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RCS MEDDH - 288 (RI)

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

Including

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

ANNUAL PROGRESS REPORT
1 July 1964 - 30 June 1965

VOLUME 2

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

9

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 1964 - 30 June 1965

Volume 2

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

SUMMARY

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

In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care as established by the National Society for Medical Research."

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PROJECT 3A014501B71Q
COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01
Communicable Diseases and Immunology

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23. KEYWORDS: Parasite; serology; immunology; fluorescent antibody; malaria; filaria; schistosome; antigen; antibody; chemotherapy

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

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

26. (U) Progress (Jul 64 - Jun 65) - The susceptibility of various mouse strains to infection with Schistosoma mansoni was studied as was the character of this infection in various species of primates. The passive transfer of immunity with serum and serum fractions was studied in rats. The multiple antibody response in S. mansoni infections was examined through studies on antigenic constituents in eggs, cercariae and adults (excretions and secretions) determined by flocculation reactions, cross absorption and double diffusion studies. Studies on the activity of TWSb in oil against S. mansoni infections in monkeys were completed while experiments using a non-antimonial compound (CIBA) were begun. Preliminary experiments for developing a fluorescent antibody test for the diagnosis of filariasis were started and the life-cycle of Dipetelonema witei was established in the laboratory.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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ANNUAL PROGRESS REPORT

Project 3A014501B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 165, Parasitic diseases

Investigators.
Principal: Elvio H. Sadun Sc.D.
Associate: John I. Bruce, M.S.; Capt Robert L. Hickman, VC; Maurice Schoenbechler, B.S.; Joseph S. Williams

Description.

The primary purpose of these investigations was to study the various immunological, physiological and ecological aspects of parasitic diseases toward the goal of gaining a better understanding of natural susceptibility, acquired resistance and the effectiveness of therapeutic agents for the prevention, suppression and treatment of some of these diseases.

Progress.

1. The multiple antibody response in Schistosoma mansoni infections. Antigenic constituents in eggs, cercariae and adults (excretions and secretions) determined by flocculation reactions, cross absorption and double diffusion studies.

Anderson described a simple and reliable slide flocculation (SF) test which utilizes lipid-free cercaria antigen coated onto cholesterol-lecithin crystals. The sensitivity of this test was greatly increased by removing the antigen-cholesterol-lecithin complex from the salt solution in which it was prepared and by re-suspending it in fresh salt solution. The sensitivity and specificity of this procedure with sera from individuals with proven S. mansoni infection was found to be excellent. All the serologically reactive antibody for this test can be removed without any dilution by simple absorption with a calculated volume of washed, packed, and essentially dry antigen-cholesterol-lecithin complex. This permits the standardization of antisera in terms of amounts of antibody nitrogen, thus introducing into the field of schistosomiasis some of the quantitative criteria which in the past existed only in the serology of some bacterial infections.

Critical evaluations and thorough understanding of immunologic and serologic observations in schistosomiasis are seriously handicapped by the lack of adequate information on the numbers and characteristics of specific antigens. Gel diffusion methods were applied by Levine for an

analysis of antigens of S. mansoni and Schistosomatium douthitti. He observed two precipitin bands with sera from infected animals and up to six bands with sera from artificially immunized rabbits. Subsequent studies by Smithers revealed that sera from Rhesus monkeys infected with S. mansoni formed one precipitin band when reacted with cercarial extract, two or three bands when reacted with adult worm extracts and four bands when reacted with egg extracts. Kent observed ten distinct fractions by electrophoresis but only two or three with human sera from proven infections. An analysis of S. mansoni antigens by agar-gel diffusion was also conducted by Kagan and Norman. They found seven precipitin bands with adult extracts, three with cercarial extracts, and five with egg extracts when these were reacted with sera from artificially immunized rabbits. Conversely, they observed only between one and three antigenic components when the extracts were reacted with sera from patients with proven infections. Extensive electrophoretic and immunoelectrophoretic studies on S. mansoni antigens were carried out by Biguet and co-workers. With sera from immunized rabbits they observed 14 antigenic fractions. Excretory and secretory products of adult worms were found to contain six components. Only two precipitin bands were identified by them when sera from infected patients were used.

One of the primary aims of the present series of investigations was to determine whether extracts from egg and adult stages of the schistosomes could be coated onto cholesterol-lecithin crystals and thus be used effectively for the serological diagnosis of schistosomiasis in the SF test. The reactions obtained with these antigens were compared with those obtained with the cercaria antigens to determine whether they stimulated or detected distinct humoral factors or whether they differed only in the relative proportions of common antigens.

Attempts were also made to isolate and define some of the soluble antigenic fractions present in egg, cercaria and adult S. mansoni extracts; to ascertain which, if any, are common to various stages; to characterize the antigenic fractions which are adsorbed onto cholesterol-lecithin crystals; and to compare results obtained with the SF test and immunoelectrophoresis before and after homologous and heterologous serum absorption.

The data presented herein indicate that egg and adult extracts can be coated onto cholesterol-lecithin crystals to provide satisfactory and consistent results in the SF test. The adult excretion and secretion extract confers to the test the great sensitivity as evidenced by an early appearance of detectable antibodies, by consistently higher titers, and by the persistence of positive reactions for years after a single exposure to infection. Immunoelectrophoretic and SF reactions, conducted before and after homologous and heterologous absorption, in sera from

artificially immunized and experimentally infected animals and from naturally infected humans show stage specific fractions which stimulate distinct humoral factors. Whereas some antigen-antibody systems were stage specific, others were found to be shared by two or more stages of the life cycle of the parasite.

Animals: Young adult mice, Charles River ICR strain, weighing approximately 20-25 grams each; white rabbits, weighing between 2 and 3 kilograms each; and Rhesus monkeys (Macaca mulatta), about two to four years of age, weighing between 1 and 3 kilograms each at the beginning of the experiment were used as test animals. All animals were fed a standard diet. The rabbits and monkeys were kept in individual cages and were bled at regular intervals from the beginning of each experiment until death.

Sources of Antisera:

a. Experimental infections: Forty mice were exposed percutaneously to 200 cercariae each. Prior to exposure some mice were exsanguinated. The serum was collected and pooled and served as a pre-infection control. Other mice were killed at weekly intervals up to eight weeks after exposure. The serum was pooled and used as a source of antiserum from infected mice.

Two rabbits were exposed once to 5,000 cercariae each. They were bled just prior to exposure to infection and again at weekly intervals for 12 weeks. One monkey was exposed to 500 cercariae and bled at weekly intervals up to 15 weeks after exposure. Another monkey was exposed to 350 cercariae and bled at various intervals up to 42 months after exposure.

b. Artificial Immunization: Six rabbits were divided into three groups of two animals each and were given intravenous injections of extracts from eggs, cercariae, and adult excretions and secretions (ES) respectively. Each rabbit was given intravenous injections of 0.5 mg of antigenic extracts every 3 days for 4 times, and thereafter, 1 mg every 5 days for 10 times. Throughout the immunization schedule the rabbit sera were tested by the quantitative Ouchterlony method and by the SF test using extracts from eggs, cercariae and adult ES as sources of antigen to determine the earliest appearance of antibodies and the increase in antibody titers. The quantitative Ouchterlony technique was conducted by placing the antigenic extract in a central well and two-fold dilutions of each antiserum in six wells surrounding the central well.

c. Human Infections: A total of 187 sera from humans was also used in these experiments. Of these 38 came from individuals with proven S. mansoni infections as detected by recovery of eggs in the stool. Seventy-seven came from individuals living in a heavily endemic area of S. mansoni and S. haematobium in Africa. Control sera were selected from healthy Americans who had applied for admission to a military academy and had been found free of parasitic or bacterial infections by a thorough physical examination. In addition, 40 sera obtained from individuals with one or more parasitic infections other than schistosomiasis, eight serum specimens from individuals with proven infections of syphilis, eight from individuals with proven leprosy and five from individuals with a diagnosis of lupus erythematosus were selected to determine the relative specificity of the SF test with the different antigens. One hundred and eight sera were tested by the SF test with egg, cercaria and adult ES and 79 sera were also tested by the CF test employing an adult extract as antigen.

Antigens: Eggs, cercariae, and adult schistosomes employed in the preparation of antigens were obtained from S. mansoni (Puerto Rican strain) maintained in albino mice and Australorbis glabratus snails. Eggs were collected as follows: intestines from mice exposed 8 weeks earlier to 200 cercariae each were removed and flushed out with 2 percent saline by injecting it into the lumen from a rubber bulb and hypodermic needle. Pools of 20 to 25 intestines were then homogenized with a Waring Blendor for 2 minutes in a small quantity of 0.4 percent trypsin (1:250 Difco) in 2 percent saline. The 0.4 percent trypsin in saline was added to the homogenate to a total of 2000 ml, and the suspension was incubated at 37°C for 90 minutes, while being mixed by a magnetic stirrer. After digestion, the contents were strained through 40 and 100 mesh brass wire sieves and then filtered through the apparatus described by Browne and Thomas. The eggs were collected in the filtering capsule and washed with 200 ml of 2 percent saline. The suspension was then centrifuged, the supernate was decanted and the packed and essentially dry eggs were lyophilized immediately. Cercariae were collected from pools of 300-400 laboratory raised and infected A. glabratus snails by allowing them to shed for two hours after exposure to light. Cercarial suspensions were concentrated in 1000 ml conical pharmaceutical flasks placed overnight in the refrigerator at 4°C. The following morning the supernatant fluid was withdrawn and the sedimented cercariae were transferred to drying ampoules and lyophilized. Adult worms were collected from mice by the Perf-o-Suction method. Preliminary experiments had indicated that extracts of adult worms could not be coated readily onto the cholesterol-lecithin crystals. Conversely, excretions and secretions from the adults could be readily coated onto the crystals providing a stable test antigen which gave consistent results. Therefore, throughout these experiments excretions and secretions of adult worms were used instead of somatic extracts. The adult ES was prepared by repeatedly washing adult worms in

saline immediately after perfusion and placing approximately 600 of them in 15 ml of distilled water overnight at 4°C. The following morning the supernate was collected, filtered, centrifuged for 30 minutes at 8,000 G., dispensed into ampoules and lyophilized.

The lyophilized eggs, cercariae and adult ES were weighed and delipidized with anhydrous ether. Subsequently, the cercariae were extracted in triethanolamine buffered saline and the eggs and adult ES in phosphate buffered saline. Twenty milliliters of cold anhydrous ether were added to 100 mg of dried antigen and homogenized with a motor driven Tenbroeck grinder in an alcohol ice bath for 10 minutes. The extracted antigen was lyophilized and was reconstituted in distilled water when used. The antigenic extracts were absorbed onto cholesterol-lecithin cyrstals. The extracting and absorbing procedures followed the method previously described in detail. Protein nitrogen was determined for several antigenic extracts before and after absorption onto cholesterol-lecithin crystals. The determination was made after digestion by the micro Kjeldahl method. The procedure was essentially that described by Kabat and Meyer. However, a modified one-piece Parnas and Wagner distillation unit was used. Furthermore, 0.2 percent bromcresol green was used as an indicator in place of those originally recommended.

Serological Tests: The SF test was performed with strict adherence to published instructions. Preliminary experiments indicated that all three antigenic extracts reacted in the flocculation test with serum of infected and artificially immunized animals. Conversely, they did not react with serum from uninfected controls. The complement fixation test using somatic adult extracts as antigen was performed according to the published instruction.

Absorption Technique: The amount of a given antigen emulsion required to absorb all of the serologically reactive antibody in the SF test was calculated on the basis of the titer obtained with the antigen and the particular serum in this test. The simplified calculation is as follows: The titer divided by 2 equals the number of milliliters of antigen emulsion required to absorb all of the serologically reactive antibody from 1 ml of serum. The serum to be absorbed was added directly onto the washed, packed and essentially dry antigen cholesterol-lecithin complex obtained by centrifuging the given antigen emulsion at 2,500 G. for 30 minutes and decanting the supernatant fluid. Once the serum to be absorbed was added to the packed antigen the mixture was placed on a slide rotating machine (220 rpm) for 1 hour temperature (22° - 24°C). To reduce the requirements for large amounts of antigens and for greater precision titrations were conducted using the Sanz pipette which could distribute as little as 2 microliters with an error of ± 1 percent. After absorption, the antibody-antigen cholesterol-lecithin aggregate was re-

moved by centrifugation at 2,500 G. for 30 minutes, and the supernatant serum was retested with all three antigenic mixtures. As an absorption technique control, a washed BSA antigen-cholesterol-lecithin emulsion was prepared in a manner similar to that previously described for the schistosome antigens. Bovine crystalline albumin (1 mg per ml) in 0.85 percent sodium chloride was substituted for the antigenic mixture. The resulting antigen emulsion was highly reactive against sera from rabbits immunized with BSA and was non-reactive with normal rabbit sera as well as with the sera from humans with schistosomiasis. As a further control, serum to which cholesterol-lecithin complex without antigen had been added, was incubated for the same amount of time, centrifuged and tested by the SF test. No significant reduction in antibody titer resulted from incubation of the serum with cholesterol-lecithin crystals.

Double diffusion studies: The method of double diffusion in agar of Ouchterlony was used in a series of preliminary experiments. However, the antigenic complexity of the individual extracts tested was such that the numerous precipitin bands which formed in agar occurred in a restricted space and could be identified only with great difficulty. Because of the laboriousness in separating and enumerating the various lines of precipitation, the results often were not reproducible. Conversely, more consistent results could be obtained when the presence of antigens in the extract was evaluated by immunoelectrophoretic analysis, according to the technique described by Graber. This technique greatly reduced the chances of error by spreading the various components according to their mobility in the electrical field. A special polyester cell based on a commercial model was utilized for these studies. This cell permits one to carry out electrophoretic separations by the method of Scheidegger as modified by Weime. The 2 percent agar was diluted in half with a veronal buffer solution at pH 8.2 final ionic strength of 0.05. It was filtered rapidly and poured into the medium compartment of the cell up to a level just above the central plateau (5 mm). After proper cooling 4 strips of agar-gel spaced 2x2 at a distance of 70 mm were removed from the central plateau. The wall side of the cell and exposed areas of the plateau were carefully sponged. The extreme compartments of this cell were filled with 200 ml of stock buffer diluted with equal parts of distilled water. For the electrophoresis, 20 microliters of buffered antigenic extracts were prepared in the following concentration: 40 mg of lyophilized cercarial extract in 1 ml of phosphate buffered saline (PBS), 50 mg each of lyophilized egg and adult ES extracts in 1 ml of PBS. These extracts were utilized directly without further lyophilization (pH 7.2, 0.01M). The extracts were mixed with one half their volume of 2 percent agar at 40°C. Preliminary experiments had shown that this treatment did not affect the results noticeably. The origin was located 50 mm. from the anodic extremity of the slide. Voltage at the margins of the slide was maintained at 27 volts. The amperage was approximately of 5-8 mA per slide. The slides were bathed in petroleum ether. This

did not affect the junction between agar surfaces and prevented the slides from being in contact with air. The electrophoresis time was 110 minutes. For the immunodiffusion, the lateral troughs of each standard size slide were filled with antiserum in the following amounts: cercarial antiserum, 80 microliters diluted with equal parts of saline in the homologous reaction, 80 microliters undiluted in the heterologous reaction; egg antiserum, 100 microliters diluted with equal parts of saline, adult ES antiserum, 100 microliters in the homologous reaction, 80 microliters in the heterologous reaction; serum from experimentally infected rabbits and monkeys, 100 microliters undiluted. The time of diffusion was 72 hours under refrigeration (4°C) after which the slides were treated by the usual method. The slides were stained by 0.3 percent thiazine R in acetate buffer and were read and drawn against a black background while still wet. For comparative purposes the same serum specimens (R/C, R/E -- M/Inf) were used throughout the immunoelectrophoretic and absorption studies.

