.A., adw; Thailand, adr; Israel, adw. Studies on arboviruses revealed that morphologically complete intracellular virions of dengue-2 and Japanese encephalitis are less infectious, and have a different polypeptide composition, than released extracellular virions. This may explain the low immunogenicity of foreign-made vaccines prepared from intracellular viruses. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1971 - 30 June 1972.

* 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 68 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 166, Viral Infections of Man

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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. Adenovirus Disease in Military Recruit Populations.

During the past year adenovirus type 4 and 7 (ADV-4, ADV-7) vaccines were used extensively in Army basic training populations. Data obtained from the adenovirus surveillance program indicate that varying degrees of success were achieved by immunization with available vaccines during the period 1 Sep '71 through 31 May '72. Studies in this laboratory pertaining to vaccine potency and type specific neutralizing (N) antibody responses following immunization provided additional data which

helped to explain the varying success that was observed. Finally, data from the first five years of the adenovirus surveillance program (FY '67 FY '71) were re-examined for the purpose of doing a cost-benefit study of ADV-4 and ADV-7 vaccines. The results of the cost-benefit analysis provide an additional data base for making decisions about when, how and where ADV vaccines should be used in recruit populations in the future.

A. Adenovirus Surveillance Program, July 1971 - May 1972

Table 1 summarizes the acute respiratory disease (ARD) hospitalization rates at BCT posts during FY '72. A median weekly ARD rate per 100 trainees for each calendar month is given for each BCT post. Wherever appropriate, the ADV vaccines used during each month and the number of months they were used, have also been indicated.

Two posts. Ft. Campbell and Ft. Lewis discontinued basic training during the past year, thus reducing the number of BCT posts to six.

At Ft. Dix, immunization was initiated on 1 Sep '71, following slight increases in the ARD rate during August. The ADV-7 vaccine used (Lot 16CV-01106) contained $10^{4.6}$ tissue culture infectious dose50 (TCID50) per tablet and the ADV-4 vaccine, approximately $10^{3.5}$ TCID50. By the end of September, ARD rates had fallen at Ft. Dix to below 2.0/100/week and they remained at this low level throughout the months of October, November and December. Immunization was begun at Ft. Wood and Ft. Lewis on 1 Oct '71 with these same vaccines with good results. Ft. Ord experienced an unusual fall epidemic of ARD, primarily associated with ADV-7, but because vaccine supplies were almost exhausted, immunization was not begun at Ft. Ord until early December.

Beginning in January 1972, two new lots of vaccine were obtained for use at all CONUS BCT posts. The ADV-4 vaccine contained only $10^{3.5}$ TCID50 per tablet, and, at the time this vaccine was released by the manufacturer, no back-up lots of ADV-4 vaccine containing an optimal immunizing dose of virus ($10^{5.0}$ TCID50 or greater) were available. Since previous dose-response studies with ADV-4 vaccine indicated that a dose of $10^{3.5}$ TCID50 might be expected to immunize approximately 75% of susceptibles, the decision was made to use the low titer vaccine, along with the new lot of ADV-7 vaccine, released at the same time. The ADV-7 vaccine (Lot 16CV02901) contained $10^{5.0}$ TCID50 per tablet when it was tested for potency in November 1971 by Wyeth Laboratories. No problems were anticipated with an ADV-7 vaccine of this potency. However, preventive medicine offices were notified about the intent to use a low titer ADV-4 vaccine since it was anticipated that if ADV associated ARD did occur, it would be associated with ADV-4 primarily.

Beginning in February, one month after the new ADV-4 and 7 vaccines had been in use, most of the BCT posts began to report modest rises in

Table 1. - Median Weekly ARD Rate per 100 Men for CONUS BCT Posts in FY '72

Month:	July	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May*
Post											
Ft. Campbell	1.1	1.5	1.2	0.5	0.9	1.4	2.4	1.7	(End BCT Training, 1 Feb '72)		
Ft. Dix	1.4	2.0	2.0	1.0	0.8	0.8	1.2	1.2	1.9	2.5	2.2
Ft. Jackson	1.2	1.3	0.8	1.2	1.8	2.8	1.7	3.3	2.9	1.8	1.2
Ft. Knox	0.3	0.3	0.2	0.5	1.0	1.6	0.7	3.2	1.8	1.2	0.7
Ft. Lewis	0.4	0.7	0.4	1.9	3.1	1.7	(End BCT Training, 1 Dec '72)				
Ft. Ord	1.0	0.9	0.9	2.4	6.0	6.5	2.5	3.3	3.1	3.0	2.3
Ft. Polk	0.4	0.3	0.4	NR	0.7	1.4	2.0	2.7	2.4	1.7	1.1
Ft. Wood	0.5	0.7	0.8	1.4	1.5	1.7	2.1	2.7	3.6	2.7	2.1

Adenovirus Vaccines:

x - Adv-7 Lot 16CV01106, titer $10^{4.6}$ $TCID_{50}$/tablet (9/71); Adv-4 Lot 16CI02901, titer $10^{3.5}$ (6/71

o - Adv-7 Lot 16CV02901, titer $10^{5.0}$ (11/71), $10^{4.0}$ (2/72, $10^{3.4}$ (5/72);
Adv-4 Lot 16CI03701, titer $10^{3.5}$ (11/71), $10^{3.2}$ (2/72)

* - Three weeks, ending 20 May 1972

ARD admission rates. This rise continued through March and April and although the ARD rates never reached the peaks that occurred in past years when no vaccines were available, the rates were nonetheless higher than anticipated. At Fts. Dix, Jackson, Ord and Wood, the majority of ADV-associated ARD was caused by ADV-7, whereas at Fts *Knox* and Polk, ADV-associated ARD was caused equally by ADV-4 and ADV-7. During several weeks, as much as 50% of ARD at these posts could be attributed to adenoviruses indicating that the vaccines were not exerting as effective control as they had in 1971 during similar periods. Furthermore, the high percent of ARD admissions associated with ADV-7 suggested that that vaccine was even less effective than the ADV-4 vaccine, a finding incongruous with the reported titer of $10^{5.0}$ $TCID_{50}$ for the ADV-7 vaccine.

The initial potency assay performed in this laboratory in late January 1972 on the ADV-7 vaccine, Lot 16CV02901 had shown a titer of $10^{4.0}$ $TCID_{50}$ per tablet. This was approximately 1 log less than the titer reported by the manufacturer three months earlier. A repeat titration in early May 1972 showed that the titer had fallen even further to $10^{3.4}$ $TCID_{50}$/tablet. The apparent rapid loss of potency *is unexplained*.

When it became apparent in late December 1971 that the low potency ADV-4 vaccine was to be used, a study was designed to assess the immunogenicity of that vaccine in a sample of recruits as well as that of the new lot of ADV-7 vaccine. Accordingly, preventive medicine officers at each BCT post were instructed to furnish this laboratory 25 or 30 acute and 21 day convalescent sera obtained from recruits who had been immunized with the new lots of vaccine during the first week of January. There sera were subsequently tested for the presence of type specific neutralizing antibody to both ADV-4 and 7 and the results are shown in Table 2.

The percent of recruits susceptible to ADV-4 (i.e. those who lacked N antibodies in acute sera diluted 1:4) was 68% and *to* ADV-7, 48%. These results are comparable to previously reported data indicating that approximately 75% of recruits lacked N antibodies to ADV-4 and 50% lacked N antibodies to ADV-7. Following immunization, 76% of those recruits susceptible to ADV-4 showed a rise in ADV-4 N antibody titer, a result which had been predicted based on previous dose-response studies of vaccines of comparable potency. However, the ADV-7 vaccine was even less effective, producing N antibody responses in only 54% of susceptible recruits. This of course corroborates the data from the Adenovirus Surveillance Program which showed more frequent recovery of ADV-7 than ADV-4 from ARD cases at four of the six BCT posts during February - March 1972. This level of immunogenicity is compatible with vaccine titers below $10^{3.5}$ $TCID_{50}$, but the percent of recruits who showed antibody responses is far lower than anticipated, had a vaccine with optimal potency been used.

Table 2. - Type Specific Neutralizing (N) Antibody Responses Following Immunization with ADV-4 (Lot 16 CI03701) and ADV-7 (Lot 16CV02901) Live, Oral Vaccines

	Number Acute Sera Tested	Number Acute Sera Lacking in Antibody	Percent Susceptible	Number Susceptibles Tested for N Antibody Response	Number Susceptibles with N Antibody Response	Percent with N Antibody Response
ADV--4 Antibody	149	98	66%	84	64	76%
ADV-7 Antibody	146	70	48%	70	38	54%

In summary, ADV-4 and 7 vaccines used during early 1972 exerted less than optimal control over adenovirus associated ARD. The ADV-4 vaccine was known to have a low potency at the time immunization was begun, but the ADV-7 vaccine was assumed to be adequate. The results indicate that potency of ADV-7 vaccine declined rapidly, for unexplained reasons, resulting in even less effective protection that that afforded by the ADV-4 vaccine.

(Note: Wyeth Laboratories, the sole supplier of adenovirus vaccines has had periodic difficulties during the past seven years producing vaccines with optimal potency ($10^{5.0}$ *TCID50* or greater). This has been due, largely, to the bulkiness of the diluent used to stabilize the virus in the core of the tablet. Most recently Wyeth Laboratories have produced several small experimental lots of ADV-4 and 7 vaccine using a different formulation which permits them to consistently manufacture vaccine tablets containing optimal potencies. Furthermore, the new formulation seems to provide for greater stability inside the core, markedly prolonging the shelf-life of the tablets. Studies at Wyeth have indicated that potency of the vaccine, stored at 4°C, will remain unchanged over a period of 18 months. ADV-4 and 7 vaccines, produced with the new formulation, were tested in recruits at Ft. Ord during March and April 1972, under the auspices of the Army Investigational Drug Review Board. If, as anticipated, those vaccines are more immunogenic than the vaccines in current use, Wyeth Laboratories will plan to use the new formulation for all subsequent adenovirus vaccine production. Thus the problems that have occurred during 1972 with ADV-4 and 7 vaccines should be avoided in the future.)

B. Adenovirus Type 4 and 7 Vaccines: A Cost-Benefit Analysis

1. General considerations

A cost-benefit analysis of preventive medicine measures such as vaccines is a comparison of the cost to develop, test, purchase, administer and monitor the vaccines as compared to the benefits obtained. Costs are relatively straightforward and can be measured, and when not measurable, at least closely estimated in terms of dollars. In order to similarly quantify the benefits of preventive measures, one should measure the cost savings in dollars of preventing the illness or disease. The costs of the illness include both direct and indirect costs (Rice, D. P., 1969).

a. Direct costs are expenditures for the delivery of prevention, detection and treatment of the illness. Such direct costs may include the costs of hospital and outpatient care, physicians' and other medical and professional care, drugs and medical supplies.

b. Indirect costs of illness are those costs that result from being away from one's work and include the individual's salary as well as the cost to the employer in lost or decreased productivity. If the illness studied causes mortality as well, an additional indirect cost is calculated by discounting that individual's earnings for the remainder of his productive life. Tables for figuring the indirect costs of mortality have been prepared for such studies and can constitute a considerable proportion of the benefits achieved (Weisbrod, B. A., 1961 and Rice, D. P., 1966)

c. Cost-benefit versus cost-effectiveness. It is important to distinguish between cost-benefit analyses and cost-effectiveness studies because the two are often confused. Cost-effectiveness analysis differs from cost-benefit analysis in that costs are calculated and alternate ways are compared for achieving a *specific* set of results (Smith, W. F., 1968). In the discussion that follows a comparison of alternative methods for control of ARD against a measurable endpoint is not possible. Thus, at this point, it can only be determined whether the benefits of the two vaccines (ADV-4 and ADV-7 vaccine) used to control ARD in military trainees outweigh their costs. A ratio of greater than one is a favorable result. If additional vaccines or control methods are developed in the future, then the ratios of various methods of control could be compared against the same end point in a cost-effectiveness study.

2. Cost of ADV-4 and ADV-7 vaccines

The total cost of ADV-4 and ADV-7 vaccines will be the sum of the cost to the Army to research and develop, test, administer and monitor the vaccines from 1966 to 1971.

Research and development costs between 1966 and 1971 occurred primarily from the development and testing of ADV-7 vaccine. As previously stated, ADV-4 vaccine was already developed and tested when made available to the Army in 1966. Although there were some ADV-4 vaccine studies carried out by the Army in 1966 and 1967, major research costs were incurred beginning in 1968 associated with the development and testing of the ADV-7 vaccine. Total five year research and development costs (1966-1971), given in 1971 dollar values, are estimated to be $684,700. This estimate was based on allocation of a proportion of known costs of operating The Department of Virus Diseases, Walter Reed Army Institute of Research, where vaccine testing and development was carried out. The estimate included costs of equipment, supplies, travel associated with field studies, and both military and civilian personnel salaries, as well as overhead.

Extensive administration, monitoring and surveillance of ADV-4 vaccine was begun in the Fall of 1966 with the creation of the Adenovirus

Surveillance Program. The costs of this activity are given by year and category in Table 3. For FY '67, ADV-4 vaccine purchase cost was $115,000 and the remaining $470,000 noted in column 1 was spent for equipment and supplies required to increase the Army Area Laboratories' capability to support the Adenovirus Surveillance Program. In subsequent years the costs of equipment and supplies decreased in proportion to vaccine costs so that by FY '71, $260,000 was spent for equipment and supplies and $100,000 for vaccine. Note here that both ADV-4 and ADV-7 vaccines were purchased in FY '71 at a cost below the purchase price for ADV-4 vaccine alone in FY '67. Each dose of vaccine (either ADV-4 or ADV-7) cost $0.225 in FY '71, resulting in an immunization cost of $0.45 for each recruit.

Throughout this paper, FY '71 dollar values are used for the cost-benefit comparisons, and these are shown in column 1-a of Table 1. These were calculated by using an inflationary rate of 6% compounded annually. Personnel costs shown in column 2 of Table 1 include the salaries of personnel at Army posts as well as personnel at Walter Reed and the Office of the Surgeon General who were involved in the Adenovirus Surveillance Program. As such, these costs include salaries from the rank of E-3 to Lieutenant Colonel as well as two civilian secretaries using FY '71 pay scales. Column 3 of Table 3 lists miscellaneous training and travel costs incurred in the Administration and Operation of the Adenovirus Surveillance Program. These costs, averaged over the five year period, include those associated with temporary duty, education and training expenses and are reported in FY '71 dollar values.

Since parts of many Army facilities were used for the Adenovirus Surveillance Program between FY '67 and FY'71, it is difficult to calculate exact overhead costs. The Army comptrollers recommended that 40% of the total costs was an acceptable estimate of overhead cost and this has been added to the total 5-year program costs in Table 3. The grand total of the costs for the Adenovirus Surveillance Program from its inception in July 1966 to July 1971, plus vaccine research and development costs is $4,233,739 plus $684,700, or $4,918,439.

3. Benefits of ADV-4 and ADV-7 vaccines

a. The cost of one ARD illness. The benefits of these vaccines are the costs of the illnesses prevented. As previously discussed, the costs of illness comprise both direct and indirect costs. Direct costs of caring for an uncomplicated hospitalized ARD illness include the cost of one dispensary visit ($8.00) and costs associated with an average 3 day (± 1 day) hospitalization at $58.50 day. The latter costs include all professional services as well as medications and supplies used. The total for one dispensary visit and three days of hospitalization is $183.50, which represents the direct cost savings of preventing one hospitalized case of ARD. Indirect costs are the wages of the trainee during hospitalization as well as the costs of lost training time. For

Table 3. - Adenovirus Vaccine and Surveillance Program Costs in Dollars for FY '67 - FY '71

Fiscal Year	1 Equipment Supplies Vaccine	1-a 1971 Dollars*	2 Personnel Salaries**	3 Training and Travel Expenses**	
FY '67	585,000	738,549	134,000	5,950	
FY '68	365,000	439,700	134,000	5,950	
FY '69	360,000	404,500	134,000	5,950	
FY '70	360,000	381,600	134,000	5,950	
FY '71	360,000	360,000	134,000	5,950	
TOTAL	2,030,000	2,324,349	670,000	29,750	3,024,099
				+ 40% overhead	1,209,640
					4,233,739

* Inflationary rate of 6% per annum

** Given according to FY '71 Salaries and Expenses

FY '71, the salary of a typical recruit was $4.42/day and the cost of training him was $27.40/day. Thus, for a 3-day hospitalization, indirect costs are $95.46. Table 4 summarizes direct and indirect cost of one hospitalized case of ARD which results in a total expenditure of $278.96 per illness in FY '71 dollars.

b. Estimate of total ARD illnesses prevented. Beginning in the Fall of 1966 each BCT post was required to report the total number of recruits hospitalized because of ARD. Total ARD admissions when divided by the number of trainees at risk and the quotient multiplied by 100 yields an ARD hospitalization rate per 100 trainees per unit time (week, month, etc.). In addition to reporting ARD rates, each BCT post was required to provide specimens (throat swabs and serum) from a portion of trainees hospitalized with ARD in order to determine the cause of their illness by appropriate virologic and/or serologic studies.

Several qualifications and assumptions underlie any estimate of ARD illnesses prevented by the use of ADV-4 and ADV-7 vaccines. First, an estimate of what the ARD rate might have been at a given BCT post during a given month had ADV-4 and 7 vaccines not been used was based on available data obtained during prior years when both vaccines were not used, or, in the case of Ft. Dix, when ADV-4 vaccine alone was used. In most instances three or four ARD rates for each month (at each post) were available from previous surveillance data, and from these a median ARD rate for that month was calculated. The monthly median ARD rate for a given post prior to both ADV-4 and 7 vaccines is the estimate of what the ARD rate would have been during that month had vaccines not been used.

Second, the monthly median ARD rate is compared with the actual ARD rate obtained during the same calendar month when both vaccines were used. The difference between the two, when multiplied by trainees at risk, yields an estimate of the number of illnesses prevented by both vaccines. Here the assumption is that any differences in rates are attributable exclusively to the use of the vaccines and this may not be entirely true. The vaccines, however, are the only variable which can be directly related to the observed differences.

Third, the number of trainees at risk (recruit training strength) varies from post to post, from month to month. Thus, an estimate of the number of illnesses prevented necessarily was determined for each month vaccines were used at each post. A monthly estimate of trainees at risk was obtained by using the actual training strength for the week ending nearest the 15th of each month. The number of prevented illnesses could be no greater nor fewer than the number of trainees at risk during the months that vaccines were used, multiplied by the difference in ARD rates for those months attributed to immunization.

Table 5 illustrates the data used to obtain an estimate of the

Table 4. - Cost of ARD Illness*
(Potential Benefits)

Direct Costs of Illness	$	Totals
Dispensary Visit	8.00/visit	
Hospitalization	58.50/day	
1 Visit & 3 days Hospitalized		183.50
Indirect Costs of Illness	**$**	
BCT Salary	4.42/day	
BCT Training	27.40/day	
For 3 days Missed		95.46
	TOTAL	$278.96

*FY '71 costs

Figure 6. Immunodiffusion reactions of absorbed anti-ay and anti-ad

Table 5. - Ft. Dix

	ARD Rates per 100 Trainees per Month						Training Strength	Estimate ARD Hospitalizations Prevented
	ADV-4 Vaccine Alone				ADV 4 & 7 Vaccines	Rate Difference		
	FY '67	FY '68	FY '69	Median	FY '70			
Feb	21.8	31.0	13.0	21.8	13.1	8.7	9,060	788
Mar	22.4	19.7	19.7	19.7	11.7	8.0	9,197	735
Apr	27.8	19.3	22.8	22.8	12.0	10.2	8,924	946
May	20.2	18.5	12.0	18.5	6.4	12.1	8,881	1,075
							TOTAL	3,562

Table 9 - Distribution of HB Ag Immunoprecipitin Patterns by Diagnosis of Carrier

Diagnosis	Precipitin Pattern			Total
	1	2	3	
Hepatitis	155	9	15	179

number of illnesses prevented at Ft. Dix, N. J., where the vaccines were first used together on a large scale in February 1970. Note that in prior years, FY '67 - '69, ADV-4 vaccine had always been used during February - May. This is the only post where monthly median ARD rates obtained from surveillance data during years before ADV-4 and 7 useage were based on the use of ADV-4 vaccine. Table 6 illustrates similarly obtained data for Ft. Knox, Kentucky during FY '71 when ADV-4 and 7 vaccines were first used together there. A brief comparison of rates in the two tables illustrates the considerable variability among all posts and the resultant differences in the estimate of total number of illnesses prevented.

Table 7 is a compilation of estimated illnesses prevented at each BCT post during FY '70 and FY '71 attributable to use of ADV-4 and ADV-7 vaccines. The number of months these vaccines were used varied from post to post. Only those months when 50% or more of trainees at risk were immunized were included in the analyses. During FY '70 when the vaccines saw limited use and FY'71 when they were used at all BCT posts, it is estimated that 26,979 cases of ARD were prevented. The benefit (cost saved) in preventing one ARD hospitalization was shown to be approximately $279; thus, the total benefits in preventing 26,979 hospitalizations are: $279.00 x 26,979, or $7,527,141.

c. Benefit-cost ratio. The estimate of dollars saved by use of vaccines during FY '70 and '71 ($7.53 million) divided by costs of the Adenovirus Surveillance Program and vaccine research and development for FY '67 - '71 ($4.92 million) yields a favorable benefit-cost ratio of approximately 1.5.

In addition to the calculation of the above ratio, it is now possible to estimate the number of ARD hospitalizations that need to be prevented at each post to assure that program costs, plus the cost of immunization, equal the benefits derived. Program costs are shared equally among BCT posts. The unequal cost variable is the number of men immunized each month. Thus, in FY '71, total program costs, less the cost of vaccines were $599,960, including overhead. Allocated equally among eight BCT posts, the cost per post was $74,995 for the year. Surveillance and monitoring costs (activities) are year-round, regardless of the ARD rate. Therefore, they may in turn be divided by 12 to yield a monthly cost of $6,250 per post.

For any single BCT post, the costs of immunization include the post's share of the program costs ($6,250/month) plus the cost of vaccine for immunizing "x" number of recruits during that month. Thus, if 3,000 men are immunized -- a typical month's recruit input, the total cost of immunization is $6,250 plus vaccine costs of $1,350 or $7,600. To achieve a benefit-cost ratio of 1.0, the vaccines need only prevent 27.2 ARD hospitalizations: ($7,600 divided by $279 -- the cost on one ARD hospitalization, equals 27.2).

Table 6. - Ft. Knox

	ARD Rates per 100 Trainees per Month						Training Strength	Estimate ARD Hospitalizations Prevented
	Without Vaccine(s)				ADV 4 & 7 Vaccines	Rate Difference		
	FY '68	FY '69	FY '70	Median				
Feb	16.0	1.4	13.0	13.0	4.8	8.2	8,923	732
Mar	12.1	10.7	18.2	12.1	5.0	7.1	9,200	653
Apr	6.0	9.0	8.2	8.2	3.5	4.7	8,200	385
May	1.6	5.6	5.6	5.6	3.7	1.9	7,375	140
							TOTAL	1,910

Table 7. - Total ARD Illnesses Prevented

Year	Post	Months of Immunization	Estimate of ARD Hospitalizations Prevented
FY '70	Ft. Dix	4	3,562
	Ft. Lewis	4	1,799
	Ft. Wood	4	989
FY '71	Ft. Campbell	4	3,228
	Ft. Dix	7	7,224
	Ft. Jackson	4	1,540
	Ft. Knox	4	1,910
	Ft. Lewis	7	3,026
	Ft. Ord	4	1,054
	Ft. Polk	4	1,075
	Ft. Wood	7	1,572
		TOTAL, FY '70 & '71	26,979

At Ft. Polk, La. in the months of April and May 1971, between 3,000 and 4,000 men were immunized each month. Total recruits hospitalized with ARD were 212 and 153 respectively and, based on previous years' admission rates, an estimate of the number of hospitalizations prevented each month was 106 (April) and 92 (May). Thus favorable benefit-cost ratios were obtained in each instance. However, had the same number of men been immunized in the following July or August when the ARD rates were very low, it is doubtful that favorable ratios would have been achieved. During those months, the numbers of recruits hospitalized with ARD were 121 and 91 respectively, and of those sampled only 3 recruits had adenoviruses isolated from their respiratory tracts. Based on these kinds of data, one might predict that immunization would not have prevented 27 or more ARD hospitalizations during either month, resulting in benefit-cost ratios of less than 1.0.

4. Discussion

Aaron Wildarsky has stated: "The great advantage of cost-benefit analyses, when pursued with integrity, is that some implicit judgments are made explicit and subject to analysis." (Lyden, J. and Miller, G., 1967). Thus, the purpose of studying the costs versus the benefits of ADV-4 and ADV-7 vaccines is not to justify their use on purely economic grounds. Rather, the purpose is to use the economic tool of cost-benefit analysis to better understand the impact of these vaccines in economic terms and to develop an economic data base for making decisions about when and how to use these vaccines in the future.

Cost-benefit studies applied to the health field first became popular in the late 50's and several detailed analyses have been done by Fein, Weisbrod, Klarman, Mushkin and Rice in recent years (Fein, R. 1958, Klarman, H. E. 1964, Mushkin, S. J. 1962). The terminology and methodology used in the present study may be found in one or more of those analyses.

For a number of reasons, the present cost-benefit analysis of ADV-4 and ADV-7 vaccines in military training populations, is unique among similar analyses of other preventive measures in the health field.

First, the costs of illness in the present study are primarily those associated with morbidity and the associated direct costs of hospitalization. Recruit ARD is so rarely associated with mortality that such costs have been ignored in this paper. In similar studies of other diseases, mortality and the associated indirect costs of lost future earnings contribute a major portion of total illness cost and benefits derived from preventing the illness.

Second, most data pertaining to the incidence of a specific disease cited in other cost-benefit studies have been based on sampling procedures rather than measuring all the disease in the entire population at risk.

Third, accurate and reliable diagnosis of certain diseases may be difficult, especially in the case of childhood exanthems such as measles. In the present study, not only were the diagnostic criteria standardized (recruits with fever >100°F and upper respiratory symptoms) but the fact that they were almost invariably hospitalized provides a relatively finite endpoint for measurement of the disease.

The direct costs of ARD hospitalization and illness not considered in this analysis were those costs associated with the standard methods used in the prevention of ARD in recruit populations. These include such things as proper ventilation, the provision of each recruit with adequate living space, and many other measures aimed at decreasing droplet transmission of disease. These costs are not added to the benefits in that such measures are essential for the prevention *of* other diseases, specifically meningitis, and would not be discontinued even if control of ARD in trainees is achieved.

Other costs of ARD hospitalization not considered are those associated with recycling individuals who are hospitalized for an extended period of time (usually more than five days). As previously noted, the average length of hospitalization for ARD is three days. However, a recruit, admitted because of ARD, who develops a more serious respiratory illness such as pneumonia, may miss two or three weeks of training. Whenever a recruit cannot "make up" the training time lost because of illness, he must be recycled (transferred) to another training company. Although the number of men recycled because of prolonged hospitalization associated with ARD is small, recycling creates a number of logistical and operational problems for the training center which result in additional costs associated with illness. And, beyond the tangible costs of recycling are the intangible costs associated with lower morale that results when a recruit cannot complete training with the company or platoon to which he was assigned initially.

In addition, the measure of the benefits of these vaccines ignored the intangible benefits. Benefits were measured only in terms of an estimate of hospitalized illnesses prevented and as such do not include any measure of illnesses prevented that did not require hospitalization. Past studies have shown that among a group of susceptible recruits who acquired adenovirus infections during training only 40% were actually hospitalized (Buescher, E. L. 1967). However, an equal percent may have symptoms of illness which are most certainly associated with discomfort and impaired function. Prevention of such discomfort is a rather specific intangible benefit. Economists are well aware of the problem of distorting the overall economic and social costs by assuming an economic value for intangibles as zero (Klarman, H. E. 1965). One cannot place dollar values on the intangible benefits of these vaccines, but they must be considered along with the cost-benefit analysis when such data is used for making any decision about when and how they are to be used.

The benefit-cost ratio of ADV-4 and ADV-7 vaccines was shown to be highly favorable for the first 5 years of the Adenovirus Surveillance Program. Total program costs included vaccine research and development costs during that period. As such, these costs are "sunken" and will not be included in future program costs, resulting in potentially lower future costs over-all. Provided the continued availability of potent ADV-4 and ADV-7 vaccines and the inability of another respiratory pathogen to produce a significant amount of ARD in recruit populations, the future benefits from using these vaccines should at least equal the benefits reported in this study. A number of decisions will have to be made in the future with regard to when, where and how ADV-4 and 7 vaccines are to be administered to achieve optimal control of ARD in recruit populations. It is anticipated that the economic tool of cost-benefit analysis may provide additional capability and rationale for making those decisions.

II. Hepatitis

During the past year there has been general acceptance of new terminology. Australia antigen (Hepatitis-associated antigen) is now known as Hepatitis B Antigen (HG Ag) since it is only found in Hepatitis B. Antibody to it is designated Hepatitis B Antibody (HB Ab). Studies of HB Ag were directed toward an analysis of antigenic subtypes, evaluation of more sensitive techniques for detecting antigen and antibody, and related clinical studies.

A. Characterization of HB Ag

1. Detection of additional antigenic determinants of Hepatitis B antigen

Using an immunodiffusion procedure, Levene and Blumberg (1969) first suggested that Hepatitis B Antigens (HB Ag, Australia Antigen) from different individuals may differ in their precipitin reactions. They designated a commonly shared antigenic determinant as a and nonidentical antigens as ab or ac. Kim and Tilles (1971) reported that HB Ag may have one, two or three determinants and that antigens differed in their electrophoretic mobility, depending upon which determinant were present. They also named their determinants a, b and c without implying identity to those of the earlier authors. Le Bouvier (1971) used d, y and x to designate determinants, which differed from the b and c of Levene and Blumberg. He suggested d and y do not occur together and may reflect differences in the genotype of the virus. The ability to detect differences in the antigenic configuration of the agent causing hepatitis may be essential to acquiring a full understanding of the routes of transmission and clinical expression of this infection. Epidemiologic studies will require techniques which are capable of distinguishing minor antigenic differences. This report describes a technique for comparing antigenic determinants by immuno-

diffusion. A total of 5 antigenic determinants has been recognized.

Hepatitis B antigen preparation. Two whole human plasmas containing Hepatitis B antigen (HB Ag) were used for antigen preparation. Each had a complement fixing titer of HB Ag of 1:256 or greater. Plasma J020 was collected from an American with icteric hepatitis on the 16th day of illness. Plasma S2794 came from a Thai blood donor. Separation of HB Ag from the normal plasma proteins was accomplished by a three-step combination of equilibrium centrifugation in cesium chloride (CsCl) and rate zonal centrifugation in sucrose gradients. All gradients were prepared in 0.02M Tris(Hydroxymethyl)Aminomethane (Tris) in distilled water, pH, 8.3. For the initial step, a discontinuous CsCl gradient was formed of four layers with densities of 1.3300 (3.0 ml), 1.24519 (3.0 ml), 1.18546 (9.0 ml) and 1.10943 (10.0 ml). Five milliliters of plasma was layered over the gradient and centrifuged at 25,000 rpm for 18 hr in a Beckman SW 25.1 rotor. Thirty fractions of equal volume were collected dropwise from the bottom of each tube. For the second step, fractions containing HB Ag but with no visible color were pooled. The density was adjusted to 1.203 with saturated CsCl solution and the pool was centrifuged to equilibrium at 39,000 rpm for 40 hr in a Beckman SW 39 rotor. HB Ag was concentrated in a visible band between densities 1.1691 to 1.2202. Gradient fractions of approximately 0.35 ml each were collected from the top and tested for HB Ag and normal serum proteins by complement fixation tests (see below). All HB Ag-containing fractions with CF antigen titers of normal serum protein of less than 1:512 were pooled for further purification. In the third step, CsCl was removed from the pooled antigen by pressure filtration through a PM 30 membrane in an Amicon pressure cell by the continuous addition of 0.02M Tris in distilled water, pH 8.3. The resulting material had a density of 1.008 or less. A 0.25 ml aliquot was layered over 4.4 ml of a 5 - 25% preformed sucrose gradient. Following centrifugation at 50,000 rpm for 100 min in a Beckman SW 50 rotor, 12 or 13 fractions were collected from the bottom of each tube. Fractions containing HB Ag but no detectable human serum protein by CF were kept separate and used for animal immunization (Fig. 1). HB Ag prepared in this manner was examined under an electron-microscope using uranyl acetate as a negative stain. Only small homogeneous particles of approximately 22 nm diameter were found. The large particles and filamentous forms described by Dane et al (1970) were not seen.

Hepatitis B antiserum. Sucrose fractions selected for use as immunizing antigens were diluted 1:2 with Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) and thoroughly emulsified. Each rabbit received 0.25 ml of emulsion injected intradermally into each of four sites on the back. Identical doses were given again after four weeks and the rabbits were exsanguinated at six weeks. Each antiserum was used in immunodiffusion tests at dilutions giving balanced reactions with standard sera containing HB Ag.

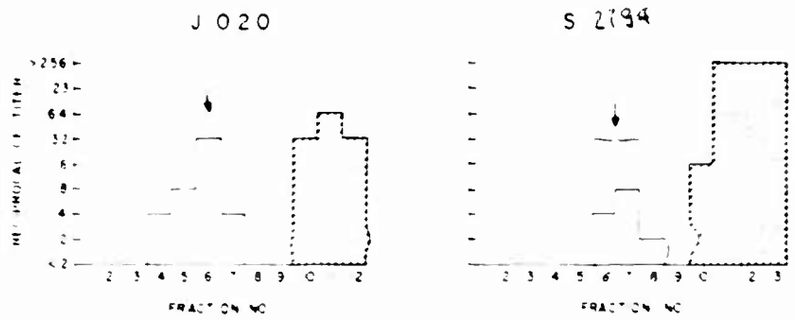

Figure 1. Distribution of HB Ag and normal serum proteins during the third step of antigen preparation. J020 is a plasma containing ay determinants; S2794 contains ad determinants. In each case a 0.25 ml aliquot of material concentrated in the second-step cesium chloride centrifugation was layered over 4.4 ml of a 5 - 25% sucrose gradient and centrifuged at 50,000 rpm for 100 min in a Beckman SW 50 rotor. Fractions were collected dropwise from the bottom of the gradient and numbered accordingly. Each fraction was tested for HB Ag and normal serum protein by complement fixation. The arrows indicate the fractions used to immunize rabbits.

Human serum protein antiserum. Rabbits were immunized with a pool of serum collected from 10 healthy adults. After the pool was diluted 1:2 with Freund's incomplete adjuvant, 0.5 ml was injected intradermally into each of four sites on the back of each animal. Four weeks later, each animal again received 0 5 ml of the same antigen mixture intradermally in each site. The animals were exsanguinated at seven weeks. In complement fixation tests, this antiserum detected normal serum diluted from 1:64 to 1:262,144.

Complement fixation tests. Complement fixation (CF) tests for HB Ag were performed in the manner of Purcell and associates (1920) using microtiter equipment and four units of guinea pig antibody. CF tests for normal human serum proteins used 16 units of rabbit antibody. Reagents were standardized by the method of Kent and Fife (1963).

Immunodiffusion test. Immunodiffusion (ID) was performed by a micro-Ouchterlony (1958) technique using 1.0% agarose dissolved in a buffer solution containing 0.01 M Tris (Hydroxymethyl) Aminomethane, 0.01 M Ethylenediaminetetraacetic acid, 0.1 M sodium chloride and 1:10000 Thimerasol for a preservative at pH 7.6. Three milliliters of agarose solution were dispensed on each 2.5 x 7.5 cm glass slide. Two seven-hole patterns were punched into each slide, forming wells 3 mm in diameter and 6 mm from center to center. Each well held approximately

6 μ liter. Slides were incubated in a moist chamber at room temperature and read unstained at 24, 48 and 72 hr.

Analysis of precipitating antigens. ID tests were used to compare the precipitation reactions of human sera containing HB Ag from different sources to reference sera containing known antigenic determinants. The reference antigens were plasma J020, S2794 and S561. J020 and S2794 were known to contain ayx and adx, respectively (G. Le Bouvier, personal communication). S561 was shown to be antigenically identical to S2794 by ID. Unabsorbed rabbit antiserum to J020 and S2794 were used as anti-ay and anti-ad reference sera. In each ID slide, J020 (ay) was placed in the top well of each pattern, S561 (ad) in the lower. Reference antiserum was placed in the center well, anti-ay on the left and anti-ad on the right (Fig. 2). Two sera containing antigen to be studied were tested on each slide with each serum in a well adjacent to every combination of reference antigen and antiserum.

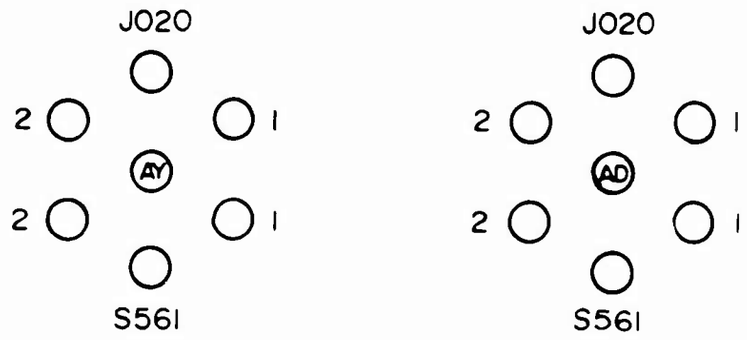

Figure 2. Well arrangement used for immunodiffusion studies of HB Ag determinants. J020 is a reference ay antigen; S561 is a reference ad. AY is a rabbit antiserum to J020, AD an antiserum to S2794, another reference ad antigen. Each test serum is placed in four peripheral wells (1 or 2).

Antiserum absorption. Absorption was accomplished by diluting each antiserum with sera containing appropriate HB Ag. The mixtures of antiserum and absorbing serum were incubated at 37° for 120 min, at 4° C for 18 hr and centrifuged at 2800 rpm for 60 min. The supernatant was decanted and used as absorbed antiserum. To determine the effect of dilution alone on reactivity, each antiserum was also diluted to an

equal extent with a normal human serum (NHuS).

Immunodiffusion patterns. The antigenic determinants of the test sera were determined by immunodiffusion reactions of complete or partial identity formed between them and the reference antigens. Three distinctive patterns were observed (Fig. 3). Some specimens showed complete identity with the reference ay antigen and partial identity (spur) with ad antigen (pattern 1); other sera showed identity with ad and partial identity to ay (pattern 2). A third group of sera contained HB Ag which gave reactions of partial identity with both reference antigens (pattern 3). Test sera which failed to react with both reference antisera or which did not form spur reactions with either reference antigen were considered to have too little antigen for adequate testing and therefore were not classified. The observed reaction patterns indicated that each antiserum recognized at least three separate antigenic determinants. Furthermore, the two reference antigens had at least three antigenic determinants each. One determinant was shared in common between antigens of all patterns and is hereafter referred to as a.

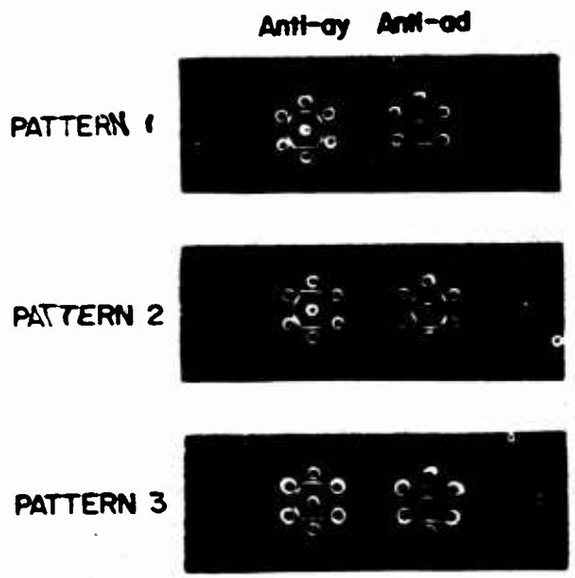

Figure 3. Three patterns of immunodiffusion reactions observed with test sera containing HB Ag. Unabsorbed rabbit anti-ay serum is in the center wells on the left and anti-ad on the right. In each set of wells, reference J020 (ay) and S561 (ad) antigens are in the top and bottom wells, respectively. Some test sera formed reactions of complete identity with one of the reference antigens (Patterns 1 and 2). Other sera showed partial identity to both reference antigens (Pattern 3).

Reactivity of absorbed antiserum. Aliquots of rabbit anti-ad serum were diluted equally with J020 (Pattern 1 antigen), Y439 (Pattern 3 antigen) and NHuS. Diluting the antiserum with NHuS did not alter its ability to detect three antigenic determinants (Fig. 4). Absorption of the anti-ad serum with J020 (ay) removed the reactivity with the common a determinant. Absorption of the anti-ad serum with Y439 also removed the anti-a reactivity and, in addition, removed the reactivity to another determinant shared by the pattern 2 and 3 antigens. Absorption with the pattern 3 antigen left only the reactivity to the homologous antigen. When both reference antisera were absorbed and

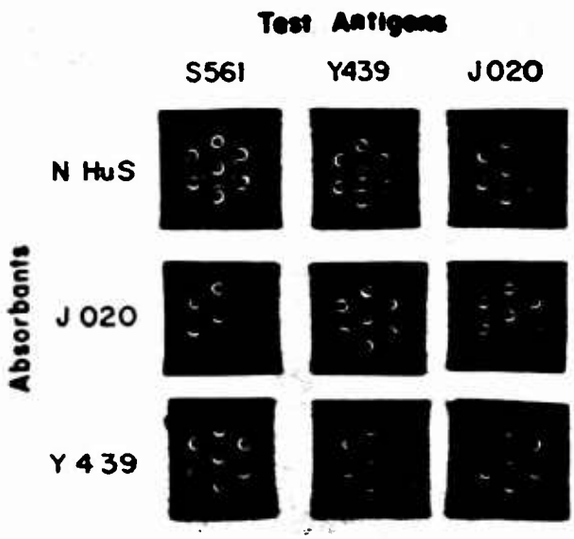

Figure 4. Immunodiffusion reactions showing the effect of absorbing one rabbit anti-ad serum made to a pattern 2 antigen with plasma containing Hb Ag with heterologous antigenic determinants. In each row the antiserum is diluted 1:3 with a normal human serum (NHuS), top row; a pattern 1 antigen (J020), middle row; and a pattern 3 antigen (Y439), bottom row. In each arrangement of wells, the top well contains standard ay antigen (J020) and the bottom well contains standard ad antigen (S561). The test antigens in the side wells for each column are indicated at the top.

compared (Fig. 5), it was apparent that the pattern 3 antigens share one common determinant with both reference antigens, another with ay alone and a third with ad alone. To show that these antisera were reacting

only with determinants on HB Ag, each absorbed antiserum was allowed to react with an antigen of each precipitin pattern (Fig. 6). The reaction lines of complete identity indicated each antiserum was reacting with the same multideterminant antigen structure.

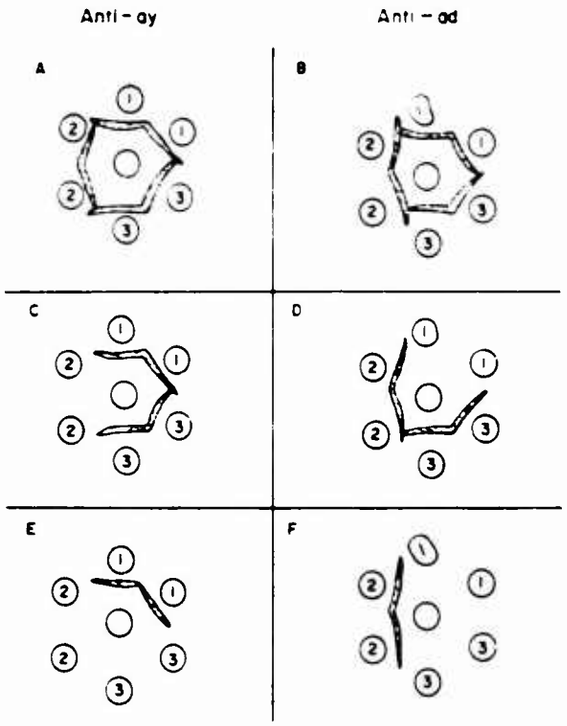

Figure 5. Reactivity of rabbit antisera by immunodiffusion after absorption with HB Ag with heterologous antigenic determinants. Anti-ay, in the center well on the left, was made to a pattern 1 antigen. Anti-ad, in the center wells on the right, was made to a pattern 2 antigen. The antisera were absorbed with NHuS (A, B); a pattern 2 antigen (C): a pattern 1 antigen (D); and a pattern 3 antigen (E, F). The final dilution after absorption was the same for each antiserum. Peripheral wells contain six different human sera containing HB Ag. The numbers indicate the immunodiffusion precipitin patterns of the antigen within the wells.

Figure 6. Immunodiffusion reactions of absorbed anti-ay and anti-ad rabbit sera with Hepatitis B Antigens having different antigenic determinants. The center wells, from left to right, contain HB Ag giving precipitin patterns 1, 2 and 3, respectively. The peripheral wells contain, in clockwise order, anti-ay + NHuS (top well), anti-ay + pattern 2 HB Ag (S561), anti-ay + pattern 3 HB Ag (Y439), anti-ad + NHuS, anti-ad + pattern 1 HB Ag (J022) and anti-ad + pattern 3 HB Ag (Y439).

Prevalence of precipitin patterns. Between August 1969 and November 1971, HB Ag was detected by CF in one or more serum samples from 628 individuals. Sera from 310 (49.4%) contained sufficient antigen to determine the precipitin patterns. In most cases there was incomplete historical information about the source of the serum. However, the majority came from American males and patients with hepatitis. There was no obvious relationship between the precipitin pattern and the sex or diagnosis of the individual (Tables 8 and 9).

Table 8 - Distribution of HB Ag Immunoprecipitin Patterns by Sex of Carrier

Sex	Precipitin Pattern			Total
	1	2	3	
Male	199	13	31	243
Female	28	0	11	39
Unknown	6	18	4	28
Total	233	31	46	310

Table 9 - Distribution of HB Ag Immunoprecipitin Patterns by Diagnosis of Carrier

Diagnosis	Precipitin Pattern			Total
	1	2	3	
Hepatitis	155	9	15	179
Blood Donor	2	11	3	16
Other	6	0	5	11
Unknown	70	11	23	104
Total	233	31	46	310

Patterns 1 and 3 were the most frequent types of antigen found in the United States (Table 10). On the other hand, pattern 2 was most common in Thai nationals and frequently found in Americans with acute hepatitis in Korea. Multiple serum specimens were tested from 45 individuals to determine if the precipitin pattern changed during the course of the antigenemia. The precipitin pattern was determined for 98 of

Table 10 - Distribution of HB Ag Immunoprecipitin Patterns by Geographic Location of Carrier

Country	Precipitin Pattern			Total
	1	2	3	
U. S. A.	228	7	45	280
Thailand	2	20	1	23
Korea	3	4	0	7
Total	233	31	46	310

126 sera. In each case, the pattern did not change or became indeterminant as the CF titer of HB Ag fell.

Discussion. The observation of three precipitin patterns was not predicted, but not unexpected, since HB Ag may have many antigenic determinants. The observed reactions are not considered to be artifacts of the test system, since they were reproducible and antibody specificities of each antiserum were selectively removed by absorption. It is also unlikely that the pattern 3 antigens represent a new configuration of the previously recognized determinants a, d, y and x. Such an arrangement would have to allow pattern 3 antigens to share one determinant with both reference antigens, and one other with each reference antigen separately. The only configurations permitting this would be ady and dyx. In either situation, absorbing anti-ad serum with Y439 (pattern 3) would be expected to remove the reactivity with the reference ad antigen. This was not observed (Fig. 4). The absorption studies indicated that d and y do not exist together, which agrees with the findings of Le Bouvier (1971).

All of the sera containing HB Ag represented in Fig. 5 were found to have the x determinant (G. Le Bouvier, personal communication). It is apparent, then, that the x determinant is not the reason for partial identity among these samples. If x is indeed an antigenic determinant of HB Ag, it cannot be distinguished from a with these antisera. It is possible that a represents multiple and, as yet, undifferentiated determinants that are shared in common by HB Ag from many individuals.

The best explanation for the observed reactions is that there are two additional antigenic determinants (w, r), one on the reference ay antigen, the other on the reference ad, and these are shared in some manner with antigen giving the pattern 3 reaction. Three combinations of determinants could give the pattern 3 reaction (Table II). On the basis of current information, adw seems most likely, since other investigators using human antisera have not been able to distinguish pattern 2 antigens from pattern 3 antigens (G. Le Bouvier, P. Holland, personal communications). The single anti-r specificity remaining after absorbing ADR antiserum with an adw antigen is demonstrated in Fig. 4, bottom row, and in Fig. 5, F. Fig. 5, E, represents anti-y specificity after absorbing AYW antiserum with adw antigen. The r determinant was detected in 20/23 (87%) of the Thai antigens but only 7/280 (2.5%) of the American antigens. With this apparent unequal geographic distribution, it is likely that the W and r determinants would not have been recognized had the reference antisera been made to ay and ad antigens from the United States. The difference in prevalence of these antigenic determinants suggests there are major geographic differences in the distribution of serotypes of HB Ag. Definitive epidemiologic studies of hepatitis virus transmission, expression of infection and chronic carrier states will require sensitive and precise techniques for detecting these serotypes.

Table 11 - Proposed Configurations of Antigenic Determinants of Hepatitis B Antigens and Antisera

	Immunodiffusion Reaction*	Standard Reagent	Proposed Determinants
Human Antigens:	Pattern 1	J020	ayw**
	Pattern 2	S561	adr
	Pattern 3	Y439	adw
			ayr
			awr
Rabbit Antisera:		anti-ay	AYW***
		anti-ad	ADR

* - Precipitin patterns observed in immunodiffusion tests

** - Small letters designate antigenic determinants

*** - Capital letters designate specific antibody populations

2. Further investigations of antigenic subtypes

Subsequent work has led to the purification of an adw antigen for the preparation of ADW rabbit antiserum. Preliminary testing of this antiserum shows it can distinguish adr antigens from adw. This should permit the production of limited amounts of specific anti-w, anti-r, anti-d and anti-y antisera. Furthermore it should help us to detect ayr and awr antigens if they exist. Subtype reagents have been exchanged with other investigators. The results of subtyping their antigens support our earlier observations on the rarity of the r determinant in the U. S. A. (Table 12). Of special interest was the human antiserum provided by Dr. Holland, NIH Clinical Center; this antiserum has strong anti-aw activity. This is the only evidence to date of human antibody to the new determinants.

Table 12 - Subtypes of Referred Reagents

Source	Specimen	Number of Sera	HB Ag Subtypes			
			ayw	adw	adr	Un-determined
ANRC (Roger Dodd)	U.S. Blood Donors	29	3	9	1	16
N. J. State Dept of Health (Martin Goldfield)	U.S. "adr" Blood Donors	2	0	0	2	0
U. S. C. (James Mosley)	Israeli Blood Donors	39	22	2	0	1
	Calif. Chronic Carriers	3	2	0	0	1
	Asian Chronic Carriers	1	0	1	0	0
NIH - DBS (Lowellys Barker)	Test Panel	34	9	10	0	15
NYBC (Alfred Prince)	"ay"	5	5	0	0	0
	"ad"	5	0	5	0	0
NIH Clin. Center (Paul Holland)	HB Ag:					
	J. M.	1	0	1	0	0
	YTR	1	1	0	0	0
	Begley	1	0	0	1	0
	HB Ab:					
	Garcia	AD				
	HAB	AW				
	Boyce	A				

B. Detection of HB Ag and HB Ab

1. A solid-phase radio immune assay (RIA-Ag) developed for HB Ag by Abbott Laboratories (Chicago, Ill.) was eveluated by Dr. Allen Ginsberg (Dept Hematology) in corroboration with this department.

This test utilizes square-bottomed polypropyline tubes coated on the inside bottom with an unknown amount of unlabelled pooled guinea-pig HB Ab. The test was performed by pipetting 0.1 ml of serum to be tested for HB Ag into a tube coated with HB Ab and incubating at room temperature for 16 hours. The contents were then aspirated and the tube was washed five times with 1 ml aliquots of 0.01 M Tris buffer, pH 7.1. One-tenth milliliter of ^{125}I labelled pooled guinea pig HB Ab (0.1 uCi) was added to the tube and incubated at room temperature for 90 minutes. The tube was again aspirated and washed 5 times with Tris buffer and the remaining radioactivity quantified in an automatic gamma radiation counter.

In each series of determinations, ten normal control samples are included for which a mean counts per minute (CPM) and standard deviation were calculated. Any test specimen having a CPM of 5 or more standard deviations (s.d.) greater than the mean normal value was considered positive for HB Ag. Specimens with CPM between 3 and 5 s.d. greater than the mean of the normals were considered questionably positive and repeated. Fig. 7 shows the range of CPM values for 1133 military blood donors from Fort Dix. Although only 3 were previously determined to have HB Ag by CF, the solid phase RIA-Ag detected antigen in 7; 3 others were questionably positive.

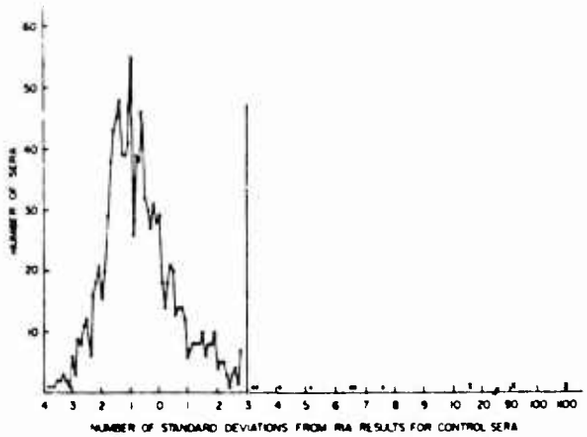

Figure 7. Amount of radioactivity detected by examination of 1133 serum specimens from normal blood donors by RIA. Values are recorded as the number of standard deviations by which they varied from the mean of 10 tests of pooled control serums. Only three blood donors were shown to have HAA by CF testing (X). These three and seven additional specimens were found to have detectable HAA by the RIA method.

2. The relative sensitivity of five tests for HB Ag

The ability of the RIA-Ag to detect HB Ag was compared to that of immunoelectro-osmophoresis (IEOP), complement fixation (CF), passive hemagglutination inhibition (HAI) and immunodiffusion (ID, AGD). Tests were run on sera from 211 patients with acute hepatitis from Korea provided by Col. M. E. Conrad. The RIA-Ag detected HB Ag in 52 individuals, nearly twice as many as the next most sensitive test (Table 13). No sera were positive by IEOP, CF, HAI or AGD that were not also positive by RIA-Ag. Thus the RIA-Ag was not found to have any false negative reactions in this study.

Table 13-Relative Sensitivity of HAA Tests in 211 Patients with Hepatitis

Test Combination*					HAA-Positive Serums
R			H		1
R	I		H		1
R		C			1
R	I	C	H		8
R	I	C	H	A	9
R	I	C		A	2
R	I	C			5
R	I				2
R					23
52	27	25	19	11	52

*R indicates RIA, I IEOP, C CF, H HAI, & A AGD.

3. Subtyping antigens by RIA-Ag

When serial dilutions of human sera containing different subtypes of HB Ag were made, concentrations of ad antigens that saturated the tubes gave higher peak CPM than ay antigens. This was due to there being more anti-d than anti-y reactivity in the Abbott guinea pig antiserum.

A preliminary study was conducted to determine the feasibility of using RIA-Ag to subtype antigens. Twenty-four sera containing HB Ag previously subtyped by ID were coded and tested in three dilutions. The three recognized antigen subtypes showed different levels of radioactivity at concentrations of antigen that saturated the tubes (Fig. 8). It is expected that when monospecific antisera is used to coat the tubes, greater distinction between individual antigenic determinants will be possible.

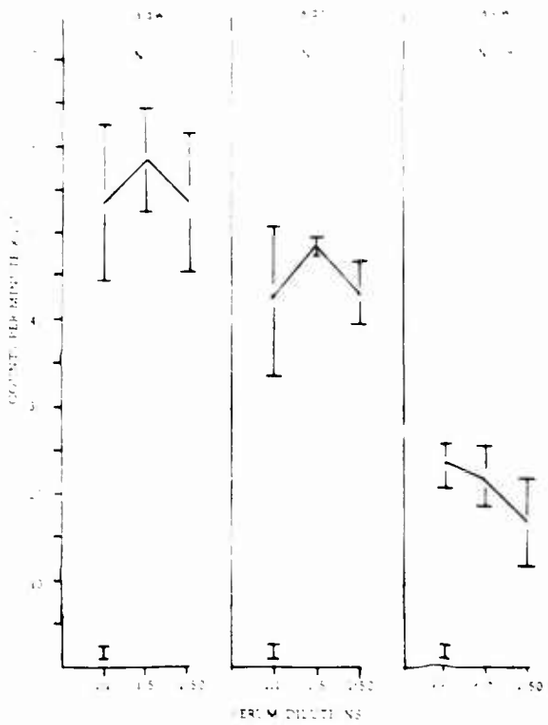

Figure 8. Differentiation of HB Ag subtypes by solid phase radioimmune assay (RIA-Ag). Twenty-four HB Ag positive sera previously subtyped by immunodiffusion were coded and tested for antigen by RIA-Ag, at three serum dilutions. The mean CPM for each dilution are connected by solid lines. The upper brackets represent ± 1 S.D. Brackets at the bottom of each section represent the mean CPM ± 3 S.D. of 10 HB Ag negative sera.

4. Rheophoresis

Another test produced by Abbott Laboratories known as "Rheophoresis" was compared to previous methods of immunodiffusion. This test utilizes a plastic dish with a seven-well immunodiffusion pattern. Around the pattern is a buffer moat. After filling the wells the moat is filled with 1 ml of buffer and the dish covered with a plastic lid which has a single hole over the center well. The dish is incubated at 37° C in a closed box for 18 - 48 hours. The principle of the test is: as moisture evaporates from the center well, buffer in the moat will promote diffusion of antigen from the peripheral wells toward the antibody in the center.

Using the Rheophoresis technique, it was possible to determine the subtype of 15 of 29 HB Ag sera which could not be subtyped by standard ID with or without preliminary concentration of the serum (Table 14).

Table 14 - Results of Three Methods for Subtyping 29 HB Ag Sera by Immunodiffusion

Method	No. Sera Subtyped	Remaining Untyped
Standard Immunodiffusion		
Serum unconcentrated	4	25
Serum concentrated 8 - 10 X*	8	17
Rheophoresis	15	2
Combined Results	27	2

* Serum concentrated with polyacrylamide gel granules (Annual Report, 1971)

C. Clinical Studies of HB Ag

1. Clinical Desk Requests

Due to an increasing number of requests to the clinical desk for HB Ag tests, a review was made of the numbers and source of specimens. Of 1,125 serum specimens from 962 individuals received over [illegible] month period, 204 (21.2%) were found to have HB Ag by CF and/or IEP (Table 15). The frequency of detection of antigen ranged from 2.3 to 44.7% among the various referring installations. This reflected differences in the selectivity of requests as well as differences in prevalence of infection. As expected, the majority of specimens came from U. S. Army installations (Table 16).

Table 15 - Hepatitis Associated Antigen

Sources of Specimens from

January to July, 1971

	Source	Serum Specimens	Persons Tested		
			No.	HAA+	%HAA+
1.	WRGH	286	266	36	13.5
2.	USNH, Bethesda	211	153	18	11.8
3.	Ft. Dix	147	118	48	40.7
4.	M.G.H.	115	108	31	28.7
5.	H.S.T. Lab	74	44	1	2.3
6.	1st USAML	51	50	16	32.0
7.	MGAFMC	42	42	4	9.5
8.	Ft. Belvoir	41	38	17	44.7
9.	5th USAML	24	23	3	13.0
10.	AFIP	21	18	1	5.6
11.	34 Others	113	102	29	28.4
	TOTAL	1,125	962	204	21.2

Table 16 - Hepatitis Associated Antigen

Sources of Specimens from

January to July, 1971

Source	Serum Samples		Patients		
	No.	%	No.	HAA+	%HAA+
Army	706	62.8	658	161	24.5
Navy	233	20.7	170	25	14.1
Air Force	76	6.8	72	11	15.3
Civilian	110	9.8	62	7	11.3
Total	1,125	100.1	962	204	

2. Hepatitis on a Hemodialysis Ward

In July 1970, a program was started to detect HB Ag and HB Ab in patients and staff members of the hemodialysis service, Ward 38, WRGH. The purpose was to identify subclinical carriers of HB Ag as well as anyone who acquired hepatitis antibody during the time they were exposed to the ward environment. This is a summary of the first 18 months of surveillance.

The study group consisted of 77 patients (40 male, 37 female) and 36 staff members (27 male, 9 female). Demographic data is completed only for the first 12 months of study. The mean ages of the first 50 patients and first 27 staff members were 35.8 and 28.6 years, respectively. Of the first 50 patients, 8 were dialyzed an average of 12.8 times (range 7-26) for acute renal failure and 42 were dialyzed an average of 47.1 times (range 5-196) for chronic renal failure. Chronic dialysis patients were usually treated for 6 hours 2 to 3 times per week. Thirteen of the chronic patients had been dialyzed an average of 3.3 months prior to the study period. The first 50 patients received an average of 7.9 units of blood during the first 12 months of surveillance. The 36 staff members included 7 physicians, 7 registered nurses and 22 technicians and students. All staff members had some exposure

to hepatitis patients, blood products and/or dialysis equipment in the course of their work.

Hemodialysis Ward. Hemodialysis was performed in one room of the ward using dialyzing machines with disposable coils. Machines were primed with normal saline. Patients known to have HB Ag were identified to all personnel but treated with the same precautionary measures as the others. After each use, the dialysis equipment was carefully washed with formalin or hypochlorite solution.

Collection of Specimens. During the first week of each calendar month, 5 to 10 ml of whole venous blood was collected from each patient and staff member present on the dialysis service. The blood was allowed to clot, centrifuged and the serum stored at -20° C until tested. Persons admitted to the ward between routine bleeding periods were usually bled within a week of admission. No attempt was made to collect blood during intervals when patients were dismissed from the hospital or staff members were assigned to other duties. Any individual who was found to have HB Ag or abnormal blood chemistries was notified and another serum specimen obtained for testing as soon as possible.

Detection of HB Ag and HB Ab. Serum specimens were tested for HBAg by CF and RIA-Ag. A serum was considered to contain HB Ag if it had a CF titer of 1:2 or more or the RIA-Ag was positive on two determinations. A serum was considered questionably positive if the CPM by RIA-Ag was between 3 - 5 s.d. above the normal mean value on two determinations. Comparative tests showed the RIA-Ag was dependably more sensitive than the CF and gave no recognizable false negative results (Table 17).

Table 17 - Comparison of CF and RIA Ag for Detecting HB Ag in Sera from 113 Individuals

RIA-Ag	Reciprocal CF titer					Total
	<2	2	4	8	≥ 16	
Positive	2	3	2	0	8	15
Questionable	12	0	0	0	0	12
Negative	509	0	0	0	0	509
Total	523	3	2	0	8	536

HB Ab was detected by passive hemagglutination (HA) and solid phase radioimmune assay (RIA-Ab). HB Ab was considered to be present if a serum had an HA titer of 1:8 or more with no detectable natural erythrocyte agglutinins. Alternatively, a serum contained HB Ab if the RIA-Ab was positive on two determinations. Of 502 sera tested, the HA titer was 1:8 or greater in 15.1% and the RIA-Ab positive in 24.3% (Table 18). Five sera were positive by hemagglutination and negative by RIA-Ab. If these have HB Ab, then the RIA-Ab gave false negative results in $^{5}/_{363}$ sera (1.4%).

Table 18 - Comparison of HA & RIA Ab for Detecting HB Ag in Sera from 113 Individuals

RIA Ab	Reciprocal HA Titer				Total
	<8	8	16	≥ 32	
Positive	52	0	13	57	122
Questionable	16	0	0	1	17
Negative	358	2	0	3	363
Total	426	2	13	61	502

Criteria for Infection. Since there are no means of isolating Hepatitis B virus from infected individuals or determining protective neutralizing antibody, arbitrary criteria were established for determining the presence of infection and the existence of immunity (Table 19).

Table 19 - Criteria for Infection with Hepatitis B

Active infection:	The detection of HB Ag in serum with or without co-existing antibody. If HB Ag persisted for more than 3 months, the individual was considered a chronic antigen carrier.
Acquired antibody:	The detection of HB Ab in 1 or more serum samples after a period of at least 3 months when HB Ab was not detected. HB Ag need not have been detected at any time.
Prior antibody:	The detection of HB Ab in any serum collected during the first three months of surveillance.
Negative:	The absence of HB Ag and HB Ab in all specimens tested.

Results.

Index case. The study was initiated when the head nurse (K. K.) of the hemodialysis service developed clinical hepatitis in May 1970. With the development of nausea, skin rash and joint pains, she was found to have a serum glutamic oxaloacetic transaminase (SGOT) of 1800 u, serum glutamic pyruvic transaminase (SGPT) of 900 u, alkaline phosphatase of 22 KA u and a total bilirubin of 1.9 mg%. Her initial CF titer of HB Ag was 1:256. By July 1970 her symptoms were gone and her blood chemistries were normal but she still had a low level of HB Ag (Table 20). HB Ab was first detected by HA and RIA-AB in November, four months after antigen was last found. However, the HA titer fluctuated over the next 12 months while the RIA-AB results were more consistently positive.

During the 18-month period 3 staff members (8.3%) and 5 patients (6.5%) were found to have HB Ag (Table 21). An additional 6 patients (7.8%) but no staff members developed detectable antibody without preceding HB Ag. In all, 3 staff members (8.3%) and 11 patients (14.3%) showed serologic evidence of Hepatitis B infection during the period of observation (Fig. 9). Furthermore, another 38.9% of the staff and 29.9% of patients had HB Ab which may have been acquired through some prior exposure.

Table 20 - Serologic Studies for HB Ag and HB Ab from One Staff Member (K. K.)

Year: Month	HB Ag		HB Ab	
	CF*	RIA-Ag**	RIA-Ab**	HA*
1970: 5	256			
6	16			
7	2	P	.	.***
8	.			.
10	.	.	.	.
11	.	.	P	≥64
12	.	.	P	.
1971: 1	.	.	P	.
2	.	.	P	32
3	.	.	P	.
4	.		Q	.
6	.		P	.
7	.		P	.
8	.	.	P	.
9	.	.	P	.
10	.	.	P	.
11	.	.	P	≥32
12	.	.	P	≥32

* CF and HA titers are reciprocal serum dilutions

** RIA results: P signifies positive
Q signifies questionably positive

*** Negative values are expressed by dots

Table 21 - Prevalence of HB Ag and HB Ab
among Hemodialysis Staff Members and Patients
during 18 Months of Surveillance

	Staff		Patients	
	No.	%	No.	%
Transient HB Ag	3	8.3	3	3.9
Chronic HB Ag	0	0.0	2	2.6
Acquired HB Ab	0	0.0	6	7.8
Prior HB Ab	14	38.9	23	29.9
Negative	19	52.8	43	55.8
Total	36	100.0	77	100.0

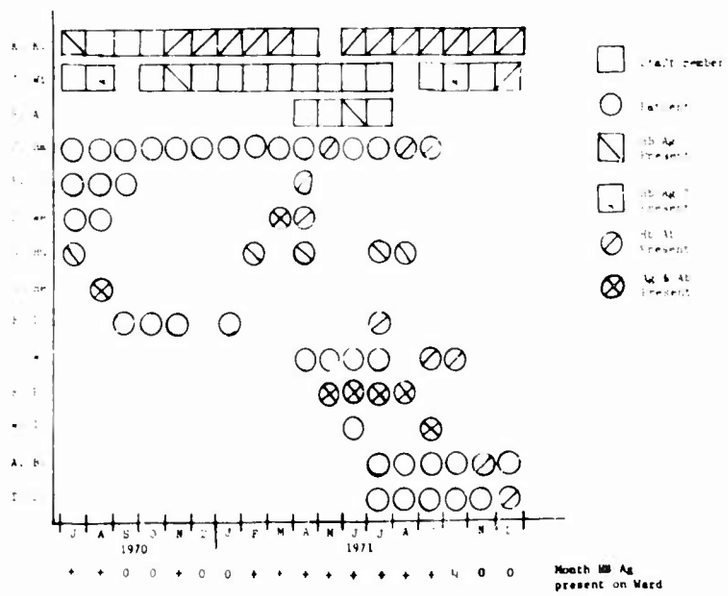

Figure 9. Temporal relationship of HB Ag and HB Ab in serial serum specimens from 14 individuals from a hemodialysis service. HB Ag was detected by CF and/or RIA-Ag; HB Ab was detected by HA and/or RIA-Ab.

Of the 8 individuals with detectable HB Ag, 6 had detectable antibody (Fig. 9). Of these, 4 patients had antigen and antibody in the same serum samples. In each case, when antibody appeared, it was best detected by RIA-Ab. Interestingly, although the 8 antigen carriers represented only 7.1% of the entire study group, one or more carrier was present on the ward during 11 of the 18 months of testing. It appears that a source of potential hepatitis infection was present on the ward most of the time.

Conclusion. The overall infection rate for this period of observation was 14/113 persons/1.5 years, or 8.3/100/year. This preliminary data suggests that Hepatitis B is an endemic infection on this hemodialysis ward.

III. Immunoglobulins: Preparation of Subclass Antigens and Antisera

A. Background and Statement of Problem

In recent years immune complexes as possible mediators of disease in microbial infections have been recognized. Based on the prototype of immune complex disease in animals, much has been learned about the interactions of the various components involved. Most of the interest and work has been focused on the antigen moiety and on the complement system, but other than concentration variations, very little has been said about the antibody component other than whether it is IgG, IgM or IgA antibody involved in particular disease states.

In recent years the technology has become available to isolate protein in pure form, utilizing a variety of methods. In addition, sophisticated studies on the structure of antibody molecules themselves have revealed several genetic differences in these molecules, forming the basis for the subtyping of antibodies. There are now known to be at least four (4) distinct subclasses of the IgG antibody (γG_1, γG_2, γG_3, γG_4), at least two (2) distinct subclasses of the IgA antibody (γA_1, γA_2) and by various techniques (patterns of digestion with enzymes, migration in electrophoretic field precipitation reactions) probably at least two (2) subclasses of the IgM antibodies. No work has been done to date on the possible genetic subclasses of the IgD and IgE antibodies.

It seemed feasible to begin to develop the reagents needed to evaluate the subclassed antibody response in infectious diseases, both associated and non-associated with immune complex disease.

B. Approach to the Problem

Collection of reference antigens. A system was set up with the help of the Department of Hematology, Walter Reed General Hospital, to collect myelomoma proteins of various types. From time to time the master list of electrophoresis done on a routine basis in the clinical laboratory of the WRGH is reviewed. Specimens with definite peaks representing large production of antibodies (IgG, IgA, IgM) are plasmaphoresed from selected patients and stored in -70° C freezer. To date we have been able to collect 9 IgG's, 5 IgA's and 6 IgM's. In addition we obtained sera from the National Immunoglobulin Reference Center known to contain large amounts of the 4 subclasses of IgG. These were designated in both IgG-K and IgG-λ types.

The immunoglobulin proteins were isolated by various techniques after development of the technical capability for salting out, column chromatography, ion exchange chromatography, and Pevicon and Starch Block electrophoresis. After many trials it was found that the easiest and most efficient method for isolating the δG myelomoma proteins as subclasses was by ion exchange chromatography. Therefore, fractions were all run in this manner on DEAE cellulose in a PO_4 buffer over a gradient equivalent electrically from 0.01M saline to 0.1M saline. Further purification may be obtained if needed by rerunning the fraction over a layer gradient (.001M to 0.1M).

The fractions were determined to be pure by Ouchterlony and by electrophoretic cross-testing for the opposite light chain in the isolate. After purity was established, immunization of rabbits was begun. Antibodies will be isolated and purified to subclassed antibodies by absorption methods.

In the future, it is hoped to evaluate reactive sera by Ouchterline precipitation techniques, utilizing subclassed antihuman antisera to determine subclassed antibody response in viral diseases.

IV. Group B Arbovirus Epidemiology, Identification and Control

A. Identification of Dengue Viruses Currently Active in the Caribbean

During 1970-1971 sporadic cases of dengue were observed in Haiti by Dr. J. Ehrenkranz and Dr. A. Ventura (University of Miami). Their investigations indicated that dengue is endemic on Hispanola (Personal communication). Serum specimens from acutely ill patients were received in this laboratory for re-isolation and identification of the agents. Seven strains of dengue were recovered from cases occurring between October 1970 and October 1971. Identification test

results are shown in Table 22; all are strains of dengue-2.

Table 22 - Identification of Dengue Viruses
from Haiti (1970 - 1971) and Colombia (1972) by Neutralization Test

Virus Strain	Immune Ascitic Fluid			
	DEN-1 HAW	DEN-2 N.G.C.	DEN-3 PR-6	DEN-4 H-241
Haiti-315	< 10*	> 640	15	< 10
Haiti-391	< 10	> 640	15	< 10
Haiti-398	< 10	> 640	10	< 10
Haiti-1300	< 10	> 320	< 10	< 10
Haiti-1301	< 10	200	< 10	< 10
Haiti-4372	< 10	600	< 10	< 10
Haiti-4390	< 10	> 600	< 10	< 10
Colombia HGL-1047	10	600	< 10	< 10
Colombia 306104	< 10	600	< 10	< 10
Colombia 306105	15	700	15	< 10

* Reciprocal of 50% plaque reduction titer

An epidemic of dengue began on the Atlantic Coast of Colombia in August 1971, and continues to spread southward. Numbers of cases have been unofficially estimated to exceed 500,000. No hemorrhagic disease or shock syndrome has been observed. Acute serum specimens collected between January and April, 1972, were received from Dr. Hernando Groot. Dengue viruses have been recovered from three patients; all are dengue-2 (Table 22). Additional isolation attempts from sera and mosquitoes are in progress in an attempt to determine if another serotype is also present in Colombia.

The Haitian and Colombian strains were readily isolated in LLC-MK_2 cells, producing small clear plaques. Attempts to isolate the Colombian strain HGL-1047 in suckling mice resulted in only 7 of 87 exhibiting symptoms by the 12th day, but typical CNS symptoms were observed by the 8th to 9th day in all the second passage mice.

B. Human Immunoglobulin Specificity Following Group B Arbovirus Infections

Type specific serologic diagnosis of group B arbovirus infections is sometimes possible in primary cases; however, precise identification of the infecting virus by usual serologic means in subsequent group B infections is precluded by the development of antibody that exhibits extensive cross reactions among members of this group. Our studies were designed to determine whether the viral specificity of IgM antibody in sera collected from patients during a dengue-2 epidemic is different from whole sera; and, if so, whether this specificity could be useful as a diagnostic tool.

Sera. Blood was collected from military personnel or their dependents presenting with clinically diagnosed dengue fever at three military clinics in San Juan, Puerto Rico, during the summer of 1969. All patients had a benign self-limiting disease with no sequelae. All military personnel but not their dependents had previously been immunized with 17-D yellow fever virus vaccine. Sera were separated and stored at -20° C.

Separation and identification of immunoglobulins. Serum samples were absorbed with goose red blood cells and 0.3 ml was applied to a 4.8 ml 10-40% linear sucrose gradient. Gradients were centrifuged in a Beckman SW-39 rotor at 100,000 xg for 18 hr; 0.3 ml fractions were collected through the bottom of the gradient tube. Single radial diffusion on commercially obtained agar plates (Hyland Laboratories) was used for identification and quantification of immunoglobulins. Sucrose fractions containing IgM were pooled and tested for antibody activity.

Hemagglutination-Inhibition (HI) test. Whole sera extracted with acetone and gradient fractions containing IgM stabilized with 1% bovine plasma albumin were tested for HI activity. Antigens made from prototype strains of yellow fever (YF), Japanese encephalitis (JE), and the four dengue (DEN-1-4) viruses were diluted with borate saline buffer to contain eight hemagglutinating units at their optimum pH. Sensitivity of HI antibody activity to reduction by 2-mercaptoethanol (2 ME) was tested by incubating 0.2 ml of each antibody sample with an equal volume of 0.2M (2 ME) in borate saline (pH 9.0) for 1 hr at room temperature, followed by standard dilution and testing for HI.

Plaque reduction neutralization test. Virus neutralizing activity of whole sera and IgM fractions were measured by a plaque reduction neutralization test (Russell and Nisalak, 1967). Viruses used were dengue-1 (Hawaii), dengue-3 (H-87), dengue-2 (PR-109), dengue-4 (23751), and yellow fever (French Neurotropic). Sensitivity of neutralizing antibodies to reduction was determined by incubating antibody dilutions for 3 hr with an equal volume of 0.3M ethanethiol, followed by heating the mixture at 56° C for 30 min to remove the volatile mercaptan prior to testing (Murray et al, 1965).

Results. Fourteen patients with significant increases in HI antibody against dengue-2 antigen were selected for study. Patients were divided into two groups on the basis of the HI antibody response to group B arboviruses. The first group consisted of four patients with low (<1:160) convalescent HI antibody titers and minimal cross reactions with JE virus. These patients were presumed to have had a primary reaction to their first group B arbovirus infection (Table 23). Dengue-2 virus was isolated from two of these patients. All four were dependents of military personnel.

Table 23 - Hemagglutination-Inhibition Antibody Titers in Sera from Patients with Primary Group B Arbovirus Infections with Dengue-2

Patient Number	Virus Recovered	Day of Disease	Antigen					
			DEN-1	DEN-2	DEN-3	DEN-4	YF	JE
PR-118	None	3	<20*	<20	40	<20	<20	<20
		30	20	80	80	20	<20	<20
PR-120	DEN-2	3	<20	<10	<20	<20	<20	<20
		15	80	160	160	80	<20	40
PR-142	None	?	<20	<20	<20	<20	<20	<20
		11+	40	160	80	20	<20	<20
PR-165	DEN-2	1	<20	<20	<20	<20	<20	<20
		14	40	80	80	<20	<20	<20

* Reciprocal HAI titer versus 8 units of antigen

The second group consisted of 10 patients (all military personnel) with high level (≥1:640) HI antibody responses and marked cross reactions among the group B antigens (Table 24). In this group the recent dengue infection was considered to be secondary to a previous group B experience, resulting in an anamnestic response. Dengue-2 virus was isolated from five acute sera in this group.

Table 24 - Hemagglutination-Inhibition Antibody Titers in Sera from Patients with Secondary Group B Infection with Dengue-2

Patient Number	Virus Recovered	Disease	Antigen					
			DEN-1	DEN-2	DEN-3	DEN-4	YF	JE
PR-110	None	1	<20*	<20	<20	<20	20	<20
		38	320	2560	1280	160	320	320
PR-122	None	1	20	20	40	20	40	20
		8	5120	10240	10240	2560	10240	1280
PR-125	None	3	40	40	80	40	80	20
		8	640	640	320	1280	2560	1280
PR-133	DEN-2	1	40	80	80	80	40	80
		14	1280	10240	5120	2560	2560	1280
PR-139	DEN-2	2	<20	<20	<20	<20	40	<20
		18	640	1280	2560	2560	5120	2560
PR-140	DEN-2	?	<20	<20	<20	<20	80	<20
		11+	1280	10240	5120	2560	5120	640
PR-147	None	6	1280	2560	2560	2560	5120	2560
		20	2560	5120	5120	2560	5120	2560
PR-148	None	?	<20	20	40	<20	80	<20
		20+	640	10240	2560	1280	5120	1280
PR-156	DEN-2	2	<20	20	<20	<20	40	<20
		13	160	2560	640	80	2560	40
PR-166	DEN-2	4	160	320	320	80	80	40
		17	5120	2560	1280	1280	1280	1280

* Reciprocal HAI titer versus 8 units of antigen

The acute sera of half of the second group contained HI antibodies against YF but not dengue or JE viruses. Since specific low level HI titers have been demonstrated to persist in persons immunized with YF and since military personnel had undergone YF immunization, this probably represented persistent YF antibody. The remaining acute sera had hetero-specific antibodies to all HI antigens tested. These individuals may

have had a previous group B arbovirus infection following the YF immunization, but prior to the dengue-2 infection, or, more likely, the heterospecific antibody might be accounted for by collection of the acute sera after the onset of the anamnestic antibody production.

When convalescent sera from the primary group was treated with 2-ME, there was a four-fold decrease in HI titers whereas 2-ME had no effect on the convalescent HI titers in the second group. Separation of serum immunoglobulins was carried out in sucrose gradients; IgM was usually found in the lower four fractions; and IgG was found above fraction four. Fractions 1-3 from each gradient were pooled and assayed for IgM and IgG content. A relatively constant proportion of IgM, ranging between 29-41% of the whole serum concentration, was extracted by this means; IgG concentrations in the IgM fractions were less than 5 mg%.

HI antibody activity of the IgM fractions showed a rise in titer against the homotypic dengue-2 antigen in all 14 individuals. In the four primary cases dengue-2 was the only *an*tigen recognized by the IgM fractions (Table 25).

Table 25 - Hemagglutination-Inhibition Antibody Titers of Serum IgM Fractions from Patients with Primary Group B Arbovirus Infections with Dengue-2

Patient Number	Disease	Ratio IgM Fraction / Whole Serum	Antigen					
			DEN-1	DEN-2	DEN-3	DEN-4	YF	JE
PR-118	3	0.34	NT*	0*	NT	NT	NT	NT
	31	0.31	0**	16***	0	0	0	0
PR-120	3	0.35	NT	0	NT	NT	NT	NT
	16	0.39	0	32	0	0	0	0
PR-142	?	0.31	NT	0	NT	NT	NT	NT
	11+	0.33	0	16	0	0	0	0
PR-165	1	0.38	NT	0	NT	NT	NT	NT
	14	0.36	0	8	0	0	0	0

* NT - Not tested

** 0 - No activity undiluted

*** Reciprocal HAI titer versus 8 units of antigen

In the convalescent sera from secondary cases there was a similar lack of cross reactivity of IgM with heterologous dengue and JE antigens. The magnitude of the dengue-2 IgM response was found to be similar in the two groups (Table 26). However, in 6 out of 10 of the patients in the secondary group, there was also IgM antibody activity against the YF antigen. In these cases anti-YF activity was found in both the acute and convalescent serum. Both the YF and dengue-2 HI antibodies in the IgM fractions were sensitive to 2-ME.

Plaque reduction neutralization studies were performed on convalescent sera from three individuals with secondary antibody responses. Whole convalescent serum was found to neutralize all four dengue serotypes and YF (Table 27). The IgM fractions obtained from these convalescent sera again showed specific activity against dengue-2, with little or no cross reactivity against the other dengue types. As expected from the HI data, YF neutralizing activity was found in the IgM fractions of the two sera tested. Neutralizing antibody titers in the IgM fractions were reduced by ethanethiol treatment.

Discussion. The most important practical aspect of these observations is the fact that the specificity of the IgM antibody can be used to make a type-specific serologic diagnosis in secondary group B arbovirus infections. Herein we have demonstrated the specificity of the IgM response in dengue-2 infections in persons immunized against yellow fever. A similar observation was made in JE infections following dengue infections in both man and gibbons (Edelman, R., personal communication). The usefulness is limited, however, to situations where the infecting group B agents have distinct antigenic differences since little or no IgM response occurs when both infections are due to very closely related agents, such as within the dengue virus group. The reasons for a higher degree of specificity for IgM antibody compared to IgG are as yet unclear. Differences in combining sites on the antigen for IgM and IgG antibodies may play a role.

The antibody response in the secondary infections with group B arboviruses appears to be a mixture of an anamnestic response to some antigenic determinants which are very similar or identical in both viruses, and a primary response to other determinants which are unique to the second virus. In the cases reported here common group B antigens to which the host had been exposed through prior YF immunization lead to an anamnestic response when the dengue infection occurred, while dengue type-specific antigenic determinants not previously seen produced primary responses with IgM antibody. Thus the specificity of IgM may be in part due to exclusion.

The observation of unchanging titers of IgM antibody directed against YF in 6 of 10 patients showing secondary HI titers is consistent with the report that IgM antibody has been found to persist for as long as 18 months following YF immunization. It is noteworthy that in spite of major rises in titers of cross reacting IgG antibody and a

Table 26 - Hemagglutination-Inhibition Antibody Titers of Serum IgM Fractions from Patients with Secondary Group B Arbovirus Infections with Dengue-2

Patient Number	Day of Disease	Ratio IgM Fraction / Whole Serum	Antigen					
			DEN-1	DEN-2	DEN-3	DEN-4	YF	JE
PR-110	1	0.37	0*	0	0	0	0	0
	38	0.30	0	8**	0	0	0	0
PR-122	1	0.34	0	0	0	0	1	0
	8	0.31	0	16	0	0	1	0
PR-125	3	0.39	0	2	0	0	0	0
	8	0.31	0	64	0	0	0	0
PR-133	1	0.41	0	0	0	0	0	0
	14	0.36	0	16	0	0	0	0
PR-139	2	0.35	0	0	0	0	4	0
	18	0.38	0	16	0	0	8	0
PR-140	?	0.36	0	0	0	0	8	0
	11+	0.31	0	16	0	0	8	0
PR-147	6	0.35	0	16	0	0	2	0
	25	0.32	0	64	0	0	4	0
PR-148	?	0.30	0	2	0	0	8	0
	20+	0.34	0	32	1	1	4	1
PR-156	2	0.31	0	2	0	0	2	0
	13	0.31	0	16	0	0	2	0
PR-166	4	0.31	0	4	0	0	0	0
	17	0.33	0	16	0	0	0	0

* 0 - No activity undiluted

** Reciprocal HAI titer versus 8 units of antigen

Table 27 - Neutralization Tests on Whole Convalescent Sera and IgM Fractions

	VIRUSES									
	DEN-1		DEN-2		DEN-3		DEN-4		YF	
	Whole Serum	IgM* Fractions	Whole Serum	IgM Fractions	Whole Serum	IgM Fractions	Whole Serum	IgM Fractions	Whole Serum	IgM Fractions
PR-139	640**	<20	780	143	<20	<20	87	<20	560	82
PR-140	460	<20	522	152	<20	<20	54	<20	460	140
PR-147	380	<20	810	150	>20	<20	-	-	-	-

* IgM concentration adjusted to that of whole serum

** Reciprocal of 50% plaque reduction titer

rise in anti-dengue IgM antibody, the anti-YF IgM antibody remained constant indicating that completely separate antigenic determinants are involved.

C. Vaccines

1. Dengue vaccine substrates

During the current report period activities are being directed toward the adaptation of dengue type 2 strain (PR 159) to growth in 5 different tissue culture substrates: (1) pre-tested, bank-frozen primary green monkey kidney (GMK 0476), (2) pre-tested, bank-frozen primary canine kidney (CK 13), (3) human fetal lung (WI 38), (4) continuous line fetal rhesus monkey lung (FRhL/DBS) and (5) continuous line fetal cercopithecus lung (FCL/DBS). The latter two tissue cultures were obtained from the Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland. The canine kidney, monkey kidney and WI 38 cells were obtained from a commercial supplier.

Although good replication was observed in the FCL/DBS cultures, it was necessary to discontinue use of this cell line as a candidate vaccine substrate because of the development of abnormal karyology characterized by excessive aneuploidy. However, the karyologic data on the FRhL/DBS line continues to look very good and passage work with the candidate virus vaccine strain PR 159 is proceeding with this substrate.

Adaptation of PR 159 dengue virus in both primary canine and WI 38 cells has proved very disappointing. Even when initiated with a comparatively heavy inoculum, after 4 or 5 passages viral replication in both substrates steadily decreases to the point of extinction.

Passage work with the PR 159 virus in the primary green monkey tissue culture is proceeding in a very encouraging manner. From the initial passage we have been able to demonstrate a consistent increase in viral yield through 5 consecutive passages. If the viral yields continue this upward trend in subsequent passages, these cells may very well be the vaccine substrate of choice.

Progress during this report period has been painfully slow since cell production at the source has been beset with technical difficulties. To mention a few, at the second passage level the entire stocks of canine and green monkey kidney cells were wiped out by a nitrogen freezer failure at the supplier. It was February before delivery of pretested cells from these two species could be initiated. On several occasions it was necessary to re-passage the virus in canine, monkey kidney and WI 38 cells because of bacterial contamination in the original cultures. The loss of these passages was unavoidable since overt contamination did not appear until several days after harvests were made. This strongly suggested a dilution effect on the residual antibiotic concentration. It was observed that transfers of original growth media from these cultures

would always show contamination in the sterility media several days prior to the time contamination made its appearance in the original unopened control flasks. Also, delivery of tissue cultures was delayed as much as 2 and 3 weeks as a result of poor growth and cultivation problems at the supplier end. At best, the production capability of this supplier has been somewhat less than ideal. A summary of the passage data for the 5 substrates is presented in Tables 28 through 31 and in Figure 10.

Table 28 - Dengue-2 Vaccine Seed Virus Passage (PR 159) in WI 38 Cells

Passage Number	Inoculum (Log_{10})	Harvest Period	Titer	Post Adsorp.	Thermal
			(Log_{10} plaque-forming units/0.2 ml)		
1	3.02	96 hr	<1	2.83	2.90
		120 hr	<1	2.91	
2	<1	96 hr	<1	<1	
		120 hr	<1	<1	
3	<1	96 hr	<1	<1	
		120 hr	<1	<1	
4	<1	96 hr	<1	<1	
		120 hr	<1	<1	

Table 29 - Dengue-2 Vaccine Seed Virus Passage (PR 159) in Canine Kidney

Passage Number	Inoculum (Log_{10})	Harvest Period	Titer	Post-Adsorp.	Thermal
			(Log_{10} plaque-forming units/0.2 ml)		
1	3.02*	96 hr	<1	2.83	2.90
		120 hr	<1	2.74	
2	3.48	96 hr	<1	1.30	
		120 hr	2.54	2.50	
3	<1	96 hr	<1	<1	
		120 hr	<1	<1	
4	<1	96 hr	<1	—	
		120 hr	<1		

* PR 159 infected serum inoculum was toxic to canine cells, so the passage 1 harvest from green monkey tissue culture was used for passage 2 inoculum.

Table 30 - Dengue-2 Vaccine Seed Virus Passage (PR 159) in Green Monkey Cells

Passage Number	Inoculum (log_{10})	Harvest Period	Titer	Post-Adsorp.	Thermal
			(Log_{10} plaque-forming units/0.2 ml)		
1	3.02	96 hr	3.48	2.88	2.90
		120 hr	3.54	2.86	
2	3.54	96 hr	4.15	3.41	3.36
		120 hr	4.23	3.30	
3	4.20	96 hr	3.45	3.18	—
		120 hr	3.60	4.23	
4	4.60	96 hr	4.43	—	—
		120 hr	4.65		
5	4.60	96 hr	5.65	—	—
		120 hr	5.54		
6 (large pool)	5.48	96 hr	—	—	—
		120 hr	5.78		

Table 31 - Dengue-2 Vaccine Seed Virus Passage (PR 159) in FRhL/DBS Cells

Passage Number	Inoculum	Harvest Period	Titer (pfu/0.2 ml)
1	4.60	120 hr	3.34
2	3.34	120 hr	2.18

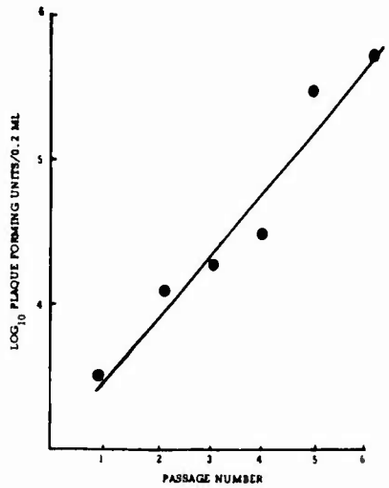

Figure 10. Yields of the candidate dengue-2 vaccine strain (PR-159) in primary green monkey kidney cell cultures during consecutive passages.

2. Comparison of Dengue-2 candidate vaccine strain (PR-159) and prototype strain (New Guinea C) in Rhesus monkey

Five adult Rhesus monkeys previously used for chikungunya virus vaccination and challenge were inoculated with two strains of Dengue-2 virus. Two received 3.5 logs of Dengue-2, PR-159 (Naral-Ortiz acute serum), while the other three monkeys received a subcutaneous inoculation with Dengue-2, New Guinea C strain suckling mouse brain seed at a dose of 4.6 logs. All monkeys were negative for Den-2 neutralizing and HI antibody before challenge with virus. All monkeys were bled on days 2, 3, 4, 5, 6 and 9 after challenge and the serum tested for live virus by plaque titration in LLC-MK_2 cell culture. A delayed plaque titration which may reveal higher titers of Dengue virus will be done in the future. Blood was also taken at 18, 31, 45 and 75 days post inoculation and assayed for neutralizing, HI and CF antibody.

Although the temperature of each monkey was recorded for each day of bleeding, no significant variation or pyrexia was noted nor were there any clinical signs of disease evident. Only the two monkeys that received Den-2, PR-159, gave positive viremias. Monkey #995 was positive for one day, and monkey #997 was positive for two days (Table 32).

Table 32 - Virus Titers of Viremic Monkeys (PFU/0.2ml)

	Day 5	Day 6
Monkey #995	41	—
Monkey #997	19	9

Neutralization, *HI* and CF antibody response of the monkeys to Den-2, New Guinea C and PR-159 strains are shown in Tables 33 and 34, respectively. Neutralization titers were low for all 5 monkeys when tested against the New Guinea C strain in the neutralization test, and strain specificity was hard to see. Neutralization tests using the PR-159 strain show a marked strain specificity with high neutralization titers in the homologous monkey sera.

Table 33 - Plaque Reduction Neutralization Tests
(Challenged monkeys were tested against both strains of Den-2 virus)

Challenge Virus	MK#	50% Plaque Reduction Endpoint; Days Post-Inoc. Strain							
		Strain: N. Gu. C.				Strain: PR-159			
		18	31	45	75	18	31	45	75
Dengue-2, N.Gu.C.	113	29*	80	135	590	80	80	48	100
	134	<10	23	150	490				
	993	13	21	23	860	120	110	92	140
Dengue-2, PR-159	995	48	120	290	480	3000	860	1450	1750
	997	42	280	390	720	750	920	1350	1250

* Reciprocal of the dilution of serum giving 50% plaque reduction against 50 to 150 plaques

Table 34 - HI and CF Tests

(Antigen for HI and CF tests was a crude Den-2, N. Guinea C. suckling mouse brain preparation employing ammonium sulfate precipitation of protamine sulfate treated and clarified suckling mouse brain).

MK#	HI/CF Titers; Days Post Inoculation			
	18	31	45	75
113	40/<4*	40/<4	40/ 64	40/4
134	20/<4	20/<4	20/ 16	40/<4
993	<10/<4	20/<4	20/ 16	160/<4
995	160/<4	80/<4	40/ 32	20/ 4
997	80/<4	40/<4	40/ 64	80/ 4

* Reciprocal of HI endpoint dilution/reciprocal of CF endpoint dilution

3. Chikungunya Vaccine

Evaluation studies relating to the immunogenic efficacy and long-term protective capacity of a formalin-killed, freeze-dried chikungunya vaccine prepared in this Department are reported here.

The neutralization indices for 26 individuals after a primary series of 0.5 ml of vaccine given 28 days apart, and a booster dose of 0.5 ml approximately 1 year later, are shown in Table 35.

Table 35 - Serum Neutralization Indices Observed in Chikungunya Vaccinees over a 12-Month Period and Following a Booster Dose

Subject	15 Days p. 1	30 Days p. 2	Pre-Boost LNI	Post Boost* LNI
GC	1.3	2.0	< 1.0	1.7
DC	1.7	2.4	1.0	2.7
LD	1.7	1.7	< 1.0	1.7
SD	3.0	3.0	< 1.0	2.7
DD	2.0	4.3	1.7	4.0
GD	2.7	4.4	2.7	No specimen
WH	2.0	2.0	< 1.0	2.3
GH	2.0	2.3	< 1.0	1.7
AK	1.6	3.0	2.0	2.7
DL	1.7	3.3	< 1.0	2.0
WL	1.3	1.7	< 1.0	1.0
DM	2.0	3.0	< 1.0	1.7
TO	3.4	4.4	1.7	2.7
RP	2.6	4.0	1.7	2.3
GP	1.7	2.7	1.7	2.0
SP	1.3	1.3	< 1.0	2.0
KR	0.7	2.4	< 1.0	1.3
RR	1.0	2.3	< 1.0	2.7
TS	1.3	3.0	< 1.0	2.7
DS	2.0	2.7	< 1.0	2.7
JS	1.3	2.7	< 1.0	1.7
WT	1.0	2.7	1.0	2.0
FV	1.0	1.7	< 1.0	2.3
EW	1.0	2.0	< 1.0	2.3
RW	2.3	3.0	< 1.0	2.3

* A booster dose of 0.5 ml was administered 1 year after primary series

From the data it may be seen that the initial sero-conversion rate was extremely good (96%). However, approximately 1 year later a sharp decline in circulating antibody levels was observed for these same

individuals. This pattern is not consistent with one observed for a similar group reported earlier (Annual Report 1971). It is strongly felt that this disparity was due either to improper handling of the serum samples prior to testing, or interference by a simian adventitious agent in the tissue culture system used for the test (the latter hypothesis is currently being investigated). After a 0.5 ml booster dose of vaccine there was a marked increase in the sero-conversion rate; however, when compared to a previously boostered group (Annual Report 1971), these neutralization indices appear abnormally low. A repeat mouse potency assay on the vaccine at this time indicated no loss in potency over a 2-year period.

The long-term protective efficacy of this vaccine was clearly demonstrated by the fact that, with one exception, all vaccinated monkeys were solidly protected against viremia when the individual groups were given a live virus challenge 6, 12, 18 and 24 months after vaccination, whereas the control monkeys challenged at the same time intervals developed viremias of 4 to 5 days duration, with titers of circulating virus reaching 5 logs. The vaccine failure, if such were the case, occurred in one monkey of the 18 month post-vaccination group. This monkey experienced a severely prolapsed rectum approximately 1 year prior to challenge. Even though complete retraction took place without surgical intervention, there obviously was some interference with the normal adsorption of food and liquids, since he never fully regained the physical status of his cage mates. At the time of challenge this monkey still exhibited signs of extreme dehydration and emaciation and expired on the 4th day after challenge.

It was of interest to note that antibody levels observed in human vaccinees were not at all consistent with those observed in monkeys after vaccination. This was particularly evidenced by our inability to detect circulating antibody in the monkeys only 5 to 6 months after vaccination. In spite of this, it is apparent from the data that complete protection was conferred by the vaccine since, with one exception, none of the vaccinated monkeys developed viremia after a live virus challenge, for periods as long as 24 months post-vaccination. Data pertaining to the 18 and 24 months post-vaccination challenge groups are summarized in Tables 36 and 37, respectively (results of the 6 and 12 month post-vaccination challenge were reported in the 1971 Annual Report).

Table 36 - Long-Term Protective Efficacy of Chikungunya Vaccine Observed in Rhesus Monkeys Challenged 18 Months after Vaccination

(a) Viremia

Monkey No.	Vaccine Status	Viremia Patterns on Day Post-Inoculation						
		1	2	3	4	5	6	7
995	Yes	0	0	0	0	0	0	0
9%	Yes[+]	0	3.5[*]	3.8	1.5	—	—	—
997	Yes	0	0	0	0	0	0	0
993	No	0.8	4.2	3.2	0	0	0	0
113	No	1.5	4.5	4.5	1.8	0	0	0
134	No	1.2	4.5	4.5	2.2	0	0	0

[+] Discussed in text

[*] $TCID_{50}$ / 0.1 ml

(b) Serological Response

Monkey Group	Vaccine Status	Test	Mean Response on Day Indicated After Challenge			
			Pre	30	60	90
Group 1 (995, 997)	Yes	CF	< 2[*]	2	2	2
		HI	<10	< 10	< 10	< 10
		BN	0	9[+]	9	8
Group 2 (993, 113, 134)	No	CF	0	40	63	32
		HI	0	50	50	50
		BN	0	17	17	17

[*] CF and HI results are expressed as reciprocals of geometric means

[+] BN, zone of plaque-inhibition measured in mm

Table 37 - Long-Term Protective Efficacy of Chikungunya Vaccine Observed in Rhesus Monkeys Challenged 24 Months after Vaccination

(a) Viremia

Monkey No.	Vaccine Status	Viremia Patterns on Day Post-Inoculation						
		1	2	3	4	5	6	7
100	Yes	0	0	0	0	0	0	0
111	Yes	0	0	0	0	0	0	0
115	Yes	0	0	0	0	0	0	0
129	No	1.5*	3.5	4.8	2.8	0.5	0	0
990	No	1.8	4.8	4.5	0.5	0	0	0

* $TCID_{50}$ / 0.1 ml

(b) Serological Response

Monkey Group	Vaccine Status	Test	Mean Response on Day Indicated After Challenge			
			Pre	30	60	90
Group 1 (100, 111, 115)	Yes	CF	< 4*	16	8	8
		HI	< 10	< 10	< 10	< 10
		BN	8+	12	13	12
Group 2 (129, 990)	No	CF	0	43	64	43
		HI	0	120	100	100
		BN	0	20	23	23

* CF and HI results are expressed as reciprocals of geometric means

+ BN, zone of plaque-inhibition measured in mm

V. Basic Studies of Arbovirus Virions, Antigens and Proteins

A. Change in Viral Membrane Glycoproteins during Morphogenesis of Group B Arboviruses

Group B Arboviruses, which include Japanese encephalitis (JE) and dengue-2 (DEN-2) viruses, mature in intimate association with membranes of the endoplasmic reticulum (Ota, 1965; Murphy et al, 1968): ill-defined, 28 nm round particles line cytoplasmic vacuoles and apparently penetrate them to form what has been thought to be mature virions within the vacuoles (Matsumura et al, 1971); subsequently the vacuoles migrate to the plasma membrane and, by unknown mechanisms, release the virus. The viral membrane (or envelope of JE or DEN-2 is composed of two polypeptides: V-3, a glycoprotein of MW 53-59,000 daltons, and V-1, a non-glycoprotein of MW 7,700-8,700 daltons (Stollar, 1969; Shapiro, et al, 1971). In addition to V-3, JE-infected chick embryo cells synthesize V-2, the virion nucleocapsid polypeptide, and at least five non-virion polypeptides; every polypeptide binds to cytoplasmic membranes (see V-B, below) and three of them are glycoproteins (see V-C, below). The biochemical events that lead to viral membrane genesis are unknown. Here we show that the viral membrane protein composition changes significantly during morphogenesis: the intracellular form of the virus contains two membrane glycoproteins, one of which disappears from the extracellularly released virus, apparently replaced by the small non-glycoprotein membrane polypeptide. We show that these processes can be distinguished from those that lead to virion release by the use of tris(hydroxymethyl)aminomethane (Tris).

We found that at least two forms of radioactively-labeled DEN-2 virions could be released by infected cells when DEN-2 was grown in LLC-MK2 cells incubated with either: 1) Medium 199 (M-199) containing ^{14}C-amino acids, or, 2) Earle's balanced salt solution media (see legend to Fig. 11) containing 6 mM tris(hydroxymethyl)aminomethane (Tris) and ^{3}H-amino acids. When the extracellular fluids were mixed and harvested, both kinds of virions had similar sedimentation rates (Fig. 11A) and densities (Fig. 12A). However, when the mixed virions were dissociated and coelectrophoresed through polyacrylamide gels, two differences were apparent (Fig. 13A). The ^{14}C-virions grown in the absence of Tris had the expected composition V-1, V-2 and V-3, with a small shoulder to the left of V-2; by contrast, the ^{3}H-virions grown in the presence of Tris had only a small amount of V-1 and a large amount of a polypeptide migrating more slowly than V-2. Since the mobility of this DEN-2 structural polypeptide was similar to the mobility of the JE-specified polypeptide NV-2, we designated it also as NV-2. We have termed these two kinds of virions as the N-form (virus grown in "normal" media) and the T-form (virus grown in Tris-containing media).

The decrease in V-1 and the presence of NV-2 (called Tris effects) resulted from the presence of Tris regardless of the media base; i.e., the T-form was produced by cells incubated with Tris and either M-199 or

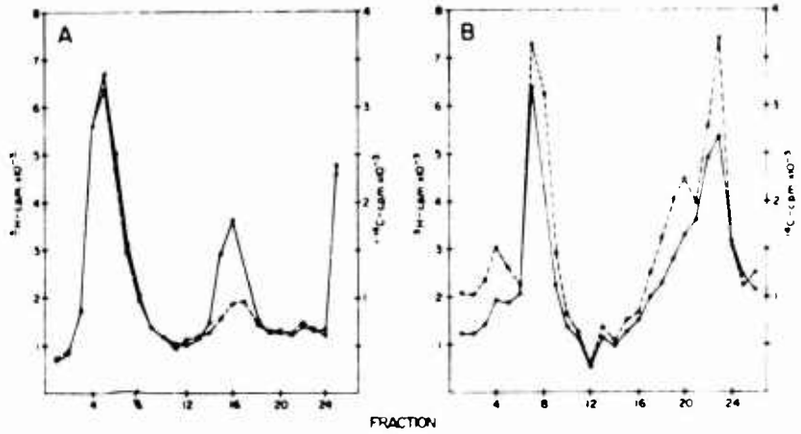

Figure 11. Rate zonal centrifugation of A. dengue-2 virions through a 5-25% sucrose gradient (virions are in fractions 4 to 6) and B. Japanese encephalitis virions through a 5-35% sucrose gradient (virions are in fraction 7). Each figure depicts co-centrifugation of virions propagated in cell culture under "normal" medium (O----O) and medium containing Tris buffer (●——●)

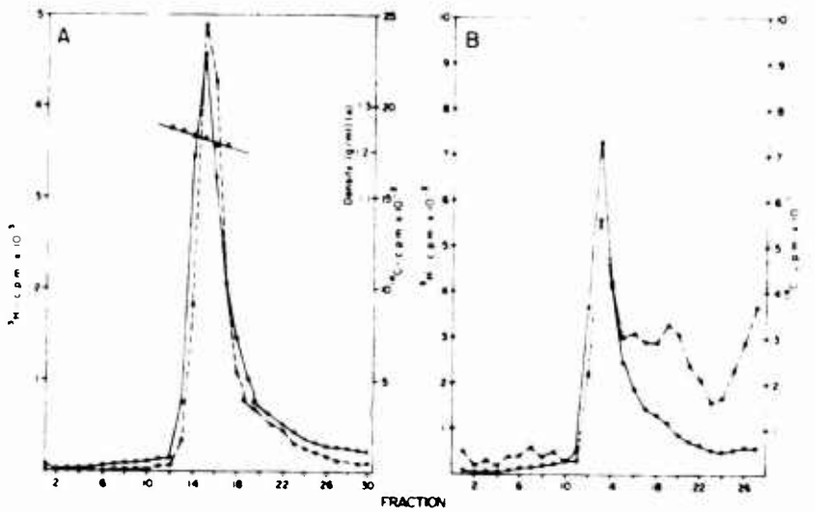

Figure 12. Isopycnic (equilibrium) centrifugation of A. dengue-2 virions and B. Japanese encephalitis virions through preformed linear 20-70% sucrose-D_2O gradients. Each figure depicts co-centrifugation of virions propagated in cell cultures under "normal" medium (O----O) and medium containing Tris buffer (●——●).

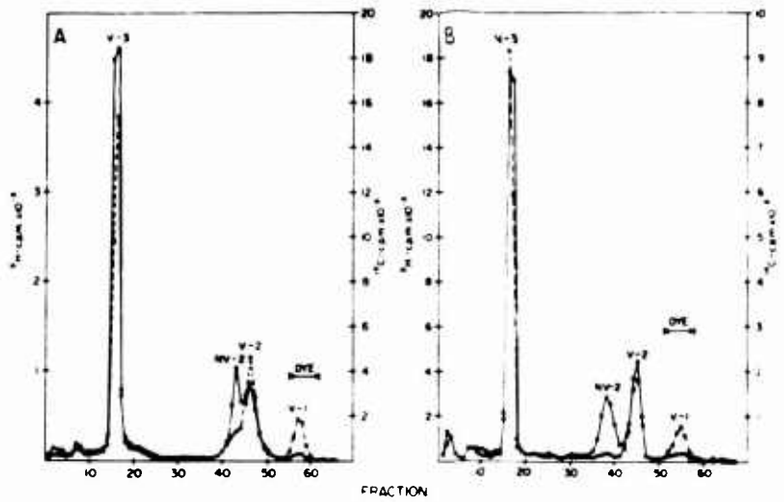

Figure 13. Polyacrylamide gel electrophoresis of A. dengue-2 virions and B. Japanese encephalitis virions dissociated with sodium lauryl sulfate and 2-mercaptoethanol at 100C for 10 minutes. Each figure depicts co-electrophoresis of virions propagated in cell cultures under "normal" medium (O----O) and in medium containing Tris buffer (●——●).

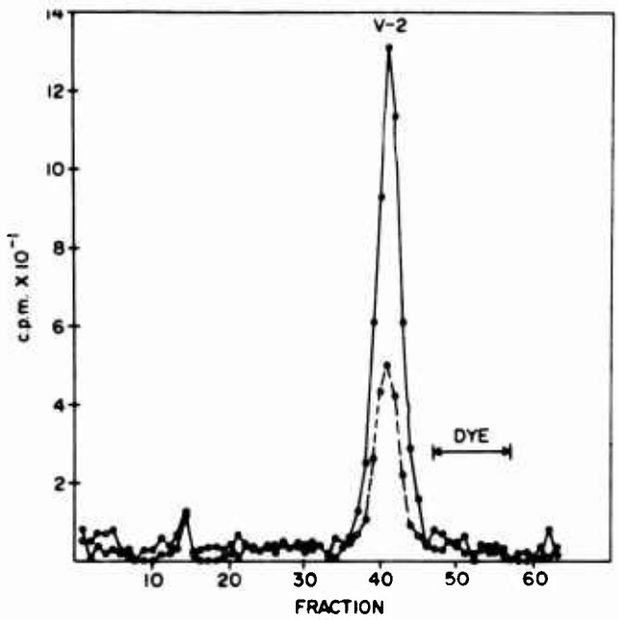

Figure 14. Polyacrylamide gel electrophoresis of dissociated (as above) dengue-2 nucleocapsids (described in 1971 Annual Report) isolated from virions ;harvested from "normal" medium (O----O) and from medium containing Tris buffer (●——●).

EBSS in a pH range of at least 7.4 to 7.8, whereas the N-form was produced in either media when lacking the Tris in the same pH range. The addition of Tris, and not of equimolar NaCl, was sufficient to produce T-forms. Tris effects were not altered when T-form virions were banded isopycnically prior to electrophoresis (as in Fig. 12) or when ^{14}C-amino acids were used as the label. However, there was some variation in the amount of NV-2 and of V-1 present in the T-form in various experiments; we did not determine which other factors were capable of modulating the ability of Tris to produce T-form virions.

When JE-infected LLC-MK$_2$ cells were grown in EBSS media in the presence or absence of Tris, T-form or N-form virions were released, respectively (Figs. 11B, 12B and 13B). We therefore suggest that the potential existence of two virion forms is a characteristic of group B arbovirus replication in LLC-MK$_2$ cells.

We determined whether NV-2 was a component of the T-form virion nucleocapsid or membrane by isolating the nucleocapsids of mixed N and T-form DEN-2 virions using the nonionic detergent NP-40 as described previously (Stollar, 1969; Shapiro et al, 1971) and by analyzing their polypeptide composition by polyacrylamide gel electrophoresis. The only polypeptide component of both nucleocapsids was V-2 (Fig. 14), indicating that V-3, V-1 and NV-2 are all virion membrane components.

Since many virion membrane proteins are glycoproteins (Burge and Strauss, 1970), we determined if NV-2 was also one by labeling the DEN-2 T-form with ^{14}C-glucosamine and co-electrophoresing it with ^{3}H-amino acid labeled T-form virions (Fig. 15). Both V-3 and NV-2 were labeled by glucosamine, indicating that they are glycoproteins; it has already been shown that V-1 and V-2 are not glycoproteins (Stollar, 1969). Two glycoproteins were also present in ^{3}H-glucosamine-labeled JE-T-forms but the distribution of the glucosamine-labeled component of NV-2 was not completely symmetrical with respect to the amino acid-labeled component (see V-C, below). At a minimum, we can conclude that T-form virions contain two membrane glycoproteins whereas N-form virions contain one membrane glycoprotein and one small membrane non-glycoprotein.

It was important to determine if the existence of both N and T-forms implied a relationship occurring during morphogenesis between them, and, in particular, between NV-2 and V-1. We have previously shown that JE-infected chick embryo cells synthesize little or no V-1 and speculated that V-1 might be formed at the time of virus release from a precursor (Shapiro et al, 1971). Since intracellular virions are probably the precursors to extracellular virions, we isolated the intracellular virions (Brandt et al, 1970) within JE-infected chick embryo cells grown in "normal" medium and determined their polypeptide composition (Fig. 16). Under such conditions, the extracellular virus was the N-form (Fig. 16A) but the peak of intracellular virus (I-form) closely resembled the T-form in containing V-3 and NV-2 as membrane

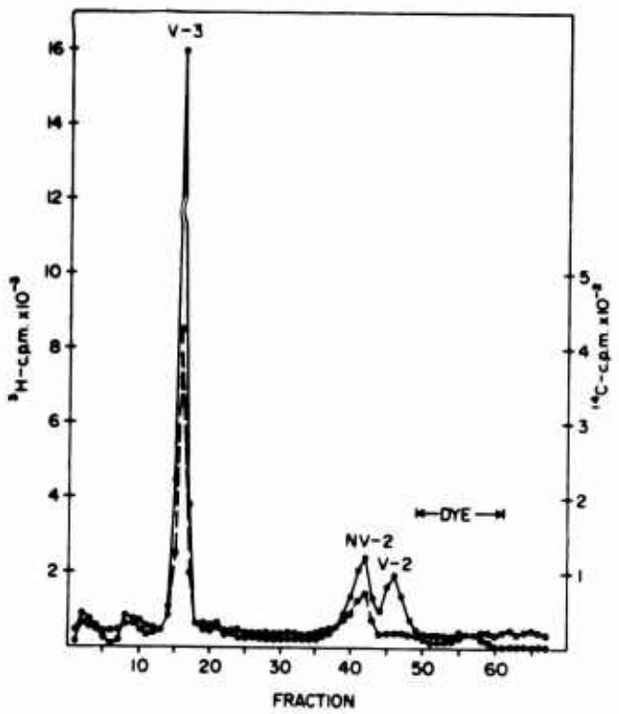

Figure 15. Polyacrylamide gel electrophoresis of ^{14}C-glucosamine (O----O) and ^{3}H-amino acid labelled (●——●) dengue-2 virions propagated in LLC-MK2 cell cultures under medium containing Tris buffer, and dissociated with sodium lauryl sulfate and 2-mercaptoethanol at 100C.

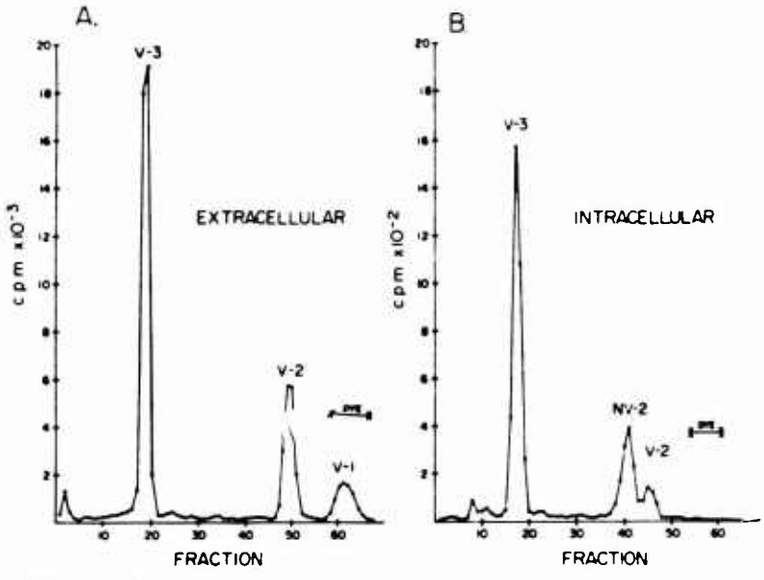

Figure 16. Polyacrylamide gel electrophoresis of A. extracellular and B. intracellular virions harvested from chick embryo cells under "normal" medium and dissociated with SLS and 2-ME as above.

proteins (Fig. 16B). V-1 was not present in the I-form.

The I-form appeared to have a reduced content of V-2 relative to that of the N-form, but I-form virions from the leading edge of the peak of rate-zonally purified virus in this experiment had less V-2 than the peak virus, whereas virions from the trailing edge had more V-2 than the peak. In some other experiments there was both less heterogeneity and increased levels of V-2. The reasons for the heterogeneity in the proportion of membrane proteins (relative to V-2) are unknown; we therefore cannot conclude that the I-form (from chick embryo cells) and the T-form (from LLC-MK_2 cells) represent identical virion forms. However, it is clear that the viral membrane and the virion are first formed in an "immature" state. Analagous processes may occur in cells infected with other membrane-containing viruses (Schlesinger et al, 1972).

When the infectivity of the supernatant fluids from Tris-treated cells was compared to that from "normal" cells, the values ranged from 0.05 to 0.7. When the specific infectivity (pfu/cpm) of T-form virions was compared to that of N-form virions, values ranged from 0.2 to 0.7. Furthermore, when the specific infectivity of I-form virions was compared to that of the N-form virions (both from chick embryo cells) the value was 0.004. Therefore, it seems possible that one result of viral membrane "maturation" is increased infectivity.

The simplest interpretation of our results is that NV-2 is the precursor to V-1; that sometime late in morphogenesis, after the I-form is assembled within cytoplasmic vacuoles, NV-2 is cleaved and deglycosylated to form V-1. Tris, then, would be an agent that directly or indirectly inhibits this cleavage enzyme in LLC-MK_2 cells, but which does not inhibit, at least to the same extent, release of the virion from the cells. The release mechanism therefore has ambiguous specificity, as does the viral membrane structure. The fact that release from the cell is not dependent on cleavage also implies that different populations of T-forms might exist with variations in the amount of NV-2 and of V-1 contained, dependent on the degree of antecedent cleavage.

If the I-form and N-form truly differ in membrane content, then a third reaction must occur during morphogenesis which eliminates excess membrane. It may be pertinent to recall that cells infected with some group B arboviruses, especially DEN-2 (Stollar, 1969; Cardiff, et al, 1971), liberate "incomplete virus particles" composed exclusively of membrane proteins.

B. Membrane-Bound Proteins of Japanese Encephalitis Virus-Infected Chick Embryo Cells

Japanese encephalitis virus (JEV) is a group B arbovirus composed of three polypeptides (Annual Report, 1971), the largest of which is a glycoprotein (Section 5-C, below). Electron microscopic studies of infected cells (Murphy et al, 1968; Ota, 1965) have indicated that viral morphogenesis occurs in intimate association with internal cellular membranes; neither "free" cytoplasmic structures, such as viral cores, have been described, nor has viral "budding" through plasma membranes been observed as in group A arbovirus-infected cells (Acheson and Tamm, 1967, 1970). Instead, electron-dense, membrane-bound, round structures, about 27 nm in diameter, form on cytoplasmic vacuoles; they penetrate the vacuoles and become enveloped to form apparently mature virions within the vacuoles.

JE-infected cells synthesize seven virus-specified polypeptides (Annual Report, 1971). Here we have examined some aspects of the binding of these polypeptides to internal cellular membranes of infected chick embryo cells.

Preparation of membranes. Methods for infection of chick embryo cells with JEV in the presence of actinomycin D, and for pulse inhibition with cycloheximide have been described (Annual Report, 1971). Briefly, infection of confluent monolayers in 32-oz prescription bottles was begun at 0 hr, actinomycin D (1 ug/ml) was added at 9 hr, cycloheximide (500 ug/ml) was added at 18 hr, medium was replaced with cycloheximide-free medium at 18.5 hr, and radioactive amino acids were added from 19 through 23 hr. The cells were scraped off with a rubber policeman into 0.25 M sucrose (unless otherwise indicated all sucrose solutions were made in RSB — 0.01 M tris(hydroxymethyl)aminomethane (Tris), 0.01 M NaCl, 0.0015 M $MgCl_2$ pH 7.4) at 4° C and broken with 25 strokes of a 7-ml tight-fitting Dounce homogenizer (Kontes Glass). The material was centrifuged at 2000 rpm (480 g) for 2 min in the Sorvall SS-34 rotor. The supernatant was then centrifuged through 0.3 M sucrose onto a 2.6 M sucrose cushion at either 65,000 rpm (300,000 g) for 45 min in the Beckman SW 65 rotor, at 50,000 rpm (203,000 g) for 1 hr in the SW 50L rotor at 40,000 rpm (106,000 g) in the type 40 rotor for 1.5 hr. The membranous material was then collected, resuspended with a Pasteur pipette and used directly for experiments as "crude membranes." When purified membrane bands were desired as starting material, "crude membranes" were made 1.33 M in sucrose and then placed on the discontinuous sucrose gradient shown in Fig. 18, which is a modification of previously described methods (Uhr and Schenkin, 1970; Bosmann et al, 1968; Caliguiri and Tamm, 1970; Spear et al, 1970). Centrifugation was at 65,000 rpm (300,000 g) for 1.5 hr in the SW 65 rotor; 0.1 ml aliquots were collected by bottom puncture.

Treatment and analysis of membranes. The various fractions were diluted with either NaCl, $NaHCO_3$ + Na_2CO_3, KCl or NP-40 (Shell Chemical

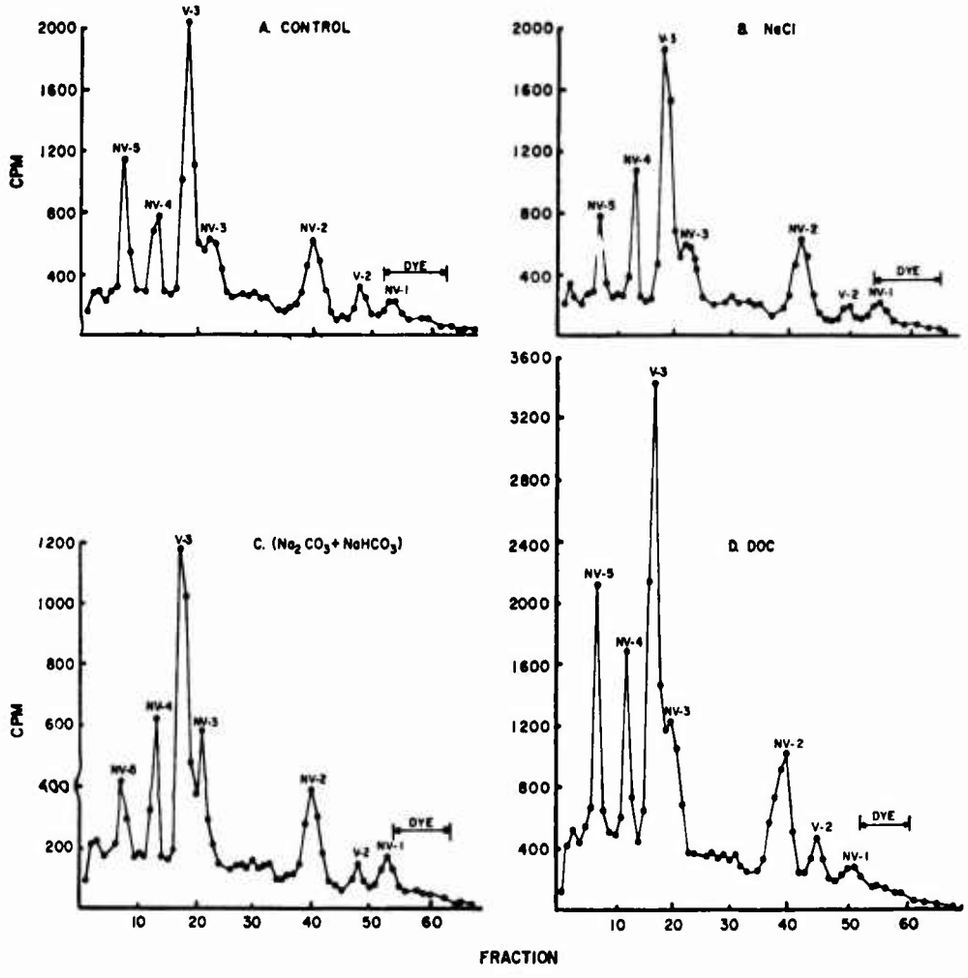

Figure 17. Polyacrylamide gel electrophoresis of band 3 membranes treated with various salts or detergent. After treatment with either 1.5 M NaCl, 0.2 M $NaHCO_3$ + Na_2CO_3 or 0.075% deoxycholate (DOC), the membranes were pelleted at 50,000 rpm (204,000 x g) for 60 minutes, dissociated with SLS and 2-ME and electrophoresed.

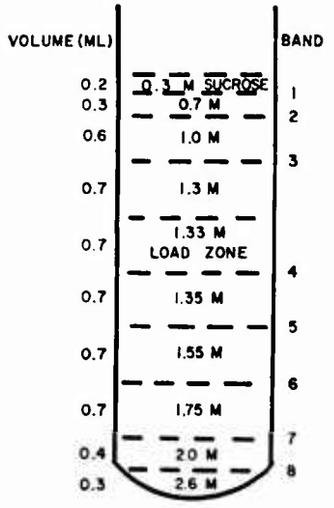

Figure 18. Discontinuous sucrose gradient for membrane fractionation. Samples were made 1.33 M sucrose with a refractometer and loaded in the middle of the gradient. Centrifugation was at 65,000 rpm (300,000 x g) for 90 minutes; 0.1 ml fractions were collected from the bottom.

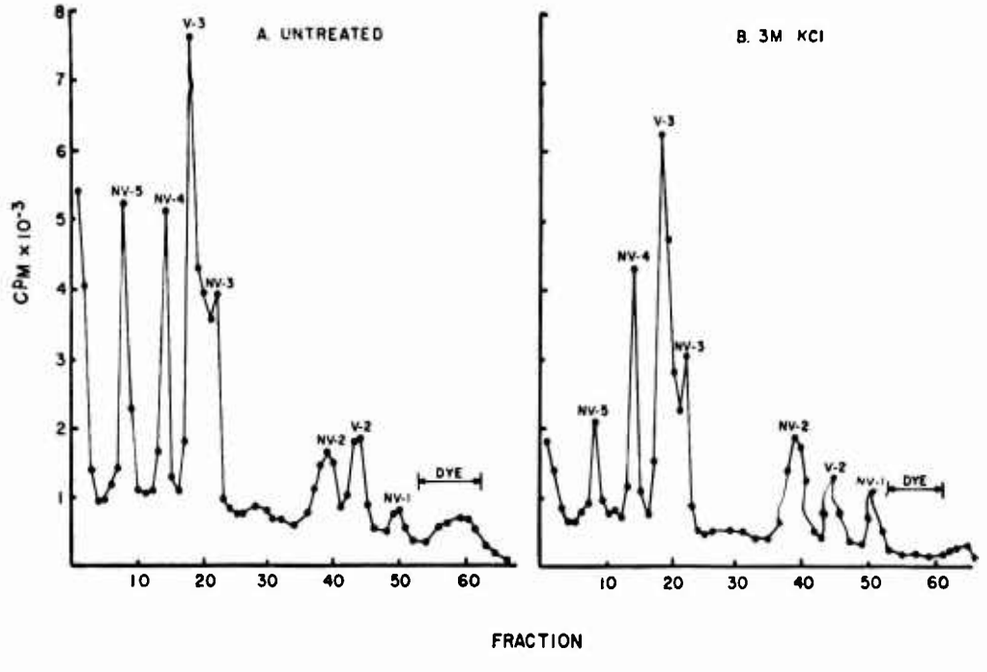

Figure 19. Polyacrylamide gel electrophoresis of crude membranes before and after extraction with 3 M KCl. Each aliquot was diluted with either buffer or KCl and incubated at 4C for 18 hours. The membranes were then pelleted twice, dissociated with sodium lauryl sulfate and 2-mercaptoethanol, and electrophoresed on an 8% gel.

Co.) or else were incubated with trypsin (2X recrystallized, lyophilized, salt-free, Mann Research) as indicated in the text. Subsequently, the membranes were diluted at least 25-fold and pelleted at 50,000 rpm for 60 min in the SW 50L rotor or else were fractionated on the discontinuous sucrose gradient shown in Fig. 18. Pelleted material was dissociated with sodium lauryl sulfate (SLS) and 2-mercaptoethanol (2-ME) and electrophoresed on 8% polyacrylamide gels (PAGE). Determination of radioactivity in the gel slices was as described previously (Annual Report, 1971), except that the NCS (Amersham Searle), Liquifluor (New England Nuclear), and toluene were added simultaneously to the vials, which were then incubated at 37° overnight and counted. All radioactive l-amino acids were obtained from New England Nuclear and consisted of a synthetic mixture of 15 l-amino acids, either ^{3}H or ^{14}C.

Production of radioactive JE virions. JEV was propagated in either LLC-MK_2 or chick embryo cells, labeled with radioactive amino acids, and purified by rate zonal centrifugation as previously described (Annual Report, 1971).

Results: Specificity of membrane binding. Most of the virus-specified radioactive proteins in JEV-infected chick embryo cells were associated with sedimentable structures that could be solubilized by treatment with a detergent (1% Nonidet P-40). Furthermore, when the non-sedimentable proteins of infected cells (those proteins not sedimenting at 300,000 g for 60 min in the absence of detergent) were analyzed by polyacrylamide gel electrophoresis (PAGE), much of the material did not coelectrophorese with marker "whole-cell extract" containing the seven previously described virus-specified proteins. We therefore concluded that the great majority of virus-specific proteins were on, or enclosed by, detergent-labile membranes.

The infected-cell membranes were obtained from Dounce-homogenized cells and were fractionated on the discontinuous sucrose gradient illustrated in Figure 18. By this technique most of the "light" membranes were located in band 3, which was predominantly smooth; the denser, "heavy" bands, 5, 6 and 7, were more heterogeneous, consisting of rough membranes, cytopathic vacuoles, and some cell organelles. We selected bands 3 and 5 for studying the binding of viral proteins to membranes. In the following, we present the data only for band 3, which is the most homogeneous membrane band isolated; however, similar results were obtained for band 5.

The polypeptide composition for untreated band 3 is presented in Fig. 17-A. All seven of the virus-specified polypeptides previously found within whole cell extracts of JEV-infected chick cells (Annual Report, 1971) were present on the membranes. These ranged from NV-5 (nonvirion, molecular weight about 94,000 daltons) down to NV-1 (molecular weight about 10,500 daltons), and included V-3 (the large virion envelope protein) and V-2 (virion core protein). Since it has recently been shown that nonspecific binding of proteins to membranes can be

greatly reduced or eliminated by washing membranes successively in 0.15 M NaCl, 1.0 M NaCl, 0.1 M ($NaHCO_3$ + Na_2CO_3), and 0.075% deoxycholate (Hinman et al, 1970), we treated band 3 membranes similarly: aliquots were diluted 25-fold with (1) 0.25 M sucrose (untreated control); (2) 1.5 M NaCl; (3) a solution of equal parts of 0.2 M $NaHCO_3$ and 0.2 M Na_2CO_3; or (4) 0.075% sodium deoxycholate (DOC). After 20 min at 4°C, the material was pelleted and analyzed by PAGE (Fig. 17). None of these treatments eliminated any of the virus-specified polypeptides from the membranes, which suggests that the binding was not adventitious.

However, there was a suggestion that both the neutral and alkaline salt partially released NV-5 and V-2 from the membranes. In order to examine this further, membranes were treated with 3 M KCl, a procedure which has been shown to extract membrane-bound transplantation antigens (Kahan and Reisfeld, 1971; Reisfeld et al, 1971). As a result of this salt treatment, NV-5 was partially extracted (Fig. 19); probably V-2 was also partially extracted. We therefore suggest that the binding of NV-5, and probably of V-2, to membranes is partially stabilized by polar or hydrogen bonds.

Trypsinization of Virions. Two recent studies have indicated that the sensitivity of 30 S ribosomal subunit proteins to tryptic digestion reflects the topographic arrangement of the proteins: those that are digested first are assembled last on to the subunit and are the most superficial (Chang and Flaks, 1970; Craven and Gupta, 1970). When we initially trypsinized infected membranes in order to study the topography of the virus-specified proteins, we found a complex pattern; it was therefore necessary to first look at the sensitivity of virions to trypsin.

When JE virions were treated with 0.1% trypsin, three discrete polypeptide fragments were produced, along with low-molecular weight material (Fig. 20). The three discrete trypsin-derived fragments were designated as TF-1 (with a mobility similar to that of NV-1), TF-2 (with a mobility corresponding to an estimated molecular weight of 32,000 daltons), and TF-3 (with a mobility similar to that of NV-3). TF-3 and TF-2 were derived ultimately from V-3; TF-1 could have been derived from V-2 but, by Occam's razor, was also probably derived from V-3. The facts that TF-1 and TF-3 were not readily distinguishable from NV-1 and NV-3, and that a high background was present in the low molecular weight region of gels of trypsinized membrane samples, and that NV-5 and NV-4 did not appear to be trypsinized to small membrane-bound fragments (see Fig. 24) indicated that readily interpretable results could be obtained only by studying the disappearance from trypsinized membranes of the relatively large peptides NV-5, NV-4 and V-3.

Trypsinization of Membranes. When crude membranes of JEV-infected chick cells were treated with trypsin and then fractionated, there were two changes (Fig. 21); some of the radioactivity was solubilized (the radioactivity remained in the load zone) and some was converted to membranous material of low density (the radioactivity floated to the top of

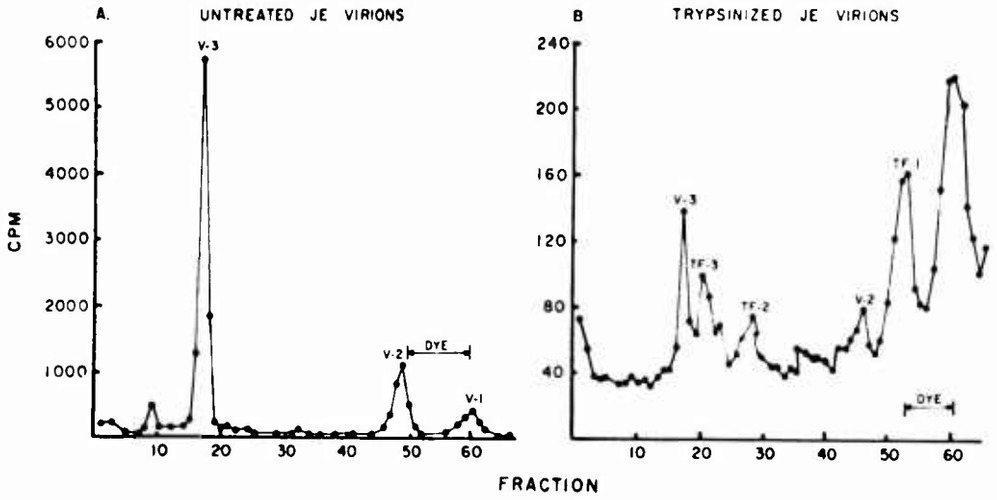

Figure 20. Polyacrylamide gel electrophoresis of Japanese encephalitis virions before and after treatment with 0.001% trypsin at 38C for 1 hour. The virions were pelleted, dissociated with SLS and 2-ME and electrophoresed on an 8% gel.

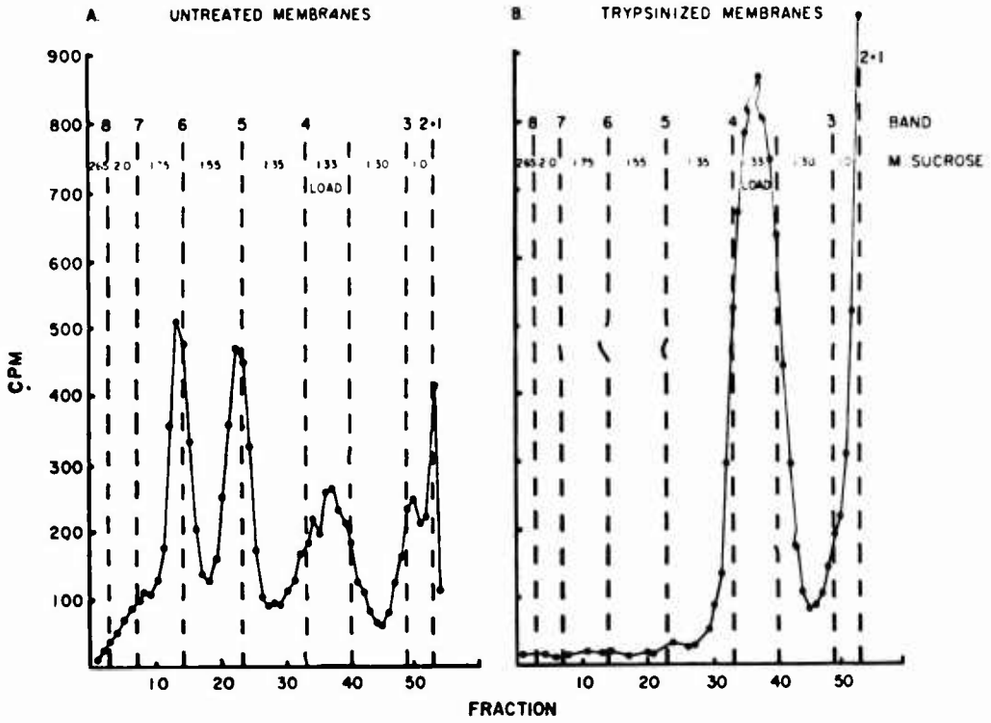

Figure 21. Fractionation of JEV-infected chick embryo cell membranes on the discontinuous sucrose gradient before and after treatment with 0.04% trypsin (37C x 4 hours) and addition of 0.05% soybean trypsin inhibitor.

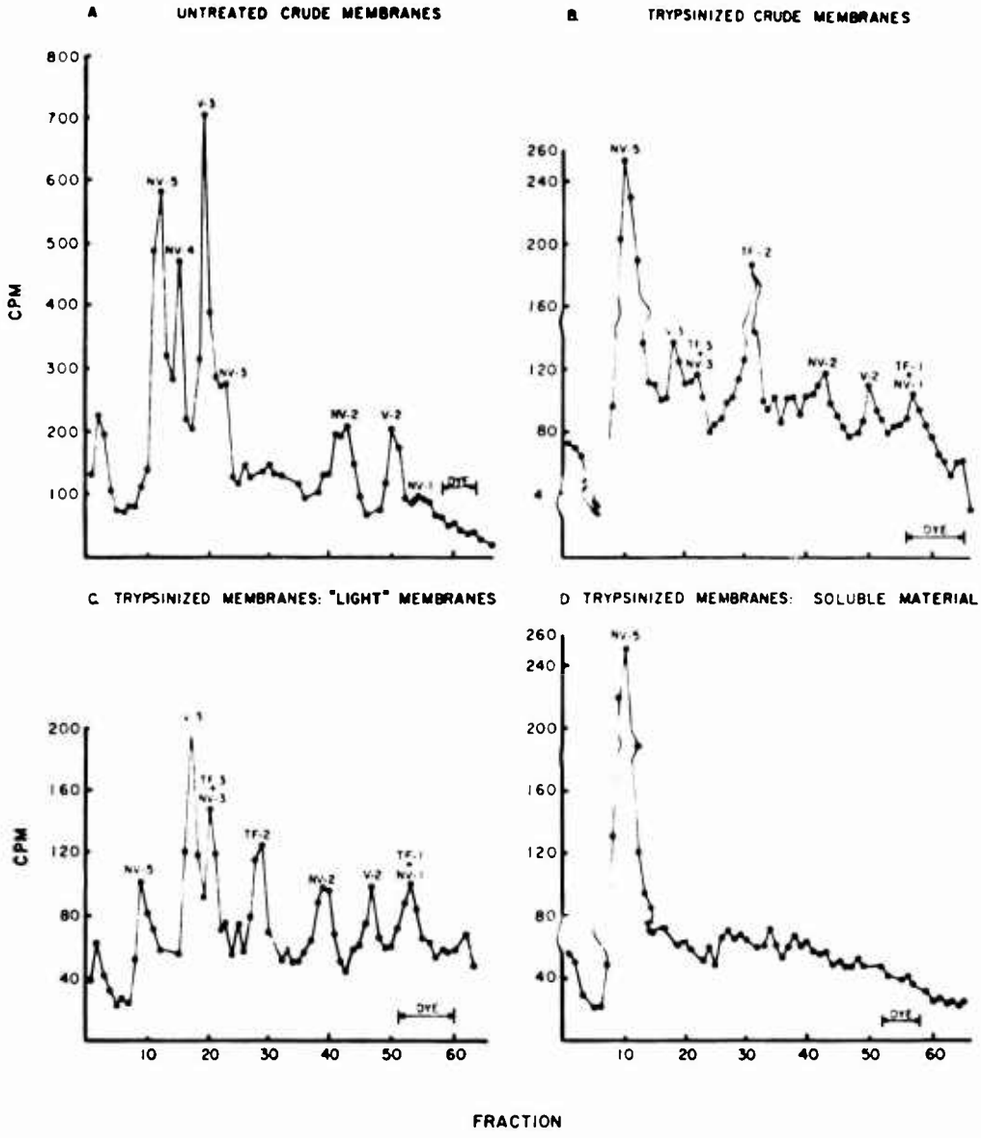

Figure 22. Polyacrylamide gel electrophoresis of trypsinized membrane fractions. Fractions from the previous experiment were heated with SLS and 2-ME and electrophoresed on 8% gels, including: A. crude membranes (control); B. crude membranes trypsinized; C. solubilized trypsinized membranes (fractions 36-38 pooled); D. low density trypsinized membranes (fraction 53).

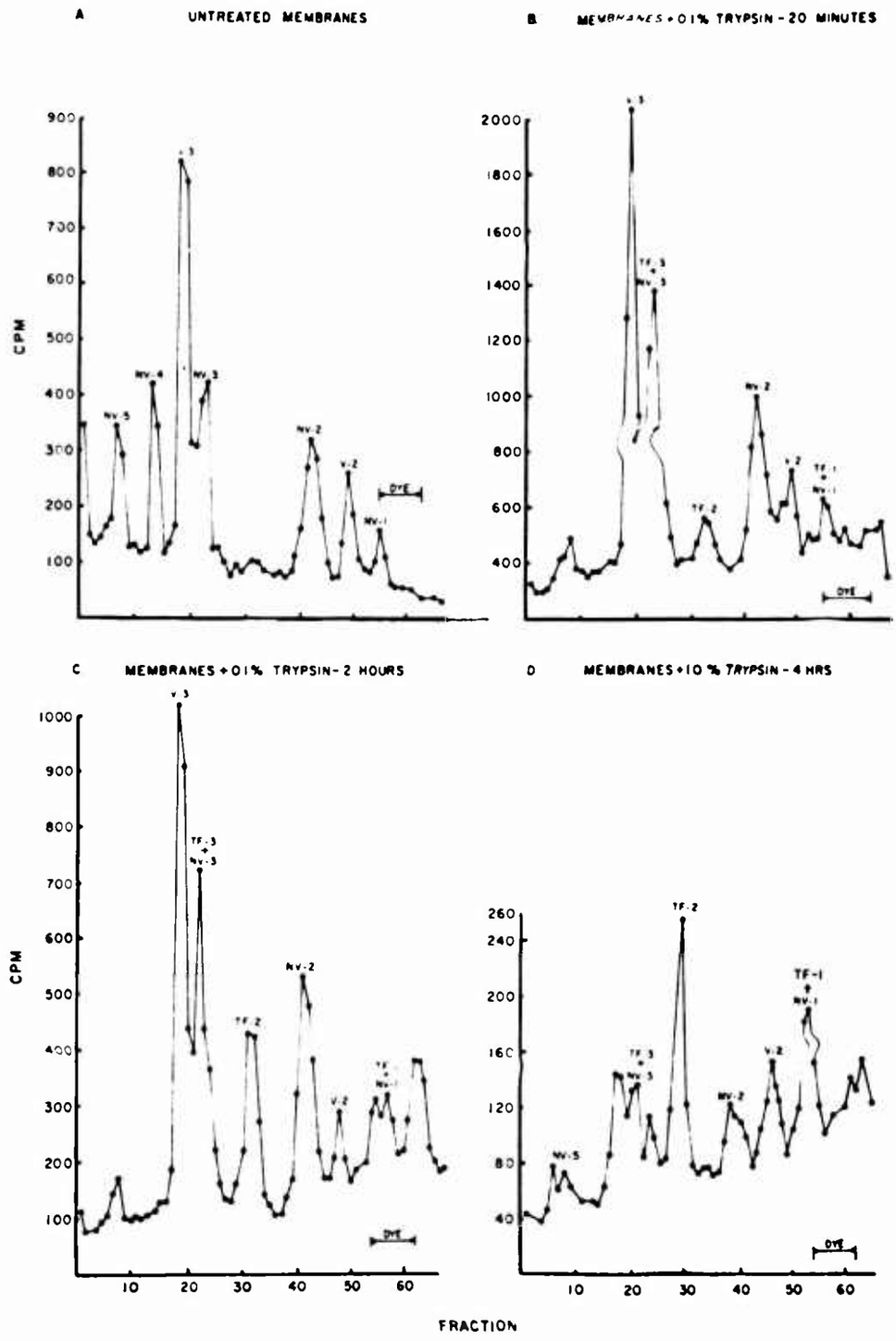

Figure 23. Polyacrylamide gel electrophoresis of JEV-infected chick embryo cell membranes after increasing treatments with trypsin.

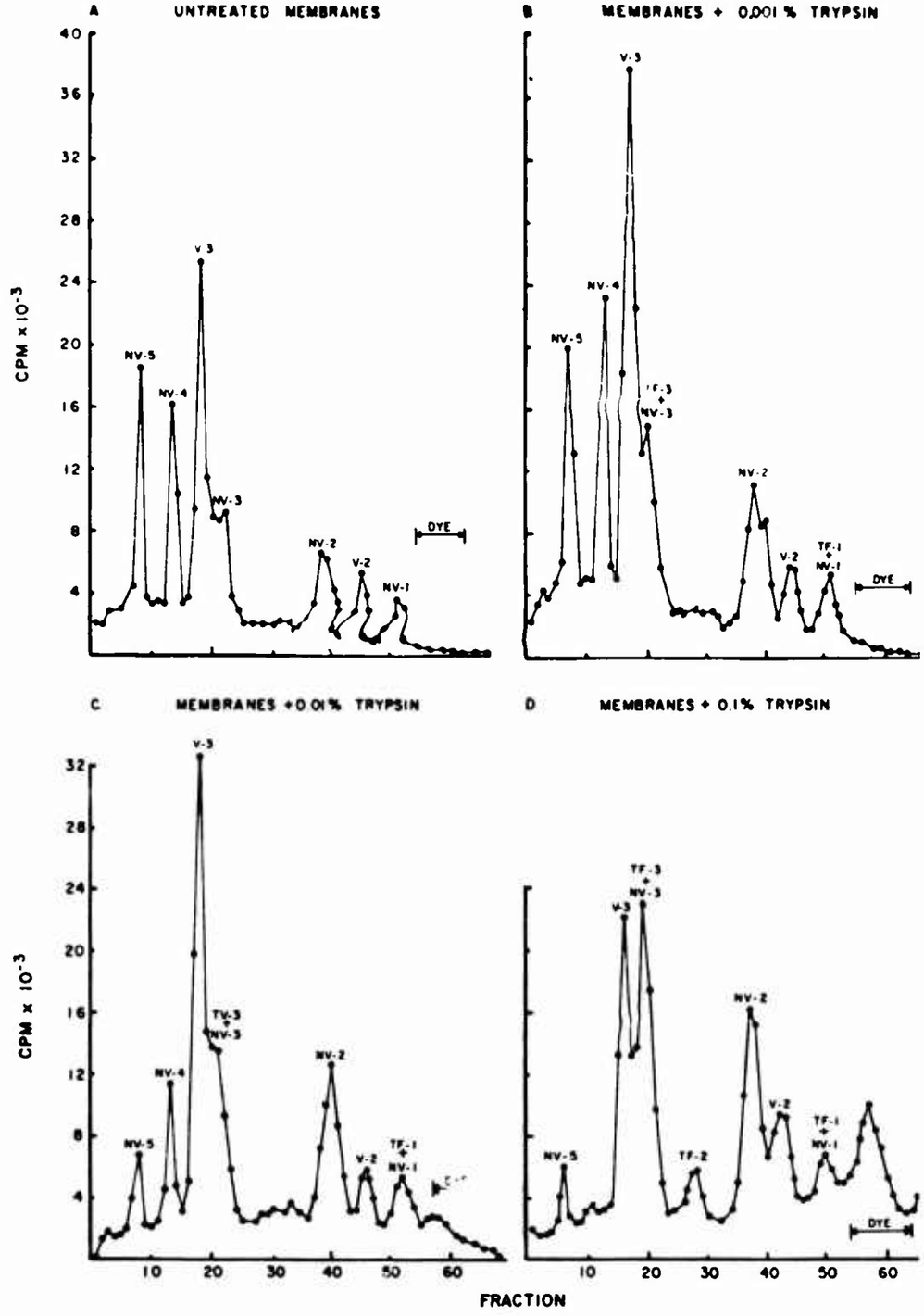

Figure 24. Relative rates of disappearance of NV-5 and NV-4 from JEV-infected chick embryo cell membranes after treatment with indicated trypsin for 30 minutes, 37C. After addition of trypsin inhibitor, the mixtures were pelleted, dissociated with SLS and 2-ME, electrophoresed.

the gradient). The various fractions were analyzed by PAGE (Fig. 22). Before trypsinization, V-3, NV-5, and NV-4 were the most prominent polypeptide peaks (Fig. 22-A). After trypsinization, before membrane fractionation, the major peak present was a broadened NV-5 peak, indicating some degradation; NV-4 disappeared (or was not resolved within the broadened NV-5 peak), and V-3 was reduced.

By contrast, the low density membranes contained a reduced amount of NV-5 relative to V-3; NV-4 was not present (Fig. 22-C). Since NV-5 was present in large amounts in the unfractionated trypsinized mixture (Fig. 22-B) and was present in only small amounts in the low density membrane fraction (Fig. 22-C, then it should have been present in *large* amounts in the solubilized material, which was confirmed (Fig. 22-D). However, by this reasoning, the recovery of the other polypeptides was not quantitative. One can conclude that NV-5 was readily released from the membranes (relative to V-3) in a relatively undegraded soluble form. By contrast, NV-4 appeared to be released and degraded. Finally, V-3 was degraded but not readily released (relative to NV-5); at least some of TF-2 (a derivative of V-3) remained membrane-bound (Figs. 22-D, 23-C, 23-D). These facts suggest that the sensitivity of a membrane-bound polypeptide to trypsin "attack" can be expressed in at least three ways: (1) It can be released in a relatively undegraded form (NV-5); (2) It can be released and degraded (NV-4); and (3) It can be degraded but still difficult to release (V-3). The initial stimulus for release under the aegis of trypsin is unclear; whether covalent bond breakage is necessary is not known. We therefore use the term "release" only to indicate disappearance of a polypeptide from trypsinized membranes regardless of the exact mechanism involved, under the assumption that the topographic arrangement of the polypeptide on the membrane determines its sensitivity while its intrinsic nature determines its response.

Kinetics. In order to obtain a clearer understanding of trypsin mediated release, kinetic studies were done (Figs. 23 and 24). With relatively high concentrations of trypsin (0.1% and 1.0%) the order of release was NV-5 $\approx$ NV-4 > V-3 (Fig. 23). In order to determine the release sequence of NV-5 relative to NV-4, lower concentrations of trypsin were used (0.001% — 0.01%) (Fig. 24), NV-5 was released at a slightly faster rate than NV-4. Moreover, both NV-5 and NV-4 were released without the production of small membrane-bound trypsin-derived fragments of V-3, indicating the reliability of determining the respective release rates of NV-5, NV-4 and V-3.

The order of trypsin-mediated release is therefore NV-5 $\geq$ NV-4 > V-3. Despite the great sensitivity of NV-5 to trypsin, a small residual peak of radioactivity remained in the region of the gel even after extensive trypsinization (Figs. 23 and 24). This suggests that either (1) the release of NV-5 plateaus before total release is achieved, or (2) a resistant subpopulation of NV-5 exists, or (3) the residual radioactive peak represents an aggregate which coincidentally migrated similarly to NV-5, similar to what we have previously observed when

electrophoresing dissociated virions.

No definite statements can be made about the release sequence of polypeptides smaller than V-3, especially NV-3 and NV-1. One puzzling feature is the apparent reversal of the order of release of V-2 with respect to NV-2 (Figs. 23-B,-C); it is possible that a trypsin-fragment migrates similarly to one of them or that a resistant subpopulation of one of them exists.

Specificity of release of NV-5 and NV-4. We have assumed that NV-5 and NV-4 were rapidly released because of their specific arrangement on membranes. However, it is possible that any other polypeptide of similar molecular weight would be similarly released. To examine this, uninfected cells were labeled with ^{3}H-amino acids and mixed with ^{14}C-amino acid labeled infected cells. Membrane band 3 was isolated, trypsinized, pelleted, and analyzed by PAGE (Fig. 25); the specific loss of NV-5 and NV-4 from viral infected membranes exceeded the loss from normal membranes of host polypeptides of molecular weight exactly equal to those of NV-5 and NV-4. Similar results were also obtained when crude membranes were obtained from cells labeled with ^{3}H-amino acids prior to infection, then infected and labeled with ^{14}C-amino acids.

Discussion. We have shown that all seven JE-specified intracellular polypeptides (both virion and nonvirion) bind to membranes. Two of them, NV-5 and probably V-2, are partially released by salt and are therefore presumably stabilized by polar or hydrogen bonds. The sequence of trypsin-mediated release from membranes is NV-5 $\geq$ NV-4 > V-3. Therefore our results suggest that the large viral proteins bind to membranes in an orderly fashion, such that the larger the polypeptide, the more superficial and "exposed" it is. Furthermore, since unequal rates of loss of radioactivity were observed for the polypeptides, mediated by either trypsin or salt, it seems likely that they are bound as monomolecular polypeptides rather than as quaternary complexes of multiple species of polypeptides, for the latter would imply simultaneous loss of radioactivity for every component. However, we cannot completely exclude the possibility that NV-5 and NV-4 are two polypeptide subunits of one membrane-bound protein.

In addition to being the two largest polypeptides, NV-5 and NV-4 are also nonvirion polypeptides. The release sequence therefore also indicates that two nonvirion polypeptides are more superficial than one virion polypeptide (V-3); whether or not this is a general pattern of organization of nonvirion versus virion polypeptides on cellular membranes is not known. Finally, we have ignored the possible contributions to the observed release sequence of factors related to target theory.

Our results may be limited by technical factors, which include (1) nonspecific binding and (2) inadequate fractionation of membranes. 1. - Nonspecific binding. This would include adventitious binding and the trapping of proteins within vesicular structures rather than actual

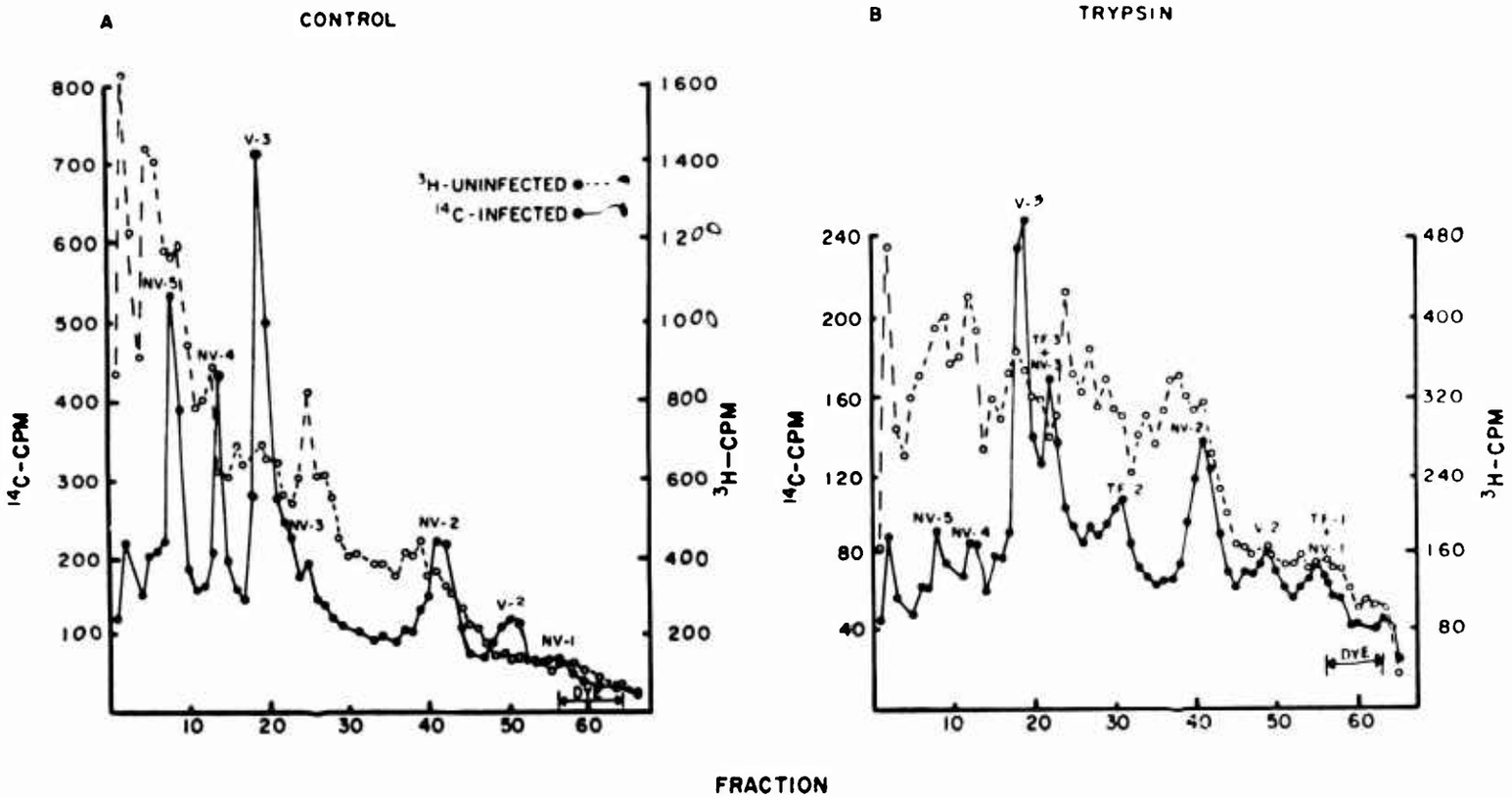

Figure 25. Trypsinization of a mixture of infected and uninfected band 3 membranes: 8 x 10^7 uninfected chick cells labeled with ^{3}H-amino acids were mixed with 8 x 10^7 JEV-infected chick cells labeled with ^{14}C-amino acids. After isolation and fractionation of membranes, band 3 was divided into two 0.3 ml aliquots; one aliquot received 3 ul of 0.1% trypsin. Both aliquots were incubated at 36C for 0.5 hour; then 2 ul of 1% trypsin inhibitor was added and the mixtures were diluted, pelleted, dissociated with sodium lauryl sulfate and 2-mercaptoethanol at 100C for 10 minutes and electrophoresed on 8% polyacrylamide gels.

binding to membranes. The washing experiments with detergent, salt, and alkali were attempts to minimize or evaluate these problems. In connection with this is a recent report demonstrating the binding of two or three vesicular stomatitis virion polypeptides to uninfected plasma membranes (Cohen et al, 1971). The authors regarded this as evidence for "nonspecific" binding and suggested that such controls are necessary for interpretation of membrane binding. However, the polypeptides that did bind in this manner were probably originally membrane bound and were solubilized by sonication (Wagner et al, 1970). Furthermore, a polypeptide that was present intracellularly in a soluble form did not bind to uninfected (or infected) membranes. Therefore, the problem of nonspecific binding can be a moot one. 2. Inadequate fractionation of membranes. Our data may represent averages of different populations of morphologically similar membranes which have different polypeptide compositions. One would have to devise other methods for membrane fractionation in order to eliminate this possibility. Related to this problem is the fact that an unknown, presumably large, proportion of our membranes are probably "inside-out." Since membrane surfaces have asymmetric compositions and structures (Bretscher, 1971), this may complicate, but should not invalidate, the interpretation of our data. Finally, band 3 has been variably contaminated with rough membranes (or membranes lined with the 27 nm particles). We do not feel this to be significant because normal smooth and rough microsomes have similar polypeptide compositions (Schnaitman, 1969; Kiehn and Holland, 1970; Fleischer and Fleischer, 1970; Hinman and Phillip, 1970; Ernster et al, 1962). Furthermore, similar results were obtained with band 5 and even with total crude membranes, both of which are morphologically quite heterogeneous, indicating that the various membranes behaved similarly regardless of their heterogeneity. In support of the last, it has been shown in influenza-infected cells that all the various membranous fractions had very similar polypeptide compositions (Holland and Kiehn, 1970). However, we cannot totally exclude the possibility that NV-5 and NV-4 are predominantly components of the 27-nm particles.

Finally, and quite apart from considerations about membrane-binding, it should be noted that two trypsin-derived polypeptide fragments have molecular weights similar to two naturally occurring polypeptides. The relationships, if any, between the two groups of polypeptides remain to be determined.

C. Japanese Encephalitis Virus: Irreversible Inhibition of the Synthesis of Non-glycosylated, Non-structural Proteins by Puromycin

It was shown in the previous Annual Report (1971) that Japanese encephalitis (JE) virus-infected chick embryo cells that were treated with actinomycin D and a pulse of cycloheximide synthesized seven virus specified polypeptides: NV-5 (non-virion, MW $\approx$ 93,000), NV-4 (non-virion, MW $\approx$ 71,000), V-3 (the virion large membrane polypeptide, MW $\approx$ 53,000), NV-3 (non-virion, MW $\approx$ 45,000), NV-2 (non-virion, MW $\approx$ 19,000), V-2 (the virion nucleocapsid polypeptide, MW $\approx$ 13,500), and NV-1 (non-virion, MW $\approx$ 10,500). The virion small membrane polypeptide V-1 (MW $\approx$ 8,700) was not found in significant quantities. We now show that pulse-inhibition of JE-infected cells with high levels of puromycin irreversibly inhibits the synthesis of a group of these polypeptides; these are the non-structural, non-glycoproteins.

Materials and methods: cell culture conditions. Methods for infection of chick embryo cells with Japanese encephalitis (JE) virus in closed 32-ounce bottles or 30 ml Falcon flasks at 35.5° C were described in the previous Annual Report (1971). The medium used was M-199; glucose and amino acid concentrations were varied as described in the text. When used, actinomycin D was at a concentration of 1 µg/ml; cycloheximide was at a concentration of 300 µg/ml; and puromycin was at concentrations of 15 to 1000 µg/ml. Infection was at 0 hours; when used, actinomycin D was added at 9 hours; cycloheximide or puromycin was added at 18 hours for 30 minutes; the cells were washed ten times with Hank's or Earle's balanced salt solution and fresh medium lacking puromycin or cycloheximide but containing actinomycin D (if added previously) was added to the cells. Isotope was added at 19 hours to 23 or 24 hours.

Processing of Extracts. The cells were washed three times with saline and dissolved in 1% sodium lauryl sulfate (SLS) in 0.01 M. phosphate buffer, pH 7.2. The material was heated at 100° C for 5 minutes and then divided into two aliquots: the first aliquot, heated at 100° C for an additional 5 minutes, was used for assaying both protein concentration by the method of Lowry, et al (1951) and incorporation of acid-insoluble radioactivity. The second aliquot was made 1% in 2-mercaptoethanol (2-ME) and then heated at 100° C for 5 minutes and either used directly for polyacrylamide gel electrophoresis or dialyzed against 0.01 M phosphate containing 0.1% SLS.

When assaying acid insoluble radioactivity, a portion of the first aliquot was suspended in cold 10% trichloroacetic acid (TCA) and left at 4° C for at least 30 minutes. The material was filtered through glass fiber filters and washed three times with 10% TCA and three times with acetone. When glycoprotein incorporation was assayed, the filter was washed three times with 2:1 (V/V) chloroform-methanol prior to the acetone wash. The filters were dried and counted in toluene-Liquifluor (New England Nuclear) as previously described (Annual Report, 1971).

Polyacrylamide gel electrophoresis. Methods for continuous polyacrylamide gel electrophoresis (PAGE), with 8% gels containing 0.1% SLS in phosphate buffer, have also been described (previous Annual Report). When samples were co-electrophoresed, and both were not whole cell extracts, they were mixed either before or after dissociation with SLS and 2-ME; no differences were apparent with either method. Whole cell extracts were mixed after dissociation. When gel slices were counted, the counts were background subtracted and corrected for spillover from ^{14}C to ^{3}H. When undialyzed material was electrophoresed, the end of the gel was sliced off (at the middle of the dye front) and the gel was placed in at least two changes of 10% TCA for at least 20 hours. The slicer for the TCA-soaked gels was modified to contain phospho-bronze blades instead of stainless steel razor blades and needed periodic treatment with a bronze cleaner.

Radioimmune precipitation. Preparation of NP-40 (Shell Chemical, New York) treated cell extracts and their use in radioimmune precipitation (RIP) assays have been described (previous Annual Report).

Harvesting of virus. Methods for radioactively labeling, harvesting, rate-zonally purifying and electrophoresing "normal"-form and "Tris"-form virions from chick embryo cells and from LLC-MK_2 cells were defined and described in the previous Annual Report and in Section V-A, above. Intracellular virus (I-form) was prepared from chick embryo cells as described for the isolation of virus from mouse brain (Brandt et al, 1970) except protamine sulfate was used at a final concentration of 2 mg/ml for clarification. After rate zonal centrifugation, ^{3}H-glucosamine labeled I-form virions were purified further by isopycnic centrifugation through a 20-70% sucrose-D_2O gradient; they banded at a density of about 1.24 g/ml.

Isotopes. Isotopes included: ^{3}H or ^{14}C-L-amino acid mixture (New England Nuclear, Boston, Mass.); D-glucosamine-6-^{3}H (from New England Nuclear or Amersham/Searle, Arlington Heights, Illinois) and D-galactose-1-^{3}H (Amersham/Searle).

Results: Effect of inhibitors of protein synthesis. When chick embryo cells were infected with JE protein synthesis decreased (line 1, Table 38). When the chick cells were treated with actinomycin D, the infected cells synthesized more protein relative to uninfected cells, than when the drug was absent (line 2, Table 38), indicating preferential inhibition of host-protein synthesis. However, when extracts of actinomycin D-treated infected and uninfected cells were electrophoresed we could not confidently distinguish viral from host proteins: infected and uninfected cells contained similar quantities of radioactively-labeled proteins (Fig. 26-A) and some viral and host proteins differed only slightly in mobility (Fig. 27). By contrast, when actinomycin D-treated cells were pulse-inhibited with cycloheximide (line 4, Table 38), infected cells synthesized more protein relative to uninfected cells, than when treated with actinomycin D alone. When extracts of

Table 38 - Effects of Drug Treatment

Treatment	Exp 1[a]	Exp 2[a]	Exp 3[a]
1. *None*	2962[b] / 4521	2744[b] / 3411	7458[b] / 8376
2. Actinomycin D	858 / 778	1337 / 1312	2458 / 2362
3. Cycloheximide pulse	2895 / 3400		1962 / 2992
4. Actinomycin D + cycloheximide pulse	698 / 427		1155 / 633
5. Cycloheximide-continuous	ND / 61		
6. Puromycin pulse - 150 µg/ml		1083 / 2832	
7. Actinomycin D+ puromycin pulse (15 µg/ml)		786 / 1166	1561 / 2089
8. Actinomycin D + puromycin pulse (112 µg/ml)			1097 / ND
9. Actinomycin D + puromycin pulse (150 µg/ml)		390 / 940	
10. Actinomycin D + puromycin pulse (225 µg/ml)			580 / 1424
11. Actinomycin D + puromycin pulse (450 µg/ml)			376 / ND
12. Actinomycin D + puromycin pulse (1000 µg/ml)			197 / 300
13. Puromycin-continuous - 15 µg/ml		ND / 233	
14. Puromycin-continuous - 150 µg/ml		ND / 32	

[a] Specific activity = $\frac{\text{cpm} \times 10^{-3}}{\text{mgm protein}}$

[b] infected / uninfected

ND - Not determined

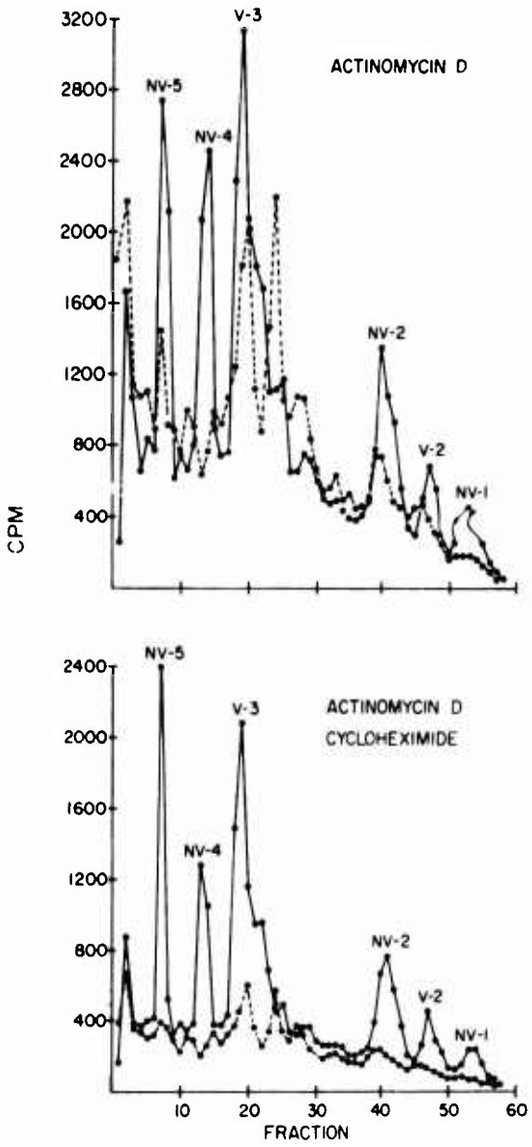

Figure 26. Polyacrylamide gel electrophoresis of actinomycin D (1 ug/ml) treated JEV-infected and uninfected cells (top), and of cycloheximide pulse-inhibited (300 ug/ml) actinomycin D treated (1 ug/ml) infected and uninfected cells. All cells were labeled with ^{14}C amino acids and electrophoresed separately.

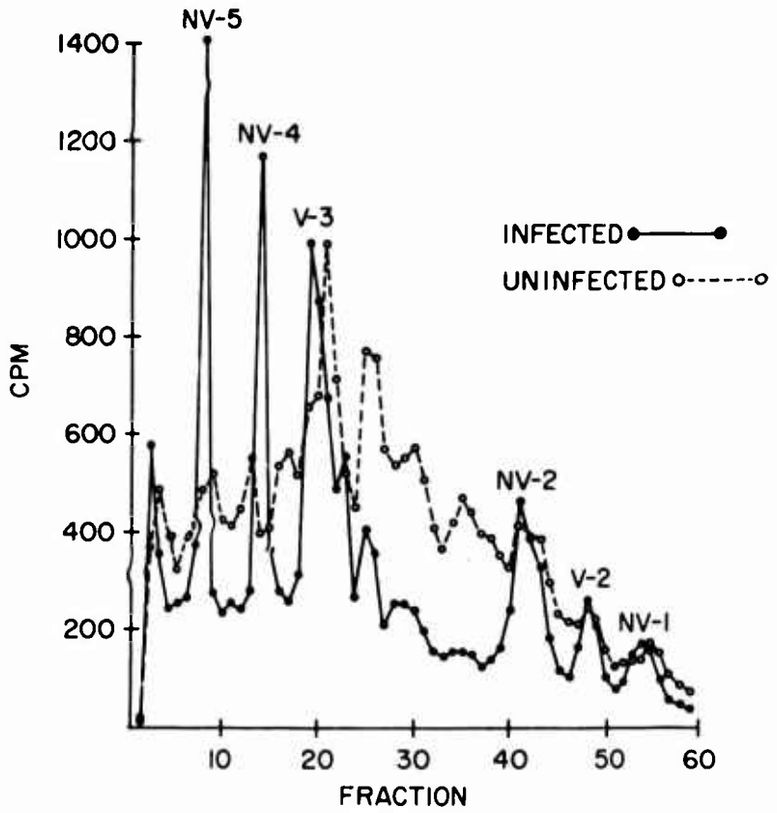

Figure 27. Polyacrylamide gel co-electrophoresis of Japanese encephalitis virus-infected (^{14}C-amino acids) and uninfected (^{3}H amino acids) chick embryo cells treated only with actinomycin D.

such pulse-inhibited cells were electrophoresed, we could clearly distinguish the proteins of infected cells from those of uninfected cells (Fig. 26-B). Qualitatively, however, the gel pattern from infected, actinomycin D-treated cells pulse-inhibited with cycloheximide, was identical to that from infected cells treated only with actinomycin D (Figs. 26-A and 26-B). In the absence of actinomycin D we could not reproducibly determine whether cycloheximide pulse-inhibition differentially affected host or viral protein synthesis.

We obtained different results with puromycin. Infected, actinomycin D-treated cells which were pulse-inhibited with high concentrations of puromycin synthesized less protein relative to uninfected cells than when treated with actinomycin D alone (Table 38). This inhibition of protein synthesis in infected cells resulted from a specific dose-dependent qualitative change in the viral-specified proteins synthesized after puromycin pulse-inhibition (Fig. 28). With relatively low levels of puromycin (15 μg/ml), which can effectively inhibit protein synthesis when continuously present (line 13, Table 38), we did not find any alteration after pulse-inhibition (Fig. 28). When higher concentrations of puromycin were used for pulse-inhibition (112 - 225 μg/ml) we found that the synthesis of three polypeptides was irreversibly inhibited relative to that of the others. Those irreversibly inhibited polypeptides were the nonvirion (NV) polypeptides NV-5, NV-4 and NV-1, whereas the relatively unhibited ones were V-3, NV-3 (to be shown below), NV-2 and V-2. With even higher concentrations of puromycin, the synthesis of the relatively uninhibited group became progressively inhibited, apparently in order of decreasing molecular weight (Fig. 28).

The gel pattern from uninfected cells treated only with actinomycin D was identical to that from uninfected cells treated with actinomycin D and pulse-inhibited with puromycin (225 μg/ml) (not shown). In the absence of actinomycin D, puromycin pulse-inhibited (225 μg/ml) uninfected cells generally had a gel pattern similar to that of untreated, uninfected cells; occasionally we noted a slight decrease in the synthesis of high molecular weight polypeptides, significantly less than that occurring in infected cells (not shown). When infected cells were pulse-inhibited with puromycin (150-225 μg/ml) in the absence of actinomycin D, the irreversible suppression of NV-5, NV-4 and NV-1 was equivalent to that occurring in actinomycin D-treated infected cells similarly pulse-inhibited with puromycin (not shown); however, in the absence of actinomycin D, the differentiation of viral from host proteins within infected cells was not unambiguous. When infected, actinomycin D-treated cells were pulse-inhibited with progressively higher concentrations of puromycin (Fig. 28), the background of presumably host proteins became more prominent. These results indicated that puromycin (150-225 μg/ml) specifically irreversibly inhibited the synthesis of the viral-specified polypeptides NV-5, NV-4 and NV-1 relative to the synthesis of both the other viral-specified polypeptides and host-specified proteins.

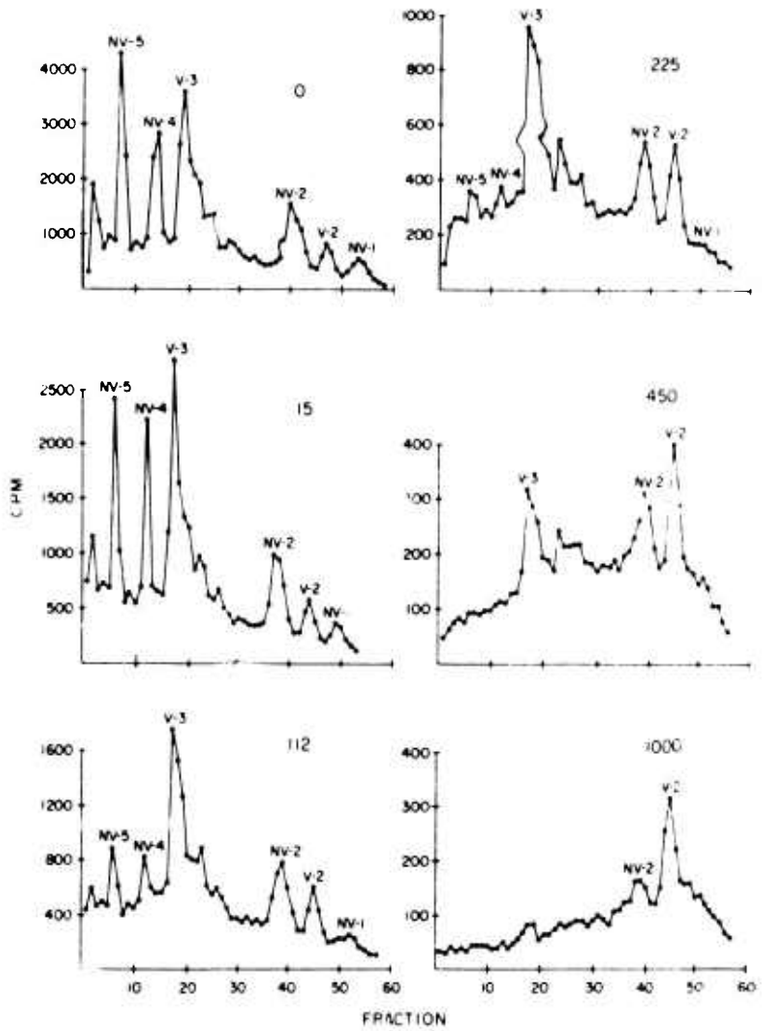

Figure 28. Polyacrylamide gel electrophoresis of actinomycin D treated Japanese encephalitis virus-infected chick embryo cells pulse-inhibited with increasing levels of puromycin. Specific activities calculated from weighing the graphs were 1.0, 0.62, 0.49, 0.31, 0.16 and 0.09.

Structural glycoproteins. In an effort to find if there were functional differences between the puromycin "resistant" and "sensitive" groups of proteins, we determined which ones were glycoproteins. We have shown that there are three forms of JE virions (see V-A, above): 1) the N-form, from infected LLC-MK2 or chick cells incubated in "normal" media, containing V-3 and V-1 as membrane polypeptides and V-2 as the nucleocapsid polypeptide; 2) the T-form, from infected LLC-MK_2 cells incubated in media containing tris(hydroxymethyl)aminomethane (Tris), which has NV-2 as a membrane polypeptide instead of V-1; and 3) the I-form, the intracellular form from infected chick embryo cells, which is similar to the T-form but is heterogeneous with respect to the amount of V-2 contained.

To determine the glycoproteins of N-form virions, ^3H-galactose was used as the label; it was incorporated into V-3, but not V-2 or V-1 (Fig. 29). We obtained similar results with radioactive glucosamine. Therefore, V-3 is a glycoprotein whereas V-2 and V-1 are not, a result consistent with earlier work by Stollar (1969) on dengue-2 virus.

T-form virions from LLC-MK_2 cells incorporated glucosamine into V-3 and NV-2, but the distribution of the carbohydrate NV-2 label was skewed slightly to the left of the amino acid label (Fig. 30). Therefore, at least one component of NV-2 is a glycoprotein (see below).

Glucosamine-labeled I-form virions from chick cells contained three peaks of radioactivity: one co-migrated with V-3 and another co-migrated with NV-2 (Fig. 31). The third peak migrated slowly, was present in variable proportions in different rate-zonally purified preparations, was decreased after isopycnic centrifugation of I-form virions, and was present in heterogeneously sedimenting material from uninfected cells, most of which sedimented more slowly than I-form virions (Fig. 31). Therefore, the third radioactive peak is a host glycoprotein contaminant. We concluded that V-3 and NV-2 are structural glycoproteins; we have previously shown (for dengue-2 virus) that they are components of the viral membrane (Section V-A, above).

Radioimmune precipitation of intracellular glycoproteins. We have previously shown that at least the three polypeptides, V-3, NV-3 and NV-2, were specifically precipitated from solubilized infected cell extracts by hyperimmune mouse ascitic fluid (1971 Annual Report). When similarly prepared radio-immune precipitates were obtained from extracts of infected, actinomycin D-treated cells labeled with ^{14}C-glucosamine and ^3H-amino acids, all three polypeptides contained glucosamine, indicating that they all are viral-specified glycoproteins (Fig. 32); the significance of the small amount of glycoprotein at fraction 30 was unclear. We concluded that NV-3 is a non-structural glycoprotein. Interestingly, under these conditions, these three glycoproteins (V-3, NV-3 and NV-2) were the principal components of JE-specific antigens.

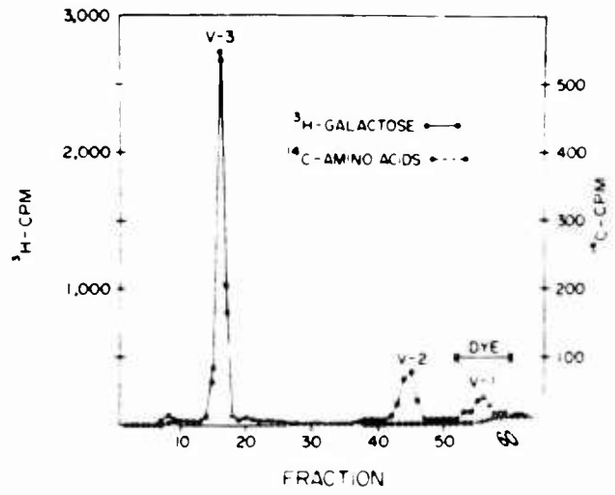

Figure 29. Polyacrylamide gel co-electrophoresis of JE virions propagated in chick embryo cells under "normal" medium 199. These are the N form virions where only the major membrane polypeptide (V-3) is a glycoprotein.

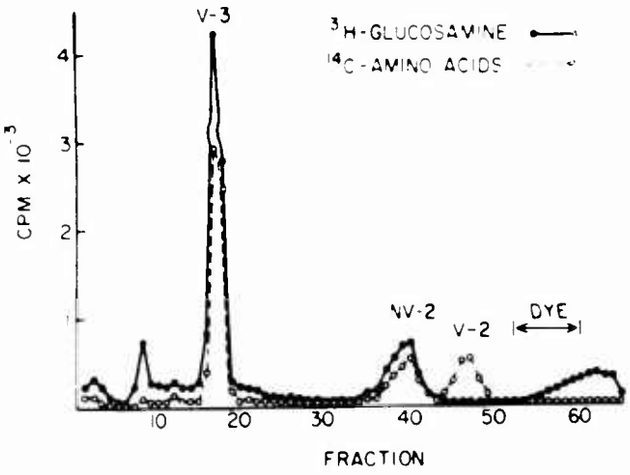

Figure 30. Polyacrylamide gel co-electrophoresis of JE virions propagated in LLC-MK2 cells under medium containing Tris buffer. These are the T-form virions that contain 2 glycoproteins (V-3 and NV-2).

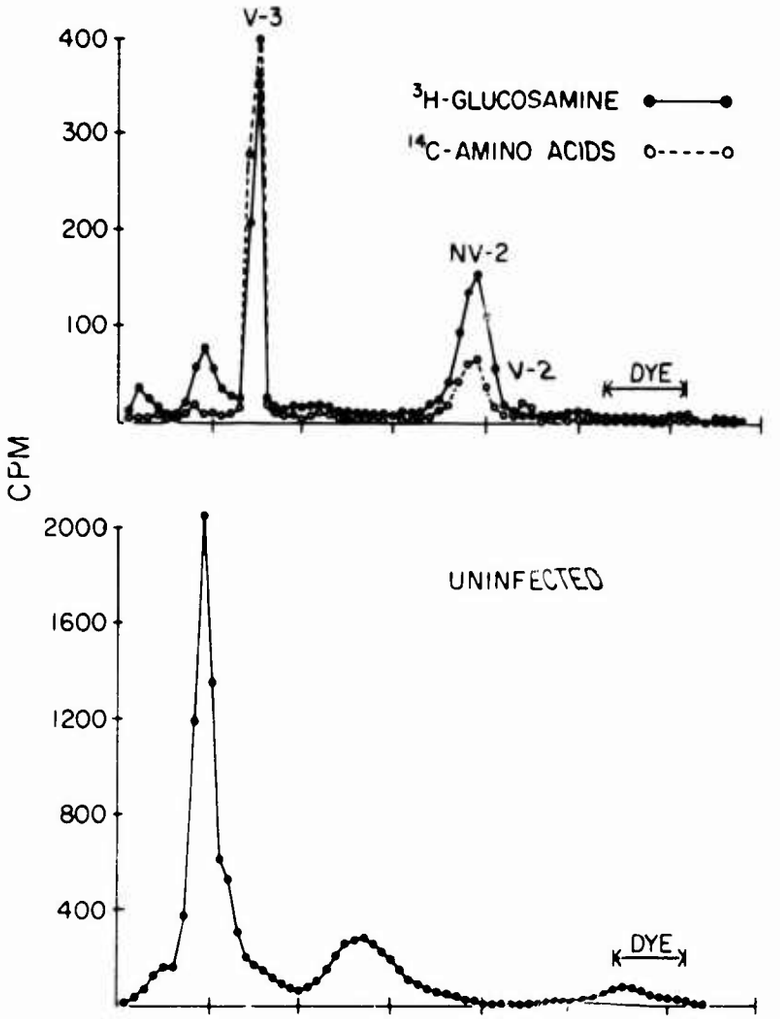

Figure 31. Polyacrylamide gel co-electrophoresis of SLS-degraded intracellular virions from Japanese encephalitis virus-infected chick embryo cells (top panel). These are the I-form virions that contain very low amounts of V-2, the nucleocapsid polypeptide. The bottom panel represents electrophoresis of SLS-degraded intracellular structures from uninfected cells that sediment slightly slower than virions.

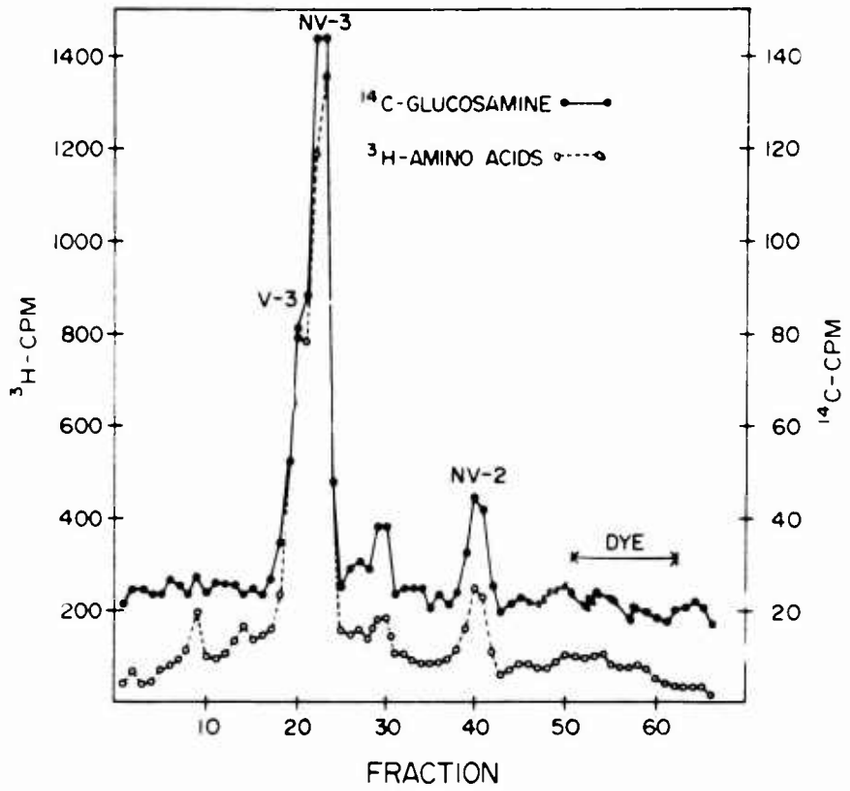

Figure 32. Polyacrylamide gel co-electrophoresis of SLS-degraded radioimmune precipitate from Japanese encephalitis virus-infected, actinomycin D treated chick embryo cells. About 15% of infected cell proteins (glycoproteins) precipitate while no precipitate is obtained from uninfected cells with Japanese encephalitis virus antibody.

Glycoproteins in whole cell extracts. Glycoproteins that were not antigenic under the conditions used for radioimmune precipitation would not have been identified. Therefore we prepared whole cell extracts from infected, ^{3}H-glucosamine-labeled, actinomycin D-treated cells and co-electrophoresed them with ^{14}C-amino acid labeled "marker" proteins through 6, 8, 10 and 12% gels (Fig. 33). The resulting gel patterns were complex: 1)Glucosamine was incorporated into a peak of radioactivity that almost always co-migrated with V-3 at the four gel concentrations used. We concluded that the glucosamine and amino acid labels were incorporated into the same polypeptide, V-3. 2) Glucosamine was incorporated into another peak that probably corresponded to NV-3; however, because the resolution of NV-3 from V-3 was incomplete, we could not be certain that the two labels co-migrated. 3) Glucosamine was incorporated into a third peak that always migrated slightly ahead of amino-acid labeled NV-2 regardless of whether the latter was from actinomycin D-treated cells or from actinomycin D-treated cells also pulse-inhibited with cycloheximide. Therefore, this glycoprotein was distinguishable from amino-acid labeled "NV-2". Because a third component of "NV-2" exists (see below), we have referred to the slowly migrating, apparently nonglycosylated component as S-NV-2 and the more rapidly migrating glycoprotein as M-NV-2 (middle component of NV-2). These two components of NV-2, S-NV-2 and M-NV-2 may be completely unrelated polypeptides. 4) Glucosamine was incorporated into a peak that co-migrated with NV-1 in the 8% gel in this experiment, but in other experiments and at different gel concentrations was clearly separable from NV-1, in a manner indicating that it is a small, relatively uncharged, molecule (Bretscher, 1971). Furthermore, it was not present in every experiment. We concluded that it is not a JE-specified glycoprotein. 5) Glucosamine was incorporated into two or three peaks that migrated in the NV-5 to V-3 region of gels. In the 6% and 8% gels, these peaks did not co-migrate with NV-5 or NV-4. Furthermore, similar peaks were present in glucosamine-labeled uninfected cells (Figs. 34, 31) and in amino acid-labeled uninfected cells (Fig. 27). We concluded that they are not JE-specified glycoproteins.

We obtained similar results with radioactive mannose and fucose (not shown). In summary, V-3, NV-3 and a component of NV-2 we designated as M-NV-2 are JE-specified glycoproteins, whereas NV-5, NV-4 and NV-1 are non-structural, non-glycoproteins.

The "NV-2" in I-form virions. Intracellular "NV-2" was heterogeneous, but I-form "NV-2" apparently consisted of a homogeneous glycoprotein (Fig. 31). We therefore examined the mobility of I-form "NV-2" more closely. Unexpectedly, it was electrophoretically distinct from both M-NV-2 and S-NV-2: it migrated slightly faster than both (Fig. 35). We designated the I-form NV-2 as F-NV-2 (fast component). Therefore, there are at least three populations of molecules with mobilities that are "NV-2-like": a "slow" non-glycoprotein, a "middle" glycoprotein, and a "fast" structural glycoprotein which is ordinarily not detectable in glucosamine-labeled whole cell extracts; we have no direct evidence

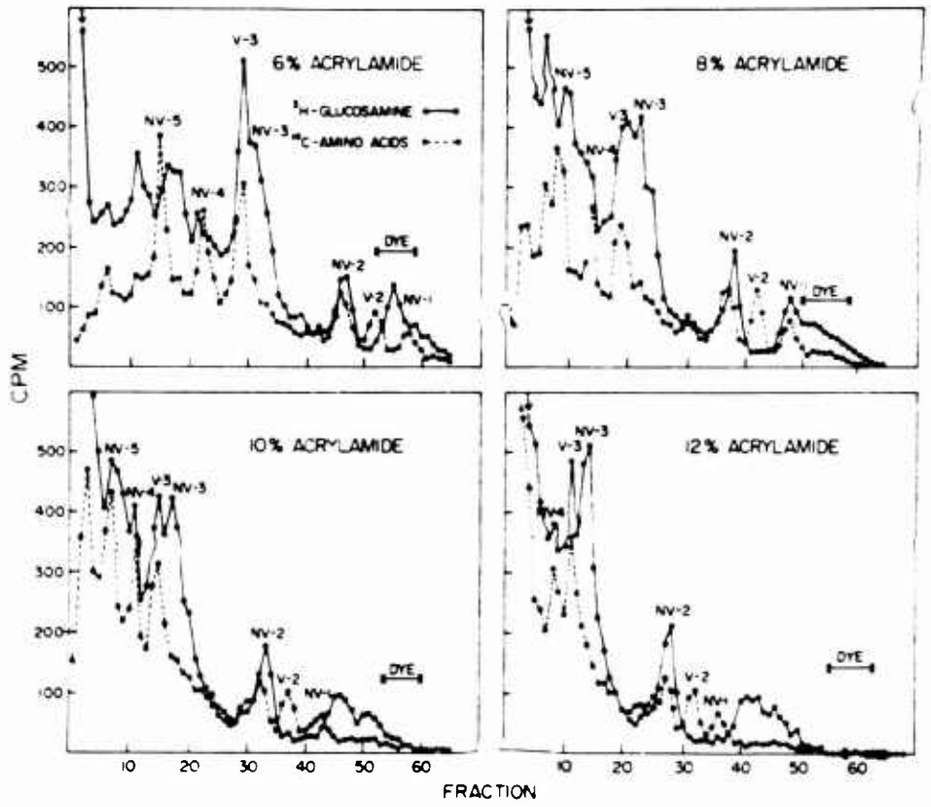

Figure 33. Analysis of glycoproteins in whole cell extracts. Infected cells treated with actinomycin D were labeled with ^{3}H-glucosamine from 14-22 hours, and infected cells pulse-inhibited with cycloheximide were labeled with ^{14}C-amino acids from 20 to 21 hours. The monolayers were dissolved in sodium lauryl sulfate and 2-mercaptoethanol, mixed, and coelectrophoresed through polyacrylamide gels.

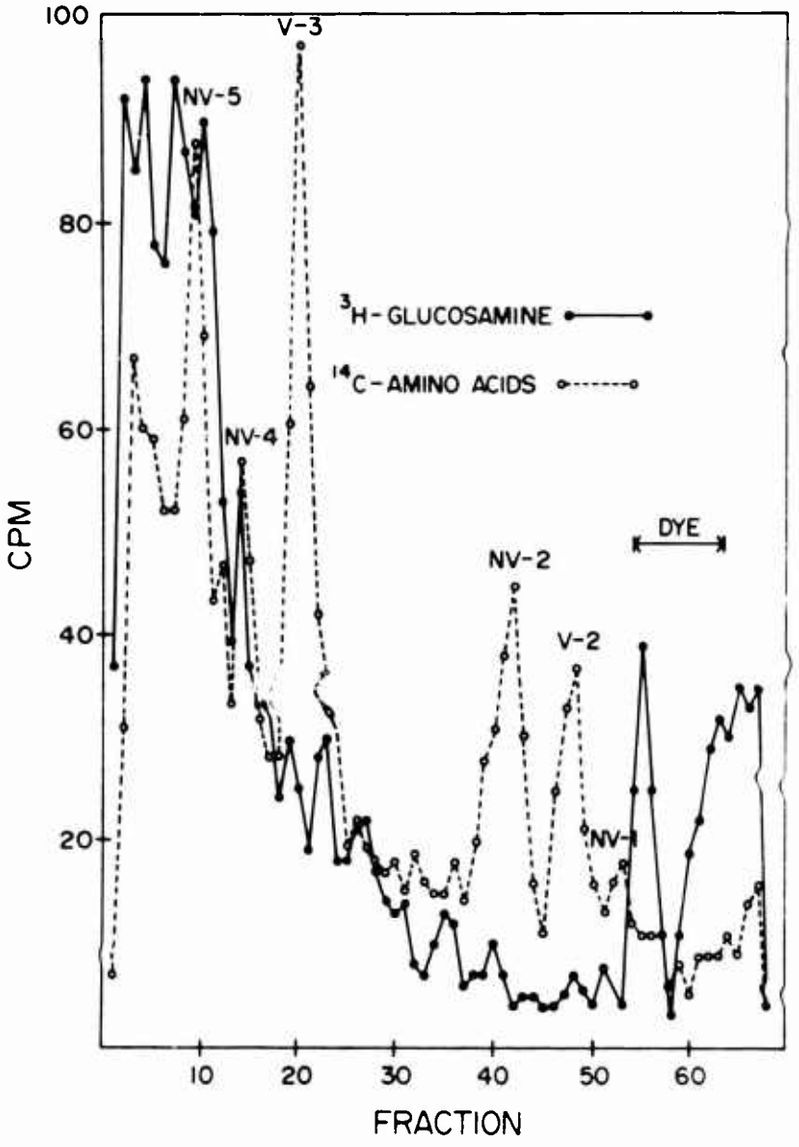

Figure 34. Polyacrylamide gel co-electrophoresis of ^{3}H-glucosamine labeled uninfected cells and ^{14}C-amino acid labeled JE-infected cells.

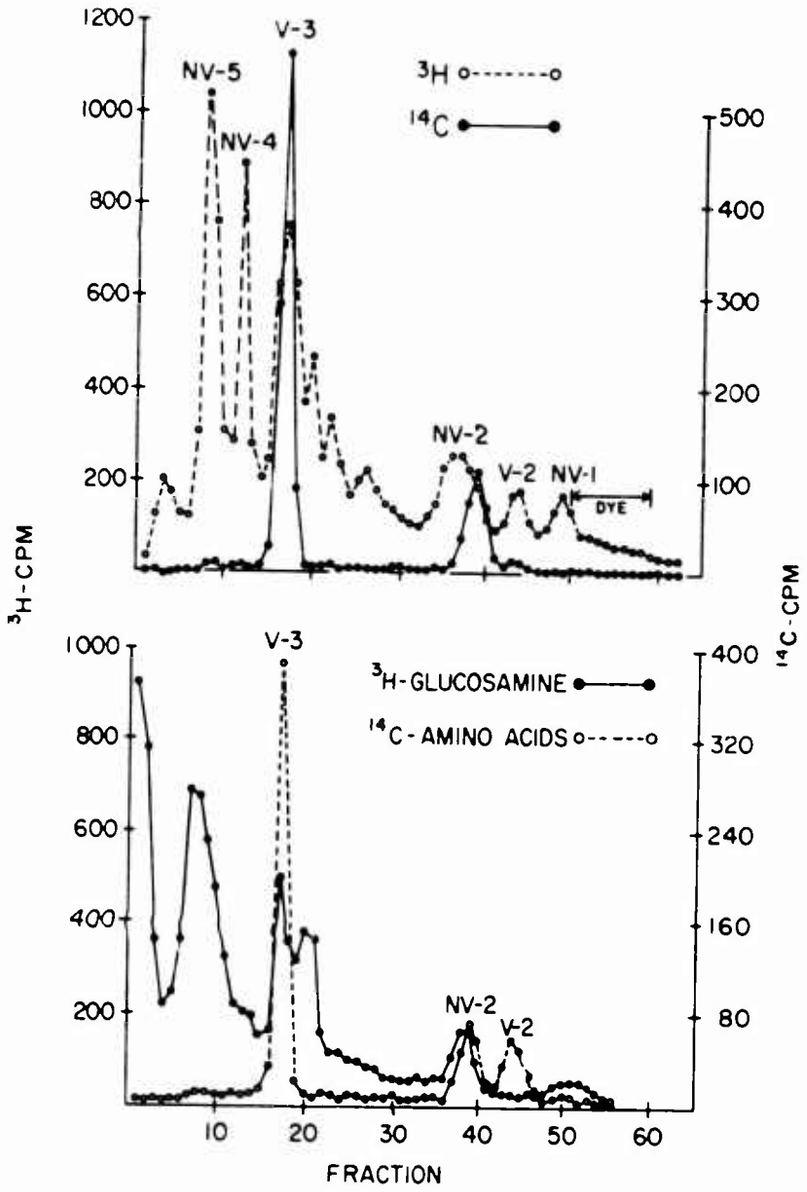

Figure **35**. Polyacrylamide gel co-electrophoresis of I-form virions (^{14}C-amino acids) with ^{3}H-intracellular proteins (top panel) and ^{3}H intracellular glycoproteins (bottom panel).

implicating any relationship among them. The "NV-2-like" glycoprotein present in T-form virions cannot be directly related to any of these components since it was derived from different infected cells (LLC-MK_2 cells).

Effect of puromycin on glucosamine incorporation. We considered if puromycin pulse-inhibition of actinomycin D-treated infected chick cells altered the mobility of "NV-2": at levels up to 150 μg/ml we detected no change in mobility; at higher levels we did not obtain consistent results and therefore could not make definite conclusions. When we studied the effect of puromycin pulse inhibition (225 μg/ml) on subsequent glucosamine incorporation (for four hours) by infected, actinomycin D-treated chick cells we found that glucosamine incorporation decreased less than did amino acid incorporation in parallel cultures; by contrast, with cycloheximide pulse-inhibition, the decrease in glucosamine incorporation equaled the decrease in amino acid incorporation (not shown). Puromycin pulse-inhibition (225 μg/ml) did not qualitatively alter the glycoprotein gel pattern from that obtained from infected cells treated only with actinomycin D; this was the best evidence that NV-3 is "puromycin-resistant", as is M-NV-2. These two polypeptides, along with the other two "puromycin-resistant" polypeptides, V-3 and V-2, comprise the detectable intracellular complement of either structural proteins or glycoproteins; the "puromycin-sensitive" polypeptides (NV-5, NV-4 and NV-1) were all non-glycosylated, non-structural proteins. We could not determine the response of S-NV-2 or T-NV-2 to puromycin.

Discussion. Puromycin (150-225 μg/ml) irreversibly inhibited the synthesis of nonglycosylated, non-structural proteins. Because comparable concentrations of cycloheximide did not, the action of puromycin was specific and not a general property of any inhibitor of protein synthesis. Puromycin could have been active at either a transcriptive or translational level. In view of the facts that: 1) puromycin interacts with ribosomes, and 2) the use of high concentrations of puromycin and other reagents can distinguish two classes of normal mammalian polyribosomes (Rosback and Penman, 1971 a & b), we propose that viral-specified, non-glycosylated, non-structural proteins are translated in a manner that differs from that of viral-specified glycosylated or structural proteins (and host proteins); this difference may result from the existence of two classes of virus-directed polyribosomes. If so, as a corollary, multiple species of m-RNA are likely to exist as is the case for vesicular stomatitis virus (Huang, 1970; Mudd, 1970; Schincariol, 1970). We have previously found that two of the non-structural, non-glycoproteins (NV-5 and NV-4) are the most superficially bound membrane-bound proteins (Section V-B, above). It appears possible, therefore, that the cell discriminates between non-glycosylated, non-structural proteins and the other proteins with respect to both origin and destination.

We have shown the unexpected existence of three polypeptides with

mobilities similar to "NV-2": a slowly migrating non-glycoprotein (S-NV-2), an intermediately-migrating glycoprotein (M-NV-2) and a more rapidly migrating structural glycoprotein that is not readily detected in glucosamine-labeled cell extracts (F-NV-2). The electrophoretic mobility differences were small, and we therefore recognize the need for confirmatory results using higher resolution electrophoretic methods (Neville, 1971). We have not presented any evidence about a relationship between any of these polypeptides. However, it is possible that a relationship exists, at least between the two glycoproteins. We have previously presented evidence that suggested that F-NV-2 is probably the precursor to V-1, the small N-form virion membrane non-glycoprotein (submitted for publication). Since F-NV-2 is apparently synthesized in small amounts, its synthesis is either tightly controlled (and rate-limiting) or else it is formed from a larger glycoprotein precursor. M-NV-2 may therefore be an uncleaved precursor of F-NV-2, since it appears to be a larger glycoprotein that is snythesized in amounts comparable to the synthesis of the two other structural polypeptides, V-3 and V-2.

Recently, a theory has been proposed that the carbohydrate moieties of glycoproteins serve as codes that determine their topographical processing (Winterburn and Phelps, 1972). If M-NV-2 is the precursor to F-NV-2, which, in turn is the precursor to V-1, then our results could indicate that different stages of viral morphogenesis are attended by modifications of at least one glycoprotein, which would be in accord with that theory, and which may occur both with myxoviruses (Lazarowitz et al, 1971) and group A arboviruses (Schlesinger et al, 197-).

D. Japanese Encephalitis Virus (JEV) Subgroup Antigens

1. JEV membrane associated antigens. Japanese encephalitis (JE) virus-infected cells contain several complement fixing (CF) antigens which can be separated physically. This is also the case for Dengue-2 infected cells where virion CF activity can be separated and characterized separately from soluble CF (SCF) activity (Cardiff et al, 1971). We found that antigens of JE subgroup viruses may be extracted from infected cell membranes using detergent or salt treatment; detergents such as NP-40 and sodium lauryl sulfate (SLS) can be used to solubilize membrane-bound antigens, and another technique used is extraction with 3M KCl.

First, Japanese encephalitis, Murray Valley encephalitis (MVE), St. Louis encephalitis (SLE), West Nile encephalitis (WNE), and Ilheus (Ilh) viruses were used to infect BHK-21 roller flask cell cultures at an MOI of 10. After 30 hr incubation, the cells were scraped from the walls of the roller bottle into phosphate buffered saline and washed. The cell pellet was resuspended in reticulocyte standard buffer (RSB) and the cells allowed to swell 10 min at room temperature before Dounce homogenizing. After separation of nuclei by

centrifugation at 1000 g for 2 min, the supernatant material was removed and layered on a biphasic gradient of 2.5 M and 0.25 M sucrose. After centrifugation for 1.5 hr at 100,000 g, the membranes were recovered from the sucrose pad and NP-40 (0.5% final concentration), or SLS (0.1% final concentration) was added. Membranes extracted with NP-40 were shaken for 18 hr at 36C; SLS extracted membranes were shaken for 1 hr at 36; and KCl extracted membranes were shaken for 18 hr at 4 C. After a final centrifugation of 100,000 g for 2 hr, the supernatant was assayed for CF activity and chromatographed on a Sephadex G-100 or G-200 column.

Salt or detergent treatment appeared to unmask or liberate antigen which is bound to cell membranes as seen by the increase in CF activity (Table 39). Supernatant culture fluids from infected cell culture roller bottles showed no increase in CF activity.

Table 39 - Complement-Fixing Activity after Treatment of JE Virus-Infected Cell Membranes and Supernatant Culture Fluids with Salt or Detergent

	Treatment	CF Titer
Membranes	Control (RSB)*	64
	NP-40	512
Membranes	Control (RSB)	64
	SLS	128
Membranes	Control (RSB)	8
	3M KCl	32
Supernatant culture fluid	Control	256
	NP-40	128

* Membranes extracted with reticulocyte standard buffer (RSB)

Gel filtration chromatography of soluble antigens derived from cell membranes by detergent treatment resulted in CF activity eluting at several different molecular weights. In addition to the void CF, an antigen with a molecular weight of 45-60 x 10^3 was eluted as well as a larger antigen with a molecular weight of 80-105 x 10^3 (Figs. 36-A, B, C & D). Salt extraction of membranes with 3M KCl resulted in an antigen that eluted in the void when chromatographed on Sehadex G-100 or G-200. Attempts to reduce aggregation by using high salt concentrations or detergent in the buffer eluant did not produce an antigen that could be resolved by gel filtration on Sephadex.

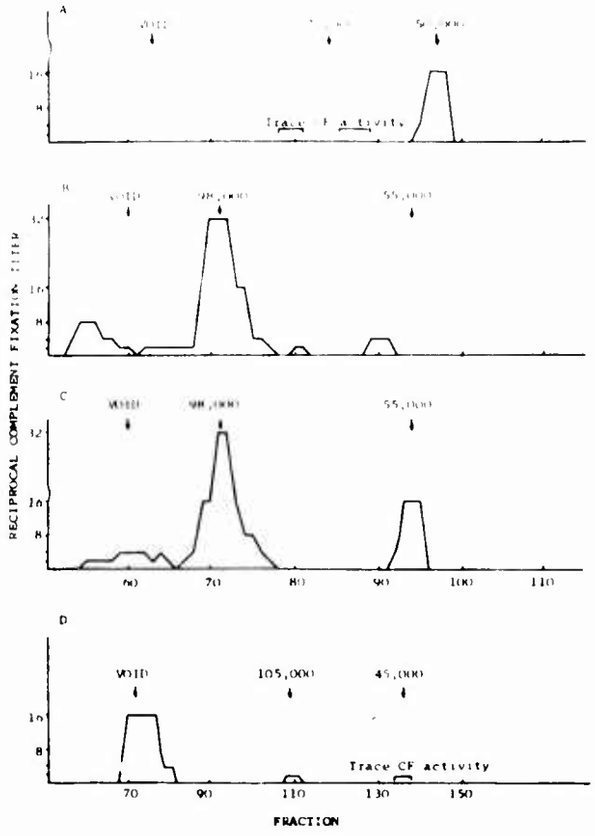

Figure 36. Sephadex gel filtration of detergent degraded group B arbovirus-infected cell membranes. A. Murray Valley encephalitis (G-100; sodium lauryl sulfate); B. Saint Louis encephalitis (G-100; sodium lauryl sulfate); C. Saint Louis encephalitis (G-100; NP-40); D. Japanese encephalitis (G-200; NP-40).

Shapiro (1971) has characterized the intracellular and virion proteins of JE virus finding molecular weights of 53, 45, 77 and 93 x 10^3 for V-3, NV-3, NV-4 and NV-5 respectively. This would be the molecular weight range of the antigens extracted from cell membranes with detergent. It would not be surprising to find a CF antigen that corresponds to the c t protein (53 x 10^3) of JE virus associated with cell membranes. However a CF antigen in the heavier 80-105 x 10^3 molecular weight class would not correspond to any structural virion protein and could possibly be similar to the non-structural Dengue SCF antigen. Complete solubilization of cell membranes and concomitant separation without aggregation has not as yet been possible with gel filtration on Sephadex and may account for the higher molecular weight antigens eluting from these columns. Future separations in the presence of detergent using radio-labelled antigens will help to resolve this problem.

2. Immunodiffusion analysis of JE subgroup viruses. Previous studies involving immunodiffusion reactions of arboviruses using crude mouse brain or cell culture antigens (Clark, 1962) points to the need for more pure viral antigens for these tests. Dengue SCF antigens purified by gel filtration chromatography were found to be useful for separating the four Dengue serotypes by immunodiffusion reactions (Russell et al, 1970). Use of the rapidly-sedimenting hemagglutinin (RHA) which can be prepared for each member of the JE subgroup was used in this study to determine its usefulness for studying the serological cross-reactivity of these viruses in the immunodiffusion test.

Suckling mouse brain RHA was prepared by methods previously described for Dengue virus (Brandt et al, 1970). The RHA pool was used unconcentrated or if the HA titer was low, concentrated in an Amicon cell under pressure. Before adding the RHA to the immunodiffusion plate, it was treated for 1 hr at room temperature with 0.5% NP-40. Agarose plates were made by filling 60 mm petri dishes with 7 ml of 0.6% agarose diluted in 0.02M Tris-HCl buffer, pH 8.2, and containing a final concentration of 0.1% NP-40. After a template was used to produce a hexagonal pattern of wells around a center well, 0.1 ml of appropriate antigens was placed in the peripheral wells while 0.1 ml of mouse ascitic fluid for the JE subgroup viruses was placed in the center well. Plates were incubated at room temperature for 3 –4 days then read and photographed.

Use of NP-40 for pretreatment of RHA and in the agarose reaction medium facilitates formation and clarity of precipitin lines. Double precipitin lines were produced by JE, MVE, and Ilh RHA against their homologous ascitic fluids (Figs. 37-A, B, D). The faster diffusing band appears to be group specific and has a broad cross-reacting spectrum. Only one band was found between SLE RHA and SLE ascitic fluid, and this appears to be group specific, cross reacting with other subgroup RHA's. The same held true for WNE, although a complete line

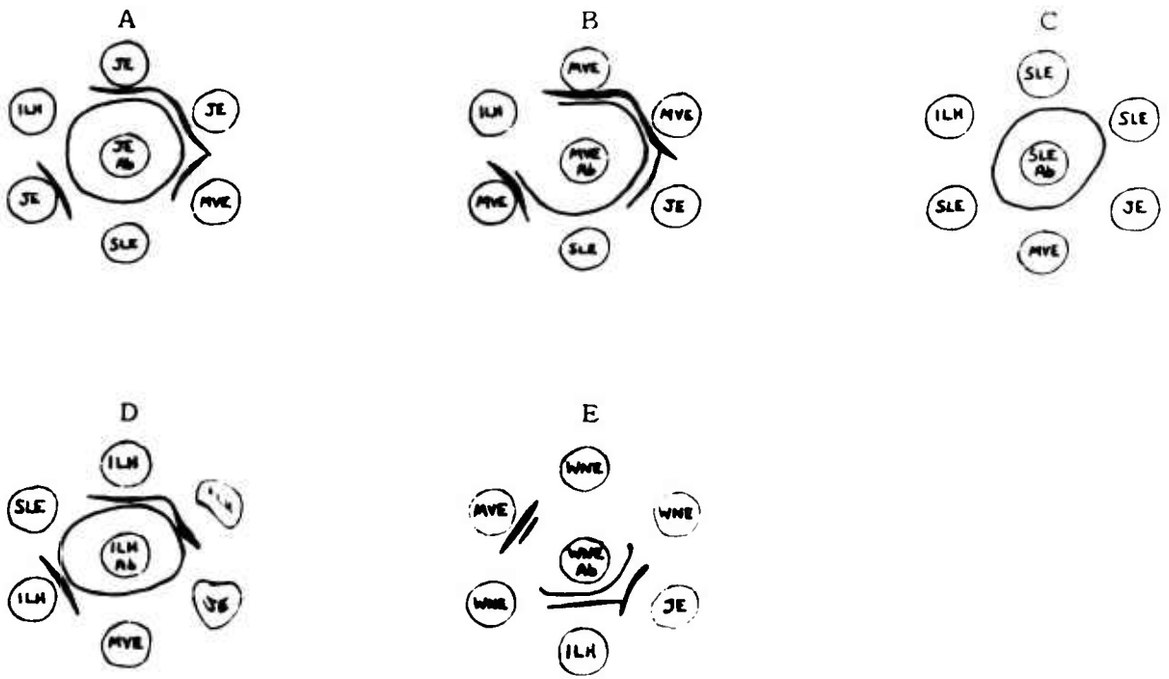

Figure 37. Immunodiffusion reactions of Japanese encephalitis (JE), Murray Valley encephalitis (MVE), Saint Louis encephalitis (SLE), West Nile encephalitis (WNE), and Ilheus (ILH) purified virions.

was absent and only a bend in the neighboring precipitin line was visible. The thickest precipitin line closest to the antigen well appears in most cases to be type specific or partially type specific. There is a partial identity reaction between JE and MVE RHA and their ascitic fluids. West Nile antibody, although not reacting with its homologous RHA, did react with JE, ILH, and MVE RHA's producing what appears to be a type specific and group specific reaction.