Flocculation tests and absorption with sera from artificially immunized animals: Preliminary experiments revealed that egg and adult ES extracts could be coated onto cholesterol-lecithin crystals for use in the SF test. Flocculations of antigen preparations were readily detected in the presence of homologous rabbit antisera and were absent in the presence of sera from normal control rabbits. The nature of the floccules varied somewhat with the antigens used. Those produced with egg-cholesterol-lecithin test antigen were intermediate in size and very well defined permitting an easy classification of negative, weakly positive and strongly positive reactions. The flocculations produced with the cercaria test antigen were relatively large, less well defined, somewhat feathery in appearance, and in strongly positive reactions they were fused together into large agglomerates. The flocculations produced with adult ES antigen were small, clearly visible in discrete and compact bodies and very easily definable. Table I shows the nitrogen content of the antigenic extracts used before and after absorption onto cholesterol-lecithin crystals. Before absorption onto the crystals the antigenic extracts contained comparable amounts of nitrogen per milliliters. After absorption and washing of the excess antigen, however, the cercaria antigen-cholesterol-lecithin emulsion contained the greatest amount of N/ml and the egg antigen-cholesterol-lecithin emulsion contained the smallest. Within the limits of the concentrations used, approximately 1/10 of the nitrogen content of egg and adult ES antigenic extracts was recovered from the antigen-cholesterol-lecithin emulsion. Between 1/3 and 1/5 of the original content was recovered when cercaria extract was coated onto the crystals.

In view of these observations experiments were designed to evaluate and compare the SF test with the three different test antigens in homologous and heterologous antisera. As indicated in Table II the egg antiserum was reactive at relatively high titers in the SF test when egg or cercaria test antigen was used but did not react when the adult ES antigen

was used. The cercaria antiserum was reactive at relatively high titers with the cercaria test antigen but only at low titers or not at all with the adult ES and egg antigens. The anti-adult ES antiserum was reactive only with adult ES antigen.

TABLE I

Nitrogen content of antigenic extracts used before and after coating them onto cholesterol-lecithin crystals

Extract	Sample number	Nitrogen determinations (mgN/ml)		B/A ratio
		A Before absorption	B After absorption onto crystals	
Egg	1	0.32	0.03	11
	2	0.35	0.03	12
Cercaria	1	0.39	0.08	5
	2	0.41	0.11	4
	3	0.41	0.12	3
	4	0.43	0.14	3
Adult ES	1	0.51	0.06	8
	2	0.50	0.05	10
	1	0.23	0.03	8
	1	0.23	0.03	8

TABLE II

Slide flocculation titers in rabbits artificially immunized with egg, cercarial and adult ES extracts

Time from first inoculation (Days)	Test antigen	SF titers in rabbits immunized with:		
		Egg	Cercaria	Adult ES
39	Egg	16	1	--
	Cercaria	32	16	--
	Adult ES	--	2	16
59	Egg	128	--	--
	Cercaria	64	16	--
	Adult ES	--	2	16
67	Egg	ND	--	--
	Cercaria	ND	16	--
	Adult ES	ND	4	32

ND - Not Done

Since these results suggested a greater degree of sensitivity in the SF test when the homologous antigen was employed, a second experiment was set up to determine whether antibodies could be absorbed selectively. The results of this experiment are summarized in Table III.

TABLE III

Slide flocculation titers of sera from artificially immunized rabbits before and after homologous and heterologous absorption

Nature of antiserum	Test antigen	SF titers with given antigen			
		Before absorption:	After absorption with:		
		Titer	Egg AG	Cercaria	Adult ES
Anti-Egg	Egg	64	--	--	ND
	Cercaria	64	--	--	ND
	Adult ES	--	--	--	ND
Anti-Cercaria	Egg	1	--	--	--
	Cercaria	16	8	--	16
	Adult ES	2	2	2	--
Anti-Adult ES	Egg	--	--	--	--
	Cercaria	--	--	--	--
	Adult ES	32	32	32	--

ND - Not Done

Before absorption the egg antiserum reacted at a high titer with egg and cercaria test antigens but did not react with the adult ES antigen. The reactivity of this serum could be removed by absorption with either egg or cercaria antigen.

Before absorption the cercaria antiserum reacted with cercaria test antigen at a titer of 1:16 and with the other two antigens at very low titers. Absorption with egg antigen removed the reactivity in the SF test with egg test antigen but not the reactivity with cercaria and adult antigens. Absorption with cercaria antigen removed the reactivity in the SF test with both egg and cercaria test antigens. Absorption with adult ES removed the reactivity in this test employing egg and adult ES test antigens but did not bring about any reduction in titer when the cercaria test antigen was used.

Before absorption the adult ES antiserum reacted only with the adult ES test antigen. Absorption with either egg or cercaria antigens failed to bring about a reduction in the reactivity with the adult ES antigen. However, absorption with the adult ES antigen removed from the serum all homologous antibodies demonstrable in the SF test.

Flocculation and absorption tests with sera from experimentally infected animals: A series of experiments was designed to determine the activity of the SF test using each of the three antigenic extracts with sera obtained from experimentally infected animals. Efforts were made to compare the 3 tests from the time of exposure up to more than 3 years after appearance of eggs in the stools. Sera from 2 monkeys exposed to a single dose of 500 and 350 cercariae, respectively, were tested at several intervals with all 3 antigens. The results are summarized and presented graphically in Figures 1, 2, 3, and 4.

The serologic response of one monkey exposed to 500 cercariae (Fig. 1) was followed for 15 weeks. All the specimens were negative with all 3 antigens for 3 weeks following exposure. The serum became reactive after 4 weeks with all 3 antigens before eggs were detectable in the stools (6 weeks). Initially, the titer obtained with the adult ES antigen was much higher than that obtained with the egg antigen and this in turn was higher than the titer obtained with the cercaria antigen. The maximum titer was reached with all three antigens 7 weeks after exposure to infection and remained high up to the end of the experiment 8 weeks later.

The serologic response of another monkey exposed to a single dose of 350 cercariae (Fig. 2) was followed for 42 months. All the specimens were negative with the 3 antigens for 5 weeks following exposure to infection but became positive to all antigens the following week coincidentally with the earliest appearance of eggs in the stools. Titers rose rapidly and reached their peak 2 months following exposure. As shown previously, higher titers were achieved with the adult and egg antigens than with cercaria antigen. Twenty-four months after exposure to infection the serum from this monkey was negative in the SF test when egg or cercaria antigens were used and remained negative up to the end of the experiment. Conversely, when adult ES antigen was used, the serum remained positive at high titers until the end of the experiment 42 months after exposure to infection and 34 months after eggs were last recovered in the stools.

Two rabbits were exposed to a single dose of 5000 cercariae each. Sera were collected weekly for 12 weeks and tested in the SF test with all 3 antigens. The results obtained with the two rabbits were very similar. Therefore, only those of one rabbit have been summarized and presented graphically in Fig. 3, as representative of both. All the specimens gave negative results with the 3 antigens for the first 2 weeks following exposure. After 3 weeks the serum reacted positively in the SF test using the adult ES antigen but was negative when the egg and cercaria antigens were used. Subsequent serum specimens from this animal gave positive reactions with increasing titers during the following 2 weeks when adult ES antigen was used. Conversely, there was a total absence of demonstrable reactivity when the egg and cercaria antigens were used. Six weeks

SEROLOGICAL RESULTS OF MONKEY EXPOSED TO 500 S. MANSONI CERCARIAE (SHORT DURATION)

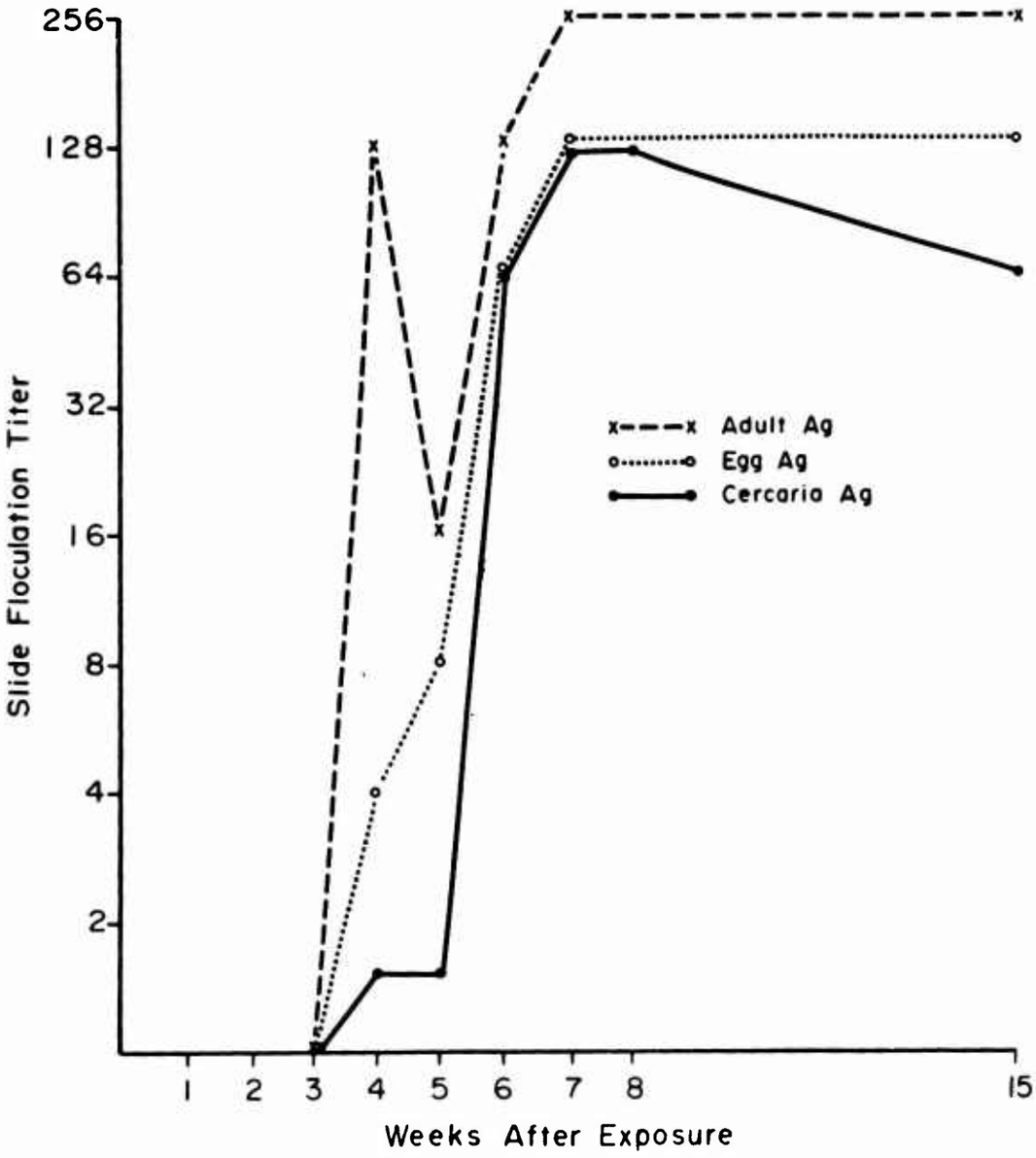
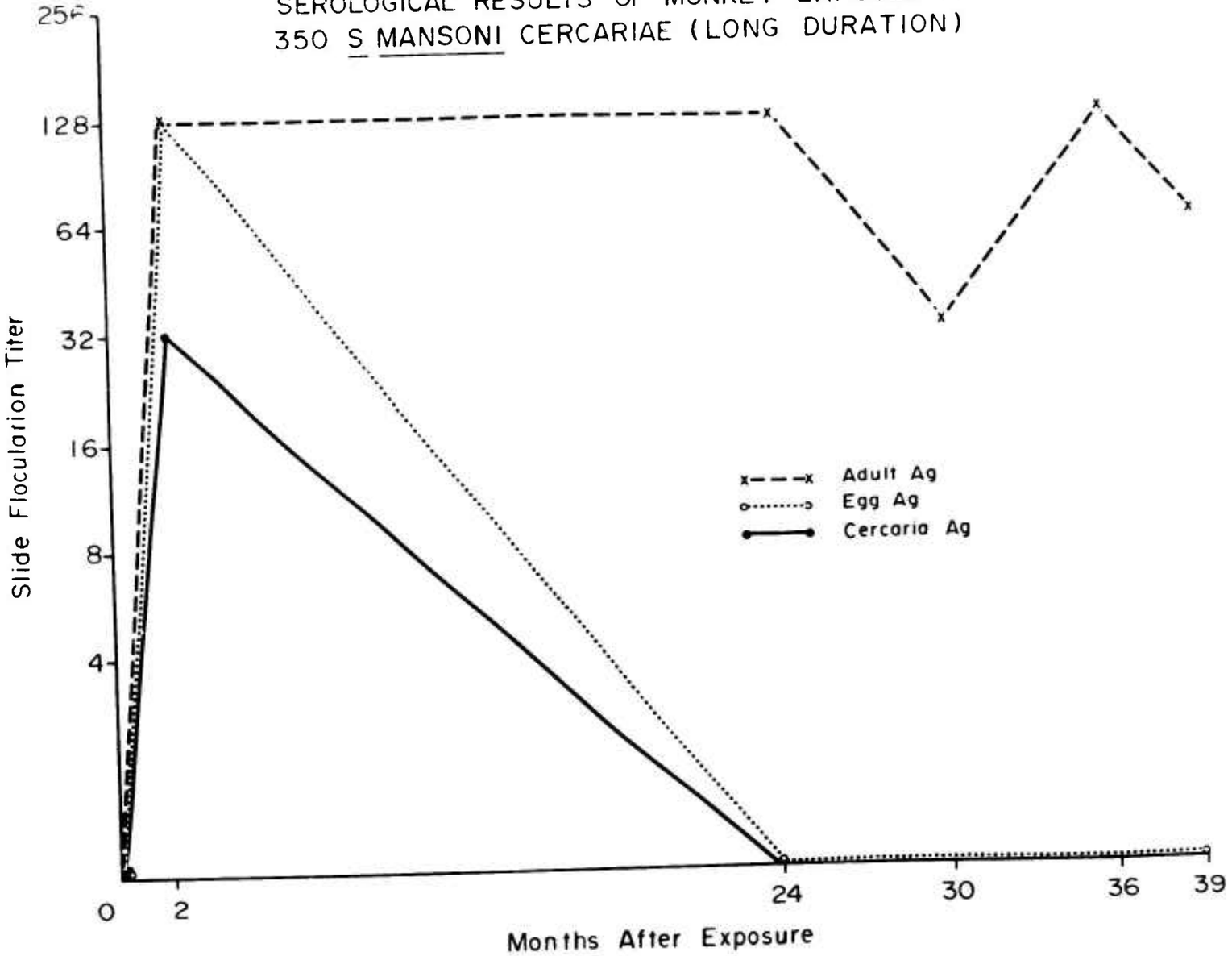


Fig. 2

SEROLOGICAL RESULTS OF MONKEY EXPOSED TO 350 S MANSONI CERCARIAE (LONG DURATION)



following exposure positive results were obtained in the SF test with each of the 3 antigens. However, when the adult ES antigen was used, much higher titers were observed. With all 3 antigens, titers in the SF test continued to increase up to the end of the experiment 12 weeks after exposure.