Each RHA except for SLE and WNE (Figs. 37-C & E) appears to contain two separately reacting precipitating antigens which are visualized after NP-40 treatment of the RHA. Detergent treatment with NP-40 probably dissociates the virion into coat and core fragments and allows more specific reactions to occur. Further characterization and separation of coat and core proteins by fore complete degradation of these fragments will make it possible to assign type and group specific determinants to specific proteins. Purification of these proteins will ultimately lead to more specific typing and characterization of the antigenic interrelationships among the group B arboviruses.

3. Solubilization of JE virus. A variety of reagents and methods have been used to solubilize viral proteins. One of the most popular methods had been treatment of virions with urea, sodium dodecyl sulfate, and mercaptoethanol (ME) and electrophoresis in a polyacrylamide gel. Although consistent separation is possible using this method, irreversible protein denaturation occurs and biological activity of the separated proteins is lost. An alternative method using guanidine hydrochloride (GuHCl) with a reducing agent to solubilize avian tumor virus proteins has been described (Fleissner, 1971). Attempts to adapt the GuHCl treatment to separate JE virus proteins will be described. Other alternative methods will also be discussed.

Japanese encephalitis virus, strain M1/311 was used to infect BHK-21 roller flasks at a multiplicity of infection of 10. After adsorption of virus for 2 hr, the inoculum was removed, the flasks washed with Hanks BSS, and 50 ml of maintenance media added. The maintenance media was Earle's BSS containing 1% human serum albumin, 1% glutamine, the normal concentration of vitamins, 1/20 concentration of amino acids, 10 mM Tris-HCl (pH 9.0), and antibiotics. After 6-8 hrs of incubation, 500 mC of H^3 amino acids or 250 mC of C^{14} amino acids were added to each inoculated flask. At 30 hr post inoculation, the supernatant culture fluids were removed and cell debris removed by centrifugation at 1000 g for 20 min. After pelleting the virus at 78,000 g for 3 hr, the pellets were resuspended in 0.02 M Tris-HCl buffer, pH 8.2, and sonicated for 3 cycles of 10 sec duration on a Branson sonicator set at maximum output. The resuspended pellets were centrifuged at 6700 g for 30 min and the supernatant fluids layered over 5 to 25% sucrose gradients and centrifuged for 2.5 hr at 25,000 rpm in the Spinco 25.1 rotor. One ml fractions were taken by draining the centrifuge tube from the bottom and

each fraction assayed for radioactivity using the appropriate settings for H^3 or C^{14} in a Picker Liquimat liquid scintillation counter. The RHA pool was pelleted at 105,000 g for 3 hr and the pellet resuspended by sonication in a denaturing solution containing 8 M GuHCl, 2% ME, 0.01 M EDTA, 0.5M Tris-HCl and 1% NP-40, pH 8.2. Denaturation was carried out at 100 C for 10 min and the solubilized RHA layered over a 1.2 x 65 cm Sepharose 4B (4% agarose) column equilibrated with 6 M GuHcl, 0.01 M dithiothreitol, and 0.02 M Na_2HPO_4, pH 6.5. After fractionation, 50 ul samples were dissolved in a toluene-Triton X-100 (2:1) cocktail containing Liquiflor and counted in a Picker Liquimat scintillation counter.

A typical chromatogram of JE RHA treated with GuHCl/ME/NP-40 is shown in Fig. 38-A. Molecular weight markers of bovine albumin, ovalbumin, and ribonuclease were used to calibrate the column, these being denatured in an identical manner to JE RHA preparations and chromatographed in GuHCl/dithiothreitol eluant. The void peak and the 62,000 and 16,000 molecular weight peaks were dialyzed for 3 changes of 250 volumes in 0.02M Tris-HCl buffer, pH 8.2. The dialyzed protein was assayed for serological activity by the radioimmune precipitation (RIP) test. When the radioactivity was high enough, the peak was subjected to polyacrylamide gel electrophoresis (PAGE) for further analysis.

Complete degradation of the JE virion was difficult to achieve even using NP-40 in the denaturant. A comparison of the use of 8M GuHCl/ME denaturant with and without NP-40 added is shown respectively in Figs. 38-B and 38-C. The void, which is the first radioactive peak eluted, contains aggregated and non-degraded viral proteins consisting mainly of coat protein. This has been confirmed by polyacrylamide gel electrophoresis (PAGE) of this material. Core protein is not found after PAGE analysis of the void peak and appears to be totally liberated by GuHCl treatment. Lipid-containing structures such as the viral envelope and cell membranes are not easily solubilized in GuHCl. Attempts to recover biological or serological activity from the isolated proteins has so far been negative. The separated proteins will auto-precipitate in the RIP test unless bovine albumin is added to keep the proteins from aggregating. However, precipitation by homologous ascitic fluid has been negative and renaturation of the proteins into an antigenically reactive form has not been achieved.

Another method that solubilizes the JE virion is treatment with 0.5% SLS. After treatment of an RHA preparation with SLS for 30 min at 36C, antigenic (CF) activity is usually not lost and chromatography on a Corning CPG 240A° column equilibrated with 0.05% SLS in PBS, pH 7.2, indicates that the lipoprotein RHA envelope is broken down to a point where the void is nearly non-existent (Fig. 38-D). Better separation is required for characterization of individual structural proteins but this can probably be achieved using longer columns or different pore size glass beads.

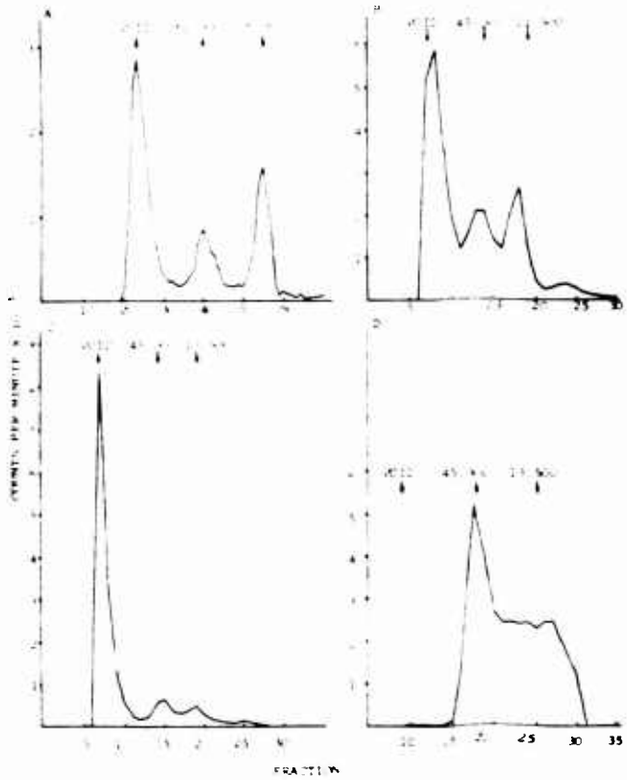

Figure 38. Chromatogram of Japanese encephalitis virions on: A. Sepharose 4B column (1.2 x 65 cm) after treatment with guanidine-HCl/2-ME/NP-40; B. Sepharose 4B column (1 x 30 cm) after treatment with guanidine-HCl/2-ME/NP-40/; C. Sepharose 4B column (1 x 30 cm) after treatment with guanidine-HCl/2-ME (no NP-40); D. Corning CPG-240°A column (0.8 x 40 cm) after treatment with sodium lauryl sulfate. Counts per minute per 50 ul in A; 500 ul in B, C, and D.

It is concluded that guanidine HCl treatment for separation of JE virion proteins offers another physical method, which depends on a molecule's physical size and shape for separation and molecular weight determination. Gel filtration in GuHCl offers the added benefit of molecular weight estimation on a fully extended peptide chain rather than depending on the variability that may exist between different proteins in their coiled and native form. Although biological activity is not recovered after treatment with GuHCl, the technique appears to have promise more as an analytical procedure which can be used in place of or in addition to PAGE. If lipid can be dissociated and viral subunits resolved by SLS treatment, then GuHCl treatment may be used as a final analytical step to check on the purity of isolated viral subunits.

E. Dengue-3 Chronic Infection of LLC-MK_2 Cells

Attempts to propagate dengue virus type-3 in cultures of LLC-MK_2 cells have yielded variable results in both the titer of infective virus released into the medium and the observed cytopathic effect. The possibility that dengue-3 virus might eventually establish chronic infection status in these cells was studied in anticipation of future investigations involving: a) recombination experiments with the dengue viruses, b) accumulation of virus antigens, structural & non-structural proteins for subsequent analysis and c) an in-detail study of the virological parameters influencing chronically infected cells.

All of these proposed studies required that the chronically infected cells be stable and consistent with respect to infectious virus release over a regular time period.

Methods. Dengue type 3 virus (J-1007/68), originally obtained from the University of the West Indies and passed five times in LLC-MK_2 cells at WRAIR, served as the infective inoculum.

Methods for the propagation and plaque assay of the dengue viruses in LLC-MK_2 cells have been extensively described in previous annual reports. Plaque assays of group A arboviruses and vesicular stomatitis virus (VSV) were performed as described for the dengue viruses with the notable exception that plaque assay cultures were stained 48 - 72 hours after infection. Fluorescent antibody studies were also done on various passages of the Den-3 chronically infected LLC-MK_2 cells.

Results. A single 25 cm^2 plaque flask of confluent LLC-MK_2 cells was infected with approximately 1×10^5 dengue type-3 PFU and the culture examined daily for CPE. Cytopathic effect was detected on days 7 and 8 but was only transient in nature and the infected culture could not be distinguished from control cultures by day 10. Cells were split on day 12 and neither cell growth nor morphology could distinguish these infected cultures from companion control cells treated in an identical manner. Samples were taken at regular intervals prior to

passage of the cells and examined for infectivity by plaque assay (Table 40). Virus replication was not detected until 88 hours post-infection. However, by 136 hours virus titers were greater than 10^5/ml and continued to increase slightly until the cells were split at 288 hours.

Table 40 - Replication of DEN-3 Virus in LLC-MK_2 Cells

Sample Time	Infectivity	(Log_{10} PFU/ml)
0 time - post adsorption (hrs)	2.5×10^3	3.40
20	$< 10^3$	< 3.00
40	$< 10^1$	< 1.00
88	2.0×10^3	3.30
136	2.6×10^5	5.41
184	2.0×10^5	5.30
240	3.1×10^5	5.49
288	3.8×10^5	5.58

Following the initial infection period, infected cells were split 3:1 at weekly intervals and infective virus in the supernatant monitored by at least three samples taken during each weekly pass. Infectivity titers of these samples are recorded in Table 41. Virus titers varied considerably in the early passages of these cells, reaching titers of 10^6PFU/ml on occasion (split #10) yet giving consecutive passages of very low virus titers (splits 3 - 8). After 15 - 20 cell passages, the virus titers appeared more consistent. Although most samples contained less than 10^2PFU/ml, the highest titer observed at a given passage level was almost always detected within 24 - 48 hours after the cells were split.

The pattern of fluorescent staining of the Den-3 chronically infected LLC-MK_2 cells was quite similar to that seen in LLC-MK_2 cells acutely infected with Den-2 virus. Materials and methods used have been previously described (Annual Report, 1971). Intense fluorescence, localized mainly in the perinuclear area with some cytoplasmic stippling, varied from 5 - 70% at different passage levels. It will be seen in Table 42 that similar observations were made when daily samples from a

Table 41 - Infectivity Titers and Passage History of Den-3 Chronically Infected LLC-MK$_2$ Cells

Passage No.	Sample Day 1	2	3	4	5	6	7
1	3.7	3.9			3.9		
2	3.8	5.4					
3	1.7			< 2.0		< 2.0	
4	1.9	2.2	2.6	2.5	< 1.0		
6							< 2.0
7						< 2.0	
8						4.6	
9	≤ 4.7	2.7			< 2.0		< 2.0
10	6.0	2.7					
11	< 2.0	3.0	< 1.0		< 1.0		< 2.0
12	1.9	2.3	< 1.0	< 1.0	2.6	2.3	2.0
13		2.4			< 1.0		< 1.0
14		2.7					1.7
15		1.7			< 1.0		< 1.0
16		1.6			< 1.0		< 1.0
17	2.7			2.0		4.8	
18		1.9				1.3	1.3
19		2.5			1.3		1.4
20					1.3		1.3
21	1.5					< 1.0	
22		2.5			1.2		1.8
23						1.2	
24		2.8			1.8		1.8
25					< 1.0		1.3
26	2.0			1.5		< 1.0	
27		2.4			1.0		1.1
28		1.7			1.4		< 1.0
29		2.3			< 1.0		1.1
30			1.9		< 1.0		
33		2.4			2.4		1.3
34		1.9					1.0
35		1.5			< 1.0		< 1.0
36		1.3			< 1.0		< 1.0
37		1.7			1.2		< 1.0
38		2.1			1.3		1.0
39		2.6			2.1		2.1
40		2.4			2.1		1.7
41		2.4			1.1		1.6
42		1.1			< 1.0		< 1.0
43		1.4			< 1.0		< 1.0
44		1.2			< 1.0		< 1.0
45		1.4			< 1.0		< 1.0
46					< 1.0		< 1.0
47		1.3			< 1.0		< 1.0

single passage level (fourth) were studied. Fluorescence was still detected in the chronically infected cell line at the 44th passage level.

Table 42 - Fourth Passage of Dengue-3 Infected LLC-MK_2 Cells: Percent of Total Cells

Day 1	3 - 5 %
Day 2	5 %
Day 3	10 - 15 %
Day 4	25 - 50 %
Day 5	25 - 50 %
Day 6	10 - 25 %
Day 7	5 - 10 %

An attempt to isolate interferon from 5 passage levels of the Den-3 chronically infected LLC-MK_2 cells was undertaken. The fluids from 32-oz bottles in which the chronically infected cells had grown at 37° C for 6 - 8 days were harvested and dialyzed against pH 2.0 buffer at 4° C for 5 days, changing the buffer once. The samples were then dialyzed against pH 8.4 buffer for 24 hrs., harvested and tested on normal LLC-MK_2 cells for the presence of interferon by the plaque-reduction technique; VSV was used as the challenge agent. No interferon was detected in the five samples as determined by their ability to reduce approximately 50 plaque-forming units of VSV.

We were not able to prepare CF and HA antigens from the Den-3 chronically infected LLC-MK_2 cells. Finally, cells chronically infected with dengue-3 virus supported the replication of eastern equine encephalitis (EEE) virus as well as normal cells, as determined by similar EEE virus yields. However, attempts to plaque EEE on the dengue-3 infected cells failed. This discrepancy is not disturbing since the study of EEE replication utilized high concentrations of virus to infect the cells, (10^{-1} dilution of EEE seed material) and the attempt to plaque EEE was carried out with very low concentrations of virus (initial 10^{-5} dilution of the seed material); infection of non-fluorescing cells in the D-3 chronically infected cell line (5 - 50%) may therefore have been greatly enhanced in the replication study where high concentrations of virus

were used. Alternatively, a larger amount of EEE virus may have been required to overcome some "intrinsic" interference conferred on the LLC-MK_2 cells by chronic infection with dengue-3, or to overcome low (undetectable) levels of interferon, or a combination of these and other factors.

F. Cell Surface Antigens Specified by Arboviruses.

Numerous electron microscopic studies have indicated that group A arboviruses undergo final assembly at the surface of the plasma membrane by budding through it. Group B arboviruses, on the other hand, appear to undergo maturation within cytoplasmic vesicles within the infected cell, and are then released from the cell by egestion or reverse pinocytosis. Thus, there might be found group A, but not group B arbovirus specified antigens on the cell surface. Fluorescent antibody studies have tended to confirm this concept: the previous annual report (1971) showed that group A arbovirus infected cells (Sindbis) exhibited intense fluorescence in the cytoplasm and on the plasma membrane, while group B arbovirus infected cells (dengue and Japanese encephalitis) exhibited discrete stained foci in the cytoplasm, extending to, but not involving, the plasma membrane. In fact, at the time of first virus release, dengue infected cells fluoresce primarily in the perinuclear zone, Because the presence or absence of cell surface antigens would affect our considerations of the immune response of the host in group B infections, we carried out additional experiments to test this concept gained from a morphological approach.

One of the most recent methods for examining the surface of cells for viral specified antigens is immune cytolysis. Cells either before or after infection are allowed to absorb radioactive chromium, and at the time they are selected for examination, they are washed and treated with viral antibody and complement. If viral antigen is present on the cell surface, a complement dependent reaction between the antibody and the viral antigen results in cellular injury and the radioactive chromium leaks out. Initially, the method of Brier, et al was used; rabbit serum served as a source of complement, and phosphate buffered saline as a diluent (Brier et al, 1971). The following report shows that this method resulted in release of chromium from Sindbis-infected cells, but not dengue-infected cells. However, when the test system was modified by using guinea pig serum as a source of complement and triethanolamine buffered saline as a diluent, there appeared to be dengue specified antigen(s) on the cell surface. but only after two viral generation times had elapsed.

The method of Brier et al (1971) was adapted to 30 ml Falcon flasks and LLC-MK_2 cells. About 1 x 10^6 infected cells were incubated at 36C with 1 to 2 microCuries of radioactive chromium ($Na_2Cr^{51}O_4$) in 0.5 ml medium 199 for 90 minutes. The cell monolayers were then washed 5 times with 0.02 M phosphate buffered saline (PBS), and reincubated

with 0.2 ml indicated dilutions of viral antibody (in hyperimmune mouse ascitic fluid) and 0.2 ml 1:2 dilution of fresh rabbit serum as the source of complement. Controls consisted of antiviral antibody with heat-inactivated (56C x 30 min) complement, normal ascitic fluid with complement, and diluent alone. An additional control of anti-LLC-MK_2 cell antibody (immune ascitic fluid) and complement on infected or uninfected cells was occasionally included. After 1 hour, antibody-complement mixtures were aspirated from the cells, clarified by centrifugation, and assayed for radioactivity (counted) in a Baird-Atomic spectrometer. The cells were dissolved in 1 percent sodium lauryl sulfate (1-2 ml) and also counted; percent release was calculated by dividing counts in the supernate by counts in the supernate plus counts in the cells and multiplying by 100. Radioactive chromium was added the previous evening to uninfected cells when experiments were designed to determine how soon viral specified antigens appeared on the cell surface. The radioactive cells were then infected and replicate monolayers were taken for immune cytolysis at the indicated intervals.

Table 43 contains percent chromium release data for LLC-MK_2 cells before and after infection with Sindbis (18 hours) and dengue-2 (48 hours) viruses. Uninfected cells exhibited a background release of chromium between 6 and 7 percent; the same background was observed in the presence of Sindbis antiserum. Sindbis infected cells had a higher level of background release (12%), perhaps due to active release of virus, but chromium release was increased considerably in the presence of Sindbis antiserum (20%). This release was dependent on the presence of complement and was similar to that obtained in the presence of anti-LLC-MK_2 cell antibody. The lower half of this table shows that there is also an increased background of released chromium from dengue infected cells. However, in this case, the presence of antibody and complement did not increase the amount of chromium released from the cell. The experiments were repeated several times with 64 to 256 CF units of antibody with the same results. It was concluded that dengue-specified cell surface antigens could not be detected with the Brier et al method of immune cytolysis.

The immune cytolysis system was then tested and modified to obtain its maximum sensitivity in this laboratory. Since the phosphate ion has an inhibiting effect on complement, triethanolamine (0.02 M) buffered saline containing the optimum quantities of calcium (1.5×10^{-4}M) and magnesium (5×10^{-4}M) for complement-fixation tests was substituted for PBS. This buffer produced a considerable increase in the percent of chromium released from Sindbis infected cells (Figure 39). Dilutions of Sindbis hyperimmune mouse ascitic fluid as high as 1:320 (about 2 CF units) resulted in as much as 50% release of chromium. One CF unit of this antibody preparation (1:512) effected about half the chromium release between maximum and background; this would suggest that the sensitivity of immune cytolysis and complement fixation is similar. The complement-fixation, plaque reduction neutralization and radioimmune precipitation titers of this immune ascitic fluid are indicated in the figure for comparison.

Table 43 - Release of Radioactive Chromium from Dengue-2 and Sindbis Infected LLC-MK_2 Cells by Immune Cytolysis

Infecting Virus	Antibody	Percent ^{51}Cr Release
None	None	6.4
None	Sindbis	6.6
Sindbis	None	11.9
Sindbis	Sindbis	20.0
None	Dengue-2	6.5
Dengue-2	None	10.8
Dengue-2	Dengue-2	10.1

The use of triethanolamine buffered saline (TBS) with rabbit serum as a source of complement also caused a considerable increase in chromium released from infected cells without the presence of antibody; background levels rose from a previous 10 percent to 26 percent. Even uninfected cells exhibited a background level of 20 percent chromium release. Since various lots of rabbit serum are known to produce non-specific reactions, guinea pig serum and rabbit serum were compared in the immune cytolysis system using a constant (1:8) dilution of anti-LLC-MK_2 cell antibody and TBS.

The results depicted in Figure 40 show that the rabbit and guinea pig serum are almost identical in terms of potency; very low dilutions, 1:2 to 1:4 are required for maximum release of chromium. However, the rabbit serum again caused a high background of released radioactivity, about 25%, as compared to the guinea pig serum, which produced a background of only 11 percent. We then re-examined the question of surface antigens on Sindbis and dengue-2 infected LLC-MK_2 cells using guinea pig serum and TBS, and designed the experiment to ask how soon after infection viral specified antigens could be detected. Sindbis specified cell surface antigens could not be detected by chromium release during the first 8 hours after infection, even though release of newly replicated virus begins at 6 to 9 hours (Figure 41). However, some chromium release was detected at 12 hours after infection, soon after release of virus. We obtained similar results with eastern equine encephalomyelitis virus (Figure 42), except that chromium release appears to occur simultaneously with release of virus, 6-9 hours after infection. An unexpected finding was the apparent inhibition of anti-LLC-MK_2 homogenized cell antibody to cause maximum release of chromium early in infection, followed by gradually increasing chromium release during the replication cycle. However, uninfected cells of the same

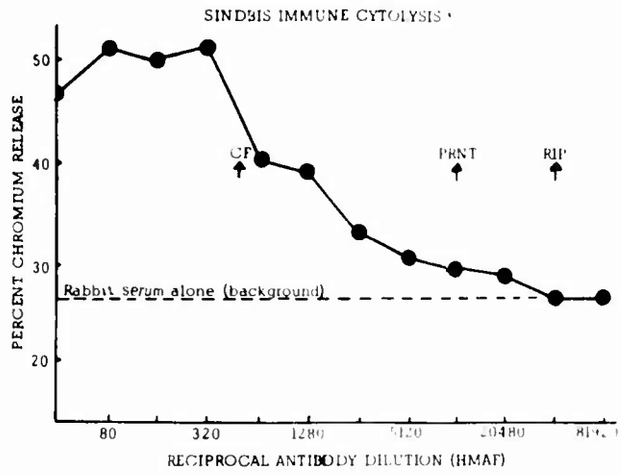

Figure 39. Radioimmune chtolysis of Sindbis-infected LLC-MK2 cells 18 hours post infection by Sindbis hyperimmune mouse ascitic fluid, rabbit complement and triethanolamine buffered saline. Titers of the immune ascitic fluid by complement fixation (CF) plaque reduction neutralization (PRNT) and radioimmune precipitation (RIP) are indicated by arrows.

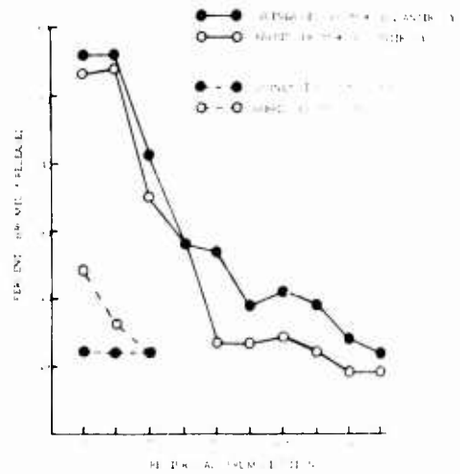

Figure 40. Effect of guinea pig and rabbit serum as a source of complement on radioimmune cytolysis. Rabbit serum alone caused *non-*specific release of chromium.

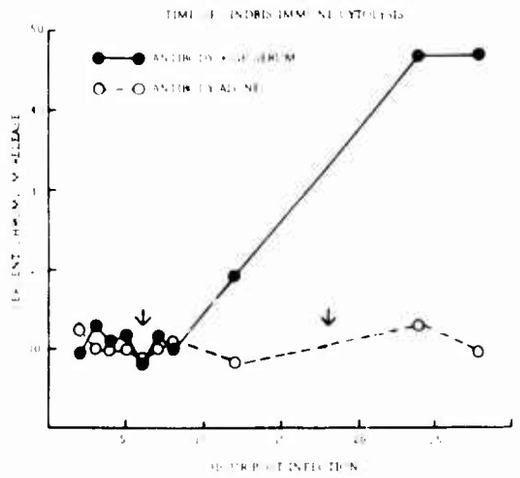

Figure 41. Appearance of Sindbis virus specified antigens on the surface of LLC-MK2 cells by radioimmune cytolysis. Arrows indicate time of first detection of new virus and time of maximum virus production.

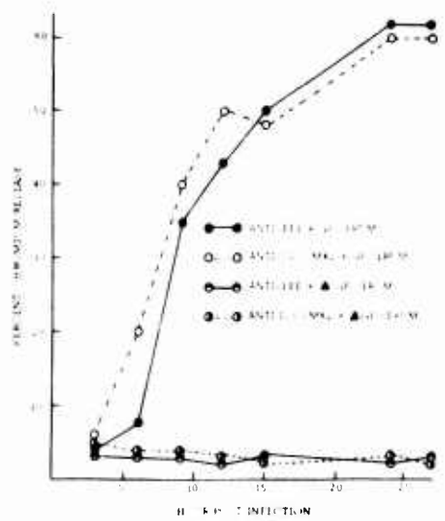

Figure 42. Appearance of Eastern equine encephalomyelitis virus specified antigens on the surface of LLC-MK2 cells by radioimmune cytolysis. Virus generation time is 6 to 7 hours. Anti-LLC-MK2 cell antibody was unexpectedly inhibited early in infection.

age after passage were not susceptible to the LLC-MK_2 antiserum either. The cells were producing acid at a faster rate than previously observed, perhaps as a consequence of increased PPLO activity. Antigenic sites on the cell may have been blocked or covered, and then uncovered only on infected cells as virus release occurred. Alternatively or in addition, exposure of internal or subsurface membranes may have occurred during virus release, making antigenic sites available to the antiserum prepared against homogenized LLC-MK_2 cells. If increased permeability of cells to antibody was another factor, it was not paralleled by increased nonspecific release of radioactive chromium. This problem will be pursued further. At any rate, immune cytolysis of EEE-infected cells is complement-dependent, does not occur with antibody prepared against dengue-2, a group B arbovirus, but does not occur until the time of virus release from the cell. Brier et al (1971) have shown that myxovirus and herpes virus specified antigens can be found on the infected cell surface at 4 to 5 hours, which would be just before virus release from the cell. Why one generation time of Sindbis virus must occur before surface antigens can be detected by substantial chromium release remains to be determined. The infected LLC-MK_2 cells were not exhibiting cytopathic effects at this time.

A re-examination of dengue-2 infected LLC-MK_2 cells using guinea pig rather than rabbit serum as a source of complement and TBS rather than PBS as a dilutent resulted in the release of chromium by immune cytolysis (Figure 43). Unlike the group A viruses Sindbis and EEE, dengue immune cytolysis occurred well into the second and third generation time (GT = 16-18 hrs), long after the first release of newly replicated virus. Dengue infected cells were not examined after the third day of infection, at which time some slight cytopathic effects were observed. The background nonspecific release was only 4 to 5 percent. An endpoint dilution curve with dengue-2 hyperimmune mouse ascitic fluid was carried out on dengue infected cells 3 days post infection. The CF titer of this immune ascitic fluid (1:256) is marked by an arrow (Figure 44) and at that dilution, about 50% of maximum possible chromium release was obtained. This again suggests that the sensitivity of complement-fixation and immune cytolysis are similar. Chromium release due to dengue antibody was complement dependent; no radioactive chromium over background levels was found in the presence of dengue antibody and heat-inactivated complement. Since dengue immune cytolysis occurs very late, the reaction might be due to viral antibody reacting with newly replicated virus emerging from the cell. However, no release of chromium occurred after saturating uninfected cells with stock seed virus, and then adding complement and antibody. The possibility that internal membranes are being exposed late in infection is considered unlikely since potent antiserum to a dengue soluble complement fixing antigen (SCF) does not react with the cell by immune cytolysis; anti-SCF has previously been shown to react only with the internal portions of the cell, including the perinuclear zone, by fluorescent antibody methods 1971 Annual Report). This would suggest that internal membranes are not exposed, and it is assumed that immune cytolysis is due to virus

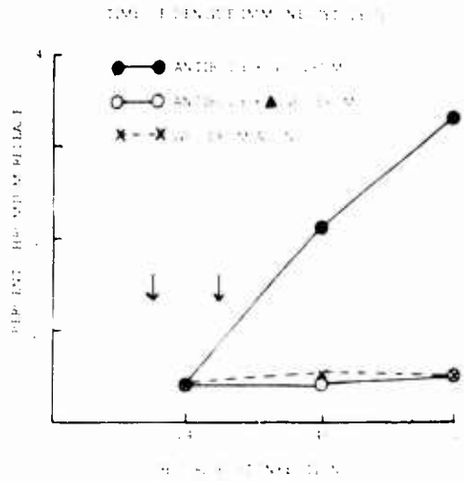

Figure 43. Appearance of dengue specified antigens on the surfa of LLC-MK2 cells by radioimmune cytolysis. Arrows indicate time o first appearance and maximum production of infectious virus.

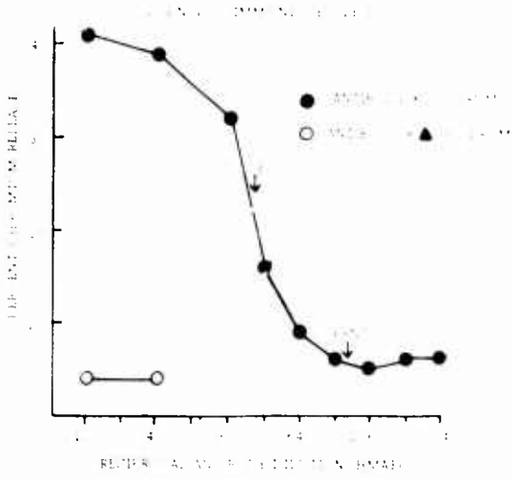

Figure 44. Potency of dengue-2 (PR-109) hyperimmune mouse ascitic fluid by radioimmune cytolysis 72 hours after infection of LLC-MK2 cells. Complement fixation (CF) and 50% plaque reduction neutralization (PRNT) titers are indicated by arrows.

specified antigens on the surface of dengue-2 infected cells. Finally, antiserum to purified virions (unlike anti-SCF) causes immune cytolysis, indicating that at least the viral membrane structural proteins are found on the cell surface. Whether the surface antigen(s) is coincidental or plays a role in the release of what appears to be (by electron microscopy) already mature virions within the cell, will also be examined. Data presented in Section V-A, above, has shown that there is a difference in the polypeptide composition between intracellular and extracellular virus; thus the plasma membrane may be involved in the final step of maturation.

G. Studies on Arbovirus Structure

1. Group C arbovirus structural proteins

The group C arbovirus, Oriboca, was propagated in cell monolayers of primary chick embryo (CEC), BHK-21 and LLC-MK_2 by the addition of a 1:5 dilution of a 20% smb suspension of virus to previously washed monolayers. After adsorption for 90 min at 36 C the inoculum was removed by washing three times with medium 199 containing 2% dialyzed fetal calf serum and 1/20 the normal level of amino acids, and then replaced with 50 ml of the same medium. After further incubation for 2 hr, ^{3}H-amino acids were added to a final concentration of 10 microcuries/ml. Virions propagated in LLC-MK_2 and BHK-21 cell cultures were harvested at 2 days post infection and virus propagated in CEC one day later, which corresponded to the appearance of detectable CPE. The radioactive infected medium was clarified by slow speed centrifugation. The virions were precipitated, resuspended, purified by rate-zonal centrifugation, reconcentrated, and analyzed by polyacrylamide gel electrophoresis, as previously described (1971 Annual Report).

When treated in this manner, Oriboca virions have sedimentation characteristics similar to those of the Bunyamwera supergroup viruses BFS-283, Bunyamwera, and Tahyna, as shown in Figure 47 of the 1971 Annual Report. Radioactive virions were obtained only from BHK-21 cell cultures. The polypeptide composition of Oriboca and Murutucu (another group C arbovirus) viruses, as determined by polyacrylamide gel electrophoresis, is shown in Figure 45. The peaks labeled 1, 3 and 4 have been observed in all Bunyamwera supergroup virions studied thus far (Bunyamwera, BFS 283, Tahyna). Peak 2 which has on occasion been observed in the Bunyamwera preparations may be either a structural viral protein or a viral specified non-structural protein which contaminates some preparation.

2. Isolation of glycopeptides from Sindbis virions

An attempt was made to develop methods and techniques to isolate and characterize arbovirus glycoproteins from Sindbis virions. Primary chick embryo cell monolayers were inoculated at an moi of 5

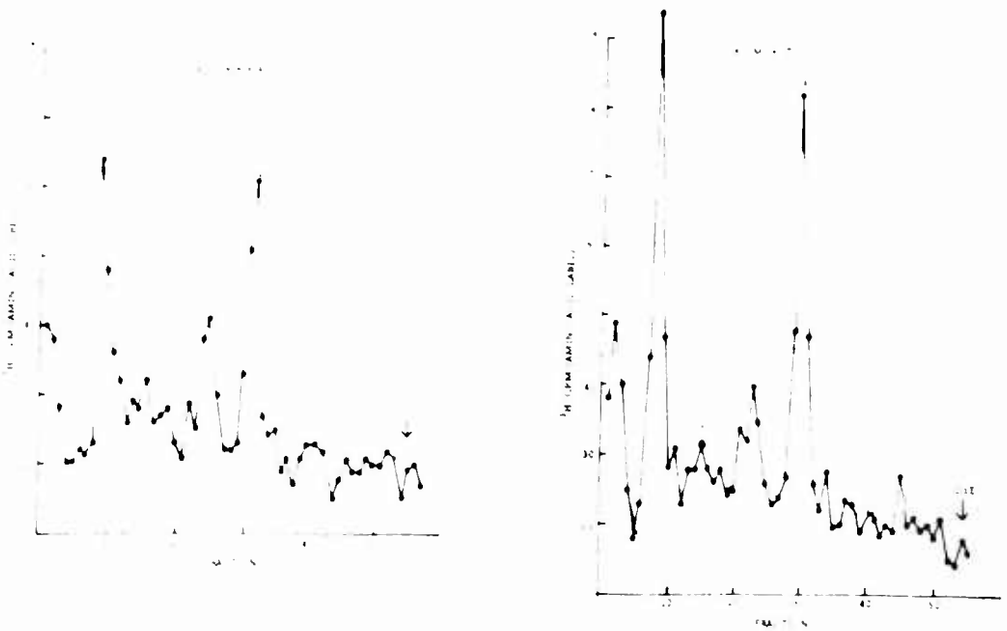

Figure 45. Polyacrylamide gel electrophoresis of SLS and 2-ME degraded Group C virions: A. Oriboca, B. Murutucu.

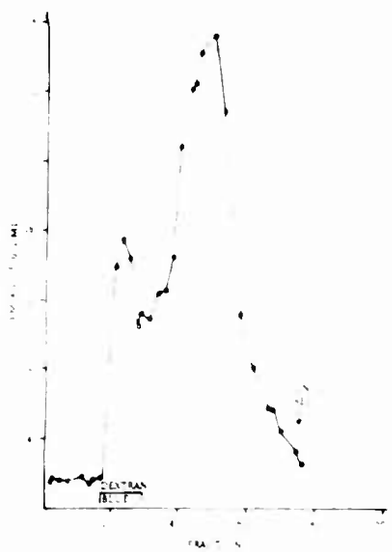

Figure 46. Elution of bromelain-released, labeled (^{3}H) virion constituents from a Biogel P-6 column.

and adsorbed for 60 - 90 min at 37° C; prior data indicated that similar levels of labelled virions were obtained with moi's of 0.001, 1, 5 50. The inoculum was removed by washing three times with medium containing 2% dialyzed fetal calf serum and antibodies, and replaced with 40 - 50 ml of the same medium. The concentrations of amino acids and/or glucose in medium 199 were adjusted depending on the radioactive substance to be incorporated. At 2 hr post adsorption either a ^{3}H amino acid mixture or ^{3}H glucosamine was added to a final concentration of 10 microcuries/ml. The virus containing medium was harvested 16 - 20 hr post adsorption, clarified by centrifugation, precipitated by ammonium sulfate, concentrated by centrifugation, resuspended in a minimal volume of TSE pH 7.2, and purified by rate-zonal centrifugation as previously described. The purified virions were determined to be free of labeled host material by examining uninfected host cells labeled with either ^{3}H glucosamine or ^{14}C-amino acids as previously described. Virions were harvested at the optimal time and both infected and uninfected culture media were clarified by one cycle of slow speed centrifugation. The virus containing supernate was divided into two portions, one of which was added to the ^{14}C labeled non-infected cell culture supernate. Both supernatants were then processed as described for the purification of virions. The results indicated that the ^{3}H labeled virions sedimented like Sindbis normally sediments in 15 - 30% sucrose (1971 Annual Report), and were found in fractions 12 - 14, whereas ^{14}C-labeled noninfected material did not significantly enter the gradient, indicating that the procedure for virion purification does not contain host cell constituents as a contaminant. The host derived material is neither trapped nor adsorbed to virions during purification when infected cell culture media is harvested early after peak titers are obtained and prior to the appearance of CPE. Incorporation of glucosamine could be increased by decreasing the glucose concentration to 1/10 the normal level while retaining the normal level of amino acids. As the level of amino acids increased, the level of labeled glucosamine decreased, presumably, by the preferential utilization of the excess amino acids rather than the glucosamine as a primary energy source. Isolation, purification and subsequent analysis of virions by polyacrylamide gel electrophoresis indicated labeling of virion coats (membrane) and cores (nucleocapsid) by ^{3}H amino acids and the specific labeling of virion coats by glucosamine.

Compans, 1971, demonstrated that the glycoprotein containing spikes of Sindbis virus, specifically labeled by glucosamine, could be removed by treatment with bromelain, a protease obtained from pineapple. Routinely, a 50 Lambda sample of either ^{3}H amino acid or ^{3}H glucosamine labeled, purified virions was treated with 150 Lambda of bromelain solution (1.3 mg/ml bromelain, in 0.1 M *Tris-HCl*, 0.001 M EDTA, 0.005 M dithiothreitol, pH 7.2) for various time intervals at 37° C and then subjected to rate-zonal centrifugation (30 - 15% linear sucrose gradient for 50 min at 50 K in 50 L rotor). Time studies (15, 30, 60 min., 48 hr.) indicated essentially complete release of incorporated glucosamine label within 60 min from the virion region of the

gradients. Prolonged reaction time (48 hr) or the addition of bromelain to increase its concentration 2-fold for 24 hr completely solubilized all incorporated glucosamine so that it remained in the top fractions. Acrylamide gel electrophoresis, as previously described, failed to resolve any reproducible peaks, indicating that bromelain may act as a nonspecific protease which produces a multitude of randomly-sized proteins from purified virions unseparable by charge. In an attempt to gain some indication of the relative size(s) of the proteins released by bromelain, a sample pool was made of bromelain released proteins, collected in top fractions from rate-zonal runs, and passed through a P-6 Biogel acrylamide column (0.9 x 20 cm elution buffer 0.1 M phosphate pH 7.8, 0.1% SLS, flow rate (3 ml/hr) from which 0.5 ml fractions were collected. Biogel P-6 (200-400 mesh) excludes molecules greater than 6000 daltons and presumably will separate the glycopeptides in the 1800, 2800, 3200 dalton range as reported by Burge and Strauss (1970) who solubilized Sindbis glycoproteins, using pronase. Separation is by molecular sieving in which dextran blue 2000 (mol. wt. 200,000) was used to define the void volume and phenol red (mol wt. 354.37) as a marker to determine the end of the run. Fig. 46 is a representative column run and closely parallels the results reported by Burge et al. The peak fractions, labeled with ^{3}H amino acids, were found in the regions of reported glycopeptides labeled with carbohydrate precursors. At the time of this run, adequate bromelain degraded virions, incorporating glucosamine, were not available; hence only labeled amino acid data is given. It is apparent that the Biogel series of polyacrylamide separating gels is usable for the separation of bromelain-released glycopeptides from purified virions, and that with the utilization of appropriate molecular weight marker standards that molecular weights will be able to be determined. Further, it seems plausible that with the advent of increased levels of specific carbohydrate precursor incorporation into virions, glycopeptides can be released by enzymatic means, separated by the Biogel column chromatography technique, and sufficient labeled material obtained to allow for antigenicity testing by radioimmune precipitation techniques.

H. Isoelectrofocusing of Group A Arboviruses

Antigenic analysis of the products of degraded group A arbovirus virions revealed marked differences in the antigenic specificity of virion components. Virion disruption with the detergent NP-40 resulted in the release of a broadly cross reactive core particle and a soluble envelope polypeptide fraction which contained both type specific and cross reactive antigens of the closely related WEE-SIN virus complex (Annual Report 1971). Further analysis of the antigens in the soluble coat polypeptide fraction was restricted by the inability of physical separation procedures to detect or resolve any components of this fraction. Isoelectricfocusing in sucrose density gradients, a technique which allows the separation of molecules with different isoelectric points, was employed in these studies in an attempt to resolve separate virus antigens in degraded virion preparations. This report

presents some preliminary attempts to separate antigens of 3 group A arboviruses (WEE, SIN & EEE) by isoelectricfocusing.

Virus preparations and immunological methods. Viruses used in these studies were propagated in primary chick embryo cell cultures, labelled with ^{3}H-amino acids, concentrated by ammonium sulfate precipitation and purified by rate zonal sucrose gradient centrifugation; virion degradation with NP-40 and sucrose gradient separation of core particles also has been described (Annual Report, 1971). The radioimmune precipitation (RIP) test was essentially the same as described previously (Annual Report 1971). Complement fixation tests were performed according to the method of Kent and Fife (1963), using hyperimmune mouse ascitic fluids (Brandt et al. 1967).

Isoelectrofocusing. Isoelectrofocusing was performed in a 110 ml capacity vertical column, jacketed for circulating coolant (LKB 8101/ column, LKB-Produkter AB, S-16125, Bromma 1, Sweden). Ampholytes (LKB) were used at a concentration of one percent in a sucrose gradient prepared by the stepwise addition of 4.6 ml mixtures of decreasing sucrose concentrations in distilled water (70% to 0%). Sulfuric acid in dense sucrose solution (70%) served as the anode buffer at the bottom of the column while ethanolamine (2% in distilled water) was used as the cathode buffer on top of the sucrose gradient. Voltage was increased stepwise in 100 volt increments over 48 hours. Voltage adjustments were made as amperage dropped below one milliamp. A maximum of 300 volts was applied to the column and a typical separation run was terminated after 96 hours. Two ml fractions were collected dropwise from the bottom of the column and the sample tubes tightly corked to prevent pH alterations due to interaction of the carrier ampholytes with the air. The pH of each sample was determined within a few hours after collection, using a Beckman Model 76 pH meter and a combination electrode. Fractions were stored at 4° C prior to analysis and when necessary, peak fractions were dialyzed against saline for removal of sucrose and carrier ampholytes.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis analysis of isoelectrofocus fractions was performed according to the methods described by Shapiro et al. (1971). Samples were heated at 100° C for 10 minutes in one percent sodium lauryl sulfate (SLS) and one percent 2-mercaptoethanol (2-ME) prior to separation on eight percent polyacrylamide gels.

Results. Initial attempts at isoelectrofocusing were primarily concerned with determining the operating conditions necessary for the establishment of a linear pH gradient. Bromphenyl blue was added to the light sucrose solution (0.05 ml of a saturated solution) as a visual marker of the focusing process. Two narrow blue bands were observed at the upper cathodal end of the column within 6-10 hours of operation, which merged into a dark blue band 0.5 cm wide. This blue band slowly migrated downward through the column over the next 48 hours, changed to

a green color, and finally completely diffused in the anode buffer coloring it a bright yellow. Although the time required for the described dye migration depended on the voltage applied, dye migration was always complete with 48-72 hours, using the stepwise voltage increases described in Materials and Methods. Using pH 3-10 carrier ampholytes at a one percent concentration, a linear pH gradient was always established following complete dye migration to the anode. Although dye migration served as a reliable marker for pH gradient linearity in early experiments, subsequent studies have indicated that proteins may not have completely migrated to their isoelectric point by this time, hence the dye marker technique was abandoned in favor of the longer running times described in Materials and Methods.

Purified radioactive labelled Sindbis virion was taken directly from the purification sucrose gradient peak and one ml focused on a pH 3-10 isoelectrofocusing run. A light band was observed forming in the upper part of the column during the early part of the run, but decreased in intensity as it migrated down the column and had virtually disappeared by the end of the run. The distribution of radioactivity in the fractions obtained from this focusing experiment is shown in Figure 47. A major peak of radioactivity was observed at pH 4.2 with a minor peak at 6.5. The peak of radioactivity was treated with sodium lauryl sulfate and 2-mercaptoethanol and examined by polyacrylamide gel electrophoresis (PAGE) in an attempt to determine which of the virion polypeptides constituted the peak. The PAGE pattern is shown in Figure 48.

The gel pattern was essentially that observed with intact Sindbis virion. The larger envelope polypeptide was observed in fraction 30. Approximately the same ratio of envelope to core existed in this preparation as has been previously reported for intact virion, suggesting that intact Sindbis virion had focused to pH 4.2 in the previous experiment.

In an attempt to obtain a more exact isoelectric point for Sindbis virus, an aliquot of the same purified Sindbis virion used in the previous experiment was run on a pH 3-6 gradient. Degraded samples were dialyzed overnight against distilled water prior to addition to the column. The distribution of virus in this system is shown in Figure 49. Sindbis virion again banded at pH 4.2 in a sharp peak, even under conditions of the more shallow pH gradient. The radioactivity observed near pH 6.0 in previous experiments was not detected on this gradient. Since virus was apparently not degraded under conditions of electrofocusing, Sindbis virus was degraded with SLS and 2-ME prior to electrofocusing on a pH 3-10 gradient. Identical treatment allowed the resolution of the envelope and core polypeptide by PAGE. In this experiment such treatment did not alter the pattern observed in previous electrofocus experiments in that a single peak at pH 4.4 was observed (Figure 50). Since SLS and 2-ME were not in the gradient other than as added with the sample, it is conceivable that reassembly (or at least

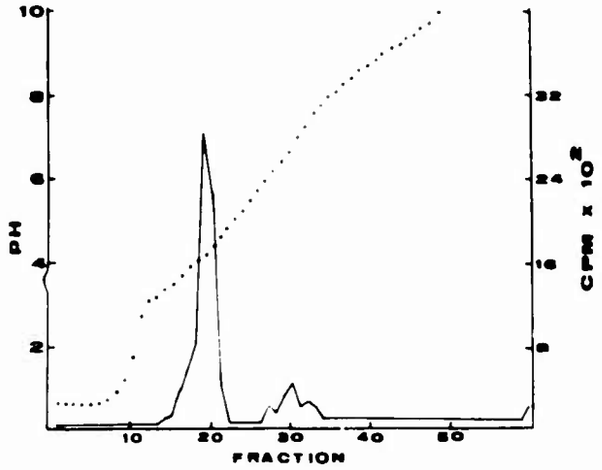

Figure 47. Isoelectrofocus of Sindbis virion on pH 3-10 gradient. Samples of 0.05 ml were counted for radioactivity.

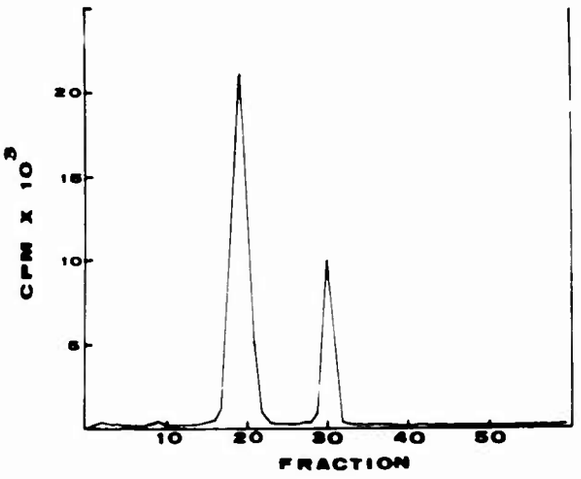

Figure 48. Polyacrylamide gel electrophoresis pattern of pH 4.2 peak from Sindbis virion isoelectrofocus experiment.

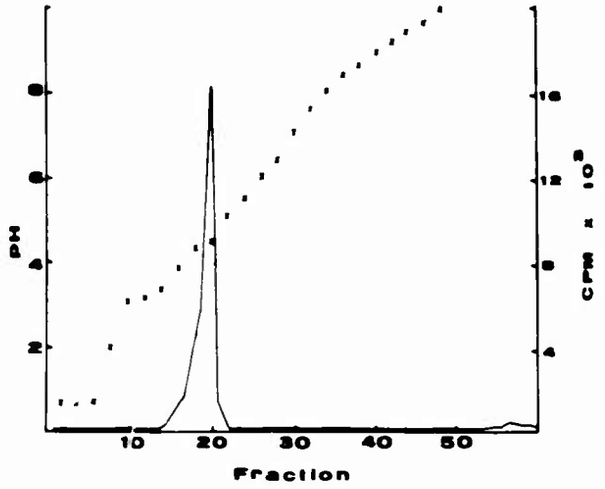

Figure 49. Isoelectrofocus of Sindbis virion on pH 3-6 gradient. Samples of 0.05 ml were counted for radioactivity.

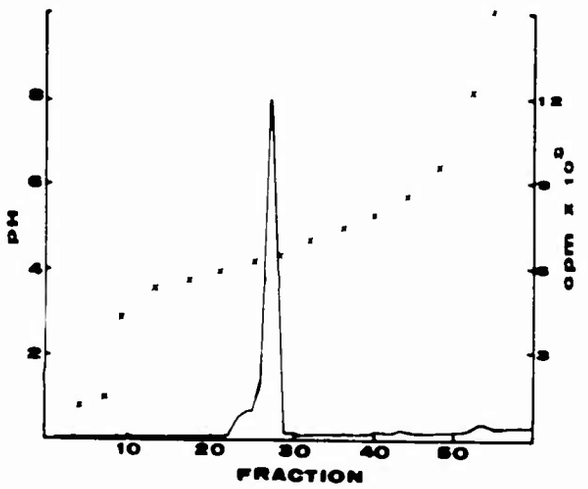

Figure 50. Isoelectrofocus of SLS-2ME degraded Sindbis virus on a Triton X-100 equilibrated pH 3-10 gradient. Samples of 0.05 ml were assayed for radioactivity.

reaggregation) of the polypeptides occurred following dilution of the detergent and the reducing agent. Experiments with added detergent in the gradient appeared impossible in this system because of the reduced solubility of SLS in the cold and the necessity for cooling during electrofocusing. In other experiments with SLS in the sample a gelatinous ring was observed soon after cooling water was applied.

The detergent Triton X-100 was investigated as a possible substitute for SLS to disrupt virus since it is non-ionic and soluble in the cold at low concentrations. It previously has been shown to release core particles and solubilize the envelope of intact virions. A Sindbis virion preparation was treated with one percent Triton X-100 for 20 minutes at 4° C and added to an isoelectrofocus gradient, pH 3-10, containing 0.1 percent Triton X-100. The radioactive protein distribution from this gradient is shown in Figure 51. Two major peaks of radioactivity were observed. A small peak was detected in the acid anode solution and another small peak observed near the load zone at pH 8.0, while the large peaks were at pH 6.1 and 9.2. Samples of each of the major peaks were treated and examined by PAGE to determine if they were of envelope or core derivation. These gels are illustrated in Figures 52 and 53. Both gels indicate the presence of only the envelope polypeptide. No core polypeptide could be detected in either sample, even though radioactivity was sufficient to allow the detection of even trace contamination.

Since the core polypeptide could not be detected in either major peak and since the minor peaks contained too little radioactivity for PAGE analysis, core particles obtained by treating intact virions with the non-ionic detergent and rate zonal sucrose gradient centrifugation (Annual Report 1971) were subsequently treated with Triton X-100 and run on a pH 3-10 gradient by procedures identical to those described for the intact virion (Figure 54). Core particles had been treated with ribonuclease to disrupt the particle and degrade any RNA which may have been bound to protein, possibly altering its isoelectric point. The majority of the radioactivity was detected in the acid portion of the gradient. Again a small peak was detected near the load zone at pH 8.0. The soluble envelope polypeptide fraction remaining on the top of the sucrose gradient following treatment of the virus with NP-40 and removal of the core particles (Annual Report, 1971) was also examined by isoelectricfocusing on a pH 3-10 gradient (Figure 55). The two major peaks were once again detected with only slightly altered isoelectric points -- pH 5.8 and 8.7. These somewhat lower pH values could be the result of a delay in measuring the pH of these fractions although linearity of the pH gradient was still maintained.

EEE and WEE virus preparations have also been examined in the Triton X-100 isoelectrofocusing system in the same manner as that described for Sindbis virus. The focus pattern of EEE virus is shown in Figure 56. Two peaks, similar to those described for Sindbis virus, were observed at pH 6.5 and 9.1. Again a peak was observed in the acid anode

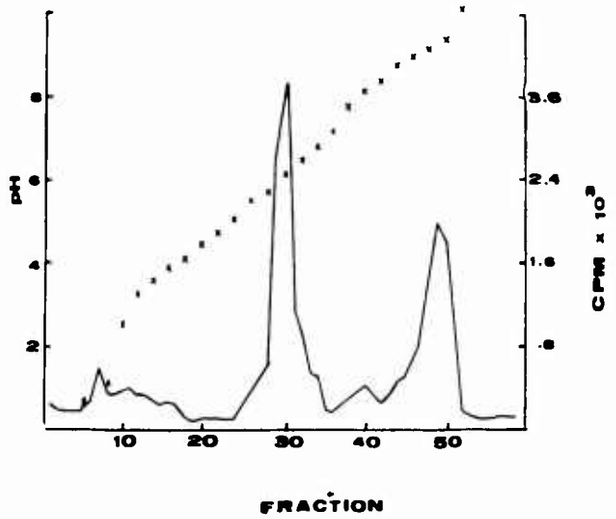

Figure 51. Isoelectrofocus of Triton X-100 degraded Sindbis virus on a Triton X-100 equilibrated pH 3-10 gradient. Samples of 0.05 ml were assayed for radioactivity.

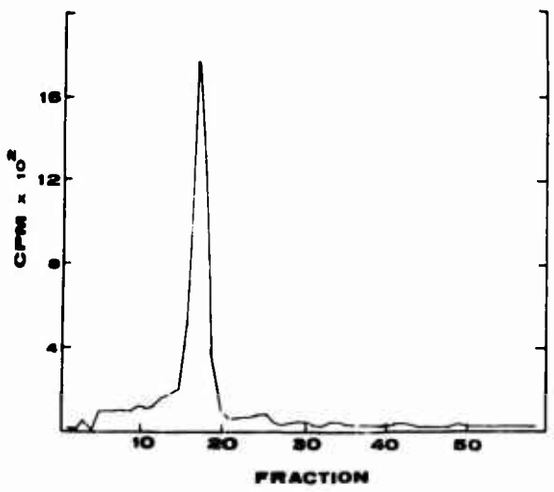

Figure 52. Polyacrylamide gel electrophoresis pattern of pH 6.1 Sindbis isoelectrofocus peak.

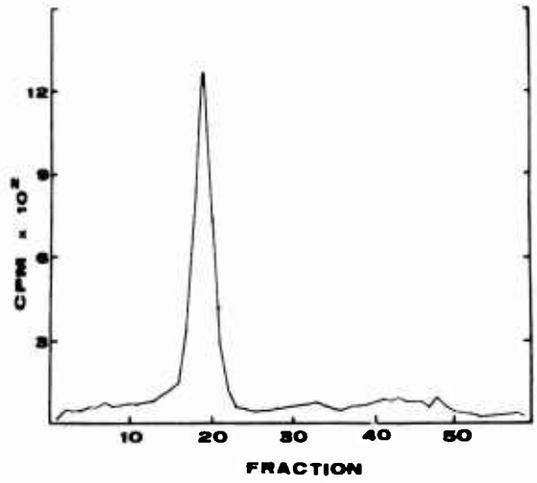

Figure 53. Polyacrylamide gel electrophoresis pattern of pH 9.2 Sindbis isoelectrofocus peak.

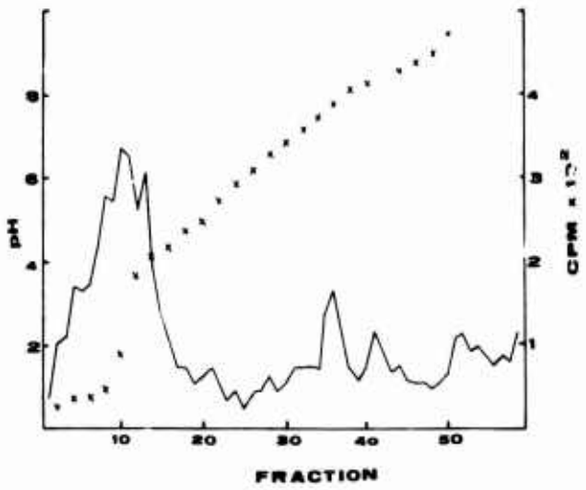

Figure 54. Isoelectrofocus of ribonuclease treated Sindbis virus core obtained by NP-40 treatment of Sindbis virion.

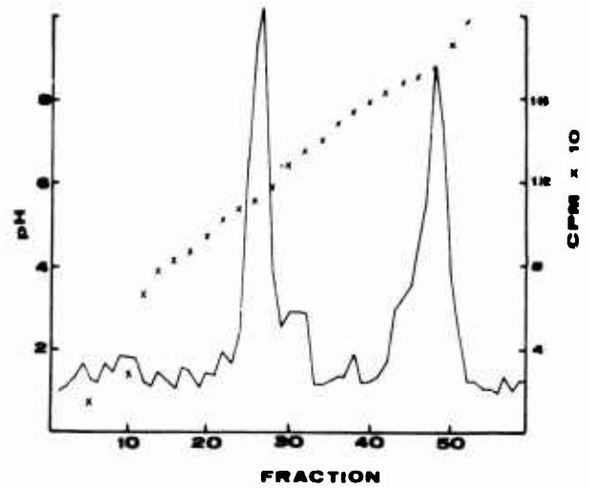

Figure 55. Isoelectrofocus of soluble envelope fraction of Sindbis virus obtained by NP-40 treatment of Sindbis virion.

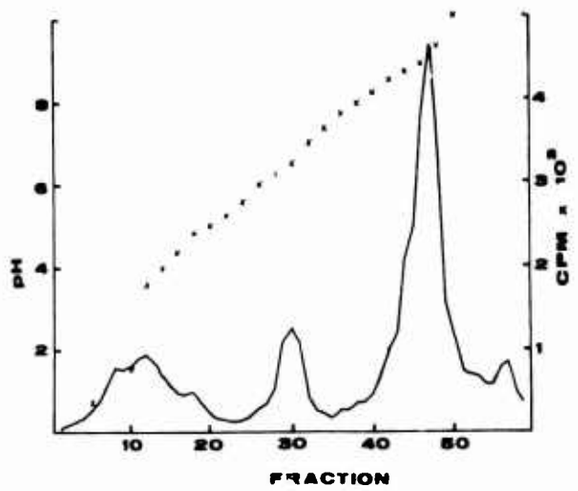

Figure 56. Isoelectrofocus of Eastern equine encephalomyelitis virion on a pH 3-10, Triton X-100 containing gradient. Samples of 0.05 ml were assayed for radioactivity.

region of the gradient and with this virus also exhibited a small peak at pH 11.5. In contrast to the profile observed with Sindbis virus, the peak at pH 6.5 was much lower than that observed at pH 9.1. To date, attempts to focus WEE virus have been unsuccessful in that no sharp peaks can be resolved reproducibly under the conditions described as optimal for SIN and EEE viruses. An added complication in the isoelectrofocus experiments with WEE virus is the low specific activity of purified virion preparations, making resolution of minor peaks difficult.

The two major peaks resolved by isoelectric analysis of Sindbis and EEE virus were further investigated as prospective virus specific antigens. Hemagglutination testing of the pH gradient fractions revealed a peak of hemagglutinin which coincided with the lower peak of radioactivity -- pI 6.1. Since samples in this experiment contained carrier ampholytes which could interfere with the hemagglutination test, aliquots of each peak were dialyzed and allowed to react with goose erythrocytes adjusted to pH 5.8, the optimal pH for hemagglutination of Sindbis virus. Only the radioactive material in the pI 6.1 peak adsorbed to the erythocytes and three successive washes failed to remove it from the cells. Complement fixation testing of these same gradient fractions revealed a major peak coincident with pI 9.2 (titer 1:32) and minor peaks of complement fixing antigen at pI 6.1 (titer 1:8) and pI 3.0 (titer 1:4). The reactivity of the antigen found at pI 3.0 must be considered suspect since erthrocytes were lysed quite rapidly at this low pH. Ouchterlony analysis of dialyzed samples of the two Sindbis antigen peaks, pI 6.1 and pI 9.2, demonstrated precipitin bands when reacted against homologous Sindbis mouse hyperimmune ascitic fluid, however insufficient antigen concentration prevented a determination of identity. Dialyzed and concentrated aliquots of the Sindbis antigen pI 6.1 were examined by RIP with both the homologous Sindbis mouse ascitic fluid, however insufficient antigen concentration prevented a determination of identity. Dialyzed and concentrated aliquots of the Sindbis antigen pI 6.1 were examined by RIP with both the homologous Sindbis and heterologous WEE antibody (Figures 57 & 58). This antigen appeared broadly cross reactive, reacting almost equally with each antiserum. An indication of a biphasic RIP curve was noted in each instance. Sindbis antigen pI 9.2 was reacted similarly in homologous and heterologous RIP tests (Figures 59 & 60). A pronounced homologous reaction was noted (again somewhat biphasic); however little if any cross-reaction with the WEE antiserum could be detected.

Discussion. Isoelectric analysis of Group A arboviruses has yielded considerable insight into the chemical composition of these viruses. At the same time it has provided a new technology for the separation of virion components in a form compatible with procedures for antigenic analysis. The disadvantages of the electrofocusing procedures described include: 1) long time periods for complete resolution, 2) precipitation of proteins at their isoelectric point, and 3) the lack of a literature investigating the effects of time,

Figure 57. Homologous radioimmune precipitation of Sindbis antigen pI = 6.1.

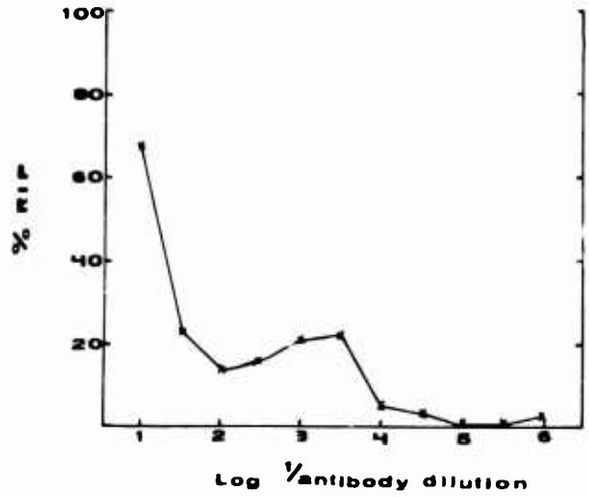

Figure 58. He erologous Western equine encephalomyelitis radioimmune precipitation of indbis antigen pI = 6.1

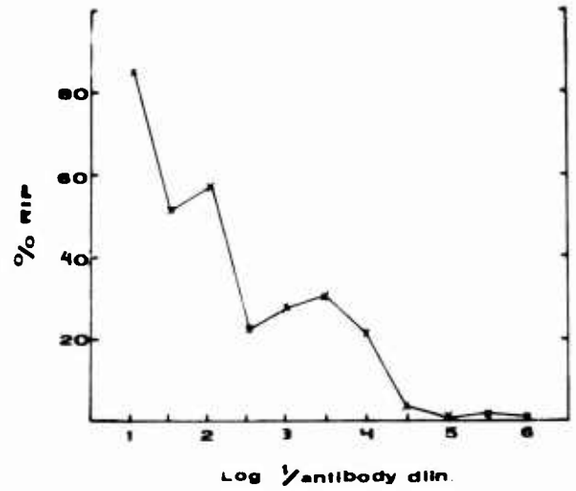

Figure 59. Homologous radioimmune precipitation of Sindbis antigen pI = 9.2.

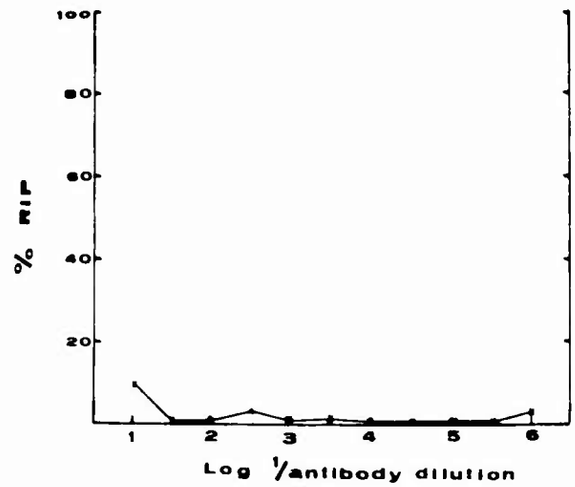

Figure 60. Heterologous Western equine encephalomyelitis radioimmune precipitation of Sindbis antigen pI = 9.2

temperature, current and other variables on the focusing of proteins. However, these were more than offset by the introduction of a new dimension to protein separation and characterization and the ability to obtain undenatured protein fractions in a form sutiable for antigenic characterization.

The observation that the intact Sindbis virion had an isoelectric point of 4.8 in repeated experiments and was not degraded for long periods at 20° C, or upon passing through the pH conditions in the other regions of the gradient suggests a stable virion architecture previously unsuspected, since such treatment should result in the rapid loss of infectivity, hemagglutinating activity, etc. The reproducibility of the technique in isoelectric point determinations immediately suggests procedures for comparing closely related arboviruses or groups of viruses and may provide a convenient method for the purification of virions from host cell proteins and constituents of the growth medium.

Our failure to resolve the constituent polypeptides of Sindbis virus following disruption with SLS and 2-ME was surprising since such treatment totally destroys the particulate structure of the virion, as evidenced by migration in eight percent polyacrylamide gels with the resolution of two separate polypeptides. It is unlikely lthat dialysis prior to isoelectric analysis resulted in reassembly of the virion components, since it is generally assumed that some SLS is irreversibly bound to protein. It is equally unlikely that both envelope and core polypeptides (even if bound with SLS) would have the same isoelectric point as described for the unmodified intact virion.

Treatment with Triton X-100 and subsequent electrofocusing in low concentrations of this non-ionic detergent resulted in the best resolution of subvirion components thus far. All evidence indicates that core polypeptide has an isoelectric point at or near the acid end of the pH gradient, pH 3.0. We have not determined if this polypeptide is denatured under these rather extreme pH conditions, however preliminary complement fixation data suggest that it may retain antigenic activity. [illegible] of two peaks from the soluble envelope fraction of de[illegible] virion, pI 6.1 and pI 9.2, was unexpected since PAGE [illegible] have shown most group A arboviruses to contain only two polyp[illegible] (Annual Report 1971). Burge et al. [illegible] have shown the separation of two glycosylated envelope polyp[illegible] using a modified PAGE system; however, these techniques apparently did not allow subsequent antigenic characterization.

Antigenic analysis of the electrofocus peaks revealed the pI 6.1 antigen to be an active hemagglutinin, while both peaks were antigenic in complement fixation and Ouchterlony precipitin tests. The higher complement fixation titer of the pI 9.2 peak may well reflect a greater concentration as indicated by higher radioactivity rather than increased antigenic efficiency. RIP testing of the two envelope antigens showed the pI 6.1 antigen lacked specificity in that it reacted equally well

with either Sindbis or WEE antiserum, while the I 9.2 antigen appeared specific under the same conditions.

Speculation as to the exact nature and antigenic specificity of these virion components must await more detailed experimentation, however it is interesting to note that: 1) the core protein, which in the intact virion is in intimate association with RNA, has an acid isoelectric point, 2) the two components of the virus envelope, presumably united by electrostatic attraction, have isoelectric points on either side of neutrality, 3) the pI of the hemagglutinin is the same as the optimal pH for hemagglutination, and 4) the pI of the other envelope component is the same as the pH at which the hemagglutinin of these viruses appears most stable, pH 9.0.

VI. Ecological Studies of Western and Eastern Encephalitis Viruses

A. Review

During 1969, ecological investigations were conducted in five major habitats of the Pocomoke Cypress Swamp (PCS), Maryland: a) swamp grid; b) peripheral swamp; c) upland; d) farmland; and e) marsh. Adult mosquitoes emerged abruptly during late April, and the following four species comprised approximately 90% of the total seasonal collection: Aedes cantator (6%), Aedes canadensis (35%), Culex salinarius (17%) and Culiseta melanura (28%). Five (5) EEE and 14 WEE viruses were isolated out of pools from 65,146 C. melanura, whereas no virus was isolated out of pools from 35 other species. Sequentially only WEE virus was isolated from 14 July to 12 August 1969, whereas 5 of 6 isolates from 18 August to 8 September 1969, were EEE virus. C. melanura were collected in variable numbers from each of five different habitats, and virus was recovered from mosquitoes of each habitat.

Bobwhite quail, exposed as sentinels in and around the swamp, were maximally infected with WEE virus during late July and early August, coinciding with peak densities of C. melanura. Infection of quail with EEE virus, as compared with WEE virus, occurred much less frequently and later in the summer. Additionally, 1 EEE and 5 WEE viruses were recovered from sentinel quail from 1 July to 8 September 1971. One quail was infected early with WEE virus, and subsequently with EEE virus during a second exposure period.

From January to December 1969, 2550 wild birds of 70 species were captured with mist nets from the swamp area. These species can be placed into four major temporal groups, based upon their time of arrival and/or duration of residence in the swamp: a) transients, b) winter residents, c) permanent residents, and d) summer residents. Permanent residents and summer residents comprised 60% of the total numbers tested, but 84% of those with demonstrable antibody. Birds of these two

groups had higher WEE infection rates than EEE, representing the greater WEE virus transmission during the 1969 breeding season. The transients (fall) and winter residents had higher EEE infection rates than WEE, reflecting the late summer transmission of EEE virus acquired either in the study area or at northern breeding sites.

Observations of EEE and WEE virus transmission in the PCS from 1967 through 1969 assisted in design of the 1970 and 1971 programs and subsequent evaluation of data. Infection rates of C. melanura with EEE and/or WEE were dissimilar each year (Table 44). Infection rates were high during 1968, with EEE virus predominating, and low during 1967 and 1969, with WEE virus predominating. During 1967, neutralizing antibody against WEE virus was demonstrable in only 15% of 1468 wild bird sera, and WEE transmission to approximately 75% of susceptible quail did not begin until the end of August. Evidence of EEE transmission during 1967 was less apparent, in that only 4% of 1468 wild bird sera and one sentinel quail showed antibody against EEE virus. By contrast, during 1968, infection of susceptible quail with either virus was initially detected at about the same time (22 July for WEE, 9 Aug for EEE) which was one month earlier than the onset of transmission during 1967. Infection of wild birds and susceptible quail was slightly higher for EEE than WEE, and bird infection with either virus greatly exceeded that observed during 1967. During spring and early summer 1969, neutralizing antibody against EEE virus in sera of wild birds was more prevalent than to WEE virus. This observation was interpreted as a consequence of the 1968 EEE epizootic. Co-incident with the appearance of the year's immature population, a rapid decline in antibody prevalence rates was observed. From mid-summer, WEE antibody prevalence rates increased rapidly, declining sharply in October with the passage of fall migrants through the swamp. EEE infection rates increased and peaked (October) after WEE infection rates were negligible. The temporal appearance and degree of wild bird infection coincided with infection rates in sentinel quail and relative density of C. melanura. The antibody prevalence rates in the White-eyed vireo, the most abundant breeding bird in the area, were consistently higher than those for the total wild bird population; however, the relationship between EEE and WEE antibody remained constant.

A review of available information characterizing EEE/WEE virus transmission in the PCS indicates that the following aspects of virus transmission in the PCS are understood poorly, or not at all:

1. Persistence or seasonal introduction of the viruses.
2. Variations in the degree of virus amplification from year to year.
3. Factor(s) influencing whether EEE or WEE will be predominately transmitted.
4. The extent of virus transmission beyond the confines of the swamp habitat.
5. The variable time of onset of virus transmission from year to year.

6. The usual late summer transmission of EEE virus as compared with earlier transmission of WEE virus.
7. Vector(s) responsible for transmission of viruses to non-avian hosts.

Table 44 - EEE and WEE Virus
Infection Ratios of Female Culiseta melanura
Collected in the Pocomoke Swamp from 1967 to 1970

Virus	Year			
	1967	1968	1969	1970
EEE	0	1:852	1:13,029	1:2,170
WEE	1:3,170	1:1,394	1:5,922	1:542
Both	1:3,170	1:529	1:3,428	1:434

B. Approach to Current Studies

Field collections were designed in 1970 - 1971 in a manner permitting comparison of data with those obtained in years past. Susceptible quail were exposed to mosquito bite in the swamp sufficiently early in the spring to monitor possible virus transmission before the onset of wild bird migration. The type and degree of virus amplification, dependent upon successful infection of avian hosts, was measured by continuous periodic exposure of sentinel quail and appropriate sampling of the wild bird population. Wild birds were captured by mist nets before spring migration and continuing through fall migration. Birds were identified by species to ascertain the proportion of each temporal group. Birds were aged to detect the introduction of susceptible young into the population and the post-breeding dispersal of adults. Plasma samples were obtained from captured wild birds and sentinel quail to permit the development of antibody profiles characterizing the type, onset, duration and level of virus transmission. Mosquitoes were periodically collected from spring through fall at those sites in the swamp which provided the best collections of C. melanura during 1969. The frequency distribution of C. melanura collected from these sites over time, and virus infection rates, permit correlation with infection rates of immature wild and sentinel birds, as well as comparison with similar data obtained in previous years.

In order to measure the extent of virus transmission beyond the confines of the swamp, during 1970 mosquitoes were collected and

sentinel quail were exposed at 1, 2, and 3-mile intervals from the center of the swamp. Wild birds were also captured at Irish Grove, a habitat located six miles southwest of the PCS. Irish Grove harbors species of wild birds common to the PCS, but without detectable presence of C. melanura. Additionally, during 1971, sentinel quail were exposed to mosquito bite on Assateague Island, a habitat devoid of C. melanura during previous years' collections.

C. Laboratory Methods

Mosquitoes. Following collection, mosquitoes were killed at -20° C, placed in labelled vials, and stored at -70° C. Mosquitoes were identified by personnel from the Department of Entomology. Females of each species were placed, 25 or less, into labelled Wasserman tubes and stored at -70° C for virus assay. Mosquito pools were triturated by adding 7 glass beads (6 mm dia.) to the tube and vortexing. A 1.5 ml volume of diluent (Basal Medium Eagles with 20 percent inactivated fetal bovine serum, and 200 units/ml and 100 ug/ml of penicillin and streptomycin, respectively) was added to each tube. The slurry was vortexed to a fine suspension and centrifuged to sediment mosquito debris. A 0.1 ml volume of supernatant fluid was inoculated into each of two tubes containing primary hamster kidney cell monolayers (Flow Lab.). Cultures were incubated at 37° C and examined daily for five days. Cultures showing cytopathic effect (CPE) were stored frozen until passed a second time in monolayers of BHK-21 cells. Second passage material producing CPE was stored at -70° C until identified by plaque reduction neutralization (PRN) test. Hyperimmune rabbit antiserum prepared against Cambridge strain EEE and California strain WEE viruses were used for identification.

Serology. Birds were bled by juglar venipuncture, using syringes wet with heparin solution (50 USP units per ml). Plasma was separated by centrifugation and stored at -70° C until diluted for assay. Plasma samples were heated at 56° C for 30 min and diluted 1:5 with growth medium containing 5 percent inactivated fetal bovine serum. Plasma from 1970 sentinel quail were assayed for antibodies against EEE and WEE viruses by PRN test in BHK-21 cells. All other plasma samples were assayed for antibody in a tube neutralization test, using primary hamster kidney cell cultures (Flow Lab.). Plasma considered positive by PRN test reduced 50 to 100 plaque-forming units (PFU) of Cambridge strain EEE and/or MacMillin strain WEE viruses by at least 80%. Plasma considered positive by tube neutralization test were associated with 25 percent or less CPE in at least 1 of 2 tubes by the fifth day post-inoculation. Plasma were assayed in the presence of 20 to 80 tissue culture infective doses -- 50 percent ($TCID_{50}$) of Cambridge strain EEE and MacMillin strain WEE viruses.

D. Results of 1970 and 1971 Studies

Sentinel Birds. Bobwhite quail (Colinus virginianus) were used in all sentinel studies. Quail used early in year 1970 and 1971 were obtained from sources in Savannah, Georgia and Forest City, Arkansas, respectively. Throughout the remainder of each year, quail were obtained from Willards, Maryland, Quail were bled before exposing them to mosquito bites in the study areas. During 1970, ten quail of mixed sex were exposed for two-week periods, placed in a mosquito-proof environment for an additional two weeks, then bled for antibody assay. Exposure periods continued from 26 May to 27 November 1970. One cage of 10 birds was located at slab road, pasture, 20Q, cemetery, and at sites 1, 2 and 3 miles distant from the center of the swamp. During 1971, 10 quail of mixed sex were exposed for weekly periods, placed in a mosquito-proof environment for an additional two weeks, then bled for antibody assay. Exposure periods continued from 13 April to 25 November 1971. One cage of 10 birds was located at each of three sites: pasture, slab road and 20 Q.

Starting 5 July 1971, 10 sentinel quail per site were exposed in cages at the north, middle and south end of Assateague Island. These birds were bled at monthly intervals through November for evidence of virus transmission. Figure 61 shows the sequence of EEE and WEE virus infections of sentinel quail exposed in the swamp during 1970 at 20 Q, pasture and slab road. Sentinel quail exposed in the swamp during 1970 were first infected with EEE virus during the period 15 to 28 June (1 of 6 birds placed at slab road). The next EEE virus infection was not detected until a month later, 13 to 26 July, from which time infection steadily increased until 20 Sept when approximately 50% of exposed quail were infected with EEE virus. EEE virus infections continued through the period 16 to 27 November, though at a much reduced rate. WEE virus transmission to quail began approximately one month later than EEE (13 to 26 July). Approximately 50% of the birds were infected through 6 September, at which time WEE transmission gradually declined to 20% by 16 October. An abrupt increase in WEE transmission was observed during 16 to 27 November, similar to that observed for EEE virus.

Figures 62 and 63 show the sequence of WEE and EEE virus infections, respectively, of sentinel quail exposed at sites one, two, and three miles distant from the center of the swamp. The onset, duration and degree of WEE transmission generally reflected the WEE transmission monitored at the three swamp sites (Fig. 61). However, EEE transmission was delayed considerably over that observed in the swamp and it was principally limited to the month of September. Following a decline in virus transmission during late September and October, a second increase in virus transmission occurred during November, similar to that observed at the swamp sites. Transmission of each virus at one, two and three mile sites was similar in time of onset, duration and degree.

Figure 64 shows the sequence of EEE and WEE virus transmission to

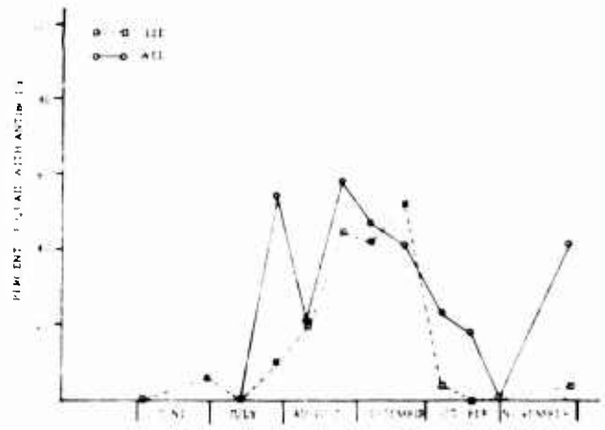

Figure 61. Period prevalence rates of Eastern equine encephalomyelitis (EEE) and Western equine encephalomyelitis (WEE) virus antibody in sentinel quail in the Pocomoke swamp during 1970.

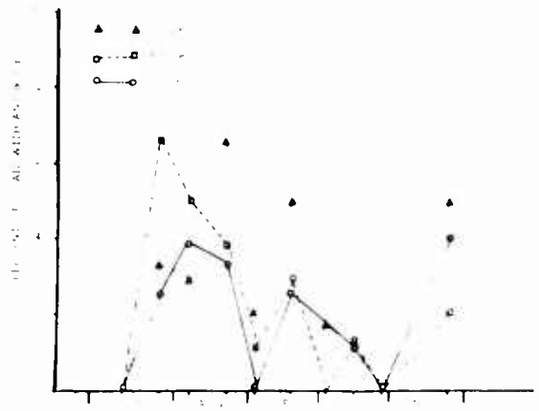

Figure 62. Period prevalence rates of Western equine encephalomyelitis virus antibody in sentinel quail at 1, 2, and 3 mile sites from the Pocomoke swamp during 1970.

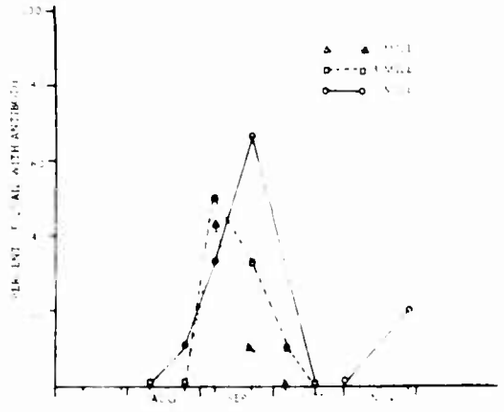

Figure 63. Period prevalence rates of Eastern equine encephalomyelitis virus antibody in sentinel quail at 1, 2, and 3 mile sites from the Pocomoke swamp during 1970.

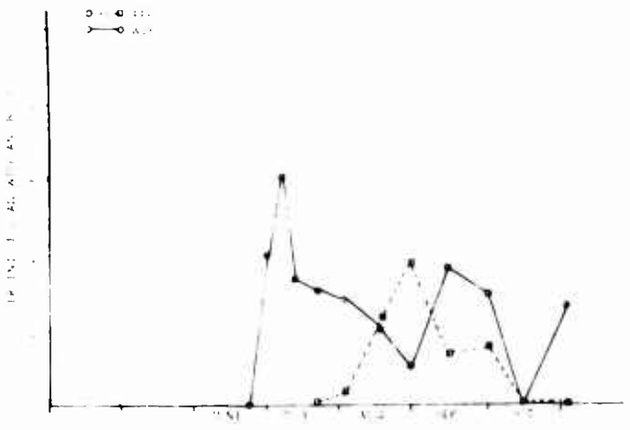

Figure 64. Period prevalence rates of Eastern and Western equine encephalomyelitis (EEE and WEE) virus antibody in sentinel quail in the Pocomoke swamp during 1971.

sentinel quail exposed in the swamp during 1971 at 20Q, pasture, and slab road. Transmission of WEE virus abruptly increased during 23 to 29 June, peaked by 6 July and gradually declined through August. Increased WEE transmission was again apparent in September and early November, separated by a two-week period in October with no apparent virus transmission. EEE virus transmission was confined to the months of August and September. The 30 sentinel quail exposed on Assateague Island from 5 July through November 1971 showed no evidence of EEE virus transmission. Whether or not WEE virus was transmitted cannot be ascertained, because plasma samples from individual birds over time showed variable neutralizing ability.

Mosquitoes. During 1970 and 1971, all mosquitoes were collected by CDC miniature light traps baited with carbon dioxide. All traps were operated from approximately one hour prior to sunset until three hours after sunrise. Mosquitoes were processed after collection, as described under "laboratory methods". From 14 April to 22 November 1970, weekly trapping for mosquitoes was conducted at the following sites: a) 20 Q; b) slab road; c) superswamp; d) pasture; e) transition 2; f) upland; and g) Bishop's. Periodic trapping throughout the summer was conducted at sites 1, 2 and 3 miles distant from the center of the swamp.

A total of 154,076 female mosquitoes from 30 species were collected. As in 1969, Aedes cantator, Aedes canadensis, Culex salinarius, and Culiseta melanura were a major proportion (94%) of the sampled population. C. melanura made up 5% (8,670) of the collection, as contrasted with 28% during 1969. The number of C. melanura captured per trap night from spring to early fall, 1970, is expressed as a three-week moving average for three swamp sites (20 Q, slab road, pasture) in Figure 65. Adult C. melanura were first observed on 26 April. Captures gradually increased through 10 May, abruptly increased and stayed at relatively high levels for three successive weeks to 31 May, and sharply decreased again to relatively low levels for three successive weeks, 1 through 21 June. This pattern is interpreted to represent the emergence and die-off of one population of C. melanura. From 21 June, relatively high captures of C. melanura were sustained through mid-August, when collections started to decline and reached negligible levels by mid-November, 1970. From 12 April to 30 October, 1971, weekly trapping for mosquitoes was conducted at the following sites: a) 20Q; b) slab road; c) pasture; d) transition 2, and e) two upland sites.

A total of 111,755 female mosquitoes from 30 species were collected. The same four species made up a major proportion (80%) of the sampled population, as in 1969 and 1970. C. melanura comprised 10% (11,236) of the sampled population, intermediate between the proportions observed in 1969 and 1970. The differences in proportion of the total catch represented by C. melanura from 1969 through 1971 may be more apparent than real, since the total collections were obtained at different sites and by different methods for the three years. Since transmission of virus

is dependent, in part, upon an above-threshold density of C. melanura at times when susceptible hosts are present, the proportion of C. melanura relative to other species would be irrelevant.

The number of C. melanura captured from spring to early fall 1971 is expressed as a three-week moving average for three swamp sites (20 Q, slab road, pasture) in Figure 66. Adult C. melanura were first observed on 4 May. Captures sharply increased from 10 May to 3 June and were sustained at a relatively high level through 21 August. Collections precipitously declined from 8 September and remained relatively low through 5 October. On 13 October, a sharp increase in collections of C. melanura was observed which continued relatively high until collection terminated on 30 October 1971. Apparently, the frequency distribution of C. melanura over time, at the same swamp sites and by the same collection methods, was remarkably different in 1970 vs. 1971.

Upon comparing the "density" of C. melanura over time (Figure 65) with observed infections in sentinel quail (Figure 61) during 1970, one observes that the onset and duration of quail infections slightly follows and corresponds rather closely to relatively high densities of C. melanura. An exception to this would be the relatively high incidence of quail infections during November 1970, when the population of C. melanura was relatively low and declining. One very interesting observation was the EEE virus infection of one quail on 28 June, the credibility of which is strengthened by isolation of EEE virus from C. melanura captured on 12 July. This limited early transmission of EEE virus was apparently related to the emergence and apparent die-off of a single large mid-May population of C. melanura.

During 1971, the onset and duration of quail infections (Figure 64) with either virus corresponded closely to those time periods when C. melanura was at peak density (Figure 66). This year, increased infection rates during November followed a remarkable late October increase in C. melanura density. Also, WEE virus infection rates over the summer months varied in the presence of relatively high and constant levels of C. melanura. Only the C. melanura pools (366) collected during 1970 were processed for virus isolation. EEE and WEE viruses were identified from 4 and 16 pools, respectively. EEE virus was isolated from the earliest collection (12 July) and was not again isolated until 20 September (Table 45).

All but one of the viruses were isolated from 12 July to 27 September, the period during which the summer density of C. melanura was maximum. No virus was isolated during October, a period during which sentinel infections were declining and C. melanura populations were low. The last virus was isolated on 1 November, which correlated well with increased quail infections but poorly with an apparent low density of C. melanura. The last column of Table 44 shows the infectivity ratios of C. melanura with EEE and/or WEE viruses for 1970 in comparison

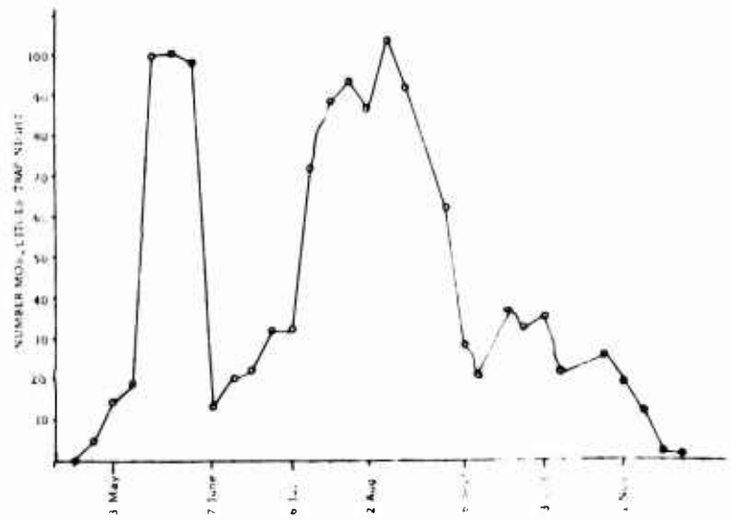

Figure 65. Number of female C. melanura per trap night collected during 1970 in the Pocomoke swamp (20 Q, pasture, slab road).

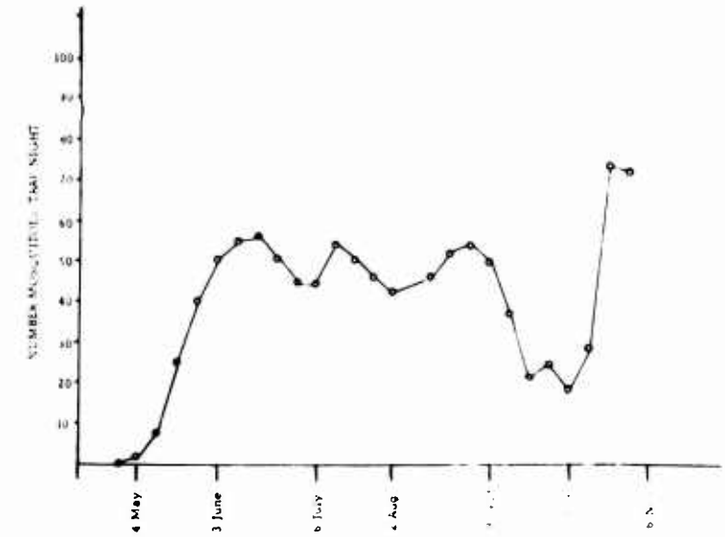

Figure 66. Number of female C. melanura per trap night collected during 1971 in the Pocomoke swamp (20 Q, pasture, slab road).

with three previous years. It is apparent that mosquito infection with combined viruses is relatively high and low on alternate years. It is equally apparent that mosquito infection with either virus is alternately high and low in consecutive years, but there is no clear cyclical pattern of one virus predominating.

Table 45 - Viruses Isolated from C. melanura
Collected in the Pocomoke Swamp during 1970

Mosquito pool no.	Capture date	Location	Virus
5354	12 July	Upland	EEE
5363	12 July	2-mile	WEE
5423	19 July	Slab road	WEE
5480	20 July	Pasture	WEE
5563	26 July	2-mile	WEE
5564	26 July	2-mile	WEE
5576	26 July	Cemetery	WEE
5601	2 Aug	Slab road	WEE
5647	9 Aug	Pasture	WEE
5701	9 Aug	2-mile	WEE
5947	11 Aug	Railroad	WEE
6010	14 Aug	Slab road	WEE
6025	14 Aug	20 Q	WEE
6088	21 Aug	Pasture	WEE
6133	23 Aug	3 mile	WEE
6300	6 Sept	Pasture	WEE
6396	20 Sept	Slab road	EEE
6449	27 Sept	Slab road	EEE
6450	27 Sept	Slab road	EEE
6516	1 Nov	20Q	WEE

Wild Birds. In 1970 and 1971, birds were captured by mist nets in four general habitat areas in and around the PCS (pasture, slab road, swamp road and grid). Approximately 32 nets were operated at various intervals from 6 Jan to 27 Nov in 1970; and 27 nets were operated from 19 Jan to 18 Nov in 1971. Table 46 summarizes wild bird captures for the two years. Based on the initial demonstration in 1969 of the possible role of immature wild birds in the amplification and selection of virus in the PCS, greater effort was expended in 1970-71 to study

the age structure of the avian population. It was also thought that the density of avian hosts over time and space might have an effect on virus amplification, so methods were developed for the collection and analysis of data which would express avian densities.

Table 46 - Captures of Wild Birds in the Pocomoke Cypress Swamp during 1970 and 1971

	1970		1971	
	No.	(%)	No.	(%)
Captured	1,234		916	
Bled & tested	1,015		730	
Immatures captured	449	(36)	302	(33)
Immatures bled & tested	404		272	
Recoveries	194	(15.7)		(16.7)

In 1969, a crude measure of avian densities was utilized, "number of birds captured per sampling period" (Figure 67). This measure, however, did not account for the variables of length of sampling period or number of nets used during sampling period. Data for 1969 was recomputed, using "birds per 100 net hours", a measure which accounts for all major sampling variables. Figure 68 combines these years into one expression, averaging the values in each 10-day period. An interpretation of Figure 68 indicates that the PCS is not in the main path of migration in the spring but is in the path of migration in the fall. An examination of the species profile for the two migration periods also substantiates this view; i.e., greater number of species in fall than in spring. These facts may mitigate against spring introduction of virus into the swamp by migrating birds. Figure 68 shows a steady decline in avian density from the latter part of June to the beginning of September. This decline could be caused by a variety of factors, possibly including high mortality, movement out of the area, or absence of young of year. Possible explanations for this observed decline in avian populations should be examined, because this decline coincides with peak virus transmission in sentinel quail and peak densities of the vector mosquito (See Figure 72).

An analysis of adult-immature ratios through time for 1970 and 1971 (Figure 69) indicates that by the end of July approximately 75% of the population was immature and remained at that level for the

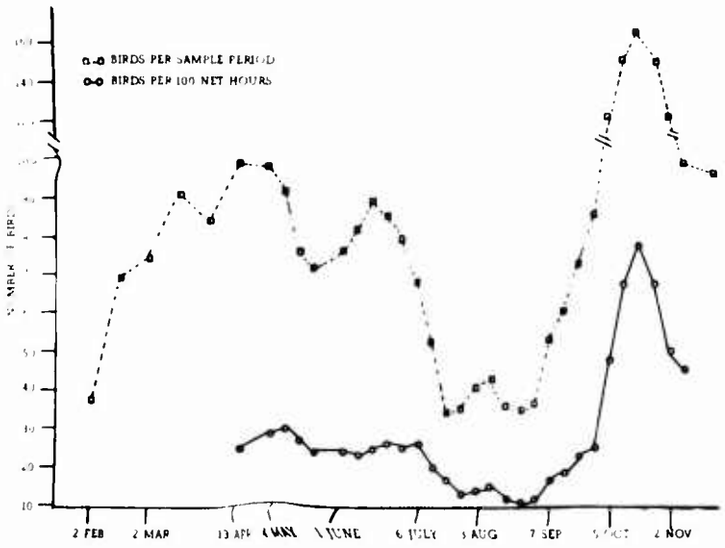

Figure 67. Comparison of number of birds per 100 net hours and number of birds per sample period by three week moving average (1969).

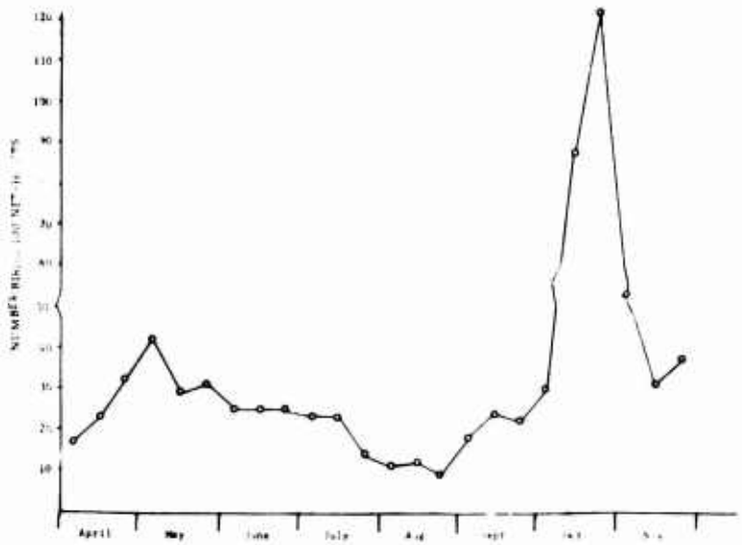

Figure 68. Average seasonal avian densities in the Pocomoke swamp from 1968 through 1971.

duration of the season. Obviously, the total population decline was not due to the absence of an immature population. In fact, with 75% of the population immature, the total population should have increased. The observed decline in total population suggests that either adults experienced a major die-off or they moved out of the area. There is no information available that a die-off of adult birds occurred in both 1970 and 1971 (or in 1968 or 1969). It is also difficult to conceive of a mechanism that would selectively kill only adults. There is a mechanism, however, that would remove the majority of adults from the area after the fledgling of young, i.e., "post-breeding dispersal". Figure 70 portrays avian densities for four separated habitats examined in 1969. The two "swamp" areas, swamp grid and slab road, show a decline in densities through June, July and August. The two peripheral, non-"swamp" areas, pasture and super swamp, show a steady increase in densities into middle July, with subsequent drop-off to lows at the end of August. This increase into mid-July could be the result of adults moving in from "swamp" areas and the appearance of immatures in the area, with parents remaining. The mid-July to late August decline in avian densities in these peripheral areas could be a combination of post-breeding dispersal and those factors which caused "swamp" birds to move earlier. Factors other than post-breeding dispersal, which might cause birds to leave the "swamp" area, are shown in Figure 71. Using 1971 data, the four weeks of August had mean weekly maximum temperatures over 80° F. and weekly minimum relative humidity values around 55%. These two factors alone made the "swamp" most uncomfortable for humans and more than likely had some affect on birds.

Correlating the decline of avian densities with other factors in the virus transmission cycle leads to the formation of several interesting hypothesis:

1. Decreasing avian densities through the summer means fewer viremic birds to provide a source of virus for vector mosquitoes. It also would become more difficult for a mosquito to find an avian blood meal. These factors would tend to decrease the probability of virus transmission as the summer progressed. Obviously, the opposite is true, because the transmission of EEE and WEE viruses amplifies during summer months (Figures 61 and 64).

2. Lowering avian densities in the face of constant or increasing vector densities would increase the number of mosquitoes/per bird but initially not increase the number of infective mosquitoes per bird. After a lag period, increased virus transmission would be expected. This association was apparent in 1970, as measured by sentinel quail (Figure 72).

3. As avian densities decline, the relative attractiveness of the sentinel quail increases, making sentinel quail perhaps a more sensitive monitor than originally believed. (Unfortunately, this increases their possible role as virus amplifiers in the study area).

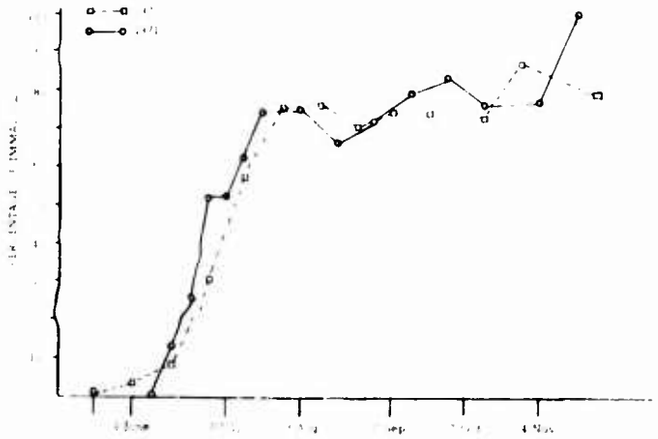

Figure 69. Percent immatures of total captured birds from Pocomoke swamp during 1970 and 1971.

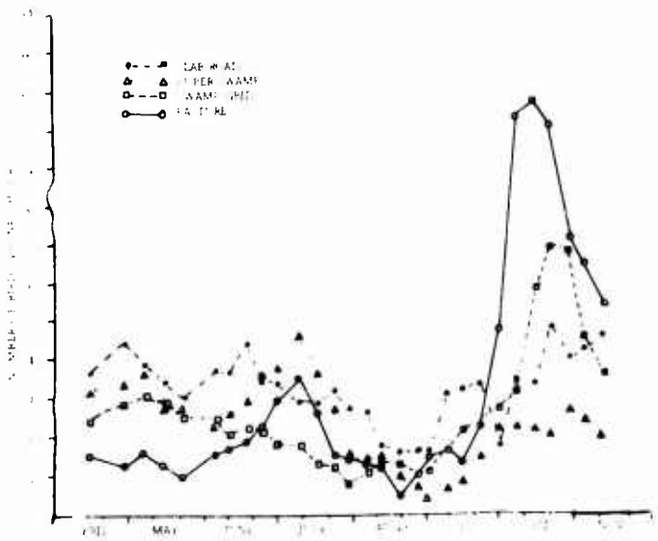

Figure 70. Three week moving average of avian densities in the Pocomoke swamp during 1969 by location.

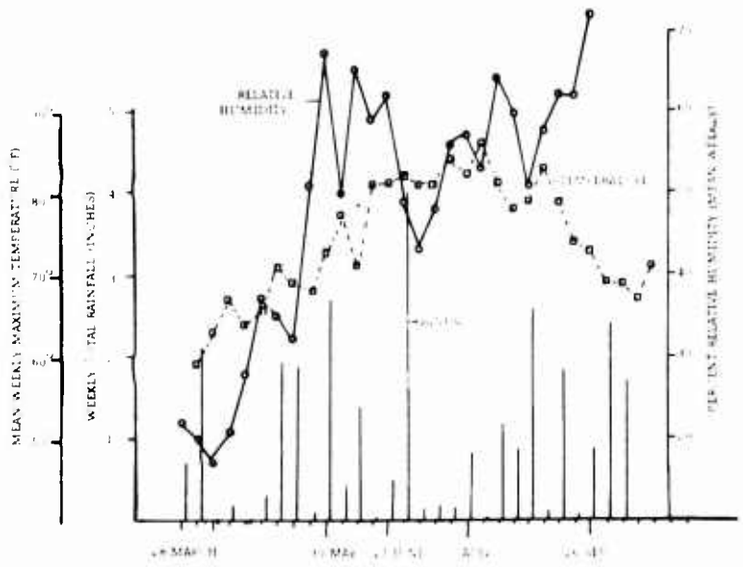

Figure 71. Mean weekly maximum temperature, percent relative humidity, and weekly total rainfall during 1971 in the Pocomoke swamp.

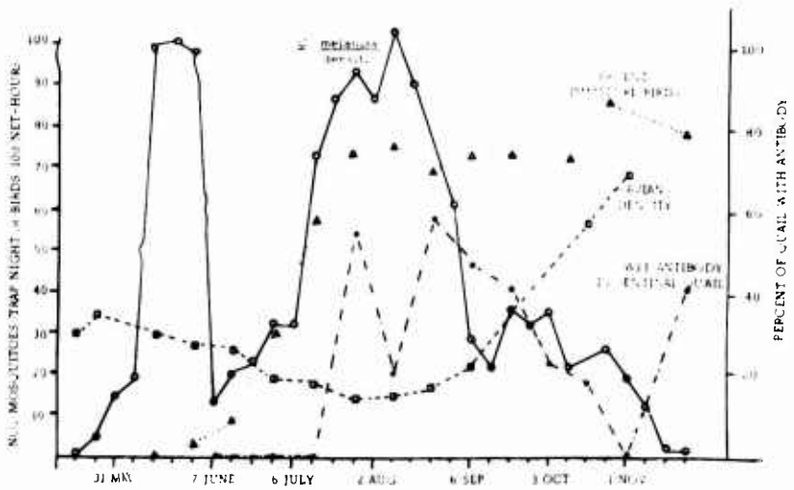

Figure 72. Temporal comparisons of Western equine encephalomyelitis antibody prevalence rates in sentinel quail, avian and female C. melanura densities by three week moving average, and percent immature of the bird population (1970).

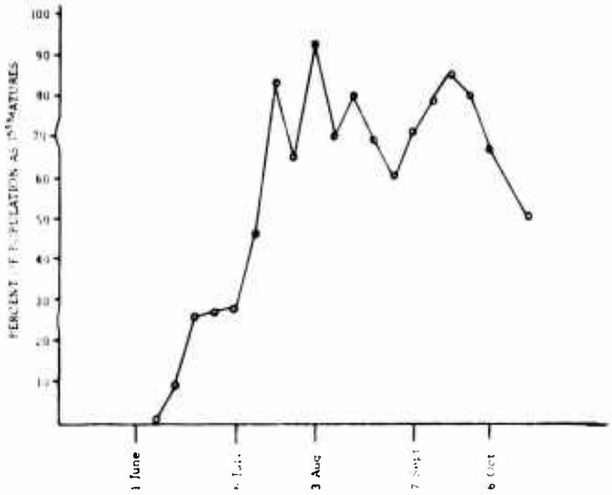

Figure 73. Five year average of White-eyed vireos as percent immatures captured in the Pocomoke swamp from 1967 through 1971.

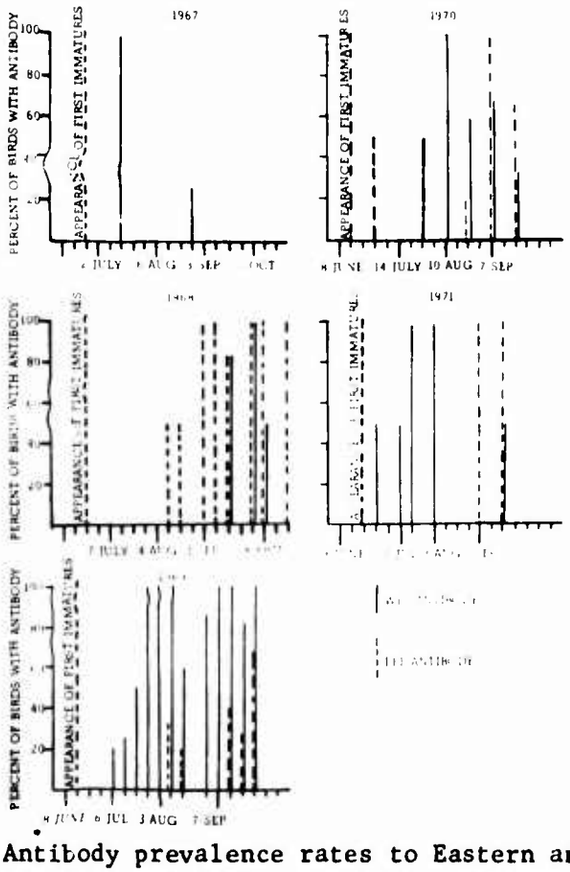

Figure 74. Antibody prevalence rates to Eastern and Western equine encephalomyelitis (EEE and WEE) viruses for immature White-eyed vireos captured in the Pocomoke swamp.

4. The fact that declining avian densities in the summer and increasing virus transmission in sentinel quail has occurred for *each* of the previous four years suggests that this is the normal enzootic situation. It is possible that for an epizootic to occur, lowered avian densities and/or increased vector densities must occur earlier in the season. This would allow for post-breeding dispersal of viremic adults into peripheral areas early enough in the season to permit virus amplification and dispersal in local avian populations.

5. Decreasing avian densities may, in fact, be a prerequisite for an enzootic (state) to be maintained in the study area. The movement out of the area of adults which are mostly immune, leaving a steadily increasing proportion of susceptibles, apparently happens each year and may be required for virus amplification to detectable levels.

Single species monitor. Continuation of studies on the White-eyed Vireo now allow multi-year comparisons:

1. Adult-immature ratios for five successive years (1967 through 1971) show immatures to eventually reach a level exceeding 50% of the total species population each year (Figure 73).

2. The immature population was primarily infected with WEE virus during 1967, 1969 and 1971, and EEE virus during 1968. Infection rates for both viruses appeared comparable in 1970 (Figure 74).

3. Very good correlation was observed in 1969, 1970 and 1971 between sentinel conversion rates and antibody levels in the immature White-eyed Vireo population (Figure 75). In 1970, immature populations show a small peak of EEE virus activity very early in the season (29 June) which corresponds to equally early evidence of transmission in sentinel quail.

4. Both 1969 and 1971 data show a short lag period (1 to 3 weeks) between appearance of first immatures and first antibody to WEE virus, indicating WEE virus was present and being transmitted when immatures appeared. Sentinel quail conversions also indicate transmission during the same period (Figure 75). In 1970, both parameters show a longer lag *period* (6 weeks) between first appearance of immatures and first *antibody* to WEE.

5. Table 47 indicates that the White-eyed Vireo has the lowest proportion of immatures to adults of any of the major species resident in the swamp during the summer months. This might indicate a higher productivity for other species, but probably represents less movement out of the area *of* the White-eyed Vireo adults. It would appear that the predominant species in the swamp, the White-eyed Vireo, has less effect on virus amplification than previously expected. With a higher percentage of the summer pop*ulation* adult, and hence probably immune from previous years, the impact of this species on virus amplification may be less than some of the other major species.

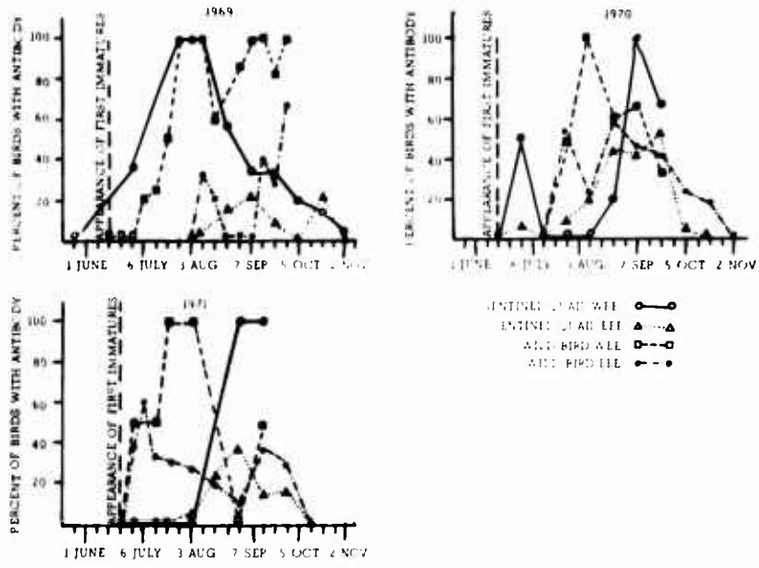

Figure 75. Antibody prevalence rates to Eastern and Western equine encephalomyelitis (EEE and WEE) viruses for sentinel quail and immature White-eyed vireos captured in the Pocomoke swamp.

Table 47 - Percent Immatures of Total Captures
(from appearance of first immature)

Species	1970	1971
White-eyed Vireo	26/61 = 43%	11/26 = 42%
Yellowthroat	30/41 = 73%	18/25 = 72%
Carolina Wren	38/46 = 83%	49/55 = 89%
Catbird	16/19 = 84	14/17 = 82
Ovenbird	25/38 = 66	12/26 = 46
Worm-eating Warbler	9/12 = 75	19/34 = 56

Viral transmission outside the PCS: Hypothesis. EEE and WEE viruses are transmitted annually in geographical areas other than the PCS by C. melanura and occasionally other vectors. Heretofore, studies have been restricted in time and place, and have not permitted comparative observations on transmission occurring the same year in different places. Such comparisons might further clarify the ecology of EEE/WEE virus transmission by relating observed differences in virus transmission to differences in climate or arthropod and vertebrate hosts.

Approach. During 1970, sentinel quail were exposed as previously described to mosquito bite at sites 1, 2 and 3 miles beyond the center of the PCS to ascertain the degree of virus transmission, if any, at these distances. Similarly, during 1970, wild birds were captured and mosquitoes collected at Irish Grove, a pine wood, salt marsh habitat located 6 miles southwest of the PCS. Limited studies were also conducted around Middleboro, Mass. and Jacksonville, North Carolina during 1971. The latter studies were conducted in an attempt to find habitats north and south of the PCS which support considerable seasonal transmission of EEE/WEE viruses. These more northerly and southerly climates might alter the spring emergence of adult C. melanura, the onset of spring bird migration, or arthropod/vertebrate host characteristics to show meaningful differences in virus transmission as compared with the PCS.

Data. As previously described, EEE and WEE viruses intected sentinel quail at sites 1, 2 and 3 miles distant from the center of the PCS, and WEE virus was isolated from C. melanura collected at the latter two sites. C. melanura larvae were collected from the 1 mile site during June 1970, and adult C. melanura, representing 2% to approximately 50% of the total collection, were periodically captured throughout the summer from each of the three sites. These observations indicate that the virus transmission which occurred at sites 1, 2 and 3 miles distant from the center of the swamp was related to the reduced but constant

presence of C. melanura associated with permanent breeding sites rather than foraging mosquitoes from the swamp.

From 30 May to 20 September 1970, during eight field trips, mosquitoes were collected and wild birds were netted at Irish Grove, and a total of 6,000 female mosquitoes were captured. Anopheles bradleyi-crucians and Culex salinarius comprised 70 and 27 percent of the total population, respectively. Only seven C. melanura were captured the entire year. Neutralizing antibody against EEE and WEE viruses were detected in plasma from 5 and 17 immature wild birds, respectively. Six immatures had acquired infection from 17 July to 11 August, before fall migration, indicating that infection had occurred locally. Also, immature cardinals and mockingbirds, which are permanent residents, were infected with EEE and WEE viruses. The absence of significant collections of C. melanura, and infection acquired locally by immature birds, suggests that EEE and WEE viruses were transmitted by a mosquito other than C. melanura.

During 1971, an attempt was made to find habitats supporting the seasonal transmission of EEE and WEE viruses sufficiently north and south of the PCS to alter the onset of adult mosquito emergence and bird migration. Preliminary inquiries enabled placing susceptible bobwhite quail in three swamp sites around Middleboro, Mass., and Jacksonville, N. C., respectively. Neutralizing antibody against WEE virus in the plasma of a single bird over time appeared variable. Antibody prevalence rates against WEE and EEE viruses over time for quail exposed in Mass. and N. C. are shown in Tables 48 through 51. WEE infection occurred earlier (June vs August) than EEE among birds exposed in N. C. and Mass., but the rate of infection with either virus appeared comparable. During June 1971, light trap collections at each of the three quail exposure sites in N. C. resulted in relatively poor collections of C. melanura. Looking for larger populations of C. melanura, a State forest (Croatan) approximately 10 miles N. E. of Jacksonville, N. C., was examined during June. No larvae of C. melanura were found, but 1900 female C. melanura were collected in one light trap placed in the Croatan Forest on 21 June. On the basis of this collection, a field trip to the Croatan Forest was conducted on 9 to 12 August 1971.

Approximately 6,000 female mosquitoes were collected from four traps operated for four nights in the Croatan, with C. melanura comprising 27 percent of the population (Table 52). EEE virus was recovered from seven pools of C. melanura and 1 pool of Anopheles bradleyi-crucians, whereas WEE virus was isolated from two pools of C. melanura (Table 53). All pools of Aedes atlanticus tormentor were toxic to primary hamster kidney cells. This material was not toxic to LLC-MK$_2$ cells, but no virus was isolated. Approximately 100 wild birds were also netted and bled from 9 to 12 August 1971 in the Croatan Forest. This sample of wild birds probably accurately reflects the bird population present, and compares favorably by species to those birds present

Table 48 - WEE Infection of Sentinel Quail Exposed around Middleboro, Massachusetts from 28 May to 20 September, 1971

Serum Obtained	Ratio of Quail with Antibody Exposed at		
	Raynham	Anderson Farm	Hocomock
17 June	0/10	0/10	1/10
2 July	1/10	0/10	new birds
15 July	0/10	0/10	1/9
25 August	2/10	1/9	3/9
20 September	7/10	1/9	0/9

Table 49 - EEE Infection of Sentinel Quail Exposed around Middleboro, Massachusetts from 28 May to 20 September, 1971

Serum Obtained	Ratio of Quail with Antibody Exposed at		
	Raynham	Anderson Farm	Hocomock
17 June	0/10	0/9	0/9
2 July	0/10	0/9	0/9
15 July	0/10	0/9	0/9
25 August	6/10	0/9	1/5
20 September	8/10	3/9	1/5

Table 50 - WEE Infection of Sentinel Quail Exposed in Onslow County, North Carolina from 18 May to 11 August, 1971

Serum Obtained	Ratio of Quail with Antibody Exposed at		
	Bear Creek	Huffmantown	Haw Branch
8 June	0/10	1/10	1/10
22 June	3/10	4/10	7/10
7 July	7/10	3/10	6/10
11 August	3/10	6/10	6/8

Table 51 - EEE Infection of Sentinel Quail Exposed in Onslow County, North Carolina from 18 May to 11 August 1971

Serum Obtained	Ratio of Quail with Antibody Exposed at		
	Bear Creek	Huffmantown	Haw Branch
8 June	0/10	0/10	0/10
22 June	0/10	0/10	0/10
7 July	0/10	0/10	0/10
11 August	4/10	7/10	0/8

Table 52 - Female Mosquitoes Collected in Croatan Forest, North Carolina during June and August, 1971

Species	Number (%)			
	22 June		9 to 12 August	
Culiseta melanura	1914	(86)	1,508	(27)
Aedes atlanticus-tormentor	158	(07)	3,342	(51)
Anopheles bradleyi-crucians	0		959	(15)
Aedes canadensis	37	(02)	266	(04)
Culex salinarius	0		54	(0.8)
Psorophora ferox	0		50	(0.8)
Mansonia perturbans	30	(01)	0	
Aedes infirmatus	21	(01)	0	
Others	61	(02)	113	(01)
Total	2221		6292	

during the summer in the PCS. Antibody prevalence rates to WEE and EEE viruses in the captured total and immature bird population, respectively, shows higher WEE than EEE antibody prevalence rates (Tables 54 and 55). Even though there was a 4:1 ratio of EEE to WEE isolates from mosquitoes during 9 to 12 August 1971, the antibody prevalence rates are commensurate with the earlier summer transmission of WEE virus in the area (Tables 50 and 51). Information obtained from the Croatan Forest, although incomplete in many respects, suggests that the annual

transmission of EEE/WEE virus is comparable to that which occurs in the PCS. The isolation of EEE virus from A. bradleyi-crucians is noteworthy when viewed in relation to the infections of wild birds at Irish Grove, Maryland, during 1970, where A. bradleyi-crucians is the predominant mosquito and C. melanura is virtually absent.

Table 53 - Viruses Isolated by Mosquito Species Collected in Croatan Forest, from 9 to 12 August, 1971

Mosquito species	Number of mosquitoes	(pools)	Viruses isolated
Aedes atlanticus tormentor	3,267	(132)	0
Culiseta melanura	1,483	(61)	7 EEE 2 WEE
Anopheles bradleyi-crucians	959	(39)	1 EEE
Aedes canadensis	266	(12)	0
10 others	200	(29)	0

Table 54 - WEE and EEE Infection of Wild Birds Netted in the Croatan Forest from 10 to 12 August, 1971

Species	Number with antibody / Number tested	
	WEE	EEE
Yellowthroat	7/13	4/13
Carolina wren	5/12	2/12
Catbird	4/8	3/8
Rufous-sided towhee	5/7	3/7
Crested flycatcher	2/5	0/5
Red-eyed vireo	1/2	2/2
White-eyed vireo	2/2	2/2
8 Others	3/9	4/9

Table 55 - WEE and EEE Infection of Immature Wild Birds Netted in the Croatan Forest from 10 to 12 August, 1971

Species	Number with antibody / Number tested	
	WEE	EEE
Carolina wren	4/10	2/10
Yellowthroat	5/7	2/7
Rufous-sided towhee	5/6	2/6
Crested flycatcher	2/4	0/4
Red-eyed vireo	1/1	1/1
White-eyed vireo	1/1	1/1
4 others	1/5	1/5

E. Evaluation

Information obtained during ecological studies of EEE and WEE virus transmission in the PCS during 1970 and 1971 has strengthened hypotheses advanced on the basis of previous data and added additional information to the transmission picture. It was previously postulated that the seasonal immunity acquired by the avian amplifying host would exert a dampening influence on transmission of the homologous virus the following year. On the basis of limited data collected during 1968 and 1968, a reasonable extension of this hypothesis suggested that EEE and WEE viruses would be predominately transmitted on alternate years. When comparing the infectivity ratios of C. melanura with EEE and WEE viruses for a four-year period (1967 to 1970) it is apparent that mosquito infection with EEE and/or WEE virus is relatively high and low on alternate years (Table 44). It is equally apparent that mosquito infection with either virus is alternately high or low in consecutive years, but there is no clear cyclical pattern of one virus predominating. Data obtained during 1970 strengthens and modifies the original postulate. Predominate WEE virus transmission during 1969 presumably favored transmission of EEE virus during 1970. Indeed, following a large May population of C. melanura, the first virus isolated from C. melanura and infecting immature wild birds and sentinel quail was EEE virus. Then, EEE virus transmission stopped for approximately one month, apparently related to an abrupt decrease in numbers of C. melanura for the first three weeks in June. From this point, transmission of WEE virus approximated (sentinel and wild bird infections) or exceeded (mosquito infection ratios) that for EEE virus. Whether or not EEE virus transmission during 1970 would have predominated with sustained optimum

densities of C. melanura during early summer can only be speculated.

Information obtained since 1965 indicates that EEE and WEE viruses amplify each year in the PCS. Data obtained during 1970 and 1971 indicate that post-breeding dispersal of adult wild birds during June decreases the total wild bird population while disproportionately decreasing the adult to immature ratio, thereby providing conditions each year for successful transmission of virus to immature wild birds. The isolation of EEE virus from Anopheles bradleyi-crucians collected in the Croatan Forest, N. C., and the transmission of EEE/WEE viruses to birds captured at Irish Grove, Maryland, in the relative absence of C. melanura indicate that C. melanura is not a sine qua non for transmission of these viruses.

Using low challenge doses of MacMillen strain WEE virus in tube neutralization tests, a high proportion of equivocal serological reactions were observed. Further, mouse hyperimmune ascitic fluid prepared against MacMillen strain of WEE virus and rabbit serum prepared against California strain of WEE virus reduced a challenge dose (approximately 100 pfu) of MacMillen virus by 80% to 90%. These immune reagents reduced, by 50% or less, comparable challenge doses of WEE virus isolated from mosquitoes. These observations suggest that recent field isolates of WEE virus are immunologically different from high passage, laboratory strains of WEE virus.

Summary

I. During the past year adenovirus type 4 and 7 (ADV-4, ADV-7) vaccines were used extensively in Army basic training populations. Data obtained from the adenovirus surveillance program indicate that varying degrees of success were achieved by immunization with available vaccines during the period 1 Sep '71 through 31 May '72. Studies in this laboratory pertaining to vaccine potency and type specific neutralizing (N) antibody responses following immunization provided data which helped to explain the varying success that was observed. Finally, data from the first five years of the adenovirus surveillance program (FY '67 - FY '71) were re-examined for the purpose of doing a cost-benefit study of ADV-4 and ADV-7 vaccines. The results of the cost-benefit analysis provide an additional data base for making decisions about when, how and where ADV vaccines should be used in recruit populations in the future.

II. The heterogeneous antigenic determinants of Hepatitis B Antigen (HB Ag) from 310 individuals were compared, using a micro-Ouchterlony immunodiffusion technique. Reference antisera were made in rabbits immunized with a purified HB Ag with ay determinants from an American with hepatitis and another purified HB Ag with ad determinants from a Thai blood donor. The immunizing antigens were prepared by a combination of equilibrium centrifugation in cesium chloride and rate zonal centrifugation in sucrose gradients. Three immunodiffusion patterns of antigen reactivity were observed. Pattern 1 antigens showed identity to the reference ay HB Ag. Pattern 2 antigens showed identity to the reference ad HB Ag. Pattern 3 antigens showed partial identity to both reference antigens. Further study after absorbing the antisera with heterologous antigens, indicated that the reference antisera were distinguishing two additional antigenic determinants, designated w and r. Preliminary data suggest w is more common than r on HB Ag in the United States, but r is most common in Thailand.

A comparative evaluation was made of a solid phase radioimmune assay for antigen (RIA-Ag) and a passive hemagglutination inhibition test (HAI) to immunoelectro-osmophoresis (IEOP) complement fixation (CF) and immunodiffusion. In tests of 1133 military blood donors and 211 patients with hepatitis, the RIA-Ag detected HB Ag in 2 to 3 times as many sera as the CF, IEOP or HAI methods. The sensitivity of the immunodiffusion technique for antigen subtyping was improved by using a "Rheophoresis" system (Abbott Lab) in which antigen migration toward antibody is augmented by buffer evaporation from the center of the plate.

Requests for hepatitis tests of clinical specimens submitted to this department increased over the previous year. A survey was made of HB Ag and HB Ab among patients and staff members of the hemodialysis service of WRGH. During an 18-month period, HB Ag was detected in 6.5%

of 77 patients and 8.3% of 36 staff members. An additional 7.8% of the patients developed detectable antibody during this period. At least one antigen carrier was present on the ward during 11 of the 18 months of surveillance, confirming the suspicion that Hepatitis B is endemic to the hemodialysis service.

III. Myeloma proteins were collected from patients selected from a master list of electrophoretic analyses carried out on a routine basis in the hospital laboratory. Specimens with definite peaks representing large production of antibodies (IgG, IgA, IgM) were plasmaphoresed from selected patients and stored at -70 C. Sera were also obtained from the National Immunoglobulin Reference Center that were known to contain large amounts of the 4 subclasses of IgG, designated in both IgG-kappa and IgG-lambda types. The gamma-G myeloma proteins were isolated by ion exchange chromatography and tested for purity by Ouchterlony diffusion methods and by electrophoretic cross-testing for the opposite light chain. Rabbit antibodies to these subclass human globulins will be purified and properly absorbed and used to determine the subclass antibody response in infectious diseases, both associated and non-associated with immune complex disease.

IV. Sporadic cases of dengue observed on Haiti through October 1971 were shown to be due to dengue type 2 by virus isolation from serum specimens sent to WRAIR. An epidemic of dengue began on the Atlantic Coast of Colombia in August, 1971 and continues to spread southward. Dengue type 2 viruses were recovered from the acute serum specimens collected between January and April, 1972. Isolation attempts from recently collected mosquitoes and additional sera are in progress to determine if other serotypes are also present in Colombia.

Sera obtained during a dengue-2 epidemic from individuals with primary or secondary group B arbovirus infections contained cross reactive antibody against several viruses in this group. Hemagglutination-inhibition (HI) antibody titers of the IgM fractions from these sera revealed a rise in antibody specific for dengue-2. The acute and convalescent sera in over half of the secondary infection group also had low level unchanging IgM antibody titers to yellow fever which implicated yellow fever immunization as the primary infection.

Growth of a candidate vaccine strain of dengue type 2 virus (human serum isolate PR 159 which produces viremia in monkeys) was tested in 5 different certified cell cultures. Good replication was observed in a continuous line of fetal cercopithecus lung (NIH), but the cell culture was discarded because of development of abnormal karyology. Replication in primary canine kidney and in fetal human lung (WI-38) cells was considered poor. Replication of dengue-2 in a continuous line of fetal rhesus monkey lung from NIH was good, and a consistent increase in viral yields through 5 passages was demonstrated in primary green monkey kidney cells. GMK cells will be used to isolate variants in the

passage material having acceptable vaccine characteristics for human use. Work on the development of a chikungunya vaccine has almost terminated with the demonstration of the safety, efficacy and long term stability of a formalin-killed, freeze-dried vaccine. The vaccine seed virus is ready for use for large-scale commercial production.

V. The viral membrane protein composition of dengue-2 and Japanese encephalitis viruses changes significantly during morphogenesis: the intracellular form of the virus contains two membrane glycoproteins, one of which disappears from the extracellularly released virus, apparently replaced by small non-glycoprotein membrane polypeptide. The change in polypeptide composition from the intracellular form to the extracellular form appears independent of virion release mechanisms since virions with polypeptide composition similar to the intracellular form can be released from cells cultured in tris(hydroxymethyl)aminomethane buffer.

The seven Japanese encephalitis virus specific polypeptides found in infected chick embryo cells were all bound to membranes. None were completely released from the membranes by treatment with neutral salt, alkaline salt, or dilute detergent, but two of them were partially released by both the neutral and alkaline salts. The polypeptides were released or attacked by trypsin at unequal rates and in the sequence: NV-5 $\geq$ NV-4 > V-3. NV-5 was released as a relatively undegraded soluble polypeptide, NV-4 was extensively degraded, and V-3 was degraded but part of its trypsin-derived fragment (TF-2) remained membrane bound. We suggest that the three largest viral polypeptides are bound in such a manner that the larger the polypeptide, the more exposed and superficial it is. Treatment of virions with trypsin produced low molecular weight material and three discrete polypeptide fragments, probably all derived from the large virion envelope protein V-3; two (TF-1 and TF-3) had electrophoretic mobilities similar to the two naturally occurring nonvirion virus-specified polypeptides, NV-1 and NV-3.

Actinomycin D-treated, JE infected chick embryo cells that were or were not pulse-inhibited with cycloheximide synthesized qualitatively identical protein patterns. By contrast, infected cells pulse-inhibited with high concentrations of puromycin were irreversibly inhibited in the synthesis of non-glycosylated, non-structural proteins. When using radioactive glucosamine as a label, one of the previously-identified "non-virion" polypeptides, NV-2, was shown to be heterogeneous.

Solubilization of JE virus RHA and virus-infected membranes using non-ionic and ionic detergents results in a recovery of several immunologically active subunits with varying degrees of cross-reactivity within the JE virus subgroup. Immunodiffusion tests using detergent-treated JE RHA reveals the existence of at least two precipitin

reactions against homologous antibody and at least one reaction against heterologous antibody to members of the JE subgroup. Type and group specificity will be assigned to individual antigens after further characterization of individual viral subunits. Another method for solubilization of JE RHA, guanidine HCl chromatography, offers an analytical tool for determining viral protein molecular weight by a physical separation of viral subunits.

Dengue Type 3 infection of LLC-MK_2 cells has resulted in a chronically infected stable cell line monitored for more than 50 weekly passages. Virus titers in the supernatant fluids were low after the first 10 passages; however the highest titer observed at a given passage level was almost always detected within 24-48 hours after the cells were split. Specific immune fluorescence was detected in the chronically infected cells even in the absence of infective virus in culture fluids. Attempts to prepare CF and HA antigens from these cells were unsuccessful.

The presence of surface antigens on group A and B arbovirus infected cells was tested by immune cytolysis; the method is based on measurements of radioactive chromium released from infected cells after cellular injury is produced by antiviral antibody in the presence of complement. Release of chromium from LLC-MK_2 cells infected with Sindbis or Eastern equine encephalomyelitis (group A viruses) occurs when mature virions are first released from the cell. Release of chromium from dengue (group B) virus infected cells by immune cytolysis occurs much later after first release of virus. Negative immune cytolysis with antibody against the dengue soluble complement fixing antigen and positive immune cytolysis with antibody against purified virions suggests that only some of the viral specified proteins (at least the surface membrane proteins of the virion) are inserted in the plasma membrane.

The group C arboviruses Oriboca and Murutucu have been examined by polyacrylamide gel electrophoresis and found to be similar to the structural polypeptide composition of the Bunyamwera supergroup viruses BFS-283, Bunyamwera and Tahyna. All have three recognizable proteins of approximately 83,000, 29,000 and 20,000 daltons when compared to the Sindbis virus structueal protein markers of 50,000 and 30,000 daltons. A fourth protein has been observed on occasion, but preparations containing higher levels of radioactivity are required to determine its significance. Preliminary studies on the isolation of bromelain released glycoproteins of Sindbis viruses by Biogel P-6 column chromatography indicate that adequately labeled glycoproteins may be obtained for antigenic analysis.

Isoelectric analysis of purified Sindbis virus has demonstrated a pI of 4.8 for the virion. Treatment of the Sindbis virion with Triton X-100 and electrofocusing in low concentrations of the non-ionic detergent resolved two major peaks of envelope derivation (pI 6.1 and 9.2) and a diffuse core polypeptide near the acid end of the gradient.

Both envelope fractions gave precipitin arcs in Ouchterlony gels, fixed complement with homologous antisera but only the pI 6.1 peak served as an efficient hemagglutinin. RIP tests showed the Sindbis pI 6.1 antigen to be cross reactive with WEE antiserum, while the pI 9.2 antigen was specific and reacted only with Sindbis antibody.

VI. The following summarizes the transmission of Eastern and Western equine encephalomyelitis (EEE and WEE) viruses in the Pocomoke Cypress Swamp (PCS) during 1970 and 1971. During 1970 the transmission of EEE virus was first detected around mid-June and was interrupted for approximately one month. The interruption in EEE virus transmission coincided with an abrupt decrease in the first early population of C. melanura. From mid-July, sentinel quail and immature wild birds showed comparable antibody prevalence rates to either virus, although four-fold more WEE virus was isolated from C. melanura. Virus transmission corrolated well with observed fluctuations in density of C. melanura, with the exception of increased virus transmission during November when densities of C. melanura were very low. During 1971, WEE virus transmission beginning during late June preceeded that of EEE virus which was confined to the months of August and September. The onset and duration of quail injections corresponded closely to those time periods when C. melanura was at peak density, including increased infection rates during November which followed a remarkable late October increase in C. melanura density. An analysis of seasonal avian densities in the PCS for 1968 to 1971 showed a steady decline in the total bird population from late June to early September. This decline was interpreted as post-breeding dispersal of adults, which would decrease the adult to immature ratio every summer and enhance virus amplification.

The following were observations on virus ecology outside the PCS during 1970 and 1971. At one, two and three mile sites outside the PCS, relatively high proportions of C. melanura were periodically captured during the summer of 1970. Sentinel quail were comparably infected with EEE and WEE viruses at each of these sites, although the level and duration of transmission was reduced from that observed in the PCS. During 1970 EEE and WEE virus infections occurred in resident immature wild birds captured in a salt marsh habitat located six miles southwest of the PCS. The predominant mosquito collected throughout the summer here was Anopheles bradleyi-crucians, and C. melanura was virtually absent. During August 1971, antibody against EEE and WEE viruses was observed in wild birds captured in the Croatan Forest, N. C. The species of birds were similar to those captured during the summer in the PCS. Seventeen EEE and two WEE viruses were isolated from female C. melanura, and EEE virus was recovered from one pool of female Anopheles bradleyi-crucians.

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
DA 0A 6442	72 07 01	DD-DR&E(AR)636

3. DATE SUMMARY ENTERED	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. GROUP INDICATOR	9. SPECIFIC DATA CONTRACTOR ACCESS	10. LEVEL OF SUM
71 07 01	D. Change	U	U	NA	NL	YES NO	A. WORK UNIT

11. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A061102B71Q	00	167
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114(f)			

12. (Rickettsial) Diseases of Military Personnel (09)

13. SCIENTIFIC AND TECHNOLOGICAL AREAS: 010100 Microbiology

14. START DATE	15. ESTIMATED COMPLETION DATE	16. FUNDING AGENCY	17. PERFORMANCE METHOD
55 08	CONT	DA	C. In-House

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 71	3	150
FISCAL YEAR CURRENT 72	3	200

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PII Redacted

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Provide each with Security Classification Code)
(U) Rickettsial Infections; (U) Laboratory Diagnosis; (U) Vaccines; (U) Epidemiology; (U) Ecology; (U) Vectors and Reservoirs

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

23. (U) 1. Prevention of scrub typhus. 2. Evaluation of the military importance of R. canada. 3. Improvement of methods for serologic diagnosis of rickettsial diseases.

24. (U) 1. (a) Formulation of a polyvalent vaccine to immunize against antigenically heterogeneous scrub typhus strains in nature. 1. (b) Development of tissue culture plaque assay system for cloning of candidate vaccine strains. 2. (a) Surveillance of enzootic rickettsial diseases at Fort Bragg, NC and Fort Leonard Wood, MO. 2. (b) Investigation of Typhus Group rickettsial infections in flying squirrels in VA. 3. Development of a screen test for laboratory detection of rickettsial infections.

25. (U) 71 07-72 06. 1. (a) Antigenic analysis of major and minor components of strains by indirect immunofluorescence has defined the extent of antigens shared among the eight prototypes. Theoretically, a vaccine containing as few as three or as many as six strains should be highly effective. 1. (b) A reliable method for plaquing of candidate vaccine strains has been developed and the process of cloning of the prototypes to insure purity is nearly completed. 2. (a) Serologic evidence of enzootic R. rickettsi was found at Fort Bragg in 5 of 14 areas surveyed. At Fort Leonard Wood, Typhus Group and Spotted Fever Group rickettsiae are enzootic. 2. (b) Flying squirrels infected with a Typhus Group rickettsia have been identified in areas of VA and MD remote from the site of the original observation. 3. A screen test antigen comprised of a mixture of representatives of all groups of rickettsiae for use in indirect fluorescent antibody tests has been successfully field-tested in Vietnam. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 71 - 30 Jun 72.

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 167 Rickettsial Diseases of Military Personnel

Investigators.

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Description.

During the current reporting period, research activities have been concerned with: (1) the scrub typhus vaccine development program including: (a) characterization of the antigenic interrelationship among three prototype and five candidate prototype strains of R. tsutsugamushi; (b) comparison of the antigenic composition of homotype strains with their respective prototype strains; (c) evaluation of the theoretical composition of polyvalent scrub typhus vaccines; (d) preparation of type-specific anti-scrub typhus fluorescein conjugates for rapid characterization of strains of R. tsutsugamushi; (e) development of a plaque assay system for cloning prototype and homotype strains of R. tsutsugamushi; (2) evaluation of the existing and potential military importance of R. canada by: (a) continued surveillance of enzootic rickettsial infections in Fort Bragg, North Carolina, and Fort Leonard Wood, Missouri; and (b) investigations of the etiology of the typhus group rickettsial infection in flying squirrels in Virginia; (3) improvement of methods for serologic diagnosis of rickettsial diseases by: (a) development of a single screen test for detection of all diseases and (b) conducting a field trial in the 9th Medical Laboratory; and (4) study of rickettsial infections of military dogs in combat areas.

Progress.

1. Scrub Typhus Vaccine Development

Prior to 1964, the prospects of immunizing man for protection against scrub typhus were believed to be slight because of (a) the unknown magnitude of antigenic heterogeneity that existed among strains of Rickettsia tsutsugamushi in nature; and (b) the finding that active infection with one strain controlled by immunochemoprophylaxis with chloramphenicol did not offer protection against subsequent disease caused by an antigenically diverse strain for more than a few months (1). When Japanese workers reported that all

strains of R. tsutsugamushi recovered from patients, vector chiggers and wild animals in Japan were classified as one of three antigenic types (2), the potential value of formulating a polyvalent vaccine with the proposed prototypes could not be disregarded. The studies undertaken to demonstrate the antigenic relationship of 78 strains of scrub typhus rickettsiae recovered in Thailand to the prototype Karp, Gilliam and Kato strains which resulted in the recognition of five other distinctive types, i. e., TA678, TA686, TA716, TA763 and TH1817, have been described in previous annual reports. Although each of the eight prototype strains contained dominant antigens which were unique, nothing was known about minor antigen components. The success of the indirect immunofluorescent test for the diagnosis of human disease using a mixture of the Karp, Gilliam and Kato strains as antigens indicated there was considerable duplication in the antigenic composition of wild strains. A polyvalent killed vaccine that would be expected to provide a reasonable degree of protection against disease should contain antigens representative of all those present in the different antigenic types. Therefore, before a polyvalent vaccine could be formulated, the minor antigenic components of the prototype strains had to be identified so that the fewest number of strains could be used to provide the broadest degree of protection.

a. Antigenic Interrelationship Among Prototype Strains of R. tsutsugamushi.

Indirect immunofluorescence was used to assess the extent of the sharing of antigens among the prototype strains. Since it was expected that the antibody response to infection would vary with different animal species, immune rabbit and guinea pig sera were employed. Groups of three rabbits were infected with yolk sac suspensions of each of the eight prototype strains of R. tsutsugamushi and serum was collected 28 days after inoculation. Since the immune rabbit serum was to be used also for the preparation of direct-staining fluorescein conjugates (see below), it was necessary that a homologous titer of 1:2560 be attained. In order to obtain this level of antibodies, the rabbits inoculated with Kato and TH1817 were given one booster dose; those inoculated with TA686 required three extra doses; and those infected with TA678, six additional doses. Booster inoculations were administered at 2 to 4 week intervals. Immune serum was collected from groups of three guinea pigs 28 days after intracerebral inoculation with spleen suspensions from mice infected with the eight prototype strains. Indirect fluorescent antibody (IFA) tests were carried out with serial 4-fold dilutions of the serum and antigens comprised of smears of suspensions of yolk sacs infected with the respective strains. In order to insure that optimum homologous and heterologous reactivity was detected, only recently prepared antigen smears were used which had not been stored for longer than one month at 4 C in sealed

containers with silica gel. Prior tests established that deterioration of the antigen smears did not occur under these conditions. Antirickettsial antibodies in rabbit and guinea pig serum were determined with a goat anti-rabbit fluorescein conjugate, and a rabbit anti-guinea pig fluorescein conjugate, respectively.

Some variation in the response of animals receiving the same inoculum was apparent. Differences were found in the magnitude of the titer of antibodies reacting with the same antigens. Also, serum from one or two animals in the group reacted with more heterologous antigens than the others.

Table 1 summarizes the results obtained. The values presented are the geometric means of the titers of the sera from animals receiving the same inoculum. In each block the top number refers to rabbit sera and the bottom number to guinea pig sera. The geometric mean titers are indicative of both the antigenicity of the strain as well as of the host responsiveness and can be used to assess antigenic interrelationship.

If it is assumed that the magnitude of the homologous antibody response is an index of the concentration of the dominant antigens, then the proportional amount of heterologous antibody reactivity is an index of the antigens shared. In Table 2, the homologous titer was assigned an arbitrary value of 1000 and the titers with heterologous antigens adjusted accordingly. A theoretical limitation of this method of analysis was encountered when the homologous geometric mean titer was less than 1000 as in the results of tests with sera from guinea pigs infected with TA678 and TA686 (see Table 1). In the course of calculating the relative antibody titer, negative results were artifically made positive suggesting possible relationships that may not have existed. However, since the assigned values were still relatively low, they were not considered significant. Some differences attributed to host species response were evident between the relative heterologous reactivity of the sera from rabbits and guinea pigs infected with the same strain. However, 4-fold or less differences between respective geometric mean titers were not considered significant. It was not possible to generalize about which animal species exhibited the more type-specific response. The finding of reactivity of the prototype immune sera with a heterologous antigen which gave a relative titer of 250 or greater was interpreted as indicating that a significant antigenic mass was shared by the two strains. Differences in the concentrations of the antigens shared by the respective strains were manifested by the reciprocal heterologous reactivity. For example, relative to the dominant homologous antigens, TA763 had a greater amount of the antigens shared with Karp than were present in the Karp strain. It was not possible to make any conclusions about the identity of the antigens shared except when the heterologous

TABLE 1

ANTIGENIC ANALYSIS OF CANDIDATE PROTOTYPE SCRUB

TYPHUS STRAINS BY INDIRECT IMMUNOFLUORESCENCE

Immune Animal* Sera	Geometric Mean Antibody Titers Antigens							
	Karp	Gilliam	Kato	TA678	TA686	TA716	TA763	TH1817
Karp	10240	426	640	426	426	1016	426	1613
	1613	108	1016	63	254	403	160	403
Gilliam	1613	25850	1613	2560	4064	1613	1613	2560
	108	6450	160	160	108	108	25	254
Kato	640	254	2560	640	1016	1016	640	640
	320	80	2560	160	320	640	80	320
TA678	320	320	320	2560	1600	320	320	640
	40	10	101	403	25	40	10	25
TA686	254	254	426	254	4064	1016	1016	426
	40	63	160	63	640	160	25	160
TA716	1613	426	1613	1016	6450	6450	2560	1613
	254	160	1016	160	1016	2560	254	160
TA763	1016	254	1613	426	6450	4064	6450	640
	640	160	254	403	640	640	1016	640
TH1817	426	160	254	160	1613	160	426	2560
	160	40	160	160	63	160	63	2560

* Top number is geometric mean of antibody titers of immune rabbit sera.
Bottom number is geometric mean of antibody titers of immune guinea pig sera.

TABLE 2

ANTIGENIC ANALYSIS OF CANDIDATE PROTOTYPE SCRUB

TYPHUS STRAINS BY INDIRECT IMMUNOFLUORESCENCE

Immune Animal* Sera	Relative Antibody Titer with Antigens							
	Karp	Gilliam	Kato	TA678	TA686	TA716	TA763	TH1817
Karp	1000	42	63	42	42	99	42	158
		67	630	39	157	250	99	250
Gilliam	62	1000	62	99	156	62	62	100
	17		25	25	17	17	[illegible]	39
Kato	250	99	1000	250	396	396	250	250
	125	31		63	125	250	31	125
TA678	125	125	125	1000	625	125	125	250
	99	23	251		62	99	23	62
TA686	62	62	104	62	1000	250	250	104
	63	98	250	98		250	39	250
TA716	250	66	250	157	1000	1000	397	250
	99	63	357	63	397		99	63
TA763	157	39	250	66	1000	630	1000	99
	630	157	250	397	630	630		630
TH1817	166	62	100	62	630	62	166	1000
	63	16	63	63	25	63	25	

* Top number is calculated from results of tests with immune rabbit sera.
Bottom number is calculated from results of tests with immune guinea pig sera.

reactivity was of the same magnitude as the homologous reactivity. If the cross-reactivity of the immune sera and antigens of TA686, TA716 and TA763 are examined, it is evident that the reactivity of the TA716 and TA763 immune sera with the TA686 antigen was essentially the same as the respective homologous reactivity. The most likely explanation for these findings is that strains TA716 and TA763 contain the dominant antigens of strain TA686.

b. Antigenic Composition of Homotypes of R. tsutsugamushi.

Strains of R. tsutsugamushi were considered prototypes when complement-fixing antigens comprised of partially purified suspensions of rickettsial organisms reacted only with the homologous immune sera produced by the intracerebral inoculation of guinea pigs and not with heterologous immune sera. The problems of producing type-specific antigens from the prototype strains other than Karp, Gilliam and Kato have been described in previous reports and they still prevail. Therefore, classification of wild strains of R. tsutsugamushi has been limited to complement fixation tests of the respective immune guinea pig sera with the Karp, Gilliam and Kato antigens. When the serum from all three guinea pigs infected with the same wild strain fixed complement to a significant titer with only one of the prototype antigens, the strain was tentatively classified as a homotype. In order to determine the similarity of the minor antigenic composition of strains having the same dominant antigens, two homotypes of Karp (TA583 and TC765), three homotypes of Gilliam (TC586, TA675 and TA688) and one Kato homotype (TH1826) were selected from among the Thai collection for further study. In addition, four homotype strains of Kato recovered from single chiggers of the naturally infected colony of Leptotrombidium (L.) akamushi maintained in Kuala Lumpur by the U. S. Army Medical Research Unit, were analyzed. Each of the homotype strains had to be adapted from propagation in mice to cultivation in the yolk sac of embryonated hens' eggs. Serial passages were made in eggs until sufficient numbers of organisms were obtained for the preparation of antigen smears for indirect immunofluorescent tests. The growth of TH1826 in eggs is not yet satisfactory and serial passages are being continued. Indirect immunofluorescent tests were carried out in the following manner. Each immune serum from the groups of three guinea pigs and three rabbits infected with each of the homotypes was examined for reactivity with antigens prepared from each of the eight prototype strains as well as from each of the nine homotypes under study. Also, the prototype immune rabbit and guinea pig sera mentioned earlier were tested for reactivity with each homotype antigen.

The results of these studies are summarized in Tables 3, 4, 5 and 6. The relative antibody titers were calculated on the basis of assigning the homologous titer a value of 1000. Before the data could be evaluated to determine if the antigenic composition of the homotype is the same as the corresponding prototype, the significance of differences between values had to be established. The reproducibility of the IFA procedure in replicate tests with the same reagents provided individual results which did not vary more than 2-fold from the geometric mean of all the results. When the geometric means of the homologous titers of the immune rabbit sera for each homotype and its corresponding prototype were compared with the geometric mean of all the individual rabbit sera in the respective groups, less than a 2-fold difference was found. Variation expected between geometric mean titers of groups of guinea pigs infected with the same strain was of the same magnitude. Therefore, differences of 4-fold or greater among the relative antibody titers were considered significant.

Table 3 summarizes the results obtained when the immune rabbit and guinea pig sera of Karp and its homotypes TA583 and TC765 were tested with the respective antigens as well as with the seven other prototype antigens. Inspection of the reactivity among Karp and its homotype immune rabbit sera and the corresponding antigens shows that although the strains share antigens in common, TA583 a d TC765 are significantly different not only from Karp, but from each other. Indeed, the cross-reactivity displayed was not unlike that found previously among the prototype strains TA686, TA716 and TH1817 (see Table 2). It is possible that TA583 and TC765 contain dominant antigens that are different from those in the eight prototype strains. The reactivity of the homotype immune rabbit sera with the other seven prototype antigens also reveals significant differences among the strains (Table 3). TC765 is differentiated from TA583 and Karp by its reactivity with the Kato antigen. The differences between the reactivity of the TA583 and Karp immune sera with the TA686 and TH1817 approaches closely the level of significance.

Tests with the guinea pig immune sera reveal only a few differences between Karp and its homotypes. It is known that immune sera from different animal species will differ in the ability to distinguish among antigenically closely related organisms by serologic means. However, the immunologic importance of the differences reported here between the antigenic composition of a prototype and its homotype remains to be evaluated.

TABLE 3
COMPARISON OF ANTIGENIC COMPOSITION OF HOMOTYPES OF KARP STRAINS

OF SCRUB TYPHUS BY INDIRECT IMMUNOFLUORESCENCE

Immune Animal Sera	Relative Antibody Titer with Antigens								
	Karp	TA583	TC765	Gilliam	Kato	TA678	TA686	[illegible]	[illegible]
Rabbit Immune Sera									
Karp	1000	395	158	42	63	42	[illegible]	[illegible]	[illegible]
TA583	157	1000	630	15	62	40	157	[illegible]	[illegible]
TC765	397	1595	1000	63	397	39	251	[illegible]	[illegible]
Guinea Pig Immune Sera									
Karp	1000	1000	1000	67	630	39	157	250	[illegible]
TA583	158	1000	396	99	158	99	99	250	158
TC765	630	1588	1000	25	250	66	158	1000	153

TABLE 4

COMPARISON OF ANTIGENIC COMPOSITION OF HOMOTYPES OF GILLIAM STRAINS OF SCRUB TYPHUS BY INDIRECT IMMUNOFLUORESCENCE

Immune Animal Sera	Relative Antibody Titer with Antigens										
	Gilliam	TC586	TA675	TA688	Karp	Kato	TA678	TA686	TA716	TA763	TH1817
Rabbit Immune Sera											
Gilliam	1000	1000	396	1000	62	62	99	156	62	62	100
TC586	500	1000	1000	1000	31	31	63	31	31	3	31
TA675	1000	1587	1000	2532	158	158	63	158	63	25	158
TA688	398	100	250	1000	15	26	9	9	15	3	15
Guinea Pig Immune Sera											
Gilliam	1000	250	1000	250	17	25	25	17	17	4	39
TC586	630	1000	630	1000	16	16	16	10	16	2	25
TA675	397	1000	1000	1000	99	63	99	63	166	63	250
TA688	1000	1000	630	1000	63	63	158	63	158	63	158

Table 4 summarizes the results of IFA tests to compare the antigenic composition of the prototype Gilliam and its homotypes, TC586, TA675 and TA688. The tests with rabbit immune sera clearly differentiate TA688 from Gilliam and the other two homotypes. Differences between TC586 and Gilliam were evident only in the heterologous reactivity of the rabbit immune sera and the TA686 and TA763 antigens. No significant differences were evident between TA675 and Gilliam when the reactivity of the respective rabbit immune sera were compared, and only differences in minor antigenic composition were revealed in tests with the immune guinea pig sera.

The results of the comparison of the antigenic composition of Kato and its homotypes, MF2334, MF2569, MF2624, MF2651 are presented in Table 5. Tests with the rabbit immune sera reveal differences between the dominant antigenic composition of MF2651 and MF2624. On the basis of minor antigenic components, MF2624 is almost identical to the prototype Kato, and the other three homotypes are more closely related to each other than to the prototype strain.

Additional information about differences in antigenic composition among the homotypes and the corresponding prototype was obtained when the prototype immune rabbit and guinea pig sera were tested with the homotype antigens (see Table 6). If all of the homotypes had the same antigenic composition as the prototype, the relative antibody titer of an immune serum with the different antigens should not vary more than 4-fold. No significant differences were found between the reactivity of the prototype immune rabbit sera and the Karp homotypes with the exception of Karp sera with TC765 antigen which was described previously. Tests with the Kato guinea pig immune sera differentiated Karp from its homotypes, and differences between TA583 and TC765 were evident in tests with the TA678 immune guinea pig sera. Similar differences were found which differentiated among the prototype and the respective homotypes of Gilliam and Kato.

c. Formulation of Experimental Scrub Typhus Vaccines.

If it is assumed that the degree of cross-reactivity exhibited by immune sera with heterologous antigens in the IFA test is indicative of the amount of heterologous antigen relative to the homologous antigen, and that the antibody response to a mixture of strains would be equal to the sum of the components, then the data summarized in Tables 2 through 6 could be used to select strains for incorporation into experimental vaccines. It was recognized that the relationship between indirect fluorescent antibody response and resistance to infection has not yet been

TABLE 5

COMPARISON OF ANTIGENIC COMPOSITION OF HOMOTYPES OF KATO STRAINS OF SCRUB TYPHUS BY INDIRECT IMMUNOFLUORESCENCE

Relative Antibody Titer with Antigens

Immune Animal Sera	Kato	MF2334	MF2569	MF2624	MF2651	Karp	Gilliam	TA678	TA686	TA716	TA763	TH1817
Rabbit Immune Sera												
Kato	1000	396	250	250	250	250	99	250	396	396	250	250
MF2334	396	1000	250	250	628	62	10	25	16	62	16	25
MF2569	396	630	1000	397	1000	157	66	66	99	66	66	66
MF2624	1000	4000	2000	1000	2000	500	31	250	250	250	500	250
MF2651	396	396	250	99	1000	39	16	39	39	62	10	39
Guinea Pig Immune Sera												
Kato	1000	2000	1000	1000	1000	125	31	63	125	250	31	125
MF2334	500	1000	1000	250	500	125	31	31	63	125	31	125
MF2569	1587	1000	1000	1000	1000	250	63	63	99	250	63	250
MF2624	2000	1000	500	1000	1000	125	31	63	63	125	31	125
MF2651	1000	1000	397	630	1000	63	25	16	63	105	105	63

TABLE 6

COMPARISON OF ANTIGENIC COMPOSITION OF SELECTED PROTOTYPE AND THEIR HOMOTYPE STRAINS OF SCRUB TYPHUS BY INDIRECT IMMUNOFLUORESCENCE

Immune Animal Sera	Relative Antibody Titer with Antigens											
	Karp Homotypes			Gilliam Homotypes				Kato Homotypes				
	Karp	TA583	TC765	Gilliam	TC586	TA675	TA688	Kato	MF2334	MF2569	MF2624	MF2651
Rabbit Immune Sera												
Karp	1000	395	158	42	99	42	42	63	42	63	42	42
Gilliam	62	39	39	1000	1000	396	1000	62	39	39	25	39
Kato	250	250	250	99	250	25	15	1000	396	250	250	250
TA678	125	34	125	125	125	63	125	125	250	250	125	250
TA686	62	104	104	62	104	62	62	104	25	25	25	25
TA716	250	627	397	66	157	99	66	250	250	99	157	250
TA763	157	157	157	39	66	15	10	250	39	157	66	66
TH1817	166	630	250	62	62	39	62	100	166	166	100	166
Guinea Pig Immune Sera												
Karp	1000	1000	1000	67	39	99	157	630	264	264	264	264
Gilliam	17	17	17	1000	250	1000	250	25	39	39	16	39
Kato	125	1000	1000	31	250	1000	250	1000	2000	1000	1000	1000
TA678	99	59	376	23	251	376	94	251	376	376	94	376
TA686	63	250	250	98	250	250	63	357	666	250	159	250
TA716	99	250	99	63	63	99	40	397	166	99	63	63
TA763	630	1588	1000	157	250	419	419	250	630	630	630	630
TH1817	63	63	99	16	42	99	63	63	397	397	63	397

established. Furthermore, there is no information available which would permit a comparison of the antibody response of man to that of the rabbit and guinea pig. Consequently, two evaluations were made: (a) one in which the higher of the antibody responses exhibited by either rabbits or guinea pigs might be representative of man; and (b) another in which the lowest antibody response was used. Table 7 lists the fewest number of strains that in combination might be expected to contain concentrations of antigens equivalent to the dominant components of the eight prototype strains. This optimistic formulation would include Karp, TA675 (a Gilliam homotype), TA678 and TA763. If the latter criterion is used, then as many as six strains might be required for a polyvalent vaccine. In this pessimistic evaluation, it would be necessary to combine Kato and TH1817 with the four previously mentioned strains illustrated in Table 8. However, this formulation might be deficient in Gilliam since the sum of the component antigens would only elicit slightly more than half of homologous relative antibody titer.

As mentioned previously, these analyses were based upon the relative concentrations of homologous and heterologous antibodies in the same immune sera. The values obtained indicated the amount of antigens shared with other prototype or homotype strains relative to the dominant antigen of the immunizing strain. The relative concentrations of antigens are an important consideration in the formulation of killed polyvalent vaccines. However, this treatment of the data does not show the relationship between the titer of the antibodies reacting with a heterologous prototype antigen, and the level of homologous antibodies produced by infection with that same prototype strain. The data obtained with the immune rabbit sera in Table 1 was re-examined in order to define this relationship, and the result of the analysis is summarized in Table 9. Again, the homologous antibody titers were assigned the value of 1000, but the values for the heterologous titers were relative to the magnitude of the homologous antibody response of the antigen strain. Thus, infection with the Gilliam strain evoked levels of antibody against TA678, TA686 and TH1817 that were identical to the homologous antibody responses of rabbits infected with each of these strains. It may be recalled that in Table 2, the concentration of antigens in Gilliam shared with these other prototype strains was found to be small relative to the amount of the dominant antigens. Selection of strains for incorporation into the polyvalent vaccines described in Tables 7 and 8 was based upon the concentrations of shared antigens relative to the dominant antigen. If performance of a polyvalent vaccine is more closely related to the magnitude of the heterologous antibody then a different formulation would be possible. As shown in Table 10, infection with a combination of Karp, Gilliam and TA763 would be

TABLE 7
EXPECTED ANTIBODY RESPONSE TO COMPONENTS AND

COMPOSITE POLYVALENT SCRUB TYPHUS VACCINE

OPTIMISTIC EVALUATION

Candidate Vaccine Strain	Relative Antibody Titers to Prototype Strains								
	Karp	Gilliam	Kato	TA675	TA678	TA686	TA716	TA763	TH1817
Karp	1000	67	630	99	42	157	250	99	250
TA675	158	1000	158	1000	99	158	166	63	250
TA678	125	125	251	376	1000	625	125	125	250
TA763	630	158	250	419	397	1000	630	1000	630
TOTAL	1913	1349	1289	1894	1538	1940	1171	1287	1380

TABLE 8

EXPECTED ANTIBODY RESPONSE TO COMPONENTS AND

COMPOSITE POLYVALENT SCRUB TYPHUS VACCINE

PESSIMISTIC EVALUATION

Candidate Vaccine Strains	Relative Antibody Titers to Prototype Strains								
	Karp	Gilliam	Kato	TA675	TA678	TA686	TA716	TA763	TH1817
Karp	1000	42	63	42	39	42	99	42	158
Kato	125	31	1000	25	63	125	250	31	125
TA675	99	397	63	1000	63	63	63	25	158
TA678	99	23	125	63	1000	62	99	23	62
TA763	157	39	250	15	66	630	630	1000	99
TH1817	63	16	63	39	63	25	63	25	1000
TOTAL	1543	548	1564	1184	1294	947	1204	1146	1602

TABLE 9
RELATIVE CONCENTRATIONS OF TYPE-SPECIFIC

ANTIGENS IN PROTOTYPE SCRUB TYPHUS STRAINS

Prototype Strains	Antibody Titer Relative to Magnitude of Homologous Response							
	Karp	Gilliam	Kato	TA678	TA686	TA716	TA763	TH1817
Karp	1000	16	250	166	105	158	66	630
Gilliam	157	1000	630	1000	1000	250	250	1000
Kato	63	10	1000	250	250	158	99	250
TA678	31	12	125	1000	394	50	50	250
TA686	24	10	166	99	1000	158	158	166
TA716	158	16	630	397	1587	1000	397	630
TA763	99	10	630	166	1587	630	1000	250
TH1817	42	6	99	63	397	25	66	1000

expected to elicit levels of antibody equal to or greater than the homologous antibody response of each of the eight prototype strains. Experiments are in progress to determine which of the three possible formulae for polyvalent vaccines offers the broadest degree of protection against other prototype strains and wild strains of R. tsutsugamushi.

d. Preparation of Type-Specific Anti-Scrub Typhus Fluorescein Conjugates.

Studies were undertaken by the U. S. Army Medical Research Unit (Malaysia) in collaboration with the Department of Rickettsial Diseases to determine if changes in antigenic composition of R. tsutsugamushi occurred during transstadial and transovarial transmission in vector mites, or during periods of chronic latent infections in vertebrate reservoirs. These investigations required development of methods for rapid, type-specific identification of strains of scrub typhus. Similar methods were required also for the antigenic characterization of clones of prototype and homotype strains selected by a tissue culture plaque assay system (see below). The limitations of the current methods for identification of an organism as a species of R. tsutsugamushi and the subsequent characterization of the antigenic relationship to prototype subspecies strains were described in detail last year and will not be repeated here. Efforts initiated previously were continued to conjugate immune sera with fluorescing dyes to produce a series of reagents that would react specifically with the homologous organisms and permit subspecies identification of each of the prototype strains of R. tsutsugamushi.

Groups of three rabbits were infected intraperitoneally with about $10^{8.3}$ 50% mouse infectious doses of each of the prototype strains. Serum was obtained 28 days later and tested for homologous antibody by indirect immunofluorescence. Previous experience had shown that rabbit serum had to have an antibody titer of 1:2560 or greater in order to prepare a satisfactory conjugate. Rabbits immunized with Kato, TA678, TA686 and TH1817 were given repeated booster doses until the desired titers were attained (see section 1.a).

The method reported last year for conjugating fluorescein to antibody was modified and the following procedure gave more uniform and predictable results: To 10 ml of cold rabbit serum was added an equal volume of cold isotonic saline. To the mixture, 20 ml of cold saturated ammonium sulfate was added, dropwise, and the precipitate that formed was mixed continually in an ice-water bath for 1 hour. The precipitated globulins were

TABLE 10
EXPECTED ANTIBODY RESPONSE TO COMPONENTS

AND COMPOSITE POLYVALENT SCRUB TYPHUS VACCINE

Candidate Vaccine Strain	Relative Antibody Titers to Prototype Strains							
	Karp	Gilliam	Kato	TA678	TA686	TA716	TA763	TH1817
Karp	1000	16	250	166	105	158	66	630
Gilliam	157	1000	630	1000	1000	250	250	1000
TA763	99	10	630	166	1587	630	1000	250
Total	1256	1026	1510	1332	2692	1038	1316	1880

sedimented by centrifugation at 9,000 rpm for 15 min. The supernatant fluid was discarded and the sediment was dissolved in 10 ml of phosphate buffered saline pH7.2 (PBS). The globulins were precipitated again by adding 5 ml cold saturated ammonium sulfate dropwise and mixing continually for 1 hour. After centrifugation as described above, the globulins were precipitated again as in the previous step. The sedimented globulins obtained after centrifugation were resuspended in 4 ml of PBS and residual ammonium sulfate was removed by dialysis overnight. The protein content of the globulin solution was determined by the Biuret reaction. The concentration of globulin was adjusted to 1% protein by addition of isotonic saline and enough 0.5 M sodium bicarbonate-sodium carbonate buffer to yield 10% of the final volume and maintain the solution at pH10. To the cold globulin solution was added 1 mg of fluorescein isothiocyanate (FITC) for each 20 mg of protein. The mixture was stirred overnight at 4 C. Unlabeled FITC was removed by gel filtration of the conjugate through a column of Sephadex G25 (coarse). On occasion when tests with the conjugate revealed undesired background staining, this could be eliminated by filtration through Sephadex G50 (fine) with relatively little loss of specific reactivity.

Table 11 presents the results obtained when 2-fold serial dilutions of the anti-prototype FITC conjugates were applied to recently prepared smears of yolk sac suspensions of each of the eight prototype strains. The extinction titers given were the highest dilution of the conjugate which permitted definite recognition of rickettsial morphology although the intensity of fluorescence was slight. When freshly prepared antigen smears were used, more heterologous reactivity was encountered than was expected on the basis of previous experience. Indeed, it was found that acetone-fixed antigen smears stored at 4 C remained stable for a period of a month but thereafter, the titer of heterologous reactivity with the conjugates declined rapidly with a slower decline in titer of homologous reactivity.

Attempts were made to improve the specificity of the anti-prototype FITC conjugates by blocking heterologous reactivity with the corresponding immune rabbit serum. Initially, immune serum prepared against each of the eight prototype strains was tested for its ability to reduce the titer of each of the conjugates with homologous and heterologous antigens in the following two-step procedure. Undilute immune serum was applied to the antigen smears and incubated at 37 C for 1 hour. After washing the smears, serial 2-fold dilutions of the conjugate were applied and the overlay incubated at 37 C for 30 min. The smear was washed again, air-dried, mounted with buffered glycerine and a coverglass and examined with the fluorescent

TABLE 11

SPECIFICITY OF ANTI-SCRUB TYPHUS FLUORESCEIN CONJUGATES

FITC** Conjugate	Blocking Immune Sera	Extinction Titer with Antigens*							
		Karp	Gilliam	Kato	TA678	TA686	TA716	TA763	TH1817
Karp	None	512	<64	256	<64	512	256	256	128
	TA678 TA686	512	<64	<64	<64	<64	<64	<64	64
Gilliam	None	<128	1024	<128	<128	<128	<128	<128	<128
Kato	None	512	<64	<64	<64	512	512	128	<64
	Karp TA686	<64	<64	<64	64	64	<64	<64	<64
TA678	None	<16	<16	<16	128	<16	<16	<16	<16
TA686	None	<16	<16	32	<16	128	128	<16	<16
	Kato TA763	<16	<16	<16	<16	<16	<16	<16	<16
TA716	None	128	<64	512	<64	512	512	256	<64
	Kato TA763	64	<64	<64	<64	<64	512	<64	<64
TA763	None	<64	<64	256	<64	512	512	512	<64
	Gilliam	<64	<64	<64	<64	256	256	256	<64
TH1817	None	256	<32	256	<32	<32	128	128	256
	Karp Gilliam	<32	<32	<32	<32	<32	<32	<32	256

*Highest dilution giving definite reactivity

**Fluorescein isothiocyanate conjugated rabbit immune sera

microscope. The results obtained from these experiments were not valid because the 1 1/2 hour period of incubation had the effect of accelerating the antigen deterioration observed with aging. Consequently, the experiments were repeated by mixing the heterologous immune sera with the FITC conjugate so that the final dilution of the former was 1:4 in the presence of each of the 2-fold serial dilutions of the conjugate. Evan's Blue in a final concentration of 0.005% was also incorporated into the mixture as a counterstain. Each mixture was applied to the antigen smear and incubated for 30 min. The remainder of the procedure was the same.

As can be seen in Table 11, it was possible in most instances by the selection of the appropriate sera to reduce heterologous activity to a titer that was 8-fold or lower than the homologous titer without appreciably decreasing the homologous titer. The notable exception was the heterologous staining of TA686 and TA716 antigen with the TA763 FITC conjugate which could not be significantly reduced by any of the prototype immune sera without causing a corresponding decrease in homologous activity. It was of interest to note that heterologous reactivity of some of the conjugates with prototype antigens could be markedly reduced by immune sera that did not correspond to the strain stained by the heterologous reaction. For example, the staining of Kato, TA686, TA716, TA763 and TH1817 antigens with the Karp FITC conjugate was diminished significantly by combining TA678 and TA686 immune sera. Although TA678 antigen did not react with the Karp conjugate, the TA678 serum blocked the staining of other heterologous antigens. This effect of immune serum in blocking homologous and heterologous reactivity of the FITC conjugates is being evaluated to determine if it will provide more specific information about the identity of the antigens shared by prototype strains.

e. Plaque Assay System for R. tsutsugamushi.

The principal requirement for a plaque assay procedure was (a) to clone all prototype and candidate prototype strains of R. tsutsugamushi in order to insure their purity; and (b) to confirm the existence of mixtures of different antigenic types in certain scrub typhus strains recovered from man, rodents and vector chiggers in Thailand. The problems that were encountered when attempts were made to duplicate the work of Weinberg et al. (3) and McDade et al. (4, 5, 6) with primary chick embryo fibroblasts were detailed in the annual report last year. Difficulties attributed to inadequate wetability of the growth surface of plastic culture vessels were identified. It was noted that significant deterioration

of the growth surface occurred during a period of 3 to 6 months after manufacture. Moderate success had been achieved with R. rickettsi which produced plaques 5 to 6 days after inoculation and to a lesser extent with R. mooseri where plaques were formed after 10 to 11 days. Comparison of the titers of seed suspensions of these organisms showed the tissue culture plaque system to be greater than 10-fold more sensitive than embryonated hens' eggs for assaying infectivity. A major problem that was yet to be resolved was the maintenance of the cell monolayers for the 16 to 18 days required for the production of plaques by R. tsutsugamushi.

All of the various conditions for preparation, growth and infection of the chick embryo cells were evaluated. In addition to an absolute requirement for recently manufactured plastic flasks, the following important changes were made in the original procedure: (a) 0.2 ml of the inoculum was incubated on the monolayer of the chick embryo fibroblasts for 30 min at 37 C (instead of 15 min at room temperature); (b) Leibovitz L-15 medium, which has a greater buffering capacity, was substituted for Medium 199 in the agarose overlay which contained also 5% calf serum; (c) the volume of the nutrient overlay was increased to 10 ml which maintained the cells better than the prescribed 5 ml; and (4) after infection and application of the overlay, the flasks were incubated at 33.5 C instead of 32 C. The monolayers were stained on day 17 or 18 by overlayering with 5 ml of 0.5% agarose and 0.1% neutral red in L-15 medium and returned to the incubator. The plaques were visible generally within 24 to 48 hours but occasionally were not seen until after 4 days. With this procedure, it has been possible to regularly produce plaques with all R. tsutsugamushi strains.

Serial 10-fold dilutions of yolk sac suspensions of each of the prototype strains of R. tsutsugamushi were prepared in L-15 medium with 5% calf serum. Each dilution was inoculated into three cell culture flasks and intraperitoneally into five mice. The 50% infectious dose of the suspensions for mice was determined by challenge of all surviving animals with 1000 LD_{50} of the lethal Karp strain. All mice not killed by this dose were presumed to have been immune as the result of a prior inapparent infection.

The plaque titers of yolk sac suspensions of each of the eight prototype strains are compared in Table 12 with the infectivity titers obtained in mice. TC586, a Gilliam homotype, was included also. Generally, tissue culture was slightly more sensitive than the mouse for assaying infectivity. With the exception of Kato, TA678 and TA763, where the titers were essentially the same, the values obtained by plaque assay were at least 0.6 log higher than the mouse ID_{50} titer. The difference between the LD_{50} and

TABLE 12
COMPARATIVE TITRATIONS OF STRAINS OF

R. TSUTSUGAMUSHI IN CHICK EMBRYO

FIBROBLASTS AND MICE

Strain	PFU*	Titer Log_{10} per ml Mouse LD_{50}**	ID_{50}***
Karp	9.0	8.2	8.4
Gilliam	9.4	7.6	8.3
Kato	8.7	8.4	8.4
TC586	9.6	8.8	8.8
TA678	8.2	<1.5	7.8
TA686	8.8	<1.5	7.4
TA716	9.3	<1.5	7.6
TA763	9.3	9.4	9.4
TH1817	8.6	5.8	7.8

* Plaque-forming units
** 50% lethal dose
*** 50% infectious dose

ID_{50} titer of TA678, TA686, TA716 and TH1817 in mice are characteristic of these strains. The first three strains are avirulent for mice and the last-mentioned is only moderately virulent.

The development of the plaque assay system has permitted the program to clone each of the strains of R. tsutsugamushi to proceed. To date, at least 2 and as many as 20 plaques have been picked from flasks infected with each of the eight prototype strains and most of the homotypes of Karp, Gilliam and Kato described in an earlier section. Each plaque was aspirated with either a Pasteur pipette or with a needle and syringe into 0.5 ml of Snyder I diluent. The suspension was inoculated intraperitoneally into a single mouse. When the animal exhibited signs of illness, or, in the case of the avirulent strains, on the 13th or 14th day, the mouse was sacrificed and the spleen harvested aseptically. A 20% suspension was prepared in Snyder I diluent and injected into 5 additional mice. When these second passage mice became sick, or on day 13 or 14, one animal was sacrificed. Peritoneal smears were made to be stained with Giemsa and by immunofluorescence and a spleen suspension was stored at -70 C for recloning. All second passage mice surviving 28 days were challenged with 1000 50% lethal doses of the Karp strain to confirm that viable organisms had been picked from the plaque.

The antigenic composition of the rickettsiae in the peritoneal smears from all mice infected with the first clones has been determined by staining with specific fluorescein conjugates prepared from sera from rabbits infected with each of the strains (see section 1.d). Conjugates that stained heterologous organisms were made strain-specific by blocking the undesired cross-reactivity by the addition of high titering heterologous antiserum. Thus far, minor differences have been observed among clones of the same strain. These were limited to a slight increase or decrease in mouse virulence and a reduced intensity of fluorescent staining in comparison with the parent strain. One problem that has been encountered during some of the second clonings has been the inability to detect rickettsiae in the peritoneal smears of infected mice by either Giemsa or fluorescent staining. A procedure is being developed that should allow the recovery of suspensions of infected exudate cells from the peritoneum of the mice. In addition to obtaining rickettsiae from infected mice more regularly for analyses, the rickettsiae will be distributed more uniformly throughout the antigen smears prepared from suspensions of cells than was obtained in the smears of peritoneum.

Future plans include completion of a total of three clonings with each strain of R. tsutsugamushi in order to select two lines;

one that most closely resembles the parent strain, and another that is most different. Thereafter, each line will be cultivated in eggs and the antigenic composition characterized for comparison with the parent strain.

Certain strains of R. tsutsugamushi recovered from man, mites or rodents were believed to be mixtures of two or more different subspecies or distinctive types because sera from guinea pigs infected with different passage levels of the same strain exhibited marked differences in complement-fixing reactivity with partially purified prototype antigens. Attempts will be made with the cloning technic to confirm that these strains are indeed mixtures. Studies are also in progress to determine if a serum neutralization test for rickettsial antibody can be developed from the plaque assay system.

2. Evaluation of Existing and Potential Military Importance of R. canada

a. Surveillance for Rickettsial Diseases at Fort Bragg, North Carolina and at Fort Leonard Wood, Missouri.

Attempts were made during the current reporting period to continue the coordinated clinical and field study initiated in 1969 in collaboration with Womack Army Hospital and the Preventive Medicine Activity, Fort Bragg. Three of four patients suspected of having experienced a severe febrile illness caused by R. canada that clinically resembled Rocky Mountain spotted fever contracted their disease at Fort Bragg (7). However, due to rapid changes in personnel as well as troop reductions last year, it was not possible to get meaningful information about the occurrence of rickettsial diseases among assigned personnel and their dependents. Last year 26% of the 403 cases of Rocky Mountain spotted fever in the U. S. reported to the Center for Disease Control, Atlanta, Georgia, occurred in North Carolina. The Weil-Felix test usually performed by State Laboratories for serologic diagnosis would not differentiate between illnesses caused by R. rickettsi and R. canada. On the basis of past experience and the 1971 incidence of disease in the state, at least five cases of Rocky Mountain spotted fever should have been admitted to Womack. Convalescent serum specimens from five patients who after retrospective review could have had a rickettsial infection were sent to WRAIR. Complement fixation and indirect immunofluorescent tests confirmed a prior R. rickettsi infection in two cases and the etiology of the other three was non-rickettsial.

As in prior years, the emphasis of the field studies was directed toward random trapping throughout the area of the post with special attention to places where the risk of tick exposure for military personnel and their dependents, at work or play, was the greatest. Animals were trapped throughout the year by the 714th Preventive Medicine Unit and during the month of March by the Post Veterinarian in connection with an annual zoonoses survey. Blood was obtained for serum from the animals and all ectoparasites were removed for identification. Complement fixation tests with spotted fever and typhus group, R. canada and Q fever antigens were performed on 259 sera collected from 16 different species during the period January through December 1971 (see Table 13). Nine of the specimens were anticomplementary and unsuitable for evaluation. Serologic evidence of prior spotted fever infection was found in approximately 5% of all the sera, implicating six species of animals as possible reservoirs; i.e., in 5 of 112 cotton rats, 1 of 29 raccoons, 1 of 18 white-footed mice, 2 of 10 foxes, 4 of 9 rabbits and 1 of 2 rice rats. No evidence of murine typhus, R. canada or Q fever infection was found.

The details about date of capture, trapping area and titer of spotted fever group antibody are summarized in Table 14. There was no relationship between season of vector tick activity and trapping of seropositive animals. The geographic distribution of seropositive animals allows the recognition of enzootic foci of infection in Fort Bragg. Of the 14 different areas of the post that were surveyed, 5 were found to be inhabited by infected animals. The identification of these foci, the species of animals trapped and the incidence of infected animals are presented in Table 15.

Last year it was possible also to collaborate with the Preventive Medicine Activity, General Leonard Wood Army Hospital, to carry out a rickettsial disease survey of the wild animals at Fort Leonard Wood, Missouri. Serum from 135 animals representative of eight different species were received during 1971. Complement fixation tests were carried out as described previously, and 25 of the specimens were anticomplementary. The results of serologic tests are summarized in Table 16. Serologic evidence of prior rickettsial infection was found in 16% of the sera. Specimens from five raccoons, five dogs, four woodchucks and four cottontail rabbits reacted with either one or more of the spotted fever, typhus group and R. canada antigens. All tests for prior Q fever infection were negative.

Details about the respective titers of complement-fixing antibodies in the positive specimens, as well as information about

TABLE 13

RESULTS OF RICKETTSIAL DISEASE SURVEY OF WILD ANIMALS

FORT BRAGG, 1971

Species	Number Specimens Received	Negative	AC*	Spotted Fever Group Positive**	Percent Positive
Cotton Rat	116	107	4	5	4
Raccoon	29	28	0	1	4
Opossum	25	25	0	0	-
White-Footed Mouse	18	17	0	1	6
Squirrel	17	17	0	0	-
Dog	16	13	3	0	-
Fox	12	8	2	2	20
Rabbit	9	5	0	4	44
Cat	5	5	0	0	-
Norway Rat	5	5	0	0	-
Rice Rat	2	1	0	1	50
Misc.***	5	5	0	0	-
TOTAL	259	236	9	14	5.4

* Anticomplementary

** CF tests for Typhus Group, R. canada, and Q Fever antibody were negative.

*** Includes 3 birds, 1 bog lemming and 1 harvest mouse.

TABLE 14
SUMMARY OF SEROLOGICALLY POSITIVE ANIMALS

FORT BRAGG, 1971

Animal Species	Designation*	Capture Date	Trap Area	RMSF CF Antibody Titer
Cotton Rat	PMU-1021-010	21 Jan	McFayden Pond	4
	PMU-1035-040	4 Feb	McFayden Pond	4
	PMU-1056-071	25 Feb	McKellars Lodge	8
	PMU-1225-164	13 Aug	McFayden Pond	8
	PMU-1294-195	21 Oct	McFayden Pond	>16
Rabbit	PMU-1028-034	28 Jan	Smith Lake	16
	PMU-1028-035	28 Jan	McFayden Pond	>32
	PMU-1060-074	1 Mar	McKellars Lodge	>16
	PMU-1120-109	30 Apr	Smith Lake	4
Fox	VET-1068-004	9 Mar	Camp McCall	8
	VET-1084-063	25 Mar	Camp McCall	8
Raccoon	VET-1084-067	25 Mar	Camp McCall	8
Field Mouse	PMU-1127-117	7 May	Smith Lake	4
Rice Rat	PMU-1167-132	15 Jun	Sanitary Landfill	>16

* PMU - 714th Preventive Medicine Unit
VET - Post Veterinarian

TABLE 15

ENZOOTIC FOCI ROCKY MOUNTAIN SPOTTED FEVER

FORT BRAGG, 1971

Species	Numbers of Animals in Trapping Areas															
	McKellars Lodge		McFayden Pond		Sanitary Landfill		Smith Lake		Camp McCall		Texas Pond		Misc.*	
	T**	P***	T	P	T	P	T	P	T	P	T	P	T	P
Cotton Rat	18	1	60	4	29	-	6	-	-		-		3	-
Raccoon	1	-	2	-	2	-	3	-	7	1	1	-	13	-
Opossum	3	-	10	-	-		1	-	2	-	2	-	7	-
White-Footed Mouse	4	-	1	-	1	-	12	1	-		-		-	
Squirrel	2	-	6	-	6	-	2	-	-		-		1	
Dog	-		-				6	-	2	-	2	-	6	-
Fox	-		-				-		5	2	4	-	3	-
Rabbit	1	1	2		-		4	2	-		1	-	1	-
Cat	-		-		-		-		1	-	-		4	-
Norway Rat	-		1	-	-		-		-		-		4	-
Rice Rat	-		-		1	1	-		-		-		1	-
Misc.	-		1	-	1	-	-		-		1	-	0	-
TOTAL	29	2	83	5	40	1	36	3	17	3	10	-	43	-
Percent Positive	6.9		6.0		2.5		8.3		17.6		0.0		0.0	

*Includes Yadkin Road and Salerno Creek studied by 714th Prev. Med. Unit and Salerno Creek and 6 other dispersed regions studied by the Post Veterinarian.

**Tested

***Positive

TABLE 16

RESULTS OF RICKETTSIAL DISEASE SURVEY OF WILD ANIMALS

FORT LEONARD WOOD, 1971

Species	Number of Specimens Received	Negative	AC	With CF Antibodies* Spotted Fever Group	Typhus Group	R. canada	Q Fever
Raccoon	47	38	4	3	2	1	-
Opossum	35	34	1	-	-	-	-
Dog	24	8	11	5	1	1	-
Woodchuck	12	6	2	4	1	1	-
Cottontail	11	1	6	4	3	3	-
Deer	3	3	-	-	-	-	-
Fox	2	1	1	-	-	-	-
Bobcat	1	1	-	-	-	-	-
TOTALS	135	92	25	16	7	6	0
Percent		69	19	15	6	5	0

*Values not mutually exclusive since some sera reacted with more than one antigen.

TABLE 17
SUMMARY OF SEROLOGICALLY POSITIVE ANIMALS

FORT LEONARD WOOD, 1971

Animals	Designation	Date Specimen Collected	CF Antibody Titer		
			Spotted Fever Group	Typhus Group	R. canada
Dog	8	31 Mar 71	4	<4	<4
	9	31 Mar 71	8	64	64
	20	28 Apr 71	16	<4	<4
	70	6 Aug 71	8	<4	<4
	71	6 Aug 71	4	<4	<4
Rabbit	2	25 Mar 71	>512	256	64
	18	21 Apr 71	128	16	4
	65	4 Aug 71	>16	4	4
	88	16 Aug 71	>16	<4	<4
Raccoon	30	9 Jun 71	8	<4	<4
	33	18 Jun 71	>16	<4	<4
	44	23 Jul 71	>16	<4	<4
	57	30 Jul 71	<4	4	<4
	61	3 Aug 71	<4	4	4
Woodchuck	39	16 Jul 71	>16	<4	<4
	51	27 Jul 71	8	<4	<4
	76	9 Aug 71	4	<4	<4
	96	19 Aug 71	>16	4	4
TOTALS	18		16	7	6

date of capture of the animals, are presented in Table 17. Ten of the sera fixed complement only in the presence of the spotted fever antigen and the titers ranged from 1:4 to >1:16. The highest titer of antibodies in specimens from Rabbits No. 2, 18 and 65 and Woodchuck No. 96 was with the spotted fever group antigen, but the sera reacted also with the typhus group and R. canada antigens. The titer of typhus group and R. canada antibodies in the serum from Dog. No. 9 was 8-fold higher than the spotted fever antibodies. This type of cross-reactivity between the spotted fever and typhus groups has not been encountered before with sera from wild animals infected with R. rickettsi or other spotted fever agents. By definition and methods of standardization of the reagents, the respective CF antigens are group-specific and do not react with control immune sera from other groups of rickettsiae. The finding of both spotted fever and typhus group antibodies suggests prior infection of the animals with members of both groups or infection with an unknown rickettsia that shares antigens with the two groups. Tests with specific R. prowazeki and R. typhi (mooseri) CF antigens will be carried out when these reagents can be produced in order to get more information about the identity of the typhus group organism that may be enzootic in Fort Leonard Wood.

b. Typhus Group Antibodies in Flying Squirrels in Virginia.

The existence of an enzootic focus of infection with a member of the Typhus Group of rickettsiae in the flying squirrel (Glaucomys volans) population near Montpelier, Virginia, was first described in the Annual Report, 1 July 1968 - 31 June 1969. Analysis of the results of complement fixation tests with specific R. prowazeki, R. typhi (mooseri) and R. canada antigens and sera from eight serologically positive animals captured between May and July 1968 indicated that the agent that had infected the flying squirrels was either R. prowazeki, R. canada or another as yet unknown microorganism closely related to both species of rickettsiae.

In January 1972, preliminary work was initiated with Dr. Daniel E. Sonenshine, Department of Biology, Old Dominion University, Norfolk, on a collaborative project in preparation for its official beginning 1 June 1972. The specific aims of the project will be (a) to isolate, identify and characterize the etiologic agent responsible for the typhus group antibodies in flying squirrels; (b) determine the method of transmission of infection in squirrels and if arthropod-borne, identify and study the bionomics of the vector(s); and (c) determine the geographic distribution of the agent, define the mechanisms of

maintenance and spread in nature, and transmission to man. The Department of Rickettsial Diseases is responsible for the laboratory studies and Old Dominion University, supported by a USAMED R&D contract, will carry out the field investigations.

The forests in the study area near Montpelier, VA, where the observations were first made had been lumbered during the interim, and the land was not available for a continuing study. A search was made for other sites inhabited by a population of infected flying squirrels and one was identified on a farm near Ashland, VA, about 13 miles from the original area. During the period January through April 1972, 30 flying squirrels were captured in the Ashland area and 3 from the original site near Montpelier. In addition, Dr. Vagn Flyger, Natural Resources Institute, University of Maryland, supplied 12 flying squirrels captured near Centerville, MD, and 11 taken near Trappe, MD. All animals were brought to WRAIR where they were weighed and measured, tagged and approximately 0.6 ml of blood was collected. As soon as the blood had clotted, the serum was removed after centrifugation. Individual clots or pools of as many as 6 clots were ground with Snyder I diluent and the suspension inoculated into the yolk sac of 6- to 8-day old embryonated hens' eggs. Smears of the yolk sacs of all eggs dying after 48 hours were examined for rickettsiae. The yolk sacs of all eggs surviving until the 12th day after inoculation were harvested, smeared and examined for rickettsiae and used to inoculate additional eggs. Two blind passages were carried out before the isolation attempt was considered negative. To date, 13 pools of clots have been processed with negative results.

It was planned to hold the animals in the laboratory and collect a second serum specimen 7 to 10 days later. If complement fixation tests revealed conversion from negative to positive, or a significant increase in titer occurred, the animal was to be sacrificed and attempts made to recover rickettsiae from liver, spleen and kidney by the inoculation of embryonated eggs and guinea pigs. These criteria were adopted after four animals with antibody titers ranging from 1:4 to 1:64 were sacrificed and isolation attempts were negative. Tissues from two sero-negative animals that died during bleeding were processed for isolation with negative results. Isolation would not be attempted with the clots from the second specimens. All animals not sacrificed were to be released into the Ashland area irrespective of where they were captured.

Complement fixation tests using the LBCF method adapted to the microtiter system were carried out with typhus and spotted

fever group and Q fever antigens. The results are summarized in Table 18. Serologic evidence of typhus group infection was found among the squirrels from each of the areas sampled varying in incidence from 33 to 75%.

No differences were noted between the serologic reactivity of the first and second specimens obtained from the animals held in the laboratory. Six squirrels released into the Ashland area that were recaptured 2 to 4 weeks later did not exhibit any change in levels of antibodies; five remained negative and the titer in the other remained the same. No serologic evidence of spotted fever or Q fever infection was found.

Because of the general suspicion of rickettsiologists about the significance of the results of complement fixation tests with sera from domestic and wild animals, it was deemed important to use other serologic methods for the identification of antibody. Rabbits are in the process of being immunized with flying squirrel serum globulins for the preparation of fluorescein conjugates for use in indirect immunofluorescent tests.

In addition, it is planned to use rickettsial agglutination tests as a third method for evaluating the significance of the typhus group complement-fixing antibodies. Antigens are currently being made for this purpose. Small portions of purified suspensions of R. prowazeki, R. canada and R. rickettsi that had been prepared for other purposes a couple of years ago were tested for suitability for use in a microagglutination test. The method of Fiset et al. in which antigens are standardized on the basis of weight was employed (8). The reactivity of these antigens with corresponding immune guinea pig serum pools is summarized in Table 19. In the microagglutination test, R. canada appears to be more closely related to R. rickettsi than to the other members of the Typhus Group. The R. canada antigen was agglutinated by 32 units of RMSF antibody but the reciprocal reaction was negative. The R. prowazeki antigen was agglutinated by 8 units of murine typhus antigen and the reciprocal reactions were essentially negative. Thus far, 27 flying squirrel sera have been examined in the microagglutination test and 10 specimens were positive (see Table 20). Eight of the sera agglutinated only the R. prowazeki antigen, one only the R. mooseri antigen, and the remaining sera agglutinated both the epidemic and murine typhus antigens. Tests with R. canada and R. rickettsi antigens were negative. Of the 10 specimens positive in the agglutination tests, 5 were positive in the complement fixation test, 3 were negative

TABLE 18
RESULTS OF COMPLEMENT FIXATION TESTS FOR

TYPHUS GROUP RICKETTSIAL INFECTION

OF GLAUCOMYS VOLANS(Jan - Apr 1972)

Trapping Location	Numbers				Percent Positive	Distribution of Titers
	Captured	Anti-Compl.	Negative	Positive		
Montpelier, VA	3	0	2	1	33	8(1)
Ashland, VA	30	4	15	11	42	4(3), 8(3), 16(2), 32(1). 64(2)
Centerville, MD	12	0	3	9	75	4(1), 8(3), 16(4), 64(1)
Trappe, MD	11	0	3	8	73	4(2), 8(2), 16(3). 32(1)
Totals	56	4	23	29	56	

TABLE 19
REACTIVITY OF MICROAGGLUTINATION

TEST REAGENTS

Guinea Pig Immune Sera	Antibody Titer with Antigens			
	R. prowazeki	*R. mooseri*	*R. canada*	*R. rickettsi*
Epidemic Typhus	128	4	<2	<2
Murine Typhus	64	512	4	<2
R. canada	4	4	128	<2
Rocky Mountain Spotted Fever	4	4	64	2048

TABLE 20
RESULTS OF MICROAGGLUTINATION TEST FOR
RICKETTSIAL INFECTION OF GLAUCOMYS VOLANS

Location	Total Number Tested	Total Number Negative	Antibody Titers of Positive Specimens				
			Serum Number	Epidemic Typhus	Murine Typhus	R. canada	RMSF
Ashland, VA	12	8	844	32	8	<8	<8
			846	<8	16	<8	<8
			856	32	<8	<8	<8
			1803	32	<8	<8	<8
Center-ville, MD	7	3	817	16	<8	<8	<8
			821	64	<8	<8	<8
			825	32	<8	<8	<8
			835	32	16	<8	<8
Trappe, MD	8	6	882	32	<8	<8	<8
			886	64	<8	<8	<8

and 2 of the sera were anticomplementary. One specimen that was positive in the complement fixation test was negative in agglutination tests. These findings will be confirmed and additional testing carried out before an attempt is made to correlate the results of the two different tests.

3. Screen Test for Serologic Diagnosis of Rickettsial Diseases

a. Development of Diagnostic Test.

The Weil-Felix test is generally performed routinely with certain other bacterial agglutination tests to assist the physician in making a diagnosis of the etiology of acute infectious disease. Although the deficiencies of this nonspecific test which employs bacterial antigens for the identification of rickettsial diseases are well established, its use continues because rickettsial antigens for complement fixation tests are either not available from commercial sources, or are of poor quality. Work previously reported by this Department established that indirect immunofluorescence could be used for rapid specific diagnosis of scrub typhus (9) as well as for the recognition of Typhus Group, Spotted Fever Group and Q fever infections (10). Because of the marked degree of antigenic heterogeneity among strains of R. tsutsugamushi in nature, the antigen employed in the indirect fluorescent antibody (IFA) test for scrub typhus was composed of a mixture of the Karp, Gilliam and Kato strains. In the evaluation of this polyvalent antigen, it was demonstrated that the antibody titer obtained with the mixture of organisms corresponded exactly with the highest titer obtained with one or more of the component antigens when these were used individually (11).

Studies were carried out to determine if a mixture of all the antigens which had been used individually for the diagnosis of Typhus Group, Spotted Fever Group, scrub typhus and Q fever could be used in an indirect fluorescent antibody screen test for all rickettsial diseases. The rickettsial screen test antigen was prepared by mixing equal portions of suspensions of yolk sacs infected with: (a) R. typhi (mooseri) for detection of Typhus Group infections; (b) the Karp, Gilliam and Kato strains of R. tsutsugamushi for detection of scrub typhus; (c) the Bitterroot strain of R. rickettsi for detection of Spotted Fever Group infections; and (d) the Henzerling strain of C. burneti principally in phase II for identification of Q fever.

Because of the great sensitivity of the indirect immunofluorescent test, R. prowazeki could be substituted for R. mooseri, R. akari or other Spotted Fever Group species could

replace R. rickettsi, and other strains of Q fever could have been used without appreciably affecting the performance of the screen test antigen.

Smears were prepared by placing two rows of five drops of 0.005 ml amounts of the suspensions of the screen test and each of the individual component antigens on glass slides. The smears were air-dried at room temperature, fixed in acetone for 10 min and stored at 4 C in a sealed container with silica gel used. IFA tests were performed with 4-fold serial dilutions of patients' sera in normal yolk sac diluent as described previously (9). Paired serum specimens from patients with representative rickettsial diseases were selected for examination from among clinical material stored in the department.

Table 21 summarizes the results of tests with specimens from patients with murine typhus and scrub typhus. Table 22 presents the results of tests with specimens from patients with spotted fever group infections and Q fever. In every instance, the antibody titer with the screen test antigen was identical to the highest titer detected by one of the individual antigens.

When evaluating the significance of the results of the tests with the individual antigens, it should be remembered that immunofluorescence, because of its sensitivity, will reveal minor interrelationships not evident by other serologic tests. This explains the reactivity of the sera from murine typhus patients WJB and RJH with the spotted fever antigen and the reciprocal situation where sera from spotted fever patients HG and HM reacted minimally with the typhus group antigen. The presence of preexisting antibody or unchanging titers was indicative of prior immunization or infection. The spotted fever infection in patient RP was acquired in the laboratory about seven weeks after he was accidently infected with R. mooseri.

The cross-reactivity of the 18 day serum of spotted fever patient JL with the scrub typhus antigen was unexpected. Antigenic relationships between these two groups of rickettsiae have never been described. The patient was a 7 year old boy who had never been out of the USA and contracted Rocky Mountain spotted fever in North Carolina. Studies are in progress to determine the significance of this finding. Attempts will be made to assess how frequently spotted fever and scrub typhus antibodies are associated in human infections as well as in laboratory animals experimentally infected.

TABLE 21

COMPARISON OF RESULTS OF INDIRECT IMMUNOFLUORESCENT TESTS

WITH RICKETTSIAL SCREEN TEST AND DIAGNOSTIC ANTIGENS

	Patient and Disease	Day of Disease	Screen Test	Final Titer* When Tested with Antigen: Typhus Group	Scrub Typhus	Spotted Fever Group	Q Fever
Murine Typhus	WJB	pre	<40	<40	<40	<40	<40
		14	640	640	<40	40	<40
	RJH	pre	40	40	<40	<40	<40
		30	10240	10240	<40	160	<40
	NAMJ	1	<40	<40	<40	<40	<40
		8	10240	10240	<40	<40	<40
	RAM	pre	40	40	<40	<40	<40
		14	640	640	<40	40	<40
Scrub Typhus	JMC	pre	40	40	<40	40	<40
		24	2560	40	2560	<40	<40
	JWH	2	160	40	<40	40	160
		15	10240	40	10240	40	160
	WRM	pre	<40	<40	<40	<40	<40
		21	2560	<40	2560	<40	<40
	CS	pre	<40	<40	<40	<40	<40
		?	2560	<40	2560	<40	<40

*Reciprocal of highest dilution which gave 1+ or minimal fluorescence.

TABLE 22

COMPARISON OF RESULTS OF INDIRECT IMMUNOFLUORESCENT TESTS

WITH RICKETTSIAL SCREEN TEST AND DIAGNOSTIC ANTIGENS

	Patient and Disease	Day of Disease	Screen Test	Final Titer* When Tested with Antigen: Typhus Group	Scrub Typhus	Spotted Fever Group	Q Fever
Spotted Fever	HG	3	<40	<40	<40	<40	<40
		27	2560	40	<40	2560	<40
	HM	6	<40	<40	<40	<40	<40
		24	10240	40	40	10240	<40
	JL	5	<40	<40	<40	<40	<40
		18	10240	40	640	10240	<40
	RP	pre	<40	<40	<40	<40	<40
		24	2560	2560	<40	160	<40
		52(6)+	2560	2560	<40	160	<40
		73(30)+	2560	2560	<40	2560	40
Q Fever	NIH	pre	160	<40	<40	160	<40
		53	2560	40	40	160	2560
	SJO	pre	<40	<40	<40	<40	<40
		58	2560	<40	<40	<40	2560
	DKP	pre	<40	<40	<40	<40	<40
		55	2560	<40	<40	<40	2560
	JRS	pre	<40	<40	<40	<40	<40
		63	2560	<40	<40	<40	2560

*Reciprocal of highest dilution which gave 1+ or minimal fluorescence.

+Day after onset of subsequent laboratory infection with a spotted fever agent.

b. Field Trial of Rickettsial Screen Test.

Since 1967, the Department of Rickettsial Diseases, WRAIR, has been supplying the 9th Medical Laboratory, South Vietnam, with all of the IFA test reagents for the diagnosis of rickettsial diseases. Last year an evaluation of the performance of the test procedure under field conditions was included in the annual report. Through the cooperation of Captain Paul D. Trainor, MSC, Chief, Immunology Division, it was possible to evaluate the performance of the screen test antigen under field laboratory conditions. The screen test antigen was shipped to the 9th Medical Laboratory and the following study was carried out. It was proposed that the IFA screen test for rickettsial diseases would be carried out with a single 1:40 dilution of the patient's serum. If the test was positive, then serial 4-fold dilutions of the serum would be tested against the individual antigens. The usual practice for testing for rickettsial diseases at the 9th Med Lab included application of aliquots of five serial 4-fold dilutions of the patient's serum to four different antigens. For the purpose of the study, the initial 1:40 dilution was tested also with the screen test antigen, and the intensity of fluorescence recorded.

Seventy-two consecutive specimens received at the 9th Med Lab were tested. Table 23 compares the intensity of the fluorescent reaction of the 1:40 serum dilutions obtained with the screen test antigen with that obtained with one of the individual antigens. The correlation between the results is remarkably good since no attempt was made to insure that the identical antigens supplied for individual tests were incorporated into the screen test antigen. Differences observed in the intensity of the fluorescence were probably due to the variation in age, storage or handling of antigens, all of which were intentionally not controlled.

The intensity of fluorescence of the 1:40 dilution of serum with the screen test antigen is compared with the antibody titer found with one of the individual antigens in Table 24. In diagnostic testing, IFA titers of 1:40 are not considered significant. The screen test antigen detected all sera which contained significant levels of antibody. Of the 24 sera with titers of 1:160 or greater, 15 contained scrub typhus antibody and 9 had murine typhus antibody. None of the sera reacted with the spotted fever or Q fever antigens. In this series of specimens, use of the rickettsial screen test would have saved more than half of the reagents and a considerable amount of the time ordinarily expended in the diagnosis of rickettsial diseases.

TABLE 23

RESULTS OF FIELD TRIAL OF RICKETTSIAL SCREEN TEST

ANTIGEN BY 9TH MEDICAL LABORATORY, SOUTH VIETNAM

Intensity of Fluorescent Reaction

Number of Specimens

Screen Test Antigen	Individual Diagnostic Antigens					TOTAL
	Negative	1+	2+	3+	4+	
Negative	36	3				39
	1	2				3
1+	3	4	4	2		13
2+			3	4		7
3+				6	2	8
4+					2	2
TOTAL	40	9	7	12	4	72

TABLE 24

RESULTS OF FIELD TRIAL OF RICKETTSIAL SCREEN TEST

ANTIGEN BY 9TH MEDICAL LABORATORY, SOUTH VIETNAM

Results of Screen Test**	Number of Specimens IFA Titer* with Diagnostic Antigens						
	Negative	1:40	1:160	1:640	1:2560	1:10240	TOTALS
Negative	36	3					39
±	1	2					3
1+	3	3	4	2	1		13
2+				6	1		7
3+				3	3	2	8
4+					2		2
TOTAL	40	8	4	11	7	2	72

*Indirect fluorescent antibody

**Intensity of fluorescence of 1:40 dilution of serum with screen test antigen

4. Rickettsial Infection of Military Dogs in Combat Areas.

A collaborative study of the zoonotic infections in military scout and tracker dogs with the Division of Veterinary Medicine, WRAIR, showed that 45% of the specimens collected from 64 animals after 2 to 36 months in Vietnam contained scrub typhus antibodies (1:[illegible]. Failure to find serologic evidence of murine typhus or tick typhus was believed to be due to limited exposure to infected arthropod vectors and the absence of Q fever to lack of exposure to the infectious agent. Murine typhus was known to be a frequent cause of febrile disease among U. S. military personnel, but tick typhus and Q fever were diagnosed only rarely.

Since the majority of the cases of murine typhus occurred in personnel assigned to base camps or support operations, in contrast to the occurrence of scrub typhus in troops involved in combat, sera from additional dogs were tested to determine if the rickettsial infections sustained by the animals were related to their type of duty. Specimens were available from 124 animals after service in Vietnam (RVN) as sentry dogs, scout, tracker and patrol dogs, and mine and tunnel dogs. In addition, sera were obtained from 64 of the animals before departing the USA for overseas duty. Single specimens from 10 sentry dogs after service in Korea and from 38 indigenous Vietnamese dogs were tested also. Canine sera diluted 1:40 were first tested by indirect immunofluorescence with the rickettsial screen test antigen (see section 3.a) and a rabbit anti-dog 7S globulin fluorescein conjugate. Positive sera were then tested at dilutions of 1:160 and 1:640 for reactivity with individual scrub typhus, typhus group, spotted fever group and Q fever antigens. Titers of 1:160 or greater were considered significant of prior infection.

The results of the tests are presented in Table 25. Scrub typhus antibodies were found in the specimens from 43% of U. S. scout, tracker and patrol dogs, and in 14% and 8%, respectively, in sentry, and mine and tunnel dogs. Serologic evidence of scrub typhus infection was demonstrated in one (10%) of the sentry dogs in Korea and in one (4%) of the indigenous Vietnamese dogs. It had been expected that typhus group infections should have occurred in both groups of sentry dogs and in the indigenous Vietnamese dogs. However, typhus group antibodies associated with overseas service were detected in only two sera from scout, tracker and patrol dogs. Similarly, it was two members of this group of animals that sustained spotted fever group infections also. The absence of typhus group antibody in the sentry and indigenous dogs was probably due to lack of exposure to infected fleas. Tests for plague antibody using

TABLE 25

RESULTS OF INDIRECT IMMUNOFLUORESCENT TESTS FOR RICKETTSIAL

ANTIBODY IN CANINE SERA

Deployment	Numbers Tested	Numbers Negative	Serologic Evidence of Infection: Scrub Typhus Group Number*	Scrub Typhus Group Percent	Typhus Group Number	Typhus Group Percent	Spotted Fever Group Number	Spotted Fever Group Percent
Sentry RVN	59	54	1(4)	8	0		0	
Korea	10	9	(1)	10	0		0	
Scout, Tracker, Patrol RVN	58	32	16(9)	43	2	3	2	3
Mine & Tunnel RVN	7	6	(1)	14	0		0	
Indigenous RVN	38	37	(1)	4	0		0	

*Number with 8-fold or greater increase in titer between pre and post service specimens. Number in brackets denote results with post-service specimen only.

an IHA technic with fraction I plague antigen had been carried out previously with the sera from the indigenous dogs and the specimens were negative.

Summary and Conclusions

1. Scrub Typhus Vaccine Development

a. Antigenic Interrelationship Among Prototype Strains of R. tsutsugamushi.

The antigenic interrelationship among the three prototype strains, Karp, Gilliam and Kato, and the five candidate prototype strains, TA678, TA686, TA716, TA763 and TH1817 was characterized by indirect immunofluorescent tests with immune rabbit and guinea pig sera. Some variations in the titer and heterogeneity of the antibody response of animals receiving the same inoculum was noted and differences were found also between the response of the two species. An index of the extent of antigens shared between strains relative to the concentration of the respective dominant antigens was calculated. The results suggest that strains of R. tsutsugamushi were comprised of a mosaic of antigens and similarities and differences were due to the presence or absence and relative concentrations of constituent components.

b. Antigenic Composition of Homotypes of R. tsutsugamushi.

Studies were carried out to determine whether wild strains of R. tsutsugamushi considered to be homotypes on the basis of complement-fixing reactivity of immune guinea pig sera with the prototype antigens had the same composition of minor antigens as the prototype. Indirect fluorescent antibody tests were carried out with homotype immune rabbit and guinea pig sera and the corresponding homotype antigens as well as with the eight prototype antigens. A comparison of Karp and two homotypes showed that TA583 differed from the other two in the composition of major antigens and differences among minor antigen components distinguished TC765. The cross-reactivity among the prototype and homotype strains suggested the presence of dominant antigens in TA583 and TC765 different for those in the eight prototype strains.

When the antigenic composition of Gilliam was compared with three homotypes (TC586, TA675 and TA688), differences between major and minor components was evident among the strains. Similarly, of four homotypes of Kato, MF2624 was almost identical

to Kato, while MF2334, MF2569 and MF2651 were more closely related to each other. The immunologic significance of the antigenic differences between homotype and prototype strains remains to be established.

c. Formulation of Experimental Scrub Typhus Vaccines.

Based on the data obtained in the antigenic analyses of prototype and homotype strains, formulae for the composition of three polyvalent vaccines that might afford protection against all eight of the prototype strains were devised. Depending upon the magnitude of the antibody response that would occur in man to the concentration of antigens shared among strains relative to the dominant components, a vaccine containing only Karp, TA675 (a Gilliam homotype), TA678 and TA763 might afford the desired extent of protection. On the other hand, if there was a poor antibody response, Kato and TH1817 also would be required. Even with the six strains the formulation might be deficient in Gilliam. However, if immunity induced by a vaccine is related to the magnitude of the heterologous antibody response, then a vaccine comprised of Karp, Gilliam and TA763 would be expected to produce titers equal to or greater than that produced by each of the eight prototypes. Experiments are in progress to evaluate the validity of the three possible formulae.

d. Preparation of Type-Specific Anti-Scrub Typhus Fluorescein Conjugates.

Globulins from rabbits infected with each of the eight prototype strains of R. tsutsugamushi have been conjugated with fluorescein isothiocyanate for the preparation of direct staining fluorescent antibody reagents. Specificity of reactivity was improved by blocking heterologous reactivity with the corresponding immune rabbit sera. These reagents are being used for the rapid specific antigenic characterization of strains of R. tsutsugamushi recovered from sibling larvae of successive generations of the naturally infected Leptotrombidium (L.) akamushi colony in Malaysia and in connection with cloning of prototype and homotype strains.

e. Plaque Assay System of R. tsutsugamushi.

The conditions required for the consistent production of plaques by R. tsutsugamushi in primary chick embryo fibroblasts have been defined. The plaque assay system was needed to clone candidate vaccine strains to insure purity. Comparison of

infectivity titers of the prototype strains in tissue culture and in mice revealed the former to be 0.3 to 1.7 logs higher. All eight prototype strains have been cloned at least once and some of the strains twice. Minor differences in mouse virulence and reactivity with direct-staining prototype conjugates have been observed among clones of the same strain. Each strain will be cloned three times in an attempt to recover one substrain most closely resembling the parent and another that is most different.

2. Evaluation of Existing and Potential Military Importance of R. canada

a. Surveillance for Rickettsial Diseases at Fort Bragg, North Carolina and Fort Leonard Wood, Missouri.

Complement fixation tests on sera from small and medium-sized mammals trapped at Fort Bragg showed that some of the cotton rats, raccoons, white-footed mice, rabbits and rice rats had been infected with R. rickettsi. Evidence of infection with Typhus Group rickettsiae including R. canada, and with Q fever was not found. Of the 14 different areas of the post surveyed, 5 were found to be inhabited by infected animals. At Fort Leonard Wood, the serum from some raccoons, feral dogs, woodchucks and cottontail rabbits contained spotted fever group, typhus group and R. canada antibodies. Tests for Q fever were negative. The finding suggests that members of the Spotted Fever Group and Typhus Group of rickettsiae are both enzootic at Fort Leonard Wood.

b. Typhus Group Antibodies in Flying Squirrels in Virginia.

Enzootic foci of infection of flying squirrels (Glaucomys volans) have been identified in areas of Virginia and Maryland other than in Montpelier, VA, where the observation was first made. Sera from animals trapped near Ashland, VA, Centerville, MD, and Trappe, MD, contained typhus group complement-fixing antibodies. The results of preliminary microagglutination tests with R. prowazeki, R. typhi (mooseri), R. canada and R. rickettsi antigen suggest the agent may be more closely related to epidemic typhus than to other known members of the typhus group. A study is in progress to attempt to isolate and identify the rickettsia causing the infection, and define the vectors and vertebrate hosts that are responsible for maintenance in nature.

3. Screen Test for Serologic Diagnosis of Rickettsial Diseases

a. Development of Diagnostic Test.

A polyvalent antigen comprised of a mixture of representatives of the Typhus Group, Spotted Fever Group, scrub typhus and Q fever rickettsiae detected the same level of antibodies in serum from patients with each of the four types of rickettsial diseases by indirect immunofluorescence as was found in tests with the respective individual component antigens. Therefore, this single antigen could be used in a screen test for detection of all rickettsial diseases.

b. Field Trial of Rickettsial Screen Test.

Personnel at the 9th Medical Laboratory in Vietnam undertook an evaluation of the screen test. The results of performing an indirect fluorescent antibody test (IFA) with a 1:40 dilution of a patient's serum and the screen test antigen compared well with the results of the standard IFA test with individual antigens. The screen test antigen detected all sera which contained significant levels of rickettsial antibody. The use of the screen test is expected to conserve time and reagents in the laboratory diagnosis of rickettsial infections particularly in areas where the incidence is relatively low.