Sera from 4 of 40 mice exposed to a single dose of 200 cercariae each were tested weekly by the SF test with the 3 antigens. Pools of sera from these 4 mice gave negative results with all 3 antigens for the first 3 weeks following exposure (Fig. 4). After 4 weeks, the serum reacted positively in the SF test using adult ES antigen but was negative when the egg and cercaria antigens were used. After 5 weeks, positive results at a high titer were observed with the adult antigen, and positive results at a relatively low titer were obtained with the egg antigen. No demonstrable reactivity was present at this time when the cercaria antigen was used. Seven weeks after exposure the pooled sera gave positive reactions in the SF test with each of the 3 antigens. Highest titers were obtained with the adult ES antigen and lowest titers with the cercaria antigen. Titers in the SF test continued to increase with all 3 antigens until the end of the experiment 8 weeks after exposure.

Representative sera from a monkey, a rabbit, and 4 mice, all of which had been experimentally infected, were selected for absorption studies. This was done in order to determine whether absorption of sera from infected animals with predetermined amounts of egg, cercaria and adult ES extracts coated onto cholesterol-lecithin crystals would remove the antibodies responsible for the SF reactions in the homologous and heterologous systems. The results have been summarized in Table IV. Before absorption the serum from the infected monkey reacted in the SF test with all 3 antigens. The reactivity to the egg and cercaria test antigens was removed after absorption with either egg or cercaria antigens. However, in both instances the serum was still reactive in the SF test using adult ES antigen. Conversely, absorption with adult ES test antigen removed the reactivity of the serum to the homologous antigen but did not diminish in any demonstrable way the reactivity of the serum to the egg and cercaria antigens. Comparable results were observed when serum from infected rabbits and mice was used (Table IV).

Flocculation tests with sera from infected humans: These studies with human sera were set up to compare the relative sensitivity and specificity conferred to the SF test by egg, cercaria, and adult ES antigens. A great similarity in the overall results was observed with all 3 antigens (Table V). However, sera which reacted in the SF test with some antigens failed to react with others, thus suggesting that they might detect different antibodies. In general, a satisfactory degree of sensitivity and specificity was observed with all 3 antigens. Attention is called to the fact that of the 7 sera from trichinosis patients, 2 reacted positively with egg antigen and 3 with cercaria antigen but none reacted with adult ES antigen indicating a greater specificity of ES antigen.

Fig. 3

SEROLOGICAL RESULTS OF RABBITS EXPOSED TO 500 *S. MANSONI* CERCARIAE

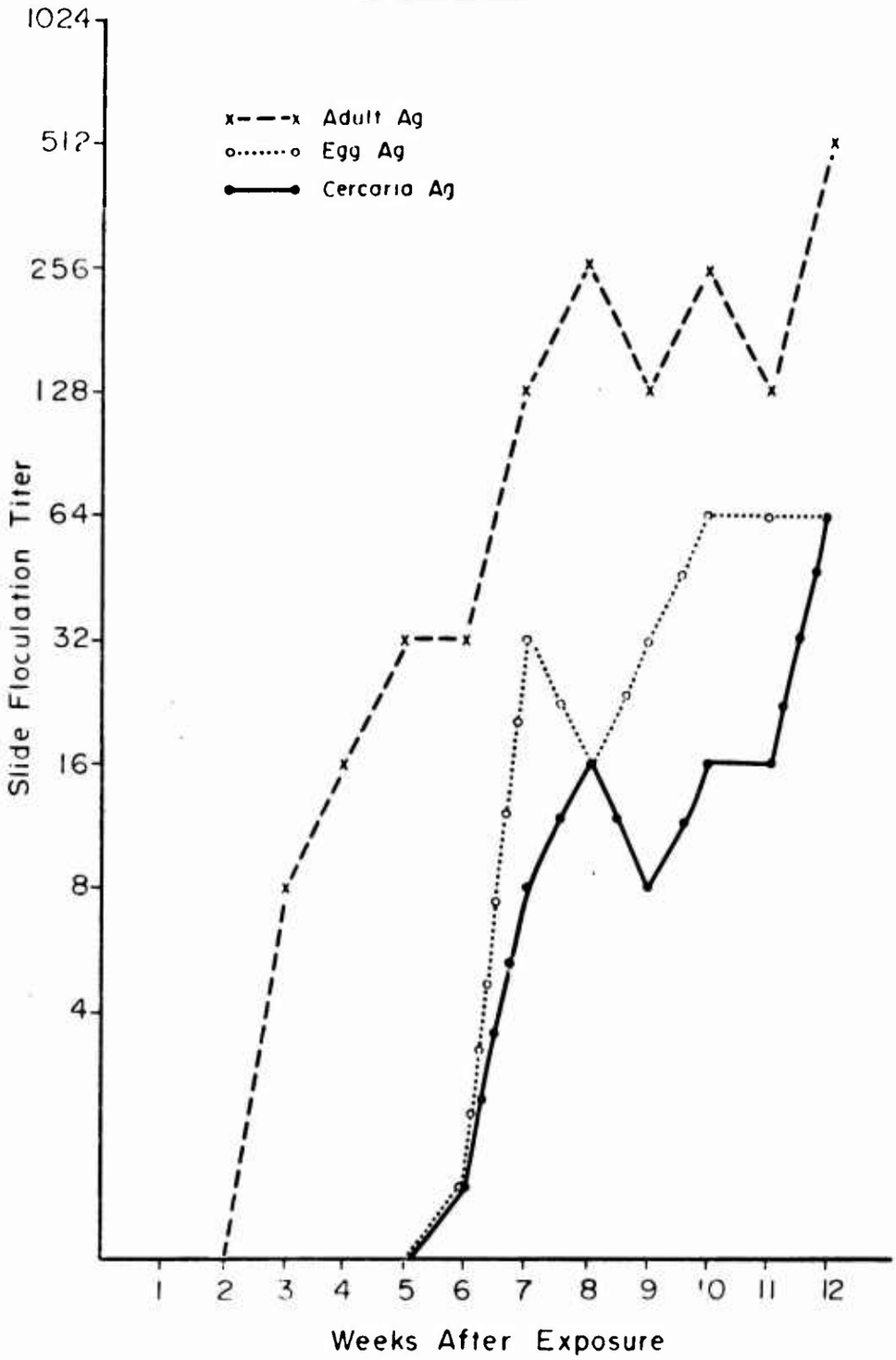


Fig. 4

SEROLOGICAL RESULTS OF MICE EXPOSED TO 200 S. MANSONI CERCARIAE

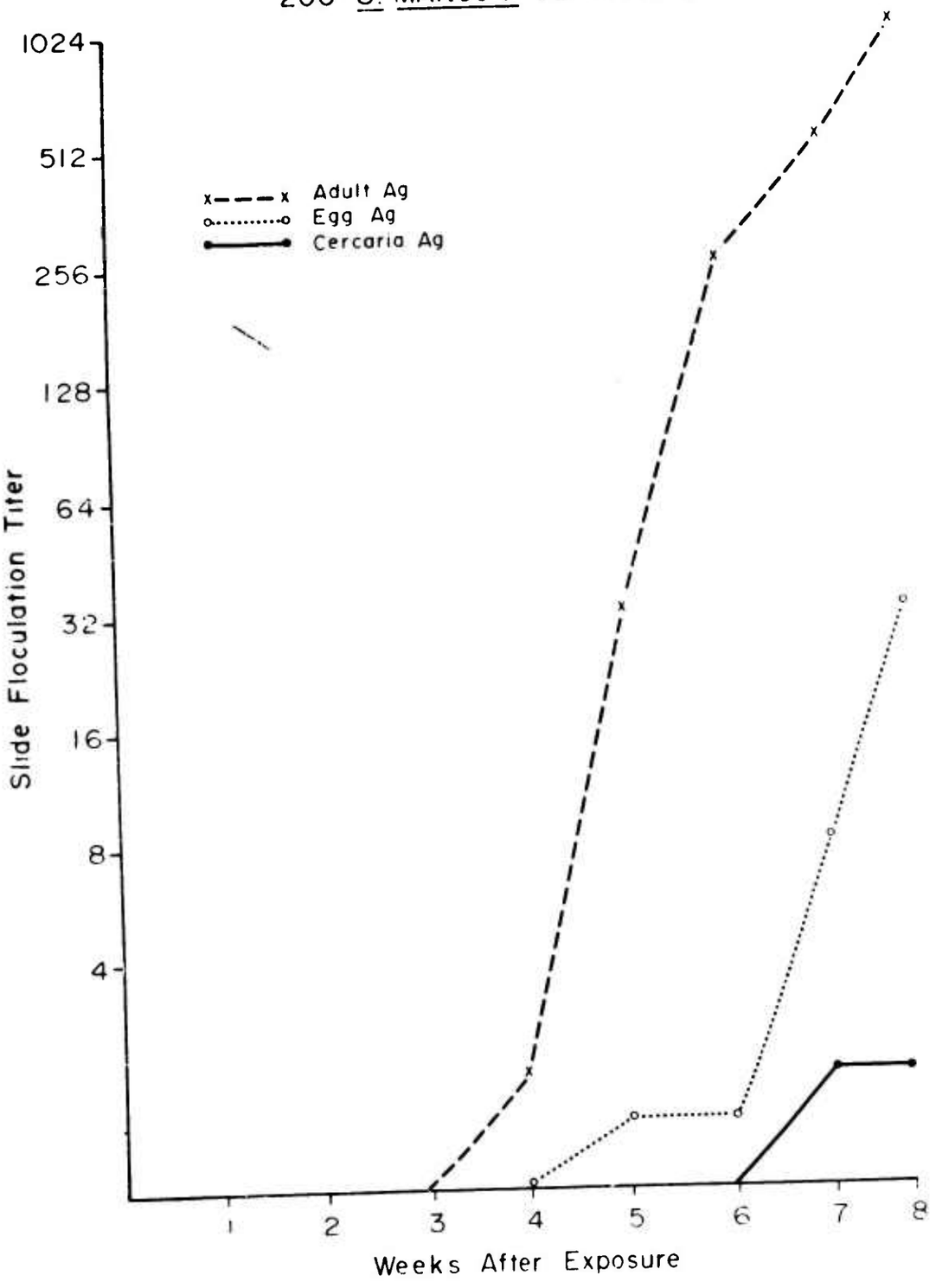


TABLE IV

Representative slide flocculation titers of sera from experimentally infected animals after homologous and heterologous absorption

Infected animal	No. of cercariae	Time after exposure (days)	Test antigen	SF titers with given antigen			
				Before absorption	After absorption with:		
					Egg	Cercaria	Adult ES
Monkey	350	56	Egg	16	--	--	16
			Cercaria	2	--	--	2
			Adult ES	32	16	16	--
Rabbit	5000	77	Egg	64	--	4	16
			Cercaria	16	--	--	8
			Adult ES	128	128	128	--
Mice	200	56	Egg	32	--	16	ND
			Cercaria	2	--	--	ND
			Adult ES	1024	1024	1024	ND

TABLE V

Sensitivity and specificity of the slide flocculation test with egg, cercaria and adult ES antigens in testing human sera

Serum	Number	Egg		Cercaria		Adult ES	
		+	-	+	-	+	-
Schistosomiasis	37	31	6	32	5	33	4
Normal	10	0	10	0	10	0	10
Leishmaniasis donovani	5	0	5	0	5	0	5
Falciparum malaria	4	0	4	0	4	0	4
Vivax malaria	2	0	2	0	2	0	2
Trypanosomiasis cruzi	1	0	1	0	1	0	1
Amoebiasis	2	0	2	0	2	0	2
Onchocerciasis	6	0	6	0	6	0	6
Strongyloidiasis	3	0	2	1	2	2	1
Paragonimiasis	3	0	3	0	3	0	3
Trichinosis	7	2	5	3	4	0	7
Filariasis	4	1	3	0	4	0	4
Loasis	2	0	2	0	2	0	2
Opisthorchiasis	1	0	1	0	1	0	1
Syphilis	8	0	8	0	8	0	8
Leprosy	8	2	6	0	8	2	6
Lupus erythematosus	5	1	4	0	5	0	5

Sera from 70 individuals living in endemic areas for schistosomiasis were tested by the complement fixation test using as antigen delipidized extract of adults, and the results were compared with those obtained in the SF test using egg, cercaria, and adult ES antigens. Although the prevalence of schistosomiasis in this particular area was very high and most individuals were presumed to be infected with S. mansoni, S. haematobium or both, no information was available to determine with accuracy which of these individuals was free from infection. The results were summarized in Tables VI, VII, VIII. A much closer agreement could be observed between the CF and SF tests when adult ES antigen was employed in the latter.

TABLE VI

Correlation of results obtained with the SF (Egg antigen) and the CF (Adult antigen) tests for schistosomiasis

Results of CF \ Results of SF	Positive	Doubt	Negative
Positive	24	5	22
Doubt	1	2	8
Negative	2	2	14

TABLE VII

Correlation of results obtained with the SF (Cercaria antigen) and the CF (Adult antigen) tests for schistosomiasis

Results of CF \ Results of SF	Positive	Doubt	Negative
Positive	30	5	17
Doubt	1	1	8
Negative	2	0	15

TABLE VIII

Correlation of results obtained with the SF (Adult ES antigen) and the CF (Adult antigen) tests for schistosomiasis

Results of CF \ Results of SF	Positive	Doubt	Negative
Positive	45	1	5
Doubt	5	1	4
Negative	6	1	11

Fig. 5

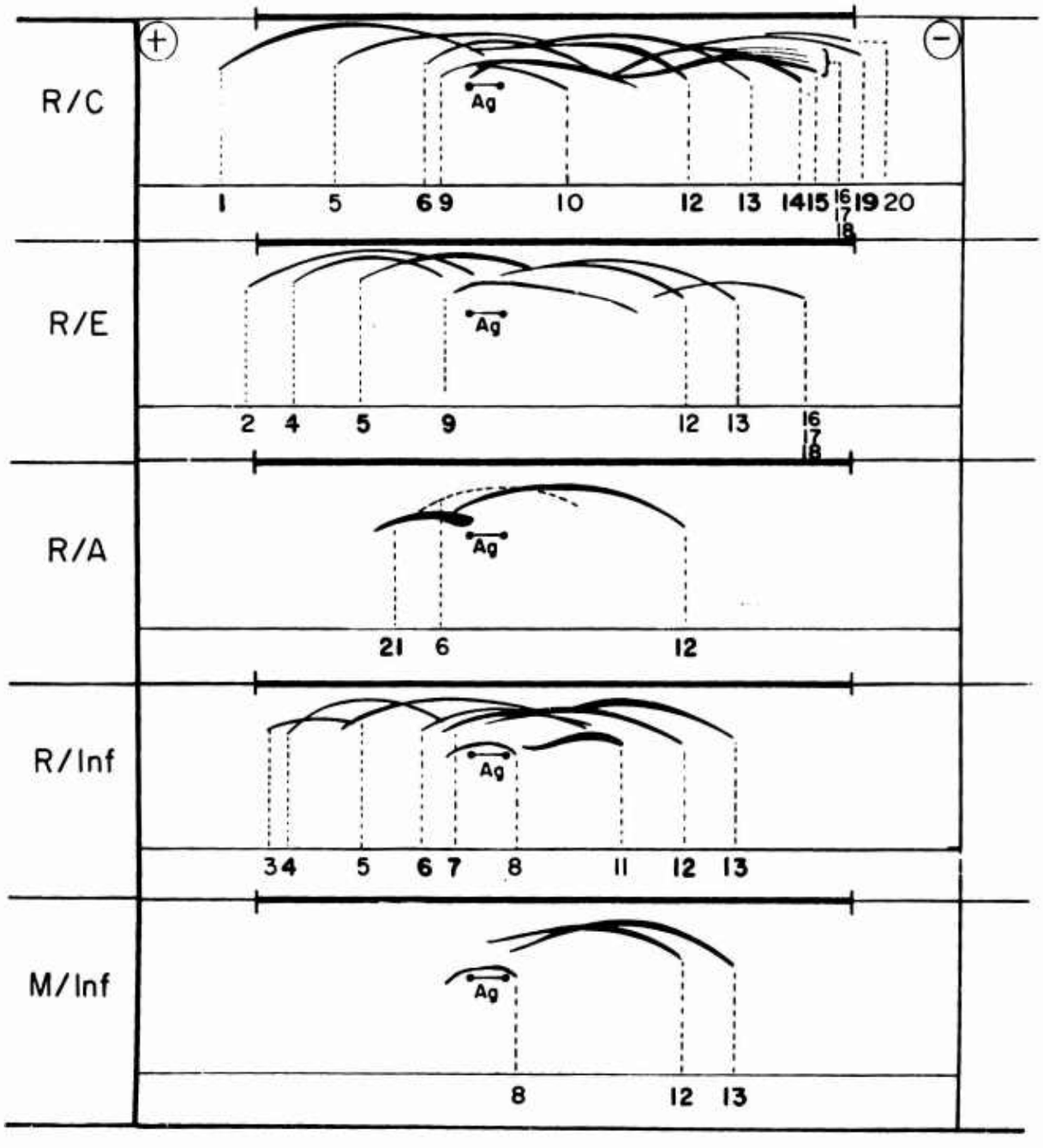

R/C
Ag
1 5 6 9 10 12 13 14 15 16 17 18 19 20
R/E
Ag
2 4 5 9 12 13 16 17 18
R/A
Ag
21 6 12
R/Inf
Ag
3 4 5 6 7 8 11 12 13
M/Inf
Ag
8 12 13

Fig. 6

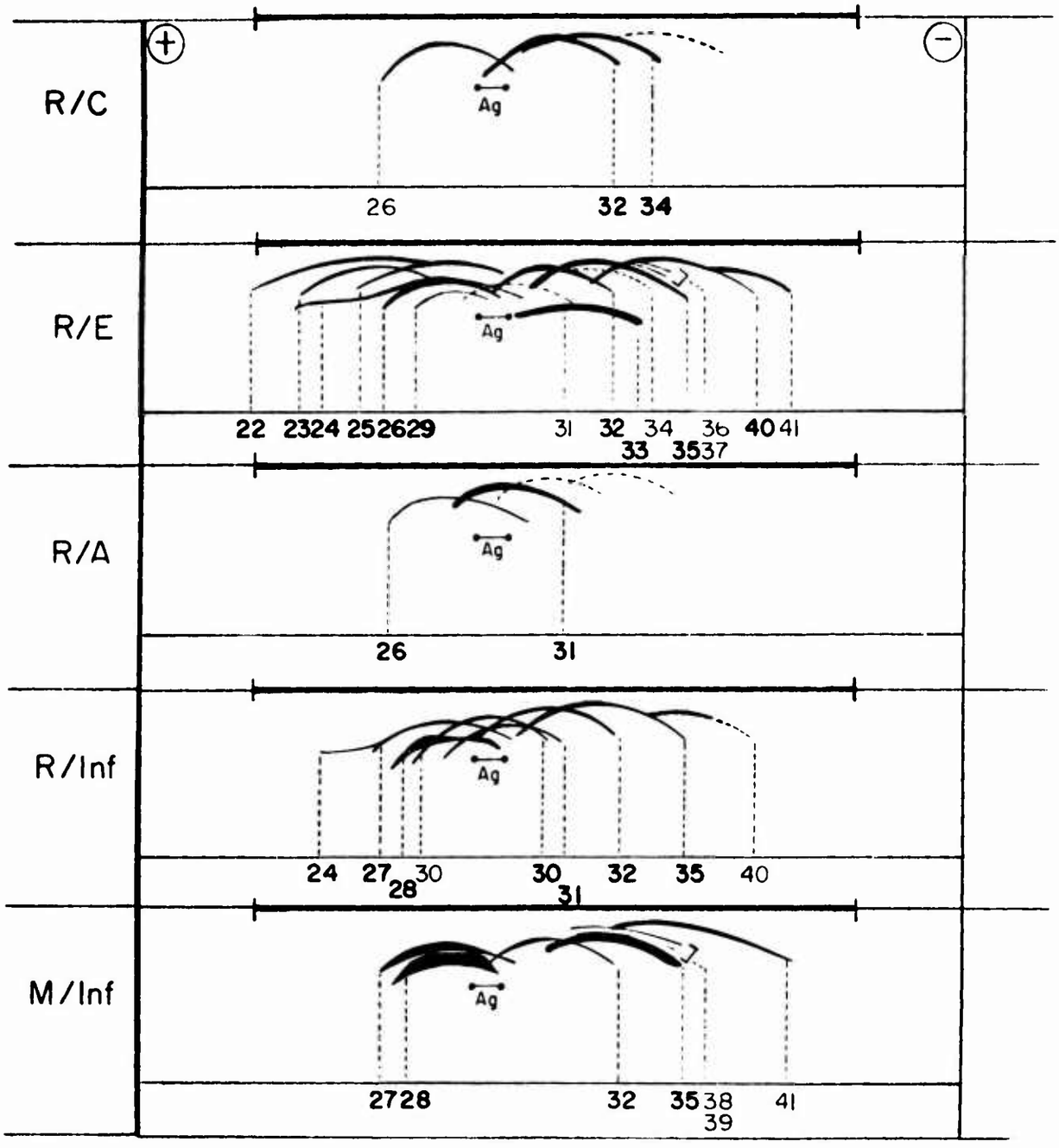
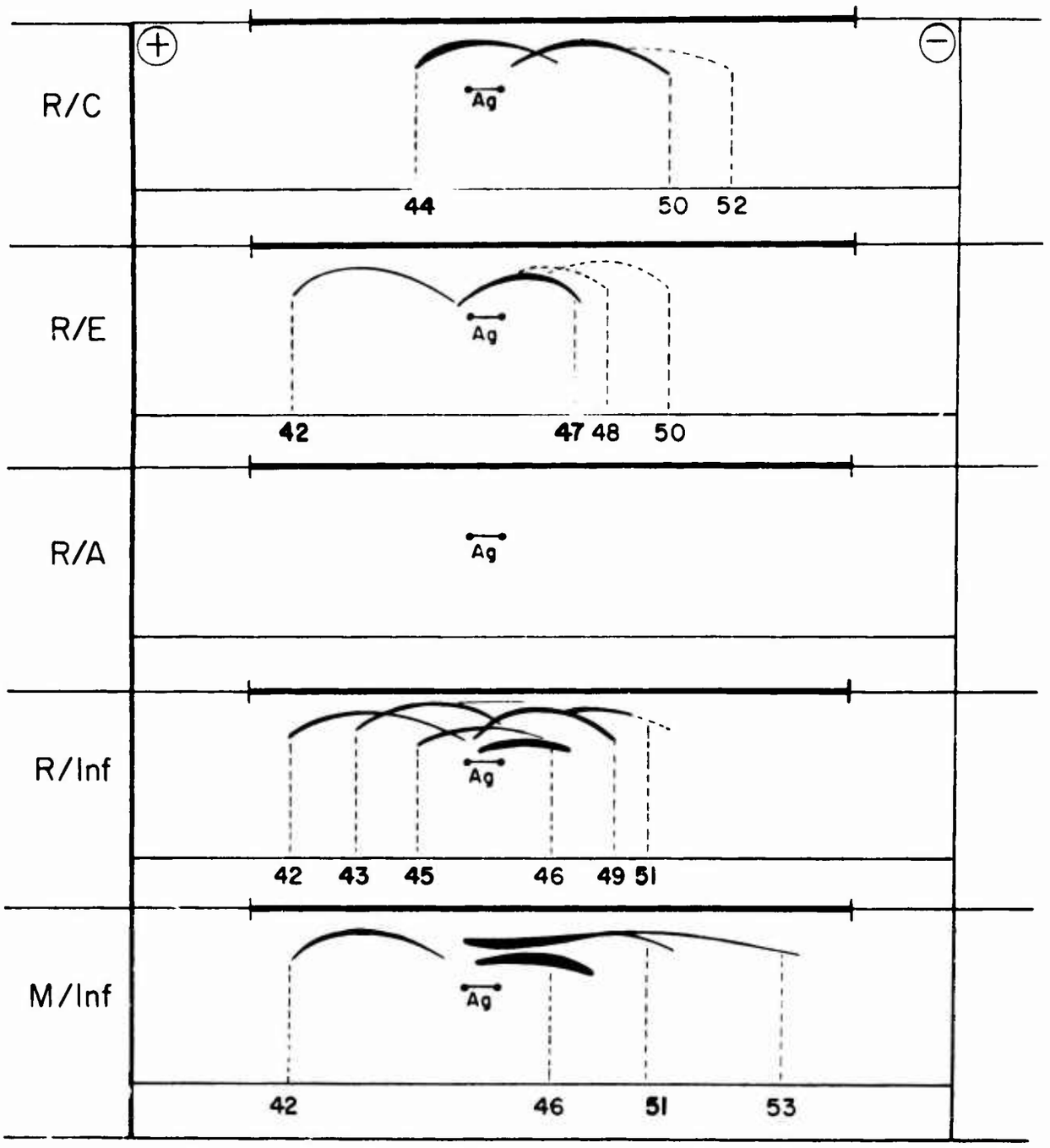

R/C
Ag
26
32 34
R/E
Ag
22 2324 252629
31 32 34 36 40 41
33 3537
R/A
Ag
26
31
R/Inf
Ag
24 27 30
28
30
31
32 35 40
M/Inf
Ag
2728
32 35 38
39
41

Fig. 7


R/C
Ag
44
50
52
R/E
Ag
42
47
48
50
R/A
Ag
R/Inf
Ag
42
43
45
46
49
51
M/Inf
Ag
42
46
51
53

Number and localization of antigenic components observed by immunoelectrophoresis (IE): In the light of the previous results, experiments were designed to enumerate and define some of the soluble antigenic fractions present in egg, cercaria, and adult ES extracts and to determine which of these fractions are shared by different stages of the parasites. These experiments were conducted only with animal sera which could be refrigerated immediately after harvesting. Human sera were not used since they might have undergone changes in their serological characteristics enroute from collection areas to our laboratory.

The results are summarized in Table IX and Figures 5, 6, and 7. The precipitin bands were given consecutive numbers according to their relative positions between the anode and the cathode. For each antigen-antiserum system, 8 individual slides were prepared and studied. Although most of the precipitation bands were easily discernible in every test performed, several bands were clearly detected in some slides but not in others. Therefore, they could not be considered as universally present. To distinguish between these two categories, bands which were observed in all tests have been listed in bold type (Table IX and Figures 5, 6, and 7). The discussion, interpretations, and conclusions have been based primarily on these most stable lines, since it is believed that when a component is readily and easily detected, its absence under changed experimental conditions is much more meaningful. Although the other precipitation bands have been listed for possible comparison with similar studies conducted by other workers, their interpretation must await further investigations.

Each of the 3 antigenic extracts formed bands by IE when run against antisera from either artificially immunized or infected animals. Comparisons can be made on the basis of the relative numbers of bands formed. Although cercaria and egg antigens were quite reactive in these 5 antisera, forming 12 and 13 consistently demonstrable bands respectively, the adult ES antigen was less reactive, forming only 8 bands. With the exception of the adult ES antigen, which did not react with its homologous antiserum from the immunized rabbit, the largest number of bands was noted when antigens were tested with homologous antisera from artificially immunized animals. Cercaria and egg extracts produced fewer bands with heterologous antisera from artificially immunized rabbits and although most of these individual bands had been noted in the homologous systems, others, e.g., number 4, appeared only in heterologous systems. Of course, since there was no reactivity with the adult ES antigen in the homologous system, all bands noted in the heterologous systems were unique.

Sera from infected rabbits and monkeys produced 5 to 7 consistently demonstrable bands with the 3 antigens. Many of these individual bands had been noted in the systems using sera from artificially immunized animals, but, on the other hand some bands noted in the latter systems were missing in systems using sera from infected animals, e.g., numbers 1, 22, and 44. The sera from infected animals did, however, produce some bands, e.g., 7, 27, and 49, against the 3 antigens which were not observed in tests using sera from artificially immunized animals.

TABLE IX

Antigenic constituents observed by immunoelectrophoresis (IE) reactory egg, cercaria and adult ES extracts with different antisera (Underlined numbers indicate fractions which were observed in every slide)

A. Cercaria extract (Fig 5)					B. Egg extract (Fig 6)					C. Adult ES extract (Fig 7)				
R/c	R/e	R/a	R/inf	M/inf	R/c	R/e	R/a	R/inf	M/inf	R/c	R/e	R/a	R/inf	M/inf
1						22					42		42	42
	2					23							43	
			3			24		24						
	4		4			25				44			45	
5	5		5		26	26	26						46	46
6		6	6					27	27					
			7					28	28		47			
			8	8		29					48			
9	9							30					49	
10						31	31	31		50	50			
			11		32	32		32					51	51
12	12	12	12	12		33				52				
13	13		13	13	34	34								53
14						35,		35	35					
						36,								
						37								
15									38,39					
16,17	16,													
or 18	17 or					40		40						
	18					41			41					
19														
20														
		21												

R/c - antiserum from rabbit immunized with cercaria extract.
R/e - antiserum from rabbit immunized with egg extract.
R/a - antiserum from rabbit immunized with adult ES extract.
R/inf - antiserum from experimentally infected rabbit.
M/inf - antiserum from experimentally infected monkey.

Serum constituents identified by homologous and heterologous absorptions: Results obtained by the SF test and by IE before and after absorption were compared to characterize serum constituents which are absorbed by homologous and heterologous antigens coated onto cholesterol-lecithin crystals. The results are summarized in Table X and Figures 8, 9, and 10. Only the precipitin bands which were found regularly prior to absorption were included in this table. As stated before, this was done in order to base the results of absorption on a more rigorous and

TABLE X

Serum constituents observed by immunoelectrophoresis (IE) and by slide flocculation (SF) before and after absorption with different antigens coated onto cholesterol - lecithin crystals*

Anti-serums	Test	Cercariae extract (Fig.8) Before absorption	After absorption with Cerc.	Egg	Adult ES	Egg extract (Fig. 9) Before absorption	After absorption with Cerc.	Egg	Adult ES	Adult ES extract (Fig.10) Before absorption	After absorption with Cerc.	Egg	Adult ES
		1	--										
		6	--										
		9	--										
		12	--	NOT	NOT	32	32	NOT	NOT	44	---	NOT	NOT
R/C	IE	13	--	DONE	DONE			DONE	DONE			DONE	DONE
		14	--			34	34(p)						
		15	15(p)										
		19	--										
	SF titer	32	--			1	--			1	1		
						22	--	--					
		4	--	4(p)		23	--	--					
						24	--	--		42	--	--	
R/E	IE	5	--	5(p)	NOT	25	--	--	NOT	47	--	47(p)	NOT
					DONE	26			DONE				DONE
		9	--	9(p)		32		32(p)					
		16,17 or 18	--	--		33	33(p)						
						35		35(p)					
						40	40(p)	--					
	SF titer	64	--	--		64	--	--		--	--		
						24	--	--		--	--		
		5	5(p)	--	--	27	27	--	--	42	--	--	--
		6	6(p)	6(p)	6(p)	28	--	--	--	43	43	43(p)	--
R/INF	IE	7	7	--	--	30	30	30	30	45	45	45	45
		12	--	--	12(p)	31	31	31	31	49	49	49	--
		13	--	--	--	32	32	--	--	51	51	51	--
						35	35(p)	35(p)	35(p)				
	SF Titer	16	--	--	8	64	4	--	16	128	128	128	--

* Only those fractions which were observed in every slide are recorded here.