4. Rickettsial Infection of Military Dogs in Combat Areas

It has been reported previously that U. S. military scout and tracker dogs were infected with R. tsutsugamushi while serving in South Vietnam. Additional groups of scout, tracker and patrol dogs as well as sentry and mine and tunnel dogs were studied to determine if deployment influenced the incidence or type of rickettsial infection sustained. Serum from a group of sentry dogs returning to the USA after duty in Korea and from a group of indigenous Vietnamese dogs were examined also. Indirect immunofluorescent tests showed that some dogs in all groups had been infected with R. tsutsugamushi. The highest incidence was in the scout, tracker and patrol dogs which were presumed to be at greatest risk of exposure to infected vector chiggers. The infection rate in sentry dogs was 20 to 25% less. Evidence of prior typhus and spotted fever group infections was found only among scout dogs. All tests with Q fever were negative.

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Publications.

1. Elisberg, B. L. The Rickettsial Diseases. Chapter in: Current Diagnosis, 3rd ed., Philadelphia, W. B. Saunders Co., pp. 151-164, 1971.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6443	72 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
71 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	168
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Bacterial Diseases (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: 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 72	11	275
	CURRENT 73	11	300

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

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 (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) - Studies on the etiology, ecology, epidemiology, pathogenesis, physiological, immunological and diagnostic aspects of diseases of microbial origin which are current or potential problems to military forces. Current emphasis on meningococcal, 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) - 71 07 - 72 06 Meningococcal antipolysaccharide antibodies were purified from whole serum by affinity chromatography. Group A and C antibodies were bactericidal for all strains of A and C tested. Group B antibodies were bactericidal for less than 50 percent of group B strains tested. Bactericidal activity of whole serum correlated best with anti-protein antibodies rather than antipolysaccharide antibodies, suggesting that protein antigens should be further studied. Gonococcal research has been directed towards development of techniques to measure antibodies and to define bacterial antigens. It has been shown that serum bactericidal reactions are not influenced by the colonial type of the strain tested. Since colonial types 1 and 2 have been correlated with genitourinary virulence for man it would appear that antigens involved in bactericidal reactions are not the same as those related to infectivity properties. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

*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 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 168 Bacterial diseases

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Description.

Investigations have been carried out which are concerned with the epidemiology, microbiology and immunology of meningococcal meningitis. The highlights of these studies have been the purification of meningococcal antibodies by affinity chromatography and the identification of antiprotein antibodies as the major bactericidal component of group B antisera. New methods have been developed for analyzing the native cell wall complex of meningococci. It is expected that this approach will provide additional data on the antigenic structure of the cell surface.

A start has been made on a program of gonococcal research. Studies of rifampin resistance and perfection of a bactericidal assay have been accomplished.

Efforts have been directed towards the establishment of isolation and identification methods for mycoplasmas and wall defective variants of bacteria. A variety of sources have been tested including hospital patients, laboratory rats, tissue cultures, virus specimens, plants and other environmental sources. A group of fastidious mycoplasma pathogenic for swine have been cultured in sufficient quantity to permit a comprehensive characterization in terms of biochemical and physiologic activities. The joint WRAIR-ATCC collection of Mollicutes and L-phase variants has been increased to 119 strains, and 10-year

viability tests show there has been very good preservation.

During the reporting period a close association with the newly established Infectious Diseases Service of the Department of Medicine, WRGH was fostered. Clinical consultation, resident and fellow teaching and clinical investigation have become important aspects of the departmental mission.

Progress.

1. Meningococcal meningitis.

a. Purification of antimeningococcal antibodies by affinity chromatography.

Using an immunoadsorbent of highly cross linked polyacrylamide containing purified meningococcal polysaccharide, antibodies were adsorbed from antisera of rabbits immunized with live meningococci. Antibody which eluted with 2.0M potassium iodide was found to be serogroup specific and functionally active in the bactericidal and hemagglutination tests. An immunoadsorbent column prepared with a meningococcal protein antigen (serotype factor II) trapped antibodies which had serotype specificity and no antipolysaccharide serogroup reactivity. The purified antibodies were found to be both IgG and IgM.

Approximately 8% of the total IgG and IgM from hyperimmune group C rabbit serum was specific anti-C polysaccharide antibody. Bactericidal activity of purified antibody (P) as compared to whole serum and serum not bound to immunoabsorbent (NB) is shown in Table 1. Purified antipolysaccharide antibodies (pool P) were bactericidal against strains of the same serogroup. Purified antiprotein II antibodies had serotype specificity (ie, purified antiprotein II antibodies killed strains 99M and 138I, both of which contained factor II antigen although 99M is serogroup B and 138I has polysaccharide of serogroup C).

b. Antigenic specificity of bactericidal antibody in meningococcal antisera.

Using a radioactive bactericidal test (RBT) and rabbit antisera prepared against a variety of group C strains (absorbed in such a fashion as to be serotype specific) cross reactions were shown with strains of serogroups A, B and Y. Thus, these strains share one or more of the seven defined type specific antigens of serogroup C organisms. Bactericidal tests with human convalescent case sera, as well as rabbit immune sera, showed that killing of 14 different group B strains was variable and correlated best with serotype antigens within the bacteria. Purified antipolysaccharide antibodies prepared against three different group B strains were active by RBT

Table icidal activity of anti-meningococcal antiserum and immunoadsorbent pools NB and vs. heterologous strains.

Antiserum to strain # (group-type)		Passed over immunoadsorbent containing	Bactericidal activity to indicated strain # (group-type)				
			2E(A-V)	99m(B-II,III,VI)	138I(C-II)	6155(B-VI)	35E(C-V)
2E	(A-V)	Asss	$\frac{4}{2} \cdot 4$*	0	0	0	$\frac{3}{3} \cdot 0$
99m	(B-II,III,VI)	Bsss	0+	$\frac{4}{4} \cdot 4$	$\frac{4}{4} \cdot 0$	$\frac{4}{4} \cdot 3$	0
138I	(C-II)	Csss	0	$\frac{4}{3} \cdot 0$	$\frac{4}{3} \cdot 3$	0	$\frac{3}{1} \cdot 3$
138I	(C-II)	Protein II	0	$\frac{4}{0} \cdot 3$	$\frac{4}{3} \cdot 3$	0	$\frac{3}{3} \cdot 0$

* Bactericidal activity is represented as $\frac{\text{pre-adsorbed antiserum}}{\text{pool NB}} \cdot \text{pool p}$

+ 0 indicates $\frac{0}{0} \cdot 0$

only against the homologous and a few other group B strains. Table 2 shows these group B antibodies to be bactericidal to at most five of the 16 different strains tested. The antibody to E. coli K antigen (which cross reacts in vitro with group B meningococcal polysaccharide) was no more effective than the antimeningococcal antibodies. However, antipolysaccharide antibodies purified from group A or C antisera were highly bactericidal to all strains tested of the homologous serogroup (Table 3). Studies of purified group B polysaccharides (inhibition of antibody binding or RBT killing) were unable to demonstrate immunological differences in the B polysaccharides to account for the observed differences in RBT killing.

Although there is no proven explanation for the failure of group B antipolysaccharide antibodies to kill a broad range of group B strains, the fact that killing by whole antisera correlated best with anti-protein (serotype) antibodies has led to a consideration of these protein antigens as vaccine candidates.

c. Local nasal secretory antibodies to meningococci.

The study of local secretory antibodies to meningococci was of interest even prior to the demonstration that group C polysaccharide immunization induced a local respiratory tract immunity to carrier acquisition. Crude freeze-thaw extracts of meningococcal suspensions produced precipitin lines with concentrated nasal washes in Ouchterlony double diffusion assay. Precipitin lines between nasal secretions (NS) and a number of meningococcal extracts from strains of different serogroups, as well as Neisseria catarrhalis, fused in a "reaction of identity". However, absorption of the NS with anti-IgA and anti-IgG antisera failed to remove the precipitating factors. Further studies have demonstrated that the antimeningococcal factor in nasal secretions is lysozyme. Egg white lysozyme was found to produce a precipitin reaction with meningococcal antigen and this line fused in a reaction of identity with the NS-meningococcal precipitate.

Previous studies to detect antibodies in NS following group C meningococcal polysaccharide vaccination have been unsuccessful using hemagglutination and immunofluorescence tests (Artenstein and Brandt, unpublished). With the development of a sensitive, specific radioactive polysaccharide binding assay (RABA) another trial was undertaken.

Five adult male volunteers were administered 50 microgram injections of both group A and group C meningococcal polysaccharide vaccines. Serum and NS were collected at weekly intervals. Each vaccinated subject developed increases in serum antipolysaccharide antibodies within one-two weeks. Calculations of peak RABA titer increases for each subject are shown in Table 4. Serum group A RABA titer rises ranged from 3.5 to 82 fold over baseline values. Group

Table 2. Bactericidal activity of purified group B rabbit antipolysaccharide antibody against group B strains.

Test strain	Degree of killing* by antibodies against group B strain with indicated HA titer			
	Purified antibodies			*E. coli* K antigen antiserum
	99M	NE	29II	
	1:32	1:16	1:8	1:256
99m	2	1	4	2
NE	0	3	0	0
29II	0	0	4	0
6249	3	0	0	0
6251	0	0	0	2
6269	3	0	4	0
6260	4	0	1	1
6587	0	0	0	0
6586	0	0	0	0
PAT	0	0	0	0
171	0	0	0	0
B-31	0	0	0	0
79I	0	0	0	0
6155	3	1	1	4
Zeuscher	0	0	0	0
B-7	0	0	0	0

* 1 = release of 5-9% *total* radioactivity; 2 = 10-14% release; 3 = 15-20%; 4 = >20%.

Table 3. Bactericidal activity of purified antipolysaccharide antibodies against groups A and C meningococci.

Test strain	Serogroup	Degree of killing by purified antibodies of an indicated serogroup and HA titer	
		A 1:256	C 1:32
2E	A	3	0
A-4	A	4	0
121m	A	4	0
693840	A	4	0
105m	A	2	0
60E	C	0	4
138I	C	0	3
126E	C	0	2
118V	C	0	2
35E	C	0	3
32I	C	0	4
89I	C	0	1
68IV	C	0	4

Table 4. Antibody increases in serum and nasal secretions following meningococcal immunization.

	Fold increase in RABA titer*			
	Group A		Group C	
Subject	Serum	NS	Serum	NS
BV	3.5	3.8	114	3.4
JW	14.1	1.8	248	4.5
FS	82.2	1.7	296	12.7
DB	10.8	3.4	21.8	8.5
JP	36.5	1.7	96.6	6.4

* Maximum increase at 28 days except BV, 14 days.

C serum binding increased from 22 to 296 fold. For the purpose of comparison, NS values were calculated as binding per mg Ig (Ig = IgA + IgG) to correct for variations in protein concentrations of secretions. From Table 4 it can be seen that increases in group A binding by NS were quite small, ranging from 1.7 to 3.8 fold over baseline. Increases in group C binding by NS were somewhat higher, being 3.4 to 12.7 times the baseline values. RABA binding does not correlate with lysozyme concentrations in NS. Thus, NS antibody rises occurred to both antigens but compared to serum responses the NS rises were very small. In interpreting these results it should be pointed out that the RABA values for group C antibody in NS were at a very low level where reproducibility is poor. Similarly, the IgA and IgG levels in NS were at the lower limits of the assay system. Therefore, these NS antibody rises are only considered to be suggestive and not definitively proven.

Table 5. Composition of the native complex from strain 99M.

Component	Percentage of native complex[a]
Protein	45-65
Group specific polysaccharide	4-10
Lipopolysaccharide	10-25
Lipid	15-30

[a] Values given represent the range of values obtained for several different preparations.

d. Meningococcal antigens: Characterization of a "native" cell wall complex from N. meningitidis.

A high MW complex was isolated from the media of N. meningitis group B cultures by ultracentrifugation (80,000-100,000 x g for two hours). This complex has been shown to be essentially identical to one separated from washed organisms by mild shear in the presence of EDTA. Equilibrium centrifugation of the complex on sucrose density gradients resulted in a single diffuse band. The components of this complex have been separated by use of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). Four components have been identified in the complex: protein,

lipopolysaccharide, loosely bound lipid, and the acidic polysaccharide associated with serogroup specificity. The relative proportions of each major component of the complex are given in Table 5; chemical analysis is shown in Table 6. The protein component produced a pattern of bands on SDS polyacrylamide gels which was simple (1-8 major bands), reproducible, and strain dependent.

Immunogenicity of the whole complex: Most of the important cell surface antigens appear to be associated with the native complex. Native complex was found to be immunogenic in rabbits when 10-50 microgram injections were given intravenously over a three week period. Sera from rabbits immunized with native complex from the group B strain 99M when tested by hemagglutination assays were shown to have at least a 16-fold rise in antibody directed against the group B polysaccharide. The same sera were shown by the bactericidal assay to have at least a 200-fold rise in antibody directed against the protein component (serotype factor II) of the complex. In addition, the presence of anti-LPS antibody in these antisera has been detected in agar immunodiffusion experiments using purified LPS. The lipopolysaccharide has been shown to have chemical and biological properties characteristic of bacterial endotoxin.

It is believed that this complex represents the outer layer of the meningococcal cell wall in its native state. This observation is supported by electron microscopic observations of the complex which demonstrated a trilaminar membrane consistent with that of the cell wall of the meningococcus. The native complex will be used for further studies such as the effect of antibody on the membrane, strain differentiation based upon protein antigens, and the role of lipopolysaccharide in meningococcal immunity and pathogenicity.

2. Gonococcal infections.

A program of investigation of various aspects of gonococcal infections was initiated during the reporting period. Study projects included search for animal or tissue culture models of infection, antigenic structure of N. gonorrhea, antibody responses to infection or immunization and antibiotic sensitivity studies. Two projects have been completed and are reported here

a. In vitro mutation of gonococci to resistance to high concentrations of rifampin.

A number of reports have described the use of rifampin in the treatment of acute gonococcal infection with cure rates approaching those achieved with penicillin. However, reports of clinical experiences with rifampin in the treatment of other bacterial infections have described the emergence of rifampin resistant organisms during or after treatment. The studies described below

Table 6. Chemical analysis of the purified components from 99M native complex.

Assay	Component			
	Protein	Lipo-polysaccharide	Lipid	Group specific polysaccharide
	Percentage of dry weight			
Protein	86	1	1.5	1.1
Esterified fatty acids	2		94	ND[a]
Carbohydrate	1		0.4	0
Sialic acid	0.2	3	0	97
Heptose	0.1	4.7	0	0
Phosphorous	0.9	3.3	3.3	ND
2-keto-3-deoxy sugar acid	0.1	7	0	ND

[a] ND = not determined

were done to learn more of the in vitro response of gonococci to concentrations of rifampin which are attainable in blood and urine following standard dose regiments.

Large numbers ($5x10^8$ to $5x10^9$) of each of 13 strains of gonococci were applied to selection agar plates containing 0.4 mcg/ml rifampin. After 48 hrs of incubation the colonies produced by rifampin resistant gonococcal mutants were enumerated and tested for resistance to greater concentrations of rifampin by the velveteen replication method.

The rates of mutation to rifampin resistance with selection made at 0.4 mcg/ml rifampin varied from $1x10^{-8}$ to $8.5x10^{-9}$. The results of six strains representative of the 13 tested initially are summarized in Table 7. In each instance all of the mutants of each strain were analyzed for the ability to grow in the presence of higher concentrations of rifampin. It can readily be seen that many of the mutants selected at 0.4 mcg/ml rifampin grew at 6.25 mcg/ml and significant numbers grew at 100 mcg/ml, a rifampin concentration 400 times greater than that present in the select agar. These data indicate that gonococci are capable of one-step mutation to resistance to high rifampin concentration. The selective pressure applied by low rifampin concentrations in vivo could very possibly result in the appearance of mutants resistant to high concentrations of antibiotic.

Similar experiments were also conducted with strains of meningococci where mutation rates 10 fold greater than the gonococcal rates were observed. The in vitro results of the mutant analysis were similar to the gonococcal results.

Clinical experiences with rifampin as four-five day chemoprophylactic regimen to eradicate meningococcal nasopharyngeal carriage have demonstrated the appearance of significant numbers of resistant meningococci which persisted in the nasopharynges of the treated carriers. In at least one study, transmission of resistant strains was shown. Viewed in this light, the in vitro experiments indicate the need for close study of strains from gonorrhea patients who fail to be cured with rifampin therapy.

b. The effect of colonial type of N. gonorrhea on the serum bactericidal reactions.

A serum bactericidal test was developed which required serum, complement and a young log phase culture of gonococci in the reaction mixture. Differing colony (T_1, T_3, etc.) types of agar grown cultures were separated and propagated using the methods of Kellogg. Antisera were then raised in rabbits by inoculating live cultures of GC strains 101 T_1, 104 T_1, 104 T_3 and 103 T_1. In the first experiment, bactericidal titers of antisera 104 T_1 and 104 T_3 were measured against 104 organisms of colonial types 1 and 3 (Table 8). Identical bactericidal titers were achieved whether sera were tested

Table 7. Mutation rates of six representative gonococcal strains to 0.4 mcg/ml rifampin resistance and analysis of such mutants for resistance to greater concentrations of rifampin.

Strain	Mutation rate	No. of mutants analyzed	No. of mutants resistant to 6.25 mcg/ml rifampin	No. of mutants resistant to 100 mcg/ml rifampin
DA	1.6×10^{-8}	110	28 (25%)	7 (6%)
7134	1.0×10^{-9}	37	25 (68%)	6 (16%)
T0	1.8×10^{-9}	32	2 (6%)	2 (6%)
CR	2.7×10^{-9}	30	16 (53%)	10 (33%)
F62	5.5×10^{-9}	22	22 (100%)	14 (67%)
1[illegible]	8.5×10^{-9}	57	17 (30%)	14 (25%)

against T_1 or T_3 colonial types. In a similar fashion (Exp. 2, Table 8) 101 T_1 antiserum showed very similar titers when tested against T_1 or T_4 organisms. In the final experiment (Exp. 3, Table 8) antiserum 103 T_1 when absorbed with 103 T_3 organisms lost all bactericidal activity against T_1 colony type. These data indicate that bactericidal activity of antisera is related to antigens other than those which are responsible for the morphologic differences between colony types. If presence of pili in T_1 colonies is the major difference between T_1 and T_3, as suggested by Swanson and Gotschlich, then the present data would suggest that this structure is not important in the cidal reaction.

Table 8. Effect of gonococcal colonial type on serum bactericidal reactions.

Exp. No.	Antiserum against strain	Organism and colonial type tested	Bactericidal titer*
1.	104 T_1	104 T_1	1:640
		104 T_3	1:640
	104 T_3	104 T_1	1:640
		104 T_3	1:640
2.	101 T_1	101 T_1	1:5120
		101 T_4	1:2560
3.	103 T_1	103 T_1	1:40,960
	103 T_1 abs. 103 T_3	103 T_1	<1:4

* 50% or > killing of inoculum.

3. Investigations on Mycoplasmas and Wall-Defective Variants of Bacteria.

a. Isolation studies on specimens from clinical and experimental sources.

(1) Specimens of blood, synovial fluid, or synovial tissue from cases of Reiter's syndrome, arthritis, or fevers of unknown origin were submitted from WRGH for cultivation for mycoplasmas or L-phase variants of bacteria. In addition to the usual heart infusion-serum-

yeast and thioglycollate-serum media employed in the past for cultivating such specimens, a variety of newer media were employed. One such medium, employed by Whittlestone for cultivation of the extremely fastidious swine pneumonia mycoplasma, contained a beef heart digest base, balanced salt solution, lactalbumin hydrolysate, yeast extract, and acid treated swine serum. Another medium, employed by Barile to test for mycoplasma contamination of biologicals, contained heart infusion-serum-yeast, glutamine, arginine, glucose, DNA and vitamins. A third medium, employed by McGee for isolation of wall-defective variants from clinical specimens, contained brain heart infusion, yeast, serum, $MgSO_4$, and increased salt and sucrose to afford osmotic stabilization to variants of both gram positive and gram negative species. A fourth medium, employed by Roberts for fastidious L-phase variants of gram negative species, contained brain heart infusion, serum, yeast and sucrose.

More recently, media employed by Shepard for differential cultivation of T-strain mycoplasmas has been prepared and tested and will be included among the test media when the nature of the case of type of specimen warrants its use.

(2) A recurrence of arthritis in the rats used for malaria transmission experiments was noted by investigators of the Department of Medical Zoology (WRAIR). Three blood, two synovial fluid and three spleen specimens from arthritic rats were submitted to the Mycoplasma Section for cultivation. Mycoplasmas were isolated from all specimens except the third blood specimen. The latter was a different stock line of malaria-infected blood that had been stored in the frozen state. The mycoplasma isolates were filtered and cloned, and the various culture lines were identified by biochemical and serologic tests as Mycoplasma pulmonis. This was the same species that produced arthritis in the malaria infected rats on a former occasion and was described in the WRAIR Annual Report of 1968.

(3) Specimens of virus harvests and of tissue cultures used for virus studies by WRAIR and area laboratories were submitted to the Mycoplasma Section for examination for contamination with mycoplasmas. Three out of nine specimens, including two simian virus preparations, yielded heavy growth of mycoplasmas. In response to a request for identification the mycoplasma isolated from one varicella virus harvest was identified as Mycoplasma orale.

(4) Four specimens of presumed mycoplasmas derived from the plant disease, aster yellows, were submitted to the Mycoplasma Section for examination prior to their being deposited in the WRAIR-ATCC collection. One specimen was too old and dry for further study and one specimen contained crystalline artifact "colonies" but no mycoplasmas. The other two specimens, one of which contained a

bacterial contaminant that was removed by filtration, contained mycoplasmas. These organisms were characterized biochemically and serologically and were identified as M. hominis. This finding was interpreted as indicating contamination of the plant specimens from a human source, and as having no relationship to disease in the plants.

Specimens from several other environmental sources were also examined for mycoplasmas, but no isolations were obtained.

b. Characterization of pathogenic mycoplasmas of swine.

A number of fastidious mycoplasma strains, which have in recent years been implicated in swine pneumonia or swine arthritis, had not been characterized biochemically because of problems in cultivating the organisms. A solution for these problems was desirable, since it could be expected that similar problems would be encountered if new isolates from the lungs or joints of human patients were obtained.

The organisms studied were M. hyopneumoniae, strains 11 and Weybridge, M. suipneumoniae, strain J, M. hyosynoviae, strains S16 and M60, and for controls M. hyorhinis BTS7, M. arthritidis Preston, M. bovirhinis PG43, and Acholeplasma laidlawii B, PG9.

Employing the methods of Aluotto et al. (I.J.S.B. 20:35, 1970) all strains were adapted to growth in heart infusion broth containing either 20 or 10% (v/v), horse serum, and 0.5% (w/v) Oxoid yeast extract (HIB + HOS + OYE). Some strains required as many as 12 passages to adapt to growth in this medium. Thus, in addition to being time consuming, this adaptation procedure exposed the isolates to risk of change in biochemical characters and loss of pathogenicity.

An alternative method was devised. Each organism was grown in the "preferred" medium (or a modification thereof) which had been recommended by the original isolator and to which the strain was already adapted. Fresh young cultures (24-48 hr) were centrifuged out of the growth medium and resuspended in HIB + HOS + OYE; this suspension was used to inoculate the various test media.

The biochemical activities of the organisms were constant for each strain regardless of which method was used to prepare the organisms for testing. However, the centrifuged and resuspended organisms yielded positive reactions earlier than did the organisms adapted to grow in HIB + HOS + OYE.

Considerable effort was spent on obtaining reliable reactions in the fermentation tests, since many mycoplasmas can produce a color change representing 0.5 to 0.8 pH units in phenol red-containing HIB

control tubes to which no fermentable sugar has been added. A positive fermentation test requires that there be a reduction of at least 0.5 pH units in the sugar-containing test below any reduction occurring in the sugar-free control. By starting with media that had a final pH of 7.8 to 8.0 instead of pH 7.4, significant acid production could be detected at a pH that was in an appropriate range for the indicator used, ie, for phenol red pH 8.4-6.8.

M. hyopneumoniae and M. suipneumoniae had been regarded as glucose fermenters according to the literature. Our test results, summarized in Table 9, showed that these strains were negative for fermentation of glucose and 13 other carbohydrates; negative for hydrolysis of arginine, urea and aesculin; negative for liquefaction of horse serum and gelatin for the digestion of casein; negative for phosphatase activity; and negative for reduction of tetrazolium. They were weakly positive aerobically and clearly positive anaerobically for reduction of methylene blue and tellurite, the latter reagent being rather inhibitory for their growth. The strains were negative for production of film and spots on HIB + HOS + OYE but positive on similar medium containing egg emulsion. The strains produced alpha hemolysis on sheep erythrocytes using a modification of the Aluotto et al. method. The growth of the organisms was inhibited by sodium polyanethol sulfonate.

The M. hyosynoviae reactions obtained are in agreement with the more limited battery of tests reported by Ross on strain S16.

A variety of other characterization tests on these strains is still in progress.

c. Type culture collection of Mollicutes and L-phase variants of bacteria.

A viability study of the earliest freeze-dried Mycoplasmatales and L-phase variant strains stored in the American Type Culture Collection (ATCC) was completed this year. Ten Mycoplasmatales and two L-phase variant strains, which had been stored for 10.3 to 10.9 years at 4°C, and eight Mycoplasmatales and two L-phase variant strains, which had been stored for 8.5 years at -30°C, were included in the study.

Originally some of the strains had been cultivated at WRAIR in a base medium that contained Oxoid beef heart infusion broth, trypticase, and DNA. For the current study that medium was modified by substituting Bacto heart infusion broth for the three ingredients used earlier. Most strains were originally cultivated in whatever medium the original contributor of the strains had designated as the preferred medium. These differed in composition to such an extent that a valid comparison of counts among different strains of a species

Table 9. Biochemical reactions displayed by newer species of swine Mycoplasma.

Test	M. hyopneumoniae Weybridge	M. hyopneumoniae T1	M. suipneumoniae J	M. hyosynoviae S16	M. hysynoviae M60
Urea hydrolysis	-	-	-	-	-
Arginine hydrolysis	-	-	-	-	+
Glucose (acid)	-	-	-	-	-
Mannose (acid)	-	-	-	-	-
Lactose (acid)	-	-	-	-	-
Mannitol (acid)	-	-	-	-	-
Galactose (acid)	-	-	-	-	-
Sucrose (acid)	-	-	-	-	-
Cellobiose (acid)	-	-	-	-	-
Glycerol (acid)	-	-	-	-	-
Inositol (acid	-	-	-	-	-
Levulose (acid	-	-	-	-	-
Sorbitol (acid)	-	-	-	-	-
Salicin (acid)	-	-	-	-	-
Xylose (acid)	-	-	-	-	-
Arabinose (acid)	-	-	-	-	-
Aesculin hydrolysis	-	-	-	-	-
Inhibition by SPS	+	+	+	+	+

Table 9 (Con't.)

Phosphatase	-	-	-	-	-
Tetrazolium reduction	-/-	-/-	-/-	-/+	-/+
Tellurite reduction	+/+	+/+	+/+	+/+	+/+
Methylene blue reduction	±/+	±/+	±/+	-/-	-/-
Film and spots (HIB-HOS-GYE)	-	-	-	-	-
Film and spots (egg yolk)	+	+	+	+(c)*	+(c)
Gelatin liquefaction	-	-	-	-	-
Casein digestion	-	-	-	-	-
Horse serum liquefaction	-	-	-	-	-
Sheep RBC hemolysis	a**	a	a	a	a

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* c = clearing

** a = alpha

was open to question. Therefore, for the current colony counts, all strains were cultivated both on the medium (or modification thereof) originally used before freeze-drying and on a standard medium (Bacto heart infusion agar (pH 7.5) + 20% (v/v) inactivated horse serum + 10% (v/v) or a 25% (w/v) fresh baker's yeast extract) now in use for a majority of strains in the collection.

The results are shown in Table 10. There was, in fact, no significant difference in counts obtained on the standard medium shown in the last column and on the other media shown in column 4. With one exception (M. salivarium stored at +4°C), the viability of the strains stored for eight to 10 years was quite good.

Beginning in 1964 freeze-dried vials of Mycoplasmatales and L-phase variants were stored at -70°C; these vials will be tested by colony counts for viability in 1974.

Sixteen strains of Mycoplasma, three of Acholeplasma, and two L-phase variants were added to the ATCC-WRAIR collection during the past year. The strains in the Mollicutes collection now number 97, and in the L-phase collection 22.

Special effort has been made this year to accession goat, sheep and equine type and representative strains. Furthermore, through the courtesy of Dr. Fabricant a reference collection of his bovine mycoplasma serotypes has been acquisitioned and another collection of his avian serotypes is in storage awaiting deposit in the near future.

Because of the specialized cultivation requirements for the genus Streptobacillus and the genus Treponema, the WRAIR Mycoplasma Section has accepted the responsibility for propagating and depositing these genera in the joint collection. The first strain of T. pallidum (human, cultivable, Kazan 8) was deposited this year, and a valuable collection of S. moniliformis strains will be deposited in the coming year.

The number of vials of Mollicutes and L-phase variants distributed by the ATCC to fulfill requests were 54 and 31 respectively, of which 64 vials were provided to military personnel free of charge. In addition, 23 vials of bacterial forms (either parent or revertants of L-phase variants) were distributed by ATCC. Seed stock of three mycoplasma strains and one L-phase variant were replenished during the year. Colony counts and cultures for detecting possible contaminants were performed on 41 mycoplasma and L-phase strains. Biochemical and serologic tests were performed on a number of strains to verify their identity.

Table 10. Colony counts showing viability after 8-10 years storage of ATCC freeze-dried Mycoplasma and L-phase variant strains.

Genus, Species, Strain and ATCC No.	Storage Temperature (°C)	Storage Time (Years)	Colony-forming units/ml On: Modification of the media (+ agar) used for freeze-drying	Colony-forming units/ml On: HIA + 20% HOS + 10% FYE[a]
Mycoplasma arthritidis H606 (ATCC 13988)	+ 4	10.9	5.6×10^{5}[b]	8.7×10^{5}
	-30	8.5	1.5×10^{6}	1.0×10^{6}
Mycoplasma arthritidis Jasmin (ATCC 14124)	+ 4	10.5	1.0×10^{6}[c]	1.0×10^{6}
	-30	8.5	3.0×10^{6}	2.0×10^{6}
Mycoplasma arthritidis Campo (ATCC 14152)	+ 4	10.5	1.4×10^{5}[c]	0.85×10^{5}
	-30	8.5	8.3×10^{5}	9.3×10^{5}
Mycoplasma bovigenitalium PG11 (ATCC 14173)	+ 4	10.4	1.9×10^{4}[c]	1.8×10^{4}
	-30	Not Done	- - -	- - -
Mycoplasma hominis 4387 (ATCC 14027)	+ 4	10.9	0.9×10^{5}[d]	1.9×10^{5}
	-30	8.5	2.4×10^{5}	2.0×10^{5}
Mycoplasma hominis Joan (ATCC 14268)	+ 4	10.3	5.3×10^{4}[e]	9.8×10^{4}
	-30	8.5	7.6×10^{4}	9.7×10^{4}
Acholeplasma laidlawii Laidlaw A (ATCC 14089)	+ 4	10.6	5.4×10^{7}[c]	2.8×10^{7}
	-30	8.5	2.3×10^{8}	2.7×10^{8}

Acholeplasma laidlawii Laidlaw B (ATCC 14192)	+ 4	10.4	4.0×10^{7}[c]	4.4×10^{7}
	-30	8.5	6.1×10^{7}	4.9×10^{7}
Mycoplasma pulmonis Kon (ATCC 14267)	+ 4	10.3	3.3×10^{4}[c]	1.3×10^{4}
	-30	Not Done	- - -	- - -
Mycoplasma salivarium Buccal (ATCC 14277)	+ 4	10.3	2.0×10^{0}[d]	0
	-30	8.5	2.4×10^{5}	1.6×10^{5}
Proteus mirabili L-9 (ATCC 14168)	+ 4	10.5	5.3×10^{4}[c]	3.0×10^{4}
	-30	8.5	1.1×10^{3}	0.2×10^{3}
Streptobacillus moniliformis L 1 Rat 30 (ATCC 14075)	+ 4	10.7	2.7×10^{3}[c]	1.7×10^{3}
	-30	8.5	1.3×10^{4}	1.4×10^{4}

[a] Bacto heart infusion agar (pH 7.5) + 20% (v/v) horse serum (inactivated) + 10% (v/v) of a 25% (w/v) fresh baker's yeast extract.

[b] Bacto veal infusion broth (pH 7.4) + 15% (v/v) horse serum (inactivated).

[c] Bacto heart infusion broth (pH 7.8) + 1% (w/v) trypticase + 20% (v/v) human serum (inactivated) + 0.5% (v/v) of a 10% (w/v) Oxoid yeast extract + 0.002% (w/v) DNA.

[d] Bacto veal infusion broth (pH 7.4) + 20% (v/v) human serum (inactivated) + 0.5% (v/v) of a 10% (w/v) Oxoid yeast extract.

[e] Bacto veal infusion broth (pH 7.4) + 25% (v/v) human ascitic fluid.

4. Clinical activities.

a. Infectious Diseases Training Program.

Two infectious disease fellows (MAJ J. M. Griffiss and LTC F. R. Stark) began a two year program in laboratory and clinical I.D. training. The program was developed with the philosophy that management of clinical infectious disease problems is best learned by developing patterns of thinking similar to those needed for controlled laboratory experimentation. In both situations a thorough knowledge of how to evaluate test data is needed. The exercises of identifying a specific problem, searching for its origin (diagnosis), designing a controlled test or trial, making precise observations and recording, interpreting and summarizing data are most readily carried out at the bench level. The lessons learned can then be relatively easily applied to clinical situations. Specialized clinical knowledge is necessary, of course, but such knowledge can be more easily acquired and applied once the analytical process is ingrained.

The following paragraphs outline the activities of the fellows:

Laboratory research: Griffiss - meningococcal and gonococcal antibodies and antigens; Stark - various topics in cellular immunity.

Clinical rounds, clinical conferences, journal club participation, clinical investigation, attendance at selected national meetings.

b. Clinical investigation.

The Nitroblue Tetrazolium (NBT) Dye Test: Reduction of nitroblue tetrazolium dye by polymorphonuclear cells was found originally to be a useful test for diagnosing and studying patients with chronic granulomatous disease, a disease caused by a selective defect in phagocytosis. Later it was adapted to the study of patients with a variety of febrile illnesses. Preliminary reports found it to be very promising in distinguishing febrile patients with acute bacterial and fungal infections from those patients with fever from other causes.

Methods:

NBT Dye Test: The nitroblue tetrazolium dye test was modified from the method of Park. Five ml. of the patient's blood was mixed with 200 USP units of sodium heparin (Upjohn Co., Kalamazoo, Mich.). Plastic syringes and plastic test tubes were used throughout.

An equal volume of heparinized blood and nitroblue tetrazolium dye were incubated for 30 min. at 37°C. A thin smear was then stained with Wright's stain and the number of neutrophils containing reduced black formazan deposits was determined after viewing 100 neutrophils.

All neutrophils with distinct deposits were considered positive.

The entire test was performed within 90 minutes. Preliminary studies had shown that there was a deterioration of neutrophil stability after 90 min. post venipuncture. Prior to each use, the dye was filtered through a 0.45 μ millipore filter (Millipore Corp., Bedford, Mass.) as recommended by Chretien. The WBC and differential was performed on each blood sample by routine methods.

A stimulated control using 200 μgm/ml endotoxin lipopolysaccharide B E. coli 026:B6 (Difco Laboratories, Detroit, Mich.) as described by Park was included in all but 20 of the tests performed.

Patient Selection: Patients were chosen for study if they had fever, leukocytosis or both. Only those patients in whom the etiology of the fever could be determined by laboratory studies or clinical means (ie, Hodgkins disease, Guillain-Barre Syndrome) were included in the study.

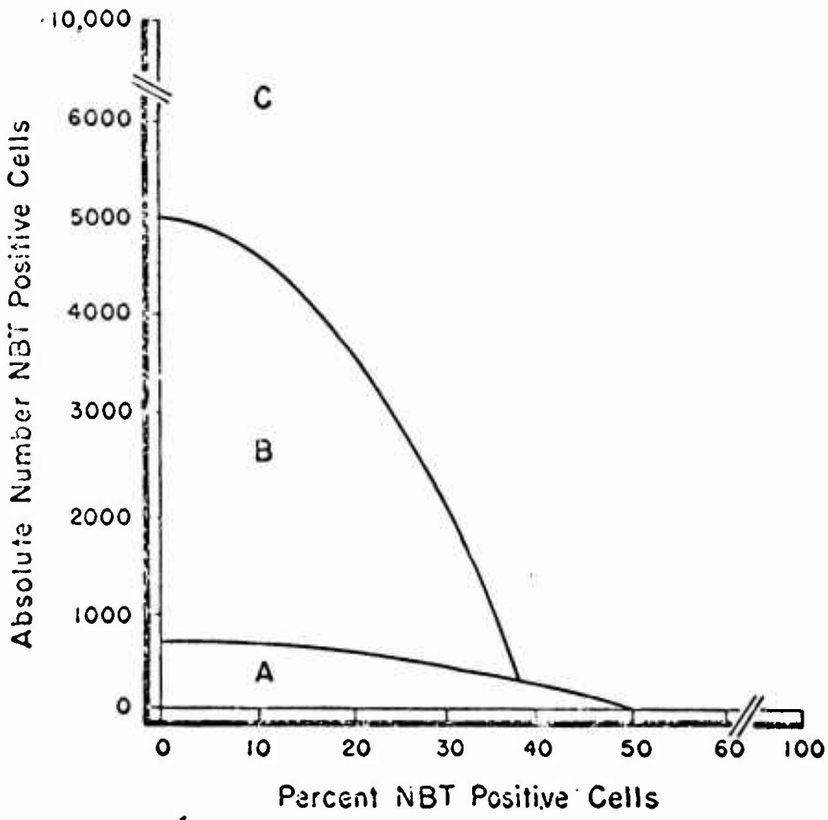
Patients were classified into three groups using a modification of Feigen's nomogram, Fig. 1. The formula for determining the total number of NBT positive PMN's was as follows: total WBC x (% PMN's + band forms) x % positive PMN's. Patients falling into the "A range" were considered normal, those in the "B range" were considered partially treated bacterial or uncomplicated viral infections, and those in the "C range" were considered to have an active untreated infection.

Salicylate Levels: Salicylate blood levels were determined by the methods of Trinder.

Results:

A total of 135 patients were categorized clinically as shown in Table II. Any infected patient who had received antibiotics prior to obtaining the test was categorized as a "partially treated infection". If a patient had been infected for more than three weeks he was categorized as a "subacute or chronic infection".

Twelve patients fell in the category of acute untreated bacterial infection. Eight of these patients had NBT scores in the "C" zone. Three patients with untreated bacterial infections had NBT scores which were in the "A" range. One of these patients had been having febrile episodes for two days. Klebsiella-enterobacter was isolated from his blood. However, when his IV catheter was removed he became afebrile and his blood cultures became sterile. His NBT test was performed 18 hrs after removal of the intravenous catheter. Another patient in the "A" range was a young woman who had fever, leucocytosis and constitutional symptoms three days post partum. A bacteroides species was isolated from her blood on two separate



A - Normal subjects

B - Viral infection, partially treated bacterial infection, non-infectious febrile illness

C - Untreated bacterial diseases

Fig. 1

Table 11. Distribution of results in patients studied with NBT dye test.

	No. of patients with indicated NBT classification			Percentage of positive neutrophils	
	A	B	C	Range	Mean
Condition with Pos. NBT					
1. Acute bacterial infection					
Untreated	3	1	8	3-74	30
Partially treated	6	21	3	0-40	16.6
2. Subacute + chronic					
Infected (untreated)	3	2	0	0-29	11.2
Condition with Neg. NBT					
1. Normal controls	31	2	0	0-17	6.6
2. Uncomplicated viral infections	14	0	0	0-28	8.4
3. CGD	1	0	0		
4. Noninfectious fever	17	1	1	0-34	6.3
5. Neutrophilia without fever	4	0	0	0-1	
6. SLE	3	0	0		
Condition of Uncertain Status					
1. Parasitic infections	3	0	1	2-38	
Hookworm	1				
Leishmaniasis (Cutaneous)			1		
Amebic abscess (Treated)	1				
Pneumocystis Carini	1				
2. Syphilis (Tertiary)	1			2	
3. Tuberculosis	2	0	0	2-3	
4. Fungal (Chronic cryptococcosis)	1	0	0	4	
5. Malaria (Treated)	1	0	0	16	

occasions. However, her blood became sterile, she became afebrile without therapy and remained asymptomatic for the next three months.

The third patient had a streptococcal pharyngitis with systemic signs including fever, petechial eruption and enlarged lymph nodes.

An additional case fell into the "B range". A blood culture was positive for beta hemolytic streptococci and an acute bacterial sinusitis was found in this renal transplant patient receiving prednisone and Imuran therapy. However, he had normal NBT dye tests prior to and after the acute episode (Table 12).

Table 12. NBT dye test before and after infection.

Date	NBT Score	WBC mm^3	Poly's	Range	Comment
Jan 12	0%	19.1	88	A	
Jan 14	5%	23.3	94	B	Pos.β strep bl. cult
Jan 17	24%	10.6	77	B	Sinus drained surgically Penicillin administered
Jan 21	0%	17.9	97	A	Clin. well
Jan 24	0%	14.5	95	A	Clin. well

Thirty patients were classified as "partially treated". Five of the six patients in the "A category" had been receiving appropriate antimicrobial therapy for 72 hours or more with good clinical response. A sixth patient died within 18 hrs of developing a bilateral aspiration pneumonia and disseminated intravascular coagulation syndrome. One of the 21 patients in the "B range" had a chronic loculated infection of the pericardium. The remaining 20 patients had been on appropriate therapy for 12 or more hours. Two of the three patients who fell into the "C range" had received appropriate treatment for less than 12 hours while the third had a gram negative (E. coli) pneumonia under "appropriate" therapy for 48 hrs.

Five cases of chronic untreated infection were studied (chronic osteomyelitis, chronic urinary tract infection, chronic liver abcess and SBE (two patients)). Three fell into the "A range" and two into the "B range".

Control patients consisted of 14 healthy adults (age 21 to 69) and 13 institutionalized elderly patients (age 55 to 96). Also

included were six patients who were clinically well, afebrile but hospitalized for evaluation of fibrous dysplasia of bone, allergic rhinitis, Gilbert's syndrome and Hodgkin's disease (three patients).

Thirteen of the 14 cases of viral infection were documented by viral isolation, antibody titer rise, or both. The 14th case had a clinical history and liver biopsy compatible with type MS-1 hepatitis.

As expected the patient with chronic granulomatous disease had a NBT dye test of 0% and his PMN's could not be stimulated with endotoxin.

Seventeen patients with noninfectious fevers were studied (Table 13).

Table 13. Noninfectious fevers.

	Diagnosis	Range A	B	C
1.	Acute myocardial infarction	2		
2.	Hyperthyroidism	1		
3.	SLE with renal involvement	2		
4.	Goodpasture's syndrome	1		
5.	Cirrhosis with alcoholic hepatitis		1	
6.	Overdose of salicylates			1
7.	Pulmonary embolism	1		
8.	Neoplastic disorders			
	Metastatic malignant melanome	2		
	Metastatic carcinoma of the rectum	1		
	Carcinoma of the rectum	1		
	Brain tumor	1		
	Metastatic breast carcinoma	1		
	Multiple Myeloma	1		
	Metastatic neuroblastoma	1		

One patient whose results were in the "C range" had taken an overdose of aspirin. His NBT remained elevated for over 38 hrs. despite recovery but he could not be followed further. Because of this experience, eight laboratory volunteers ingested aspirin to study its

effect on the NBT test. In Table 14 it can be seen that despite significant salicylate blood levels, none of the volunteers developed abnormal NBT dye tests.

Table 14. Effect of salicylates on the NBT dye test.

Patient	Salicylate level	NBT Score
Overdose	85% mg %	34%
	61 "	49%
	13 "	54%
Volunteer 1	19* mg %	3%
2	40 "	3%
3	18 "	2%
4	25 "	2%
5	19 "	6%
6	23 "	2%
7	22 "	4%
8	24 "	8%

* Highest level of salicylate reached.

Three patients were classified as having neutrophilia because of persistently elevated neutrophil counts (>10,000/mm^3) for a period of seven or more days. All of these patients had NBT scores within the "A range".

The NBT test was also evaluated as a means of following the response to therapy in some patients. One of these cases has been presented above (Table 12). Five other patients with acute untreated infection were also studied and in each case their NBT dye tests returned to normal.

Discussion:

The NBT dye test appears to be helpful in alerting the physician to the possibility of an acute bacterial infection. In this sense, it appears to be more helpful than the white blood count and differential, or the fever pattern. However, it has to be stressed that the NBT dye test must be interpreted in each individual case in the light of the clinical setting, clinical examination and other laboratory findings.

In considering the entire group of untreated bacterial infections, half of our cases fell into the "A" and "B" ranges. However, when we separated the patients into acute vs. subacute and chronic infections all of the latter group of patients had results in the "A" or "B" range. We speculate that this may have been due to the development of a tolerance of the neutrophil to the infecting microorganism. In each case the patients' PMN's were stimulated well with endotoxin suggesting that no underlying defect of the PMN's was present. Also, one of these patients who was studied sequentially developed a significant NBT rise shortly after therapy was instituted.

There were four patients with acute untreated bacterial infections who were classified in the "A" or "B" ranges and thus represented NBT "failures". Two of the patients in the "A" range had an acute bacteremia. However, in one the NBT dye test was done after the probable source of infection was removed and the patient had become afebrile. Matula has shown that the NBT dye test may return to normal before the clinical response has returned to baseline. Perhaps removal of the infected IV had already cured this patient prior to his NBT dye test. The second case had a positive blood culture at the time the patient was clinically well and had a normal WBC and NBT dye test. She never received antibiotics during or after her hospitalization. As was mentioned, subsequent blood cultures were sterile and she has remained clinically well for at least three months. Her clinical course suggests that her bacteremic episodes were transient and without significant residua. The third patient may have had a viral infection and may have been an incidental carrier of group A beta-hemolytic streptococci. No viral studies were done.

As mentioned, the infected untreated patient who fell into the "B" range was on prednisone therapy. It is reported that steroids tend to "normalize" the NBT dye test. However, this patient had a previous NBT study performed in which there was a significant rise and then fall after therapy. In selected patients receiving steroids serial NBT determinations may be a useful way of monitoring for infection. None of the other patients in the infected groups were receiving steroids. However, one of the patients with a documented influenza viral infection was being treated with steroids. In preliminary studies with patients receiving steroids, we have found that their WBC's were able to be stimulated with endotoxin to the same extent as normal patients.

We also have found that the nomogram is extremely useful in interpreting the results. For example, one of our control patients had an NBT value of 17% with a WBC of 6,000, 26% of which were PMN's, while a patient with a documented varicella infection had an NBT score of 28% with a WBC of 2,900 and 34% PMN's. If these NBT scores were taken alone, both of these patients would have been considered positive. Using the nomogram, both results were in the "A" category.

Park suggested that stressing the PMN's with endotoxin would be helpful in interpretation, particularly in ruling out underlying PMN defects. As was previously mentioned, this technique may also help to shed some light in the underlying pathophysiology of some types of infections. Other investigators have studied this aspect and have found it useful. We have now made this part of our routine NBT dye test.

Patients with malaria, various parasitic and fungal infections have been found to be positive by other investigators. The situation with tuberculosis is unclear. Early reports suggested that these patients had normal NBT dye tests. However, Mandell using a different method found patients with tuberculosis to have significantly higher NBT scores. In selected patients these returned toward normal after treatment.

When we found such high NBT values in our patient with salicylism and recognized the fact that salicylates are used commonly as antipyretics, we investigated the effects of salicylates on the NBT dye test in normal volunteers. The results in Table 14 indicate that there was no appreciable salicylate affect on the NBT test by the method we employed. The elevated NBT in one individual may represent an idiosyncratic reaction, a biological false positive or a missed bacterial infection.

Our results have not been as conclusive as those previously reported in evaluating the NBT test as a diagnostic measure of bacterial infection. However, our false negative cases all appeared to have plausible explanations for their low values. Also, the incidence of false positive reactions was very low.

Dividing the patients into categories as we and others have done has obvious drawbacks when faced with the acutely ill patient. Nevertheless, if it is kept in mind that this test measures certain metabolic functions of leukocytes and will be positive whenever these metabolic systems are stimulated and since acute bacterial infection is a very common stimulus, the NBT dye test can be extremely useful as an adjunct to diagnosis and treatment of bacterial infection.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 168 Bacterial diseases

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6447	72 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
71 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	170
b. CONTRIBUTING				
c. XXXXXXXX	CDOG 114(f)			

11. TITLE (Procede with Security Classification Code)

(U) Militarily Important Diseases Transmissible Between Animals and Man (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 06	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PRECEDING 72	1.5	35
	CURRENT 73	4.0	80

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered.

22. KEYWORDS (Procede EACH with Security Classification Code) (U) Zoonoses; (U) Military Dogs; (U) Canine Zoonoses; (U) Leptospirosis; (U) Melioidosis; (U) Bacterial genetics; (U) Pseudomonads

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 zoonoses of real or potential military significance, to determine the natural history, biological characteristics of agents, to develop suitable diagnostic procedures, treatment and control measures.

24. (U) Conventional microbiological and chemical technics are used. New procedures are developed as needed.

25. (U) 71 07 - 72 06 A high percentage of scout dogs returning from RVN had antibodies for scrub typhus, melioidosis, leptospirosis and Group B arbovirus. The military dog has potential usefulness as an indicator of these diseases. Genetic characterization of leptospiras by DNA annealing tests were completed. Pathogenic and saprophytic leptospiras each comprise 3 distinct genetic groups of organisms. A strain isolated from a bull is genetically unrelated to any group and apparently belongs to a third complex of leptospires. Cultural and serological studies on pseudomonads were continued. The presence and absence of autoplaquing in agar lawns of P. aeruginosa was correlated with ammonia production, the pH and alpha ketoacids. The presence and activity of specific Krebs and glyoxalate cycles enzymes were assayed. P. cepacia, a species which has been associated with wound infection, comprises serologically homologous strains. Unlike P. pseudomallei strainsof P. cepacia are avirulent for hamsters. High antibody titer sera from human melioidosis patients elicit no bactericidal activity for P. pseudomallei in vitro tests. No intracellular bacteria could be seen microscopically in dog kidney tissue cultures infected with P. pseudomallei, although cell sheet was destroyed in 24 hours. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*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 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 170 Militarily important diseases transmissible between animals and man

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Description.

Studies are conducted on zoonotic diseases of real or potential military importance and entail epidemiological investigations to determine natural history and occurrence of diseases, basic and applied research which bear on development of suitable diagnostic, treatment, and control measures. Principal efforts are on agents of leptospirosis, melioidosis and related pseudomonads.

1. Zoonoses in Military Scout and Tracker Dogs in Vietnam.

In operational areas military dogs and troops may be exposed to the same environmental hazards of infections with various zoonoses. Questions were posed as to whether military dogs were infected with various zoonotic agents which were significant causes of febrile illness among troops in Republic of Vietnam (RVN). Serological tests were conducted on 64 healthy military dogs which were returned from RVN to the United States for reassignment.

Scrub typhus antibodies were found in 45% of dogs, melioidosis in 19%, group B arbovirus in 49% and leptospirosis in 62%. Only 38% of the seropositive reactions for leptospirosis could be related to overseas infections. Antibodies for spotted fever rickettsia, Rickettsia canada, group A arbovirus and plague were found in single dogs respectively. Tests were conducted on paired sera obtained from 32 of the dogs before service in RVN. Significant increases in titer were demonstrated with scrub typhus, melioidosis, leptospirosis, plague, group B arbovirus and R. canada antigens. After 2 to 6 months of service in RVN, 8 of 19 dogs developed antibody titers to at least one of four zoonotic diseases. Only 3 of 45 dogs with 7 or more months of service failed to develop antibodies to one or more of the agents.

Tests are being done on 156 additional dogs which were returned to the U.S. Rickettsial antibody findings in these dogs (reported separately by the Department of Rickettsial Diseases) affirm initial observations.

The demonstration of leptospirosis, melioidosis, Group B arbovirus infections in dogs was consistant with previous findings[1-3]. The high

incidence of infection in dogs with R. tsutsugamushi was unexpected. The course of scrub typhus infection in experimentally infected dogs is now being studied. The findings in the initial study are detailed in a published report (7).

2. Genetic Characteristics of leptospiras.

Additional DNA annealing tests were carried out to complete projected genetic studies on selected strains differing in phenotypic characteristics from each other and from prototype strains of previously disclosed genetic groups (See Annual Report 1970-1971). Tests were done on a frog isolate (strain ICF) and a turtle isolate (strain A-183). These strains were compared with each other and with other known genetic types using previously described procedures (4).

A summary of DNA annealing test findings to date is shown in Table 1. The findings indicate the presence of 7 distinct genetic groups of leptospiras which could be assembled into 3 clusters on the basis of partial DNA relatedness. Clusters of "pathogenic" and "saprophytic" leptospiras each comprised 3 genetic groups. A third complex comprised one strain, 3055 (isolated from a bull) which was genetically distinct.

Table 1. Summary of Comparative Homologies of DNA from Selected Pathogenic and Saprophytic Leptospiral Serotype Strains

Complex	Radio-active DNA from strain	Percent DNA homology with strain						
		batav.	jav.	ICF	patoc	cdc	183	3055
"pathogens"	bataviae	100	31	30	3	2	4	0
	javanica	34	100	38	0	2	4	3
	frog (ICF)	ND[1]	38	100	ND	1	2	0
"saprophytes"	patoc	6	6	6	100	30	29	0
	cdc	6	3	3	28	100	48	0
	turtle 183	4	ND	7	52	37	100	7
New	bull 3055	3	ND	3	5	7	5	100

[1]ND = not done.

3. Bacteriocidal tests on Pseudomonas pseudomallei antiserum.

These tests were done to determine the cidal activity of sera from convalescent melioidosis patient for Pseudomonas pseudomallei. The procedures of both McIntyre and Feeley[5] and Finkelstein[6] were used. Tests were carried out with high titer (by complement-fixation and indirect hemagglutination tests) serum and isolate from a human patient.

Generally serum was diluted 4-fold from 1:5 thru 1:20.480. To aliquots of each dilution were added complement and bacterial cells. These mixtures were read and sampled for cell counts after various intervals of incubation. No bacterial antibody could be demonstrated in patient serum by either of the two methods.

Attempts were made to enhance antibody cell-wall interaction by pretreatment of cells with tetrasodium EDTA and $MgCl_2$. Final concentrations of EDTA ranged from 9×10^{-4}M to 2.4×10^{-2}M and that of $MgCl_2$ from 1.9×10^{-3}M to 5.8×10^{-2}M. The serum had no killing effect under any of the above conditions.

4. Pseudomonas pseudomallei in tissue culture.

A major problem in management of melioidosis cases is persistance of infection not only in patients with prolonged disease but in clinically cured patients. Studies were initiated to clarify the nature of bacterial persistance by the use of tissue culture systems. An immediate question posed was whether this organism occurs and grows intracellularly in tissue culture cells.

Initial tests were carried out in primary dog kidney tissue culture which were planted in upright screw-capped test tubes and in Leighton tubes. Cells were maintained in Eagle basal medium containing 1% glutamine. Replicate tubes were inoculated with varying 10-fold concentrations of cells ranging from 1-10 to 10^5 organisms and then incubated at 37 C. At 4, 8 and 24 hours, media and bacterial cells were drawn off. Cells in upright tubes were overlayed with 1-2 ml of a 1.5% solution of Nobel agar in M199 medium containing 3% fetal bovine serum and neutral red dye. The cells in Leighton tubes were fixed with Bouins solution and stained with hemotoxylin and eosin. The tubes with the agar overlay were further incubated at 37 C and read after 48 hours.

The tissue culture cells in agar overlay tubes failed to take up neutral red dye irrespective of the size of the P. pseudomallei inoculum. P. pseudomallei grew luxuriently in the agar overlay. Therefore, the non-viability of the tissue culture cells (manifest by dye uptake failure) could have been related to nutritional starvation, pH changes, etc., rather than to intracellular infection. The efforts to control the extracellular infection with various high concentrations of penicillin, streptomycin, aureomycin, kanomycin sulfate, and rifampin used singly and in several combinations in the agar overlay, failed. Four hours after infection the cell sheet in Leighton tubes was intact, Cytopathogenic effects became apparent 8 hours after infection with 10^4 or more organisms. Tissue culture sheets were partially or completely destroyed 24 hours after infection with all dilutions. Although many bacteria were seen on slides, none were seen intracellularly.

Additional tissue culture infectivity studies were carried out on dog lung macrophage cells. The alveolar macrophages were extracted, seeded, and maintained according to the methods of M. Appel of Cornell University

(unpublished data). The macrophage cells were planted in Leighton tubes, fed 24 hours later and thereafter at 48 hour intervals. After 4 to 6 days the cell sheet was washed twice and fed with M-199 medium containing 5% lactalbumin hydrolysate and 20% agamma calf serum (no antibiotic). Each tube was then seeded with approximately 10^7 colony forming units (CFU) of P. pseudomallei. In one series the inoculum was 2 x 10^3 CFU. The seeded tubes were incubated 1 hour. Then the supernatant fluid was removed and the cell sheet was washed twice. One ml of a sustaining medium containing antibiotics (M-199 + 5% lactalbumin hydrolysate + 20% agamma calf serum + antibiotics) was added to each tube. The purpose of the antibiotics was to control extracellular growth of P. pseudomallei. The tubes were incubated and cell sheets were fixed and stained after 1, 4 and 24 hours. Plate counts were performed on the original inoculum as well as on infected and control supernatant fluids immediately before cell sheets were fixed.

In all tests rod shaped bacteria were seen intracellularly. Four hours after infection the macrophages were more vacuolated and irregular in outline than the controls. The majority of macrophages were still intact and contained organisms 8 and 24 hours after infection.

Ampicillin, aureomycin, kanomycin, and rifampin used in concentrations as high as 200 ug/ml and in various combinations did not kill extracellular P. pseudomallei. Under these conditions it has not been possible to determine if the organisms were multiplying intracellularly

5. Serological and virulence tests on Pseudomonas cepacia.

Addition studies were done on P. cepacia, a species which has close biological affinities with P. pseudomallei and which has been increasingly recognized as a cause of wound, skin, and nosocomial infections (See Annual Report 1970 - 1971).

In previous studies P. cepacia was found to be serological distinct from P. pseudomallei in agglutination tests (Annual Report 1970 - 1971). Serological relationships of strains of P. cepacia were studied further. Antisera were prepared in rabbits from 6 strains of P. cepacia derived from different geographic areas and sources. "Box" titrations with the agglutinin technic were done with these six strains. Also cross agglutination tests were done on 5 additional strains of P. cepacia. On the basis of serologic reactions all strains of P. cepacia appeared to be serologically homologous except for 1 strain (17460) from the American Type Culture Collection.

Fourteen P. cepacia strains derived from soil, water, and human sources were tested for virulence for hamsters. Cells of each strain were obtained from overnight growth on 3% glycerol Brain Heart Infusion Agar and suspended in physiological salt solution to a concentration of approximately 2 x 10^8 colony forming units per ml. Each suspension was inoculated into five 4-to six-week old hamsters in a 0.5 ml dose. Animals were observed daily for a period of one month. Sporadic deaths

occurred on the 9th and 15th day with 3 strains. One strain provoked death in one hamster 24 hours after inoculation. The 4 other hamsters in the group had swollen testes at this time but were normal on the second day. P. cepacia was not found to be virulent for hamsters.

6. Cultural and biochemical characteristics of pseudomonads.

Further studies were done to define biochemical factors which effect colonial variations in pseudomonads and which bear on laboratory identification of pseudomonas diseases.

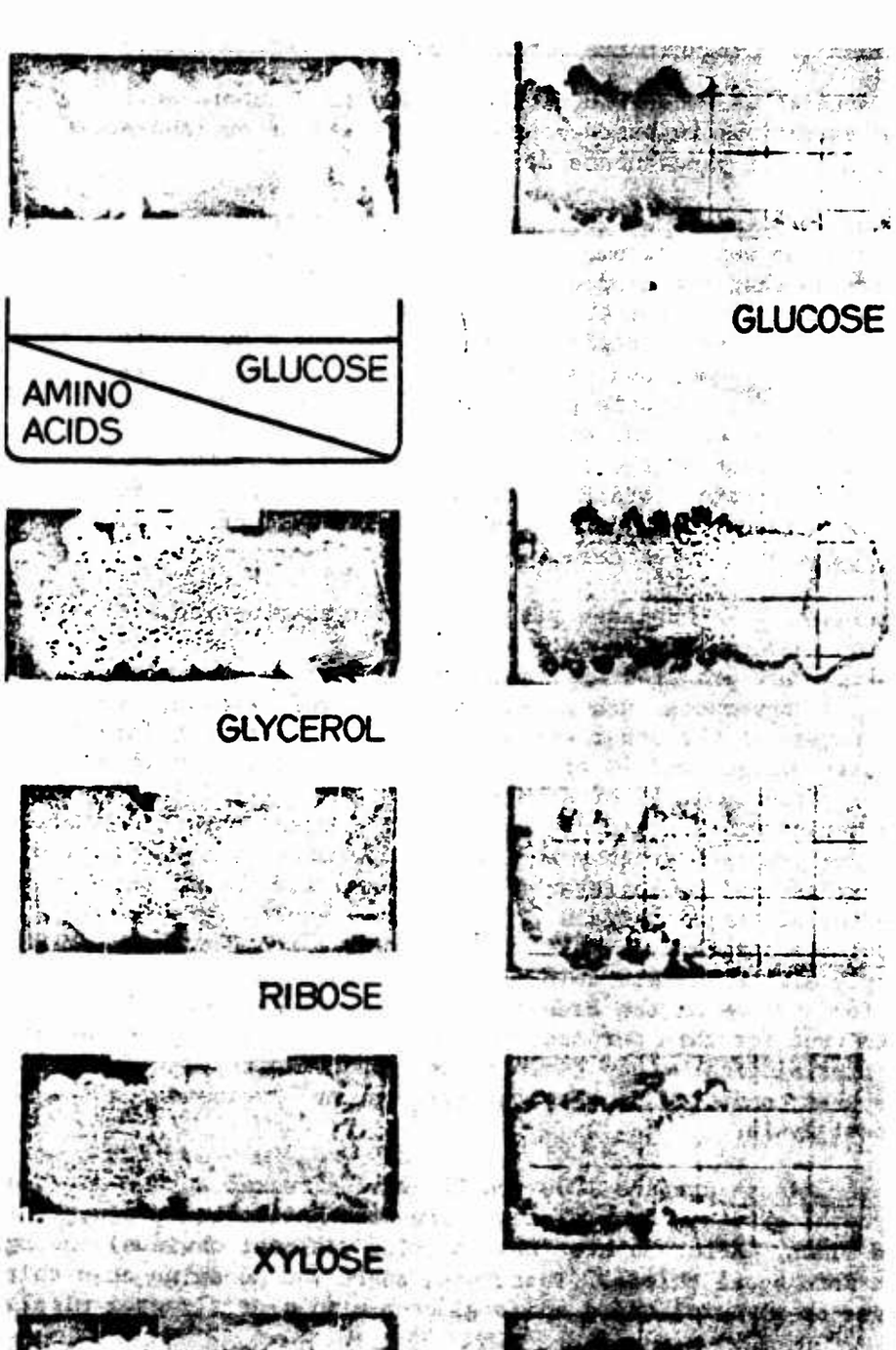
Agar lawns of Pseudomonas aeruginosa sometimes exhibit eroded or pitted localized areas which resemble phage plaques. The unique feature of this phenomenon is that the eroded areas are covered by an iridescent or metalic sheen. The cause or control of this condition have not been reported. A similar condition seemed to be caused by smooth strains of P. pseudomallei when grown on media containing a high N:C ratio. The lysis was caused by ammonia production. Rough strains produced oxalic acid on the same media and meutralized the ammonia toxicity. Increasing the glucose content of the media completely obviated the ammonia lysis of the smooth strains (WRAIR Annual report 1971 - 1972). For these reasons an attempt was made to control the iridescent plaquing of P. aeruginosa by nutritional means.

Two strains of P. aeruginosa were chosen for examination. Strain 227 plaqued on many common laboratory media, strain 1000 did not. Gradient agar plates were prepared with Difco Casamino acids (amino acids) on one side and carbohydrates such as glucose, glycerol, ribose, xylose and 2-oxoglutarate at the other extreme. Each gradient contained 1.5% agar, 0.05% yeast extract and 1% of either casamino acids or carbohydrate. Thirty six hour cultures of strain 227 are illustrated in Fig. 1. In this series casamino acids consistently caused some auto-plaquing. The glucose gradient greatly reduced the plaquing, whereas glycerol, ribose, xylose and 2-oxoglutarate (not shown) all seemed to allow or enhance the pitting in strain 227. Similar results were found in a third P. aeruginosa strain 1. Visually, phage lysis and autoplaquing look strikingly similar. This coincidence has led a number of investigators to search for a virus as the causal agent of iridescent plaquing. Strain 1 has been used for this purpose, but to no avail. It was our opinion that a physiological study (even though fraught with quantitative difficulties when conducted on agar cultures) might elucidate the cause of iridescent lysis.

The P. aeruginosa strains 1000 and 227 were streaked across glucose and glycerol gradient agar plates. The streaked lawns were approximately 2 cm in width. After 36 hrs (when pitting was most obvious) the agar was cut into equal thirds. Therefore, there was an amino acid third, a glucose or glycerol third and a glucose-middle or glycerol middle third.

The thirds were collected in 250 ml centrifuge cups, frozen overnight, thawed and centrifuged. The expressed fluids were collected. Formalin was added to a final concentration of 1.1% formaldehyde. The pH of

Fig 1.


REFLECTED LIGHT
TRANSMITTED LIGHT
GLUCOSE
AMINO ACIDS
GLUCOSE
GLYCEROL
RIBOSE
XYLOSE
AMINO ACIDS

each third was determined by a Beckman zeromatic pH meter. Alpha ketoacids were determined by their 2, 4 dinitrophenylhydrazones (Friedmann, T. E. Methods in Enzymology 3: 414-418, 1957) and ammonia was determined by titration methods. The data are listed in Table 2.

Table 2. Characteristics of Fluids Expressed from Gradient Plate Agar Cultures

Culture Fluid Thirds	pH	NH_3 gms% x 10^{-4}	Alpha Keto Acids monocarboxylic	dicarboxylic
			nanomoles	
Blank - glucose				
glucose/glucose	5.7	60.04	-	-
" /middle	5.6	66.93	-	-
" /amino acids	5.5	75.52	-	-
Blank - glycerol				
glycerol/glycerol	5.9	42.07	-	-
/middle	5.7	53.54	-	-
/amino acids	5.4	45.89	-	-
P. aeruginosa 227				
glycose/glucose	5.1	96.18	-	20
/middle	6.9	147.87	-	trace -
/amino acids	7.1	101.34	-	-
P. aeruginosa 227				
glycerol/glycerol	6.3	97.52	-	-
/middle	6.7	133.84	-	-
/amino acids	6.9	108.99	-	-
P. aeruginosa 1000				
glucose/glucose	6.6	95.60	-	-
/middle	6.3	182.22	3	-
/amino acids	7.0	114.15	-	-
P. aeruginosa 1000				
glycerol/glycerol	6.6	59.28	-	-
/middle	6.9	91.78	-	-
/amino acids	6.9	107.08	-	-

Generally, both strains increased the pH of the culture media. This correlated with increased ammonia production except when the alpha ketoacids were found in the media. Then the pH was depressed. The alpha keto acids were only found in the glucose gradient cultures. The amounts of ammonia evolved increased toward the amino acid gradient extreme but were usually (with the exception of the P. aeruginosa 1000 - glycerol middle) higher in the middle portions of glucose or glycerol

gradients the pH indicators phenol red and phenolphthalein were incorporated into the gradient agar to a final concentration of 0.0018%. Only the phenophthalein seemed to indicate that the pH was more alkaline around the edges of pitted areas, however the alkaline indication was not intense. The 2,4 dinitrophenol hydrozones were unidentifiable spectrophotometrically and too meager and unstable to identify by thin layer chromatography. Formaldehyde and media components interfered with the identification of glyoxylic acid by formazan formation. At present we have had some small amount of success in extracting, isolating and making volatile methyl esters of Kreb's and glyoxalate cycle metabolites for identifying and quantifying by gas chromatography. The accuracy and reproducibility of the method has not been ascertained. The lipoidal nature of the iridescent material suggested that the cells within the plaqued areas might manifest a difference in their catabolic and biosynthetic pathways. The operations of the Kreb's and glyoxalate cycles were ascertained by the enzyme content of P. aeruginosa 227 grown on media containing 0.5% casamino acids, 0.05% yeast extract, 1.5% agar and 0.5% glucose or 0.5% glycerol. At 36 hrs incubation the pitted glycerol agar lawn and nonpitted glucose agar lawns were scraped from the agar surfaces and suspended in 0.02 M tris buffer pH 7.2 containing 0.2 mg% glutathione. The cell sap was extracted in a French press at -20 C. The extracts were centrifuged to remove debris and sequentially filtered until filtered thru an 0.3u filter. The filtrate was stored in liquid nitrogen vapor until used.

The Krebs cycle was identified by isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase and the glyoxalate cycle enzymes of isocitrate lyase and malate synthase were identified by modifying the methods of Charles (Canad. J. Microbiol. 17: 617-624, 1971).

Although the enzyme assays of the cells did not define the cause of iridescent plaquing, the results listed in Table 3 point out some interesting characteristics and differences between glycerol and glucose grown cells. The glyoxalate cycle was very active on both media. At the isocitrate pivotal point the isocitrate dehydrogenase activity on glucose and glycerol was very high and suggested a highly active Krebs cycle, however the almost non-existant activity of 2-oxoglutaric dehydrogenase indicates that the cycle is interupted at this point. This is a condition which is more expected and found in obligate chemo-and photoautotrophs because they cannot use exogenous organic substrates and therefore do not need the catabolic function of the Krebs cycle. However, it is also possible that this is an adaptive mechanism that is more prominent only under these conditions. Otherwise the only other difference appears to be that the first step of the glyoxalate pathway is more active in glucose grown cells. There is almost twice as much activity of isocitrate lyase in glucose grown cells when compared to glycerol cultures. These results do not define the cause of plaquing and further studies will be conducted on other metabolic pathways and the iridescent lipid.

Table 3. Specific Activities of Enzymes from P. aeruginosa 227 grown on Glucose or Glycerol agar

Enzyme	Specific activity[a] glucose	glycerol	Activity ratio glucose/glycerol
Isocitric dehydrogenase	118.28	164.58	0.72
Isocitrate lyase	53.27	27.18	1.96
Malate synthase	54.00	51.80	1.04
2-oxoglutaric dehydrogenase	7.30	5.60	1.30

[a]Specific activity as nanomoles substrate oxidized, reduced or formed per milligram of protein per minute.

Summary and Conclusions.

1. Zoonoses in Military Scout and Tracker Dogs in Vietnam.

A high percentage of 64 U.S. military acout dogs returning from Vietnam had significant antibodies for scrub typhus, leptospirosis, melioidosis and Group B arbovirus. The findings direct attention to potential public health and veterinary importance of these diseases in dogs and to the possible usefulness of dogs as indicators for the presence of these infections in operational areas.

2. Genetic Characteristics of Leptospiras.

Pathogenic and saprophytic leptospiras each comprise 3 distinct genetic groups of organisms. A strain isolated from a bull is genetically unrelated to any group and apparently belongs to a third complex of leptospiras. Superficially, the genetic characteristics of various groups correlate with immunological and other phenotypic characteristics. The findings provide a basis for selection and development of appropriate leptospiral polyvalent vaccine.

3. Bacteriocidal tests on Pseudomonas pseudomallei Antiserum.

High antibody titer sera from a human melioidosis patient had no bacteriocidal activity for P. pseudomallei in in vitro tests.

4. Pseudomonas pseudomallei in Tissue Culture.

Attempts to demonstrate intracellular localization and growth of P. pseudomallei in dog kidney tissue culture were unsuccessful. Alveolar macrophages in lung tissue cultures engluf P. pseudomallei cells. Viability and possible multiplication of engulfed cells are being studied further.

5. Serological and Virulence Tests on Pseudomonas cepacia.

On the basis of cross-agglutination tests ten strains of Pseudomonas cepacia appeared to be serologically homologous. An eleventh strain was serologically distinct. Unlike P. pseudomallei strains of P. cepacia are avirulent for hamsters. The distinct serological characteristics of P. cepacia may afford a serological tool for diagnosis of the infections and for identifying these organisms.

6. Cultural and Biochemical Characteristics of Pseudomonads.

The iridescent autoplaquing of P. aeruginosa strains has been controlled by nutritional means. A number of biochemical and physiological mechanisms have been associated with this phenomenon. The techniques used would be especially applicable in the study of smooth and rough cultures of P. pseudomallei.

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.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6448	72 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	9. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 06 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	61102A	3A061102B71Q	00	171
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114 (f)			

11. TITLE (Precede 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: 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 72	6	260
FISCAL YEAR CURRENT 73	6	350

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Biological Products; (U) Formalin Inactivation; (U) Freeze-Drying: (U) Meningococcus Vaccine; (U) Typhus 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) 71 06 - 72 06 Investigations have continued on the development of new and improved biological products for military use. - 1. Purified high molecular weight polysaccharides derived from the B-NE strain of N. meningitidis and from the 07:K1(L)NM strain of E. coli, and purified protein antigens from the B-11 strain of N. meningitidis were prepared for human use. These are being evaluated for immunogenicity. 2. Laboratory studies have been initiated on the development of a method for the production of 800,000 doses of freeze-dried living strain E typhus fever vaccine, for use in human field studies and to provide a stockpile for emergency use. 3. Studies on formalin inactivation of bacterial and viral suspensions have shown that the binding of formalin to a constituent of the suspending medium results in a significant reduction in the rate of inactivation of infectious organisms. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

* 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; Calvin Powell, MS; Doria Dubois, MS; 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. Meningococcal Vaccine.

During this period studies have continued on the development of pilot scale methods for the preparation of purified antigens from Neisseria meningitidis, group B, for use in the immunization of man against this type of meningococcal meningitis.

a. Several attempts have been made to prepare high molecular weight purified polysaccharides from the B-11 strain of N. meningitidis, by the methods used successfully for the preparation of the groups A and C polysaccharide vaccines (1). However, unlike the A and C preparations, these group B products failed to elicit immunological responses in humans (Annual Report, 1971). Attention was therefore turned to other group B strains of N. meningitidis, as well as other organisms which possess a similar polysaccharide antigen. Accordingly, purified high molecular weight polysaccharides, derived from the B-NE strain of N. meningitidis and from the 07:K1(L)NM strain of Escherichia coli, were prepared suitable for human use. These preparations are now available for evaluation of their immunogenicity.

b. The observation that crude group B polysaccharide preparations, containing approximately 40 to 50% protein, produced immunogenic responses in rabbits prompted a series of studies directed towards the preparation of a specific protein antigen from the B-11 strain of N. meningitidis, for use as a possible immunogen for human use. Initially, the protein preparation was produced by the method described by Menzel and Rake in 1942 (2). This material proved to be antigenic when injected into rabbits, and protective for mice against a challenge with virulent group B meningococci. However, the protein antigen prepared by the Menzel and Rake procedure was a suspension rather than a solution, and standard membrane filtration methods, employed to obtain sterile preparations for human use, removed a considerable proportion of the antigenic activity. Experimental studies were therefore directed towards the development of techniques for solubilizing the protein antigen to permit passage through sterilizing membrane filters without reducing the antigenic activity. As the result of these studies the following procedure evolved and was used to prepare a lot of purified protein antigen which is suitable for human use.

Fifteen liters of a 21 hour culture of the B-11 strain of N. meningitidis, grown in a modification of Franz's medium (1) in which the $Na_2PO_4.H_2O$ concentration was increased 4-fold, was inactivated by the addition of phenol to a final concentration of 0.5%. After 1 hour at room temperature, the phenolized culture was filtered through gauze and then passed at a flow rate of approximately 400 ml per minute through a Sharples centrifuge operating at 48,000 g. The sediment in the Sharples bowl was collected, washed 3 times with cold physiological saline and then extracted 4 times with a 0.43 M phosphate buffered saline solution, pH 7.1. The supernatant fluids from the extractions were pooled, and the pool was dialyzed for 22 hours. The dialyzed material was centrifuged at 43,500 g for 30 minutes and the supernatant fluid was collected. Sufficient 50% trichloroacetic acid (TCA) was then added to the supernatant fluid to give a final concentration of 10% TCA and the mixture was held in a boiling water bath for 15 minutes. The heated mixture was then cooled. The resulting precipitate was collected by centrifugation and then washed once with 10% TCA and 3 times with cold water. The precipitate was resuspended in 150 ml of distilled water and brought into

solution by the addition of 0.6 ml of 10 N NaOH. The pH was then adjusted to 7.3 with 4 ml 1 N HCl and the resulting suspension was centrifuged at 43,500 g for 30 minutes. The sediment was discarded and the supernatant fluid at this point was considered to be the final product. The following table gives results of measurements and assays obtained during the processing procedure:

TABLE I

Summary of Assays During Preparation of Antigen from N. meningitidis, Strain B-11

Preparation	Assay	Result
Culture Fluid	pH	6.85
Sharples Sediment	Net Weight	135 gms*
Final Product	Yield	1.653 gms*
	Protein	88.0%
	Nucleic Acid	9.8%

*Yield obtained from 15 liters of culture fluid.

This protein antigen is currently being evaluated in animals prior to human use.

2. Typhus Fever Vaccine.

During this period laboratory studies have been initiated on the development of practical production methods for the preparation of 800,000 doses of freeze-dried living strain E typhus fever vaccine. This vaccine was requested by the Commission on Rickettsial Diseases of the Armed Forces Epidemiological Board for use in further field studies to evaluate the efficacy of this product, and also to provide a stockpile of typhus vaccine for use in the military in the event of an emergency.

Seven day old embryonated hen's eggs, certified free of all adventitious agents, are being inoculated with an appropriate dilution of seed material (lot CRD-2 of strain E typhus vaccine, freeze-dried in this laboratory in October 1965, and stored at -20 C) which will kill approximately 50% of the surviving embryos on or about the 10th post-inoculation day. At that time,

infected yolk sac membranes from living embryos are being collected (based on laboratory data provided by Dr. Bennett Elisberg and Miss Marilyn Bozeman of the Dept. of Rickettsial Diseases, WRAIR) and combined in sufficient numbers to provide a series of pools containing 300 ml of 20% blendorized membranes. These small pools will be stored at -70 C to await the completion of sterility and other tests to insure their suitability for use in preparing larger pools for filling and freeze-drying. It is anticipated that two freeze-drying runs will be required to provide the quantity of vaccine requested.

3. Formalin Inactivation of Bacterial and Viral Suspensions.

a. Previous studies in this laboratory (Annual Report, 1971) have demonstrated that, when formalin is employed to inactivate the organisms in certain bacterial vaccines, an equilibrium is established between the "bound formalin" and "free formalin" in the preparation. When sufficient sodium bisulfite is added to completely neutralize all of the "free formalin" (as measured by the NIH method), some of the "bound formalin" is released and becomes available as "free formalin". The presence of this "free formalin", which is concentrated during freeze-drying of the preparation, can have a deleterious effect on the antigen(s) and adversely affect the immunogenicity of the product.

b. Recent experimental studies have shown that reversibly bound formalin is also found in certain formalin inactivated virus preparations derived from tissue culture. Since the major portion of the nitrogenous material in these preparations comes from the synthetic medium in which the tissue cultures are grown, a study was made of the fate of formalin in growth medium alone. Formalin was added to a final concentration of 0.1% to each of three commonly used tissue culture media and to normal saline. These formalinized solutions were stored at 5, 22 and 37 C for 5 days, and were then assayed for free formalin by the NIH method. The results are shown in the following table:

TABLE II

Uptake of Free Formalin by Tissue Culture Media

Medium	% Free Formalin after 5 Days at 5 C	22 C	37 C
Medium 199	0.100	0.096	0.095
MEM Medium	0.103	0.098	0.093
Normal Saline	0.102	0.101	0.108
L-15 Medium	0.047	0.042	0.037

These results show that over 50% of the formalin added is bound by a constituent of the L-15 medium, while no appreciable uptake of formalin is detected in Medium 199, MEM or normal saline.

c. The demonstration of the binding of formalin in the L-15 medium, and the corresponding reduction in the amount of free formalin available, suggested a possible significant reduction in the rate of formalin inactivation of an infectious agent suspended in the L-15 medium. To elucidate this point, freshly harvested Salmonella typhi, strain Ty 2, were suspended in Medium 199 and in L-15 medium, and formalin was added (to a final concentration of 0.05%) to the two suspensions, as well as to the sterile media. All solutions and suspensions were held at 22 C for 48 hours and then at 5 C for an additional 3 days. Samples were removed periodically for assay of free formalin and for viable bacterial counts. The results are shown in the table below:

TABLE III

Relationship Between Uptake of Formalin and Inactivation of S. typhi Organisms

Time	199 % Formalin	199+Ty2 % Formalin	Plate Count	L-15 % Formalin	L-15+Ty2 % Formalin	Plate Count
0 hour	0.047	0.040	$1.4X10^9$	0.042	0.044	$1.7X10^9$
2 hour	0.046	0.037	$1.4X10^7$	0.043	0.040	$2.0X10^8$
4 hour	0.046	0.035	$4.6X10^5$	0.041	0.039	$1.3X10^7$
6 hour	0.046	0.033	$2.6X10^4$	0.041	0.037	$1.8X10^6$
8 hour	0.051	0.034	$>10^1$	0.043	0.039	$3.0X10^5$
24 hour	0.050	0.027	$>10^1$	0.041	0.035	$>10^1$
28 hour	0.049	0.025	80	0.041	0.033	$>10^1$
32 hour	0.050	0.024	7	0.041	0.034	$>10^1$
48 hour	0.044	0.014	10	0.031	0.027	97
5 days	0.047	0.017	0	0.036	0.030	0

The data again demonstrate the binding of formalin by a constituent of the L-15 medium, with considerably less free formalin available to combine with the bacterial cells. The result is a significant reduction in the rate of inactivation of the organisms suspended in the L-15 medium, when compared with the rate of inactivation of the organisms in Medium 199.

d. Following the 5 day inactivation period, the bacterial suspensions in the 199 and L-15 media were each divided into two portions. In one, the formalin was neutralized with sodium bisulfite on the basis of the amount of formalin originally added, and in the other portion, on the basis of the free formalin assay. After the 5 day inactivation period the four preparations were then dispensed in 1 ml aliquots into 10 ml vials, and were frozen and freeze-dried. The results of the formalin assays obtained on the rehydrated freeze-dried material are shown in column (b) of the table below.

TABLE IV

Formalin Assays on "Neutralized" Freeze-Dried
S. typhi Preparations

Suspension	% Formalin Neutralized	% Formalin in Rehydrated Freeze-Dried Product	Total % Formalin
	(a)	(b)	(a)+(b)
199+Ty2 I	0.040	<0.01	0.040
II	0.017	0.022	0.039
L-15+Ty2 I	0.044	<0.01	0.044
II	0.030	0.018	0.048

Thus in both preparations, neutralization of the free formalin, as determined by assay immediately prior to freeze drying, failed to eliminate all of the available formalin.

The results of this study to date indicate that, in certain formalin inactivated bacterial suspensions and in formalin inactivated virus and rickettsial preparations in L-15 medium, the bound

formalin is readily released and becomes available as free formalin. Therefore, in order to avoid concentration of formalin during freeze-drying, it is essential to add sufficient sodium bisulfite to neutralize all of the formalin originally added to the preparation, and not only the free formalin as measured by assay prior to freeze-drying.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 171, Development of biological products of military importance

Literature Cited.

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Publications:

(1). White, A., Berman, S., and Lowenthal, J. P.: Inactivated Eastern Equine Encephalomyelitis vaccines prepared in monolayer and concentrated suspension chick embryo cultures. Appl. Microbiol. 22:909-913, 1971.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6449	72 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
71 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	172
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114(f)			

11. TITLE (Procede 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	72	6	120
FISCAL YEAR CURRENT	73	6	120

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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. Procede text of each with Security Classification Code.)

23. (U) During OCONUS military operations, troops regularly are exposed to a variety of infectious disease 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 are urgently *required* in diseases in which causative agent is difficult to demonstrate.

24. (U) CF, FA, and HA technics used to determine efficacy of antigen fractionation procedures and to evaluate 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) 71 07 - 72 06. Work accomplished to date has provided specific, sensitive serodiagnostic tests for variety of important diseases (parasitic, treponemal, bacterial, mycotic). SAFA test more specific than tuberculin test for serodiagnosis of simian TB. Neither test alone detects all infections but when used in parallel will identify essentially all infected animals. Studies on efficacy of SAFA test for detecting infection and appraising effectiveness of therapy in human TB have been continued. Although test not sufficiently sensitive for screening, a reactive test highly indicative of current or recent active disease. Reagents for new FTA-ABS (IgM) test for congenital syphilis in neonates procured and standardized. Preliminary studies indicate drug abuse may impair serologic response. For technical report see Walter Reed *Army Institute* of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*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 172, Sero-recognition of microbial infections

Investigators.
Principal: Earl H. Fife, Jr., M.S.
Associate: Andre J. Toussaint, M.S.; Lois A. Simonton, B.S.; Barbara J. Flemmings, B.S.; Robert W. Sanders; Albert E. vonDoenhoff, B.S.

Description.

This task is concerned with the mechanisms and patterns of immune responses, and the development and improvement of immunodiagnostic methods. In vitro and in vivo procedures 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, and (2) production of specific antisera by infection and/or experimental antigens or antigen fractions. Antigens that 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 technic 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. The methodology is now well standardized and there have been no further technical modifications of the procedure introduced since the last reporting period.

a. Simian tuberculosis. Tuberculosis in non-human primate colonies is a serious problem and the need for a reliable test for early detection of active disease is readily apparent. Results of initial studies on experimentally infected monkeys suggested that the SAFA procedure using purified M. tuberculosis antigens might be of value in this respect(1). Although the SAFA test was superior to the intrapalpebral tuberculin (IDP) test for early detection of infection, it was recognized that the animals used in these experiments were challenged with an unusually large number of organisms (5,000) and that the results did not necessarily reflect the relationships that would obtain in animals receiving a minimum infective

dose.

In the succeeding studies, this deficiency was overcome by challenging the monkeys with graded doses ranging in log increments from 5000 to 5 colony forming units (cfu) of tubercle bacilli. A preliminary analysis of the results again indicated that the SAFA test was considerably more specific and sensitive than the palpebral tuberculin test, detecting 39 of the 41 infected monkeys, whereas only 31 of this group developed tuberculin hypersensitivity. Moreover, all 7 of the noninfected animals were nonreactive in the SAFA test, but 4 of these showed conversion of the tuberculin test. (See preceeding report on this Work Unit for details). A more recent comprehensive analysis of the data revealed additional findings of interest. As might be anticipated, there was a definite correlation between the size of the inoculum and the time of appearance of reactions in the various diagnostic tests. These findings are summarized in Table 1 which lists the time of appearance of reactivity in the SAFA and palpebral intradermal tests in the animals of the different exposure groups. It is noteworthy that with the monkeys receiving the smaller numbers of organisms (i.e. 5-50 cfu), the SAFA and the IDP tests became positive at approximately the same time. In contrast, the SAFA test became reactive much sooner than did the IDP test in the animals receiving the larger number of organisms (500-5000 cfu). These latter observations corroborate the findings reported for the initial studies[1]. A similar pattern of test reactivity was observed in the control animals that acquired their infection by exposure to the experimentally infected monkeys. In the animals exposed to monkeys receiving inocula of 5, 50, and 500 cfu of tubercle bacilli, the SAFA and IDP tests became reactive at approximately the same time. However, in the control monkeys exposed those receiving 5000 organisms, the SAFA test became reactive much earlier than did the IDP test, suggesting that animals receiving the larger inocula shed greater numbers of organisms in their excreta.

Since early detection of infected animals is essential in the control of tuberculosis in non-human primate colonies, the data were examined to determine the frequency with which the SAFA or IDP test reacted first. These findings are summarized in Table 2. It was apparent that with monkeys infected with modest numbers of tubercle bacilli (5-500 cfu), neither test was superior to the other for early detection of infection; the frequency with which one test was the first to react was identical for each. The SAFA test, however, always reacted first in the animals challenged with the larger number of tubercle bacilli (5000 cfu). A similar relationship was observed in the cage mate controls that acquired their infections by exposure to the monkeys receiving the graded challenge doses.

These latter studies have provided information necessary for critical evaluation of the advantages and limitations of the SAFA

Table 1

Time of appearance of SAFA* and IDP* reactions in monkeys receiving graded doses of viable M. tuberculosis.

Inoculum (cfu)**	Days after challenge test became positive+ SAFA	IDP
5	239-322 (287)	210-322 (275)
50	77-365 (200)	91-239 (195)
[illegible]	49-239 (119)	70-210 (133)
5000	28-56 (43)	70-154 (112)

*SAFA = Soluble antigen fluorescent antibody.
IDP = Palpebral intradermal tuberculin.

**cfu = Colony forming unit

+ Figures in parentheses indicate mean value

Table 2

Frequency with which SAFA* or IDP* was first to react with monkeys infected with graded doses of viable M. tuberculosis.

Inoculum (cfu)**	Number+	No. first reacting in indicated test		X++
		SAFA	IDP	
5	4	2	2	
50	6	2	3	1
500	5	3	2	
5000	6	6	0	

*SAFA = Soluble antigen fluorescent antibody test.
IDP = Palpebral intradermal tuberculin test.

**cfu = Colony forming unit

+Number of animals reacting in at least one of the diagnostic tests.

++X = Both tests became reactive on the same day.

and IDP tests for the early detection of tuberculosis in nonhuman primate colonies. It is readily apparent that use of either test alone will not assure detection of all animals with active tuberculosis. On the other hand, the data indicate that the tests do complement each other, and parallel use of both in routine screening should detect all but a very occasional infected animal at a relatively early stage of infection. The results also provide a basis for interpreting the significance of the screening test results. Thus far, no false positive SAFA test reactions have been obtained in any animal in which tuberculosis could not be established at necropsy. Therefore, it appears that a positive SAFA test is a strong indication of either current or recent active disease. On the other hand, a negative SAFA alone does not necessarily exclude the possibility of early infection in an occasional animal. The specificity of the IDP test was somewhat less than that of the SAFA test; some conversions of the IDP have been noted in animals that were free from tuberculosis by all other diagnostic criteria. Additionally, it has been observed that some monkeys with a confirmed diagnosis of tuberculosis have failed to react in the IDP test. In view of the information to date, it is suggested that the results of the SAFA and IDP tests be interpreted as follows:

Test	Result	Significance
SAFA IDP	+ +	Very probably tuberculosis. Animal should be sacrificed, or quarantined and treated.
SAFA IDP	+ -	Tuberculosis very likely. Animal should be sacrificed, or quarantined and treated.
SAFA IDP	- +	Tuberculosis possible. Animal should be quarantined and monitored.
SAFA IDP	- -	Tuberculosis unlikely. If animal appears to be clinically ill, quarantine and retest in 2 weeks.

The practice of performing SAFA tests on all non-human primates during their quarantine period at WRAIR has been continued. During this reporting period, sera from 1192 Rhesus monkeys and 4 chimpanzees were examined in the SAFA test for tuberculosis using "A" and "C" protein antigens and a cell wall polysaccharide antigen derived from M. tuberculosis. Review of the results revealed that 989 of the monkey sera and the 4 chimpanzee sera were nonreactive in the tests with all 3 antigens. Within this group of SAFA-negative animals, 24 gave positive IDP reactions. However, on necropsy only 4 of the latter showed evidence of tuberculosis. It is noteworthy that 3 of the infected monkeys giving false-negative SAFA tests belonged to a

group received from India and containing one animal that had advanced tuberculosis on arrival. In an effort to explain the repeated failures to obtain SAFA reactions with sera from the monkey that initiated the outbreak, or with the animals that subsequently acquired the infection, it was postulated that the etiologic agent in this outbreak might be an atypical Mycobacterium and that the antigens derived from the typical M. tuberculosis did not react well with the antibodies produced by the atypical organism. To test this hypothesis, these sera were tested with "C" protein antigens prepared from M. kansasii and with antigens obtained from atypical Mycobacteria belonging to the Battey and Gause groups. Again the sera were uniformly nonreactive. The causative organism was isolated from a lesion in the monkey initiating the outbreak and is now being cultivated in liquid medium. When growth is sufficient, antigens will be prepared and the SAFA tests repeated on these sera. These results will be of particular interest since it is not known at this time whether antigens derived from typical M. tuberculosis will react with antibodies produced during infection with certain atypical Mycobacterium species.

6. Human tuberculosis. It was noted in the previous report on this Work Unit that a comprehensive evaluation of the efficacy of the SAFA test for human tuberculosis was being initiated in collaboration with investigators from the Department of Epidemiology, Division of Preventive Medicine, WRAIR, and from the Tuberculosis Service, Fitzsimons General Hospital; a detailed outline of the protocol also was presented. These studies were initiated in May 1971 and will continue through December 1972.

It should be recognized that in studies such as these involving human patients, a definitive diagnosis cannot always be made because recourse to necropsy with attendant histopathological and cultural examinations is not possible as is the case in studies on experimental animals. Positive cultures or demonstration of the tubercle bacillus by microscopic methods provide the only basis for unequivocal diagnosis of tuberculosis. However, failure to demonstrate the organism does not necessarily exclude infection and a diagnosis often must be made solely on clinical bases. In the latter cases, the diagnosis may be subject to some degree of error. Nevertheless, in the interest of sound medical practice, patients with negative cultures, but presenting clinical evidence of active tuberculosis must be considered infected and accordingly treated. Approximately 50% of the Tuberculosis Inpatient Group of the present studies were culture-negative and the diagnosis of tuberculosis was made on clinical and/or radiological grounds.

Although comprehensive analysis of the findings cannot be made until the studies are completed, certain general trends can be observed at this time. The efficacy of the SAFA test and the culture procedure was evaluated in examinations on 93 patients who had been diagnosed and treated as clinical tuberculosis. Results of the findings are summarized as follows:

SAFA	T.B. Culture	Total
-	-	26
+	+	26
-	+	20
+	-	26* (21)
		98* (93)

*Includes 5 patients initially admitted as tuberculosis in-patients but later, on clinical grounds, were considered not to have active tuberculosis.

If the clinical diagnosis is accepted as a reliable indication of active tuberculosis, it appears that the SAFA test or culture procedure will detect only 50% of the cases, although use of the two procedures in parallel would increase the efficacy of diagnosis to 75% in patients presenting clinical evidence of active disease. Nevertheless, the SAFA and culture procedures appear to be under sensitive, even when used in conjunction with each other. On the other hand, the specificity of the procedures was excellent. The culture procedure unquestionably was 100% specific, whereas the SAFA test gave presumed false positive reactions with 5 patients that originally were admitted as tuberculosis inpatients, but later were considered clinically not to have active tuberculosis. It is possible, however, that the original diagnosis may have been correct in some of the latter cases.

The group of culture-positive SAFA-negative cases warrants special comment. In the initial studies on use of the SAFA test for the serodiagnosis of tuberculosis in humans[2], it was observed that individuals with advanced or far advanced disease were in a severe anergic condition and did not react in the SAFA test. However, the majority of these patients became reactive in the SAFA test 60-90 days after the initiation of therapy. The clinical status of the 20 patients giving culture-positive SAFA-negative results in the present studies is not known at this time. However, this information will be available at the conclusion of the investigations, and will be considered in the comprehensive evaluation that will be made at that time.

The SAFA test also showed a high degree of specificity in tests on the group of 104 non-tuberculosis inpatients, patients with pulmonary diseases other than tuberculosis. Only 1 of this group reacted, and although no active tuberculosis was found, this patient had been PPD-positive for more than 20 years. The results obtained on the large group of outpatients reporting for routine tuberculin tests or on the group undergoing INH prophylaxis have

not been analyzed at this time. These results will be included in the detailed analysis to be presented in the following report on this Work Unit.

On the basis of the findings thus far, it appears that the SAFA test for tuberculosis is highly specific, but is not sufficiently sensitive for use as a screening procedure. The data indicate that a positive SAFA test is highly indicative of active tuberculosis whereas a negative SAFA test does not necessarily exclude infection in all cases. More detailed analyses of the duration and patterns of positive SAFA reactions in relationship to the clinical course, severity of disease, and therapy should define the advantages and limitations of the procedure as clinical tool.

2. Serodiagnosis of American trypanosomiasis (Chagas' Disease). During the present reporting period, the Department of Serology, WRAIR, has continued its participation in the evaluation of T. cruzi antigens being conducted by a PAHO Study Group on Chagas' Disease Antigens. The principal objective of this Study Group is to select a standard antigen for universal use in the serodiagnosis of Chagas' disease, and for reference in evaluating new antigens and methods. From the original 8 candidate antigens, 2 are still under consideration for selection as the Reference. One of the latter is the purified protein antigen submitted by the Department of Serology, WRAIR. Large lots of the 2 remaining candidate antigens have been prepared and currently are undergoing extensive critical evaluation for specificity, sensitivity and reproducibility. The results of this evaluation will provide a basis for selecting one as the Standard Reference Antigen.

3. Development and improvement of serologic methods and reagents. Efforts to further improve serologic methods and reagents were continued during this reporting period.

a. A modified complement fixation procedure for tests on dog sera. As was noted in the previous report on this Work Unit, dog sera frequently become anticomplementary during the heat inactivation (56°C for 30 min) necessary to destroy the native complement. This problem can be avoided by decomplementing the sera by absorption with immune complex (sensitized sheep erythrocyte stromata), thus eliminating the requirement for heat inactivation. Details of the methodology for decomplementation of the serum were given in the previous report.

In continued studies along these lines, it was observed that fresh dog serum had the capacity to lyse unsensitized sheep red cells. This indicated that dog sera contain natural Forssman antibodies at relatively high concentrations and suggested that sensitization of the stromata with rabbit anti-sheep cell antibody

(hemolysin) prior to introduction to the dog serum probably was not a prerequisite for the decomplementation of the serum. Subsequent studies proved that this was the case; unsensitized stromata decomplemented the serum with the same efficacy as that shown by the hemolysin-sensitized stromata. Thus, the unsensitized stromata reacted with the Forssman antibodies in the serum to form immune complex, which in turn fixed the complement. It has been well established that treatment with glutaraldehyde or pyruvic aldehyde renders sheep erythrocytes impervious to severe physical or mechanical damage; these aldehyde-treated cells can be suspended indefinitely in distilled water, frozen and thawed, or lyophilized without apparent morphological change[3]. In recent years, glutaraldehyde and pyruvic aldehyde has been successfully used for the preservation and long-term storage of sensitized cells used in IHA tests for a variety of infectious diseases[4]. In view of the relatively impervious nature of the aldehyde treated cells, consideration was given to the possibility that the aldehyde treatment would not destroy the Forssman antibody receptor sites and that the treated cells could be used to decomplement the dog serum, thus eliminating the labor and time involved in preparing the stromata. Attempts to utilize glutaraldehyde or pyruvic aldehyde treated cells for decomplementation uniformly were unsuccessful; the cells rapidly lysed when incubated with the fresh dog serum. In view of the foregoing experiences, the standard procedure for decomplementing dog serum entails the use of unsensitized sheep erythrocyte stromata, and will be used in evaluating the Babesia antigens described elsewhere in this report.

b. Preservation of Treponema pallidum in the frozen state. A method for preserving T. pallidum in the frozen state was developed and reported in detail in previous reports on this Work Unit. It was noted that a glycerolized suspension of T. pallidum could be shell-frozen and stored at -70°C for 13 months without loss of motility of infectivity. Studies on the stability of the treponemes under these conditions of storage were continued during the present reporting period. The most recent observations were made on a sample that had been preserved and stored for more than 25 months under the prescribed conditions. The suspension was thawed and examination with the dark-field microscope revealed that approximately 10% of the organisms exhibited the vigorous motility characteristic of freshly harvested specimens; the remaining treponemes were motile but appeared somewhat sluggish. The suspension was inoculated intratesticularly into an adult male rabbit and a firm orchitis was observed 12 days after inoculation. The animal was sacrificed at this time, the testes removed, and the treponemes harvested according to the procedures normally used for preparing antigen for the TPI test. Dark-field examination of the harvest revealed ca. 10 treponemes/hdf with 98% showing vigorous motility. These results showed that at least some of the treponemes had retained their infectivity during this long period of storage, but the yield on sub-inoculation was only

10% of that normally expected with a fresh inoculum. The next sub-inoculation of the preserved organisms, however, produced an orchitis in 7 days and yielded a harvest containing 100 treponemes/hdf with 98% motility. Moreover, valid TPI tests were obtained with the latter harvest. These findings have shown that T. pallidum suspensions preserved in glycerine and stored at -70°C maintain their infectivity for more than 2 years, thus providing insurance against loss of the strain due to unavoidable accidents to the infected animals.

c. FTA-ABS (IgM) test for congenital syphilis in the newborn. Conventional serologic tests do not provide a basis for differentiating maternal and neonatal antibodies. Therefore, unless an infant delivered from an infected mother exhibits clinical evidence of the disease, it is not possible to determine whether the neonate was infected in utero. It has long been recognized that maternal IgG antibodies readily cross the placental barrier whereas the IgM antibodies do not. Remington et al [5] postulated that the presence of specific IgM antibodies in the infant would be indicative of congenital infection. Subsequent investigations revealed that this was correct. These authors employed the IFA procedure with a fluorescein-conjugated anti-human IgM antiserum in a large group of neonates delivered from healthy mothers and from mothers with serological evidence of toxoplasmosis. Analysis of the results revealed that infants with congenital infection could be readily identified, often before frank clinical symptoms had developed.

Scotti and Logan [6] demonstrated that these methods also could be used for detection of congenital syphilis in neonates. In their initial and succeeding studies, all infants with clinical evidence of congenital infection reacted in the FTA-ABS (IgM) test. Additionally, in a small group of neonates that reacted in the IgM test but showed no clinical evidence of syphilis at birth, the majority developed clinical symptoms within 30 days. Alford et al [7] studied the changes of serologic patterns following treatment of FTA-ABS (IgM) reactive infants. In most cases, periodic testing showed a conversion to FTA-ABS (IgM) nonreactivity in 2 to 9 months following treatment. Moreover, with successful treatment, total serum IgM levels, FTA-ABS (IgM) test values and STS antibodies often decreased in parallel. On the other hand, low levels of FTA-ABS (IgM) and STS antibodies, and higher levels of FTA-ABS (IgG) antibodies occasionally persisted for long periods, even after treatment was considered fully adequate. Finally, in rare cases, low levels of FTA-ABS (IgM) reactivity were intermittently detected in samples as long as 14 months post-treatment.

It should be emphasized that the reliability of the FTA-ABS (IgM) test is dependent on the specificity of the anti-IgM antiserum. Unfortunately, one cannot simply rely on the claims of the manufacturer concerning the specificity of his product. In our experience, the majority of presumably mono-specific anti-IgM

antisera now on the market are contaminated with anti-IgG antibodies at concentrations sufficient to invalidate the FTA-ABS (IgM) test. During this reporting period, the Department of Serology, WRAIR, has evaluated a large number of commercially prepared fluorescein-conjugated anti-human IgM antisera and from this group, located one lot exhibiting the required monospecificity. This reagent has been standardized for use in the FTA-ABS (IgM) test and the Department now is in a position to provide this special diagnostic service to medical installations of the Armed Forces.

d. Evaluation of the complement fixation test for malaria. Methods for effective separation of malaria parasites from host erythrocytes and for isolation of specific complement fixing antigens from the parasite harvests have been developed by the Department of Serology, WRAIR. Details of these procedures have been reported in the scientific literature [8]. During the previous reporting period, an opportunity to critically evaluate the efficacy of the complement fixation test for malaria was afforded in a collaborative study conducted with investigators at the CDC. In these investigations, complement fixation (CF), indirect fluorescent antibody (IFA) and indirect hemagglutination (IHA) tests were performed on sera from military returnees who developed clinical malaria after return to CONUS. From the results of these studies summarized in Table 3, it appeared that the IFA test was slightly more sensitive than the others; with the sera collected 15-58 days after onset of clinical symptoms, the IFA detected 98% of the cases whereas the CF and IHA detected 93% and 91% respectively.

During the present reporting period, the tests were further evaluated with sera from a group of drug addicts that had acquired malaria by sharing hypodermic needles. The results of these tests are summarized in Table 4. The most striking observation in the latter studies was the decrease of sensitivity of the three serologic tests. The greatest loss of sensitivity was observed in the CF test; in contrast to the earlier group with naturally acquired (sporozoite induced) infections and which showed a sensitivity of 93% in the CF test, the group of addicts with artificially acquired (blood induced) infections showed a sensitivity of only 64%. There was also a reduction in the sensitivity of the IFA and IHA tests, but not as great as with the CF test; the sensitivity of the IFA was 15% less than that of the earlier group and the IHA showed a reduction of 6%. A similar reduction of sensitivity was evident in the geometric mean titers (GMT) of the sera in the two groups. Again, the greatest reduction occurred in the CF tests; the CF GMT of the returnee group was 16.8 whereas the GMT of the addict group was only 4.9 representing a 70% reduction. The GMT of the IFA tests also was reduced in the addict group, but not to the extent shown in the CF tests.

Table 3

Results of CF, IFA and IHA tests on sera from returnees developing malaria within CONUS (Sera collected 15-58 days after onset)

Test performed	Total	Results with indicated test: Positive No.	Positive %	Negative No.	Negative %	GMT*
CF	57	53	93	4	7	16.8
IFA	58	57	98	1	2	117.2
IHA	58	53	91	5	9	172.2

* Geometric mean titer of positive sera.

Table 4

Results of CF, IFA and IHA tests on sera from drug addicts who acquired malaria by sharing needles.

Test performed	Total*	Results with indicated test: Positive No.	Positive %	Negative No.	Negative %	GMT**
CF	44†	28	64	16	36	4.9
IFA	47	39	83	8	17	91.6
IHA	47	40	85	7	15	---

* All patients slide positive

** Geometric mean titer of positive sera.

† 3 additional sera AC.

Detailed analyses have revealed that the two malaria groups share a number of things in common. All individuals in both groups represented smear-positive cases of P. vivax infection. The majority of sera in both groups were collected 15-60 days after the onset of clinical symptoms. The sensitivity controls of the tests performed on the addict group were identical with those of the tests on the returnee group. Thus, the differences could not be attributed to faulty reagents (e.g. antigen deterioration, complement deterioration, etc.). It is true that different strains of P. vivax may have been responsible for the infections in the two groups. However, it is deemed unlikely that this could account for the marked differences of reactivity observed in these studies. In previous studies in this laboratory on volunteers infected with Chesson and St. Elizabeth strains of P. vivax, no strain differences were observed with the CF tests. Additionally, the serologic response, as measured by the CF test, was the same with sporozoite and blood induced infections. The most obvious difference between the individuals of the two groups was the fact that all patients of the addict group were using hard drugs at the time the sera were collected.