(p) Partial absorption - Only minimal precipitation observed.

R/C Antiserum from rabbit immunized with cercaria extract.

R/E Antiserum from rabbit immunized with egg extract.

R/INF Antiserum from experimentally infected rabbit.

possibly more meaningful basis. The results obtained with the SF test for each of the sera employed in this experiment have been included in these tables and figures for comparison. The antiserum from rabbits immunized artificially with cercaria extract (R/C) was used only for absorption studies by its homologous antigenic extract. This was done because the undiluted serum reacted only weakly in the SF test with heterologous antigens, and, therefore, negative results after absorption would not have been significant.

Absorption tests showed that all 3 test antigenic extracts had some common antigens observable in the IE and SF tests (Table X). Thus, immunoelectrophoresis with sera from artificially immunized animals, R/C and R/E, and their homologous antigens consistently produced bands previously summarized in Table IX. Homologous and heterologous absorptions of these 2 sera uniformly reduced their reactivity in the IE test, i.e., fewer bands were formed when such absorbed sera were later tested with either homologous or heterologous antigens. After only a single absorption reactivity was not completely eliminated, however, and such sera frequently retained IE activity against individual antigenic components. On the other hand, single absorptions uniformly eliminated all activity of these sera against cercaria and egg antigens in the SF test.

The results in Table X also suggest that cercaria and egg antigens had more in common with each other than either had in common with the adult ES antigen. This is most evident in tests using serum from the infected rabbit which was quite reactive with all 3 antigens. This serum formed 5 bands with the adult ES antigen and had an SF titer of 1:128 with this same antigen. Preliminary absorption of this serum with adult ES antigen markedly reduced or eliminated its reactivity with adult ES antigen in both IE and SF tests. On the other hand, negligible losses in reactivity against adult ES antigen were noted in both tests after preliminary absorption of this serum with either cercaria or egg antigen. However, certain cercaria and egg antigens were also found in the adult ES antigen. Thus, absorption of this serum from infected rabbits with adult antigen reduced the reactivity against the other 2 antigens in both IE and SF tests.

These results, in general, indicated that egg somatic extracts and adult excretion and secretion extracts can be coated onto cholesterol-lecithin crystals to permit the development of SF tests. The serologic reactions of monkeys, rabbits and mice infected with S. mansoni showed that antibodies are produced in sufficient amounts to be detectable by the SF test with either egg, cercaria or adult ES extracts. The adult ES extract seemed to give the test a greater sensitivity than the egg extract and this, in turn, seemed to confer on the test a greater sensitivity than the cercaria extract. Whether these differences in sensitivity were due entirely to qualitative differences in the antigenic components of the 3 antigen preparations or to the relative amounts of common antigens, could not be determined with certainty.

Fig. 8

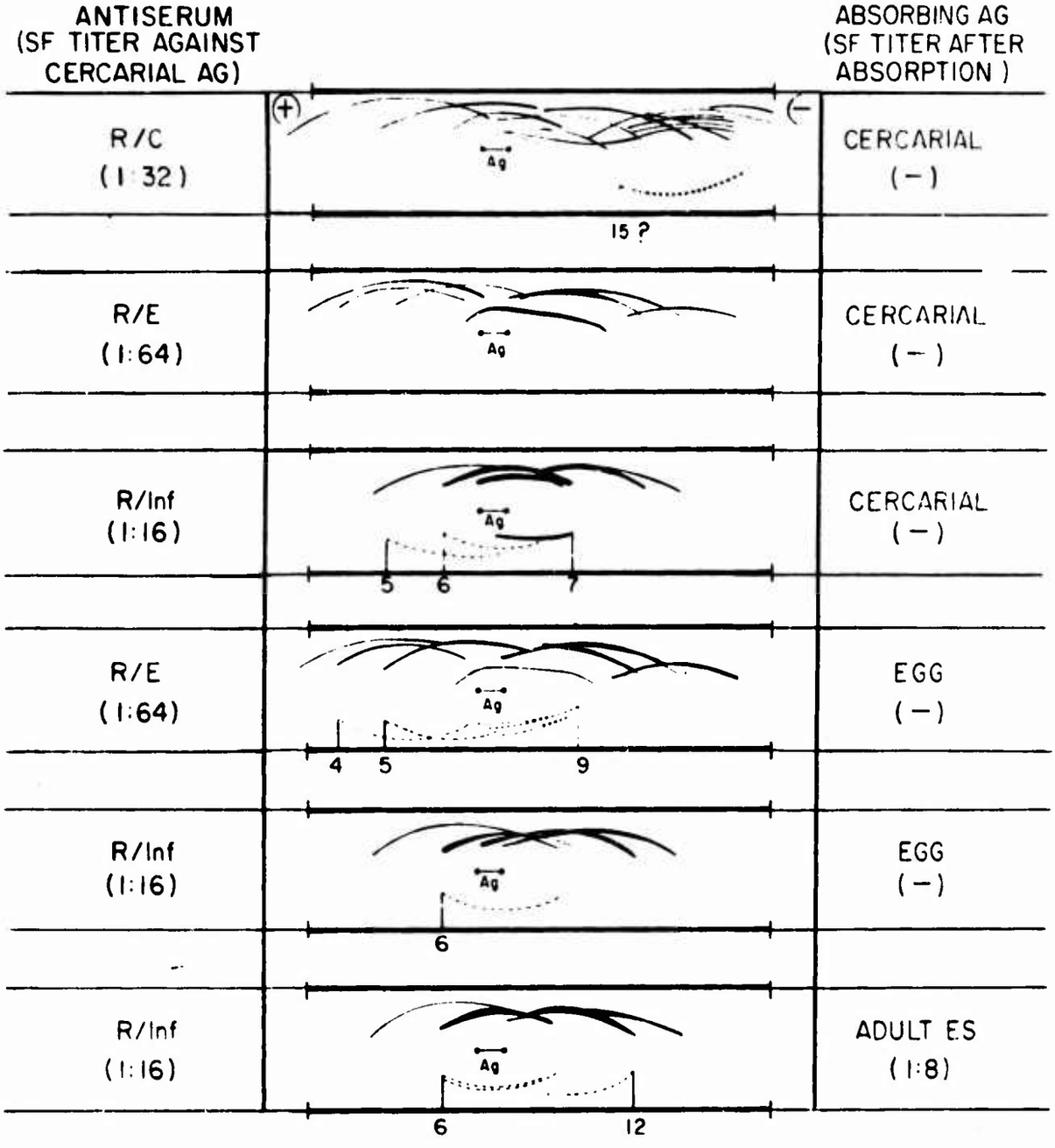

ANTISERUM
(SF TITER AGAINST
CERCARIAL AG)
ABSORBING AG
(SF TITER AFTER
ABSORPTION)
R/C
(1:32)
(+)
(−)
Ag
CERCARIAL
(−)
15 ?
R/E
(1:64)
Ag
CERCARIAL
(−)
R/Inf
(1:16)
Ag
5
6
7
CERCARIAL
(−)
R/E
(1:64)
Ag
4
5
9
EGG
(−)
R/Inf
(1:16)
Ag
6
EGG
(−)
R/Inf
(1:16)
Ag
6
12
ADULT ES
(1:8)

Fig 9

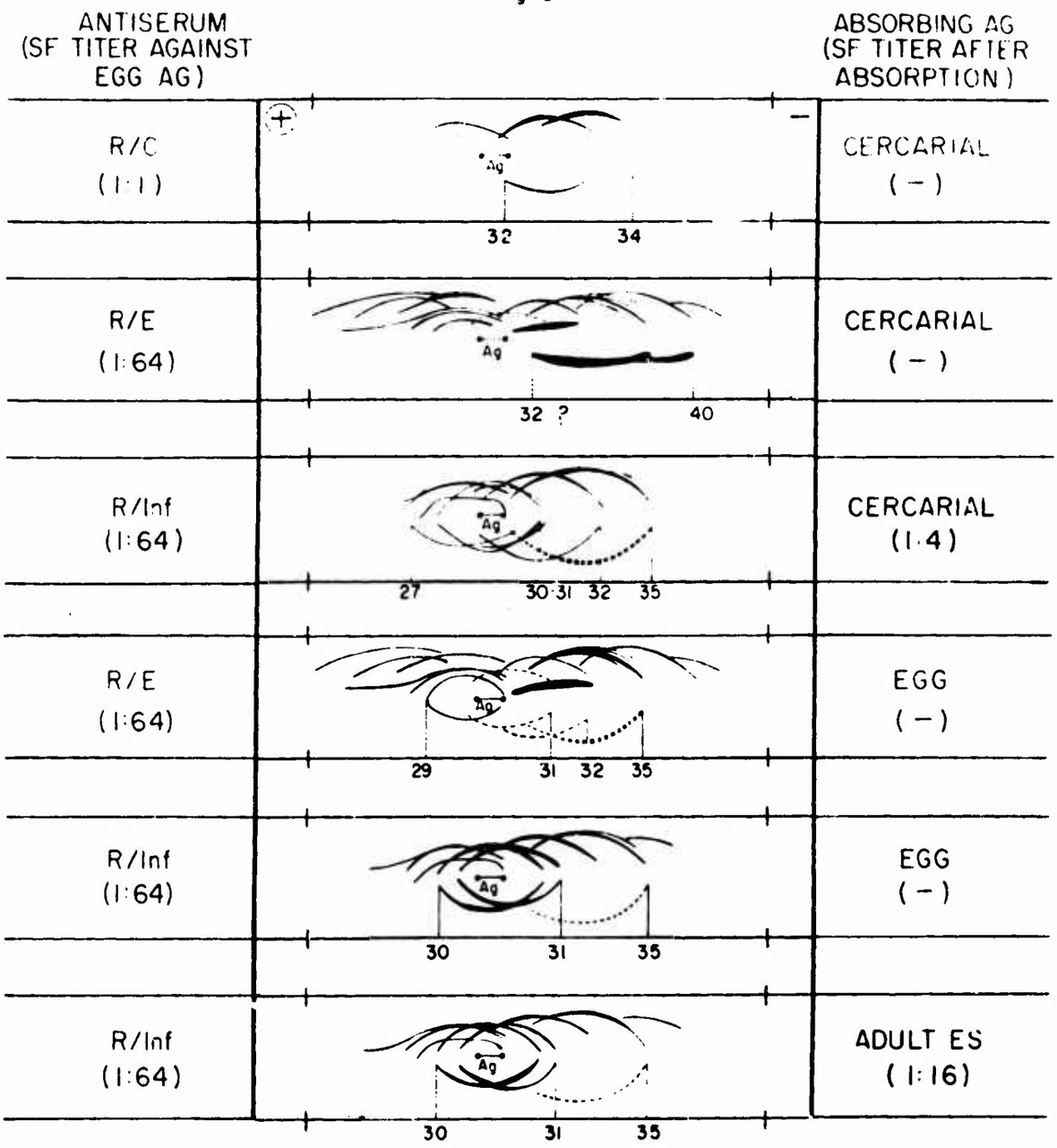
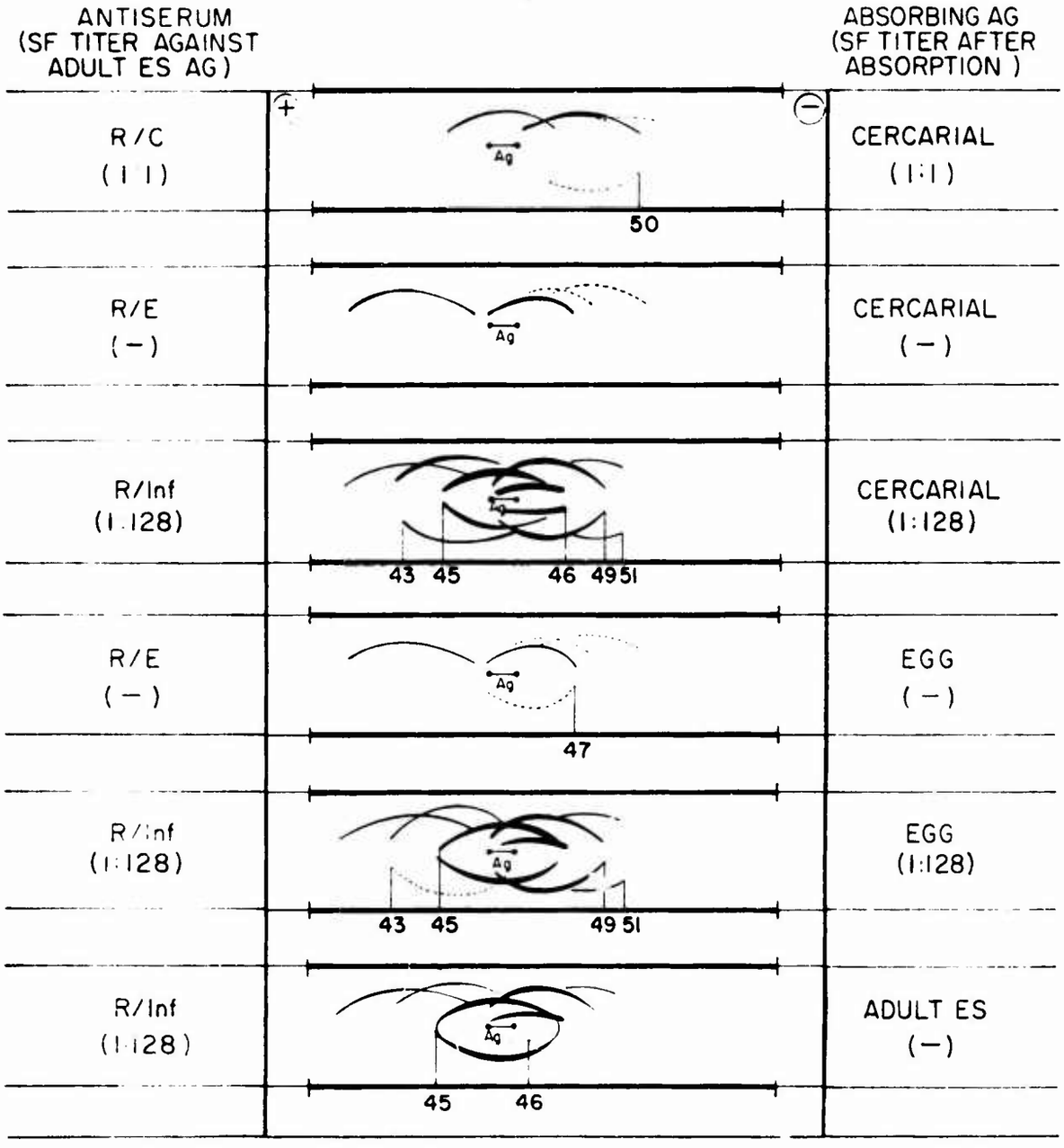

ANTISERUM
(SF TITER AGAINST
EGG AG)
ABSORBING AG
(SF TITER AFTER
ABSORPTION)
+
−
R/C
(1:1)
Ag
32
34
CERCARIAL
(−)
R/E
(1:64)
Ag
32 ?
40
CERCARIAL
(−)
R/Inf
(1:64)
Ag
27
30
31
32
35
CERCARIAL
(1:4)
R/E
(1:64)
Ag
29
31
32
35
EGG
(−)
R/Inf
(1:64)
Ag
30
31
35
EGG
(−)
R/Inf
(1:64)
Ag
30
31
35
ADULT ES
(1:16)

Fig. 10


ANTISERUM (SF TITER AGAINST ADULT ES AG)
ABSORBING AG (SF TITER AFTER ABSORPTION)
+
−
R/C (1:1)
Ag
50
CERCARIAL (1:1)
R/E (−)
Ag
CERCARIAL (−)
R/Inf (1:128)
Ag
43 45 46 49 51
CERCARIAL (1:128)
R/E (−)
Ag
47
EGG (−)
R/Inf (1:128)
Ag
43 45 49 51
EGG (1:128)
R/Inf (1:128)
Ag
45 46
ADULT ES (−)

Nitrogen determinations showed that the antigenic extracts were readily absorbed onto cholesterol-lecithin crystals. However, since the products used in these tests are not pure antigens but relatively complex extracts of heterologous composition representing in reality a mosaic of antigens, the total nitrogen content may not necessarily be related to the content of those antigens which are specifically reacting in these tests.