The unexpected results obtained in these studies raise two important questions: 1) Does drug abuse cause impairment of immune response?; and 2) Does the presence of a "hard"drug in the blood interfere with the reactions of standard serodiagnostic tests? It is recognized that the curious findings of these studies may have been simply coincidental. Nevertheless, until the above questions are answered and additional basic information is obtained, it is suggested that the results of serodiagnostic tests on drug addicts be interpreted with caution. The feasibility of investigating these problems under controlled conditions is being determined.

e. Isolation and fractionation of serologically active antigens from Babesia canis.

Babesiosis is a common infection in the dogs of certain areas of the U.S. and is highly endemic in other parts of the world. Occasional human infections also have been reported. At the present time, the serodiagnostic tests for babesiosis leave much to be desired; the antigens are crude cryolysates of parasitized blood, and the specificity of IHA tests using these products is relatively poor. In view of the inadequacies of the current serodiagnostic procedures, it is possible that human as well as canine babesiosis may be more common than presently is realized.

During recent years, investigators in the Department of Serology, WRAIR, developed methods for effectively separating malaria parasites from the host erythrocytes, and prepared highly specific serologically active antigen fractions from the parasite

harvests.[8] Since Babesia and Plasmodium share many characteristics in common, it was considered likely that the methodology employed for isolating purified malaria antigens could be used to obtain specific antigen fractions from Babesia canis. Such studies have been initiated in collaboration with investigators in the Department of Diagnostic Services, Division of Veterinary Medicine, WRAIR. A splenectomized dog has been infected with B. canis. When the parasitemia reaches the desired level, the animal will be exsanguinated, the erythrocytes washed, and the red cells passed through a French pressure cell under carefully controlled pressure. (Preliminary experiments have shown that normal dog erythrocytes are effectively fragmented when passed through the pressure cell at 1250 psi.) After passing the parasitized blood through the pressure cell, the parasite harvest will be washed in saline to remove the hemoglobin and red cell fragments, and then solubilized by repassage through the pressure cell at 20,000 psi. The solubilized parasite material finally will be freed from extraneous debris by centrifugation at 10,000 rcf, and fractionated by filtration through a Sephadex G-200 gel column. The various eluate fractions will be examined for serologic activity in CF and IHA tests.

These studies have just been initiated and no experimental results are available at this time. However, details of the investigations will be given in the succeeding report on this Work Unit.

Summary and Conclusions.

1. The soluble antigen fluroescent antibody (SAFA) procedure continues to show considerable potential for the serodiagnosis of a variety of infectious diseases. The major effort during this reporting period has been directed toward further evaluation of the SAFA test for the serodiagnosis of simian and human tuberculosis.

a. Studies on the efficacy of the SAFA test for the serodiagnosis of simian tuberculosis have been continued. In monkeys infected with a relatively small number of tubercle bacilli (5-500 cfu), it was observed that the SAFA and the palpebral tuberculin tests became reactive at approximately the same time. In contrast, in monkeys receiving a large number of organisms (5,000 cfu), the SAFA test always became reactive before the tuberculin test. The studies indicate that use of the SAFA or palpebral tuberculin test alone for screening purposes will not assure detection of all animals with active tuberculosis. On the other hand, it is apparent that the tests complement each other, and parallel use of both will detect all but a very occasional animal at a relatively early stage of infection. Both procedures, the SAFA and the palpebral tuberculin tests, currently are being used to monitor tuberculosis in the WRAIR non-human primate colony.

b. Comprehensive investigations on the potential of the SAFA test for human tuberculosis are in progress. Preliminary analysis of the findings to date indicate that the SAFA test will be reactive in approximately 50% of the patients with a clinical diagnosis of tuberculosis. This lack of sensitivity would preclude use of the SAFA procedure as a screening test. On the other hand, the SAFA test proved to be highly specific in tests on patients with pulmonary diseases other than tuberculosis. In view of these findings, it is believed that a positive SAFA test is highly indicative of current or recent active tuberculosis, whereas a negative SAFA test does not necessarily exclude the possibility of infection. At the conclusion of these studies, detailed analyses of the duration and patterns of positive SAFA reactions in relation to the clinical course, severity of disease, and therapy should define the advantages and limitations of the procedure as a clinical tool.

2. Critical evaluation of antigens for the serodiagnosis of American trypanosomiasis (Chagas' disease) has been continued. Large lots of the 2 antigens being considered for use as the Standard Reference Antigen have been prepared and currently are undergoing extensive critical evaluation for specificity, sensitivity and reproducibility.

3. Efforts to further improve serologic methods and reagents were continued during the present reporting period.

a. Studies on the decomplementation of dog serum by absorption with sensitized sheep erythrocyte stromata were continued. During the course of these investigations, it was observed that dog serum contains natural Forssman antibodies and that sensitization of the stromata was not necessary for decomplementation. Recent investigations have revealed that treatment of the dog serum with unsensitized stromata decomplemented the serum with the same efficacy as that shown by the hemolysin-sensitized stromata, indicating that the unsensitized stromata reacted with the Forssman antibodies in the serum to form immune complex which in turn fixed the complement. Attempts to use pyruvic aldehyde-treated sheep cells rather than stromata were uniformly unsuccessful. Although the aldehyde-cells could be suspended for long periods in distilled water, lyophilized, or freeze-thawed without undergoing morphological change, the cells rapidly lysed when incubated with fresh dog serum. Thus, the current standard procedure for decomplementing dog serum prior to use in CF tests entails the use of unsensitized sheep cell stromata.

b. Studies on the preservation of T. pallidum in the frozen state were extended during the present reporting period. Glycerolized suspensions that had been stored at -70°C for 25 months remained infective and provided a satisfactory inoculum for preparing TPI antigen. Storage in this manner provides insurance against loss of the strain due to unavoidable accidents to the infected rabbits.

c. Reagents necessary for performing the FTA-ABS (IgM) test for congenital syphilis in the newborn have been acquired and standardized. During this reporting period, a large number of commercially prepared fluorescein-conjugated anti-human IgM antisera were evaluated. All but one lot showed contamination with anti-IgG antibodies at concentration sufficient to invalidate the FTA-ABS (IgM) test. The IgM test is the only serodiagnostic procedure by which maternal and neonatal antibodies can be differentiated, and provides a basis for determining whether a neonate without clinical signs of disease was infected in utero. A supply of the satisfactory lot of tagged anti-IgM antiserum has been procured and standardized for use in the FTA-ABS (IgM) test. This Department now is in a position to provide this special diagnostic service to medical installations of the Armed Forces.

d. The CF test for malaria was further evaluated in tests with sera from a group of drug addicts that had acquired P. vivax infections by sharing hypodermic needles. Curiously, it was observed that the sensitivity of the serologic tests on this group was significantly less than that exhibited in non-addict malaria patients. This reduced sensitivity was most apparent in the CF tests. Although the precise cause for these unexpected findings is not known at this time, the results suggest that there may be some here-to-fore unrecognized problems in performing serodiagnostic tests on drug addicts. Therefore, until more basic definitive information along these lines is obtained, it is recommended that serodiagnostic tests on drug addicts be interpreted with caution.

e. Attempts are being made to prepare specific serologically active antigens from B. canis, using the methodology developed earlier for preparing plasmodial antigens. These studies have just been initiated and the results will be presented in the succeeding report on this Work Unit.

Project 3A061102B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 172, Sero-recognition of microbial infections

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Publications:

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2. Colwell, E.J., Bernier, G.M., and Fife, E.H.: Serum immuno-globulin D and malaria antibodies in South Vietnamese residents. Trans. Royal Soc. Trop. Med. & Hyg. 65 : 310-314, 1971.

3. Fife, E.H.: Current state of serological tests used to detect blood parasite infections. Exptl. Parasitol. 31 : 136-152, 1972.

4. Ziegler, J.L., Bluming, A.Z., Morrow, R.H., Cohen, M.H., Fife, E.H., Finerty, J.F., and Woods, R.: Burkitt's lymphoma and malaria. Trans. Royal Soc. Trop. Med. & Hyg. 66 : 285-291, 1972.

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 OA 6527	72 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	9A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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 114(f)			

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 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)
PRECEDING FISCAL YEAR 72	5	140
CURRENT FISCAL YEAR 73	4	125

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Hobson, LTC R. W., II

NAME: Croom, MAJ, M.D.; Shatsky, CPT S.A. DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Venous Reconstruction; (U) Arteriovenous Fistula; (U) Venous Occlusion; (U) Cerebral Trauma; (U) High Speed X-ray; (U) Combat Injury

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

23 (U) To define and quantitate the physiologic and metabolic derangements following combat injury and resuscitation.

24 (U) With the closure of the field research units in Vietnam and Japan attention has been focused on areas of peripheral vascular, neurosurgical, and metabolic complications to wounding and shock.

25 (U) 71 07 - 72 06. Hemodynamic studies of the constructed distal arteriovenous fistula in venous reconstruction in dogs showed a significant drop in distal arterial pressure and flow and drop in limb temperature thereby establishing a caveat in the clinical application of this technique. Heparin and low molecular weight dextran were shown to marginally improve the patency rates of canine autogenous vein grafts in the dog, but meticulous surgical technique is the most important factor in patency. Prolonged femoral venous occlusion resulted in persistent low femoral arterial flow until release of the occlusion in the dog. A rapid cerebral angiographic technique has been developed utilizing high speed x-ray pulse generators, together with rapid decay image intensifier phosfors and a pin registered camera; this can detect motion of cerebral vessels following impact trauma in the monkey in the order of 1000 frames per second.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

* 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 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01 Surgery

Work Unit 092 Clinical evaluation of responses of the body to combat injury

Investigators.
Principal: COL Robert T. Cutting, MC
Associate: MAJ Creighton B. Wright, MC; MAJ Robert D. Croom, III, MC; CPT Stanley A. Shatsky, MC; LTC Robert W. Hobson, II, MC

1. Peripheral Arterial Flow and Venous Occlusion in the Dog.

a. Statement of the problem and background: Venous injuries occurred in 38% of 1000 Vietnam casualties with major arterial injuries (1). Venous repairs were done in only one-third of these injuries. Highes, Rich, and others have favored venous reconstruction, but this has not yet been widely accepted (1-3). Although reconstruction seems the better choice, physiological evidence for this is not available. Preliminary studies at this and other institutions show consistently a 60-90% decrease in femoral arterial flow with concomitant superficial femoral venous occlusion (3).

b. Approach to the problem: Femoral arterial flow was measured bilaterally using electromagnetic flowmeters, and Statham transducers were used for arterial and venous pressure measurements.

c. Results: Prolonged femoral venous occlusion (4 hours) results in persistent low femoral arterial flow until release of the occlusion. Although there is a general impression that venous collaterals develop rapidly, the exact time sequence for this is unclear. If the venous occlusion is 6 hours or more, arterial occlusion can result from the low flow state.

In addition the placement of autogenous jugular vein grafts in the femoral vein allowed femoral artery flow restitution to near normal levels.

Studies are currently in progress on the effects of alpha and beta adrenergic manipulation on the femoral hemodynamics during venous occlusion.

d. Conclusions: Femoral venous ligation should be avoided in traumatic cases, and that autogenous vein grafting is the method of choice for repair if a primary anastomosis is not feasible. Prolonged venous occlusion is potentially disastrous.

2. Peripheral Venous Reconstruction Using Autogenous Connective Tissue Grafts Grown in Response to Implanted Silastic Rods.

a. Statement of the problem: Autogenous vein is uperior to other materials for use in venous reconstruction, but availability of a suitable vein for use in secondary venous reconstruction is not assured. Inadequate vein length and diameter are frequently encountered on an anatomical basis or may result from previous injury or disease. The need for a dependable source of autogenous tissue for use as a graft in secondary venous reconstruction is apparent.

b. Background: Following implantation of medical grade silicone (Silastic) rod or tubing into the soft tissue of the dog and man, a tissue reaction occurs which consists of a fibrous tissue proliferation. This culminates in the formation of a pliable, smooth, endothelial-like, connective tissue encasement of the silicone implant corresponding to its shape. Excision of the intact encasing mass of tissue with removal of the silicone mold provides a vein-like connective tissue tube of predetermined length and diameter that then can be employed as a graft for venous reconstruction.

c. Approach to the problem: In a series of pilot experiments, silastic rods were implanted in the anterior abdominal wall of ten dogs. The connective tissue tubes were harvested after a period of six to eight weeks and utilized as grafts to replace excised segments of femoral vein. In several animals, an arteriovenous fistula was constructed distal to the graft.

d. Results: Considerable variation in the quality of the connective tissue tubes was encountered. In two animals the tubes were very thin and flimsy, easily torn by suture placement, and completely unsuitable for use. In two animals the tubes were edematous, thickened, irregular, and of suboptimal quality. Very satisfactory tubes were present in the other animals and were easily handled, held sutures well, and closely resembled autogenous vein. In the absence of previous infection at the tube site, it was impossible to determine the quality of the tube until its excision.

Early thrombus formation was demonstrated at suture lines by immediate postoperative venography. This occurred even in association with a distal arteriovenous fistula in several animals. Progression to complete occlusion had usually occurred by the second postoperative day. Recanalization of the occluded connective tissue grafts did not occur. Dense scarring developed about the thrombosed grafts and obliterated tissue planes in several dogs.

e. Conclusion: Connective tissue tubes grown in response to implanted silastic are probably unsuitable for venous reconstruction due to unpredictable variability in texture, thickness, and strength, and and a likely enhanced thrombogenicity.

f. Recommendation: Further limited studies with connective tissue tubes reinforced with synthetic mesh should be carried out in an attempt to overcome the problem of qualitative variability. In addition, the question of enhanced thrombogenicity should be further investigated.

3. Neuroradiological Studies of Experimental Head Injury.

a. Statement of the problem: Head injury remains a major cause of morbidity and mortality for the Army in both war and peace. For example, in 18,000 consecutive hospitalized U. S. wounded in Vietnam, head injury accounted for 20% of admissions, for 38% of deaths and 49% of deaths occurring in the first 24 hours. Unconsciousness from any trauma ("concussion") includes a wide and bewildering variety of neurophysiologic changes accompanied by sharp disagreement among experimentalists and pathologist studying the problem.

b. Background: Previous studies of the biomechanics of intrabrain motion have shown some of the events associated with skull fracture and coup injury, ballistic cavitation phenomena in gelatin brain models as well as rotations, shear strains and transitional movements. However, the state of the arts has not heretofore permitted high speed cineradiographic studies.

c. Approach: X-ray tubes newly developed allow pulses to 1200 per second, although limited by heat production to 100 pulse sequences. Image intensifiers newly available permit high brightness and extinction in the 20-50 microsecond range utilizing new phosphors. Pin registered cameras are now available to 1000 frames per second.

d. Results: Preliminary studies involved assembling the separate pieces of apparatus: sled, automatic angiography injection apparatus, pulse generator, x-ray tube, image intensifier, and camera switched to the pulse generator. Current resolution is 2 - 3 line pairs/min. for a 4" x 4" image size (rhesus skull). Preliminary studies show that cine film grain size to be the present resolution limiting factor.

e. Conclusion and Recommendations: We have demonstrated the technical feasibility of a high speed angiographic system to record intrabrain events during impact trauma. Utilizing bifurcation of vessels as landmarks, local movement can be plotted with respect to each other and, to bony landmarks during trauma and the whole related to observed neurological events. Software remains to be developed. The system is now ready for permanent assemblage and installation for pilot studies to proceed.

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. Rich, N. M., Hughes, C.W., and Baugh, J.H.: Management of venous injuries. Ann. Surg. 171:724, 1970.

2. Rich, N.M., Baugh, J. H., and Hughes, C.W.: Popliteal artery injuries in Vietnam. Amer. J. Surg. 118:531, 1969.

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Publications:

None

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	72 07 01	DD-DR&E/AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DOD/E INTEREST	8B. SPECIFIC DATA: CONTRACTOR ACCESS	9. LEVEL OF SUM / 10. WORK UNIT
71 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A061102B71R	02	085
b. CONTRIBUTING				
c. [illegible]	(2)(3) 11.(f)			

11. TITLE (Include title 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)
PRECEDING FISCAL YEAR 72	11	275
CURRENT FISCAL YEAR 73	10	260

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Olsson, COL R. A. DA

NAME: Elliot, Dr. E. C.

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide 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 (Provide brief but specific paragraphs identified by number. Provide 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) 71 07 - 72 06. The binding of adenosine to the cardiac sarcolemmal transport carrier requires the 6-amino group, the 2- and 3-hydroxyls of the ribosyl moiety in the erythro configuration, and an anti conformation at the glycosidic bond. Cardiac adenosine transport may be an example of active site-directed stereospecificity. A more sensitive beta ray detector has been developed for the study by the isotope clearance technique of capillary blood flow in the heart. Attempts are being made to increase the longevity of the device for use in long term chronic experiments. During gradual or following abrupt coronary artery occlusion in the conscious dog, directly measured coronary collateral flow to the ischemic myocardium increases rapidly (within a few hours) to protect the heart. For echnical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

26. Available in computer 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 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: COL Ray A. Olsson, MC; MAJ Jerry A. Snow, MC; MAJ Harvey L. Wray, MC; Eric C. Elliot, M.D., Ph.D.; Edward M. Khouri; Howard S. Lowensohn, Ph.D.; R. Richard Gray; Mary K. Gentry; Charles E. Cain; George P. Frick; Stanislaw Pasyk, M.D.; David E. Donald, M.D. (Mayo Clinic); R. Lee Pyle and D. F. Patterson (Univ. of Pennsylvania School of Veterinary Medicine).

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.

Techniques have been developed for the encapsulation of the avalanche diode semiconductor beta ray detector, for use in the study of capillary blood flow by tissue clearance of isotope indicators, in chronic experiments. Such a device has been implanted on the surface of the heart in one dog and performed satisfactorily for a period of 21 days yielding low noise clearance curves in agreement with a precordial counter. In order to increase the longevity of the unit, a study has been made of the effects on the diodes of mechanical and environmental stresses and of various types of polymers. A prototype unit applying the latest information thus obtained is now under construction and evaluation.

A method for measuring coronary retrograde blood flow in the chronic conscious dog preparation was successfully achieved by surgically creating an autogenous arterial connection between the aortic arch and the central circumflex coronary artery. A side branch of this connection was left intact. Retrograde flow was measured by opening this side branch to atmospheric pressure while the circumflex branch was temporarily occluded by a pneumatic cuff central to the graft. The effect of myocardial ischemia on retrograde flow was studied as the inflow into the circumflex artery was progressively reduced by a Hg constrictor placed at the origin of the circumflex.

2. Metabolic Control of Left Coronary Blood Flow.

Adenosine appears to enter the cardiac cell by facilitated diffusion, the rate of uptake being rapid enough to constitute an important mechanism for regulation of the concentration of this nucleoside in the vicinity of the coronary resistance vessels. The erythro configuration of the sugar hydroxyls, the 6-amino group, and the anti conformation at the glycosidic bond are the major determinants of the binding affinity of this nucleoside and the putative membrane carrier. Papers reporting these findings have been submitted for publication.

Other workers have shown that aminophyllin inhibits the coronary vasodilatory effects of adenosine but does not influence the reactive hyperemia response. This methylxanthine has no effect on cellular uptake of adenosine. Preliminary studies on isolated coronary artery strips suggest that tissue levels of cyclic AMP are not influenced by adenosine, theophyllin, or the two in combination.

3. Cyclic AMP-Stimulated Protein Kinase Activity on Sarcoplasmic Reticulum.

A cyclic AMP-stimulated protein kinase associated with a "sarcoplasmic reticulum" preparation for dog heart has been demonstrated. Its activity is stimulated two to threefold by maximal concentration of cyclic AMP with an apparent Km of about 1×10^{-7} M. This membrane fraction contains a protein substrate for its protein kinase as well as a substrate for a soluble protein kinase from beef heart. This membrane-associated enzyme can not be fully activated by cyclic GMP and is not inhibited by the heat-stable protein inhibitor for all other known mammalian protein kinases.

4. Normal Coronary Circulation. Excitement.

Following excitement in the resting dog with a denervated heart or with intact cardiac nerves, coronary sinus oxygen content rises markedly indicating a plethora of left coronary inflow beyond the needs of the left myocardium (1971 Annual Report). Since this could be due to contamination of coronary sinus blood by blood from the right atrium, catheters were implanted in the right atrium for injection and in the coronary sinus for sampling of blood. During excitement, while coronary sinus blood was continuously sampled with a cuvette oximeter, cardiogreen or saline saturated with H_2 gas and injected into the right atrium did not appear in the coronary sinus.

5. Regulation of the Coronary Collateral Circulation.

Three successful experiments were performed in which retrograde blood flow was measured in chronic dogs beyond the point of constriction of the inflow to the circumflex artery. It was demonstrated that a rise in retrograde flow above the control value occurred during the period of progressive reduction of circumflex inflow. However, owing to the fact these measurements were made against atmospheric pressure, the results are difficult to interpret. These experiments led to the next set of experiments in which an electromagnetic flowmeter was placed on a branch of the circumflex beyond the point where the circumflex artery was constricted. It has been demonstrated by this technique in five chronic dogs that the rapid development of collateral blood flow occurs during gradual (4-7 days) and rapid occlusion (5-24 hours) of the central circumflex coronary artery, that is, before coronary inflow reaches zero. It has also been shown with this method that collateral flow will increase in response to mild exercise, excitement, and to coronary dilator drugs.

6. Effects of Pulmonic Stenosis on the Right Coronary Circulation.

In continuation of a joint project with the Comparative Cardiovascular Studies Section, University of Pennsylvania (see 1971 Annual Report), a third dog with congenital pulmonic stenosis was studied. Peak right ventricular systolic pressure was much less in this dog (76 mm Hg) than in those previously reported(>140 mm Hg). Resting right coronary artery systolic blood flow was considerable and forward with some restriction in the latter half of this cycle. In reactive hyperemia, systolic and diastolic right coronary blood flow increased markedly, systolic enhancement being only slightly less than that seen in the normal dog.

Conclusions.

Experiments are under way to increase the longevity of a semiconductor beta ray detector.

Adenosine uptake by canine myocardium appears to occur by facilitated diffusion at a rate rapid enough to constitute an important mechanism for regulating the concentration of this nucleoside in the cardiac interstitial space.

A unique protein kinase has been isolated with a sarcoplasmic reticulum of dog heart.

The rise in oxygen saturation of the coronary sinus blood which occurs during excitement has been shown to be a functional response.

Retrograde blood flow, an indicator of collateral blood flow, has been measured in the conscious dog before, during, and after reduction of circumflex coronary artery inflow, and a rise above control found to occur even before complete circumflex occlusion. Collateral blood flow has also been directly measured by means of an electromagnetic flowmeter in a distal branch of the circumflex coronary artery beyond the site of occlusion of the central circumflex. The collateral flow has been shown to rise following rapid and gradual central circumflex occlusion, and to increase following exercise, excitement, and coronary dilator drugs.

Right coronary artery blood flow is restricted during systole in congenital pulmonic stenosis dogs with right ventricular hypertension. The degree of systolic coronary artery blood flow impedance was related inversely to the level of right ventricular hypertension.

Project 3A061102B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 085, The heart under abnormal and pathological stresses

Literature Cited.

Publications:

1. Pyle, R. L., Lowensohn, H. S., Khouri, E. M., Gregg, D. E., and Patterson, D. F.: Coronary artery hemodynamics in dogs with congenital subaortic stenosis. Circulation 44:II-115, 1971.

2. Olsson, R. A., Snow, J. A., and Gentry, M. K.: Steric requirements for adenosine transport by canine heart. Federation Proc. 31:815, 1972.

3. Gregg, D. E., Khouri, E. M., Donald, D. E., Lowensohn, H. S., and Pasyk, S.: The coronary circulation in the conscious dog with cardiac neural ablation. Circulation Res. Vol. 31, #2, 1972.

4. Gregg, D. E.: Coronary vasodilator effects of nitroglycerin during coronary insufficiency. Chapter 20 in, The Study of The Systemic, Coronary and Myocardial Effects of Nitrates. Charles C. Thomas, Springfield, Illinois, 1972.

5. Gregg, D. E.: Factors influencing coronary blood flow in the presence of coronary obstructive disease. In, Atherosclerosis and Coronary Heart Disease. Grune and Stratton, New York, N. Y., 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 0450	72 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
71 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	086
b. CONTRIBUTING				
c. [illegible]	CDOG 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Military Hematology (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

0800 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

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 72	7	130
FISCAL YEAR CURRENT 73	7	130

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

ASSOCIATE INVESTIGATORS

NAME:

NAME: 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 (Punish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Studies of the pathophysiology of diseases with hematologic manifestations in soldiers and the investigation of those that occur because of their occupation.

24. (U) Studies of hematologic abnormalities produced by chemicals, drugs, and infectious agents 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) 71 07 - 72 06 The mechanism for destruction of aged and injured erythrocytes in vivo is important in blood banking, hemolytic disorders such as sickle cell disease and malaria, and in trauma and infection. Correlation between cell deformability, surface charge, and red cell lifespan is being made in normal and disease states, and before and after treatment of cells with compounds believed to affect erythrocyte survival. The effect of antimalarial drugs upon hemolysis and the production of methemoglobinemia was quantified and biochemical mechanisms identified. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*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 3A061102B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 086, Military Hematology

Investigators.

Principal: COL Marcel E. Conrad, MC

Associate: LTC Donald E. Dillon, MC; LTC George M. Bernier, MC; MAJ Thomas A. Bensinger, MC; MAJ Jeffrey L. Berenberg, MC; MAJ Bertil E. Glader, MC; MAJ Jon P. Gockerman, MC; MAJ Daniel B. Kimball, MC; LTC M. Jeffrey Maisels, MC; MAJ Frederick R. Rickles, MC; MAJ Phillip P. Toskes, MC

Description.

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

Results.

Urea therapy has been proposed as a treatment for patients with sickle cell anemia both to prevent and cure crisis. This form of treatment was based on in vitro observations that high concentrations of urea prevented sickling. In vivo studies of the effect of intravenous doses of urea in patients with sickle cell anemia were initiated in our laboratories. Studies of endogenous carbon monoxide production, an index of heme degradation, in patients before and after the intravenous infusion of 80 to 90 grams of urea in invert sugar solution showed a significant increase in carbon monoxide production following therapy. This indicated that the therapy caused hemolysis of red blood cells. Recent studies suggest that this increased distinction of red blood cells probably occurs at the site of infusion of the urea and is mediated by the production of markedly increased osmolality with crenation of erythrocytes in the vein.

Studies of the effect of oral urea therapy (40-120 grams per day) in patients with sickle cell anemia were performed to ascertain if the rate of hemolysis was affected by treatment. Red blood cell survival was measured before and after therapy using both radiochromium and DFP^{32}. No significant change in the rate of red blood cell survival was observed even though patients had an increased hematocrit following therapy. Measurements of the red blood cell mass and plasma volume showed that the improved hematocrit was caused by a decrease in the plasma volume without a significant change in the red blood cell mass; this was attributed to the diuresis and dehydration produced by urea therapy.

Recent reports from Rockefeller University have indicated that sodium cyanate therapy was useful in the treatment of patients with sickle cell anemia. They demonstrated that cyanate treatment of erythrocytes in vitro prevented sickling and that when these cells were autotransfused, they had a nearly normal red blood cell survival. In collaboration with investigators at Rockefeller University, we are studying the rate of heme degradation in patients with sickle cell anemia before and after cyanate therapy. In addition, in vitro studies of the effect of cyanate on normal erythrocytes and sickle cells have been initiated in our laboratory. Presently it appears that cyanate treatment of red blood cells causes an irreversible inactivation of the enzyme glucose-6-phosphate dehydrogenase with a concentration and time-dependent depression of the hexosemonophosphate shunt. This metabolic abnormality may limit the usefulness of cyanate therapy in patients with sickle cell disease and most particularly if they also are deficient in glucose-6-phosphate dehydrogenase. Likewise, it suggests that many other proteins may be functionally altered which will limit the clinical usefulness of this agent. Studies of the surface charge and deformability of cyante-treated red blood cells have been initiated. Preliminary observations show that cyanate treatment causes an increase in the electronegativity of both normal and sickle erythrocytes, suggesting that cyanate affects the erythrocyte membrane in addition to carbamylation of hemoglobin.

Studies of the mechanism by which the reticuloendothelial system recognizes aged and injured red blood cells were continued. Erythrocytes were treated with neuraminidase to decrease the surface charge. These cells are rapidly sequestered by the reticuloendothelial system. Studies of their lactate generation, ATP production, and osmotic fragility were normal. Studies of the deformability of neuraminidase-treated erythrocytes showed diminished passage of these red blood cells through nucleopore filters. ATP levels of both normal cells and neuraminidase-treated cells were either maintained by incubation of the cells in glucose or depleted by incubation in saline. A decrease in ATP levels resulted in decreased filterability. A combined decrease in both the surface charge and ATP levels resulted in a marked diminution in filterability. These studies suggested that a relationship exists between red blood cell surface charge and deformability and that either mechanism or both may serve as the signal to the reticuloendothelial cell for hemolysis.

White patients with glucose-6-phosp ate dehydrogenase (G6PD) deficiency were studied. Three new variants of this enzyme abnormality were found and named G6PD Puerto Rico, G6PD Benevento (B), and G6PD Johnstown (J). The effects of repeated doses of primaquine were studied in these patients to determine the degree of hemolysis and its relation to properties of the G6PD enzyme. Hemolysis was quantified by obtaining periodic samples of blood from patients in whom

aged cells were labeled with iron-55, young cells were labeled with iron-59, and chromium-51 was used to radiolabel erythrocytes of all ages. Baseline ^{51}Cr red blood cell survival ($T_{\frac{1}{2}}$) varied from 18 to 29 days in various patients. After the initial dose of primaquine (45 mg of base), 10 to 30 percent of radiolabeled cells were hemolyzed. All patients showed a diminished hemolysis with subsequent doses of primaquine. Patients with evidence of hemolysis before primaquine administration had a greater proportion of their red blood cells hemolyzed than those with a normal red blood cell survival. Older red blood cells were destroyed in greater numbers than young erythrocytes. Contrary to previous reports, White patients may have G6PD variants with self-limited hemolysis to weekly doses of primaquine similar to that observed in Black patients with G6PD variant A-.

Studies of heme turnover by measurements of carbon monoxide production were performed in patients with G6PD deficiency before and at intervals after the administration of 15 mg of primaquine. Hemolysis was found to occur within 18 hours after the ingestion of drug instead of 3-4 days as determined previously by ^{51}Cr red blood cell survival studies. Using smaller initial doses of primaquine (7.5 mg) and gradually increasing them in G6PD-deficient patients produced a decreased hemolytic rate and less anemia at the time when the larger doses were employed. Studies of heme turnover were performed in patients with Gilbert's disease before and after fasting because fasting produces a marked increase in the indirect serum bilirubin concentration in patients with this disease. Failure to find an increase in carbon monoxide production after fasting suggested that the enhanced bilirubinemia was not caused by hemolysis.

Dapsone is known to cause both hemolysis and methemoglobinemia in patients but has no adverse effects when the drug is added to mixtures of red blood cells in vitro. A derivative of dapsone--hydroxylamine dapsone--was found that caused these adverse effects in vitro and is presumed to be the oxidant agent that affects red blood cells in patients receiving this chemotherapeutic agent. The method by which hydroxylamine dapsone causes methemoglobinemia was reported previously in collaboration with the Division of Biochemistry, WRAIR. During the current year we demonstrated that hydroxylamine dapsone--but not dapsone--irreversibly inhibited catalase with the generation of hydrogen peroxide and diminished concentrations of reduced glutathione in treated red blood cells. The treated erythrocytes had diminished metabolism of glucose in hexosemonophosphate pathway and decreased ^{51}Cr surivival in animal studies.

Reports of the production of a procoagulant in leukocyte tissue cultures led to the hypothesis that white blood cells participate in the coagulopathy of the Schwartzman reaction. Likewise the lymphocyte has been implicated in the production of factor VIII. Therefore we undertook to characterize the procoagulant material in the particulate fraction of lysed lymphocytes in tissue culture. This procoagulant shortened the standard recalcification time of citrated normal plasma, the unactivated partial thromboplastin time, the activation of factor X, and all one-stage correction assays. It neutralized the activity of antibody prepared against human tissue factor. The addition of either phytohemagglutinin or the purified protein derivative of the tuberculosis bacillus to lymphocytes in tissue culture media stimulated the production of procoagulant. Although this material appeared to have factor VIII activity in a one-stage system, significant amounts of factor VIII were not demonstrated by radioimmune assay. This suggested that the factor VIII production reported in lymphocyte and mixed leukocyte cultures and spleen cell cultures was tissue factor rather than factor VIII. Similarly the factor VIII activity observed in hemophiliacs following splenic transplantation was probably tissue factor and not factor VIII.

The sudden rejection of a transplanted kidney after the administration of antirabbit thymocyte serum with a kidney that showed extensive arterial thrombosis led to a study of the antilymphocyte preparation for procoagulant effects. Laboratory studies showed that the antilymphocyte serum shortened the recalcification time and inactivated partial thromboplastin time of citrated plasma. This activity was unaffected by dialysis, adsorption with barium sulfate, or heating for 30 minutes at 56°C. In addition, it caused platelet aggregation that was found on a Sephadex G-200 column to be associated with the 170,000 molecular weight fraction (gamma G) and neutralized by antihorse gamma G antisera. Survey of other lots of antilymphocyte serum showed that some contained platelet aggregating factor whereas others contained a procoagulant similar to tissue factor, and a few contained both. These data suggest that all preparations of antilymphocyte sera should be tested in a coagulation laboratory before they are used in humans.

Coagulation abnormalities were studied in Aotus trivirgatus monkeys infected with human strains of P. falciparum malaria. These animals showed evidence of disseminated intravascular coagulation during the periods of marked parasitemia. Studies of tracker dogs with tropical canine pancytopenia showed that the bleeding of this disorder was caused by a paragglutinin against thrombocytes rather than a disseminated intravascular coagulopathy. Recent studies of canine trypanosomiasis documented the occurrence of disseminated intravascular

coagulation in these animals. The latter studies will be extended to monkeys infected with trypanosomiasis and humans if appropriate specimens can be obtained for study.

The presence of chemotactic responses of white blood cells were studied to identify factors affecting the response of leukocytes in inflammatory reactions. Using in vitro micropore filter techniques and neutrophils as indicator cells, chemotactic responses of leukocytes to the complement-derived chemotactic factors (C3a, C5a, C567) and a chemotactic factor derived from E. coli were studied. A chemotactic inhibitor was isolated from human sera that blocked the activity of all chemotactic factors tested, was soluble in 45% saturated ammonium sulfate, and appeared in a biphasic distribution with activity near the IgG and albumin markers in sucrose density gradient ultracentrifugation and gel filtration studies. The inhibitor acted directly upon chemotactic factor rather than on the leukocyte and seemed to be present in increased amounts in serum from patients with either Hodgkin's disease or agammaglobulinemia than in normal subjects. No evidence was found that the inhibitor bound to chemotactic factor. The inhibitor in sera from patients seemed similar to that found in normal sera. The amount of chemotactic activity generated in serum by the addition of zymosan was significantly less in inhibitor-rich serum than normal sera. It is believed that the presence of serum inhibitors of chemotactic factors may represent a control mechanism of these inhibitors. While the chemotactic factor inhibitor differs from anaphylatoxin inactivator, a conclusive statement about the relationship of these two inactivators cannot be made as yet from available data.

Studies were initiated related to the role of neutrophils in fungus infections. Neutrophils were found to phagocytize and destroy Candida albicans effectively. Since neutrophils participate in the host response in acute Candida infections, we undertook to quantify the production of neutrophil chemoattractants by C. albicans. Cell-free filtrates of Candida albicans cultures were prepared by centrifugation and filtered through a 0.2 micron membrane. The filtrates contained a chemotactic factor for neutrophils but not monocytes which did not require serum for activation. This factor was heat stable for one hour at 56°C and inactivated by boiling for 15 minutes. It was nondialyzable and on Sephadex G-200 had a molecular weight of about 220,000. This factor may be important in the mediation of the acute host response to Candida infections.

Some insight of the biological significance of the two light chains found in human serum may be obtained from studies of a four-year-old girl with recurrent respiratory infections and diarrhea. She had a

marked decrease in the serum concentration of immunoglobulin molecules with κ-chain. In addition she had decreased γA and γε globulin with partial albinism and intestinal lactase deficiency. No eosinophils were found in the blood, bone marrow, or nasal secretions. Radiolabeled studies of the rate of κ and γ globulins showed normal survival, suggesting that the synthesis of molecules containing κ-chains was decreased.

Recommendations.

Further studies of the mechanism of hemolysis are needed to determine the method by which the reticuloendothelial system identifies effete erythrocytes; this is important in many human diseases to include malaria, sickle cell anemia, and drug-induced hemolysis. Additional studies of coagulation abnormalities are needed to define their role in the etiology of a number of infectious diseases and to provide more appropriate therapy when accelerated utilization of clotting factors causing both thrombosis and hemorrhage is found. Continued studies of humoral mechanisms related to host response provide a better understanding of the methods by which man naturally protects himself from invasion by microorganisms.

Project 3A061102B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 086, Military Hematology

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6452	72 07 01	DD-DR&E-AR-636

3. DATE PREV SUMRY	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
71 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	087
b. CONTRIB'G				
c. [redacted]	CDOC 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) GASTROINTESTINAL DISEASE (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012900 Physiology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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 72	8	100
FISCAL YEAR CURRENT 73	6	80

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

DA

22. KEYWORDS (Precede Each with Security Classification Code) (U) Diarrheal Disease; (U) Intestinal Blood Flow; (U) Intestinal Absorption; (U) Intestinal Enzymes; (U) Narcotic Metabolism

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

23. (U) Research efforts in this department are directed toward defining the pathophysiology of diarrheal disease, assessing the role of the splanchnic vasculature in shock, and developing an assay for morphine metabolites in urine.

24. (U) Animal models are being used to evaluate the effect of diarrhea on intestinal transport and enzyme activities. Regional splanchnic blood flow is being studied in experimental shock. A radioimmunoassay is being developed to detect narcotic metabolites in urine.

25. (U) 71 07 - 72 06. In experimental diarrhea, there is a reversal of salt and water absorption to secretion thus providing diarrheal volume. Intestinal flora overgrowth is accompanied by reduction of vitamin B_{12}, glucose, and amino acid absorption and disaccharidase enzyme activity. In experimental endotoxin shock, the baboon behaves unlike the dog in that mesenteric arterial and hepatic arterial blood flow is maintained during hypotension. The spleen exhibits vasoconstriction and the renal blood flow falls passively without constriction. Intestinal microvascular studies demonstrate a direct constrictor effect of endotoxin in dogs that does not occur in baboons. This observation might account for some of the species differences. A radioimmunoassay has been developed that is more effective in detecting morphine and its metabolites in urine than any of the currently used assays. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 71 - 30 June 72.

(Available to contractors upon originator's approval)

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 087, Gastrointestinal disease

Investigators
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Description.

The research activity in the Department of Gastroenterology this year has continued in three areas of gastrointestinal pathophysiology. These areas include work completed on the pathophysiology of experimental diarrheal disease, the pathogenesis of gastric stress ulcer, and the involvement of the regional splanchnic circulations in the pathophysiology of cardiovascular shock. In addition, investigation has continued in the area of drug abuse. A laboratory test has been established for narcotic screening in urine specimens.

Progress and Results.

1. Pathophysiology of Diarrheal Disease.

a. Intestinal Transport. Three papers dealing with intestinal transport in salmonella infected rats have been published (1-3). In the first, the ilea of infected rats were shown to secrete water, sodium, and chloride. The second paper reports that, unlike cholera, glucose did not reverse the ileal secretion. These data suggest that oral fluids will not be useful to rehydrate salmonella infected patients. The third paper suggests that in the infected ileum, as in the control jejunum, H^+ secretion and Na^+ absorption are associated with the mechanism of HCO_3^- absorption.

A study dealing with intestinal fluid and electrolyte transport in the salmonella infected rhesus monkeys has been completed, and a manuscript is in preparation. The data illustrate that both the ileum and colon secrete water and electrolytes and that these abnormalities are corrected in 10 to 24 days.

Examination of the effect of salmonellosis on the gastrointestinal bacterial flora of the rhesus monkey revealed that the ileal and colonic flora are profoundly disturbed and remain so long after the diarrhea has ceased, and the physiologic water and electrolyte transport has returned to normal. A manuscript is in preparation.

A study dealing with intestinal transport in shigellosis in the rhesus monkey is underway. The goals of the study are to determine the sites

of diarrheal fluid formation, to transport mechanisms involved, and to ascertain whether the adenyl-cyclase-cyclic AMP system are important to this form of diarrhea.

A study of the mechanisms of salmonella diarrhea in the rabbit ileum has been completed. It has been published in abstract (4), and a manuscript has been submitted for publication. The findings demonstrate that only invasive strains evoke diarrhea and that this is not related to an enterotoxin. Furthermore, invasion of the mucosa and intramucosal multiplication of salmonellae are not the stimulus for fluid secretion but that a bacterial factor, in addition to invasiveness, is responsible.

Studies dealing with the effects of proliferation of normal intestinal flora on gut physiology are in progress. Preliminary results indicate that mucosal function is abnormal in that uptake of glucose and amino acids is disturbed.

A study dealing with the effect of dietary potassium deprivation on small intestinal function in the rat is in progress. An abstract has been published (5). Preliminary results demonstrate changes in small intestinal potential difference and abnormalities of salt and water transport. Further studies are in progress to determine the mechanisms of these abnormalities.

Dr. C. S. Tidball, George Washington University School of Medicine, worked in this department while on sabbatical leave. During this period, he wrote a review article dealing with the nature of the intestinal epithelial barrier. This manuscript has been published (6).

b. Intestinal Disaccharidase Activity. A manuscript describing the distribution of disaccharidases in human fetal intestine has been published (7). It demonstrated that maltase and sucrase levels reached adult value by four months of fetal development. However, lactase activity remained low beyond six months of development.

A study has been completed in which fecal disaccharidase enzyme activity was correlated with mucosal disaccharidase activity in germ free mice. The results indicate that the potential exists of employing analyses of these enzymes in stool specimens for clinical screening of possible malabsorption conditions. This study is being prepared for publication.

2. Pathogenesis of Gastric Stress Ulcer.

The urinary excretion of endogenous catecholamines is being studied in stressed rats and correlated with the development of gastric stress ulcer. A method has been devised to collect uncontaminated urine specimens by creating a permanent cystostomy. Preliminary data indicate that urinary excretion of catecholamines is increased in rats subjected

to two hours of rotational stress. The increase in urinary epinephrine is significantly greater than that of norepinephrine. Tritiated catecholamines are currently being used to define urinary washout curves of these agents in stressed animals.

3. Shock.

The splanchnic vasculature has been implicated in the pathogenesis of the irreversible phase of shock. The endogenous catecholamines released by hypotension reputedly cause an intense constriction of these vascular beds and leads to ischemia and ultimate tissue necrosis. A series of studies has been completed that question these traditional concepts.

Mesenteric hemodynamics were studied in baboons subjected to endotoxic shock and the results compared to the classical canine responses as described above. The intravenous injection of endotoxin (LD_{80}) caused profound hypotension without a change in either mesenteric blood flow or portal pressure. Thus, there is an obvious species difference between canine and subhuman primate responses to endotoxin. This study has been published (8). A similar study was done in which hemorrhage was employed to reduce arterial pressure. When baboons were hemorrhaged to an extent that would be lethal to dogs within 24 hours of reinfusing the shed blood, the mesenteric blood flow fell, the gut was ischemic, but the animals survived. This study was presented before the American Physiological Society (9) and has been published (10). The effects of endotoxin on hepatic and splenic arterial blood flow were studied in baboons. The results revealed no change in blood flow to the liver whereas splenic blood flow fell in parallel with arterial pressure. This study has been abstracted (11) and is currently in press in Surgery.

The silicone-rubber injection technique was used to describe the effects of endotoxin on canine and subhuman primate intestinal microvascular architecture. Endotoxemia caused the following vascular changes in the canine mucosa: a 50% shortening of villi; coiling of subepithelial capillaries and core vessels; congestion of subepithelial capillaries; and hemorrhage through core vessels as evidenced by rubber extravasation. These changes might explain the increased mesenteric vascular resistance and decreased mesenteric blood flow characteristic of canine endotoxemia. The mucosa of baboons treated in the same manner revealed evidence of dilation of subepithelial capillaries, a change that might contribute to maintainance of mesenteric blood flow during endotoxin shock. This study has been abstracted (12), presented before the American Federation for Clinical Research, and published (13). Abstracts have been published (14,15) that report epinephrine to cause mesenteric dilation in baboons when infused at low dosage. The same dose caused constriction in the dog mesentery. The study suggests that the elevation of blood epinephrine content during hypotension could cause the vasodilation seen in baboons. Similar injection studies were

conducted on dogs and baboons subjected to hemorrhagic shock. There was no change in mucosal vascular architecture in either species after four hours of hypovolemia. One and one half hours after the shed blood was reinfused there was evidence of hemorrhage into the cores of the villi in the dog studies. However, there were no changes observable in the baboons studied. This observation might explain in part the survival of baboons and the death of dogs when subjected to experimental hemorrhagic shock. This study has been abstracted (16) and presented at the American Gastroenterological Association.

The above series of studies on the pathophysiology of shock in baboons has seriously questioned the concept of the splanchnic viscera being target organs in shock and has demonstrated specific species differences in splanchnic hemodynamics between canine and subhuman primate models of shock. This material has been presented at the following international conferences: The European Society of Experimental Surgery (17). The 1971 Oklahoma Shock Symposium (18), and The Third International Conference on Experimental Medicine and Surgery in Primates.

Three papers have been published that describe the action of catecholamines in the splanchnic circulation (19,20,21). The endogenous catecholamines are vasoconstrictors in the mesenteric vascular bed. However, when infused for a ten minute period, the mesentery vasculature exhibits autoregulatory escape to a degree that questions the premise that the catecholamines elaborated during shock can cause prolonged intestinal ischemia.

The effects of sublethal doses of endotoxin on mesenteric hemodynamics were studied in dogs. Abstracts have been published that describe the action of the endotoxin when it was delivered through either the vena cava, portal vein, or superior mesenteric artery (22,23,24). The results suggest that endotoxin has a direct constrictor influence on the mesenteric vascular bed. This study is in press in the American Journal of Physiology. The effects of a sublethal dose of endotoxin on epinephrine responses in the mesenteric circulation was studied and abstracted (25). The overall results suggest that small amounts of endotoxin do not alter the constrictor potential of epinephrine.

A paper has been published that describes the action of the new Walter Reed alpha adrenergic antagonist (WR-2823) in the mesenteric circulation (26). The agent was demonstrated to produce an effective alpha adrenergic blockade in terms of aortic pressure response to epinephrine. However, within the mesenteric circulation, it was of minimal effectiveness.

4. Drug Abuse.

Progress was made in the following three areas of drug abuse research: development of a hemagglutination inhibition (HI) assay for

morphine; development of a radioimmunoassay (RIA) for morphine; and a clinical trial comparing five different assay techniques for morphine.

The HI assay, as modified and improved, is currently the least expensive, rapid screening test available. Sensitivity studies indicate an absolute detectability of 0.025 ng morphine. When applied to undiluted urine, the sensitivity is 25 ng morphine/ml. Specificity studies revealed major cross reactions with codeine and other opiates except methadone, and minor cross reactivity with poppy seeds and dextromethorphan. This study is in press in Clinical Immunology and Immunopathology and has been abstracted (27).

In collaboration with Hoffman LaRoche, Inc., a RIA for morphine was further developed and tested. This test is equal in sensitivity to HI, slightly more specific, but more expensive. Modification in reagent concentration has improved stability over time, reduced analysis time to one hour, and improved standard curves. The assay was tested on specimens received from Dr. C. Gorodetzky, NIMH, Addiction Research Center in Lexington, Kentucky. The study demonstrated the RIA to be superior in validity and sensitivity when compared to FRAT and thin layer chromatography (TLC) analysis for morphine, This work is being prepared for publication.

Serum and urine samples were obtained from a methadone clinic and were subjected to a double blind analysis with FRAT, RIA, HI, TLC, and Technicon auto spectrofluorometric techniques. The results, which are being prepared for publication, indicate that the RIA and HI are superior for drug screening.

5. Miscellaneous.

A member of the Research Fellowship Program worked in this department during the year. Two studies were completed that dealt with the pharmacology of genitourinary smooth muscle. The first was a characterization of some pharmacological properties in isolated human vasa deferentia and suggested that motor control of this organ is mediated via alpha adrenergic receptors. In addition, glucose was shown to have an inhibitory effect on this smooth muscle. The study is in press in The Journal of Investigative Urology. The second described the adrenergic mechanisms in the canine ureter. The results revealed both alpha and beta adrenergic mechanisms present. Alpha stimulation increased while beta stimulation decreased ureteral motility. These data are being prepared for publication.

A paper was published describing the construction of a miniature occlusive device to be used in studying blood flow through small arteries (28). The device proved effective in accurately determining blood flow in rat superior mesenteric arteries.

Conclusions and Recommendations

Work done on animal models of diarrhea indicate that in monkeys infected with salmonella the ileum and colon both secrete water and electrolytes and suppress the normal population of intestinal flora for prolonged periods. However, an overgrowth of intestinal flora causes a decrease in glucose and amino acid absorption that can lead to diarrhea. Studies in rabbits showed that only invasive strains of salmonella caused diarrhea and that the effect was independent of any enterotoxin. These findings will be incorporated into the presently planned clinical study of diarrhea in human volunteers.

The splanchnic circulation has been shown to differ markedly in terms of physiology, pharmacology, and morphology in dogs and baboons subjected to experimental cardiovascular shock. The data have been presented as an argument against the validity of current concepts of the pathophysiology of shock and secondarily against current therapeutic management of shock. These studies will be continued to evaluate regional circulatory responses to shock in canine and sub-human primate models to further define the pathophysiology of this condition.

An improved method of detecting morphine and morphine metabolites in body fluids has been perfected. Research efforts will continue to be directed toward defining the nature of narcotic addiction.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 087, Gastrointestinal disease

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15. Swan, K. G. and D. G. Reynolds. The vasodilator action of epinephrine in the mesenteric circulation of the baboon. Gastroenterology 62: 819, 1972.

16. Reynolds, D. G., L. W. Evans, and K. G. Swan. Intestinal microvascular architecture in experimental shock. Gastroenterology 62: 798, 1972.

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PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6453	72 07 01	DD-DRAE-AR(A)...

3. DATE PREV SUMM	4. KIND OF SUMMARY	5. SUMMARY LEVEL	6. WORK UNIT	7. REGRADING	8. DISPOSITION	8a. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
71 07 01	D Change	U	U	N/A	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. [illegible]	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
A. Primary	61102A	3A061102B71R	02	088
B. [illegible]				
C. [illegible]	CDOG 114(f)			

11. TYPE (Precede with Security Classification Code)
(U) Military Nursing (09)

12. FIELD AND TECHNOLOGICAL AREAS
003500 Internal Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
58 05	CONT	DA			C In-House

17. CONTRACT/GRANT

a. EFFECTIVE	EXPIRATION	18. RESOURCES ESTIMATE	b. PROFESSIONAL MAN YRS	c. FUNDS (In thousands)
d. NUMBER N/A		PRECEDING FISCAL YEAR 72	4	250
e. TYPE	f. AMOUNT	CURRENT FISCAL YEAR 73	4	200
g. KIND OF AWARD	h. CUM. AMT.			

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Military Nursing; (U) Guidelines for Procedures; (U) Sterile Items; (U) COMPSY; (U) Drug Abuse

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 and guidelines for nursing procedures; determine the sterility of intravenous therapy equipment; use of computers in military psychiatry; and study of drug abuse.

24. (U) Assessment of thermometer placement times, methods of enhancing sleep, and the accuracy of recorded intake; bacteriological testing of sterile items; testing of the use of computerized nursing notes; and assessing normal and drug abuse subjects.

25. (U) 71 07 - 72 06 Guidelines for adult rectal temperatures derived; protocol for methods of enhancing sleep being prepared; data collection on accuracy of intake recording begun; sterile items have been tested; nursing notes continue to be assessed; and protocols for drug abuse are being prepared. Portions of this work unit have been reported in DD-1498's submitted to Walter Reed General Hospital under Project 3A062110A826, Work Units 481, 485, and 704. For technical reports, see Walter Reed General Hospital and Walter Reed Army Institute of Research Annual Progress Reports, 1 July 1971 - 30 June 1972.

*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 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 088, Military Nursing

Investigators,
Principal: LTC Glennadee A. Nichols, ANC
Associates: LTC Margaret L. O'Dell, ANC
MAJ Anna Koneck, ANC
MAJ Nancy M. Christ, ANC
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CPT Waltraut M. Hurd, ANC

Description.

Research in military nursing is concerned with both direct and indirect patient care, and with identifying and testing principles underlying nursing care. The areas being reported are concerned with the following: two studies regard thermometer placement times for adults and differences in times for febrile and afebrile temperature measurements; the effects of sleep producing procedures; fluid intake recording; bacteriological testing of intravenous therapy equipment; questions pre-operative patients have; an evaluation of two methods of warming post-operative patients; vital signs in hospital admissions; computer support in military psychiatry; and study of drug abuse.

Progress and Results.

1. Thermometer Placement Time Studies.

Nursing and medical texts do not agree on the amount of time required to measure a person's body temperature accurately by clinical thermometers. Recommended rectal thermometer placement times vary from one to five minutes. These times were not derived from systematic investigations, but they have been used traditionally in nursing practice. Also, the amounts of time recommended are usually recommended for all individuals regardless of the variables of age, sex, and room temperature. The purpose was to ascertain the maximum and the optimum placement times. The following were the operational definitions: (1) maximum temperature - the highest registration on a clinical thermometer during the testing period; (2) optimum temperature - temperature reading 0.2° Fahrenheit (F) lower than the maximum temperature; (3) maximum placement time - the time required for 90% of the subjects to register their maximum temperatures, and (4) optimum placement time - the time required for 90% of the subjects to reach their optimum temperatures. One study utilizes a secondary analysis of data of different studies on adult rectal thermometer placement times. The other study analyzes the amount of time required for measuring febrile and afebrile temperatures of adults.

Data analyzed for 403 adults showed that the maximum placement time for rectal thermometers was four minutes and the optimum placement time was two. After examining the conjoint influence of sex and environmental temperature on the optimum placement time, the following guidelines for measuring adults' rectal temperature with glass clinical thermometers were derived: (1) the optimum placement time is two minutes in rooms of at least 72° F, and (2) the placement time of three minutes should be used in rooms under 72° F.

The mean time for measuring 47 febrile oral temperature readings of adults was compared to that required for 47 afebrile readings matched for room temperature. Afebrile readings required an average of 8.21 minutes and febrile readings required 6.21 minutes. The difference was significant at the .001 level. For 42 matched rectal temperature readings, there was no significant difference in the average time for measuring febrile and afebrile readings which were 2.09 and 2.29 minutes, respectively. There were considerable differences in the individual times for measuring oral readings, but less variation was present for rectal readings.

2. Comfort Measures to Promote A Physiological State.

Nurses use both physical and mental comfort measures in giving patient care. This study is designed to observe the effectiveness of two relaxation techniques in promoting sleep. A skeletal muscle relaxation technique (representing peripheral entry into relaxation) and relaxation via imagery (representing central entry into relaxation) will be compared. Three audio tapes, a muscle relaxation, an imagery, and a control, are proposed to be used on a population with sleep problems. Subjective evaluation of sleep will be used.

Content for the tapes has been derived from literature review and a word selection Q-sort. The tapes for this project are in the process of being made and validated.

3. Fluid Intake Recording.

Nursing service personnel are responsible for accurately recording fluid intake of patients. The recording of intravenous fluids is no problem because solution bottles are marked in milliliters. The recording of oral intake, however, is problematic because graduated containers are not available. Estimation errors may arise from the unsymmetrical contour of containers, the meniscus of the liquid, and optical illusions based on parallax. This investigation was designed to assess accuracy in determining the amount of liquid ingested by hypothetical patients. The primary objective was to determine the accuracy accompanying the use of unmarked and marked containers. A second objective was to find whether individuals underestimated or over-estimated the amount of liquid removed from unmarked containers.

A glass, cup, and soup bowl were calibrated for use in collecting data. Data collection is complete on 40 nursing service personnel and data collection is planned for 40 more subjects.

4. Intravenous Therapy Solutions and Equipment.

Intravenous therapy, an integral part of the medical treatment of hospitalized patients, is used by all medical specialties and for a variety of reasons. Increased interest has grown concerning its safe administration because it has been cited along with urinary catheters and inhalation equipment as sources of hospital-acquired infections. Studies have indicated that commercial equipment is usually sterile, but it may become contaminated during the therapy. It has been suggested that the incidence of infections might be reduced if the injection sets were changed daily. Under normal management, an infusion unit might be manipulated (opened and closed) several times in 24 hours. Changing of the injection set would require additional manipulations. At the present time no study has ascertained whether, under normal operational conditions, an injection set can remain longer than 24 hours without possibly becoming contaminated. The purpose of this study was to determine if sterility of intravenous equipment and solutions can be maintained over a 72-hour period using four methods of manipulating the equipment and the solution.

The following manipulations were completed on four infusion units, two standard and two additive units: (1) injection sets were not changed on one standard and one additive unit; (2) injection sets were changed at 24 and 48 hours on the two remaining units; (3) additional solution was added to the additive units; and (4) solution bottles were changed every eight hours. Data were obtained from cultures of the equipment and solutions prior to being operational, during the 72 hours at spaced intervals, and at the end of 72 hours. Data collection is completed and the data are being analyzed.

5. Pre-Operative Preparation Study.

Health care personnel traditionally provide patients with information they think patients should have. There have been few studies dealing with asking patients themselves what they would like to know. This study was designed to elicit patients' queries about their surgical event and immediate post-operative recovery. It is being reported under Project 3A062110A826, Task 00, Work Unit 485.

Open-end interviews were conducted with 20 male patients to elicit queries they still had a few hours before surgery under general anesthesia. Fifteen of the 20 subjects had 79 queries 9 to 11 hours before their surgery even though they had been in contact with nursing service personnel, physicians, and professional persons from the anesthesia department. Thirty-three queries pertained to the surgical event, 13 to the immediate post-operative recovery, and 33 regarded other aspects of the patients' hospitalization and convalescence. It can be concluded that many of the patients either were not provided with the information they needed, or they did not hear and understand the information given by health personnel.

6. Hypothermic Study.

Of the patients arriving in the recovery room from the operating room daily, many are hypothermic. The contributing causes may include: decreased body metabolism from anesthetic agents, depressed thermoregulating mechanism, peripheral vasodilation, prolonged operation time, and transfusion of cold blood. Prolonged hypothermia is not a desirable state for patients to continue in, and therefore, various methods of warming them have been used. In this study these two methods of warming patients were used: (1) three blankets covering the patient and (2) Emerson Mobile Heat Lamp. As soon as the warming method was instituted in the recovery room on each patient, both skin and rectal temperatures were taken every ten minutes until 30 minutes after normothermia was reached. It is being reported under Project 3A062110A826, Task 00, Work Unit 481.

Data were collected on 28 hypothermic patients, 10 men and 18 women ranging in age from 20 to 55. The analysis of data is in progress.

7. Vital Signs on Hospital Admission.

Many patients entering a hospital may have some degree of anxiety. Routinely, nursing service personnel measure patients' vital signs on admission, and these readings may be used thereafter as a baseline for the patient. It would seem that a more accurate assessment of patients' vital signs would be possible after they have had some time to adjust to a new environment. The purpose of this study is to determine if a significant difference exists between vital signs taken on admission and those taken three and six hours post-admission. This study is being reported under Project 3A062110A826, Task 00, Work Unit 704.

Data collection is complete on 21 adult subjects having routine hospital admissions. A total of 60 subjects will be needed, with an equal number of men and women.

8. Computer Support in Military Psychiatry.

The Computer Support in Military Psychiatry (COMPSY) study is a project in Walter Reed General Hospital's Department of Psychiatry and Neurology with full-time nurse support from the Division of Nursing, Walter Reed Army Institute of Research. It is being reported under Project 6215601A-3A025601A823, Task 048.

9. Drug Abuse in Military Personnel.

This program is under the direction of the Department of Experimental Psychophysiology, Division of Neuropsychiatry, Walter Reed Army Institute of Research, with full-time support from the Division of Nursing. It is being reported under Project 3A062110A823, Task 032.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 088, Military Medicine

Publications

1. Nichols, G. A.: Young nurses' reasons for joining, remaining in and leaving the army. Military Medicine 136:715-722, 1971.

2. Kucha, D. H.: An evaluation of traditional and programmed instruction to teach medical management to patients and their families. Educational Technology Research 50, 1971.

3. Nichols, G. A. and Dobek, A. S.: Sterility of items sealed in plastic, Hospital Topics 49:84-86, 1971.

4. Nichols, G. A.; Kucha, D. H.; and Mahoney, R. P.: Rectal thermometer placement times for febrile adults. Nursing Research 21:76-77, 1972.

5. Nichols, G. A. and Kucha, D. H.: Taking adult temperatures; oral measurements. American Journal of Nursing 72:1090-93, 1972.

6. Nichols, G. A.: Taking adult temperatures; rectal measurements. American Journal of Nursing 72:1092-93, 1972.

7. Hurd, W. M.: Using computers for patient care. Southern Hospitals 40, No. 5:10-11, 1972.

8. Nichols, G. A., et al: Measuring oral and rectal temperature of febrile children. Nursing Research 21, No. 3:261-264, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6464	72 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
71 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				
c. CONTRIBUTING XXXXXXX	CDOG 114(f)			

11. TITLE (Precede 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

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 72	2	50
	CURRENT 73	4	100

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research	NAME: Walter Reed Army Institute of Research Division of Medicine
ADDRESS: Washington, D. C. 20012	ADDRESS: Washington, D. C. 20012
RESPONSIBLE INDIVIDUAL NAME: Buescher, COL E. L. TELEPHONE: 202-576-3551	PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME: Knepshield, MAJ J. H. TELEPHONE: 202-576-2265 SOCIAL SECURITY ACCOUNT NUMBER: [redacted]
21. GENERAL USE Foreign Intelligence Not Considered	ASSOCIATE INVESTIGATORS NAME: Briggs, MAJ W. A. NAME: Flamenbaum, MAJ W. DA

PII Redacted

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Immunopathology; (U) Heat Stress; (U) Renal Failure; (U) Dialysis; (U) Acid-base; (U) Electron Microscopy; (U) Kidney Function; (U) Renal Hemodynamics; (U) Fluid and Solute Homeostasis; (U) Shock

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede 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 dilutions, experimental models, in vivo renal micropuncture, in vitro renal microperfusion, membrane transport, light and electron microscopy and immunopathology.

25. (U) 71 07 - 72 06 Uranyl nitrate induced acute renal failure in the dog and rat is characterized by renal hemodynamic alterations, perhaps mediated by the renin-angiotensin system. Potassium suppresses renal renin synthesis and release. Suppression of peripheral renin by renin immunization or DOCA/saline does not influence the development of acute renal failure. A correlation between immuno and bioassayable renin activity was demonstrated. Methods for evaluating the immune defect in chronic renal disease are being developed. A model for rat transplantation is underway. The effect of hypoxia-ischemia on the isolated perfused dog kidney was determined. Inability to concentrate urine in the isolated dog kidney was related to medullary washout by altered renal hemodynamics. Various immunosuppressive regimens have been evaluated in murine lupus. Optimum protein intake, calcium homeostasis, vitamin D metabolism, red cell enzymes are being studied in patients with chronic renal disease and after renal transplantation. In vitro hydrogen ion secretion by rabbit gastric mucosa has been characterized and the effects of salicylates, bile salts and hemorrhagic shock are being determined. Hydrogen ion secretion by turtle bladder epithelium has been characterized and the effects of uranyl nitrate on turtle bladder epithelium are being studied. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

* Available to contractors upon requester'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 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 089, Body fluid and solute and renal homeostasis

Investigators:
Principal: MAJ James H. Knepshield, MC
Associates: MAJ Walter Flamenbaum, MC; MAJ William A. Briggs, MC; MAJ Andrew Saladino, MC; MAJ Charles H. Wallas, MC; MAJ Vincent W. Dennis, MC; MAJ John H. Schwartz, MC; MAJ Robert J. Hamburger, MC; John A. Gagnon; Natalie L. Lawson; James S. McNeil; Amy C. Lee; Martha Huddleston and Doug Grove

Description: Studies are directed at investigations 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, light and electron microscopy, and immunopathology. 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. Acute Renal Failure:

a. Despite the availability of modern blood banking and renal dialysis facilities, there continues to be a significant mortality after acute renal failure. Indeed, in comparing the mortality after acute renal failure in the Korean War and Viet Nam conflict, no significant alteration has been observed (1). In order to establish a more efficacious method of therapy for acute renal failure, the mechanisms responsible for its pathogenesis must be determined. To study these mechanisms, the

model of uranyl nitrate induced acute renal failure has been studied in the dog and rat. Using external monitoring of inert radioactive gas washout (^{133}Xenon) it was determined that marked alterations in total renal blood flow, intrarenal hemodynamics and glomerular filtration rate precede any evidence of overt tubular necrosis or tubular obstruction (2,3). These data indicate that the primary mechanism responsible for the pathophysiologic manifestations of acute renal failure is related to an alteration in renal hemodynamics. Parallel studies of uranyl nitrate induced acute renal failure in the rat were carried out using renal micropuncture techniques 6 and 48 hours after uranyl nitrate. A modification of the ^{133}Xenon washout technique was developed in order to study renal blood flow in this model of acute renal failure. Within 6 hours, effective filtration pressure and renal blood flow were reduced without evidence of tubular necrosis or obstruction. By 48 hours physiologic and pathologic evidence of severe tubule epithelium damage was evident. These studies indicate again a primary hemodynamic alteration in the development of acute renal failure (4,5).

Having identified a primary hemodynamic abnormality, current efforts are being directed towards evaluating the efficacy of various potent vasodilator agents on renal blood flow in acute renal failure. Efforts directed towards establishing radioxenon washout for study of human acute renal failure, as currently performed at other institutions (1,2), have been impeded by the nonavailability of suitable fluoroscopic facilities.

b. The mechanism responsible for the altered renal hemodynamics in acute renal failure is also being evaluated. Several reports have indicated that circulating plasma renin activity is elevated during the oliguric phase of acute renal failure. Similarly, plasma renin activity is elevated in uranyl nitrate induced acute renal failure in both the dog and the rat (2-5). We have obtained additional evidence, however, that it is renal renin rather than circulating renin that is responsible for the renal hemodynamic alterations in acute renal failure. When rats are immunized with renin, plasma renin activity is severely depressed whereas renal renin content is unchanged and the rats are not protected against the development of mercuric chloride or glycerol induced acute renal failure (6). Rats that are renal renin depleted by saline loading are protected. Similarly,

the acute reduction of plasma renin activity by 30 hours of DOCA and saline without alteration in renal renin does not alter the course of glycerol induced acute renal failure in the rat (7). Inhibition by KCl loading similarly protects against the development of mercuric chloride induced acute renal failure (8,9,10).

Although the renin-angiotensin system may be responsible for the renal hemodynamic alterations in acute renal failure, the mechanisms responsible for stimulation of renin release and synthesis must be determined. In clinical acute renal failure associated with blood loss or volume depletion, an obvious stimulus for renin release is apparent. However, in many instances of both clinical and experimental acute renal failure, a definitive stimulus is not apparent. In order to better define the relationships between nephrotoxins, renin, renal hemodynamics and acute renal failure assessment of factors such as individual nephron renin release and vascular response in shock and acute renal failure are required (3).

c. In conjunction with studies of experimental acute renal failure, a variety of clinical studies are in progress. Since patients with acute renal failure, especially after surgery or trauma, are hypercatabolic, hyperalimentation with essential amino acids is being evaluated. This study will determine if hyperalimentation will induce a positive nitrogen balance, faster wound healing, decreased dialysis requirements and diminished morbidity.

d. Additional evaluations of frank hypercalcemia in acute renal failure are being carried out since this may result in extensive soft tissue and/or cardiac calcification precipitating fatal infections or cardiac disturbances. In order to evaluate calcium homeostasis in patients with acute renal failure, studies of serum calcium, phosphorus, alkaline phosphotase and parathormone levels are being carried out.

2. Chronic Renal Disease and Transplantation:

a. Renal Hemodynamics:

Organ preservation and function of the isotransplanted kidney have been investigated using the isolated perfused dog kidney. Progressive loss of urinary concentration by the

isolated perfused kidney, even in the presence of adequate anti-diuretic hormone, has been the most commonly observed abnormality in this preparation. We have demonstrated increases in renal blood flow to supra-normal levels in the absence of altered perfusion pressure. Using various radioactive labeled microspheres (15 ± 5 u in diameter) to determine anatomic blood flow distribution, we have shown a redistribution of renal blood flow from the cortex towards the medulla with a concomittant washout of the papillary osmotic gradient. This results in increasing volumes of nearly isotonic urine. The vascular nature of the hemodynamic alterations resulting in diminished urine concentration has been confirmed using radio-xenon washout techniques (11).

The structure-function inter-relationships of the transplanted or shock kidney have been evaluated using the isolated perfused dog kidney under conditions of hypoxia and ischemia. For the first time, the morphology of the isolated kidney after 3-7 hours of blood perfusion has been characterized and correlated with functional physiologic alterations. These studies are preparatory to a full evaluation of the fixed pathologic-physiologic alterations in the end stage, failing shock kidney or transplant failure. The effects of reperfusion or reoxygenation on these alterations are being evaluated (12,13).

b. Immunology, Immunopathology:

A laboratory to probe and evaluate abnormalities in immunology defense in chronic renal insufficiency and in renal transplant rejection has been established. Techniques used include, in addition to standard immunologic techniques, the mixed-lymphocyte culture and isologous or heterologous rat renal transplantation. Efforts are being directed towards evaluating the immune alterations responsible for the great morbidity and mortality in chronic and acute renal failure.

Evaluation is underway of the poor kidney survival of living related donor renal transplants in patients with chronic renal disease due to bilateral renal cortical necrosis. This study determines the presence of circulating antiplatelet antibodies in the sera of these patients to determine its relation to the accelerated transplant rejection. Preliminary results in twelve patients have demonstrated elevated titers of antiplatelet antibody in 80% and lymphocyto-toxic-antibodies in 25%. The NZB/W mice have been used as a model for human systemic lupus

erythematosis. The model is characterized by anti-DNA antibodies, antigen-antibody complex deposition in the kidney and death from renal failure. Combination immunosuppressive regimens were found superior to single or double agent regimens in preventing the nephritis (14, 15, 16). Combination immunosuppressive therapy was also found to be superior in murine models of cellular and humoral immunity (17). Age dependent impaired cellular immune phenomena occur in NZB/W mice. Using skin graft rejection, splenectomy and re-injection of "young" spleen cells, a population of steroid sensitive spleen cells was shown to be lacking or ineffectual in "old" mice (18).

c. Coagulation and Anemia:

Anemia is a universal feature of diuretic renal failure and is most often primarily due to relative failure of erythropoesis. However, decreased red cell survival has been repeatedly demonstrated in uremic patients in hematocrit and inappropriately high blood transfusion requirements. Evaluation of glycolytic erythrocyte enzymes in patients with chronic renal disease has indicated that uremic plasma itself is not responsible for the increased glycolysis observed in uremic red cells. The pattern of enzyme changes suggests the presence of a younger red cell population (19). An increased rate of ATP production has also been observed in red cells from patients with chronic renal failure, once again suggesting the presence of an immature red cell population (20). Correlative ferrokinetic studies in patients with uremia are also underway.

A number of patients with fulminant and progressive renal disease requiring renal hemodialysis are seen each year. These patients have various forms of glomerulonephritis characterized pathologically by marked glomerular proliferation and crescent formation, which is related fibrin deposition. We are evaluating the effect of heparin anticoagulation on the progress and manifestation of this disease process.

d. Dietary Therapy:

Despite marked improvement in a variety of parameters patients undergoing hemodialysis often show marked losses in lean body mass which is difficult to correct and contributes

significantly to the increased morbidity of chronic renal disease. These patients lose essential amino acids during dialysis, are catabolic and may be in negative nitrogen balance. Patients were studied on diets of from 0.75 to 1.25 grams of protein per kilogram of body weight and nitrogen balance followed preliminary results indicated that increased dietary protein may be required for optimum management and minimal morbidity in these patients.

e. Calcium Homeostasis:

Evaluation of calcium homeostasis and vitamin D metabolism in patients with chronic renal disease undergoing transplantation is underway. Secondary and tertiary hyperparathyroidism contributes to the morbidity and transplant failure in these patients. These studies involve endocrinologic evaluation of the calcium-parathyroid-renal axis as well as determinations of parathormone activity and radiocalcium balance procedures. On the basis of results from studies such as these, vigorous therapeutic support can be undertaken in preventing the progressive skeletal abnormalities which are the cause of the increased morbidity.

3. Membrane Physiology:

a. Mechanism of Gastric Acidification in Vitro:

In order to understand gastric mucosal alterations after shock, trauma, ischemia or stress, an in vitro model using rabbit gastric mucosa was developed. Thus far, it has been shown that in vitro acidification occurs independent of the transport of sodium, potassium or chloride but dependent of the luminal pH gradient. In the absence of hormonal agents which stimulate acidification, the rate of acid secretion does not require enzymatic hydration of carbon dioxide (21). Current efforts are directed towards evaluating agents known to promote ulcer formation such as salicylates and bile salts, with and without hemorrhagic shock.

b. Urinary Acidification:

The isolated turtle urinary bladder is physiologically analogous to the distal tubule of the nephron, a site difficult to directly study. Using a titragraphic method of measuring the change in bicarbonate concentration, the rate of acidification has been measured. The mechanism seems to be independent of bicarbonate reabsorption (22-25).

c. Effect of Uranyl Nitrate on Urinary Mucosal Epithelial Tissue:

Uranyl nitrate, in addition to causing acute renal failure, produces renal tubular acidosis. To assess the direct effect of uranyl nitrate, it was studied in the isolated turtle bladder model. Preliminary evidence suggests that uranyl nitrate directly inhibits the active transport of sodium and hydrogen ions only on the urinary side of the bladder.

d. Fluid Reabsorption:

Using the isolated perfused rabbit tubule, the effect of cyclic-AMP, dibutyrylcyclic-AMP, AMP and theophyline on fluid reabsorption were studied. Radioactive polyvinyl pyrrolodine was used as the marker for fluid reabsorption. It was determined that a cyclic-AMP responsibe mechanism is available in the proximal tubule epithelium of the rabbit kidney for possible hormonal regulation (26, 27).

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 089, Body fluid and solute and renal homeostasis

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6462	72 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
71 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	090
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Cellular Mechanisms of Diseases in Military Personnel

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: 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	72	4	125
FISCAL YEAR CURRENT	73	4	125

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

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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) Fibroblast Suspension Culture; (U) Oxygen Microenvironment; (U) Amino Acid Metabolism; (U) Collagen Synthesis

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 following by tissue regeneration, healing and repair. As the restoration of the soldier's health and combat capability depends on these repair processes, this study aims to uncover the underlying mechanisms and to increase their effectiveness.
24. (U) The essential sequence of wound healing, i.e. active proliferation of initially sparse fibroblasts leading to a dense stable population synthesizing collagen was reproduced in a suspension culture model used to study the underlying metabolic interactions.
25. (U) 71 07 - 72 06 With cell contacts thus minimized, the evidence obtained suggests humoral regulation with oxygen tension and glutamine concentration among the microenvironmental factors involved. Thus, within 7 hours after media renewal of the high density stable cultures, pO2 and glutamine declined from 140 mmHg and 1400 mM, respectively, to 1 mmHg and .048 mM; for low density growing cultures these values remained above 120 mmHG and.700 mM. These quantitative environmental changes, obviously due to differences in cell density, resulted in striking qualitative changes in metabolism as seen by the high intracellular free alanine and low glutamate and aspartate found in dense stable populations; exactly the opposite was observed in sparse growing cultures. These three amino acids represent key intermediates in the utilization of glutamine in either energy production or collagen biosynthesis. As the former was found to decrease in the model while the relative rate of the latter increased, further work relating these changes to amino acid metabolism is expected to lead to the eventual understanding and clinical control of the essential wound healing sequence. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

*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 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 090 Cellular mechanisms of diseases in military personnel

Investigators.
Principal: Andre D. Glinos, M.D.
Associate: B. Taylor, CPT. CmlC; E.M. Bartos, Ph.D.; J.M. Vail, Ph.D.; R.J. Werrlein, M.S.

Description

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. 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 proceed with 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, first induce fibroblasts to proliferate and later limit cell division to the maintenance of a collagen producing steady state cell population? 2) What is the nature of intracellular molecular interactions which occur in response to these extracellular changes and result in the early phase of DNA replication and general protein synthesis followed later by a marked reduction of these activities while the synthesis of collagen continues at a relatively high rate?

Approach

The reason that in the past a great number of clinical and experimental

studies have failed to provide answers to the two questions defined above, lies in the great complexities of the clinical situation in man and of the experimental conditions in the whole animal. Even the discovery that cultures of fibroblasts attached on glass or plastic surfaces reproduce the wound healing sequence pronounced decline of DNA replication, general protein synthesis and cell division while collagen synthesis is maintained at relatively high levels as they proceed from low to high cell densities, has failed to provide the desired answers. This is due to the fact that in attached cultures, it is impossible to distinguish between possible physical growth regulatory mechanisms necessitating cell to cell contact and humoral regulation operating through decreased uptake of substances essential for growth by dense cultures.

We resolved this difficulty by using suspension cultures of L-929 fibroblasts where cell to cell contact is minimal. During the period covered by this report we completed the demonstration that, under proper conditions, active proliferation of initially low density suspension cultures leads to the development of dense stable populations where DNA synthesis and mitosis virtually cease, with the small fraction of cells which continue to divide replacing cells rendered nonviable or lost as a result of culture manipulation(1).

Having thus reproduced the essential sequence of wound healing as far as cell growth kinetics are concerned, we questioned next whether this is also true in regard to collagen synthesis as suggested by our preliminary results (cf. WRAIR Annual Progress Report, Work Unit 094, 1 June 70-30 July 71), and whether oxygen tension and amino acid availability could be among the environmental regulatory factors responsible for the sequence observed. Oxygen tension and amino acids were selected among other alternatives because of the frequent but inconclusive clinical and whole animal observations implicating them in the control of wound healing[1,2]. Intracellular ATP and free amino acid levels expected to respond promptly to extracellular oxygen and amino acid concentration changes were also investigated.

The methods used are as follows:

I. Collagen synthesis

General protein and collagen synthesis and release were determined in paired low density, exponentially-growing and high-density, stable cultures as follows: One hour after medium renewal, 3,4 ^{3}H L-proline (specific activity 5.9 Ci/mM, New England Nuclear) was added to the culture to yield a final concentration of 2 μCi/ml. At desired time

intervals, 5 ml samples were withdrawn and the cells separated from the medium by centrifugation at 500 X g for 10 minutes. The cell pellet was washed twice with 5 ml of Eagle's spinner medium and the washings added to the original supernatant. The combined medium was then incubated for 15 minutes at 35°C in the presence of 8 X 10^{-2}M added cold proline to minimize artefacts resulting from the exchange of unbound, labelled proline with serum proteins contained in the medium. High molecular-weight compounds were isolated by precipitation in ice cold 0.5N perchloric acid (PCA) containing 9 X 10^{-2}M unlabelled proline for 20 minutes followed by centrifugation and one wash with the same solution. The resulting precipitate was digested with 6N HCl at 110°C overnight, evaporated to dryness with filtered compressed air and dissolved in 0.2M citrate buffer, pH 3.41, and with carrier proline and hydroxyproline added.

Radioactively-labelled amino acids in the hydrolysates were injected on a 0.9 X 90 cm column containing Type A Chromo-Beads (Technicon Chemical Co., Inc.), a cation exchange resin having an average particle diameter of 16 μ. The absorbed amino acids were eluted from the column with 0.2M sodium citrate buffer, pH 3.42 delivered at a rate of 0.9 ml/min by digital pumps (Nester/Faust Manufacturing Corp.), and collected in 2 ml fractions. One ml aliquots of the collected samples were added to 14 ml volumes of a scintillation cocktail formulated by adding 11 g PPO, 250 mg dimethyl POPOP and 500 ml of Triton X-100 to 1500 ml of toluene. Radioactive disintegrations were measured in a liquid scintillation counter (Picker Nuclear) and expressed as dpm by use of external standard, channel ratio, correction techniques.

Labelled proline and hydroxyproline present in the column effluent were identified by their elution position compared to chromatograms obtained with known standards and quantified by integrating the total number of counts contained in each peak, correcting for loss of one-half of the tritium atoms occurring during the hydroxylation process. On the basis of the values thus obtained, the relative rate of collagen synthesis and release was calculated and expressed through the ratio $\Delta C/\Delta P$ first utilized by Green and Goldberg[3], with the modification that here both values refer to the acid precipitable material of the medium only, and ΔP refers to the sum total of medium protein including collagen.

II Oxygen measurements

Determinations of the oxygen content of the gas phase of the incubation chamber were performed with a paramagnetic oxygen analyzer (Beckman Instruments Inc.) calibrated with chromatographic-grade N_2 and laboratory air (Fig. 1). At the beginning of the incubation period, with the

culture in place, the chamber was gassed for 10 minutes with a mixture of 5 percent CO_2 in air and then sealed by closing the exit and entry valves, in that order. This procedure, together with a 20-minute wait for water vapor and thermal equilibration produced a slight positive pressure in the chamber. The analyzer was connected to the outlet valve and successive samples of the gas were then measured for oxygen content after first passing through a $CaCl_2$ column to remove water vapor. The pressure inside the chamber was then reduced to atmospheric, and the culture allowed to incubate. After incubating for slightly less than 24 hours in this closed system, the balloon was inflated in order to produce the small positive pressure necessary for the introduction of several gas samples into the oxygen analyzer.

The partial pressure of dissolved oxygen in the culture media was measured directly by immersing a galvanic oxygen probe (New Brunswick Scientific Co., Inc.) directly in the fluid after sterilization by autoclaving. Electrical currents produced by the probes were measured by an electrometer (Keithley Instruments, Inc.). The electrodes were periodically examined for residual current, linearity and drift while mounted in the incubation chamber with the culture removed. Their response at atmospheric pressure, while either in the gas phase or in stirred cell-free medium equilibrated with the gas phase, was related to oxygen values obtained by the paramagnetic analyzer, after gassing the chamber with various concentrations of oxygen. These galvanic-type electrodes were proven to be stable and allowed continuous monitoring of dissolved oxygen, by connecting the electrometer to a servo recorder (Texas Instruments, Inc.).

III. Free Amino Acid Determinations

Free amino acids in the cells and media of paired low density, exponentially growing and high density stable cultures were determined as follows: At desired time intervals following medium renewal, samples of the suspension cultures corresponding to 4×10^7 cells - found previously to be required for the analysis - were obtained, and the cells separated from the media through centrifugation. Five ml of media were treated with an equal volume of cold 1N PCA and the resulting precipitate washed with 4 ml of 0.5N PCA which was subsequently added to the original supernatant yielding a final 9 ml sample containing the acid soluble material of the original 5 ml of the culture medium. The cell pellet was treated with 5 ml of cold 0.5N PCA and the resulting precipitate washed with 1 ml of .5N PCA which was subsequently added to the original supernatant yielding a final 6 ml sample containing the acid soluble material of the original 4×10^7 cells sample of the culture. The media and cell PCA samples were then neutralized with KOH and

adjusted to the desirable volume with cold H_2O. A two ml aliquot of either a media or a cell sample was then acidified with 5 drops of concentrated HCl, and .0625 μM of nor leucine added to serve as an internal standard. The aliquot was then placed directly on a chromatogrphic column consisting of a mixture of type A and B Chromo-Beads (Technicon Chemical Co., Inc.), both cation exchange resins having an average particle diameter of 16 μ. The separation of the amino acids was carried out in two steps. In the first, the amino acids were absorbed on the column using .2N sodium citrate buffer (pH 3.41), for two hours. Elution was started at this point using a 9 chamber autograd which contained 125 ml of buffer per chamber. Chambers 1 and 2 contained the .2N citrate buffer (pH 3.41) and the other chambers contained .6N sodium citrate buffer (pH 6.30). Following the two hour absorption period the stopcock on chamber 3 was opened and formation of the eluting gradient begun. The system delivered 54 ml/hr and a one percent ninhydrin solution was used for color development.

The intensity of the color was read at 570 nm in a colorimeter (Technicon Chemical Co.) and identification and quantitation of the individual amino acids in the resulting chromatogram was carried out on the basis of a comparison with a similar chromatogram obtained from a mixture containing known amounts of the physiological amino acids plus the norleucine internal standard. The results were expressed in terms of molar concentrations in the media and number of micromoles per 10^7 cells.

IV Analysis of ATP

Cellular ATP was measured by a modification of the luciferin-luciferase method which depends on the measurement of light produced when ATP reacts with the extract of firefly tails.

In order to allow both a rapid and a sensitive measure of the ATP present in the sample, a small light-measuring device was constructed. This device consisted of two concentric cork-boring tubes cemented to a photomultiplier tube, which in turn was connected to a microameter. The inner tube, which held the test tube containing the sample and firefly enzymes, had a piece cut out of one side so that it acted as a shutter when rotated, thus exposing the glow from the sample to the phototube.

Two ml samples from the cultures were used and the ATP was liberated from the cells by the sonic vibrations produced by a Branson Sonifier. The increase in temperature to 82°C which occurred during treatment destroyed released ATPases, thus permitting storage of sonified samples in an icebath for as long as 24 hours without detectable ATP loss.

Most of the cell samples, however, were analyzed immediately after sonification.

The luciferin-luciferase reagents were prepared according to standard procedures. Varying concentrations of stock ATP solutions added to the luciferin-luciferase mixture showed that there was a linear relationship between the amount of ATP added and the amount of light produced 15 seconds after mixing, up to 15 X 10^{-3} micromoles ATP, the maximum total concentration measured in any experiment.

Assay of ATP in the 2-ml sonified samples was performed in triplicate by mixing 0.5 ml of the sample with 0.5 ml of firefly enzymes in a 12 X 17 mm glass test tube, which was then placed in the light-measuring device. The inner tube was sealed with a rubber stopper to prevent extraneous light from interfering with the measurement. The tube was then rotated, opening the shutter. The reading on the ammeter was recorded exactly 15 seconds after the addition of the cell sonicate. The amount of light emitted was then related to the ATP content of the sample using an internal standard. The quantity of ATP measured was expressed on a per cell basis by dividing the total amount of ATP by the number of cells originally present in the 0.5 ml sample of the sonicate.

Results and Discussion

Following the addition of tritiated proline to 2 pairs of low density, exponentially-growing and high density, stable cultures, samples were withdrawn at 2-hour intervals and the relative quantities of labelled proline and hydroxyproline incorporated into extracellular macromolecular material were determined as described previously. The values thus obtained were used to calculate the ratio $\Delta C/\Delta P$, i.e. the rate of collagen synthesis and release relative to the rate of the total, newly synthesized protein released into the medium. The results are shown in Fig. 2 where it can be seen that the $\Delta C/\Delta P$ ratio obtained from actively-growing cultures 3 hours after medium renewal was 1.2 percent, increasing subsequently to 1.5 percent at 5 hours and to 1.9 percent at 7 hours. The values from the high density, stable cultures were 1.6 percent at 3 hours following medium renewal, sharply increasing to 4.3 percent at 7 hours, an almost 3-fold increase. Thus, in both phases of growth, there was a near-linear increase in the $\Delta C/\Delta P$ ratio after medium renewal, this increase being significantly greater in the high density, stable cultures(2).

This is a most important finding especially if considered in conjunction with the contrasting daily medium pO_2 kinetics of low density,

exponentially-growing and high density, stable cultures, shown in detail by means of the continous tracings of medium pO_2 obtained with a galvanic oxygen probe (Fig. 3). In the growing culture, medium pO_2 declined in a somewhat irregular manner during the first half of the incubation period, beginning at 132 mmHg recorded 1-1/2 hours after medium renewal and decreasing to 122 mmHg at 12 hours. The rate of decline increased during the second half, as evidenced by the steeper slope, so that by 23-1/2 hours after renewal of the medium, the pO_2 had dropped to 97 mmHg. The average rate of oxygen tension decrease of the medium during the 22 hour interval was 1.6 mmHg/hr, during which time the density of the culture increased from 4.5 to 8.5 X 10^5 cells/ml.

Starting with an initial value of 82 mmHg recorded one hour after medium renewal, the medium pO_2 of the high density culture rapidly declined to 60 mmHg within 2 hours, followed by a period of maximum decrease of 25 mmHg per hour observed between the 4th and 5th hour. Beyond 5 hours the rate of oxygen decline lessened, with the curve asymptotically approaching the non-detectable level from the 14th hour until the end of the incubation period, some 10 hours later. Cell counts taken at the beginning and end of the incubation period were 6.97 and 6.81 x 10^6 cells per ml, indicating that cell population density remained stable during the experiment.

Assay of the gas phase of the sealed chamber with the paramagnetic gas analyzer revealed that oxygen tensions decreased during incubation, reflecting oxygen utilization by the cells. Initial gas phase pO_2 determinations 30 minutes after the cultures were sealed in the incubation chamber and gassed with 5 percent CO_2 in air, showed that cultures in both phases of growth were exposed to the same oxygen tensions, approximately 142 mmHg. At the completion of the experiment, about 23-1/2 hours post medium renewal, the pO_2 in the incubation chamber had declined to 136.7 mmHg in the case of the low density culture and to 122 mmHg in the high density culture.

This clearly indicates that the reduction of the partial pressure of oxygen in the medium cannot be attributed to its exhaustion but to the fact that the total respiratory demand by the cells exceeds the capacity of oxygen to diffuse across the gas/liquid interface. This becomes most pronounced in the stable populations where cell density is approximately one order of magnitude greater than in exponentially-growing cultures. Although with the type of probe used, medium pO_2 in high density cultures is not measurable for about half of the daily medium renewal cycle, the very fact that this level is maintained indicates that the cells are not anoxic but that they promptly utilize any oxygen made available through diffusion. Tissue pO_2 values obtained with high resolution microelecrodes *in vivo*[4] and in attached cultures *in vitro*(3), are also extremely low suggesting that the oxygen microenvironment of dense suspension cultures is within the physiological range.

In addition to its role in energy metabolism, molecular oxygen has been shown to be necessary for the hydroxylation of proline, with anoxia inhibiting collagen synthesis to a greater extent than general protein synthesis. The importance of the findings shown in Fig. 2 can now be fully grasped because they indicate that even under conditions of decreasing oxygen tension there was a preferential synthesis of hydroxyproline containing proteins, i.e. collagen, released into the media of the high density cultures(4). This clearly indicates, that, paradoxical as this may appear from a purely chemical viewpoint, the metabobited by such cultures are regulated physiologically in essentially the same way as in wound healing, where the early formation of new capillaries and the resulting increased oxygen supply is associated with the proliferation of granulation tissue while their subsequent atrophy and involution results in decreased oxygenation and deposition of collagen fibers.

A first approximation of one of the mechanisms involved in this regulation can be obtained from the free amino acid analysis data shown in Table I. Besides the expected lower final concentrations of certain amino acids in the media of the high density cultures, these data reveal a striking difference in the pathways used by the two types of cultures to metabolize glutamine. Thus, the free cellular amino acid pool of low density cultures at 5 hours after medium renewal is rich in glutamine and even richer in glutamic and aspartic acid suggesting a relatively slow glutamine hydrolysis and transamination with alpha-ketoglutaric acid, a process known to be involved in the oxidative metabolism of the cell. On the other hand, at the same time interval, cells of the high density stable cultures show low concentrations of glutamine, glutamic and aspartic acids and a high concentration of alanine, which they also release in great quantities into the medium. In this case then, glutamine appears to undergo rapid hydrolysis to glutamic acid and subsequent transamination with pyruvate. The resulting alanine is known to represent an intermediate amino group storage compound, pending further transamination and utilization of the slowly reforming glutamic acid either for energy production through oxidative deamination, or, for protein synthesis including collagen, the latter involving conversion of glutamate to proline in a multistep process. Some indication that this is indeed occurring, is provided by the composition of the cellular free amino acid pools 24 hours after media renewal, when both glutamic acid and proline are higher in the high density stable cultures by a factor of 3 while the levels of glutamine and alanine are very similar in high and low density cultures. This interpretation of the amino acid data of Table I will be verified during the coming year by supplying the cells with radioactively-labeled glutamine and following the fate of the label in the two types of cultures as a function of time.

Pending this, we confirmed the possible shift-down of the energy metabolism of the cells, suggested by the low pO_2 and the change of the transamination partner of glutamic acid from alpha-ketoglutaric to pyruvate found in the high density stable cultures. ATP concentration of cells in the exponential phase of growth, sampled two hours after medium renewal and cell dilution, averaged 7.9×10^{-9} μmoles/cell as determined by the luciferin-luciferase method described previously. Fig. 4 shows that during the evolution of a culture toward the high density stable phase ATP values declined from characteristic exponential phase levels to a mean of approximately 3×10^{-9} μmoles/cell or about 35 percent of the ATP measured in growing cells. It may be noted that cellular ATP was higher immediately prior to rapid population growth, i.e. on days 6 and 8 of Fig. 4. The detailed mechanism of this energy shift down and the relationship of the observed low ATP values to the decline of cell division and the changes in amino acid metabolism and collagen synthesis previously described is under current investigation.

Conclusions and Recommendations

The essential sequence of wound healing, i.e. early proliferation of initially sparse fibroblasts leading to a dense stable population synthesizing collagen, was reproduced for the first time in a model suspension culture system. By eliminating physical factors such as cell to cell contact, the system offers unique opportunities for investigating humoral regulation. Thus, changes in microenvironmental oxygen tension and glutamine concentration in dense stable populations were found to be associated with qualitative changes in the metabolic pathways of key amino acids involved both in energy production and collagen synthesis. Significantly, the former was found to decrease in such cultures while the latter increased.

It is recommended that the research effort concerned with these metabolic interactions be intensified and expanded as the humoral type of regulation implied, is the one most easily translatable into clinical control of the course of wound healing in the injured soldier through pharmacological means.

LEGEND TO FIGURES

Fig. 1 Diagram of the incubation chamber and 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 0.45μ pore sterile filter. (G) Gas outlet valve with connections to atmosphere or paramagnetic oxygen analyzer. (H) Removable lid. (I) Thermostat controlling water bath. (J) Movable oxygen electrode permitting calibration in the gas phase and medium pO_2 determinations by immersion into the culture. (K) Valve with attached inflatable balloon.

Fig. 2 Fraction of labelled collagen relative to total labelled protein appearing in the media of low and high density L cell suspension cultures following addition of radioactive proline. As indicated by the arrow, the isotope was added to paired low and high density cultures one hour after medium renewal and samples withdrawn for analysis at 3, 5 and 7 hours. The bar graph shows the mean $\Delta C/\Delta P$ ratios obtained in two experiments from 2 different pairs of high and low density cultures.

Fig. 3 Continuous recording of medium oxygen tension in low density, exponentially growing and high density, stable suspension cultures following daily medium renewal. Cultures were incubated in the chamber shown in Fig. 1. Liquid pO_2 was monitored by recording the output of a sterile calibrated galvanic oxygen probe deeply immersed in the stirred cultures. Figures in parentheses refer to oxygen tensions in the gas phase of the sealed incubation chamber, measured by the paramagnetic gas analyzer at the times indicated and expressed as mmHg.

Fig. 4 Energy metabolism during development of the high density stable phase. The solid line indicates cell counts taken from cultures treated by daily medium renewal without cell dilution, starting with a culture density of 4.3×10^5 cells/ml. ATP samples (dashed line) were taken at T = 0 hours and are expressed as μmoles $\times 10^{-9}$/cell.

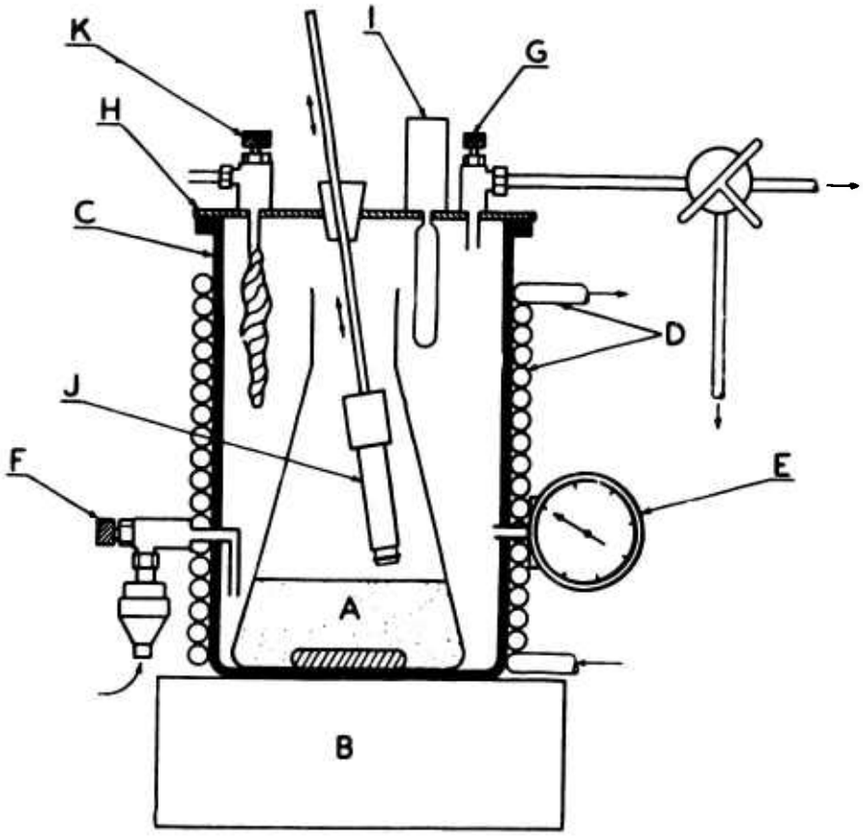

FIGURE 1

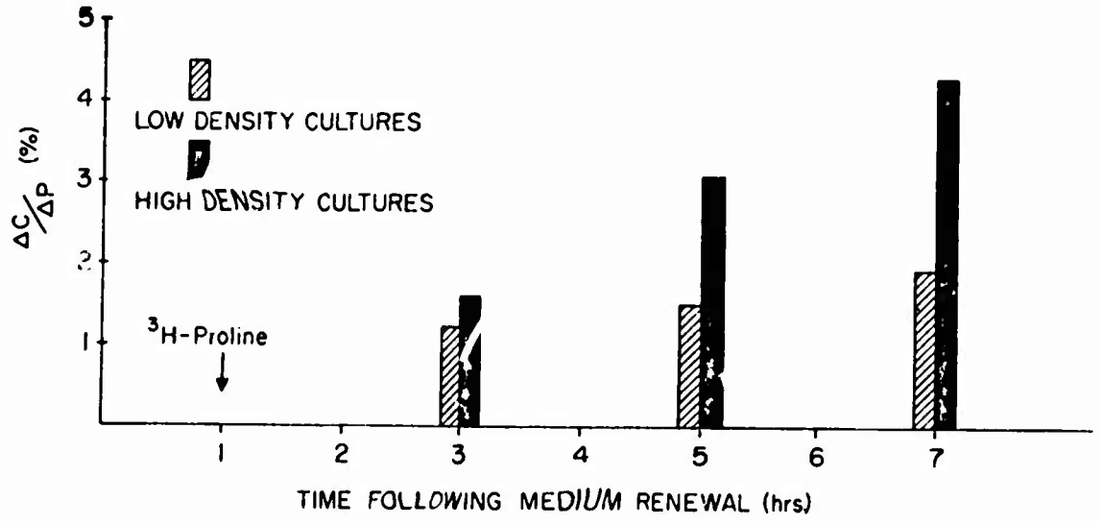

FIGURE 2

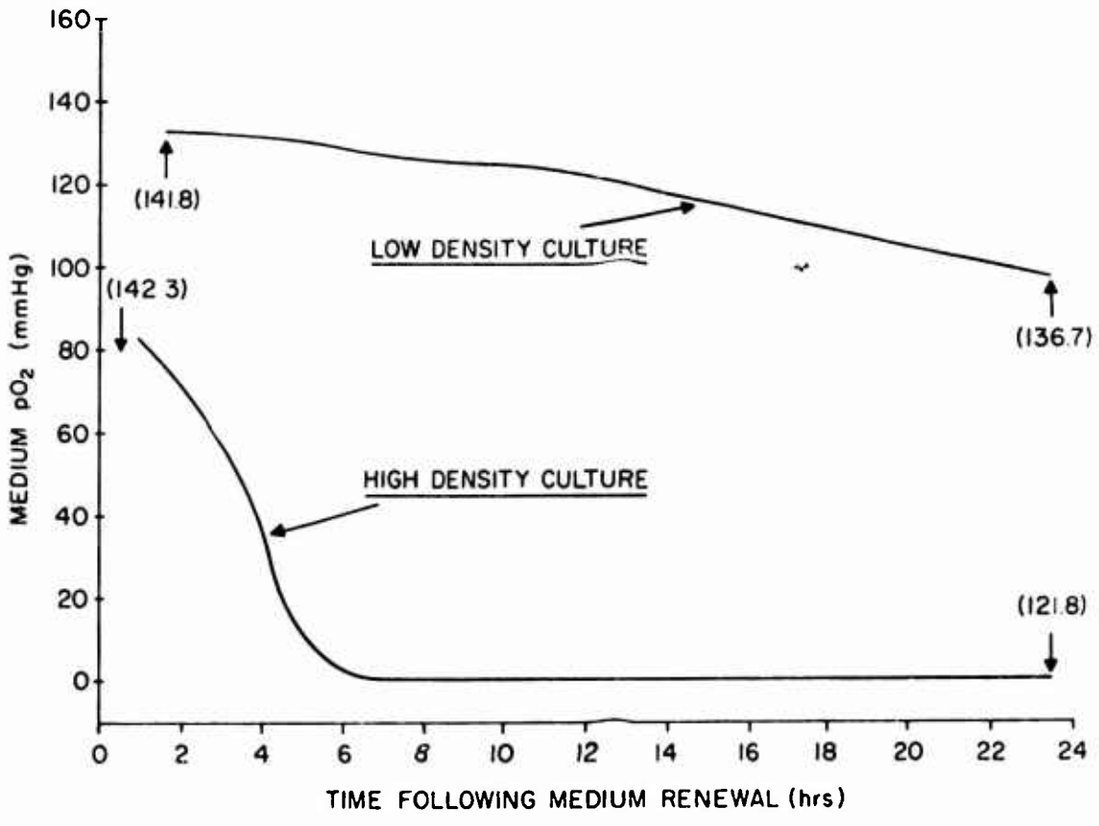

FIGURE 3

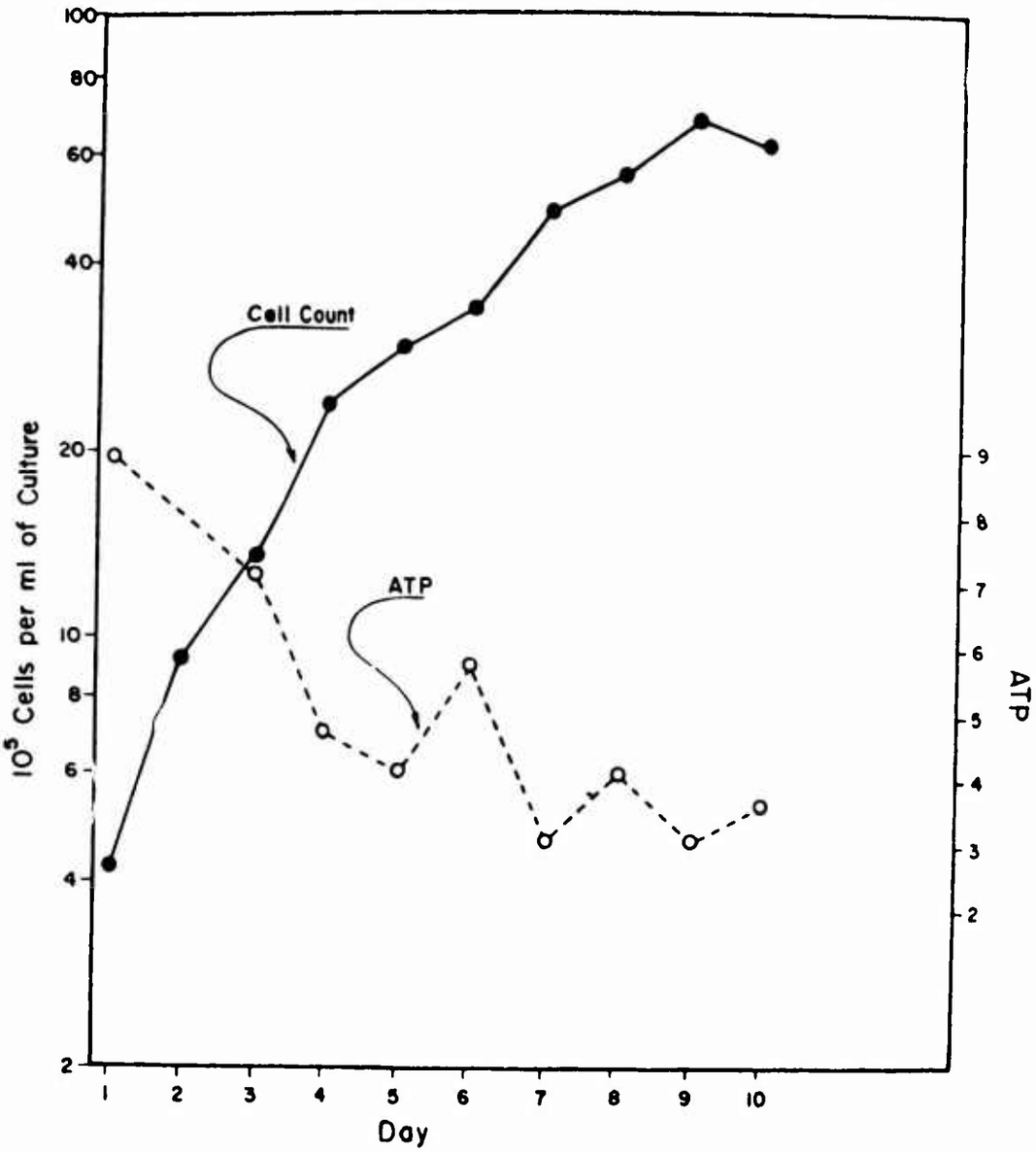
100
80
60
40
20
10
8
6
4
2
Cell Count
ATP
10^5 Cells per ml of Culture
9
8
7
6
5
4
3
2
ATP
1
2
3
4
5
6
7
8
9
10
Day

Figure 4

AMINO ACID	T=0	T=5				T•24			
	mM in unused Media	mM in Media		μM/10^7 Cells		mM in Media		μM/10^7 Cells	
		Exp	Sta	Exp	Sta	Exp	Sta	Exp	Sta
Alanine	.033	.029	.402	.060	.256	.084	.117	.072	.098
Arginine	.507	.485	.391	.021	.019	.408	.283	.037	.073
Aspartic Acid	.010	.014	.015	.264	.051	.045	.019	.009	.021
Cystine	.135	.074	.056	0	.006	.074	.035	-	.011
Glutamic Acid	.067	.043	.084	.393	.094	.456	.167	.026	.072
Glu-NH_2 + Asp-NH_2	1.448	.747	.048	.148	.007	.004	.005	.002	.005
Glycine	.053	.036	.104	.051	.088	.081	.206	.113	.180
Histidine	.151	.139	.124	.025	.016	.110	.084	.038	.032
Isoleucine	.369	.248	.108	.031	.011	.069	.003	.039	.003
Leucine	.334	.244	.081	.032	.013	.064	.006	.035	.007
Lysine	.409	.292	.252	.017	.021	.235	.179	.016	.061
Methionine	.064	.052	.052	.011	.007	.028	.009	.014	.004
Phenylalanine	.171	.137	.122	.022	.015	.105	.079	.039	.030
Proline	.101	.118	.078	.019	.018	.075	.070	.008	.024
Serine	.029	.016	.016	.021	.018	.017	.010	.020	.018
Taurine									
Threonine	.277	.206	.193	.082	.089	.268	.162	.225	.096
Tyrosine	.166	.136	.131	.026	.016	.112	.081	.042	.033
Tryptophan									
Valine	.369	.246	.168	.031	.016	.355	.041	.047	.017

TABLE I

Free amino acid levels in the media and cells of low density exponentially growing cultures (Exp) and stable high density populations (Sta). T represents time after medium renewal in hours. Values discussed in the text at some length are underlined.