The results of absorption studies in the SF test correlated very well with those obtained by IE analysis and clearly indicate some degree of stage specificity. Attention is called to the fact that greater sensitivity and specificity of the SF test with adult ES antigen was observed in all experiments regardless of whether the tests were conducted with sera from artificial immunization, experimental single infections or natural and possibly repeated infections. In this connection it is of particular interest to notice that antibodies against adult ES extracts could be detected in monkeys more than 3 years following a single exposure to infection and more than a year after antibodies against egg and cercaria extracts were no longer detectable. The SF test using adult ES antigen might also be of value in early infections as demonstrated in the experimentally infected animals. Further studies should also be conducted to determine whether the SF test employing egg extracts might offer some promise as a possible means of evaluating the results of chemotherapy. However, negative results obtained so far along these lines with other serological tests for schistosomiasis do not justify great optimism. The demonstration of stage specific antibodies in schistosomiasis is also of great interest in the study of possible mechanisms of acquired immunity to this infection.

In general these studies demonstrate the antigenic complexity of each of the 3 tested stages in the life cycle of S. mansoni. In fact, since the IE technique has relatively a low sensitivity, it is quite probable that a much greater number of bands might have been obtained under ideal conditions. This suggestion is further strengthened by the observation that although most precipitin bands were consistently found in every reaction performed with a given system, a few were in some slides and not in others. In general, the number of bands observed with sera from animal infections was considerably smaller than that from artificially immunized animals. However, the different bands obtained with sera from infected animals were more typical and clear cut and assumed a much more intense staining. Furthermore, these bands were more detached from each other and were further from the trough in which the antiserum was deposited. The results indicate that although several antigenic fractions were common among the 3 extracts obtained from the 3 different stages in the life cycle of the schistosomes, distinct antigenic fractions were also present in each of these 3 extracts. However, some of the so-called "stage specificity" may be only

apparent since immunizing extracts from different stages may stimulate distinct demonstrable antibodies by the fact they they differ in the relative portion of common antigens. The possibility of quantitative antigenic differences has been explored in early observations with other parasites and may also apply to the interpretation of the early results obtained by Oliver-Gonzalez and his co-workers on the "stage specificity" of S. mansoni antigens. The problem of "stage specificity" may also be a function of the time at which serum has been obtained. As stated by Oliver-Gonzalez, "It is to be expected that if immunization had been continued the concentration of antibodies against other antigens would have increased, and therefore more cross reactivity would have been observed." Further studies using Ouchterlony and IE techniques possibly with the addition of the technique of discontinuous troughs combined with homologous and heterologous absorption may provide additional information on this subject.

In artificially immunized animals cercaria and egg extracts elicited a much greater number of antibodies than the adult ES extracts. Conversely, in the sera of infected animals the adult ES extract produced a relatively greater number of bands than did either the egg or cercaria extracts. In general much greater similarities were obtained between the egg and cercaria extracts than with the adult ES extract. This is in agreement with the "cercarial factor in the egg" observed by Oliver-Gonzalez, et al. However, the fact that egg and cercaria extracts had greater antigenic similarities than the adult extract is not surprising since the first two are of somatic and the last of metabolic origin.

In general, the results of absorption studies indicate that the cholesterol-lecithin crystals are not very selective and that many antigenic fractions are adsorbed onto cholesterol-lecithin crystals. This is clearly shown in the adsorption of cercarial antiserum (R/C). Whereas before absorption 8 fractions were visible by immunoelectrophoresis, after absorption only 1 partially absorbed fraction was present. The absorption studies revealed once again that the egg and the cercaria extracts contained many antigenic fractions in common. This is also in agreement with studies conducted with the SF test. Partial absorption observed in many instances, was probably due to the fact that antigens coated onto the cholesterol-lecithin crystals were not sufficient to eliminate all of the corresponding antibodies. A second absorption would probably have eliminated these portions as well. Attention should be called to the fact that this work was carried out primarily to compare the results of immunoelectrophoresis with those obtained by the SF test before and after absorption. It was therefore necessary to utilize the same technique of absorption in the IE that had been used for the SF test.

Although many similarities were observed between the results obtained by the SF and IE techniques, differences were also evident. For instance, when the serum from an infected rabbit (R/inf) was absorbed with egg extract and tested again with egg antigen, the titer in the SF test dropped from 1:64 to 0. However, in the IE analysis 3 bands (30, 31, 35) were still present. When the same serum was absorbed with the adult ES extract, the titer remained unchanged when later tested with cercaria or egg antigen; however, in the IE analysis, band 42 disappeared in both instances. When tested with the adult ES antigen, the SF titer dropped from 1:128 to 0 but 1 band (45) remained in the IE analysis. These differences could be interpreted as resulting from the fact that some fractions are not adsorbed onto cholesterol-lecithin crystals (examples 30, 31, 45). Another difference may result from the fact that certain fractions were adsorbed onto the cholesterol-lecithin crystals but did not play a demonstrable role in the SF test (42).

In spite of the complexity of their interpretation, the comparisons between the results obtained by the SF test and by IE before and after serum absorption are quite interesting from several points of view. The persistence of bands following absorption may indicate incomplete absorption with a particular antigen. The fact that a number of common antigens are shared by cercaria, egg, and adult ES extracts was indicated by the number of common bands which appeared when heterologous antigen-antibody systems were tested. This probably accounts for the lack of serological specificity observed in some of the SF tests with the 3 extracts, since any one of the cross reacting antigenic components may be capable of producing a positive flocculation test.

Although a considerable amount of information was revealed by these studies it seems unlikely that a complete understanding of the relationship of stage specificity in schistosomiasis and other helminths will be achieved until individual antigenic components are isolated and biochemically characterized.

2. Schistosoma mansoni infection in splenectomized chimpanzees.

Many facets of patho-physiology of schistosomiasis mansoni in man are still unexplored because there is no suitable animal model approximating the degree of susceptibility and host response of man to this parasite. The natural history of this infection was studied in five splenectomized chimpanzees 3 to 4 years old. All had previously been infected experimentally and/or naturally with malaria. The animals were exposed to 0, 0, 250, 1000, and 2000 cercariae of S. mansoni, respectively. Detailed parasitological, clinical, hematological, serological and biochemical observations were made throughout the studies until necropsy seven months after exposure to infection.

The results of parasitological investigations indicate that the chimpanzee is susceptible to experimental infections with S. mansoni. Eggs first appeared in the feces of each animal during the sixth week after exposure (Figures 12-14). The eggs were viable, the miracidia were infective for snails and the emerging cercariae were infective for laboratory mice. The pattern of egg production remained relatively constant for the duration of the experiment. At necropsy the animal exposed to 2000 cercariae harbored 83 worms. The same number of worms was recovered from the animal exposed to 1000 cercariae and the chimpanzee exposed to 250 cercariae had 13 worms (Table XI). Both male and female worms appeared to be normal in all respects. There was widespread distribution of eggs in the various organs (Table XII).

TABLE XI
The number and location of adult Schistosoma mansoni worms in splenectomized chimpanzee (210 days after exposure)

Chimpanzee No.	No. of cercariae	Results of necropsy				
		No. of live worms				
		Liver		Mesentery		Total
		M	F	M	F	
8	2000	28	3	28	24	83
5	1000	13	4	37	29	83
1	250	2	1	6	4	13
4	*	-	-	-	-	-
12	*	-	-	-	-	-

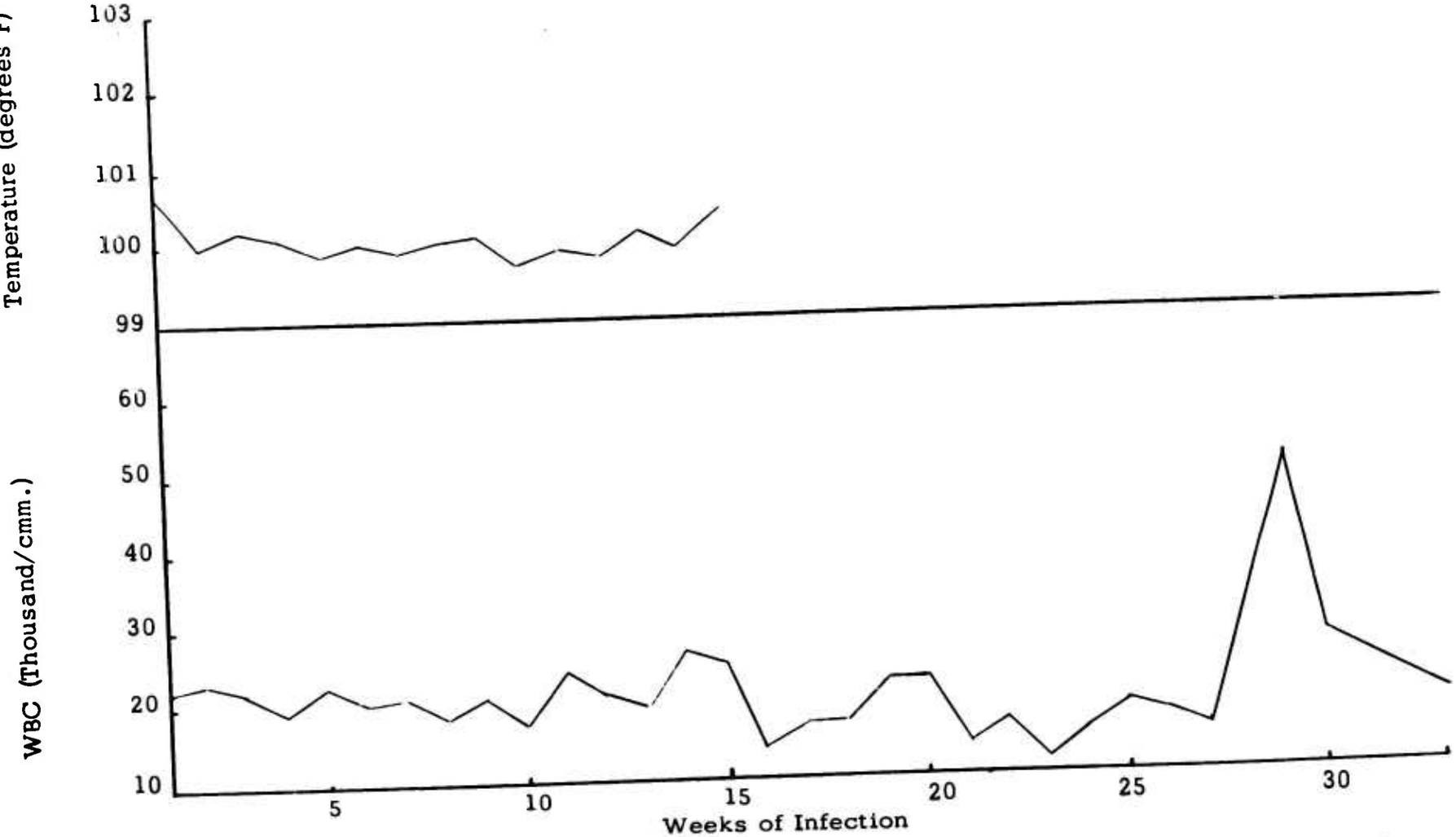
* Uninfected controls.

TABLE XII
The number of Schistosoma mansoni eggs in various organs from splenectomized chimpanzees

Chimpanzee No.	No. of cercariae	Organs (no. of eggs/gm. tissue)								*NEPGF	
		Lung	Liver	Pancreas	Kidney	Sm. Int.	Lg. Int.	Urinary bladder	Accesory spleen	Maximum	Mean
8	2000	6	687	5	1	0	3000	20	4	85	34
5	1000	13	250	10	1	195	500	21	-	104	35
1	250	6	48	13	14	1	0	30	-	11	2
4	**	-	-	-	-	-	-	-	-	-	-
12	**	-	-	-	-	-	-	-	-	-	-

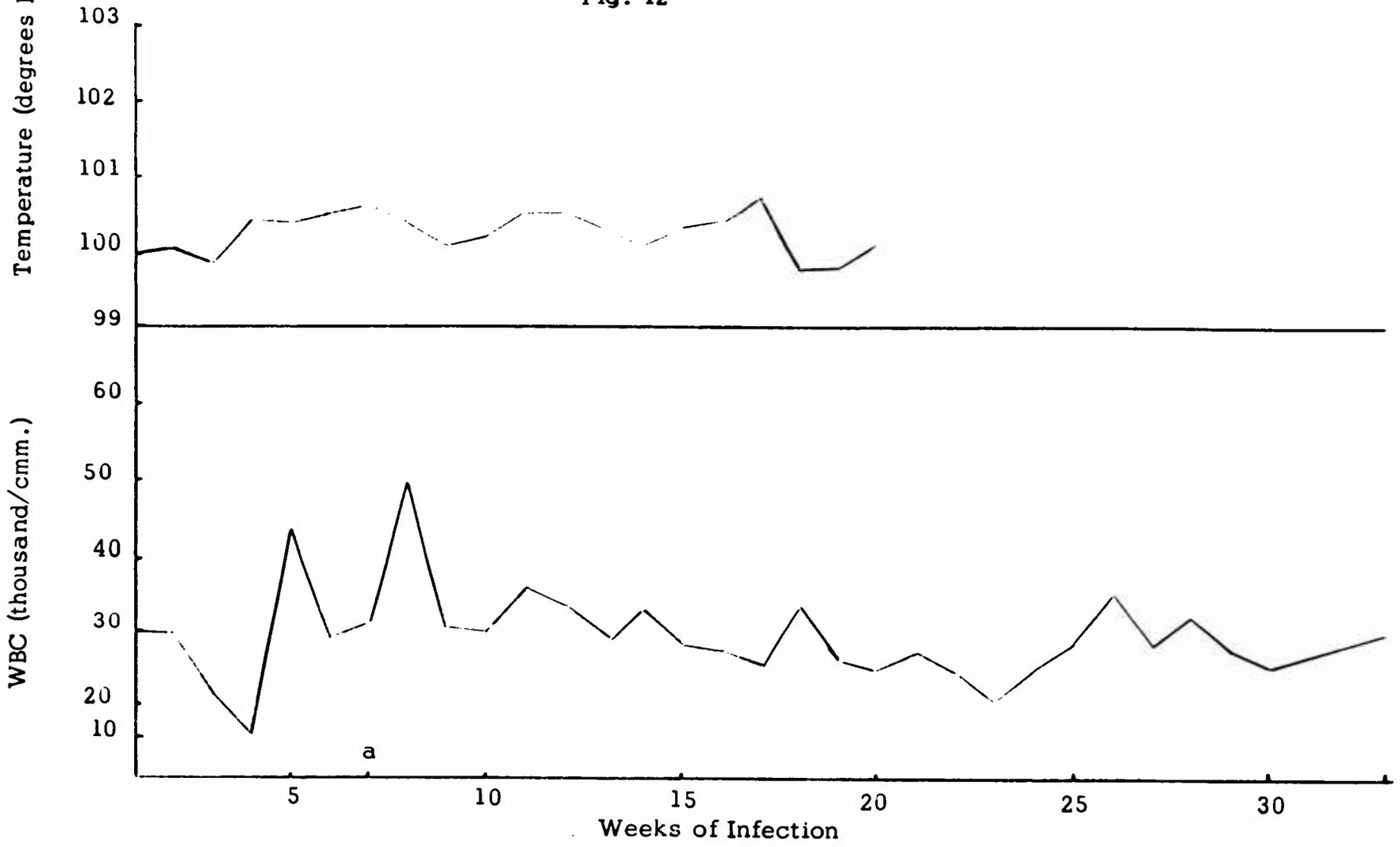
* Number of eggs per gram feces.
** Uninfected controls.