Literature Cited.

References:

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[4] Silver, I.A.: Some observations on the cerebral cortex with an ultramicro membrane-covered, oxygen electrode. Med. Electron. Biol. Engng. 3: 377, 1965.

Publications

(1) Glinos, A.D., and Werrlein, R.J.: Density dependent regulation of growth in suspension cultures of L-929 cells. J. Cell. Physiol. 79: 79, 1972.

(2) Bartos, E.M., Taylor, B., and Glinos, A.D.: Amino acid pools and collagen synthesis in high density L cell suspension cultures. Fed. Proc. 31: 647, 1972.

(3) Werrlein, R.J., and Glinos, A.D.: Approaches to the study of the oxygen microenvironment in attached cultures of mammalian cells. In Vitro 7: 266, 1972.

(4) Vail, J.M., Taylor, B., and Glinos, A.D.: Density dependent inhibition of growth in L cell suspension cultures: Regulation of collagen biosynthesis in the presence of rapidly declining oxygen tensions. Am. Soc. Cell Biol. Abstr. 11: 310, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6461	72 06 30	DD-DR&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
71 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	61102A	3A061102B71R	02	091
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114 (F)			

11. TITLE (Precede with Security Classification Code): (U) Response of Cells Derived from Subjects Injured by Combat 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	72 06	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 71	5	125
	CURRENT 72	5	125

19a. RESPONSIBLE DOD ORGANIZATION
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TELEPHONE: 202-576-3551

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Johnson, COL M.
NAME: Miller, MAJ J. DI

PII Redacted

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code)
(U)Military Operations; (U)Wound healing; (U)Cytogenetics; (U)Radiobiology; (U)Cellular hypersensitivity; (U)Host response; (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 reactions of cultured white blood cells (lymphocytes) as they pertain to antigenic, infectious, radiobiological, drug, biochemical and combat injuries encountered in military operations.

24. (U) Cytogenetic, histologic, 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 nonexperimental variables are well-controlled, is used to test hypotheses concerning the nature and therapeutic modification of whole body responses.

25. (U) 71 07 - 72 06 Thirty-four antimalarial drugs were tested with dilution studies in a lymphocyte culture system. Two drugs from the Walter Reed program were found to inhibit lymphocyte growth to a greater extent than equimolar concentrations of chloroquine. Inhibition of growth of these drugs was approximately equal to that achieved using six mercaptopurine in equimolar concentration. Use of these antimalarials may be associated with reduction of cellular immunization. These studies have been terminated because of the reassignment of all investigators.

* 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 3A061102B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 03
Psychiatry

543-CL

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6454	72 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
71 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	30A61102B71R	03	025
b. CONTRIBUTING				
c. CONTRIBUTING	C00G 114(f)			

11. TITLE (Precede with Security Classification Code): (U) Analysis and Management of Behavior and Stress in Military Environments (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 013400 Psychology 012600 Pharmacology 012900 Physiology 016200 Stress Physiology

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 72	5.0	220
FISCAL YEAR CURRENT 73	6.0	250

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
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NAME: Manning, F.J. CPT DA

PII Redacted

21. GENERAL USE: Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Operant Behavior; (U) Military Psychiatry; (U) Drug Abuse; (U) Motivation; (U) Conditioning; (U) Performance Decrement (U) Reinforcement

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

23. (U) Complex behavioral models are developed simulating conditions likely to lead to ineffective performance or psychiatric decompensation in the military environment. The interaction of physiological and psychological variables relevant to the individual environment interaction are studied (e.g. the behavioral toxicity of therapeutic and non-therapeutic drugs, the effects of stress and fatigue upon alertness and performance, the interaction of autonomic and environmental variables, and updating of operant technology as it applies to the modification of behavior).

24. (U) Techniques of experimental psychology, especially operant conditioning, combined with those of endocrinology, pharmacology, physiology, and anatomy are used to define variables that maintain and control both adaptive and dysfunctional behavior.

25. (U) 71 07-72 06 Animal studies of acute behavioral toxicity of marihuana indicated reductions in work output, decrements in timing behavior, decreased food intake, and decreased aggression. Chronic administration studies indicate weight loss and attenuation of growth and disruptions in performance attendant to termination of drug administration. Protocols and facilities have been developed and pilot studies are underway to examine conditions under which heroin self-administration can be established, maintained and eliminated. Studies of effects of environmental stimuli and stressors superimposed on on-going operant performances indicate that cardiovascular responses to stress differ as a function of on-going behavior. Neuroanatomical substrates of complex behavioral repertoires are being isolated to dissect variables that underlie behavioral dysfunction. Development of behavioral methodology has focused on techniques for the laboratory study of drug abuse. A study of behavioral effects of transmeridian air deployment is continuing. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

(Available to contractors upon completion of research)

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 03 Psychiatry

Work Unit 025 Analysis and management of behavior and stress

Investigators
Principal: CPT Frank J. Sodetz, MSC
Associate: James C. Bonbright, Jr., M.A.; Donald G. Conrad, M.A. Timothy F. Elsmore, Ph.D.; CPT Frederick J. Manning, MSC; MAJ Benjamin H. Natelson, MC.

DESCRIPTION.

Research during the reporting period can be organized into four major areas of concentration: (1) The development and application of laboratory models of stress and psychosomatic disease; (2) The experimental analysis of behavior; (3) The objective laboratory analysis of the variables that underly the prevention, establishment, maintenance, and elimination of drug abuse; (4) Evaluation of the behavioral toxicity of non-therapeutic drug use with objective laboratory models. However, it should be pointed out that the distinction among these research areas is organizational in that all of the areas overlap considerably with advances in any one area likely to stem from work in any of the others.

LABORATORY MODELS OF STRESS AND PSYCHOSOMATIC DISEASE.

Systematic environmental perturbations have been shown to produce disruptions of on-going behavior as well as changes in patterns of visceral function. With repeated exposure to environmental perturbations, these disruptions of both behavior and physiology can be elicited by more subtle components of the original eliciting events and may represent the kinds of changes which take place early in the etiology of pathological responses to stress as well as psychosomatic disorders. The military environment, of necessity, presents the organism with the kinds of environmental contingencies which can lead to the relatively permanent behavioral and visceral changes representative of those involved in psychiatric decompensation and psychosomatic disorders. The studies reported in this section are directed at identifying classes of relevant variables and developing treatment models that can capitalize on the same behavioral principles that underly the disorder.

Analysis of the behavioral variables contributing to the development of gastro-intestinal ulcers in the rhesus monkeys. It has been demonstrated that exposure to behavioral stress can result in the development of gastro-intestinal ulcers in the rhesus monkeys. To date little work has been directed at identifying the specific behavioral variables that contributed to this finding. Preparations have been made to undertake a study designed to expose monkeys to behavioral stress in such a way as to permit identification of the procedural variables requisite to the production of gastro-intestinal

pathology in the monkey. The purpose of this study will be to provide a useful model for examining the relationship of behavior to psychosomatic disease.

Patterns of hormone secretion and behavioral stress. Previous studies have shown that exposure to behavioral stress is accompanied by complex changes in the patterns of endocrine function. To date much of this research remains observational in nature in that both changes in performance and changes in endocrine patterns have been measured. The purpose of the present studies is to extend earlier work by experimentally manipulating both behavior and patterns of endocrine function by examining the interaction of both classes of variables with each other. The basic paradigm to be used in these studies is the Sidman avoidance procedure. Initial behavioral manipulation will involve the addition of free shock to a Sidman avoidance schedule. Addition of free shock to a Sidman avoidance schedule usually results in a substantial increase in overall response rate. Blood samples collected at frequent intervals during Sidman avoidance performance will permit an assessment to the impact of the introduction of free shock on endocrinological function. The impact of altered patterns of hormone secretion on behavior will be studied by using response rate during extinction of avoidance behavior as the dependent measure, and intravenous infusion of exogenous ACTH, as the independent variable. The preliminary data collected to date suggests that admnistration of exogenous ACTH results in an increase in response rate during extinction.

Omission of reward as to a frustrative stress. Laboratory models of stress have produced advances in understanding neuronal and hormonal mechanisms at work in the individual under stress. Most models have employed such potent stressors as fatigue, cold, heat, or electric shock. While none of these are without psychological consequences, other more psychological forms of stress, have seldom been employed with a view toward characterizing their physiological consequences for the organism. One such stressor, frustration, operationally defined as the omission of an expected reward, is being studied. The model being used requires that subjects be trained on schedules of reinforcement which lead the organism to behaviorally anticipate the delivery of reward and then to omit the expected reward. The lesion method has been used to document differences in neuronal systems mediating such responses; however, it has been demonstrated that this more psychological stress is not handled by the central nervous system in the same way as the more physiological stressors. These data appear to suggest that therapeutic measures designed for coping with stress-related behavioral phenomena may not be universally effective for all forms of stress. It may be necessary to consider the nature of the stressor as well as the overt symptoms of stress-related behavior disorders before prescribing courses of treatment involving drugs wtih CNS effects.

Effects of drug administration on the response to frustrative stress. A number of theories have been proposed to account for the increased rate of response observed following the omission of an expected reward. One of the most widely accepted interpretations proposes that motivational variables underly the enhanced responding seen following non-reward. For this reason several drugs generally believed to have effects on motivational variables were administered to rats being run in the frustrative stress procedure. Delta-9-tetrahydrocannabinol, ethanol and chlordiazepoxide in a wide range of doses all failed to produce consistent changes in the frustration effect even at doses which produced marked changes in overall rate of responding. The traditional motivational interpretation of enhanced responding following non-reward would appear to predict substantial effects from these drugs as would traditional motivational explanations of drug effects. These data suggest that either a motivational interpretation of the frustration effect or of the effect of these drugs or both may be inappropriate.

EXPERIMENTAL ANALYSIS OF BEHAVIOR.

Studies to be described in this section are in progress in order to obtain data or to improve technology required in other aspects of the overall research program. Therefore, these studies are related to studies described in other sections of this report.

Circadian variations in the discrimination of response force in rats and monkeys. When considering the problem of drug abuse it is necessary to address questions related to the disruption of biorhythmicities in both physiologic and behavioral function whether these stem from the effects of the drug itself or from the somewhat atypical lifestyle of the drug user. To address the question of the disruption of biorhymicities in performance in the laboratory it is necessary to identify behaviors which show Circadian variations. Several studies are under way in an attempt to develop baselines suitable to the purpose of examining drug related shifts in Circadian patterns of performance. One such baseline involves the precise discrimination of response force. The value of such a measure, should it prove feasible, would be the relative simplicity of the response itself. In many previous attempts to establish baselines sensitive to Circadian variations it has been necessary to develop extremely complex repertoires. In the two studies presently on-going rats and monkeys are being trained to exert force on transducers in order to obtain food reward. By a method of successive approximations specific force requirements can be placed upon the animal. Independent variables to be examined are schedule of reinforcement, reinforced band size, that is, the precision required of the response and dependent variables in initial studies will be accuracy, differentiation, sequential effects and tremor. When initial work is complete the response-force paradigm should provide a relatively simple method of collecting data on an essentially continuous response over long periods

of time and the effects of drugs upon this performance will be measured.

Circadian variations in conventional schedule performance. Because no data are presently available on Circadian variations in performance maintained under different schedules of reinforcement and because studies of drug self-administration discussed under the section on drug abuse will involve schedules of reinforcement in effect over 24 hour periods, data are being collected which will permit us to anticipate fluctuations in performance as a result of Circadian variation in schedule controlled behavior. Completion of these studies is essential to the drug self-adminsitration research outlined below.

Circadian variation in performance on a four component multiple FR schedule. In this study monkeys were trained to perform around the clock on a schedule in which one of four different fixed-ratio values produced a single food pellet. Circadian rhythms were evident in the performance of these animals with the larger effect being on the higher fixed-ratios. When supplemental feedings were given at the same time each day Circadian effects were accentuated with the minimum work output occurring several hours after feeding rather than immediately following the feeding as might have been anticipated.

Patterns of food and water intake in primates. Because a number of studies have shown weight loss and decreased food intake in association with the use of a number of drugs, and because weight loss has been observed in heroin abusers, it will be necessary to address questions related to changes in food and water intake patterns in our studies of drug abuse. Feeding and drinking patterns have been fairly well documented for the rat, but our studies, of necessity, are being conducted in the primate, for which little objective data is available. Feeding data is being collected from a photocell operated device which replenishes food pellets each time one is consumed by the animal and a drinking tube which allows continual flow of water as long as the animal's mouth is in contact with the the tube. Data related to the distribution of food pellet and water consumption is being collected on paper tape for later analysis by a computer. These data should provide information about the normal pattern of food and water consumption in the primate and a baseline against which to measure any drug related changes.

Response to frustrative stress in monkeys differing in social status. During the reporting period it has been possible to obtain monkeys of both high and low social status measured in terms of their position in a dominance heirarchy. Using fixed-interval schedule of reinforcement and omission of an expected reward as a frustration procedure it has been possible to demonstrate that high status monkeys show a larger effect than similarly experienced low status animals. These preliminary data suggest the possiblity

that social status plays a significant role in the occurrence and extent of the response to frustrative stress. Although the present study is correlational in nature, if these preliminary studies are supported by further data, alterations of status can be achieved by changes in group composition or by endocrine manipulations and causal relationships elucidated.

Schedule induced polydypsia in the monkey. In the course of a study in the relationship of social status to frustrative non-reward the animals were permitted free access to drinking water. It was noted that several of the animals began to drink excessive quantities of water during the 90 minute experimental sessions. In nearly all cases these have been the lower status or submissive animals. In addition the introduction of frustration trials appeared to be a significant factor in differentiating the drinking patterns of high and low status animals. Excessive consumption of water as a result of exposure to certain schedules of reinforcement has been called schedule-induced polydypsia and the phenomenon has been implicated in the production of alcoholism in laboratory animals. The relationship of this phenomenon to the establishment of drug self-administration will be examined in our studies of drug abuse. However, what is unique about the present study is the finding of an apparent relationship between social status and the tendency to emit this kind of behavior. The study is being continued both because of a possible link between social status and the occurrence of schedule-induced behavior and because this kind of behavior has been implicated in at least one form of drug abuse.

Improvement without practice in Sidman avoidance performance. Sidman avoidance schedules have become a relatively standard procedure for examining the effects of behavioral stress on neuroendocrine and other physiologic systems. Although a relatively standard tool the variables that maintain Sidman avoidance performance are not as well understood as they are for other avoidance procedures. One recent and heretofore unreported observation was an improvement in performance in a series of rats following a 21 day period during which no avoidance sessions were run. As yet these data fit none of the standard interpretations for improved performance following an extended period with no practice.

Proprioceptive information processing after limited frontal lobe lesions. Work during the last decade has revealed a far sharper localization of function within the cerebral cortex than previously assumed. Several workers have proposed that the area of the prefrontal cortex around the principle sulcus is crucially involved in behaviors guided solely by interoceptive cues. This assessment has been analyzed using two different types of interoceptive discriminations. One experiment analyzed performance on two tasks commonly believed to assess timing ability which presumably depends upon some unknown internal stimulus. Monkeys with subpial aspiration of either dorso-

lateral or orbitofrontal cortex were compared with each other and with intact controls on both fixed-interval and DRL performances. Monkeys with dorsolateral lesions were similar to controls in all phases of the experiment, but those with orbital lesions showed only minimal timing under the FI schedule. However, they were unimpaired in both acquisition and terminal performance under the DRL contingency, suggesting that the two tasks do not tap a common ability and that the perseverative deficits known to follow orbital frontal lesions may be closely related to the consequences of such perseveration. A second experiment utilized a different type of interoceptive stimulus. Monkeys were trained to make a simple choice response following an extended series of lever presses (FR). One choice was reinforced only after a series of 64 lever presses while the other choice was reinforced only after fewer than 64 presses. Typical psychophysical functions were then generated by systematically changing the size of the shorter FR. Normal monkeys showed highly reliable thresholds when tested in this manner. Large dorsolateral frontal ablations produced a severe disruption in performance in even the simplest discrimination, that is FR 10 from FR 64, but recovery was seen after extensive training and post-operative thresholds were similar to pre-operative ones. The frontal animals' difficulty, in this interoceptive discrimination at least, seems to be a learning retardation rather than a sensory loss. This retardation may be in the form of a memory defect. Since introduction of even a small delay between the end of the lever press series and the onset of the choice trial produces a severe impairment for the frontal animals control studies are now under way to insure that the critical feature in producing the retardation is the interoceptive nature of the discrimination.

Limbic system and responses to non-reward. In two studies the effects of limbic system lesions on responses to non-reward are being examined. The first study is a study of behavioral contrast. In a behavioral contrast design, an animal is run on a multiple VI 30 VI 30 schedule until performance is stable. A multiple schedule is one in which components of the schedule are in effect only in the presence of a specific exteroceptive stimulus. In such a study an animal might be run on a VI 30 for 10 minutes in the presence of a red light and a VI 30 for 10 minutes in the presence of a green light. The color of the light would be changed according to a predetermined sequence for schedule component presentation. In a behavioral contrast procedure one component of the schedule is changed to extinction, thus, a light which had previously signaled delivery of food reward on a VI 30 schedule comes to signal the absence of reward or non-reward. The contrast effect is seen in a change in performance from the other component of the multiple schedule. It is the equivalent of the frustration non-reward procedure described in other studies. To date only normal animals have been run in this procedure and a substantial increase in response rate has been recorded during the first VI 30 component when extinction replaces the second component of the schedule.

ANALYSIS OF THE CONTINGENCIES UNDERLYING PREVENTION, ESTABLISHMENT, MAINTENANCE AND ELIMINATION OF SELF-ADMINISTRATION OF DRUGS IN LABORATORY PRIMATES.

During the reporting period a protocol was developed which outlined an approach to the objective behavioral analysis of the problem of drug abuse. The purpose of the research as outlined was to identify those variables which contribute to the establishment and maintenance of drug self-administration behavior and, given identification of such variables, to use that information to begin a systematic analysis directed at the problem of prevention and elimination of drug self-administration behavior. Within the reporting period clearance and approval of the protocol was obtained from all agencies and at all levels as required. In January actions were initiated requesting remodeling of existing laboratory space to facilitate the implementation of the proposed research. It is anticipated that remodeling will be completed during FY 73. Actions were initiated to hire new personnel qualified to provide the professional and technical support necessary to undertake the proposed research. It is anticipated that these actions will be completed during FY 73. In the meantime studies of the behavioral toxicity of non-therapeutic drug use have been continuing.

THE BEHAVIORAL TOXICITY OF NON-THERAPEUTIC DRUG USE.

During the reporting period studies directed at the behavioral toxicity of synthetic marihuana (delta-9-tetrahydrocannabinol or THC) have been continuing. The studies reported below represent a continuation and extention of work reported previously in the Annual Report for FY 71 and a summary of previously completed studies can be obtained from that document. In addition, studies have been undertaken addressing selected questions related to the behavioral toxicity of heroin. The purpose of these studies is to provide data required in conjunction with other studies directed at the variables underlying prevention, establishment, maintenance and elimination of drug self-administraton behavior.

Effect of chronic administration of THC on work rate, accuracy of performance and timing behavior in chimpanzees. Three chimpanzees were trained on a task that required precise timing behavior as well as sustained performance for nearly 16 hours each day in order to obtain their daily ration of food. THC was administered orally on each of 35 successive days. At the present time the data indicate that doses of THC comparable to those reported effective in humans resulted in an overestimation of time and a reduction in work output. Gradual recovery from this initial deficit occurred during the first 14 days of THC administration. Throughout the remainder of the chronic administration period, behavior was essentially normal. When the drug was no longer administered a disruption in performance was seen which lasted approximately 14 days and was gradually eliminated with successive post-drug sessions. Thus, with chronic administration of delta-9-THC a

deficit in performance is observed which is gradually eliminated as the organism continues to perform in a manner consistent with the schedule of reinforcement. When drug is no longer administered another disruption in performance occurs which is also eliminated with continued exposure to the schedule of reinforcement. The transience of both of these disruptions in performance suggests the notion of behavioral tolerance to THC may merely reflect the continued operation of the schedule of reinforcement used to maintain performance.

Effects of THC on temporal and auditory discrimination performance in the monkey. In two experiments monkeys were trained to respond differentially to either the duration of a visual stimulus or to the frequency of an auditory clicker. In the duration discrimination experiment THC was administered acutely in doses from 1 to 16 mg/kg producing dose-related decrements in accuracy of the discrimination performance and a reduction in the rate at which animals initiated discrimination trials. In both the duration and auditory discrimination experiments chronic daily administrations of 2 to 16 mg/kg produced an initial decrement in accuracy and rate of trial initiation. Both measures showed some development of tolerance tending to return to baseline levels. However, the time course of tolerance was different for the measures with the rate of trial initiation recovering more quickly than accuracy. No differences in response to the drug were attributable to the different modalities used in these two experiments. Differences in the rate in which accuracy and total work output recovered from the disruptive effects of delta-9-THC are further evidence that the concept of tolerance to the effects of the drug might better be replaced by an operationally based description of behavioral changes in terms of the contingencies controlling behavior.

Conditioned suppression following administration of THC in the monkey. A conditioned suppression procedure widely accepted as a laboratory model for the study of the effects of psychoactive compounds on fear is being used to assess the similarities in the effects of THC and other compounds such as amphetamine and tranquilizers. Initial data suggests increased suppression of responding during the fear eliciting stimulus. In this regard, the effects of THC differ from those produced by tranquilizers which tend to eliminate suppression of responding during the conditioned stimulus.

THC as a discriminatory cue in the monkey. The dose levels of THC required to produce behavioral effects in animals tend to be relatively high compared to those used by humans. This raises a question as to the generality of results obtained in animal studies. The present study was designed to answer the question of what minimal dose could be discriminated from a placebo. When a monkey is working for food and a stimulus is presented indicating that the animal is about to receive an electric shock there is an abrupt disruption of ongoing performance. This procedure is known as conditioned suppression. In the present study placebo as well as varying doses of THC are

administered intravenously via chronic venous catheters to monkeys. Placebos never signal that shock is to be presented but an administration of THC is always followed two minutes later by electric shock. If an animal can discriminate the difference between a placebo and a given dose of THC then it can reliably anticipate the presentation of shock and the characteristic anticipatory disruption of on-going behavior can be observed. To date no reliable data has been obtained from this study. Technical difficulties related to the intravenous administration of THC and to direct effects of THC on the baseline performance against which conditioned suppression is measured continue to hamper progress in this important study. A similar study employing heroin as the conditioned stimulus will be initiated in the near future. This study will provide data as the minimal discriminable dose of intravenously administered heroin and will serve as an essential adjunct to our studies related to the self-administration of heroin in primates.

Conditioned taste-aversion in rats following THC administration. In an earlier study it was found that rats would show an aversion to saccharine, normally a preferred substance, if exposure to saccharine was followed by THC administration. Aversion to normally preferred novel substances is seen in rats whenever an illness inducing agent is administered following exposure to the substance. The THC data suggests that at least upon initial administration THC has aversive properties for the rat. The present study was designed to follow up the initial observation of aversion to saccharine following THC administration. In the present study it was still possible to obtain aversion to saccharine after as many as 8 doses of THC had been administered to the animal before exposure to saccharine suggesting that the novelty of the drug effect is not critical in producing the aversion.

Conditioned taste-aversion in the rat following heroin administration. In an attempt to extend the THC data to other drugs with abuse potential ethanol, methyamphetamine, pentabarbitol, and heroin were all tested using the saccharine aversion paradigm. All of the drugs except heroin produced an aversion to saccharine. Heroin, even at a dose of 32 mg/kg administered intraperitoneally, failed to produce a significant aversion to saccharine. These heroin data are an essential adjunct to our studies of heroin abuse.

THC administration and delayed matching-to-sample in the monkey. One of the consistently reported effects of THC administration in man is a decrement in recent memory. Delayed matching-to-sample procedure is an objective laboratory model of recent memory. For such a procedure a stimulus is presented briefly to an animal and after a specified period of delay a series of three or more stimuli are simultaneously presented to the animal. The animal's task, in order to obtain food reward, is to select from the group of stimuli the one identical to the original stimulus. Thus, the stimulus is presented, a delay period follows during which the stimulus is absent, and then an array of stimuli is presented, and the animal's task is to select from

the array the stimulus which matches the original stimulus. No data as yet are available from this experiment. However, data from other laboratories indicate a persistent decrement in accuracy will be obtained in this procedure.

Effects of heroin self-administration in feeding and drinking patterns in the monkey and baboon. Because our studies of the variables which contribute to the establishment, maintenance and elimination of heroin self-administration will rely on the maintenance of operant performance using food or water reward, it is important to identify any effect of the drug on normal feeding and drinking patterns. For this reason animals are being prepared with in-dwelling venous catheters and will be permitted to self-administer heroin by pressing a lever. Initial studies will investigate the effects of heroin self-administration on overall food and water intake and will also permit acquisition of data on the relationship of different response requirements to the frequency of drug self-administration.

Effects of THC on predatory aggression in rats. In a previously reported study no effect of delta-9-THC on shock elicited aggression was observed following acute administration of the drug. In the present study which evaluated predatory rather than shock elicited aggression, it was found that THC decreased the frequency of aggressive behavior. The discrepency between this finding and the previously reported finding is not surprising in light of evidence that operationally distinct kinds of aggression exist and that these operationally distinct kinds of aggression may have differing neuronal substrates.

Effects of THC administration on operant responding maintained by nutritive and non-nutritive reinforcers. In a previous study decreased food intake attendant to THC administration has been demonstrated. Another frequently observed effect of THC is a decrease in overall response rate when responding is maintained by food reward. It is possible that these two observations, decreased food intake and a decrease in the rate of responding for food reward, are linked and that decrements in performance maintained by food reward are but a reflection of the decrease in the efficacy of food as a reinforcer. One way in which this hypothesis was tested is to maintain responding with both nutritive and non-nutritive reinforcers and to examine the effects of THC on both. In the present study, rats were trained to stability on a multiple FI FI schedule of reinforcement in which one component ended with food pellet reinforcement and the other with saccharine solution reinforcements. THC produced dose-related decreases in both rate of responding and index of curvature in both the food and saccharine components. Effects were more marked on the saccharine component. Pre-feeding produced similar decreases in response rate but these were more marked than the food component. The results of this experiment indicate that previously reported rate-depressing effects of THC for a variety of food reinforced behaviors are not entirely secondary to the drugs hypophagic effects.

Effects of THC administration on DRL performance in the monkey. A recently completed experiment on the effects of THC on timing behavior in the chimpanzee indicated a decrement in timing behavior at dose levels comparable to those used in man and significantly lower than those used in most studies of animal behavior. Given such a finding the possiblity existed that either the chimpanzee is a subject more sensitive to the pharmacologic effects of THC or that the task employed in this study was one that was particularly sensitive to THC. For that reason certain features of the study have been replicated in the rhesus monkey. The rhesus monkeys have been required to work on a schedule of reinforcement that requires responses to be spaced at least 60 seconds apart in order to obtain food reward. Data collected to date indicate a drug related increase in the frequency of short interresponse times at doses equivalent to those effective in the chimp study. The tentative conclusion can be drawn that the effects observed in the chimp study were the result of the sensitivity of the particular task used rather than any difference in the pharmacological sensitivity to the drug.

Effects of THC on food intake and body weight in the rat. The present experiment centering on food intake depression focuses on the failure of rats chronically administered THC to show expected compensatory surge in food intake and body weight when drug administration was terminated. The initial study included no isocaloric controls so it is being replicated in a yoked-control design which will allow positive verification of this effect. Dietary and CNS manipulations as well as measurement of O_2 consumption would appear to be likely routes to follow if the present study confirms previously reported observations.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 03 Psychiatry

Work Unit 025 Analysis and management of behavior and stress

Literature Cited.

Publications:

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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 OA 6430	72 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. GRP'S INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
71 07 01	B. 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. CONTRIBUTING	CDOG 114(f)			

11. TITLE (Provide 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	72 06	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 72	13	195
FISCAL YEAR CURRENT 72	13	195

19. RESPONSIBLE DOD ORGANIZATION

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20. GENERAL USE

Foreign Intelligence Not Considered

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21. KEYWORDS (Provide Each with Security Classification Code) (U) Gene Isolation; (U) Enzymes; (U) Protein Synthesis; (U) Receptor Sites; (U) Genetic Interrelationships

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

23. (U) The technical objective of this work unit is to provide a program of differentiating the response between the host and infecting organism of those diseases of military medical importance.

24. (U) Macromolecular separations, purification, characterization and structural determination of macromolecules by physico-chemical methods and their effects on biological functions are being determined. Isotopic precursor incorporation and analysis of isotopes will be used to study host-infecting agent interrelationships. Studies on the receptor sites of metabolic antagonists and drugs will be pursued and the modification of chemical functional groups of these compounds will be attempted to selectively alleviate its effects of biological function.

25. (U) 71 07 - 72 06 All the major Enterobacteriaceae groups have been studied for their nucleic acid relatedness to strains of E. coli. Several genera have been studied in detail, including Klebsiella, Enterobacter, Shigella, Serratia and Citrobacter. Pathogenic and nonpathogenic strains of an individual genera are grouped according to their nucleic acid sequence relatedness on the basis that pathogens exhibit less divergence than the nonpathogens. A reference system for identification of enteric bacteria in the clinical laboratory is being developed. The in vitro transcription of purified tRNA and 5s RNA genes to obtain the precursor molecules is being investigated. The effect of immunological response on biochemical processes during and after organ transplantation is being studied. Project 3A061102B71P 01 070 will be consolidated with 3A061102B71P 01 075 under the work unit title Metabolic Problems and Biochemical Variations Associated with Disease and Injury. For technical reports, see the Walter Reed Army Institute of Research Annual Progress Report, 1 July 1971 - 30 June 1972.

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 variations during disease and treatment

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Description.

The technical objective of this work unit is to examine the response of the host and the infecting organism in disease of military medical importance at the cellular level. Employing technology such as chromatography, electrophoresis, ultracentrifugation and electron spin resonance, the following areas have been examined during the reporting period:

1. Cellular protein synthesis.

2. Mechanism of replication, transcription, translation and transfer of genetic information with respect to the role of nucleic acids.

3. Homology and divergence of nucleotide sequences among various species of bacterial DNA along with its correlation to pathogenicity.

4. In Vitro isolation and characterization of structural and functional genes.

5. Interaction of small molecules and ions with biological membranes.

Progress.

The areas of work accomplished during the reporting period can best be collected and summarized under the following headings:

1. Methodological advances in separation of low molecular weight ribonucleic acids and RNA-DNA hybrids from DNA on hydroxyapatite.

Previously we reported studies instrumental in developing a "DNA" grade of hydroxyapatite. This new product is ideal for separating single and double-stranded DNA. Continuing studies have concentrated on eliminating batch variation of hydroxyapatite with respect to quantitative adsorption of low molecular weight RNA and RNA-DNA hybrids. The problem is to adsorb these molecules under conditions where single-stranded DNA is not bound to hydroxyapatite. Thus far 80% or more of the RNA or hybrid and less than 5% single-stranded DNA is bound by adjusting sodium dodecyl sulfate and salt concentration and preheating the hydroxyapatite.

The first cycles of gene isolation in large scale preparations have required several large, time consuming hydroxyapatite columns. We have now switched to a batch procedure that allows the initial cycles to be carried out in 2-3 hrs, instead of 3-4 days. In addition to saving time, this rapid modification results in some 3-4 days less decay of radioactive phosphorous; thus increasing the specific activity of the final gene product by 20-25%.

The importance of genome size in interpreting interspecies DNA relatedness was determined in our laboratory using reciprocal reactions between organisms and in DeLey's laboratory in Belgium using an optical reassociation assay. Genome size must be identical or nearly identical in order to obtain reciprocal relatedness values between strains. In association with Dr. Falkow's laboratory at Georgetown University, we have confirmed DeLey's optical genome size determinations and also confirmed the use of optical reassociation to assess relatedness between organisms. Extensive genome size determinations have been carried out on members of Escherichieae; Salmonelleae and Klebsielleae. Optical relatedness assays have been used as a screening technique to assess general relatedness between members of the above groups. This technique is not nearly as sensitive or reproducible as the hydroxyapatite method and one cannot assess divergence within related sequences. Nonetheless, the method is useful in selecting strains of interest for more detail and investigation.

2. Nucleic acid relationship studies.

Using interspecies DNA reassociation followed by thermal chromatography on hydroxyapatite, we have assessed genetic, evolutionary, molecular or DNA relatedness among a substantial number of enteric bacteria. The methodology employed allows the quantification of both closely and distantly related nucleotide sequences between bacterial species. A determination of stability in duplexes formed between species relative to stability of duplexes formed by DNA from one reference species enables one to assay the amount of unpaired nucleotide bases (divergence) present in DNA held in common between organisms.

Using these methods and a rapid batch procedure developed in this laboratory, relatedness has been assessed between all major groups of enteric bacteria. Progress is outlined in the following sections.

a. Studies on Escherichia coli and Shigella strains.

DNA relatedness data from more than 100 strains of E. coli, Alkalescens-Dispar group and Shigella species confirm earlier indications that these organisms comprise a highly related group of enterobacteria. The relationships between these organisms have been used to formulate a molecular definition of a species. This definition includes variation in molecular weight of DNA (genome size), percent guanine + cytosine content in DNA and frequency distribution of DNA relatedness between strains of a given species. Using these parameters, the species of E. coli contains organisms with genome sizes between 2×10^9 and 3×10^9 Daltons, with a guanine + cytosine content between 48.5% and 52% and exhibiting a Gaussian distribution of interstrain relatedness with the mode of 90%. Relatedness values range between 70% and 100% and related sequences rarely contain more than 3% unpaired bases. The Alkalescens-Dispar group clearly belongs to the species E. coli.

Frits-Ørskov (Statens Seruminstitut, Denmark) has tested O and K antigen mobility patterns using electrophoresis. On this basis pathogenic E. coli strains show three patterns of electrophoresis antigen mobility that coincide with the type of disease they cause: infantile diarrheae, disentery-like disease and enteric fever. In collaboration with Dr. Ørskov we have tested a number of his strains and our own strains for gross DNA relatedness. Results indicated that at least two of these pathogenic groups were preferentially related. The data show 80% overall intergroup relatedness between all strains

and 90% intragroup relatedness in two of the groups. These data are an aid in distiguishing pathogenic E. coli from normal resident flora and provide additional insight into the molecular basis of pathogenicity.

Shigella species cluster at the low relatedness end of the E. coli frequency distribution. Studies on Shigella relatedness were carried out partly in collaboration with Dr. Sam Formal, DCD&I. These studies reaffirmed the closeness of E. coli strains and Shigella species. Interspecies Shigella reactions indicate 75% or higher relatedness. There is, however, one major exception. Three serotypes of S. boydii were tested. Two of these, C8 and C10 exhibited the expected high levels of relatedness to shigellae and E. coli strains. S. boydii C13, however, exhibited only 60-70% relatedness to shigellae, including other serotypes of S. boydii and to E. coli. Furthermore the degree of divergence in related sequences is some 5-8% as opposed to an average of 1-2% in all other reactions. Four C13 strains behave similarly. The reasons for the divergence of these strains from the mainstream of Shigellae is not readily apparent, although the serotypes are rare and may have been subjected to atypical selection pressures.

b. Klebsiella-Enterobacter-Serratia relatedness.

The differentiation of Enterobacter aerogenes from Klebsiella species, especially K. pneumoniae is not routinely carried out in clinical laboratories. These organisms are a source of taxonomic as well as clinical confusion. Various investigators have suggested including nonmotile E. aerogenes strains as K. pneumoniae strains or placing E. aerogenes as a new species within the genus Klebsiella. Our studies indicate that Klebsiella species share about 80% or more of their DNA sequences. E. aerogenes also form a highly related group, sharing 80-100% of their genomes. Motile E. aerogenes strains could not be distinguished from nonmotile strains on the basis of DNA relatedness. Our data indicated that E. aerogenes and all Klebsiella species have significantly diverged. They exhibit an average 56% DNA relatedness with evidence of 9% unpaired bases within related sequences. These data indicate that significant differences exist between E. aerogenes and Klebsiella species and point to the clinical importance of differentiating isolates of these organisms . The taxonomist should also be aware that these organisms are substantially different and that one can neither "lump" K. pneumoniae and E. aerogenes strains nor separate motile from nonmotile E. aerogenes strains.

Additional studies indicate that E. cloacae is about 40% related to both E. aerogenes and Klebsiella species. In both cases the related sequences contain some 12% unpaired bases. Other

Enterobacter species fall into two additional groups, typified by E. hafniae and E. liquifaciens. These groups exhibit only a core of 25% relatedness to each other, to other Enterobacter species, Klebsiella species and other genera of enterobacteria. E. liquifaciens does exhibit 65% relatedness to Serratia marcescens strains and probably should be placed with Serratia.

c. The place of Levinea species in the enteric bacteria.

DNA relatedness studies should be routinely carried out with newly described organisms. A good deal of taxonomic and nomenclatural confusion and duplication will be avoided if this is done before the proposal of new species or genera. An example of the use of DNA relatedness in newly described organisms is given with Levinea species.

A proposed new genus of enterobacteria, Levinea, was tested for relatedness to established genera and to other biochemically similar and newly described strains. The two species, L. malonatica and L. amalonatica share 60% of their DNA with evidence of 10-12% divergence within related sequences. Both organisms exhibit some 35-55% relatedness to representative genera of enteric bacteria. L. malonatica is identical to a new species, Citrobacter diversus, described by Ewing. L. amalonatica, which is biochemically similar to a biotype of C. freundii, suprisingly shows only 50% relatedness to this biotype. These studies were carried out in collaboration with Dr. V. M. Young (Baltimore Cancer Hospital).

d. DNA relatedness in Erwinia species.

We have previously shown that the phytopathogenic true Erwinia and soft rot Erwinia or Pectobacterium species each are diverse groups, both of which are significantly related to enterobacteria. The increased recognition of human infection due to Erwiniae, their agricultural importance, the horrendous state of their taxonomy and nomenclature, and interesting preliminary data combined to stimulate detailed study of these organisms. We have collected some 100 strains of Erwiniae from diverse sources. DNA has been isolated from these strains and conditions for labeling strains have been worked out. This study will occupy a significant portion of our effort in the coming year. Parts of the study will be collaboratory with Dr. Kelman (University of Wisconsin) and Dr. Starr (University of California at Davis).

e. DNA relatedness among Salmonelleae.

The tribe Salmonelleae contains the genera Salmonella, Citrobacter, and Edwardsiella, and also the Arizona and Bethesda organisms. We have studied these organisms, especially Arizona and Salmonella, only superficially. Part of the reason for this is the staggering number of serologically designated species and types. We are now studying genome sizes and relatedness in these organisms in collaboration with Dr. Falkow's group (Georgetown University). Preliminary data indicate that genome sizes in Salmonella and Arizona strains are 10-20% larger than that of E. coli strain K12. The Arizona strains tested appear a more diverse group than Salmonella species. The single-most interesting result is the low relatedness of two S. cholera-suis strains to strains of S. typhi and S. typhimurium.

3. Relatedness between episomes and chromosomal DNA.

An episome is a self-replicating, transmissable piece of DNA that can integrate into the bacterial DNA or exist free in the cytoplasm. In the past we determined relatedness among coliphages and between their bacterial host and also described the lack of relatedness between subtilis phages and their bacterial host. We are presently collaborating with Dr. Wohlhieter, DCD&I, in describing relatedness of sex factors to Vibrio cholera and E. coli. The P factor in V. cholera shows some 40% relatedness to host DNA. Similar studies are underway with the F factor in E. coli. These studies are designed to further the understanding of episome-chromosome interaction and episome origin.

4. Identification of atypical bacterial isolates by DNA reassociation as a diagnostic tool in the clinical bacteriology laboratory.

The identification of pathogenic bacteria and the determination of their frequency of occurrence, especially for atypical organisms and/or organisms not usually associated with disease, is an extremely important aspect of medical microbiology. The clinical importance of this area is exemplified by the recent epidemic due to intravenous solutions contaminated with Erwinia herbicola and by the increased incidence of enteric fevers and dysentery-like infections caused by pathogenic strains of E. coli. Furthermore the increased clinical incidence of strains with atypical metabolic patterns has made identification of these isolates more difficult. Finally, differentiation between typical isolates of certain pathogens is often a difficult

task in the clinical laboratory. Most general hospitals have neither the facilities, manpower nor funds to attempt the identification of atypical or difficult isolates. In most cases the identification is never made. In other instances, problem cultures are sent to a reference laboratory for identification. This procedure is time-consuming and not foolproof.

To our knowledge, the only technique that is applicable to the identification of all groups of bacteria and not significantly affected by atypical biochemical or serological reactions is interspecies deoxyribonucleic acid reassociation. We have often been asked whether DNA reassociation techniques can be adapted to routine clinical use. The answer always has been and continues to be no. It is felt, however, that these procedures can be altered in order to provide a diagnostic reference system for the reference laboratories and for many of the larger hospitals.

We are presently testing all the parameters in our system in hopes of developing a fast (48 hr) test for identification that is accurate and simple, in addition to being realistic on a cost per identification basis. Preliminary results are encouraging. We are able to use reasonably small culture volumes to obtain adequate amounts of DNA for test purposes. The first attempts at greatly simplifying and shortening DNA purification techniques are also encouraging. This project will be a major effort in our laboratory in the coming year.

5. Transfer RNA and 5s RNA gene studies.

The physico-chemical, structural and biological characterization of tRNA and 5s RNA genes continues. As far as it can be determined from the present investigations, many of these cistrons are clustered on E. coli genome with 40-80 nucleotide gaps between them. The E. coli tRNA genes are completely non-homologous to E. coli 5s RNA, rRNA, yeast tRNA and rat liver tRNA. The tRNA and 5s RNA cistron sequences are highly conserved among the Enterobacteriaceae as shown in Table 1.

Table 1. Conservation of tRNA cistrons and 5S RNA cistrons

Source of unlabeled DNA	Unfractionated *E. coli* B DNA		Source of Labeled DNA *E. coli* B tRNA cistrons		*E. coli* B 5S RNA cistrons	
	Relative % binding 60°C	% unpaired bases 60°C	Relative % binding 60°C	% unpaired bases 60°C	Relative % binding 60°C	% unpaired bases 60°C
E. coli B	100	---	100	---	100	---
E. coli 0128	97	---	100	---	---	---
E. coli 07	91	---	100	---	---	---
E. coli AD-06	88	---	96	---	---	---
S. flexneri	84	2.4	86	2.2	82	3.7
S. typhimurium	46	12.3	70	4.0	79	4.4
E. aerogenes	46	---	78	---	---	---
E. hafniae	19	---	67	---	---	---
P. mirabilis	7	13.8	67	8.8	71	5.0

6. Studies of the species specificity of protein biosynthesis.

Several years ago it was reported from this laboratory that the amino acylation of a tRNA from a given species is not only dependent upon the source of tRNA and the source of the synthetase but also on amino acid in question. The recent studies appear to show that one of the reasons for this fact is that the heterologous reactions for protein synthesis (i.e., when macromolecules from two different species are allowed to react in protein synthesis steps), the equilibrium constants are quite different from homologous reactions. At present we are using this information to isolate purified protein-nucleic acid complex made by heterologous reaction to ascertain the nature of their interactions. The complex thus formed is more stable than that in homologous systems.

7. Studies on the immunological stimulation of biochemical processes in human lymphocytes.

In collaboration with the organ transplant department the following biochemical changes that occur in conjunction with immunological stimulation are being carried out.

The model for study of this concept is the mixed lymphocyte culture system. Briefly, irradiated lymphocytes from one donor are placed in a 7-day tissue culture system with either peripheral blood lymphocytes or spleen cells from another donor. Drugs known to stimulate or inhibit cyclic 3'5' adenosine monophosphate (cAMP) synthesis are added to the culture system at various times in various concentrations and the degree of inhibition or stimulation of DNA synthesis is measured by the uptake of tritiated thymidine.

Indirect evidence of adenyl cyclase and cAMP involvement in the metabolism of the lymphocyte is obtained via the above model. Biochemically, the proof of alteration comes with actual measurement of the compounds.

Primarily the establishment of a rapid highly sensitive and easily reproducible method for the measurement of adenyl cyclase and cAMP has to be accomplished. A procedure for measuring cAMP using an isotopic dilution principle with a cAMP dependent protein kinase and protein kinase inhibitor was developed. It allows the measurement of 0.05 picomoles of cAMP. Three methods for the assay of adenyl cyclase have been established. The conversion of isotopically labeled ATP (^{14}C, ^{32}P, ^{3}H) to radioactive cAMP is quantitatively measured on Dowex 50 H+ columns or ^{32}P ATP

conversion to cAMP ^{32}P and measured on aluminum oxide columns. The method of conversion of ^{14}C adenine to labeled cyclic AMP has also been established. These methods are easily adaptable to measuring cyclic 6MP, guanyl cyclase and phosphodiesterase.

We have shown that lymphocyte cyclic AMP and adenyl cyclase are altered by antigenic stimulation. We have further shown that drugs which are known to act on adenyl cyclase and cyclic AMP do in fact modulate the immune response of the lymphocyte.

These studies will be further extended to better understand the ability to alter the immune response. These findings may be significant in altering the immune-rejection phenomenon in transplantation.

8. Studies on the receptor sites on membranes and macromolecules.

The diversity of function of biological membranes has been widely investigated. Non-destructive methods, such as probe-molecule spectroscopic analysis, have proven to be good indicators of configurational changes of proteins and proteolipid complexes as well as indicators of structural and functional influences of perturbant molecules. Concern with addictive drug changes in the chemistry of normal "receptor sites" of various membranes adds importance to such studies.

Previously, the erythrocyte ghost was chosen as a model membrane. More recently investigations have shown that this membrane does not necessarily relate to the intact erythrocyte, because of protein and lipid losses brought about by disruptive mechanical and osmotic forces inherent in preparation procedures. Because of this, the synaptic vesicle membrane was examined. The postulated functions of this membrane have been outlined by DeRobertis and Whittaker.

2-p-Toluidinylnaphthylene-6 sulfonate (TNS) interaction with synaptic vesicle membrane has shown an excellent consistency between preparations as determined by dye binding. The maximum fluorescence intensity per 10^{-9} moles of TNS has been found to be 0.038 ± 0.011 (95% confidence limits), by regression analysis. The equation for regression being:

$$\tilde{Y} = 0.53 + 0.17X$$

where $\tilde{Y}$ = expected fluorescence intensity (arbitrary units)
$X = (\text{mg protein})^{-1}$

Scatchard plots of TNS titration data shows the number of binding sites, n, equals 3.0×10^{-7} moles/mg protein, and the statistical binding constant, $K_D = 5.0 \times 10^{-7}$ moles/mg protein. Vesicle membrane changes with time was evident after 18 hours at 4°C. Membranes stored under these conditions showed a maximum fluorescence of 0.17 units per 10^{-9} moles TNS.

Preliminary work using vesicles from rat brain hypothalmus, predominantly noradrenergic in nature, indicates that heroin may decrease the number of binding sites available to TNS with no apparent change in the statistical binding constant.

9. Metallocomplex formations of compounds of biological interest.

Adrenergic neurotransmitters have been shown to form complexes with adenosine triphosphate, (ATP), and transition metal ions. The concentration of transition element ions in the synaptic regions of nervous tissue is sufficient to support the formation of these complexes in vivo. The stereochemistry of morphine suggests that it may form metal complexes as well, and, therefore, possibly interfere with normal biogenic amine-metal complexes.

Using the Cary 14 scanning spectrophotometer, solutions of biogenic amine and Iron II and III were examined, as well as ATP and morphine. Difference spectra confirm the presence of a morphine-Fe^{3+} complex with λ_{max} = 234 nm. ATP-Fe^{3+} complex has a λ_{max} = 280 nm, whereas, ATP shows λ_{max} = 259 nm. 3,4-dihydroxyphenylethylamine, (DOPAMINE), gives an Fe^{2+} complex at λ_{max} = 290 nm. Work is in progress to elucidate the effects of morphine on biogenic amine complex formation.

Summary and Conclusions.

The progress under this work unit has been directed toward the study and amplification of the fundamental processes that occur at the cellular level in particular the nucleic acids. The following conclusions have been reached:

1. Methodology for the separation of nucleic acids has been improved.

2. Biochemical screening procedures to be used in strain selection have been developed.

3. A large number of pathogenic strains of bacteria have been studied and characterized with respect to relatedness of their DNA.

4. A system for the identification of atypical bacterial isolates to be used in the hospital clinical laboratory is being developed.

5. Cistrons on the E. coli genome are separated by 40-80 nucleotides. tRNA and 5s RNA cistron sequences are highly conserved among the Enterobacteriaceae.

6. Lymphocytic cyclic AMP and adenyl cyclase are altered by antigenic stimulation.

7. Membrane models have been established and are being used to study drug receptor binding.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biochemistry

Work Unit 070 Biochemical variations during disease and treatment

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6452	7 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	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
71 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	073
b. CONTRIBUTING				
c. [redacted]	CDOG 114(f)			

11. TITLE (Procede with Security Classification Code)

(U) Molecular Pharmacology of Chemotherapeutic Drugs (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

004300 BIOCHEMISTRY

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)
PRECEDING FISCAL YEAR 72	7	180
CURRENT FISCAL YEAR 73	7	180

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide Each with Security Classification Code)

(U) Primaquine; (U) Distamycin A; (U) L-cycloserine; (U) Chloroquine

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

23. (U) The objective of this work is the explanation of the modes of action of clinical or investigational chemotherapeutic drugs with a view to premeditated drug design.

24. (U) Biophysical studies of the binding of drugs to their bioreceptors; biochemical studies of resulting inhibitions; physiological/microbiological studies of the resulting effects on populations of micro-organisms.

25. (U) 71 07 - 72 06 Primaquine is bactericidal for B. megaterium and, specifically, inhibits protein biosynthesis; a cell-free B. megaterium-derived ribosome-poly U system is inhibited in competition with poly U. - The antiviral antibiotic, distamycin A, is liberated from its DNA bioreceptor only partly by successive hydrolyses with deoxyribonuclease and phosphodiesterase. It gives a negative viscometric test for intercalation into circular supercoiled DNA. Reported to bind preferentially to A-T rich regions of DNA, distamycin was found to bind strongly to poly dA-T, poly dA-dT and poly dI-dC but much less strongly to poly dG-dC; the absence of guanine appears to be a determinent of strong receptor binding. - The synthetic L-enantiomer of the antibiotic, D-cycloserine, resembles L-alanine. It inhibits growth of E. coli in synthetic medium; this inhibition is reversed by L-alanine. The drug does not inhibit the enzymatic synthesis of L-alanyl-tRNA. - Nuclear magnetic resonance studies on chloroquine have shown that the side chain of the drug in solution is flexible and implicate the ring N and the aliphatic side chain N as participants in drug binding to the DNA bioreceptor. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

* 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 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 073, Molecular pharmacology of chemotherapeutic drugs

Investigators.
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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. We have reported previously that duplex DNA is a bioreceptor for the antimalarial drug, chloroquine, and that the formation of the chloroquine-DNA complex poisons DNA as a template for its own replication as well as for the transcription of RNA. The structure of the DNA-chloroquine complex has been inferred from a variety of biophysical studies but neither the structure of chloroquine itself in aqueous solution *nor that* of the drug-DNA complex can be determined directly by optical and hydrodynamic methods. Nuclear magnetic resonance techniques have been developed which can be used to determine the three-dimensional structure and molecular properties of drugs in physiological solution. These techniques can *be extended* to determine directly the structure of the chloroquine-DNA complex. The methods for determining the structure of drugs in solution were reported last year. Since that time, a three-dimensional structure has been reported for chloroquine (CQ), in acetone solution. This was deduced from the results of detailed proton magnetic resonance studies employing a paramagnetic ion probe, Tris-(2,2,6,6-tetramethylheptane-3,5-dionato)praseodymium(III), [Pr(tmh)$_3$], known also as a "shift reagent." Pseudocontact shifts induced by Pr(tmh)$_3$ in the proton magnetic resonance (pmr) spectrum of CQ were measured at 220 MHz for all of the CQ protons up to a 1:1 CQ:Pr(tmh)$_3$ concentration ratio at 20°C and 48°C. An extensive analysis of the pseudocontact shift data was then carried out to obtain a set of coordinates for that time-averaged CQ structure which gave the best overall least squares fit

between calculated and observed chemical shifts for all protons in the molecule. A stereoscopic representation of the time-averaged CQ structure was constructed from the final sets of coordinates at each temperature. The three-dimensional structure of CQ in solution is compact with the side-chain curled over the plane of the quinoline ring. An indication of the compactness is given by the rather short distance of 7.4 Å between the N1 and N3 positions at 20°C. The structure is opened up slightly at 48°C but its essential features are not altered. Chloroquine's structure in solution differs markedly from that reported for diprotonated CQ in the solid crystalline state.

Structural studies of monoprotonated CQ in methylene chloride and methanol have been carried out and the data are currently being analyzed. Indications are that the aliphatic chain in this species forms a semi-rigid ring, stabilized by a hydrogen bond between the N3 proton and the N2 atom.

Definitive structural studies of chloroquine require the capability of describing its 3-dimensional structure in physiological, i.e. aqueous solution. This presented a technical problem since the available shift reagents were insoluble in water. Use of paramagnetic lanthanide ions in aqueous solutions can not solve the problem since these ions will not interact with positive charges of chloroquine. Therefore, we synthesized stable lanthanide chelates with negative charges. To obtain such substances, some of the basic chemical methods used in the preparation of first-row transition metal ion-ligand complexes were applied to the rare-earth metal ion-shift reagent complexes. A general method has been devised to prepare water soluble lanthanide chelates with different charges. This method involves, first, the preparation of a hydrated lanthanide sulfate complex by adding stoichiometric amounts of silver sulfate to the anhydrous lanthanide chloride (1).

$$2LnCl_3 + 3Ag_2SO_4 \rightarrow Ln_2(SO_4)_3 + \underline{6AgCl} \quad (1)$$

The barium complex of the appropriate ligand, i.e. ethylenediaminetetraacetic acid (EDTA), [ethylenebis(oxyethylene-nitrilo)] tetraacetic acid (EGTA), nitrilotriacetic acid (NTA), nitrilodiacetic acid (NDA) or glycolic acid, is then prepared. The barium-legand complex is stoichiometrically added to $Ln_2(SO_4)_3$ until no further precipitation of $BaSO_4$ is observed. The solution is then filtered and sodium sulfate added to form the sodium salt of the complex in instances where the final complex is negatively charged.

Using this methodology $(Ho\ EDTA)^{(-)}\ Na^{(+)}$ was prepared. Three-dimensional structural studies of diprotonated CQ in aqueous solution have been performed and the data accumulated are presently being analyzed. Using the structural information obtained, the individual bond dipole moments of the different ionic species of CQ will be calculated as well as atomic charge and electron densities using approximate molecular orbital theory. These calculated properties

will be validated by comparing energy parameters obtained in the quantum mechanical calculations with measured values from the proton magnetic spectroscopy measurements.

2. Mode of action of primaquine. We reported last year that primaquine is bactericidal for Bacillus megaterium at concentrations of $>5 \times 10^{-4}$ M and, in this test organism, inhibits protein biosynthesis instantaneously and completely while other macromolecular biosyntheses (DNA replication, RNA transcription, formation of cell-wall polymer) continue. Inhibition of protein synthesis usually results in cytostasis; the only known bactericidal inhibitors of protein biosynthesis are the aminoglycoside antibiotics of the streptomycin group. In order to elucidate the biochemical mechanism underlying primaquine's inhibition of protein synthesis, a cell-free ribosome-enzyme system of B. megaterium origin was developed which polymerizes phenylalanine as directed by polyuridylic acid (poly U) used as a messenger RNA. The polycondensation of phenylalanine was, indeed, inhibited by primaquine although to a lesser extent than protein biosynthesis in intact B. megaterium. The ribosome-poly U system differs mechanistically from protein biosynthesis by the absence of a natural initiation step and the enforcement of artificial initiation by high concentrations of Mg^{++}. When poly U was preincubated with primaquine in the absence of Mg^{++} and this messenger-drug mixture then supplied to the other components of the reaction system, a strong inhibition of the polycondensation of phenylalanine was obtained. Experiments were, consequently, carried out with graded concentrations of poly U, either preincubated with primaquine as before or supplied to the drug-containing reaction mixture. When v is the rate of formation of polyphenylalanine and c the concentration of poly U, a double reciprocal plot of 1/v as a function of 1/c produced straight lines for a drug-free control series as well as for series in which primaquine had either been preincubated with poly U or merely added to the complete reaction mixture. These lines had different slopes but one common intercept on the ordinate where $1/c = 0$. This means that at a poly U concentration $c = \infty$, no inhibition by primaquine occurs and that the drug inhibits the ribosome-poly U system in competition with poly U. Such a result might be expected if the drug were to form a molecular complex with poly U (or with natural messenger RNAs in vivo). Indeed, binding of primaquine to ribopolynucleotides has been observed by others (Morris et al., Mol. Pharmacol. 6, 240, 1970). However, this binding is relatively weak, and we have recently discovered that the inhibition of the ribosome-poly U system by primaquine is also improved when the ribosomes (rather than poly U) are preincubated with the drug. At this phase of the study, the best hypothesis to fit our data would be one which assumes that primaquine interferes with the binding of messenger RNA to ribosomes.

3. Mode of action of distamycin A. The antibiotic distamycin A has activity against DNA-containing mammalian and bacterial viruses, also has antitumor activity and inhibits the induction of enzyme syntheses in bacteria. Certain of these activities can be attributed to the property

of the antibiotic to form very strong complexes with DNA. In one-step growth experiments we have confirmed and refined observations of others on the anti-T1 phage activity of distamycin A in E. coli. The antibiotic prevents the initial adsorption of the virus to the bacterial surface; when added to infected cells during the eclipse phase, the antibiotic acts as a lysis-inhibitor which prevents the "burst" of the progeny phage from occurring. We reported last year that a variety of experimental procedures failed to dissociate distamycin A from its complex with DNA, including digestion of the DNA-antibiotic complex with deoxyribonuclease I. However, when these digestion products were subjected to the subsequent action of snake venom phosphodiesterase, the absorption spectrum of the drug changed to exhibit a new peak which was resolved on an analog computer (Dupont 310 Curve Resolver) into two spectrophotometric components, one the free antibiotic and the other the still bound distamycin A. An extraction of the DNA-distamycin A complex with a biphasic aqueous phenol system resulted in the dissolution of DNA in the phenol-poor phase and that of distamycin A in the phenol-rich phase. We conclude that the complex of DNA with distamycin A is not held together by covalent bonds as we and others had speculated in the past. Others have reported that biophysical indications of the binding of distamycin A to DNAs of different base compositions suggest a preference of the antibiotic for A-T pairs or for A-T rich regions of DNA. Such a specificity is already known to exist for the (structurally unrelated) antibiotic nogalamycin (Bhuyan & Smith, PNAS 54, 566, 1965). We have studied the specificity of interactions of distamycin A with the synthetic DNA-like duplex polymers poly dA-dT, poly dA-T, poly dI-dC and poly dG-dC. The three polymers, not containing G, produced the same red-shift in the absorption spectrum of the antibiotic as calf-thymus DNA; poly dG-dC produced a lesser shift but decreased the intensity of distamycin A's absorption. The three polymers, not containing G, gave rise to Cotton effects in the ORD spectrum of distamycin with large molecular amplitudes; poly dG-dC produced a Cotton effect with small molecular amplitude. The three polymers not containing G, were strongly stabilized by distamycin A to strand separation by heat; poly dG-dC showed a much smaller displacement to higher temperatures of its "melting curve" by distamycin A. We conclude that the antibiotic formed complexes with all four synthetic double helices studied but that the presence of guanine decreased the strength of complex formation. These results might signal (1) A preference of distamycin A for A or T or for single A-T pairs, (2) An exclusion of strong interaction by G, or (3) A preference for a peculiar secondary structure of conformation existing in stretches of DNA with an abundance of A-T pairs. Distamycin A also interacts with denatured or genuine single-stranded DNA by spectrophotometric and spectropolarimetric criteria. Most interesting is the observation that the antitiotic (with temperature increases or decreases) gives rise to reversible and cooperative hyper- or hypochromic changes in the absorbance of denatured DNA at 260 nm. This suggests the spontaneous formation or dissolution of a duplex structure of single-stranded DNA with a regular array of antibiotic molecules. The studies of the biophysical and biochemical interactions

and consequences of complex formation with single-stranded polydeoxyribonucleotides are now being extended to synthetic homopolymers.

4. Mode of action of L-cycloserine. We reported last year that the synthetic L-enantimer of the antibiotic cycloserine inhibits the growth of bacteria in competition with L-alanine. L-cycloserine can be regarded as a structural analogue and antimetabolite of L-alanine. The effect of this compound upon the growth of E. coli in a salts-glucose medium was not antagonized by the two other amino acids which are biosynthetically derived from the Krebs Cycle, viz., glutamic acid and aspartic acid. Antimetabolism between amino acids and their analogues usually occurs at the activation step, i.e. the enzymatic synthesis of amino acyl transfer-RNA. A cell-free system from E. coli which synthesized alanyl transfer RNA was surprisingly not inhibited by L-cycloserine. This suggests that the compound does not antagonize the processing of L-alanine for protein synthesis but, instead, may inhibit the formation of L-alanine, i.e. it may convert prototrophic bacteria into phenocopies of alanine-requiring organisms. A search for a naturally alanine-requiring organism (in order to carry out exact competition experiments of alanine vs. L-cycloserine) has met with limited success: a strain of Leuconostoc citrovorum was eventually found to have among its multiple nutritional requirements also that for L-alanine. The growth of this bacterium in a defined medium is so slow and scanty that it does not lend itself easily to competition experiments based upon measurements of growth rates. The effects of L-cycloserine on the major macromolecular biosyntheses of E. coli are currently under study, and efforts at developing a working system to measure competitive inhibition (in order to arrive at the standard double-reciprocal test plot for this phenomenon) continue.

5. Testing for intercalation of drugs into DNA by measuring conformational changes in supercoiled DNA. The binding of ethidium bromide to superhelical DNA and its effect on superhelical density had been sufficiently described theoretically and experimentally to establish this binding as intercalation and ethidium bromide as a model intercalant. Many dyes and drugs,including antimalarials which bind to DNA, have a lesser potential to intercalate, a greater potential to bind by secondary modes or a tendency to precipitate DNA, thus making it difficult to describe the contribution made by each factor to observed changes in biophysical properties. A combination of centrifugational and viscometric techniques has been used to study representative drugs under experimental conditions chosen to minimize secondary binding effects.

Quinacrine. A detailed study has been made on the binding of quinacrine to PM2 DNA by viscometric titration in 0.02 M Tris, 0.01 M EDTA, 0.2 M NaCl, pH 7.36. No evidence of secondary binding was observed up to a Drug/DNA (nucleotide) ratio of 0.1. Quinacrine has an apparent affinity constant (intercalation) of 2.7×10^4 M^{-1} and an unwinding angle equal to that of ethidium bromide ($\pi/15$ radians). The affinity constant for ethidium bromide under similar conditions was greater than 10^6 M^{-1}.

Methylene blue. This is an example of a dye capable of precipitating DNA at a dye to nucleotide ratio lower than needed to cause conversion of superhelical DNA to an open circular form. Low concentrations of methylene blue were able to reduce the superhelical density of PM2 DNA as measured by a reduced requirement for known intercalants to complete the conversion to an open circular form. The apparent affinity constant was estimated to be 5.6×10^3 M^{-1} for intercalant binding under these ionic conditions.

Distamycin. The high affinity of this drug for DNA would predict that, if the primary mode of binding involved intercalation, the conversion to an open circular form would occur prior to the ratios at which precipitation is observed. The effect of distamycin on PM2 DNA was studied in conjunction with ethidium bromide. The presence of distamycin caused the amount of ethidium bromide required to convert to an open circular form to be increased. An increased requirement is in opposition to the expected result if distamycin were intercalanted.

Conclusions.

NMR measurements, using lanthanide shift reagents, have shown that the side-chain of chloroquine *in* solution is highly flexible as is necessary for binding to its DNA bioreceptor. Primaquine inhibits a cell-free model system of protein biosynthesis, i.e. polycondensation of phenylalanine, in competition with the synthetic messenger RNA, polyuridylic acid. The antibiotic distamycin A binds preferentially to synthetic duplex polydeoxyribonucleotides which do not contain guanine but some binding to poly dG-dC was demonstrated. L-cycloserine (a synthetic enantiomer of the antibiotic D-cycloserine) inhibits growth of E. coli in synthetic medium in competition with L-alanine to which the drug is an antimetabolite. Other amino acids do not reverse this inhibition. Direct biophysical tests for intercalation binding of drugs to DNA have proved that quinacrine and methylene blue are intercalated but distamycin A is not.

Project 3A061102B72D BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 073, Molecular pharmacology of chemotherapeutic drugs

Literature Cited.

Publications:

1. Olenick, J.G., Cook, T.M., and Hahn, F.E.: Bactericidal action of 2-hydroxy-3-(cyclohexylpropyl)-1,4-naththoquinone on Bacillus megaterium. J. Bact. 107:528, 1971.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 0453	72 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
71 07 01	D. Change	U	U	NA	NI	☒ 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. XXXXXXX	CDOG 114(F)			

11. TITLE (Precede with Security Classification Code)

(U) Molecular Basis of Biological Regulation (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 BIOCHEMISTRY

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: — AMOUNT:
d. KIND OF AWARD: — CUM. AMT.:

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 72	2	50
FISCAL YEAR CURRENT 73	2	50

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) R-factors; (U) Bacterial Drug Resistance; (U) Tolerance to Narcotics

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

23. (U) Exploratory studies with the objectives of (1) improving chemotherapy of bacterial infections through the restoration of drug-sensitivity to pathogens which owe their drug resistance to the presence of R-factors and (2) elucidating the biochemical basis of development of tolerance to morphine-like addictive drugs.

24. (U) Continued experimental studies on (1) the elimination of R-factors from bacteria by exposure to DNA-complexing compounds, and (2) newly initiated studies on the development of tolerance to addictive analgesics in bacterial populations.

25. (U) 71 07 - 72 06 A set of 15 DNA-complexing compounds as well as nalidixic acid eliminated resistance determinants for kanamycin, chloramphenicol, ampicillin and streptomycin at high frequencies in R-factor-containing Salmonella typhimurium. Since this genetic segregation requires growth of bacteria in the presence of test compounds, it is thought that the elimination of resistance determinants is a result of differential inhibitions of different DNA replicons. - Cultures of E. coli B whose growth was arrested by etorphine, NIH 7591 (a meperidine), or phenazocine (a benzmorphan) resumed exponential growth after several hours. Moreover, bacteria which had been exposed to the drugs and were resuspended in fresh drug-containing media grew exponentially after lag periods which were much shorter than those which preceded the first escape from inhibition. These observations signal a phenotypic adaptation of bacterial populations rather than a selective propagation of a few drug-resistant mutants. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

*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 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 074, Molecular basis of biological regulation

Investigators.
Principal: Fred E. Hahn, Ph.D.
Associate: Jennie Ciak, M.S.; Alan D. Wolfe, Ph.D.

Description.

Experimental research studies in molecular biology concerned with the physiology, biochemistry, biophysics and molecular genetics of selected processes with a view to elucidating mechanisms underlying biological events of special importance to military medicine, for example, currently, R-factor mediated bacterial resistance to chemotherapeutic drugs and the restoration of drug sensitivity to such organisms and molecular processes underlying the development of tolerance to morphine-like analgesics.

Progress and Results.

1. Elimination of episomal resistance determinants by drugs or dyes which intercalate into DNA. We reported last year the elimination of episomal elements such as the lac marker of the F'lac factor and a series of drug-resistance determinants from an R-factor carried by E. coli. The study has been extended to the gentamicin-resistance determinant of an R-factor in Klebsiella pneumonia which had given rise to an outbreak of drug-resistant infections at Georgetown University Hospital. The gentamicin-resistance determinant was eliminated by ethidium bromide, quinacrine, acridine orange and berberine, listed in decreasing order of potency at 10^{-4} M concentration. The originally studied R-factor was genetically transferred from E. coli into Salmonella typhimurium and the elimination of resistance determinants for kanamycin, chloramphenicol, ampicillin and streptomycin again studied. Since the recipient organism was naturally insensitive to sulfadiazine, the elimination of the sulfadiazine R-factor could not be investigated. R-factors, when in S. typhimurium, are less stable than in E. coli and exhibit a significant extent of spontaneous segregation during normal cultural passage. At 10^{-4} M, the most active compounds eliminating all four resistance determinants were ethidium bromide, miracil D, quinacrine and propidium iodide but substances which formerly had shown little or no R-factor eliminating capacity, such as p-rosaniline, methylene blue, quinine and chloroquine, produced significant elimination of the kanamycin-resistance determinant in S. typhimurium. The highest frequency of elimination was seen with ethidium bromide for this kanamycin determinant and exceeded 95 per cent of the cells in culture. Recently the study has been extended to

the testing of chlorpromazine, Nitroakridin 3582, coumadin and nalidixic acid (the last an inhibitor of DNA replication but not a DNA-complexing substance). This work is based on our hypothesis that substances which form complexes with DNA will selectively alter the conformation of closed-circular R-factor DNA and, thereby, render such DNA incapable of being replicated. Specifically, intercalating compounds such as ethidium, propidium, quinacrine and miracil D, will tie up circular DNA into unnatural supercoils and it is thought that in this close and compact conformation, DNA can not function as a template for its own replication. We have determined experimentally that our most active compound, ethidium bromide, does not eliminate the kanamycin-resistance determinant from E. coli upon contact for 30 min but that the organism must grow and divide in the presence of ethidium; this supports our assumption of a selective inhibition of R-factor DNA replication as the molecular mechanism underlying the elimination phenomenon. The structural requirements for intercalating eliminators of episomal elements are logically the same as for intercalation in general: (1) A flat aromatic ring system with a planar area of minimally 28 Å. (2) The presence of protonated centers on the molecule which, at physiological pH, must be positively charged to 50 per cent or more. (3) A critical geometrical arrangement of these protonated centers which requires some additional study (for example, 8-aminoquinolines such as primaquine do not intercalate while 4-aminoquinolines such as chloroquine do). Nalidixic acid is not a DNA-complexing substance but inhibits bacterial DNA biosynthesis by an unknown mechanism which possibly involves the bacterial membrane and its structural relationship with the DNA-synthesizing machinery. At 6×10^{-6} M, nalidixic acid had no influence on the growth or viability of S. typhimurium but showed strong elimination of resistance determinants. This result points, again, to a selective suppression of episomal DNA synthesis as the basis of the elimination phenomenon even if this is brought about by a molecular process other than complexing with episomal DNA. Finally, it should be noted that some of the eliminating substances which we have tested (spermine, quinacrine, chlorpromazine, coumadin) are antimutagens by virtue of their ability to stabilize DNA by complex formation. We have received additional experimental substances with demonstrated high antimutagenic activity and are currently testing them for their capacity of eliminating resistance determinants from S. typhimurium.

2. Effects of morphine-like analgesics on bacterial cultures. High concentrations of the morphine derivative, levorphanol, and of its pharmacological antagonist, levallorphan, are known to inhibit the growth of E. coli; this effect is attended by the non-occurrence of ribosomal RNA biosynthesis in drug-exposed bacteria. Morphine itself does not produce these effects. The analgesic potency of levorphanol is approximately four times that of morphine. We speculated that the antibacterial potency of morphine-like analgesics may parallel their analgetic potency and that selected such substances may become useful

in the study of addictive drug effects in bacteria, especially phenomena of tolerance development and physical dependence. Among seven pharmacopeial or experimental narcotics, phenazocine, etorphine, NIH 7564 and NIH 7591 were found to inhibit the growth of E. coli B at low concentrations in a glucose-citrate-salts liquid medium. The etorphine antagonist, diprenorphine also inhibited bacterial growth. The effects of NIH 7591 and of phenazocine have been studied in detail. Growth inhibition of E. coli by NIH 7591 was a function of the drug concentration and an inverse function of the bacterial concentration. At 5×10^7 bacteria per ml, the lowest concentration of NIH 7591 to produce a significant decrease in growth rate was 5×10^{-5} M, while complete suppression of growth required 10^{-3} M. These concentrations are comparable to those of certain synthetic chemotherapeutic drugs which inhibit bacterial growth. Growth inhibition by NIH 7591 or by phenazocine was paralleled by proportionate decreases in the rates of RNA, DNA and protein biosyntheses as studied by incorporation of appropriate radioactive precursors into the bacterial polymers. The nonspecific across-the-board character of these biochemical lesions suggests the involvement of one common physiological denominator. Many authors believe this to be the cell membranes. We have obtained evidence which supports this view by measuring the effects of NIH 7591 on DNA and RNA biosynthesis in toluene-treated E. coli. Toluinized bacteria, although no longer viable, are permeable to the nucleoside-triphosphate building blocks of both categories of nucleic acids and do synthesize DNA as well as RNA to limited extents for limited periods of time. Toluene treatment most likely involves some disorganization of the lipoprotein membrane. NIH 7591 did not inhibit DNA and RNA synthesis in the toluinized cell system, although these biosyntheses fail in intact bacteria under the influence of this narcotic. We assume that toluene treatment inactivates or eliminates the membrane bioreceptor for the narcotic.

Most important, cultures of E. coli B, whose growth was inhibited in the presence of NIH 7591 or of phenazocine, resumed exponential growth after a few hours at rates which approached those of drug-free control cultures. When these bacteria were collected and resuspended in fresh media containing either drug, the organisms went into exponential growth after lag times much shorter than those observed upon first exposure to the drugs. The time element and the magnitude of resumed growth rates eliminates from consideration the idea of a selective propagation of a few drug-resistant mutants and suggests that entire test populations can adapt to either drug to become resistant as well as cross-resistant to these narcotics. These observations bear a formal resemblance to the phenomenon of tolerance development to narcotics in man, animals or mammalian cell cultures. Media in which E. coli B had resumed growth after initial inhibition by the two drugs supported growth of fresh bacterial inocula without delay indicating that such media no longer contained growth-inhibitory concentrations of the drugs under study. While such observations suggest that the first escape of test cultures from growth inhibition by the narcotics was the result of

a biochemical modification or inactivation of the drugs, the acquired ability of the bacteria to overcome inhibitory drug effects readily upon second exposure indicates some adaptive change in the organisms themselves. In summation: exposure of E. coli to strongly analgesic and growth inhibitory morphine-like compounds produces two effects, (1) An acquisition of bacterial resistance to the original and related drugs, and (2) Inactivation of these drugs.

Conclusions.

Substances which form complexes with DNA, foremost by intercalation, eliminate resistance determinants from R-factors harbored by Klebsiella pneumonia and Salmonella typhimurium. This effect is thought to involve a selective inhibition of episomal DNA replication by template poisons. Nalidixic acid which inhibits DNA biosynthesis by a different mechanism, also eliminates resistance determinants from an R-factor in S. typhimurium. A meperidine analogue (NIH 7591) and a benzmorphan, phenazocine, inhibit growth of E. coli at low concentrations. The bacteria can escape from this inhibition probably by biochemical inactivation of the drugs. Such bacteria, when reexposed to fresh drugs, show resistance or cross-resistance to either drug. It is thought that this is the result of a phenotypic adaptation of bacterial test populations and formally resembles tolerance development to narcotics in man, animals or mammalian cell cultures.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 074, Molecular basis of biological regulation

Literature Cited.

Publications:

1. Hahn, F.E., and Ciak, J.: Elimination of bacterial episomes by DNA-complexing compounds. Ann. N.Y. Acad. Sci. 182:295, 1971.

2. Hahn, F.E., and Ciak, J.: Elimination of episomal determinants by DNA-complexing substances. Abstr. First Intern. Symp. Infect. Antibiot. Resist., p 36, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION: DA OA 6465	2. DATE OF SUMMARY: 72 07 01	REPORT CONTROL SYMBOL: DD-DR&E(AR)636
3. DATE PREV SUMRY: 71 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	3A061102B71P	01	075
b. CONTRIBUTING				
[redacted]	CDOG 114(f)			

11. TITLE (Precede with Security Classification Code): (U)Metabolic Problems and Biochemical Variations Associated with Disease and Injury in Military Personnel

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
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: 72	6	170
FISCAL YEAR CURRENT: 73	14	394

19. RESPONSIBLE DOD ORGANIZATION
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ASSOCIATE INVESTIGATORS
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NAME: Sleeman, H. K. Ph.D. DA

21. GENERAL USE: Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Enzymology; (U) Hemorrhage; (U) Trauma; (U) Biochemical Variation

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 relate factors responsible for metabolic injury and tissue damage produced by trauma and disease to morbidity and mortality. 2. To investigate metabolic problems associated with oxygen and electron transport.

24. (U) 1. Establish and study animal models for use in experiments. 2. Study biochemical, histological and physiologic systems for altered metabolic processes. 3. Evaluate pharmacological doses of various compounds in treatment. 4. Determine levels of various compounds and their effects on metabolic systems.

25. (U) 71 07 - 72 06 During the reporting period, efforts under this work unit have been reoriented to examine the effects of drugs and other agents on the physiological balance of the mammal. Major contributions to the progress are as follows: 1) Paregoric is absorbed in the rat through the gut, rises to a maximal level in the blood at 12 hours and is excreted in equal quantities in urine and feces. Total excretion is essentially complete in 48 hours. 2) Experiments have been carried out to determine the binding properties of spin-labeled morphine in vitro and in vivo. The spin-labeled morphine analog does not pass the blood brain barrier, in vivo. It does reach circulation after IP injection and readily deposits in heart and liver. Rotational correlation times have been found to be consistently higher in vitro in hypothalamic synaptosomes than in other CNS components. 3) C-14 morphine distribution in rat brain after a single dose indicates that after one week C-14 morphine appears in all brain fractions. After 30 days the highest levels were found in synaptosomic membranes. 4) Continuing studies with DDS have demonstrated a major effect on the adrenal in the rat with a concomitant reduction in blood corticosterone levels.

For technical reports see the Walter Reed Army Institute of Research Annual Progress Report, 1 July 1971 - 30 June 1972.

*Available to contractors upon originator's approval

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PII Redacted

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biochemistry

Work Unit 075 Metabolic problems and biochemical variations associated with disease and injury in military personnel

\nvestigators.
Principal: COL Charles R. Angel, MSC
Associate: LTC Douglas J. Beach, MSC; MAJ Michael Boykin, MC; John Diggs, PhD; Earl Richardson, M.S.; H. Kenneth Sleeman, PhD; Patrick M. L. Siu, PhD; CPT Nicholas Weber, MSC

Description.

This work unit is specifically designed to examine factors responsible for abberant biochemical and physiological changes as a result of either metabolic disease or tissue injury due to trauma. With the increase in emphasis on the drugs of abuse, a greater portion of the effort during the reporting period has been devoted to the biochemical and physiological factors occurring after administration of drugs.

Progress.

During the reporting period, studies have been accomplished in the following areas:

1. Cellular response to morphine and other drugs.

2. Uncoupling of oxidative phosphorylation in rat brain subfractions.

3. Action of heroin and morphine in rat brain synaptosomes.

4. Localization of morphine binding sites in rat brain.

5. The effect of heroin on intracellular 3'5' cyclic AMP.

6. The uptake and excretion of paregoric in the rat.

7. Studies on spin labeled morphine in the rat.

8. The conjugation of morphine with glucuronic acid in the human.

1. Cellular response to morphine and other drugs.

Two areas were investigated in the study of cellular response to drugs. One area of interest was the effect of drugs on human cell cultures. The second study used bacteriological assays to detect drugs of abuse.

a. Studies with human peripheral blood lymphocytes.

When a substance that stimulates cell division (mitogen), such as phytohemagglutinin is added to lymphocyte cultures, the cells are activated and transformed into lymphoblasts. These lymphoblasts are metabolically active and undergo cellular division. Various drug concentrations were added to these cultures and the cellular response was assayed by measuring glucose uptake, lactic acid production and increase in cell number. The effect of drugs on lymphoblast stimulation and on non-stimulated cells was also noted,

Initial experiments were designed to examine the effects of antimalarial drugs, however most of the drugs (DFD, WR33063 and WR61112) that are used prophylactically or experimentally are insoluble in solvents at pH values non-toxic to the cultured lymphocytes. Chloroquine hydrochloride was tested at 1×10^{-5} gm/ml and was shown to inhibit glucose uptake, lactic acid production and cell reproduction in stimulated cells. It has a lytic effect on non-stimulated cells.

Morphine sulfate at 1×10^{-2} M was also inhibitory to metabolic activity and cell proliferation. Since there are opportunities for individuals undergoing malaria prophylaxis to be exposed to drugs of abuse, a combination of chloroquine hydrochloride and morphine sulfate was tested. It was found that there is an additive inhibitory effect with these two drugs, in that the combination of both drugs produced a greater degree of inhibition than either drug alone. This result indicates the probability of separate mechanisms of action for these drugs.

The system is being modified in order to increase the recovery of lymphocytes from the blood and to improve the survival rate of stimulated cells. A collaborative study has been initiated with the investigators at the Addict Treatment Center in Lexington, Ky. They will supply blood from patients being maintained on various doses of narcotics as well as control blood from these patients before the onset of their maintenance.

b. Detection and biochemical assay of drugs of abuse in bacteriological systems.

Representative drugs of abuse, including morphine derivatives, barbiturates and cocaine are being tested in a variety of bacteriological test systems. The objectives of these studies are to 1) determine if any of the drugs of abuse can be detected at physiological levels using a toxicity assay in bacteria, 2) to distinguish between drugs of abuse based on their effect in induction of bacterial viruses, 3) to develop resistant or dependent bacteria as an aid in the study of drug metabolism and mode of action. Assay systems have been developed and preliminary data have been obtained with certain drugs.

Concentrations of greater than 1×10^{-3} M morphine sulfate or diacetylmorphine hydrochloride reduce the growth rate of Escherichia coli. Diacetylmorphine hydrochloride is completely bacteriostatic at a concentration of 1×10^{-2} M. Parallel studies are being carried out in several groups of bacteria in order to find an organism that is more sensitive to these and other drugs.

2. Uncoupling of Oxidative Phosphorylation in Rat Brain Subfractions.

Steady state levels of ATP are required for normal functioning of all cells. Uncouplers of oxidative phosphorylation inhibit ATP formation in mitochondria. Although glycolysis would be increased in the presence of an uncoupler, the amount of ATP produced by substrate level phosphorylation will not make up the deficit in ATP brought about by removal of 90% of the cell's normal source of ATP, mitochondrial oxidative phosphorylation. Dinitrophenol is a classical uncoupler of oxidative phosphorylation. At a concentration of 5×10^{-5} M it maximally stimultes respiration jointly with its inhibition of oxidative phosphorlylation. Higher levels (10^{-4} M) of this compound inhibit respiration and phosphorylation in isolated mitochondria from most tissues. We have found that the major pharmacologically active component in marijuana (Δ^9-tetrahydrocannabinol) is also an uncoupler of oxidative phosphorylation in isolated rat brain mitochondria and synaptosomes. These results confirm the reported uncoupling effect of Δ^9-THC in rat liver mitochondria. In addition, the effects on mitochondrial respiration and phosphorylation due to Δ^9-THC were similar to those reported for dinitrophenol. These results strongly suggest that at least one important mode of action of Δ^9-THC (e.g., uncoupling of

oxidative phosphorylation) may result in decreased levels of ATP which in turn is intimately involved with all facets of nerve cell function.

3. Action of Heroin and Morphine in Rat Brain Synaptosomes.

The in vivo injection of heroin (10 mg/kg) and morphine (50 mg/kg) into male rats resulted in significant alterations in in vitro catecholamine uptake in synaptosomes from the brains of treated animals. Heroin resulted in a more striking alteration of uptake than morphine when both drugs were given in equivalent pharmacological doses. Under oxygenated incubation conditions, heroin inhibited norephinephrine-H^3 uptake to a greater degree than morphine. Under anaerobic incubation conditions heroin stimulated norepinephrine-H^3 uptake to a greater degree than morphine. These data suggest a generalized response rather than specific regionalization such as hypothalmus.

4. Localization of Morphine Binding Sites in Rat Brain.

It has been reported that after a single acute dose of morphine, a certain amount of the drug remains bound in brain. This has been reported to last for three weeks and longer. The present study was directed at identifying the site or sites of morphine-binding in subfractions of brain at 7 days and 30 days after a single injection of C^{14} morphine. After 7 days, the largest amount (approx. 33%) was found in free (unbound) form. The remaining labeled drug was found in various membraneous brain subfractions with the microsomes and synaptosomes having the highest specific activity. The lowest specific activity was found with the nuclear pellet and intermediate values were found in the myelin, mitochondrial and soluble protein fractions.

Thirty days after a single dose of C^{14} labeled morphine, the microsomes were still highly labeled with somewhat lower specific activity for myelin and synaptosomes. No activity was found in the protein-free 105,000xg supernate in contrast to the 7 day experiment. Perhaps the most significant part of the study is the subfractionation of synaptosomes where no label was found to be associated with synaptic vesicles. The synaptosome membrane fragments yielded the highest specific activity of any fraction tested on a mg. protein basis. Additional studies are being carried out to characterize this apparent morphine membrane interaction.

5. The Effect of Heroin on Intracellular 3'5' cyclic AMP.

Intracellular 3'5' cyclic adenosine monophosphate has been demonstrated to mediate many functional cellular responses to chemical and hormonal stimuli. A series of experiments has been designed to analyze the possible role of 3'5' cAMP in hypothalamic function, and changes found with administration of heroin. These experiments include structural localization of 3'5' cAMP, Adenyl cyclase and phosphodiesterase, comparisons of in vivo and in vitro responses, and determination of the mechanism inducing the changes.

Preliminary experiments have shown a significant increase in hypothalamic 3'5' cAMP after stimulation by heroin administration. The results suggest a picture of the changes in metabolic homeostasis induced simultaneously by heroin administration, and potential clues as to mechanisms of physiological addiction.

6. The Uptake and Excretion of Paregoric in the Rat.

Paregoric, which is used therapeutically in intestinal disorders, contains powdered opium (15 mg/fluid oz) of which 10 to 10.5 percent is morphine. Morphine can be detected in the urine after paregoric administration and could complicate the interpretation of results in the drug screening programs. Knowledge of morphine absorbed and excreted per dose of paregoric, the retention time of a known dose of paregoric, and its pattern of excretion would aid in differentiating between the therapeutic use of paregoric and drug addiction.

Paregoric was administered orally (2 or 3 ml by intubation) to Sprague Dawley rats. The animals were housed in metabolic cages to permit the separate collection of urine and feces. Baseline specimens of urine, feces, and blood (tail vein) were collected prior to the administration of paregoric. Urine and blood were collected at 6,12,24,48,72 and 96 hours and feces at 24,48,72 and 96 hours following administration of paregoric. Urine volumes and fecal weights were recorded, and all specimens frozen until analyzed. In one experiment, radioactive morphine (1.52 μCi/ml) was added to the paregoric before administration. Urine was analyzed by the FRAT method for total morphine and by the fluorometric method for free morphine. The radioactivity of urine, feces and blood was determined by liquid scintillation counting. The chemical analysis for fecal morphine is currently in progress.

The results showed: (1) Morphine was detectable in urine by chemical procedures up to 48 hours but not at 72 hours after the administration of paregoric. (2) Radioactivity studies showed that 97 percent of the labeled morphine was excreted in 48 hours. (3) The peak levels of morphine-^{14}C occurred in the blood and urine at 12 hours. (4) The excreted morphine-^{14}C was distributed about equally between the urine and feces. (5) The amount of unbound morphine in the urine was maximal (about 40 percent) at 6 hours after the administration of paregoric, and progressively decreased after that time.

TABLE 1

EXCRETION OF MORPHINE-^{14}C ADDED TO PAREGORIC AND ADMINISTERED ORALLY TO RATS

Time (Hours) after Administration	Percent of Administered Dose (Average of 3 animals)		
	Urine	Feces	Total Excreted
24	39.2*	41.3	80.5
48	9.7	12.2	15.9**
72	1.2	0.8	2.0
96	0.5	0.2	0.7

*Cumulative urine excretion up to 12 hours (29.5%); maximum urine excretion occurred between 6 to 12 hours (20.4%).

**Over 97% of the morphine-^{14}C was excreted in 48 hours.

Blood levels were maximal at 12 hours (0.6%).

7. Studies on Spin Labeled Morphine in the Rat.

Spin labeled morphine (SLM) has been used in in vivo and in vitro studies to elucidate the binding characteristics of this morphine analog in the CNS. In vivo studies have indicated that although SLM does not pass the blood brain barrier, it does reach the circulatory system after IP injection and migrates to heart and liver tissues. After intracerebral injection, it was retained by brain tissue and appeared to have a transitory physiological effect.

The conformation of SLM is sufficiently similar to that of morphine that it shows selective uptake by the hypothalamic synaptosomal fraction in in vitro studies. The active site of free morphine is reported to be in this synaptosomal fraction. Rotational correlation times (t) have been found by ESR to be consistently higher (indicating greater immobilization of the spin label) for SLM with hypothalamic synaptosomes and their subfractions than with other CNS components including myelin, microsomes and mitochondria. The degree of association between SLM and synaptosomal junctional complexes was significantly reduced when isolated from rats previously treated with morphine or heroin.

The association of SLM with synaptosomes and their subfractions is quite weak; no strong immobilization of the label was observed. Correlation times (t) of about 3×10^{-10} sec were the highest observed whereas free SLM has t values of about 1×10^{-10} sec. SLM dissociates easily from synaptosomal fractions during washing.

8. The Conjugation of Morphine with Glucuronic Acid in Humans

The production of glucuronic acid and its subsequent utilization in the liver as a major detoxification process is well documented. However, the variation in individual ability to conjugate morphine has not been documented. This factor may have considerable bearing upon the time at which physical addiction could take place based upon the assumption that free morphine produces the major pharmacological effect.

In a series of 16 patients given morphine as the principal analgesic for open heart surgery, the ratios of free to bound morphine were computed during the first twenty-four hours after initial administration. The results expressed as percent free morphine per ml of urine ranged from 5.9% to 81% suggesting a wide biological capability to utilize the glucuronic acid conjugation system. These studies are being continued.

Summary and Conclusions

The efforts accomplished during the reporting period have been largely devoted to the study of biochemical and physiological changes after the administration of morphine and heroin. Studies were accomplished at the cellular, organ and whole animal level. Several conclusions can be reached as a result of these studies:

1) Certain drugs of abuse such as Δ^9-Tetrahydrocannibinol may influence the uncoupling of oxidative phosphorylation to decrease available adenosine triphosphate; 2) heroin and morphine influence *in vitro* uptake of catecholamines; 3) paregoric (camphorated tincture of opium) is cleared from the rat in forty-eight hours and appears equally distributed between urine and feces; and 4) conjugation of morphine with glucuronic acid in humans appears to be highly variable.

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
DA 0A 6431	72 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
71 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]	CDOG 114(f)			

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

a. DATES/EFFECTIVE Not Applicable 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 72	3	60
CURRENT FISCAL YEAR 73	3	60

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Arboviruses; (U) Trypanosomes; (U) Ecology; (U) Mosquitoes; (U) Disease Vectors; (U) Tsetse flies; (U) Control

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) 71 07 - 72 06. Larval and adult mosquito collections were made from areas in Maryland, North Carolina, and Louisiana. Twenty isolations of Group A arbovirus were made from mosquitoes collected in Maryland, several more from North Carolina. Bloodmeals have been identified as to source from all collections. Basic biological studies of two potential arbovirus vectors, Culex salinarius and C. restuans showed striking differences in the effect of temperature upon various physiological processes. These differences help explain their phenology and geographic distribution. Studies on the relative susceptibility and transmission efficiency of C. tritaeniorhynchus and C. salinarius are complete. A detailed analysis of body parameters and flight potential of Anopheles stephensi was made. Transmission of Trypanosoma congolense to experimental animals by tsetse flies was studied. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 71 - 30 Jun 72.

...ible to contractors upon originator's approval

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PII Redacted

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03 Entomology

Work Unit 035 Ecology and control of disease vectors and reservoirs

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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 organisms pathogenic to man, and to hosts and reservoirs of such reservoirs. Included are ecological and physiological studies of arthropods, studies of transmission mechanisms and the development of improved methods of arthropod control.

Progress

1. Ecology of arboviruses in the eastern United States

a. Introduction. The field aspects of a program to investigate the ecology of arthropod-borne virus diseases in freshwater swamp habitats in eastern Maryland were completed at the end of CY71, except for a specialized study of overwintering mechanisms of potential vectors. Also completed was a 1-year study of a freshwater swamp in Cartaret County, North Carolina: the Croatan Forest. In the spring of CY 72, a new program was begun having as its objective the study of California encephalitis virus (CEV) in various ecological habitats in Maryland and Virginia. A related biosystematic study of the Anopheles crucians complex is also still in progress. This report covers field data accumulated since last year's annual progress report and also results of data analyses made since that time. 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 descriptions of the study sites, see WRAIR annual reports for previous years.

b. Pocomoke Swamp studies

(1) Mosquito population dynamics. The 1971 mosquito collecting season ended three years of systematic collecting of larvae and adults of potential Group A arbovirus vectors in and around the Pocomoke Swamp, Worcester County, Maryland. Description of the study area and

the objectives of this phase of the study are given in the WRAIR Annual Progress Report for the period July 1970 - June 1971. During 1971, 12,991 larvae were collected (Table 1). Three species, Aedes canadensis, Culiseta melanura and Culex restuans, were most commonly collected and comprised about 79% of the total larvae collected. Aedes canadensis was most frequently collected.

The data show that Aedes larvae predominated during the first half of the year. Aedes grossbecki and Aedes canadensis eggs hatch in the spring when the larval habitats are flooded with water. Ae. grossbecki appears to be strictly univoltine whereas Ae. canadensis, depending on rainfall in July and August, may have a partial second hatch later in the summer.

Culex, Culiseta melanura and Psorophora species larvae are found most abundantly during the second half of the year. Culex and Anopheles adults overwinter in the adult stage. Females do not become active until the air temperature becomes favorable for activity, perhaps by mid-May or later, and increased numbers of larvae for these species probably reflects the growing mosquito populations. The relatively large number of larvae collected for Culex restuans and Culiseta melanura during October to December, 1971, may be due to the mild temperatures during this period, and should be considered unusual.

Light trap sites utilized during the 1970 season were essentially the same as those for 1969. Whereas in 1969 at least two traps were operated in each ecological habitat type, in 1970 only one often was run. The collection frequency was also reduced from five times per week to once a week. Three sites were added in 1970. In order to assess the amount of movement of swamp species such as Culiseta melanura from the swamp to adjoining farmlands, sites were established at one, two, and three miles from the eastern edge of the swamp. The ecological description of these sites is given in Table 5.

To date, all adult and larval mosquito collections made through the end of the 1971 collecting season have been identified and pooled for virus isolation. All pools have been tested for virus in the hamster kidney cell system from mosquitoes collected in 1970.

During 1970, 195,958 adult mosquitoes were collected (Table 2). As in 1969, Aedes canadensis was the most abundant mosquito, comprising 82% of all mosquitoes collected. The collections of Culiseta melanura were down from *1969*, both in number and in percentage of total catch. In 1969, nearly 100,000 adult C. melanura were captured, amounting to 29% of the total catch. In 1970, however, 10,590 C. melanura were captured, comprising only 5% of the total catch. This drop is partially attributable to the reduction in trapping frequency between 1969 and 1970. In addition, the collection rate for this species was also down, indicating that the population levels were actually lower in 1970 than in 1969. The peak collection rate in 1969 was nearly 600 females per trap night -- in 1970 it was less than 200 females per trap night.

In 1971, 115,651 adult mosquitoes were collected (Table 2). Aedes canadensis again led all species (44,161; 38%), and although more C. melanura were collected than in 1970 (11,236 vs. 10,590) and they made up 10% of the total, they were only the fourth most abundant mosquito collected in 1971, behind A. canadensis, Culex salinarius and A. cantator. In 1969 and 1970, C. melanura was second in abundance, behind A. canadensis.

Three-week moving averages of collection rates for the four most abundant mosquito species, and precipitation data are shown in Figures 1-3 for 1969, 1970, and 1971. The relationship between precipitation and population levels is evident. In 1969, the large peak of population density of Culiseta melanura, Culex salinarius, and Aedes canadensis coincides with a period of abnormally high rainfall. The univoltine A. cantator did not respond to this, however. In 1970, precipitation was normal throughout the breeding season, and population levels of C. melanura were lower. Although there was no midsummer peak of A. canadensis, the spring emergence produced enormous numbers of adults, so that in spite of heavy rainfall in late July, no corresponding emergence took place. It is difficult to explain the reason for the large emergence of A. canadensis in early 1970, at least from the examination of rainfall data. There was actually more precipitation during March and April of 1969 than for these two months of 1970, yet no such emergence took place (Fig. 1). During the period of 20 March - 3 April 1970, however, 8.13 cm. of rain was recorded, and on 2 April 1970 the largest tide was recorded at the swamp of any since the studies began there in 1964. As a result of the examination of the precipitation data and the population data for the period 1969-71 and also previous years' data, the following generalizations are warranted:

Culiseta melanura. Larvae present every month of the year. Adults active during warmer months. Population size increases with rising water table, which in turn rises with abnormally high amount of precipitation. Little day to day fluctuation as a result of precipitation; responds more to longer trends in rainfall.

Aedes canadensis. Overwinters in egg stage. Apparently but one batch of eggs deposited per year, in summer. If oviposition sites are all flooded in spring because of very high tides or rainfall, entire brood will hatch in spring and population level of adults will be low in summer. If all sites are not flooded for a substantial period of time, however, additional hatching may take place later in the season after heavy rainfall. This apparently happened in 1969.

Aedes cantator. Overwinters in egg stage, and strictly univoltine, regardless of rainfall paterns.

Culex salinarius. Overwinters as adult. During warmer periods of year, population levels of adults appear directly dependent upon fluctuations in rainfall, with heavy emergences of adults following by about ten days increases in the amount of standing water.

(2) Mosquito bloodmeal identifications. Serological identification of mosquito bloodmeals has been completed for the 1970 Pocomoke Swamp collections. The results of the precipitin screening test of engorged mosquitoes is shown in Table 3. A detailed breakdown of the specific bloodmeal identifications for four important mosquito species in the swamp is presented in Table 4. Of significance are the unusually large number of engorged Anopheles crucians complex females tested with the bulk of the positives coming from goats or other bovine hosts. The data also confirm the strong preference of Culiseta melanura for avian blood. Aedes canadensis and Culex salinarius blood feeds show across the board host feeding, again contrasting their potential vector status from the standpoint of feeding habits with the lack of evidence of their vector status based on the almost complete lack of virus isolations in nature.

(3) Virus isolations. There were, from mosquitoes collected in 1970, twenty isolations of eastern equine encephalitis (EEE) and western equine encephalitis (WEE) (Table 5). Four isolates of EEE were from mosquitoes collected between 12 July and 27 September. Sixteen isolates of WEE were from mosquitoes collected between 12 July and 1 November. All isolations were from Culiseta melanura. Although the number of isolates for 1970 was almost identical with the number for 1969 (20 vs. 19), the isolation rate was much higher for 1970. In 1969, the rate for EEE was 1/14,747 Culiseta melanura females tested; for WEE 1/5,267. In 1970, the rates were EEE: 1/2,170; WEE 1/542. In 1970, mosquitoes were trapped at considerable distances from the swamp, whereas in 1969 all collections were made within a few thousand feet of the swamp. Significantly, four of the WEE isolations were made at a distance of two miles from the swamp, one at a distance of three miles. Culiseta melanura larvae were collected at sites 1 mile from the swamp, but none were collected at sites 2 nor 3 miles from the swamp. Thus these isolates are probably from adult female C. melanura which had flown from areas closer to the swamp, again suggesting a possible mechanism for movement of virus from swamp habitats into other areas.

In order to screen mosquito pools for arboviruses which can not be picked up in the routine hamster kidney cell system (i.e. California encephalitis virus) aliquots of pools of selected species were inoculated intercerebrally into suckling mice. The testing by this method of pools collected in 1969 has been completed; no viruses were recovered from 1,691 pools. Isolation attempts are now being carried out on a more selective basis from 1970 pools. To date, 521 pools of Aedes canadensis and 1 pool of A. atlanticus have been screened with negative results.

c. Croatan Forest study. During 1971, several trips were made to the Croatan Forest, Cartaret County, North Carolina. The purpose of these trips was to study freshwater swamps in that area and to compare the mosquito fauna, virus activity, and other ecological factors with the Pocomoke Cypress Swamp. An initial collecting trip was made 21 June, and followup trips 9-12 August. Adult mosquitoes were collected with CDC miniature light traps augmented with solid CO_2. Mosquitoes captured 9-12 August are shown in Table 6. In contrast with Pocomoke Swamp

collections, Aedes atlanticus-tormentor was by far the most abundant mosquito collected. Of 6,600 mosquitoes collected on this trip, 3,343 (51%) were this species. Twenty-seven per cent of the collection (1,785) were Culiseta melanura. About 15% of the collections were Anopheles crucians complex, a group of mosquitoes of very minor importance in the Pocomoke Cypress Swamp. Virus isolations, tentatively identified as WEE have been made from Culiseta melanura and a single isolate of EEE has been made from Anopheles crucians complex. This last species is poorly known ecologically and deserves further study. Although Aedes atlanticus-tormentor (the two species cannot be separated in the adult stage) furnished no isolates, its involvement as an arbovirus vector elsewhere indicates its potential importance. Apparently this species group, of generally southern U.S. distribution assumes dominance here over the northern ranging A. canadensis. The opposite is true in eastern Maryland.

The results of the precipitin screening tests of engorged female mosquitoes collected in the Croatan Forest in 1971 are shown in Table 7. Interestingly, 10 of 28 Aedes atlanticus-tormentor females tested fed on reptilian blood. In contrast, no reptilian blood meals were noted out of 12 individuals of this species collected in the Pocomoke Swamp and tested in 1970, and only 1 of 26 tested in 1969. It may be that the species present in the Pocomoke Cypress Swamp are Aedes atlanticus, whereas the species present in North Carolina swamps is A. tormentor, this accounting for the difference in feeding pattern.

d, California encephalitis virus survey. CEV is known to occur in at least some of its serotypical forms in Maryland. The Keystone strain of the virus has been isolated from Aedes atlanticus, A. canadensis, and A. vexans (Sudia, et al. 1971). Last year data were presented showing that racoons, rabbits, and deer had Keystone antibody conversion rates of from 10 to 40%, based on collections made at Snow Hill, Md., Wallops Island, Va., Assateague Island, Va., and Pocomoke Cypress Swamp, Md. (WRAIR *Annual* Progress Report for 1971, p. 697 and Table 11).

In order to determine the extent of Keystone virus activity in eastern Maryland, and to determine if the more important LaCrosse and Trivittatus strains are present in this area, collections of mammals and mosquitoes were begun 1 May 1972. Collections were begun in four ecologically diverse habitats separated by considerable distance geographically. These areas are: an upland forest near Shad Landing State Park, Snow Hill, Maryland; a coastal pine forest on Assateague Island, Virginia; and upland forest at Wallops Station, Virginia; and an upland forest adjacent to the Pocomoke Cypress Swamp, Maryland. Collections will be made at the four sites on a rotational basis so that each site will be sampled monthly during the summer and fall period. Additional studies are planned for sites at higher elevations in mid- and western Maryland later in 1972. These sites are probably better suited for the presence of LaCrosse and Trivittatus viruses.

2. VEE surveillance in the southwestern United States

As a result of the 1971 epidemic of Venezuelan equine encephalitis in southern Texas in 1971 and the resulting national emergency, the U.S. Public Health Service and the U.S. Department of Agriculture requested assistance from the Department of Defense in the form of ten teams of entomologists and technicians plus necessary laboratory support to conduct surveillance of mosquitoes for infection with VEE in a four state area, forming a band surrounding the known epidemic area. Five entomologists from WRAIR were involved in the effort full time during the month of August and September. The implementation, supervision, identification and pooling of mosquitoes, and reporting were accomplished by WRAIR personnel (See Eldridge, B.F., 1971; VEE Surveillance Team Final Report, WRAIR, Wash., D.C., 27 Oct 71).

As a followup to an isolation of VEE made at the U.S. Army Medical Research Unit of Infectious Diseases (USAMRIID) from a pool of mosquitoes collected at Basile, Evangeline Parish, Louisiana, a team from WRAIR returned to Basile in November and collected both mosquitoes and mammals. The isolate was eventually determined to be the TC-83 (vaccine) strain of virus, inducing speculation that for the first time in nature, a mosquito had been found infected as a result of having fed on a recently vaccinated animal. A total of 40,446 mosquitoes were collected, all of which proved negative for virus.

The results of precipitin screening tests of 700 engorged female mosquitoes collected during the VEE surveillance are shown in Table 8. These specimens were all collected with CO_2 baited light traps. They illustrate again the few number of species that feed with appreciable frequency on both birds and mammals. This is particularly true of the species from which most isolates of epidemic VEE were obtained -- Psorophora confinnis and other species of this genus, Aedes sollicitans and A. taeniorhynchus -- all engorged females were found to have fed only on mammals, usually of the large domestic variety.

3. Basic biology studies

a. Overwintering of culicine mosquitoes. Research has continued on the overwintering mechanisms and phenology of two common eastern U.S. mosquitoes: Culex restuans Theobald and C. salinarius Coquillett. Interest in these two species centers around the possibility that one of them may serve as an overwintering host for Group A arboviruses. Preliminary results reported last year indicated that both species underwent true diapause in the adult stage, but that C. restuans had an entirely different response, both quantitatively and qualitatively, than did C. salinarius (Eldridge, et al., 1972). Results reported last year included test temperatures no lower than 20°C, at which point C. salinarius showed no reduction in blood feeding drive in response to short photoperiod. When both species were subjected to various photoperiods at 15°C, however, C. salinarius females showed some reduction in blood-feeding at shorter photoperiods, but never any gonotrophic dissociation (ovaries

failing to develop after a full bloodmeal). C. restuans, on the other hand, showed a drastic and almost complete cessation of bloodfeeding at short photoperiods at 15°C and those which did feed showed gonotrophic dissociation. These studies also showed that C. restuans ranged considerable farther north and west in the U.S. than did C. salinarius.