Fig. 11



Average weekly temperature and leucocyte count of splenectomized chimpanzee exposed to 0 Schistosoma mansoni cercariae

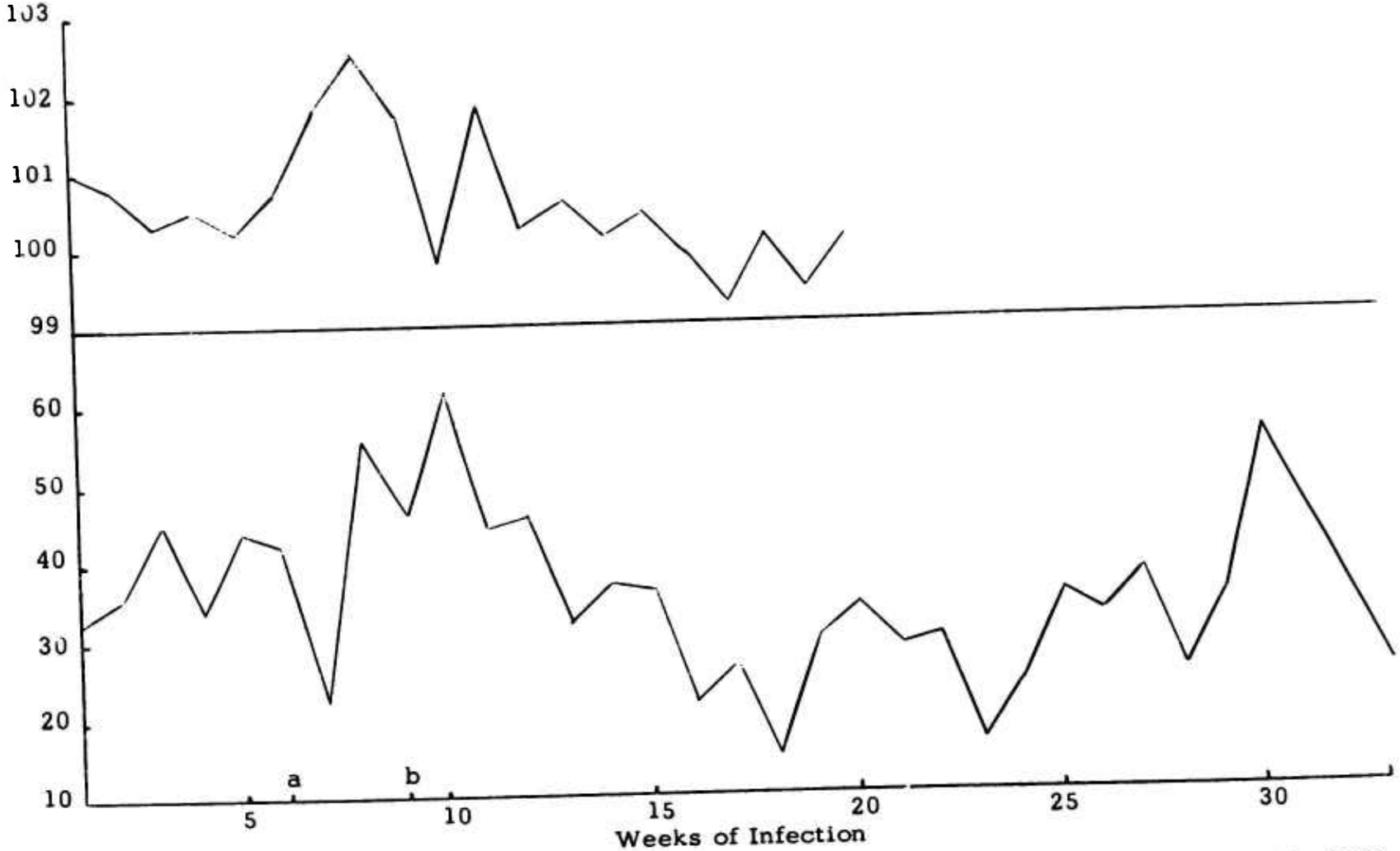
Fig. 12



Average weekly temperature and leucocyte count of splenectomized chimpanzee exposed to 250 Schistosoma mansoni cercariae

a - initial eggs in feces

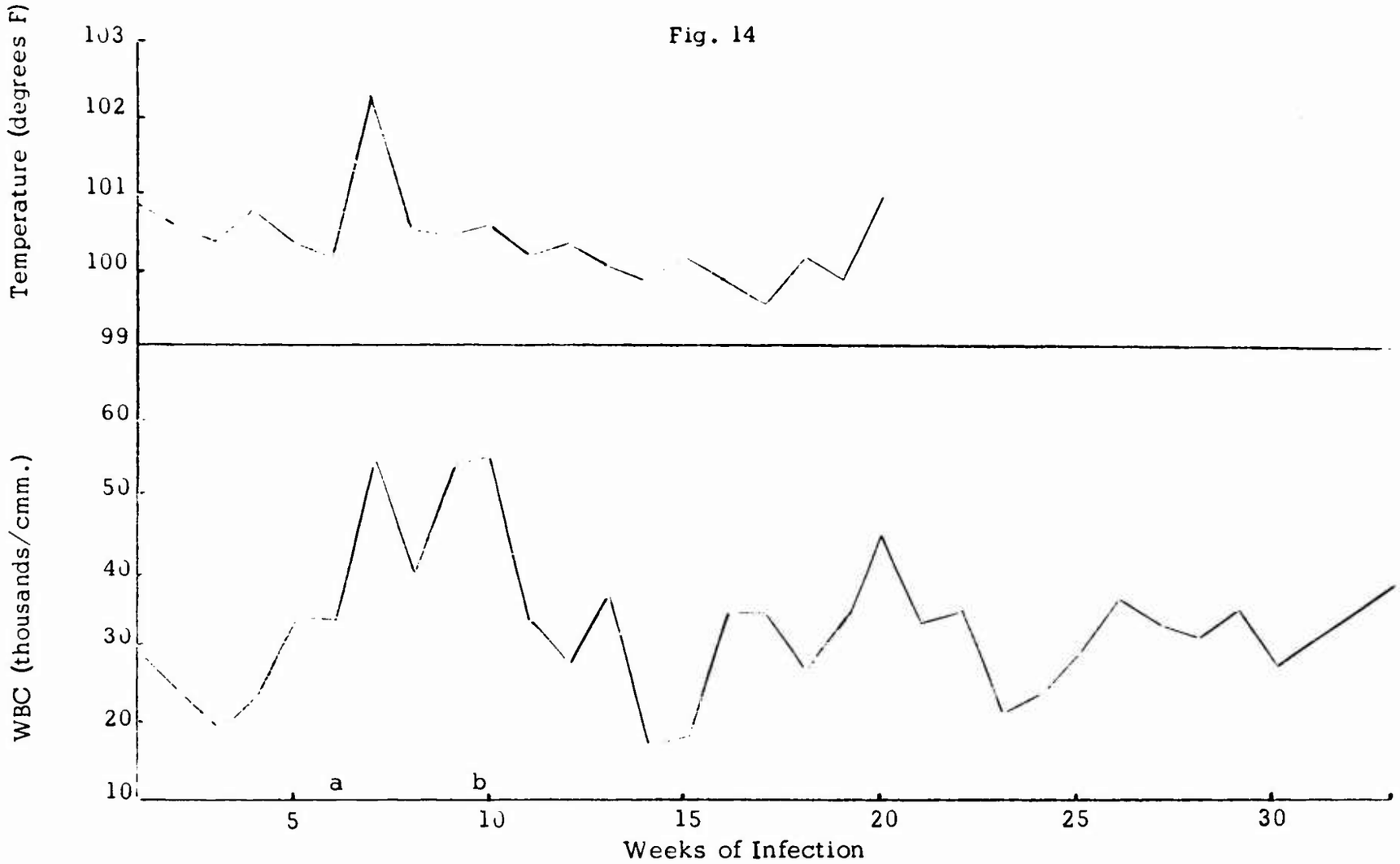
Fig. 13



Average weekly temperature and leucocyte count of splenectomized chimpanzee exposed to 1000 Schistosoma mansoni cercariae

a - initial eggs in feces
b - ascites and hepatomegaly

Fig. 14



Average weekly temperature and leucocyte count of splenectomized chimpanzee exposed to 2000 Schistosoma mansoni cercariae

a - Initial eggs in feces
b - probable ascites and hepatomegaly

Clinical observations were made daily by a veterinarian during the entire course of the study. Daily temperature recordings were taken for 15 weeks following exposure (Figures 11-14). Clinical symptoms accompanied the first appearance of eggs in the feces of the two animals exposed to 1000 and 2000 cercariae. These included a marked decrease in activity and responsiveness, anorexia, elevation of body temperature and diarrhea with mucous and frank hemorrhage. These symptoms were transient and were followed in three weeks by hepatomegaly and ascites in the chimpanzee exposed to 1000 cercariae and indications of similar findings in the animal receiving 2000 cercariae. Following regression of the hepatomegaly and for the remainder of the study, no clinical signs that could be directly related to S. mansoni were noted.

Blood samples were drawn weekly for leucocyte enumeration, hematocrit determination and differential white cell count. A leucocytosis characterized by an absolute lymphocytosis accompanied the first appearance of eggs in the feces and the onset of clinical symptoms (Figures 2-4). At no time during the study was an eosinophilia noted. Hematocrit values remained constant.

Serological tests (Table XIII) were performed at approximately two-week intervals from the date of exposure to the end of the experiment. The slide flocculation test was utilized with somatic extracts of eggs and cercariae and excretions and secretions from adults as antigens. The animals exposed to 1000 and 2000 cercariae developed detectable antibodies against all three antigens by the seventh week after exposure to infection. Antibodies were detected in the serum of the animal which was exposed to 250 cercariae two weeks later. Maximum titers were observed in the two animals receiving the larger number of cercariae on the ninth week after exposure. Titers in the animal receiving 250 cercariae were lower and did not reach a peak until the twelfth week after exposure. In all cases the titers remained relatively constant for the duration of the experiment.

The following biochemical tests were performed on all animals at approximately two-week intervals: total serum protein and electrophoresis; serum-glutamic-oxalacetic-transaminase; and bromsulfphthalein retention determinations. Only the total protein values were markedly altered (Table XIII). Elevations occurred shortly after the recovery of eggs in the feces in the animals exposed to 1000 and 2000 cercariae. These elevations were consistent for the duration of the experiment and were almost entirely a function of the gamma globulin fraction.

In addition to detailed necropsies performed at the termination of the experiment, five needle biopsies of the liver were obtained from each animal at monthly intervals. Gross pathological findings after seven months of infection in the animal exposed to 250 cercariae were

limited to numerous pseudo-tubercles in the liver. More extensive liver pathology, the presence of portocaval collateral circulation and evidence of esophogeal varices was noted in the animal exposed to 1000 cercariae. The animal exposed to 2000 cercariae was grossly similar to that exposed to 1000 cercariae with the addition of numerous lung lesions associated with eggs found in that organ. A complete histo-pathological study is currently in progress.

This experiment indicates that the patho-physiology of Schistosoma mansoni infection in the splenectomized chimpanzee is similar to that in man. Therefore, further studies using intact chimpanzees are contemplated.

3. The activity of selected chemicals against Schistosoma mansoni in monkeys.

Sodium antimony dimercapto succinate (TWSb) has been used in the therapy of schistosomiasis since 1954. Recently, Stohler and Frey have shown that antimony (III) dimercapto succinic acid administered as an olive oil suspension had a significantly higher prophylactic and therapeutic effect on infection with this parasite in mice and hamsters. Studies were conducted in mice and hamsters to determine the distribution of antimony in the blood, liver, kidneys and intestinal tracts over a period of 72 hours following a single subcutaneous injection of labeled antimony (III) dimercapto succinic acid in oil and sodium antimony dimercapto succinate in water. The results showed that the acid form in oil produced higher and longer lasting antimony levels than the sodium salt in water.

Lammler reported that a dicarbonic acid hydrazide, which he referred to as S-201 was highly effective against S. mansoni in mice. Subsequent studies in hamsters and a few monkeys seemed to confirm his earlier observations. This compound has neither color nor smell, is soluble in water in high concentrations and was reported to be effective against S. mansoni in mice and hamsters only by parenteral application in large doses. No significant therapeutic activity was observed in experimental animals against S. japonicum. Conversely, when given orally, this compound was found to be completely ineffective.

Although S-201 in higher doses succeeded in eliminating mature S. mansoni in mice, this compound appeared to have no prophylactic activity and was ineffective against the immature stages of schistosomes. Acute and chronic toxicity studies conducted in mice, hamsters, monkeys, rats and dogs revealed that this drug is well tolerated in doses up to 1,000 mg per kg of body weight. This dose was considerably higher than the recommended therapeutic dose.