Recent studies have examined the effect of temperature on various physiological processes such as ovarian development and larval growth. The relationship between temperature, and ovarian development and blood digestion for the two species is shown in Fig. 4. The degree of ovarian development and blood digestion is based on the system of Sella (1920) which estimates these processes according to the visual appearance of the mosquitoes' abdomen. The difference in response between the two species is marked. At all temperatures, these processes proceeded at a more rapid pace in C. restuans than in C. salinarius and generally took about two days longer for completion in the latter species. Interestingly, complete ovarian development eventually was completed in both species, even at 10°C, in contrast with C. pipiens, where these processes are completely suspended at this temperature (Eldridge, B.F., unpublished data). The ability of C. restuans to complete ovarian development after a blood meal in a relatively short time at temperatures as low as 10°C helps explain its more northern distribution and also its earlier appearance in the season. The difference in response is not due to geographical adaptation, since the mosquitoes used both came from the same location (eastern Maryland).

A more detailed study of the response of C. salinarius to temperature and photoperiod is almost completed (Fig. 5). These results largely confirm our preliminary results, but suggest a non-linearity of the photoperiod response. Apparently, a photoperiod of L:D 11:13 induces a drop in bloodfeeding response, whereas L:D 9:15 does not. Although this type of response has not previously been shown in mosquitoes, it is known in other insects (Beck, 1968).

b. Comparative mosquito infection and transmission of Sindbis virus. The invasion and replication of arboviruses in various mosquito species is poorly understood. Electron microscope studies of the replication in midgut tissues of two viruses are in progress in a collaborative study with the Institute of Cell Research, University of Texas. Initially, Sindbis and mosquito irridescent virus (MIV) are being studied in susceptible) and C. salinarius (resistant) for Sindbis virus and Aedes taeniorhynchus (susceptible) and A. aegypti (resistant) for MIV. Work with MIV was temporarily suspended after selecting the appropriate mosquito hosts due to problems in producing stocks of this virus. The studies now reported will describe the mosquito infectivity and transmission of Sindbis virus.

The Ar 339 strain of Sindbis virus was used in its 10th suckling mouse brain passage to prepare virus seed stock. Viruses were passed by intracerebral passage in suckling white mice (ICR)BR strain. Sick mice were sacrificed at 48 and 72 hours and brains prepared as 20% smb in 4% BAPS. The infectivity of this seed stock showed a titer of 8.0/0.02 ml.

expressed as $\log_{10}$ LD_{50}. Sindbis hyperimmune serum for identification of viruses by neutralization tests was prepared by intraperitoneal injection of rabbits with 1 ml of 20% smb Sindbis Ar 339 strain followed by 2 boosters at weekly intervals.

An initial infectivity comparison of 14 day-old Culex tritaeniorhynchus and 7 day-old C. salinarius was made by feeding mosquitoes on a 1:3 mixture of stock virus in defibrinated chicken blood on a membrane feeder at 37°C. Titers of the blood-virus mixture did not differ significantly before and after mosquito feeding (C. tritaeniorhynchus averaged 6.1 logs and C. salinarius, 5.5 logs/0.02ml.). Two weeks after the infectious meal, 15 females of each species were killed, individually triturated and inoculated into suckling mice to determine infectivity levels. 93.3% of the C. tritaeniorhynchus and 46.7% of the C. salinarius were positive for Sindbis virus. C. tritaeniorhynchus was definitely more susceptible than C. salinarius to this virus. It was thought that greater differences between the species might be observed if a more dilute suspension of virus was used in the membrane feeder.

A second experiment was conducted under similar conditions with 5-6 day-old mosquitoes feeding for 45-60 minutes on a 1:4 blood-virus mixture. Pre and post-feed titers of this feeding mixture averaged 6.4 logs. Three mosquitoes of each species were frozen 1 hour after feeding and 15 mosquitoes were removed at 7 day intervals for 3 weeks to determine percent infection and virus titer levels, The ratio of infected C. tritaeniorhynchus to C. salinarius remained approximately 2:1 (Table 9). Virus titers of both species was maximal on day 7. No appreciable differences were evident over the following 2 weeks.

Comparative studies on transmission rates of the two culicines were made with 3 day-old white leghorn chicks as recipient hosts. Three weeks after an infective meal, mosquitoes were fed singly on the normal chicks. After feeding, the mosquitoes were frozen to determine infectivity and the chicks were bled (0.1 ml) on days 2, 3 and 4 for detection of viremia. A 10% suspension of this blood was inoculated into 1-3 day old mice. The transmission rates of both Culex species were lower than their respective infection rates (Table 10), but in the same 2:1 ratio as the infection rates. It may be concluded that both culicines can serve as vectors of Sindbis virus as some birds can circulate more than $\log_{10}$ LD_{50} 6.0 of virus.

The possibility of transovarian transmission of Sindbis virus was investigated in one experiment. Eggs were collected from infected C. tritaeniorhynchus and C. salinarius at 7, 14 and 21 days post-exposure to $\log_{10}$ LD_{50} 6.4 of Sindbis virus. Their infection rates were previously shown to be 93.3% respectively. Twenty-one day old mosquitoes were pooled in lots of 25 and inoculated into baby mice. All pools were negative for virus.

An attempt was made to infect 1st stage larvae of both Culex species with a purified suspension of Sindbis virus to study the developmental cycle of the virus in the vector. Previous work of Peleg (1965) indicated that larval Aedes aegypti could be infected with Sindbis virus and subsequently transmit it. Groups of 100 larvae were placed in small containers with 40 µl purified Sindbis virus (furnished by Department of Virus Diseases, WRAIR) and 20 µl water for 24 hours. The resultant mosquitoes were allowed to develop and were killed on day 7 for virus determination by individual inoculation into suckling mice. No evidence of virus was present. Four other combinations of this virus with a fixed tracer virus (granulosus virus of the cabbage looper) also failed to disclose the presence of Sindbis virus when material was studied with the electron microscope. This experiment will be repeated upon receipt of more purified Sindbis virus.

c. Mosquito flight studies. The relationship between body parameters and flight potential is important in considering the vector potential of various species. In addition, various environmental factors, either external, such as temperature and humidity, or internal, such as the presence of insect pathogens or infectious agents may exert an effect upon the flight potential of a vector. This study examined the role of some of these factors in the flight performance of Anopheles stephensi.

Female A. stephensi (India strain) were mass reared under standard conditions in the WRAIR Insectary (see Annual Progress Report FY71, pp. 1101-02). Mosquitoes were starved for periods from 2-5 days after which time 20 females were removed, weighed and placed in individual vials. They were then allowed access to a 10% sucrose solution for 24 hours. The surviving mosquitoes were reweighed to determine weight gain and then attached to the flight mill arm. Mosquitoes were flown on flight mills, slightly modified from those designed by Rowley, et al. (1968). Attachment of mosquitoes to the arm of the flight mill was made with a low melting point wax and 0.005" nichrome wire. Twenty-five mosquitoes of ages 3, 4, 5 and 6 days were flown for a 24 hour period in light at an average temperature of 24.2°C and a relative humidity of 74.7%. Flight distance was recorded at the end of 24 hours and the mosquitoes reweighed to determine weight lost during flight. Later these mosquitoes were placed in a drying oven for subsequent determination of dry weight. Analyses consisted of multiple correlation and analysis of variance of flight distance to age, pre-feed initial weight, pre-flight initial weight, weight gain, post-flight weight, weight lost, dry weight, wing length, wing loading ratios, temperature and relative humidity during flight.

There was no significant effect of age on flight distance (Table 11). This confirms previous work reported by Rowley and Graham (1968a) for Aedes aegypti whose day to day flight distance differences were not evident. The combined multiple correlation of age to flight distance was slightly negative and non-significant.

The initial weight of mosquitoes, prior to food intake, was significantly different for the 4 ages tested. All combinations of age contrasts were significant at the 1% level. Three day old mosquitoes were heaviest (Table 12). The combined correlation of initial pre-feed weight to distance indicated that this variable had no overall significant affect on flight distance. Significant correlations with distance were obtained for 3 and 4 day old mosquitoes. The decreasing correlations with age suggest a significance of carbohydrate reserves carried over from the pupal stage. Nayar and Van Handel (1971) indicated that 5-6 days of starvation were required to eliminate larval reserves of glycogen carried over from the pupal stage in Aedes sollicitans and A. taeniorhynchus.

The initial pre-flight weights (Table 12) were not different for the ages tested. These weights showed a positive correlation with flight distance (Table 13) with days 3 and 4 being the most significant. This contrasts to the flight performance of A. aegypti where Rowley and Graham (1968a) found very little correlation (approximately 20%) between initial weight and their overall flight performance.

The amount of weight gained by mosquitoes was not significantly different for age groups. This weight is a measure of the difference between the initial (pre-feed) weight and the weight immediately before flight and represents the relative intake of 10% sucrose solution. This variable was highly significant when correlated with flight distance. This positive correlation was expected as it represents the primary source of flight energy.

Post-flight mosquito weight (Table 14) did not substantially vary by age. The correlation of 3 day old mosquitoes indicated a significant positive coefficient while other ages showed increasing negative correlation of post-flight weight to flight distance. It is believed that the greater amounts of body water and carbohydrate reserves present with the younger mosquitoes caused this effect.

The amounts of weight lost are shown in Table 14. The weights were significantly different for the ages tested at the 5% level, (Table 11). Multiple comparisons of weight loss means were made using the "q" statistic. This test statistic showed that all age comparisons were significantly different at the 1% level except days 4 and 5 which were not different. In all cases the amount of weight lost during flight was greater than the amount gained prior to flight. Mosquitoes lost approximately 51% of their pre-flight weight during the 24 hour flight period. The correlation of weight lost to flight distance had an overall significant effect with the earlier ages showing greater correlation. Weight lost expresses the difference between initial (pre-flight) weight and post-flight weight and represents the loss of hydrated glycogen during the 24 hour flight period as well as additional water lost due to respiration.

Mean mosquito dry weight for the ages tested are shown in Table 14. All ages were significantly different (Table 11). No significant

correlation between dry weight and distance flown was discernible. The higher coefficients for the 3 and 4 day old mosquitoes might be expected due to relative greater amounts of triglycerides remaining after flight which are used during resting metabolism and which might cause the higher dry weights in these mosquitoes. Triglyceride reserves would be much lower by the 4th and 5th day of starvation and although they have no affect on flight distance would affect the correlation coefficients.

On purely aerodynamic grounds it has long been recognized that wing length and its relation to some aspect of body weight affects flight. Wing length is extremely variable and may be affected by rearing temperature. In addition it is also affected by air temperature at the hour of emergence (Van Den Heuvel, 1963). This variable was measured to help account for flight distance variance and to allow computation of wing loading ratios. No overall significant correlation with flight distance occurred.

Wing loading ratios (Table 15) were not different for the different age groups nor did they have a significant correlation with flight distance (Table 13). These were calculated by dividing the dry weight (µg) by the wing length^3. Lower wing loading is thought to make flight more efficient, however, in this study it was observed that this pattern did not exist with the ages tested.

The distance flown (Table 15) by mosquitoes of the 4 ages compared did not significantly differ (Table 11). However analysis of variance (Table 16) showed that flight distance is significantly affected by weight gain and can be significantly correlated with mosquito weight prior to an after flight. Initial (pre-feed) weight, dry weight, wing length, wing loading ratios, temperature and relative humidity within the ranges flown had no significant affect or correlation with distance flown.

The average temperature during the 24 hour flight periods was 75.58°F $\pm$ 2.09 standard deviations and the relative humidity was 74.70% $\pm$ 4.28. These variations could account for some of the unexplained flight distance variance. It has been shown by Rowley and Graham (1968b) that temperature markedly influences flight ability of A. aegypti by showing increased flight distance to decreases in temperature but that relative humidity within the range of 30-90% had little demonstrable affect on their flight activity.

During previous flight studies (Schiefer, unpublished data) an extremely high amount of variation in flight distance was observed in Anopheles stephensi reared under similar environmental conditions. The multiple regression technique allows the determination of the percent variance of the dependent variable accounted for by each of the independent variables. These percentages are shown in Table 17. It can be seen that less than half of the variation in flight distance can be

accounted for by the variables measured. Other known variables affecting flight distance which were not measured are carbohydrate reserves carried over from the pupal stage and time between feeding and flight within the 24 hour feeding period (Nayar and Van Handel, 1971). There are many other variable factors that could affect flight indirectly such as larval rearing temperature (Van Den Heuvel, 1963, larval density, larval diet, water conditions, pupal density and length of pupal period, photoperiod during immature stages, temperature at the time of emergence, metabolism rates (Nayar and Van Handel, 1971), wingbeat frequency, thorax length, uniformity of attachment to flight mill, disease organisms, appetential stimuli and genetic selection. Certain factors affecting flight which were held constant are food availability, air currents, photoperiod during flight, light intensity, wave length, sex, virginity and oogenesis.

4. Tsetse fly studies

It was recently demonstrated that colonized Glossina morsitans could cyclically transmit Trypanosoma congolense to white mice (Elce, 1971). This investigation was initiated to provide infected tsetse flies for use in immunization studies. Several preliminary studies were conducted with the Trans-Mara I strain of T. congolense, 2 tsetse species (G. austeni and G. morsitans) and several experimental host species to determine the most suitable combination to be used as a model for the transmission of this parasite.

Although the Trans-Mara I strain was maintained only by blood transfer in cattle and mice since its isolation in 1966, this apparently did not affect tsetse infectivity levels. Unlike the T. brucei complex, there was no indication that age of the adult tsetse was related to infection by T. congolense. Flies which were one day or younger in age exhibited the same level of infection as flies 1-2 weeks of age. Dissection of samples of the 2 tsetse species which fed on parasitemic hosts revealed similar levels of infection: 8/58 or 13.8% of G. austeni were infected while 5/17 or 29.4% of G. morsitans developed infections. There were no significant differences in the infection level between male and female tsetse flies. White mice, calves, and white rabbits have served as donor hosts; all of which have produced infective tsetse flies. The rabbit appears to be the most practical species as a source to infect large numbers of flies.

G. austeni apparently do not become non-infective as they age and transmission has been effected to mice with single flies 100 days old.

Summary and Conclusions

1. The field data collection phase of a study of the ecology of Group A arboviruses in eastern U.S. freshwater swamps has been completed. A 3-year study of vector populations failed to implicate any mosquito other than Culiseta melanura in the endemic phase of the

transmission of eastern and western equine encephalitis. Blood meal identification studies and mosquito population studies indicate that various ecological barriers exist tending to confine these diseases to swamp habitats in years of normal or near normal weather conditions. Much of the information obtained on other mosquito species which are not involved in EEE and WEE transmission can be used in studies on the ecology of California encephalitis. Such studies are now underway. The blood feeding preferences and population dynamics of species such as Aedes atlanticus, A. cantator, A. canadensis, and A. triseriatus should be examined in the context of their role as possible CEV vectors.

2. Studies on the winter biology of Culex restuans and C. salinarius show that these two species differ considerably in the response of various physiological processes to temperature and other environmental factors. Studies should continue on the overwintering habits of the species in nature as well as the artificial overwintering of virus-infected individuals in simulated winter environments.

3. Preliminary studies on the susceptibility of various species of mosquitoes to arboviruses (Sindbis) and mosquito viruses (mosquito irridescent virus) suggest that adequate models are available for the study of virus replication in susceptible and resistant mosquito species. Such studies should be carried out, using techniques such as electron microscopy.

4. A comprehensive study of the effect of 11 variables on flight performance on a mechanical flight mill by female Anopheles stephensi was done using a computer program which performed multiple correlation and multivariate analysis of variance. Variables analyzed were: age, pre-feed initial weight, pre-flight initial weight, weight gain, post-flight total (wet) weight, weight lost, dry weight, wing length, wing loading ratio, temperature during flight, and relative humidity during flight. Although some variables showed significant correlation for certain ages of mosquitoes, the only variables which showed a significant correlation at all ages tested were pre-flight weight (after adult feeding), weight gain, and post-flight weight. These variables are clearly related to the amount of carbohydrate ingested prior to flight. The study of variation will permit sound experimental designs for the study of other factors such as infection, diapause, and other ecological factors. The difficulty in measuring carbohydrate intake and metabolism may preclude this method of assessing the effect of other variables.

5. Studies on the transmission of Trypanosoma congolense by various species of tsetse flies showed that the age of the fly did not alter susceptibility to infection.

TABLE 1

Larval mosquito collections, 1971, Pocomoke Cypress Swamp, Maryland (Number of Larvae)

Species	Month												
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
Aedes canadensis	48	315	2334	2367	0	17	607	0	57	13	9	71	5838
Culiseta melsnura	55	47	184	56	0	197	386	32	720	271	41	236	2225
Culex restuans	0	0	0	0	0	202	144	0	303	464	828	248	2189
Culex territans	0	0	0	0	0	154	246	77	341	47	18	20	903
Orthopodomyia signifera	0	1	0	0	0	16	49	99	177	73	0	0	415
Aedes triseriatus	0	16	45	31	0	26	72	4	0	0	0	0	194
Culex spp.	0	0	0	0	0	3	5	0	92	80	0	0	180
Culex salinarius	0	0	0	0	0	5	92	3	46	3	9	1	159
Aedes vexans	0	1	0	0	0	8	97	8	19	11	5	0	149
Aedes spp.	0	0	129	0	0	2	0	0	0	1	1	0	133
Anopheles spp.	0	0	0	0	0	0	13	6	46	56	4	2	127
Culex pipiens	0	0	0	0	0	0	0	12	97	3	0	0	112
Psorophora ferox	0	0	0	0	0	0	75	2	5	19	1	0	102
Other species*	0	2	13	9	0	1	23	8	42	3	0	0	101
Aedes grossbecki	4	5	72	9	0	0	0	0	0	0	0	0	90
Aedes atlanticus	0	0	0	0	0	1	58	0	1	13	1	0	74
	107	387	2777	2472	0	632	1867	251	1946	1057	917	578	12,991

* Other Species - Uranotaenia sapphirina (20), Toxorhynchites rutilis septentrionalis (16), Aedes cantator (14), Aedes aurifer (10), Anopheles quadrimaculatus (5), Aedes sollicitans (5), Anopheles punctipennis (2), Anopheles barberi (1), Psorophora howardi (1), Aedes infirmatus (1).

TABLE 2

Mosquitoes collected by light trap, Pocomoke Cypress Swamp, Maryland
1970 and 1971

Species	1970 Number collected	1970 Per cent	1971 Number collected	1971 Per cent
Aedes canadensis	161,164	82.24	44,161	38.19
Culiseta melanura	10,590	5.41	11,236	9.72
Culex salinarius	10,487	5.35	21,688	18.75
A. cantator	4,338	2.21	15,398	13.31
Mansonia perturbans	2,280	1.16	6,653	5.75
Aedes spp. (damaged)	1,666	0.85	573	0.50
Anopheles crucians complex	1,823	0.93	2,541	2.20
A. atlanticus	1,129	0.58	8,683	7.51
A. vexans	458	0.23	1,639	1.42
A. sollicitans	126	0.06	1,053	0.90
Other species*	1,897	0.98	2,026	1.75
Totals	195,958	100.00	115,651	100.00

*Uranotaenia sapphirina, A. triseriatus, C. restuans, A. aurifer, Psorophora ferox, An. punctipennis, A. taeniorhynchus, P. confinnis, A. infirmatus, C. pipiens, An. quadrimaculatus, C. territans, A. grossbecki, P. howardii, Orthopodomyia signifera, C. erraticus, A. cinereus, A. thibaulti, P. ciliata, Culiseta inornata, A. fulvus-pallens and A. trivittatus (In decreasing abundance for 1970).

TABLE 3

Precipitin screening tests of engorged mosquitoes, Pocomoke Cypress Swamp, 1970

Mosquito species	Number tested	Number positive to anti-sera of:			Number negative
		Bird	Mammal	Reptile	
Aedes atlanticus-tormentor	12	0	11	0	1
A. aurifer	1	0	1	0	0
A. canadensis	477	15	292	162	8
A. cantator	9	0	9	0	0
A. infirmatus	2	0	2	0	0
A. sollicitans	22	1	21	0	0
A. taeniorhynchus	13	0	13	0	0
A. triseriatus	6	0	5	1	0
A. vexans	23	0	23	0	0
Anopheles crucians complex	355	2	353	0	0
An. punctipennis	16	1	15	0	0
An. quadrimaculatus	4	0	4	0	0
Culex pipiens	3	3	0	0	0
C. restuans	2	2	0	0	0
C. salinarius	909	99	798	12	0
Culiseta melanura	29	29	0	0	0
Mansonia perturbans	93	14	79	0	0
Psorophora confinnis	8	2	6	0	0
P. ferox	2	0	2	0	0
Totals	1,986	168	1,634	175	9

TABLE 4

Host feeding patterns of four important species of mosquitoes
Pocomoke Cypress Swamp, 1970

Host	Percentage of precipitin tests positive			
	Aedes canadensis	*Anopheles crucians* complex	*Culex salinarius*	*Culiseta melanura*
Mammal Feedings				
Deer	23.9	9.9	4.4	0
Goat	0	45.9	35.5	0
Bovine	0.4	41.4	41.7	0
Horse	0	0	0.7	0
Human	14.6	0	0.3	0
Opossum	5.9	0	1.4	0
Pig	0.6	2.0	2.3	0
Rabbit	8.0	0	0.1	0
Racoon	8.0	0	0.4	0
Unknown	0.8	0.3	1.0	0
Mammal subtotal	62.5	99.4	87.8	0
Bird Feedings				
Ciconiiformes	0.6	0	0	3.5
Columbiformes	1.9	0	0	3.5
Galliformes	0.2	0.3	10.9	34.5
Gruiformes	0.4	0	0	6.9
Passeriformes	0	0	0	51.7
Unknown	0	0.3	0	0
Bird subtotal	3.2	0.6	10.9	100.0
Reptile and Amphibian Feedings				
Snake	0.2	0	0.2	0
Turtle	34.1	0	1.1	0
Rept./Amphib. subtotal	34.3	0	1.3	0
Total number of positive tests	472	353	910	29

TABLE 5

Viruses isolated from Culiseta melanura collected in Worcester County, Maryland -- 1970

Date of Mosquito Capture	Location (Site number*)	Habitat Type	Virus
12 July	11	Upland forest	EEE
12 July	22	Early secondary forest	WEE
19 July	7	Closed root mat swamp	WEE
20 July	14	Cultivated area	WEE
26 July	22	Early secondary forest	WEE
26 July	22	Early secondary forest	WEE
28 July	20	Cultivated area	WEE
2 August	7	Closed root mat swamp	WEE
9 August	14	Cultivated area	WEE
9 August	22	Early secondary forest	WEE
11 August	6	Closed root mat swamp	WEE
14 August	7	Closed root mat swamp	WEE
14 August	3	Closed root mat swamp	WEE
21 August	14	Cultivated area	WEE
23 August	23	Upland forest	WEE
6 September	14	Cultivated area	WEE
20 September	7	Closed root mat swamp	EEE
27 September	7	Closed root mat swamp	EEE
27 September	7	Closed root mat swamp	EEE
1 November	3	Closed root mat swamp	WEE

*For description of sites 1-17, see 1971 WRAIR Annual Progress Report, p. 718. Site 20 is adjacent to the eastern boundry of the swamp. Sites 22 and 23 are 2 and 3 miles to the east of the swamp, respectively.

TABLE 6

Mosquitoes collected by light trap, Croatan Forest, Cartaret County North Carolina, 9-12 August 1971

Species	Number collected	Per cent
Aedes atlanticus-tormentor	3,343	50.64
Culiseta melanura	1,758	27.04
Anopheles crucians complex	975	14.77
Aedes canadensis	269	4.08
Culex salinarius	54	0.82
Psorophora ferox	50	0.76
Other species*	125	0.89
Totals	6,601	100.00

*_Uranotaenia sapphirina_, _Mansonia perturbans_, _Aedes triseriatus_, _A. infirmatus_, _A. dupreei_, _Culex territans_, _C. pipiens_, _Psorophora ciliata_. (In order of decreasing abundance).

TABLE 7

Precipitin screening tests of *engorged* mosquitoes, Croatan Forest North Carolina, 1971

Mosquito species	Number tested	Number positive to anti-sera of: Bird	Mammal	Reptile	Number negative
Aedes atlanticus-tormentor	28	1	16	10	1
A. canadensis	1	0	1	0	0
A. infirmatus	1	0	1	0	0
Culiseta melanura	1	1	0	0	0
Psorophora ferox	1	0	1	0	0
Total	32	2	19	10	1

TABLE 8

Precipitin screening tests of engorged mosquitoes, Southwestern U.S., 1971

Mosquito species	Number tested	Number positive to anti-sera of:			Number Negative
		Bird	Mammal	Reptile	
Aedes atlanticus-tormentor	18	2	14	0	2
A. atropalpus	1	0	1	0	0
A. dorsalis	1	0	1	0	0
A. dupreei	2	0	2	0	0
A. fulvus pallens	1	0	1	0	0
A. nigromaculus	66	0	66	0	0
A. sollicitans	10	0	9	1	0
A. sticticus	2	0	2	0	0
A. taeniorhynchus	3	0	3	0	0
A. thelcter	9	0	9	0	0
A. vexans	111	0	111	0	0
A. spp.	3	0	3	0	0
Anopheles crucians complex	2	0	2	0	0
An. pseudopunctipennis	1	0	1	0	0
An. punctipennis	1	0	1	0	0
An. quadrimaculatus	3	0	3	0	0
Culex coronator	7	0	7	0	0
C. quinquefasciatus	9	5	4	0	0
C. salinarius	137	2	132	3	0
C. tarsalis	26	6	20	0	0
C. (Culex) sp.	31	2	29	0	0
C. (Melanoconion) sp.	31	5	26	0	0
Culiseta melanura	1	1	0	0	0
Mansonia perturbans	1	0	1	0	0
Psorophora ciliata	3	0	3	0	0
P. confinnis	72	0	67	4	1
P. cyanescens	114	0	108	0	6
P. discolor	1	0	1	0	0
P. ferox	3	0	2	0	1
P. signipennis	13	0	13	0	0
P. spp.	4	0	4	0	0
Culicidae spp.	13	0	13	0	0
Totals	700	23	659	8	10

TABLE 9

Comparative levels of infectivity and virus titers of Sindbis virus in Culex species

Days post-infection	Culex tritaeniorhynchus		Culex salinarius	
	Positive mosquitoes No. tested	Virus titers	Positive mosquitoes No. tested	Virus titers
0	3/3	3.6,3.5,3.4	3/3	4.1,4.2,3.7
7	15/15	3.3,4.6,3.8	10/15	5.0,4.2,4.7
14	15/15	3.7,4.1,3.6	8/15	3.7,3.8
21	14/15	3.6,3.9,3.4	9/15	3.0,3.7

*Expressed as mouse IC $\log_{10}$ LD_{50} per 0.02 ml.

TABLE 10

Comparison of transmission rates of Sindbis virus by two Culex species after 21 days extrinsic incubation

Species	Positive mosquitoes/ no. tested	Detection of viremia in chicks bled at following intervals:		
		Day 2	Day 3	Day 4
C. tritaeniorhynchus	14/15	9/15	10/15	1/14
C. salinarius	9/15	9/15	4/15	4/15

TABLE 11

Univariate analyses of variance for eight measured variables for 4 ages of female Anopheles stephensi

Source	SS	DF	MS	F
Days	0.480	3	0.160	4.706**
Initial Wt (pre-feed)	3.257	96	0.034	
Days	0.647	3	0.216	1.279
Initial Wt (pre-flight)	16.217	96	0.169	
Days	0.973	3	0.324	2.204
Weight gain	14.150	96	0.147	
Days	0.300	3	0.100	1.563
Post-flight weight	6.160	96	0.064	
Days	0.917	3	0.306	2.833*
Weight lost	10.370	96	0.108	
Days	0.031	3	0.010	5.000**
Dry Weight	0.190	96	0.002	
Days	2.292	3	0.764	0.507
Wing loading ratio	144.792	96	1.508	
Days	12.998	3	4.333	0.828
Distance	502.353	96	5.233	

* significant at 5% level
** significant at 1% level

TABLE 12

Initial (pre-feed) weight, initial (pre-flight) weight and weight gained during exhaustive flight by 100 female Anopheles stephensi

Day	Initial(pre-feed)wt. mg ± S.D.	Initial(pre-flight)wt. mg ± S.D.	Wt. gained mg ± S.D.
3	1.704 ± 0.197	2.542 ± 0.466	0.838 ± 0.372
4	1.518 ± 0.239	2.579 ± 0.381	1.062 ± 0.345
5	1.620 ± 0.149	2.655 ± 0.392	1.035 ± 0.449
6	1.664 ± 0.132	2.753 ± 0.399	1.089 ± 0.361

TABLE 13

Interdependent correlation coefficients for twelve variables for flight distance

Age	Age in days 3	4	5	6	Combined Age
Initial (pre-feed) weight	.546**	.561**	.325	.149	.132
Initial (pre-flight)weight	.784**	.727**	.318	.071	.483**
Weight gain	.692	.415*	.170	.024	.445**
Post flight weight	.545**	-.067	-.378	-.486*	.403**
Weight lost	.839**	.614**	.616**	.300	.254*
Dry weight	.330	.247	.083	.104	.171
Wing length	.624**	.244	.575**	.137	.108
Wing Loading Ratio	-.206	.589**	.084	-.324	.095
Temperature	.006	-.485*	.059	.253	-.037
Relative humidity	.129	.534**	.353	.461*	.079

* significant at 5% level
** significant at 1% level

TABLE 14

Post-flight weight, weight lost, and dry weight after exhaustive flight by 100 female Anopheles stephensi

Day	Post-flight wt. mg ± S.D.	Weight lost mg ± S.D.	Dry weight mg ± S.D.
3	1.404 ± 0.177	1.138 ± 0.345	0.398 ± 0.051
4	1.252 ± 0.294	1.327 ± 0.262	0.368 ± 0.031
5	1.345 ± 0.276	1.310 ± 0.401	0.414 ± 0.060
6	1.353 ± 0.250	1.399 ± 0.456	0.407 ± 0.028

TABLE 15

Wing loading ratios, and distance flown during exhaustive flight by 100 female Anopheles stephensi

Day	Wing loading ratio Ratio ± S.D.	Distance flown Km ± S.D.
3	10.812 ± 1.270	5.479 ± 1.949
4	10.391 ± 1.163	4.753 ± 2.113
5	10.619 ± 1.473	4.666 ± 1.943
6	10.573 ± 0.952	5.368 ± 2.982

TABLE 16

Analyses of variance "F" values for multiple linear regression for various variables 1/ to flight distance

Variables	Days 3	4	5	6	Combined Ages
2,3,8	2.093	4.647*	5.046**	3.843*	----
2,3,4,8	1.448	3.392*	3.608**	2.669*	----
2-11	1.282	2.368	1.417	3.809*	----
1,2,3,8	--	--	--	--	7.866**
1,2,3,9	--	--	--	--	7.884**
1,2,3,7,8	--	--	--	--	6.238**
1-11	--	--	--	--	4.329**

* significant at 5% level
** significant at 1% level

1/ 1-Age
2-Initial(pre-feed)weight
3-Initial(pre-flight)weight
4-Weight gain
5-Post-flight weight
6-weight lost
7-Dry weight
8-Wing length
9-Wing loading ratio
10-Temperature
11-Relative humidity

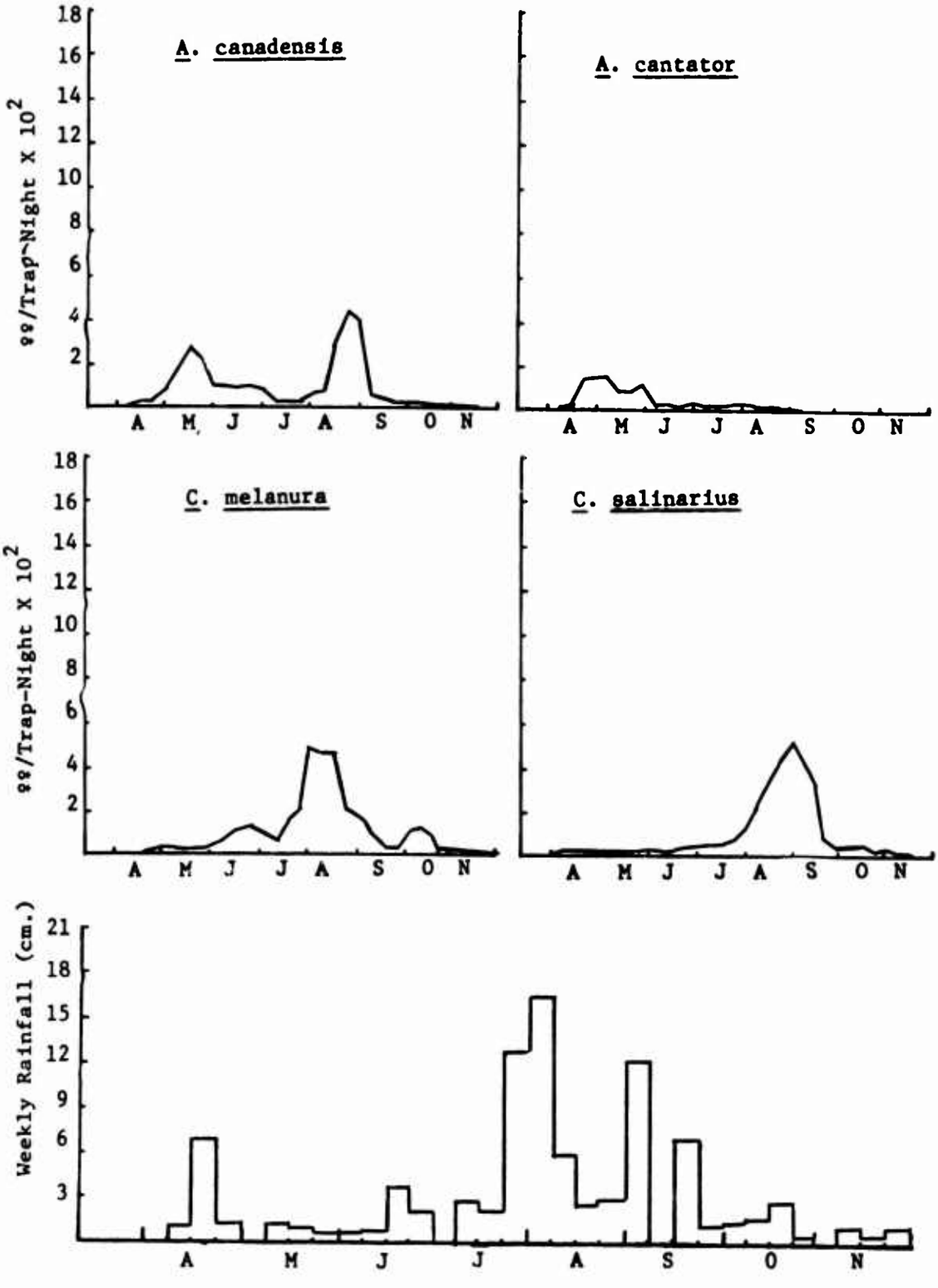
TABLE 17

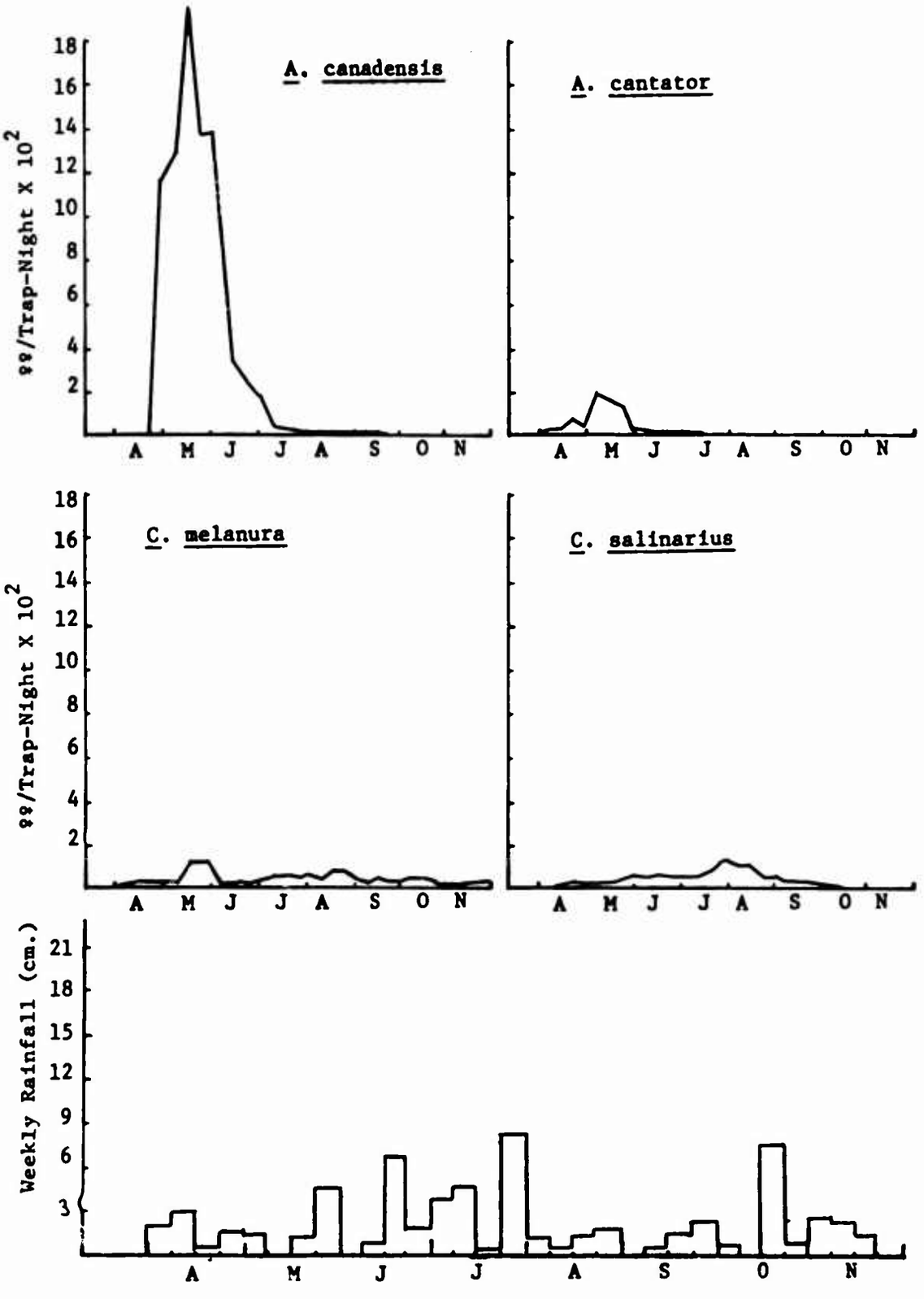
Percent accountable variance for flight distance

Variable	Days				Combined Age
Age	3	4	5	6	0.042
Initial (pre-feed) weight	8.378	6.967	0.024	5.428	1.724
Initial (pre-flight) weight	13.632	32.011	41.844	29.958	23.113
Post flight weight	14.748	1.833	1.089	24.788	5.433
Dry weight	2.474	0.349	0.095	9.700	0.635
Wing length	0.337	2.680	0.002	1.093	0.049
Wing loading ratio	2.189	1.671	1.993	0.738	1.062
Temperature	6.873	7.370	0.438	1.251	2.150
Relative humidity	0.127	7.553	5.331	0.880	1.398
Total	48.758	60.434	50.816	73.836	35.606

FIGURES 1 - 3 (FOLLOWING)

SEASONAL DISTRIBUTION OF FOUR MOST ABUNDANT MOSQUITO SPECIES IN RELATION TO WEEKLY RAINFALL, POCOMOKE SWAMP, MARYLAND. FIG. 1: 1969; FIG. 2: 1970; FIG. 3: 1971.


A. canadensis
A. cantator
C. melanura
C. salinarius
♀♀/Trap~Night X 10²
♀♀/Trap-Night X 10²
Weekly Rainfall (cm.)
A M J J A S O N


A. canadensis
A. cantator
C. melanura
C. salinarius
♀♀/Trap-Night X 10²
Weekly Rainfall (cm.)
A M J J A S O N

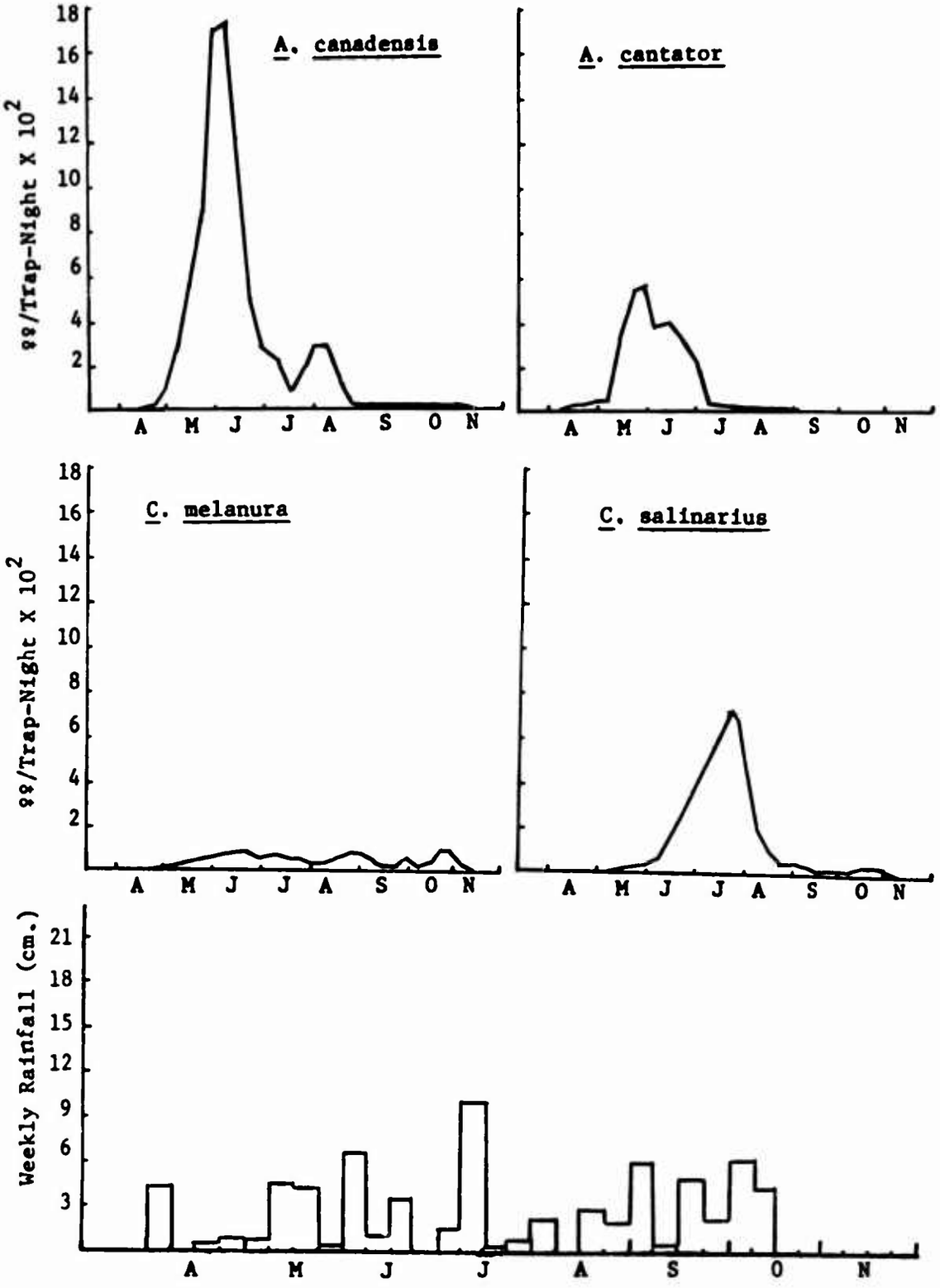
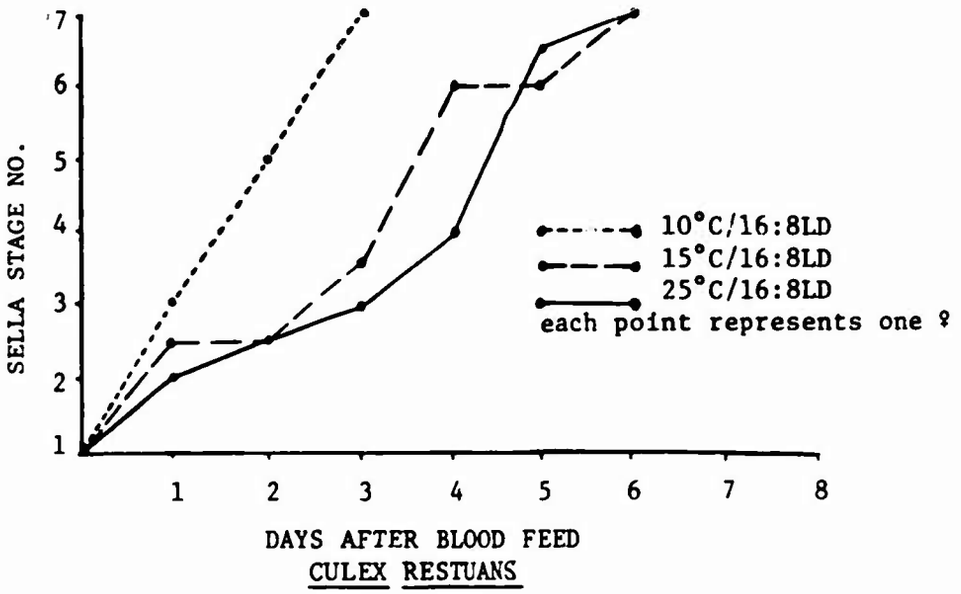
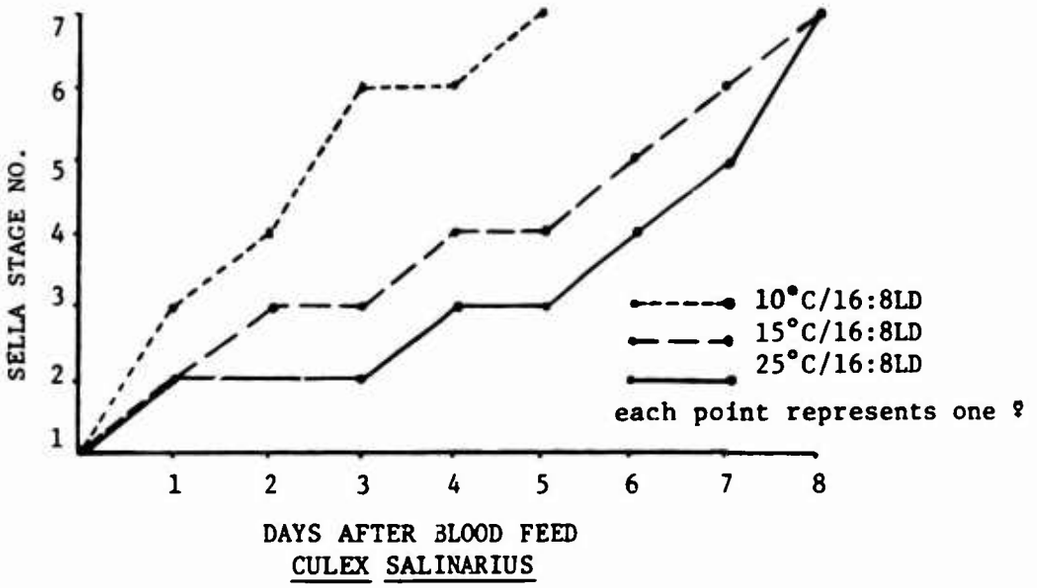

A. canadensis
A. cantator
C. melanura
C. salinarius
♀♀/Trap-Night X 10²
Weekly Rainfall (cm.)
A M J J A S O N

FIGURE 4

RATE OF EGG MATURATION IN CULEX RESTUANS AND CULEX SALINARIUS AT 10, 15 AND 25°C



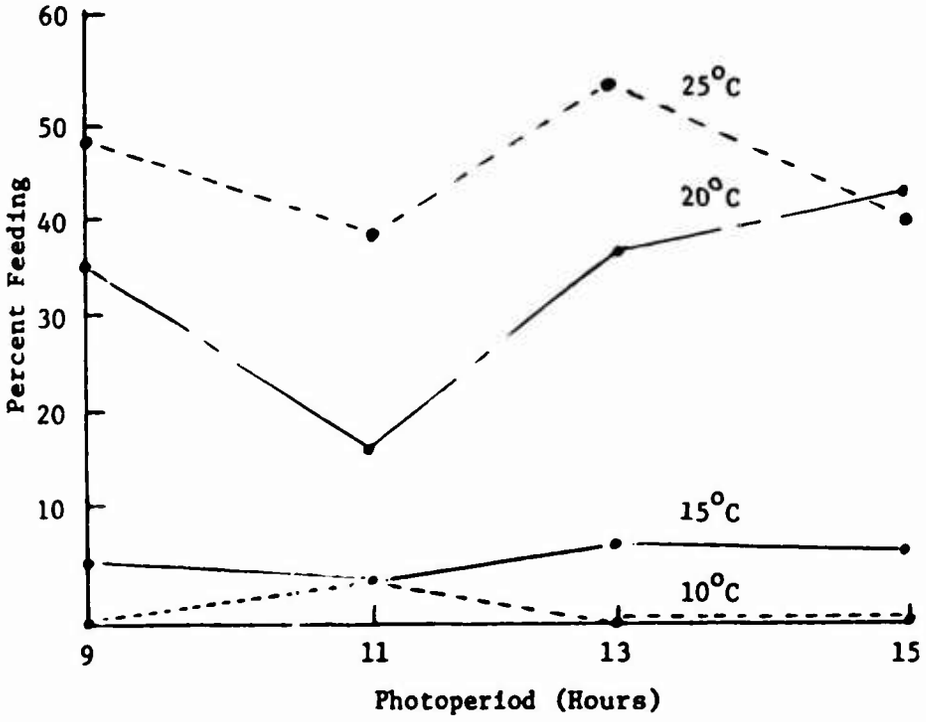
CULEX RESTUANS



CULEX SALINARIUS

FIGURE 5

BLOOD FEEDING BY CULEX SALINARIUS FEMALES AFTER CONDITONING AT FOUR COMBINATIONS OF TEMPERATURE AND PHOTOPERIOD



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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6432	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. GROUP MEMBER	9. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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	015
b. CONTRIBUTING				
c. [redacted]	CDOG 114 (f)			

11. TITLE (Provide with Security Classification Code)
(U) Antigen-Antibody In Vivo and In Vitro

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
010100 Microbiology

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: 72	7	160
FISCAL YEAR CURRENT: 73	7	160

19. RESPONSIBLE DOD ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: DA
NAME:

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Provide 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. Provide text of each with Security Classification Code.)

23 (U) This work unit is concerned with the study of basic mechanisms of the immediate type allergies, the development of methods for the isolation and the characterization of antibodies and enzymes involved in hypersensitivity reactions. This looks to the ultimate control of the hypersensitivity reaction in military personnel suffering from allergies or hypersensitivity reaction to parasite infection by a specific inhibition of these enzymes.

24 (U) The antibodies and mechanism of the leukocyte-dependent histamine release from platelets of the immediate hypersensitivity reaction to various parasitic diseases are being studied. 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, towards the recognition of the dangerous universal donor.

25 (U) 71 07-72 06 The release of histamine from normal platelets is brought about by the release of a platelet activating factor through the interaction of antigen with leukocytes obtained from Schistosoma mansoni infected rabbits. The platelet activating factor can be demonstrated only in the presence of serum albumin which acts as a carrier. The nature and characterization of this factor, as well as its significance, is under investigation. It is concluded that differences in the binding affinities of naturally occurring anti-B isoagglutinins are characteristic of the ABO genotype. The methods which must be employed to demonstrate these differences are complicated and exacting. For technical report see Walter Reed Army Institute Annual Progress Report, 1 Jul 71 - 30 Jun 72.

* Available in unclassified open publication's summary.

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 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

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

Investigators.
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Associate: D.T.O. Wong, Ph.D.; M.B. Gibbs, Ph.D.; LTC Kenneth Hedlund, MC; MAJ Carl Alving, MC; J. Fowble, M.S.; E.T. Seno, M.S.; J.P. Bingham; M.J. Schoenbechler; MAJ Robert T. Solis, MC

Description.

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

Progress.

1. Leukocyte-dependent histamine release

a. The previous annual report described the in vivo sensitization of normal leukocytes with antiserum demonstrating homocytotropic (reaginic) antibodies. Investigation involving this leukocyte-dependent histamine reaction has continued along two lines, the demonstration of first, the class of immunoglobulin involved in this reaction and second, the type of leukocyte and mechanisms of its interaction with platelets.

b. In order to test what immunoglobulin was responsible for the release of histamine in the leukocyte-dependent reaction, use was made of various specific anti-rabbit immunoglobulins. Leukocytes obtained from rabbits infected with Schistosoma mansoni were pre-incubated with varying concentrations of goat anti-IgE; guinea pig anti-IgG; sheep anti-IgA and sheep anti-light chains. The treated leukocytes were then tested for their ability to release histamine from normal platelets as well as their ability to still release histamine with the schistosomal antigen. The sheep anti-light chain clearly established the presence of immunoglobulin on the leukocytes by their capacity to cause histamine release from normal platelets. The results with anti-IgE; anti-IgG and anti-IgA demonstrated that the most prevalent immunoglobulin on the leukocyte was gamma E. Although there was no histamine releasing activity with leukocytes treated with anti-IgG, these leukocytes were unable to release histamine with the schistosomal antigen which indicates that there may be some gamma G present on these leukocytes. However, pre-adsorption with purified

rabbit light chains substantially reduced the inhibitory effect of anti-IgG without changing its inability to activate the leukocytes. Leukocytes treated with sheep anti-IgA were capable of activating the leukocyte, but only at very high concentrations in comparison to the anti-IgE treated cells. This activity was not reduced by pre-adsorption with purified light chains, which suggests the possibility that gamma-A may also be present on the leukocyte. However, an alternative explanation for the cause of this release could be the presence of anti-IgE in this antiserum. This is plausible since the gamma-A was obtained from rabbit colostrum which probably contains gamma-E also. Experiments are in progress to determine whether or not anti-IgE is present in this antiserum.

c. The release of histamine from normal platelets is brought about by the release of a platelet activating factor (PAF) through the interaction of antigen with leukocytes obtained from S. mansoni infected rabbits. The leukocyte type that has been shown to correlate with the production of PAF is the basophil. The nature and characterization of PAF has not been accomplished as yet, but is currently being investigated in several laboratories. The PAF can be demonstrated in the fluid phase only if there is sufficient protein (albumin) present in the reaction mixture. Failure to incorporate albumin in the buffer results in the fixation of PAF to the blood cell elements. The optimal concentration of albumin for maximum fixation of PAF was found to be 2.5 mg per ml. Concentrations below and above this result in a decrease of binding and subsequent decrease of histamine release. It is interesting that an excess of albumin results in a decreased histamine release. The reason for this inhibition of histamine release by PAF in the presence of excess albumin is not clear presently and is under investigation.

d. Preliminary results indicate that PAF retains its activity towards platelets after storage at -70°C for 8 days. Its activity was only slightly decreased when incubated at 56°C for 60 minutes. It was non-dialyzable and did not affect normal rabbit basophils. It should be mentioned that these results apply to PAF bound to serum albumin and may be somewhat different if PAF is free of serum albumin.

2. Production and characterization of guinea pig homocytotropic antibodies

a. The previous annual report described the production of reaginic-like antibody in guinea pigs immunized with p-amino benzoic acid conjugated to homologous serum albumin emulsified in Freund's complete adjuvant. The titers after primary injection were extremely low and only 10 percent of the animals produced the antibody. The unique aspect of this reaginic antibody is its narrow specificity which is directed neither towards the carrier protein nor hapten determinant

but appears to be link-specific i.e., a positive reaction is obtained only when the homologous antigen used to elicit the antibody is used for challenge. These studies have been extended to determine whether it was possible to increase the production of this antibody by varying several parameters, such as, 1) the type of adjuvant 2) concentration of antigen and 3) time course of immunization.

b. In an attempt to determine whether alum used as an adjuvant evoked a better link-specific response than Freund's complete adjuvant used previously the following experiments were done. Four groups of 10 guinea pigs each were injected with either 10 ug, 100 ug, 500 ug or 1000 ug of p-benzoate azo-GPA with 1 mg of alum given intraperitoneally at intervals of 4 to 6 weeks. The sera was collected 7 days after each injection and tested for 10 day PCA activity. Only one animal from each group had measurable link-specific antibody after the first boost, while over half of those animals receiving 500 or 1000 ug of antigen demonstrated link-specific antibodies of moderate titer after the second boost. However, by the third boost, with the exception of one animal, no detectable 10 day PCA antibody could be observed. In contrast those animals injected with 10 or 100 ug of antigen demonstrated rising titers and an increased number of animals showed antibody activity that could be sustained with each successive boost. It should be noted that there were only 2 guinea pigs out of 40 that produced reaginic antibody other than link-specific which was directed toward the homologous serum albumin carrier. One of these guinea pigs also produced hapten specific antibody. These results indicate the importance of antigen concentration with regard to the temporal appearance and sustained production of reaginic-like activity. These preliminary studies provide an opportunity to assess the role of the carrier protein in the production of IgE immunoglobulin and the extent to which it defines the specificity of the antibody produced. This is currently being investigated in guinea pigs as well as other animal species.

3. Immune reactions with liposomes

a. A variety of important proteins, including hormones, immunoglobulins and complement, have been shown to interact with phospholipids. Using artificial membrane models (liposomes), several of these proteins have been shown to cause permeability changes with the subsequent release of various trapped markers within the liposomes. It was found that serum-dependent damage to liposomes sometimes occurred in the absence of any recognizable antigens. Studies were initiated to determine the prevalence and specificity of permeability promoting proteins in normal serum. Sphingomyelin liposomes lacking any incorporation of antigen were selected for study with various normal sera because of their high degree of stability. Small amounts of damage to these liposomes are generally more significant than to those liposomes containing other phospholipids.

b. It was found that all normal rabbit sera had at least some degree of activity and the amount of glucose release was a function of the serum concentration. In contrast, sera from humans and pooled guinea pig serum had either low levels of activity or none at all. The activity of normal rabbit serum could be synergistically enhanced 2-3 fold by the addition of normal human or pooled normal guinea pig serum.

c. The glucose-releasing ability of normal rabbit serum was found to be completely destroyed by prior incubation at 56° for 60 minutes. However, the heated normal rabbit serum still retained the capacity to be synergistically enhanced in the presence of fresh normal human serum. Heated normal human serum invariably lost the capacity to synergistically enhance the activity of fresh normal rabbit serum.

d. These results demonstrated the participation of heat labile components in the synergistic enhancement. The question of involvement of complement in the phenomenon was next investigated. The role of complement was initially studied using rabbit serum totally deficient in C6. Pooled C6 deficient rabbit serum had approximately the same type and degree of activity as other normal rabbit serum. Although the results clearly demonstrated that this complement component is not required, it does not eliminate the possibility of activation of earlier complement components. The effects of decomplementation of normal rabbit serum and/or normal human serum with a heterologus immune precipitate was investigated. The results obtained by decomplementation were very similar to those obtained by heating normal rabbit and normal human serum. Work is now in progress to determine if this phenomenon of glucose release from liposomes by normal serum and the synergistic enhancement by the heterologous system involves naturally occurring antibodies.

e. Sixty human sera were assayed for their ability to release trapped glucose from liposomes composed of various phospholipids. Approximately 15 percent of the sera from normal individuals and patients had striking permeability-promoting activity. Experiments are in progress to determine the significance of these findings with regard to the possible occurrence of abnormal circulating serum proteins and to relate these permeability changes to surface charge, fatty acid and phospholipid composition of the liposome.

f. The previous annual report demonstrated that anti-cerebroside antibodies could be produced in rabbits and that this antibody activity was associated only with the IgM fraction. Attempts were undertaken to correlate the uptake of antibody and complement with the cerebroside concentration incorporated into sphingomyelin liposome. When heated rabbit anti-cerebroside antibody was incubated with sphingomyelin liposomes containing varying amounts of cerebroside

antigen, the amount of protein bound was minimal. At concentrations of 100 micrograms of cerebroside, only 50 micrograms of protein per micromole of liposomal phasphate was adsorbed, and the amount of protein bound remained constant up to concentrations of 200 micrograms of cerebroside. Concentrations below 100 micrograms of cerebroside per micromole of liposomal phosphate, as well as liposomes containing no cerebroside bound 25 micrograms of protein per micromole of liposomal phosphate. These results suggest that there was non-specific binding of protein to liposomes. It was then found that liposomes containing no cerebroside antigen incorporated into the sphingomyelin matrix had the ability to non-specifically bind a wide spectrum of serum proteins (such as IgG, IgM and albumin) when incubated with heated rabbit anti-cerebroside antibody. Similar results were obtained wnen guinea pig serum was used instead of anti-cerebroside antibody. These findings strongly suggest that non-specific binding of protein is a function of the sphingomyelin matrix of the liposomes rather than the cerebroside antigen incorporated into the liposomes.

g. The amount of protein bound was found to depend on the cerebroside concentration when a constant amount of heated anti-cerebroside antiserum and fresh guinea pig serum as a source of complement were added to varying concentrations of cerebroside containing liposomes. At a concentration of 100 micrograms of cerebroside the total amount of protein bound was 1311 micrograms of protein per micromole of liposomal phosphate, while cerebroside concentrations above and below 100 micrograms resulted in a sharp decrease in the amount of protein bound. This pattern of protein binding as a function of antigen when antibody is kept constant resembles the quantitative precipitin and complement fixation reactions where peak values are obtained at slight antigen excess. Therefore, it is felt that this increase in protein binding represents the specific binding of complement as a consequence of immune complex formation.

h. The percent release of trapped glucose increased with increasing amounts of cerebroside containing liposomes and a constant amount of anti-cerebroside antibody, reaching maximal at approximately the same concentration of cerebroside that causes maximal protein binding. However, unlike the protein binding experiments, the percent glucose released reaches a plateau and does not decrease with further increases of antigen. These results suggest that the amount of glucose release is directly related to the amount of complement bound. This is currently under investigation.

i. Work has continued with the use of liposomes as a model for studying immune phagocytosis. The previous report demonstrated that although phagocytosis did occur in the absence of complement, the process was greatly enhanced by antibody and complement. More recent experiments demonstrate that phagocytosis can occur in the absence of antibodies and/or complement suggesting that antibody-complement

dependent phagocytosis is not a specific phenomenon for these proteins alone. The mechanisms involved may be regulated simply by protein density and charge at the surface of the liposome. In addition, the importance of antibody, or protein, may play a more significant role in the immune-phagocytosis than complement does. The degree of phagocytosis appears to be dependent upon the concentration of antibody or protein, rather than complement. In preliminary experiments where the amount of protein per liposome is constant, there is a greater degree of phagocytosis in those liposomes that contain larger amounts of antibody than liposomes containing more bound complement. Work is underway to test the specific requirements of certain proteins, antibody and complement to determine whether the mechanism of phagocytosis is due to specific information in these proteins or merely a matter of protein density.

4. Studies on blood group antigens and antibodies

Investigations directed towards the recognition of the "dangerous" universal group O 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, a collaborative study was initiated with Dr. Salmon of the Central Blood Transfusion Service of Paris to resolve the discrepancies in binding properties of anti-B agglutinins from random group O and A_1O sera as demonstrated with the log-probit and the Wurmsers' assay procedure. When the binding strengths of anti-B agglutinins in 6 known group O, 2 known group A_1O and 4 known group A_2O were compared using the modification of the Wurmsers' procedure recommended by Dr. Salmon and the hemocytometer measurements of hemagglutination, they were found to be in excellent agreement with the results published by the Wurmsers. However, attempts to simplify the laborious Wurmsers' assay by employing the model B Coulter counter to measure hemagglutination have not been successful. The agglutinates formed with weak binding antibodies remaining in the supernatant after adsorption of some group O sera with high concentrations (5- 8 x $10^5/mm^3$) of B red cells dissociate upon dilution for counting. It was concluded that differences in the binding affinities of naturally occurring anti-B isoagglutinins from genotypes OO, A_1O and A_2O individuals do exist. However, the methods which must be employed to demonstrate these differences are too complicated and exacting to be of use clinically.

5. Studies of Microaggregates of human blood

a. The collaborative investigations with Major Solis, Division of Surgery and Major Zeller, Division of Pathology, WRAIR, on the formation and removal of microaggregates (MA) were continued. Electron microscopic examination of ACD-anticoagulated blood demonstrates that during the first few days of storage at 4°C only platelets are aggregated, while later in storage the granulocytes begin to adhere

together and to the aggregated platelets. By the 16th day of storage, the MA consist of platelets, granulocytes and nuclear material of decomposed granulocytes. These findings are in agreement with size distribution data obtained with the model T Particle Counter. In addition, the filtration characteristic of the MA changes during storage. Platelet aggregates (40 u in diameter) formed during the first few days of storage are still present in the blood after passage through the 40 u pore mesh blood filter. However, the same size MA formed after prolonged storage were effectively removed by the filter. Studies of platelet-poor and rich plasmas, buffy coat and saline washed red cells established that both platelets and leukocytes must be present before aggregates of 40 u adhere to the filter. The immunological effects of MA present in blood stored over 16 days has not been determined.

b. The effect of temperature and anticoagulants on the formation of the MA in stored blood was also investigation. Microaggregates of 13 to 80 u in size which developed in ACD blood during the first 5 days of storage at 4°C did not develop when the blood was stored at 25°C. ACD anticoagulated blood formed less platelet aggregates than when heparin was used as an anticoagulant. Treatment of whole blood with prostaglandin E1 (PGE1), an inhibitor of platelet aggregation, reduced the formation of MA and improved the recovery of platelets.

c. Quantitation of the size and number of platelet aggregates in blood or plasma has not been feasible because of the marked instability and wide range of size of these particles. The model T Coulter Counter has made it possible to obtain reproducible measurements of 13 to 161 u platelet aggregates in plasma and hemolyzed blood because of the rapidity with which size distribution analyses can be obtained. The process of platelet aggregation can be induced in vitro by the addition of adenosine diphosphate (ADP) to platelet rich plasma. The velocity of aggregation and the modal size of the aggregates increased as the ADP concentration was varied from 2×10^{-8} to 2×10^{-6}M, with no further increased at 2×10^{-5}M ADP. These findings agree with those obtained with the conventional turbidimetric studies of the kinetics of platelet aggregation.

d. The size distribution of platelet aggregates induced in vivo by the injection of ADP into anesthesized rats was also investigated. Platelet aggregation appeared quickly and the modal size of the aggregates in the inferior vena cava blood increased as the concentration of ADP was increased from 5 to 100 mg/kg. No platelet aggregates could be demonstrated in blood drawn from the descending aorta, suggesting that these aggregates may have been trapped or deaggregated in the pulmonary vascular bed. Pretreatment of rats with 100 ug PGE1/kg completely prevented the formation of platelet aggregates when 5 mg

ADP/kg was injected. However, when the dose of ADP was increased to 25 mg/kg the total volume of platelet aggregates in the inferior vena cava of PGE1 treated was the same as untreated rats. The modal size of the aggregates was much smaller in the treated rats. The in vivo induction and inhibition of platelet aggregation demonstrates that the rat provides an excellent model for quantitative studies of the effects of various pharmacologic agents on in vivo platelet aggregation.

Summary and Conclusions

1. The immunoglobulin responsible for the leukocyte-dependent histamine release belongs to the gamma-E class, similar to the human reaginic antibody.

2. The platelet activating factor was demonstrated by the interaction of antigen with leukocyte bound antibody.

3. The platelet activating factor is bound to serum albumin and cellular material. Its activity is diminished in the presence of excess serum albumin.

4. The importance of antigen concentration with regard to the temporal appearance and sustained production of guinea pig reaginic-like antibody has been demonstrated.

5. Serum dependent release of glucose from liposomes sometimes occurs in the absence of any recognizable antigen. This activity is always found in rabbit serum, while normal human or guinea pig serum has either low levels or none.

6. The permeability damaging properties of normal rabbit serum can be synergistically enhanced by either normal human or guinea pig serum. This activity can be abolished by heating or adsorption with an immune precipitate.

7. Pooled C6-deficient rabbit serum had approximately the same degree of permeability damaging properties as normal rabbit serum.

8. The non-specific binding of serum proteins to cerebroside-containing liposomes appears to be bound to the sphingomyelin matrix of the liposomes.

9. The specific binding of protein (complement) is dependent upon the cerebroside concentrations and is the consequence of immune complex formation.

10. Phagocytosis of liposomes can occur in the absence of antibodies and/or complement which suggests that antibody-complement dependent phagocytosis is not a specific phenomenon of these proteins alone.

11. The formation of microaggregates in blood during the first five days of storage is greater in blood stored at 4°C than in blood stored at 25°C.

12. The *in vivo* and *in vitro* induction and inhibition of platelet aggregation was studies using the Model T Coulter particle counter.

Project 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

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

Literature Cited.

Publications.

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2. Solis, R.T., C. B. Wright and M. B. Gibbs. A model for quantitating platelet aggregation *in vivo*: (Abstract) Proceedings at the 7th European Congress on Microcirculation. Aberdeen, Scotland,1972.

3. Zeller, J., D. Gerard, M. B. Gibbs and R. T. Solis. An electron microscopic study of microaggregates in ACD stored blood. (Abstract). Proceedings of the III Congress, International Society on Thrombosis and Haemostasis, Washington, D.C., 1972.

PROJECT 3A061102B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 07
Pharmacology

64x-C

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6448	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GROUP MARK'G	8b. SCIEN DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
71 07 01	D. CHANGE	U	U	NA	NL	YES NO	A. WORK UNIT

10a. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A061102B71P	07	036
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114 (f)			

11. TITLE (Include 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. DATE/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR (PREVIOUS) 72	2	40
FISCAL YEAR (CURRENT) 73	2	40

19a. RESPONSIBLE DOD ORGANIZATION

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20. GENERAL USE

Foreign Intelligence Not Considered

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ASSOCIATE INVESTIGATORS

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NAME: EINHERER, Dr. Albert

21. KEYWORDS (Include Security Classification Code)

(U) Pharmacology;(U) Medicinals;(U) Shock Therapy; (U) Drugs; (U) Stress

22a. TECHNICAL OBJECTIVE. 22b. APPROACH. 22c. PROGRESS (Include Individual paragraphs identified by number. Include test of each with security Classification Code.)

23. (U) Research is directed toward *investi*gating the pharmacology of promising medicinal agents, drug interactions, developing and refining animal models for the study of hemorrhagic, septicemic, 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 chemicals 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 hemorrhage, endotoxin, traumatic injury, and anaphylactic stress.

25. (U) 71 07 - 72 06 IND for Phase I human tolerance studies was approved by Clinical and Preclinical Pharmacology Advisory Committee for WR 149,024. A toxicity model for testing antidotal effects of aminothiols against heavy metal poisoning was standardized. Patent for the use of WR 2823 in an alpha-adrenergic blocking agent in shock was issued (pat No. 3,629,410). Pharmacodynamics studies on WR 2823 and WR 149,024 were continued. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71 - 30 Jun 72.

* Applicable 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 07, Pharmacology

Work Unit 036, Pharmacological studies

Investigators
Principal: Melvin H. Heiffer, Ph.D.
Associate: LTC G. E. Demaree, CPT R. Caldwell, Dr. A. Einheber, Dr. R. Rozman

1. Further definition of receptor morphology.

An extention of the work by Reynolds et al (1967) on the atypical excitatory adrenergic response in terminal quinea pig ilia was continued.

Concentration response curves were constructed for phenylephrine and epinephrine. Phenylephrine produced concentration-dependent increases in tension in the terminal ileum and also in sections taken 1 to 1-1/2 cm proximal to the caecum. Epinephrine produced variable effects on terminal ileum (3/7 contractions, 4/7 relaxation). The contractile responses were antagonized by phentolamine. Propranolol blocked the relaxation produced by epinephrine.

2. Effect of aminothiol compounds on the toxicity of inorganic mercury in anesthetized mice.

Background:

Chelating agents, dimercaprol (BAL) and ethylenediamine tetraacetic acid (EDTA) have been used clinically to reverse the effects of heavy metal poisoning. The aminothiol, cysteamine phosphate (WR 638) reduced the serum copper levels in normal human volunteers receiving the drug subacutely for several weeks. These observations prompted the speculation that other potential aminothiol chelating agents might be efficacious in reversing or preventing the toxic effects of heavy metals. This hypothesis was tested using mercuric chloride as the prototype heavy metal and aminopropylaminoethyl phosphorothioic acid (WR 2721) and aminopentylaminoethylthiol (WR 1729) as the prototype aminothiols.

Methods:

Adult female mice weighing 25-36 gm from the Walter Reed ICR colony were treated with WR 2721 at doses of 75,

150, or 300 mg/kg intraperitoneally as an aqueous solution in distilled water. The drug concentration was adjusted so that the volume of the injection was 10 ul/gm. At 1/4, 1, 2, 3, 4 and 5 hours after the injection of WR 2721, the mice were anesthetized with a mixture of phenobarbital and urethane. The left femoral vein was exposed and cannulated for injection of mercuric chloride. Mercuric chloride was injected as a 0.1% solution in normal saline as a bolus at the rate of 4 mg/kg every two minutes until death. EKG and respiration were monitored.

In a second experiment, adult female mice were anesthetized and a cannula inserted into the left femoral vein for administration of one of three solutions as a bolus at the rate of 4 ul/gm every two minutes: (1) Mercuric Chloride (0.1%); (2) Mercuric Chloride (0.1%) plus WR 1729 (0.1%); (3) WR 1729 (0.1%). Administration was continued until death or for 30 minutes.

Results:

Pretreatment of female mice with WR 2721 up to two hours before the administration of mercuric chloride intravenously did not increase the amount of heavy metal required to produce death under these experimental conditions (Table 1). There also appeared to be a qualitative difference in the mode of death between those mice pretreated with WR 2721 and the control mice. The control mice appeared to die in cardiac failure with respiration continuing as gasps after all detectable electrical activity of the heart had ceased, whereas those animals pretreated with WR 2721 appeared to die of respiratory failure with the EKG changes to be secondary to hypoxia.

Mixing WR 1729 with mercuric chloride likewise did not appear to decrease the toxicity of the heavy metal; in fact, in terms of the amount of mercury required to produce death acutely, WR 1729 increases the toxicity of the heavy metal (Table 2). As reported above, there was a similar qualitative difference in the mode of death between the control and treated animals.

Discussion:

These data clearly do not support the proposed hypothesis that aminothiols might prevent or reverse the toxicity of heavy metals acutely. In fact, they suggest that there may be some type of synergism between the aminothiols and the toxic effects of inorganic mercury. Anderson [A. B. Anderson, The effect of dimercaprol in lead poison-

ing in mice, Brit. J. Pharmacol. 4:348 (1949)], reported a similar finding using BAL in lead poisoned mice. He speculated that the lead-BAL complex may be more toxic than lead alone. Our data with mercury-aminothiol complexes would be consistent with a similar hypothesis regarding inorganic mercury. Certainly, other organic mercurials are more toxic than inorganic mercury. It remains to be seen whether these aminothiols can reverse or prevent the toxicity of organic mercurial compounds such as methyl mercury.

3. General Pharmacology of WR 149,024.

a. WR 149,024 produced an initial hypotension and bradycardia in the anesthetized dog which was not the product of increased vagal tone. Cardiac output was not decreased during this time when doses of 5 to 24 mg/kg were administered although cardiac contractile force was decreased. There was a secondary tachycardia, increase in cardiac contractile force and pulse pressure occurring 15 to 30 minutes after the administration of WR 149,024. This was probably the result of increased circulating catecholamines. This drug did not affect the isolated perfused dog heart in doses of 12.5 or 25 mg.

b. WR 149,024 reversed the epinephrine pressor response and antagonized the pressor response to norepinephrine without affecting the vascular responses to isoproterenol or angiotensin. This compound did not antagonize the inhibition of gut motility produced by phenylephrine. This indicates that this compound may exert a selective blocking effect on the vascular alpha adrenergic receptors.

c. Infusion of WR 149,024 (1 mg/kg) into the carotid artery of dogs produced a greater degree of peripheral alpha adrenergic blockage than when administered intravenously. This suggests that there may be a central nervous system component in the effect of this drug to produce peripheral alpha adrenergic blockage.

d. WR 149,024 (10 mg/kg) given intravenously to normotensive anesthetized dogs reduced effective renal plasma flow (ERPF) by 40% and glomerular filtration rate (GFR) by 35%. GFR and ERPF returned to pre-drug levels within 3 hours of drug administration. Hemorrhagic hypotension also reduces GFR and ERPF. Infusion of the shed blood causes GFR and ERPF to return to normal values. Infusion of WR 149,024 (10 mg/kg) simultaneously with the blood further augments the GFR and ERPF in these previously hemorrhaged

dogs. These observations suggest the hypothesis that the effect of WR 149,024 on renal function in shock may contribute to the life-saving properties of this drug in the treatment of hemorrhagic shock.

TABLE 1

Mercuric Chloride required to produce death acutely in non-treated anesthetized mice and in anesthetized mice pretreated with WR 2721

Control	Dose of WR 2721	Pretreatment time (hours) 0.25	1	2	3	4	5
48*,20 24,36,32	300**	8,8,8*	16,16	20,28	20,20, 20	16,20, 16,16	24,16 16
24,20, 20,32,20	150	24,24, 28	16,16 20				
24,16 20,20	75	20,24, 24					

* Dose of Mercuric Chloride required to produce cardiac arrest (mg/kg) acutely, individual values.

** Dose in mg/kg.

TABLE 2

Mercuric Chloride required to produce death acutely alone and in combination with WR 1729 in the anesthetized mouse

Mercuric Chloride alone	Mercuric Chloride plus WR 1729	WR 1729 alone
20±3[a]	13±2[b]	54±15

a - mean ± S.D. (n=4) mg/kg

b - mean ± S.D. (n=4) different from $HgCl_2$ alone ($p < .025$)

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

Task 07, Pharmacology

Work Unit 036, Pharmacological studies

Investigators
Principal: Melvin H. Heiffer, Ph.D.
Associate: LTC G. E. Demaree, CPT R. Caldwell, Dr. A. Einheber, Dr. R. Rozman

Literature Cited.

Reference:

Reynolds, D. G., Demaree, G. E. and Heiffer, M. H.: An Excitatory Adrenergic Alpha Receptor Mechanism of Terminal Guinea-Pig Ileum. Proc Soc Exper Biol and Med, 125:73-78, 1967.

Publications:

Demaree, G. E., Brockenton, R. E. and Heiffer, M. H.: Adrenergic Blockage in Rabbit Aorta by a Series of Sulfur-containing Aliphatic Amines. The Pharmacologist 13:200, 1971.

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Demaree, G. E., Brockenton, R. E. and Heiffer, M. H.: Antagonism of Norepinephrine Responses of Isolated Rabbit Aorta by Aliphatic Sulfur-containing Amines. J Pharm Sci 60:1743-1745, 1971.

Caldwell, R. W. and Heiffer, M. H.: Cardiac Aspects of Initial Blood Pressure Responses to Catecholamines. The Pharmacologist 13:249, 1971.

Caldwell, R. W., Heiffer, M. H. and Demaree, G. E.: Dissociation of Alpha Adrenergic Blockage in Gut and Vasculature Seen with Two Sulfur-containing Aliphatic Amines. Fifth International Congress on Pharmacology 14:102, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6438	72 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. GROUP	9A. SPECIFIC DATA-CONTRACTOR ACCESS	B. LEVEL OF SUM
71 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	076
b. CONTRIBUTING				
c. [redacted]	CDOG 114(f)			

11. TITLE: Brain Mechanisms Maintaining Vital Functions During Stress: Anatomical, Physiological, and Behavioral Correlates.

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 002400 Bioengineering 016200 Stress 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 ($ Thousands)
FISCAL YEAR — PRECEDING 72	6	210
FISCAL YEAR — CURRENT 73	6	210

19. RESPONSIBLE DOD ORGANIZATION
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PII Redacted

21. GENERAL USE: Foreign Intelligence not considered

22. KEYWORDS: (U) Neurophysiology;(U) Neuropsychiatry;(U) Neuroanatomy; (U) Combat Shock;(U) Autonomic Nervous System;(U) Biomedical Engineering; (U) Stress

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

23. (U) The principal objectives are to find the anatomical and physiological bases of central nervous system (CNS) responses to various environmental challenges; to find the CNS correlates of autonomic and behavioral activity during health, disease, trauma, and stress, as applicable in battle and non-battle military environments.

24. (U) Determination of CNS pathways in control of fine movement and autonomic responses by histological and histochemical techniques, with light and electron microscopy. Recording and analysis of cardiovascular and other autonomic responses to various types of CNS stimulation in anesthetized and in awake, free moving animals. Recording and analysis of single nerve cell and whole brain electrical activity under diverse types of peripheral and central stimulation. Development of new instrumentation for biomedical and neurophysiological research.

25. (U) 71 07-72 06 New techniques were developed (a) for fast and accurate collection and chemical analysis of cerebrospinal fluid, (b) for precision electromagnetic measurements of blood flow and computer analysis of results, and (c) a high precision signal pattern generator. Corticospinal pathways involved in control of visceral and motor functions in primates and other mammals were elucidated by light and electron microscope studies. Corticofugal fiber systems within the mammalian brain were studied in detail. Electrophysiological studies of vestibular afferents demonstrated for the first time two types of synapses on the same neuron in the mammalian brainstem. Cardiac arrythmias induced by diencephalic stimulations in the rhesus monkey were experimentally produced and analyzed. These and other neurophysiological studies are being continued, and provide basic information needed for favorably modifying life-threatening response in stress, trauma, and disease. For detailed technical report, see WRAIR Annual Progress Report, 1 Jul 71 to 30 Jun 72.

* 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 68 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 Brain mechanisms maintaining vital functions during stress: anatomical, physiological, and behavioral correlates.

Investigators.
Principal: N. H. Spector, Ph.D.
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DESCRIPTION

The research program of the Department of Neurophysiology attempts to: (1) provide fundamental neuroanatomical and physiological information, both basic and applied, regarding behavioral functions of the limbic system and the physiological and behavioral functions of the autonomic nervous system acting to integrate and regulate, within normal limits, the body's temperature, heart rate, blood pressure, blood flow, water balance, exocrine glands, endocrine balance, gastrointestinal motility, digestion and absorption, and other vital functions, particularly as these functions may be related to the medical, surgical or psychiatric care of military patients; (2) define the environmental or physiological circumstances contributing to or causing trauma, stress, and shock; (3) suggest applied methods of corrective therapy for stress avoidance and recovery from surgical or medical shock. These studies have applied cardiovascular, physiological, neurological and neurosurgical implications.

The knowledge and research methods of neuroanatomy, neurophysiology, neuroendocrinology, cardiovascular physiology, and experimental psychology are utilized in the department's studies. In some cases the expertise of one discipline is applied to a particular research task, but whenever possible multidisciplinary approaches are utilized to study stress. The following problem areas are under study:

(A) The anatomy and physiology of sensory pathways to the autonomic nervous system, and the anatomical basis of reflex functions in the visceral nervous system;

(B) The physiologic mechanisms controlling heart rate and rhythm, blood pressure, shifts in blood volume throughout the vascular bed during stress and snock;

* N.I.H. Post-doctoral Fellow

(C) The physiology of the vestibular system: multiple forms of synapses in the brain-stem;
(D) The physiological behavior of cortical neurons and their relationship to somatic, visceral or multimodal afferent signals: neuronal units in the reticular formation;
(E) Localization within the diencephalon and telecephalon of ethyl alcohol: physiological and autoradiographic studies;
(F) Intracranial self-stimulation, morphine addiction, and neurotransmitters;
(G) New biomedical and bioengineering techniques in support of physiological and behavioral research.

PROGRESS

A. Neuroanatomical Studies.

1. Sympathetic division of the spinal cord.

a. Identification and description of spinal cord sympathetic nuclei. Using the method of axonal reaction following sympathectomy, the location of preganglionic nuclei in the thoraco-lumbar region of the spinal cord was established. A description of the normal cell complement of these nuclear groups was also described in detail. The experimental results show that three separate nuclear groups send axons into the sympathetic chains. These studies were performed in rhesus monkeys. The findings are being extended by comparison with the occurrence of the same cell groups in other primates and in the cat.

The results in the rhesus monkey demonstrate a wider dispersal and greater number of preganglionic neurons in primates than was known before. Some of these cells are very large neurons. This finding opens the possibility of studying visceral motor neurons using single-unit neurophysiological techniques. Single-unit analysis has been attempted before in the cat, but all efforts ended in failure. The presence of such large visceral cells in the rhesus monkey was unknown at the time; this discovery should provide better opportunities for cell behavioral analysis.

b. The ultrastructural morphology of preganglionic sympathetic neurons and their synaptic complexes. A new study was initiated to extend our light microscopic observations to the electron microscopic level. These pilot experiments are being done with cats prior to more intensive study of the preganglionic cell groups in the rhesus monkey. The perfusion fixation techniques suitable for the preservation of cells for study with the electron microscope are now being developed. Our histological preparation techniques have progressed to the point that our osmication, embedding, and staining procedures are substantially improved and we are beginning to see some early results with these new techniques.

2. Parasympathetic division of the spinal cord.

a. Experimental identification and description of the parasympathetic nuclear groups. Studies are in progress to determine the nuclei of origin for the outflow of the parasympathetic division of the nervous system. These cells in man innervate the gastrointestinal tract distal to the splenic flexure and supply efferent fibers which innervate the pelvic viscera and the external genitalia. Axons of the parasympathetic neurons are selectively cut to produce central chromatolysis of the nerve cell body. The axonal reaction displayed by the nerve cell bodies will permit identification of the nuclei of origin for parasympathetic fibers.

3. Afferent fiber connections of sympathetic and related cell groups of the spinal cord.

a. The relationship of reticulospinal fibers to sympathetic cell groups. Wide areas over the medullary and pontine reticular formation project to the spinal cord. Nerve cells of this region are known to participate in respiratory and cardiovascular events. The efferent fiber connections of this region of the tegmentum with sympathetic neurons has not been carefully studied. The possibility of a monosynaptic connection, or the presence of polysynaptic pathways to sympathetic motor neurons is now being investigated.

b. The vestibulospinal projection and its relationship with sympathetic motor neurons. Labyrinthine stimulation occuring during air, land or sea travel is known to influence heart rate, blood pressure, exocrine glandular secretions and sometimes results in nausea and vomiting. The pathways over which vestibular sensory signals may reach and influence spinal autonomic centers are being investigated.

c. The corticospinal projection and its relationship to sympathetic nuclei. Abdominal sympathectomies were performed unilaterally and followed by cortical lesions in the same rhesus monkeys. As in the above studies, the brains and spinal cords will be serially cut. The tissues will be stained according to the several variants of the Nauta and the Fink-Heimer methods in order to study the resulting fibers of passage and terminal degeneration. A prominent direct projection to select somatic motor neurons has been previously described and its role in rapid, graded, limb movements and fine finger dexterity in rhesus monkeys has been implicated. The current investigation attempts to compare the cortical input to somatic motor neurons with the cortical input to visceral motor neurons, with the view of describing the means by which the cerebral cortex may exert its effects at segmental levels of the autonomic nervous system.

4. Functional anatomy of the corticospinal tract.

Cerebral cortical ablations made with suction were placed unilaterally in the motor cortex of the California sea lion, Zalophus californianus. The resulting fiber degeneration to the spinal cord was mapped utilizing the Nauta and Fink-Hemier methods.

Corticospinal fibers were found bilaterally in all spinal cord segments. The principal descending pathway appears to be located in the contralateral lateral funiculus. In cervical, thoracic, lumbar and sacral regions fiber degeneration in the gray matter is: (i) massive contralaterally in the external basilar region of the dorsal horn, the zona intermedia, and among nucleus proprius cornus ventralis neurons located along the dorsal and medial borders of the appendicular motoneuronal cell population; (ii) abundant ipsilaterally in the nucleus proprius cornus ventralis; (iii) abundant bilaterally in nucleus cornucommissuralis ventralis; and (iv) present in the nucleus cornucommissuralis dorsalis. Corticospinal fibers appear not to be present in the nucleus posteromarginalis (Waldeyer), the nucleus sensibilis proprius (substantia gelantinosa of Rolando), the nucleus proprius cornus dorsalis, the column of Clarke, the nucleus cervicalis centralis, and the nucleus cervicalis lateralis.

Additional corticospinal fibers are found among the large population of somatic motor neurons which innervate forelimb and hindlimb skeletal muscles. Such fibers are present in great abundance in all segments comprising both the cervical and the lumbosacral enlargements. Fewer pyramidal tract fibers appear to be distributed among somatic motor neurons which innervate axial skeletal muscles. Comparative neuroanatomical observations suggest that the monosynaptic proprioneuronal and somatic motoneuronal connections in Zalophus more closely resemble the motor cortical projections in New World and Old World monkeys and anthropoid apes (Petras, 1968) than the same cortical system of carnivores such as the cat, dog, raccoon, and kinkajou (Petras and Lehman, 1966; Petras, 1969).

In view of the new anatomical evidence in Zalophus, an attempt is being made to review the functional properties of the corticospinal tract by comparing the anatomical and physiological evidence in the rhesus monkey which has recently been proposed by Lawrence and Kuypers (1968). As a result of our finding we offer the speculation that perhaps fundamentally similar functions may be provided by the pyramidal tract in these two dissimilar mammals. The agile and skilled hand movements of Macaca are of course absent in Zalophus; the possession of flippers precludes the possibility of individual digital movements. However, agile swimming movements may perhaps be attributed to an influence of the pyramidal system on spinal mechanisms playing upon lower motor neurons and skeletal muscles of limb segments proximal to hands and feet.

5. Organization of the Spinal Motor System.

Experiments were begun to determine the functional organization of the primate motor system at the level of the spinal cord. The purpose of this study was twofold: (a) to locate the position of spinal motoneurons innervating specific functional muscle groups in the forelimbs and hindlimbs; (b) to determine which of the above identified cell groups receive abundant corticospinal fibers, which receive more moderate cortical input and whether some motor cell groups fail to receive monosynaptic input from the motor cortex. The evidence in the literature (Philips and Porter, 1964; Lawrence and Kuypers, 1968; Petras, 1968; Amann, 1971) suggests that those cells groups innervating the muscles of the forearm and hand are the principal recipients of direct corticomotoneuronal connections.

Myectomies or neurectomies of specific limb muscles were performed in eleven rhesus monkeys. Motoneurons whose axons were injured in this procedure responded by the process know as chromatolysis. This is characterized by a disappearance of the clumped Nissl bodies, a marked eccentricity of the cell nucleus, swelling of the cell, and a condensation of darkly stained cresylechtviolett material on the nuclear membrane.

Chromatolytic neurons were identified and plotted in three dimensional reconstructions of the spinal cord. The results of seven of these experiments demonstrate that the motor centers of more proximally located muscles (biceps, triceps) occur in more rostral spinal cord segments. On transverse sections, extensor motoneurons are seen to be located lateral to those of the flexors.

In eight of these animals, spinal cord sections have been stained to study the fiber degeneration patterns among the motoneuronal groups of the spinal cord following motor cortex ablation. It is planned to study and chart this material in order to reveal a differential input of direct cortical connections to motoneurons of specific functional muscle groups.

6. Tectothalamic and tectomesencephalic connections in the rhesus monkey. Lesions of the superior colliculus were made utilizing stereotaxic surgical methods for the placement of electrodes, and by suction ablation performed by direct visualization of the midbrain roof. This constitutes a long-term research project and we have continued the mapping of the efferent fiber connections. Tectothalamic connections have been found with the intralaminar nuclei limitans, parafascicularis, centralis lateralis and paracentralis; with medial thalamic nuclei medialis dorsalis pars parvocellularis, medialis dorsalis pars densocellularis and medialis dorsalis pars multiformis. Projections to the nucleus pulvinaris inferior and the nucleus pulvinaris lateralis were also seen. The tectal projection to the intralaminar nuclei may provide a pathway for visual impulses

to reach the striatum and extrapyramidal motor system via a transthalamic trajectory. A similar transthalamic route utlilizing the nucleus medialis dorsalis to connect the superior colliculus with the frontal eye fields is also suggested by these new data.

Tectomesencephalic connections have been partially analyzed. Ipsilateral connections are established with the nuclei griseum pontis and the nucleus paralemniscalis of Olszewski and Baxter (1954). A large contralateral projection is seen to the nucleus reticularis tegmenti pontis of Bechterew. Afferent connections to this tegmental nucleus appear not to have been identified before.

B. Left Ventricular Strain Pattern.

It has been previously demonstrated (Spector, et al, 1968; Spector, 1970) that the syndrome known in man as "left ventricular strain pattern", marked by an inverted t-wave in the electrocardiogram and associated with essential hypertension, can be replicated in dog or cat by stimulation of certain diencephalic nuclei. This observation has now been extended to the rhesus monkey. It has been postulated by Spector (1970) that the inverted t-wave is produced by a central nervous system feedback loop via the thalamus and hypothalamus, and involving the beta-adrenergic sympathetic innervation to the ventricles of the heart, and not, as had been previously thought, the result of a simple mechanical back-pressure on the heart due to increased peripheral vascular resistance.

This experimental model will be used to examine the relationships of ectopic foci and abnormal repolarization patterns in the ventricles to changes in stroke volume and cardiac muscle contractility.

C. Multiple Forms of Synapses in the Vestibular System.

Intracellular recording from neurons in the vestibular nuclei of the rat has provided evidence that vestibular afferent fibers are electrically coupled to their target cells. The excitatory post-synaptic potentials (EPSPs) recorded consist of short latency, rapidly rising potentials followed by longer latencies and more slowly rising potentials. The early component of this complex EPSP is unaffected by changing the membrane potential of the cell whereas the later component is decreased by depolarizing the cell. These results indicate that the early component is generated in the afferent fibers whereas the later component is generated by chemical transmitter agents acting on the permeability of the cell membrane to certain ion species. The average latency, in a sample of 18 cells, to onset of the first component of the EPSP was 0.50 msec., only 0.25 msec. after the arrival of the afferent volley; the average time to peak of this component was 0.22 msec.; the short latency and rapid rise-time agree with the conclusion that it is generated in the

afferent fibers. Studies of the effect of graded stimulus intensities on the amplitude of the early component of the EPSP indicate that many afferent fibers are electrically coupled to a single neuron: the amplitude of the EPSP increases in a finely graded fashion as more afferent fibers are activated by stonger and stronger stimuli. Collision experiments, in which either a directly evoked spike or a spontaneous spike immediately precedes the electrical stimulus applied to the vestibular nerve show that these potentials are generated in post-synaptic units: spike generation is delayed but not blocked and the underlying EPSP is unaffected. The amplitude of the electrically coupled EPSP or coupling potential is smaller than the chemically coupled EPSP; although the coupling potential often generates an action potential during an intracellular recording, it remains problematical whether the coupling potential plays an important role in normal synaptic transmission. Extracellular recordings indicate that action potentials are rarely generated at latencies compatible with the time course of the coupling potential. These findings (Wylie, 1972) provide the first thorough electrophysiological evidence for electrical coupling in the vestibular system of the rat, although previous studies (Korn, et al, 1972) have indicated the probable presence of this type of synapse.

D. Physiology of Neurons in Telencephalon and Brainstem.

1. Corticofugal Systems. A pilot project was begun this past year and is yielding valuable data on the details of the motor pathway from the cerebral cortex to the spinal cord. Anatomists who have investigated this subject in the past have generally done so by destroying relatively large parts of the cortex and then seeking fiber degeneration in the spinal cord (Nyberg-Hansen and Brodal, 1963). We are pursuing the question at a more refined level, by making very discrete cortical lesions which approximate the size of a single cortical column. This will enable us to plot the origins of the corticospinal tract with much greater precision and to investigate anatomically the dispersion of output of a single cortical column.

2. Medullary Reticular Formation. With the use of electrophysiological techniques, a new study is yielding data on the neuronal organization of the medullary reticular formation. The sample of cells collected is still small relative to the volume needed, but is expanding steadily. A field potential mapping of the nucleus reticularis gigantocellularis is underway as part of the project and is also proceeding smoothly. Although cell characterizations have not yet been completed, it is clear that the properties of these cells (latency of response after peripheral stimulation, receptive field size, natural stimulus sensitivity, etc.) are such that the majority of these cells cannot reasonably serve as a relay for activity passing from the skin to motor cortex, although this has been a hypothesis popular with some investigators (Buser, 1966).

E. Localization of Ethanol in the Brain. Physiological studies of the changes in various autonomic responses to electrical stimulation of various central nervous system nuclei, after graded doses of intravenous ethanol injection have led to the suggestion (Spector, 1972) that not all parts of the brain are uniformly affected by alcohol, contrary to previous dogma. A study is in progress using intravenous injections of radioactive labelled ^{14}C-ethanol and subsequent radiographic analysis of brain tissues, to determine more exactly where, and in what quantities, ethanol concentrates in the brain.

F. Morphine Addiction in Rats. With Dr. Zoltan Annau and Dr. George Koob of Johns Hopkins University, we are studying the effects of morphine upon intracranial electrical self-stimulation (ICSS) patterns in rats with electrodes implanted in several areas in the diencephalon. Changes in biogenic amines in the post-addictive state will be analyzed by Dr. J. Meyerhoff of our Microwave Research Department. Changes in the electroencephalogram following long bouts of sustained ICSS activity will be analyzed in our department for ratios of various types of sleep and rhythmic waking activity.

G. New Techniques.

1. Relationship of Brain Neurotransmitters and their Metabolites with Morphine and Other Narcotics in the Central Nervous System. A procedure for catheterizing the cistera magna has been perfected in conjunction with morphine studies in primates. This has resulted in the ability to sample cerebrospinal fluid at a maximum rate of 1.8 cc/2hrs. for six hours without apparent injury to the monkey. Morphine injections have tentatively been shown to cause a six-fold increase in cyclic 3', 5'-adenosine monophosphate ("cyclic AMP"). This effect may be related to the development of drug tolerance in these animals.

2. Low-cost Sound Pattern Generator. A low-cost sound pattern generator that is remotely programmable has been developed to produce pure-tone auditory patterns. The device, which is compatible with relay programming equipment, can generate either of two auditory patterns in the sequence ABA or BAB. The generator features an independently adjustable range of ON and OFF durations (0.1 to 1.0 sec) for pure-tone bursts and a selectable range (0.2 to 2.0 sec) of intercycle intervals. The inexpensive construction cost coupled with the flexibility of the timing characteristics of the generator should make it of interest to a wide variety of auditory researchers (for details see Swinnen, 1972).

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08 Physiology

Work Unit 076 Brain mechanisms maintaining vital functions during stress: anatomical, physiological, and behavioral correlates.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6439	72 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'S	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 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 114(f)			

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. AM

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 72	6	240
CURRENT FISCAL YEAR 73	6	240

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

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; (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) 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 humans and monkeys in a variety of acute and chronic stress situations, with the conceptual approach developed by our earlier work that the organization of endocrine regulation can best be understood by viewing "overall" hormonal balance, or multihormonal patterns, rather than by focussing on single endocrine systems, as has previously been customary in the stress field.

25. (U) 71 07 - 72 06 Collaborative studies of physical stress with ARIEM (Natick) have continued. Consistent TSH and thyroxine, as well as cortisol and norepinephrine, elevations were observed in anticipation of exhausting exercise, suggesting that the pituitary-thyroid axis responds to emotional stimuli also. Studies of humans at 12,000 feet simulated altitude revealed evidence of altered peripheral metabolism of thyroxine and showed renin-aldosterone suppression in the face of catecholamine elevations. Psychoendocrine studies of hypertensive patients (WRGH) have shown a consistent absence of the usual 17-OHCS elevations to stressful experiences in the hospital and the relationship of psychological defenses and coping style to this lack of endocrine lability is being evaluated. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 71-30 Jun 72.

*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

Investigators.

Principal: John W. Mason, M.D.; MAJ Theodore A. Kotchen, MC

Associate: MAJ Scott E. Monroe, 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.

Description.

This program is concerned with the role of the central nervous system in the coordination 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 the past year has shifted from animal work to the study of human adaptation under certain explicit environmental conditions and in disease. Particular attention has been given to the hormonal correlates of endurance during various forms of physical stress. A major portion of our efforts has been the continued collaboration with the Army Research Institute for 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. Further data has been obtained on the pituitary-thyroid system responses during exercise in a collaborative study with LTC Hartley and COL Jones at ARIEM. During sustained exercise, there

are rather consistent elevations in TSH, total thyroxine, and free thyroxine levels, but the percentage changes are relatively small, probably due partly to increased T_4 peripheral turnover. Hormonal measurements are now completed in this study except for plasma testosterone and prolactin determinations.

b. Altitude. In collaboration with MAJ R. Hogan at ARIEM, 11 normal volunteer subjects were exposed to a simulated altitude of 12,000 feet for three days. At high altitude thyroid hormone levels were increased in the absence of a TSH elevation, suggesting an alteration in the peripheral metabolism of thyroxine. Plasma and urinary catecholamines were elevated while the renin-angiotensin system was suppressed. The latter combination of changes suggests that, while catecholamines are known to be capable of stimulating renin release, some other regulatory influence takes precedence over this effect at high altitude.

c. Prolonged Bed Rest. In cooperation with COL J. Earll, an endocrine study of normal volunteer subjects exposed to ten days of bed rest has shown substantial decreases in urinary epinephrine and norepinephrine levels in the face of unchanged urinary 17-OHCS levels. These findings have significance not only in relation to stress theory, but also suggest that postural and activity factors may be relatively important independent variables in clinical studies which are concerned with functions affected by hormonal balance.

2. Hormonal Balance in Emotional Stress.

Further studies have shown that plasma total thyroxine increases accompanied the consistent plasma TSH, cortisol, and norepinephrine elevations that occurred during a 20-minute period when eight subjects were anticipating their first experience with exercising to exhaustion on a bicycle ergometer. These findings provide new support for the tentative conclusion that the pituitary-thyroid system is responsive to psychological influences in the human.

In collaboration with MAJ Natelson, Department of Experimental Psychology, a study of ultradian hormonal patterns under basal conditions and in relation to conditioned emotional disturbances in monkeys has been initiated. Preliminary data indicate a relatively stable base line pattern for plasma cortisol and TSH levels, measured at 20-minute intervals for six hours, as compared to more labile patterns reported in certain human subjects.

3. Hormonal Balance in Medical Patients.

Psychoendocrine studies of hypertensive patients have been continued, with psychiatric collaboration from Dr. M. Belfer of the National Institute of Mental Health. In eight patients evaluated for about a two-week period, there was a consistent absence of 17-OHCS elevations

to stressful experiences, even to hospital admission which is known to elicit corticosteroid responses in most people. The possibility that this psychoendocrine "unresponsiveness" or "refractoriness" may be related to a distinctive style of organization of psychological defenses is being evaluated. It is somewhat reminiscent of observations made earlier by our laboratory in normal subjects exposed to chronic psychological stress over many months.

4. Special Studies.

a. The Renin-Angiotensin System.

In collaboration with MAJ W. Flamenbaum, Department of Nephrology, WRAIR, several projects studying various aspects of the renin-angiotensin system were completed by MAJ Kotchen this year. In rats, a high potassium intake has been shown to suppress both renin synthesis and release. This renin suppression protects against experimental acute renal failure. Similarly, renin suppression by either deoxycorticosterone or renin immunization has also been shown to protect against experimental acute renal failure. The results of all these studies suggest that protection is related to suppression of renal renin content rather than circulating renin.

Renin was measured in newborn infants, in collaboration with LTC A. Strickland, Department of Pediatrics, WRGH, and was found to be elevated compared to adults. Evidence was presented indicating that the kidney of the newborn infant secretes renin.

Studies of renin kinetics were continued, and the results indicate that plasma from uremic patients and some hypertensives may lack an inhibitor for the renin-renin substrate reaction that is present in plasma of normal subjects.

A study has almost been completed comparing a bioassay and an immunoassay method for measuring renin activity. Results with both methods are comparable in a variety situation known to affect the renin-angiotensin system.

b. Regulation of Pituitary Gonadotropins.

During the past year, MAJ Monroe, in collaboration with Dr. R. B. Jaffe, University of Michigan, has investigated the mechanisms responsible for the regulation of human gonadotropins. Eight female volunteers have been given 2.5 to 10.0 ug estradiol benzoate every 12 hours for a total of five doses. In some of the volunteers, there was a rapid rise in serum gonadotropins following the estradiol injections. These data suggest that estrogen can, under certain conditions, either inhibit or promote pituitary gonadotropin release.

In another study, five female volunteers have received clomiphene citrate, an anti-estrogenic compound. In three of the five subjects,

this drug temporarily blocked the mid-cycle release of pituitary gonadotropins presumably via estrogen antagonism at the level of the hypothalamic-pituitary complex. These two studies strongly suggest that the cyclic release of pituitary gonadotropins is regulated by the gonad (ovary) and not by intrinsic mechanisms in the central nervous system.

5. Hormone Assay Methodology.

A sensitive new radioimmunoassay method for plasma testosterone measurement, capable of quantitating testosterone in less than 0.2 ml of plasma, has been set up and validated by MAJ Monroe. The procedure is now ready for application to stress experiments and clinical studies.

MAJ Monroe is also developing a radioimmunoassay for serum morphine concentrations which will be utilized in studies of drug addiction. With the assistance of Dr. B. Boone, Department of Biochemistry, WRAIR, dihydromorphinone-(O-carboxyl methyl oxime)-bovine serum albumen was synthesized and used to immunize rabbits. These rabbits have subsequently produced antibodies which will probably be suitable for use in the radioimmunoassay for morphine.

During the past year, two new data processing systems have been developed by MAJ Monroe. These two systems automatically process radioimmunoassay data directly from counting equipment without human intervention, thus eliminating technician bias and reducing the likelihood of error.

Summary and Conclusions.

Our long-term program has continued to shift considerable emphasis from work with laboratory primates to basic neuroendocrine and stress studies in normal human subjects and to clinical psychoendocrine studies. In regard to our goal of defining the "overall" patterns of hormone responses to various physical "stressors," the collaborative arrangement with physiological investigators at ARIEM has proven especially fruitful and plans for continuation of this cooperative effort have been made, particularly with regard to the study of heat, cold, and hypoxia. The data accumulating in our laboratory on broadly and distinctively organized patterns of multiple endocrine responses to different physical and psychological stimuli are providing a new basis for the revision of "stress" theory and the recognition of some basic fallacies which have led to confusion and delayed progress in stress research. Our clinical studies are also beginning to provide some guidelines as to how psychoendocrine approaches may be applied to the study of the pathogenesis of disease in patients with psychosomatic disorders.

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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* U.S. GOVERNMENT PRINTING OFFICE 1973 O—494-065