TABLE XIII

Results of biochemical and serological tests performed on splenectomized chimpanzees (Pan satyrus) exposed to Schistosoma mansoni cercariae

Tests	No. of cercariae	Duration of infection (in weeks)													
		0	2	5	7	9	12	14	17	19	23	26	28	30	33
Total Protein (gm. %)	0	7.7	9.2	7.4	7.4	7.8	7.9	7.3	7.0	6.8	7.7	7.3	8.1	8.7	9.4
	250	7.0	7.9	8.0	7.1	8.5	8.3	7.8	6.9	7.2	7.8	7.6	8.1	7.8	8.2
	1000	8.3	8.8	8.6	8.0	8.5	10.3	10.3	10.4	9.8	---	10.2	10.6	9.8	11.1
	2000	6.8	9.7	7.4	7.0	9.3	10.4	9.8	8.9	7.9	8.0	9.1	9.0	9.0	10.9
Antibody titer (somatic extract of eggs)	0	0	0	0	1	0	0	0	1	1	0	<1	0		
	250	0	0	0	0	1	4	4	2	4	4	4	4		
	1000	0	0	0	8	64	64	64	64	64	128	64	64		
	2000	0	0	0	8	16	32	16	16	16	8	8	4		
Antibody titer (somatic extract of cercariae)	0	0	0	0	1	0	0	0	1	2	0	<1	0		
	250	0	0	0	0	8	16	16	8	8	8	16	16		
	1000	0	0	0	16	64	64	128	64	64	128	64	128		
	2000	0	0	1	16	32	32	32	16	16	16	16	8		
Antibody titer (excretions and secretions of adults)	0	0	0	0	0	0	0	0	0	0	0	0	0		
	250	0	0	0	0	8	64	64	32	32	64	64	32		
	1000	0	0	0	8	---	128	128	64	128	64	128	128		
	2000	0	0	2	256	1024	1024	256	128	1024	512	256	256		

Titers are expressed as reciprocal of serum dilution.

Ronnell is an organo-phosphorus compound offered as a systemic insecticide. This drug is low in acute toxicity when given by oral administration. No morphological changes resulted from long term feeding studies on rats at 15 mg per kilogram per day, nor in dogs at 10 or 25 mg per kilogram per day. Plasma cholinesterase levels were depressed to a greater extent than those of the red blood cells or the brain, although substantial amounts of enzyme remained even after the feeding of large dosages of this drug. Preliminary studies conducted on the curative activity of this drug against S. mansoni and hookworm infections in men indicated a large number of apparent cures following treatment. No information was available on the prophylactic activity of this drug in men or in experimental animals.

Recently a non-antimonial compound was synthesized in the research laboratories of CIBA Limited, Basle, Switzerland. This compound designated as CIBA 32-644-Ba was reported to have an effective schistosomicidal activity against adult Schistosoma mansoni in vitro in a concentration of 10 micrograms per ml and to inhibit egg laying by female schistosomes in a concentration of 1 microgram/ml. In the course of preliminary studies conducted in mice infected with S. mansoni, no living worms were found when the drug was administered orally at a dose of 100 mg/kg daily for 10 consecutive days. The full therapeutic course of this compound appeared to have no significant hepatotoxic action in normal or infected mice. The only toxic action observed in therapeutic doses was a temporary and reversible inhibition of spermatogenesis. The serum of rabbits which were given the drug orally was found to exhibit a maximal schistosomicidal action in vitro when collected 6 hours after administration.

The drug was found to be concentrated in the vitellaria of the schistosomes as well as in the eggs deposited in the liver and intestinal wall of the host. Morphological changes in the ootype and vitellaria of the female and the testes of the male worms have been observed.

In view of the above considerations, studies were designed to determine the prophylactic, suppressive and therapeutic activity of these four compounds in Rhesus monkeys experimentally infected with S. mansoni and, whenever possible, to gain some information on the mode of action and the relative efficacy of these compounds.

A total of 80 monkeys (macaca mulatta) weighing between 2-1/2 and 3-1/2 kilograms at the beginning of each experiment were used for these studies. The cercariae used for animal exposures in the various experiments were obtained from pools of 300 Australorbis glabratus snails of the Puerto Rican strain. The life cycle of S. mansoni (Puerto Rican strain) was regularly maintained in these snails and albino mice. The antimony dimercaptosuccinic acid was administered intramuscularly in a 25% olive oil suspension. The compound S-201 was prepared by dissolving

TABLE XIV

The prophylactic effect of antimony dimercapto succinic acid in olive oil on S. mansoni infections in monkeys

Group No.	Monkey No.	Dose (mg)	Rx started (day)	Results of necropsy		NEPGF*	
				No. of live worms recovered	Percent recovery	Maximum	Mean
I	1	100 once	0	99	25	33.	17.
	2			40	10	25.	19.
	3			141	37	34.	23.
	4			65	16	25.	14.
II	5	25 x 4 days	-2	50	12	2.	1.
	6			75	19	8.	4.
III	7	75 x 4 days	-2	2	<	--	--
	8			0	0	--	--
	9			0	0	--	--
	10			0	0	--	--
IV	11	No Rx	--	178	44	49.	31.
	12			97	24	21.	14.
	13			61	17	20.	11.
	14			131	33	32.	22.
V	15	100 x 3 days	-2	--	--	--	--
	16			--	--	--	--

* Number of eggs per gram of feces from earliest egg appearance to necropsy.

the calculated amount of drug in 1.5 ml of sterile pyrogen free water for injection and administered intramuscularly. Ronnel was given orally in the form of enteric coated tablets. The nitrothiazole derivative was given orally by stomach tube. Throughout the experiments observations were made for obvious changes in body weight and reactions such as weakness, ataxia, paralysis, marked changes in appetite or other obvious signs of intoxication. Stool examinations were performed on all animals at regular intervals from the beginning of each experiment. All animals except those which were used as drug toxicity controls were each exposed percutaneously to a single dose of 400 cercariae. The monkeys were kept in individual cages and the feces were examined every other day for the presence of schistosome eggs. This was done by direct smear method and the AMS III concentration technique. At necropsy, the Perf-O-Suction method was used for recovery of schistosomes. Following perfusion the intestine and liver were removed and examined for the presence of living or dead worms which might not have been recovered by perfusion. As in previous similar experiments, determinations of the therapeutic effectiveness of the drug were based on the presence, number and viability of schistosome eggs in the feces, intestine and liver of the animals and on the number, sex ratio and appearance of living schistosomes recovered, and the gross appearance of the liver, spleen and intestine. Immediately after perfusion the recovered worms were placed in warm alcohol, formalin, acetic acid (AFA) fixative, cleared, stained with Delafield and Ehrlich hematoxylin, mounted and studied microscopically for possible morphological changes. The testes and ovaries of the monkeys were removed at necropsy, fixed in buffered 10 percent formalin, sectioned at 7 microns thickness and stained with hematoxylin and eosin for histopathological studies.

a. Antimony Dimercapto Succinic Acid.

A total of 16 monkeys was used to determine the prophylactic effect of sodium antimony dimercapto succinic acid in olive oil on the number of S. mansoni recovered. The monkeys were divided into five groups (Table XIV). Those of the first group received a single dose of 100 mg of the drug on the day of exposure. Those of the 2nd group received 4 daily doses of 25 mg each starting two days prior to exposure. Those of the 3rd group received 3 daily doses of 75 mg starting two days before exposure. Those of the 4th group were used as untreated controls and 2 monkeys (5th group) were used as drug control animals.

No obvious signs of toxicity were observed in any of the treated monkeys. At necropsy, 60 days after exposure no worms were observed in 3 animals of Group III and, 2 worms were found in a single monkey (No. 7). No marked reduction in the worm burden of the animals treated with a single dose of 100 mg (Group I) or with 4 daily doses of 25 mg (Group II) was observed. Eggs were never found in the stools of animals of Group III and only occasionally in those of Group II.

In a second experiment, a total of 15 monkeys, divided in 5 groups, was used to determine the suppressive and curative effect of antimony dimercapto succinic acid in olive oil. All animals of Groups VI to IX (Table XV) were treated starting with the 56th day after exposure. The monkeys of one group (VI) received 3 daily doses of 100 mg each, those of Group VII received 3 daily doses of 75 mg each, those of Group VIII received 4 daily doses of 25 mg each and those of Group IX received a single dose of 100 mg. The monkeys of Group X served as untreated controls.

TABLE XV

The curative effect of antimony dimercapto succinic acid in olive oil on S. mansoni infections in monkeys

Group No.	Monkey No.	Dose (mg)	Results of necropsy		NEPGF*	
			No. of live worms recovered	Percent recovery	Maximum	Mean
VI	17	100	0	0	0	0
	18	x	0	0	0	0
	19	3 days	0	0	0	0
VII	20	75	0	0	0	0
	21	x	8	2	1	0.5
	22	3 days	19	5	4	2
VIII	23	25	74	18	9	5
	24	x	100	25	12	8
	25	4 days	47	12	6	4
IX	26	100 once	8	2	1	0.5
	27		42	10	15	7
X	28	No Rx	84	21	18	12
	29		188	47	51	34
	30		84	21	40	26
	31		179	45	48	23

* Number of eggs per gram of feces from 2 weeks after completion of treatment to necropsy.

TABLE XVI

The curative effect of dicarbonic acid hydrazide (S-201) on S. mansoni infections in monkeys

Group No.	Monkey No.	Dose (mg/Kg)	Results of necropsy		NEPGF*	
			No. of live worms recovered	Percent recovery	Maximum	Mean
XI	32	250 x 6 days	4	1	0	0
	33		14	3	0	0
	34		29	7	0	0
	35		30	8	0	0
	36		31	8	0	0
	37		33	8	0	0
	38		37	9	0	0
	39		50	12	0	0
XII	40	No Rx	118	29	65	51
	41		185	46	58	36
	42		100	25	39	17
	43		84	21	38	28
	44		82	20	24	12
XIII	45	250 x 6 days	-	-	-	-
	46		-	-	-	-

* Number of eggs per gram of feces from 2 weeks after completion of treatment to necropsy.

As before, no obvious signs of toxicity were observed in any of the treated monkeys. At necropsy, 133 days after exposure, no worms were found in the animals of Group VI and a significant reduction of the worm burden was observed in the animals of Groups VII and IX. Conversely, no marked reduction in the worm burden was found in the monkeys of Group VIII. Repeated stool examinations conducted at regular intervals showed eggs in the feces of all monkeys which were not treated prophylactically, between the fifth and sixth week following exposure to cercariae. The untreated control monkeys showed the typical egg production pattern of S. mansoni in M. mulatta: a peak of approximately 7-8 weeks; a marked reduction for 4 weeks and then a moderate number persisting for the duration of the experiment. Fewer eggs were found in the stools of

treated animals already one week after termination of treatment. From the third week after therapy to the end of the experiment, no eggs were found in the stools of the treated animals except for occasional eggs in those receiving four doses of 25 mg each and those receiving a single dose of 100 mg each.

b. Dicarbonic acid hydrazide (S-201).

A third experiment was designed to determine the suppressive and curative effect of S-201 on S. mansoni infections. A total of 15 monkeys was divided into three groups. Those of one group (XI) received six daily doses of 250 mg of S-201 per kilogram of body weight. Those of another group (XII) were left untreated and served as controls. Two monkeys in Group XIII were unexposed to infection and used as drug controls. The results are summarized in Table XVI. Although a marked reduction in the number of worms was observed in the treated monkeys at necropsy, 133 days after exposure, some living worms were present in everyone of the treated animals (Group XI). However, the ratio of males versus female worms observed was much greater in the treated animals than in controls thus indicating a possible selective activity of this drug against female S. mansoni. Eggs in the stools of these animals followed the same pattern observed in the previous experiment. From the end of the second week after termination of therapy up to the end of the experiment only occasional eggs were found in the stools of the animals which had been treated. Most of these eggs appeared distorted and underdeveloped.

c. Organo-phosphorus (Ronnel).

A fourth experiment was set up to determine the prophylactic and curative effect of Ronnel on S. mansoni infections in monkeys. A total of 12 monkeys was used in this experiment. The animals were divided into 4 groups (Table XVII). Those of the one group (XIV) received 3 daily doses of 100 mg each starting 2 days prior to exposure. Those of another group (XV) received the same treatment starting with the 56th day after exposure. Those of another group (XVI) were not treated and served as uninfected controls. One monkey (40-65) was used as drug control and was not exposed to infection.

The results, summarized in Table XVII indicate no significant prophylactic suppressive or curative effect of this drug in monkeys under these experimental conditions.

d. Nitro-thiazole derivative (CIBA 32-644-Ba).

A total of 25 monkeys, divided in 5 groups, was used to determine the prophylactic and curative effect of CIBA 32-644-Ba.

The monkeys of Groups XVIII, XIX and XX were treated with various regimens of the drug at different times during the course of infection. Animals of Group XXI were exposed but not treated and served as untreated controls; those of Group XXII were treated and not exposed to infection and served as drug controls. In the animals of Group XVIII, the drug was administered in daily doses of 100 mg/kg for 4 days beginning with the day before exposure. In the animals of Group XIX, the drug was given in the amount of 50 mg/kg twice daily for 6 consecutive days beginning on the 56th day following exposure. (The first of the daily treatments was given between 0900 and 1000 hours and the second one was given between 1600 and 1700 hours.) The animals of Group XX were given 6 doses of 100 mg/kg beginning with the 56th day following exposure. Likewise, the animals of Group XXII were given 6 consecutive daily doses of 100 mg/kg.

TABLE XVII

The prophylactic and curative effect of an organic phosphorus compound (Ronnel) on S. mansoni infections in monkeys

Group No.	Monkey No.	Dose (mg)	Rx started (from day of exposure)	Results of necropsy		NEPGF	
				No. of live worms recovered	Percent recovery	Maximum	Mean
XIV	47	100 x 3 days	-2	183	46	36*	22*
	48			160	40	27*	8*
	49			57	14	9*	2*
	50			179	45	34	16
XV	51	100 x 3 days	56	133	33	18**	9**
	52			118	29	19**	10**
	53			72	18	10**	4**
	54			131	32	15**	6**
XVI	28	-	-	84	21	18	12
	29	-	-	188	47	51	34
	30	-	-	84	21	40	26
	31	-	-	179	45	48	23
XVII	55	100 x 3 days	-	-	-		

* Number of eggs per gram of feces from earliest egg appearance to necropsy.

** Number of eggs per gram of feces from 2 weeks after completion of treatment to necropsy.

No obvious signs of toxicity were observed in any of the treated animals. There were no significant changes in the weights of any of the monkeys during the course of this experiment. Histopathologic studies of the testes and ovaries of treated and untreated monkeys showed no obvious differences. The testes and ovaries of all animals, treated and untreated, showed a histologic pattern consistent with normal prepubertal or early pubertal stages and gave no evidence of degenerative changes.

The results of the effect of treatment on the number of the living schistosomes found at necropsy have been summarized in Table XVIII. Forty-nine days after exposure, a significant reduction in the worm burden was observed in the animals which received the drug prophylactically (Group XVIII). No eggs appeared in the stools of these animals and no gross pathological changes attributable to the infection were seen in any of the organs.

The control monkeys showed the typical egg production pattern of S. mansoni in M. mulatta: a peak at approximately 8 weeks; a marked reduction for 4 weeks; and then a moderate number persisting for the duration of the experiment. The animals treated with 2 daily doses of 50 mg/kg each for a total of 6 days (Group XIX) showed a parallel course for the first two periods but the eggs disappeared during the third. Essentially the same results were obtained in the animals which received 100 mg/kg each.

In both of these groups the passage of eggs was completely suppressed in all but 4 monkeys and the worm burden was significantly reduced. Only a few worms, almost all of them males, were found in 2 of 6 monkeys of Group XIX and 5 of 7 monkeys of Group XX. Both male and female worms appeared to be stunted and considerable morphological changes, particularly in the reproductive organs, were observed. Hatchability studies conducted each week throughout the experiment showed that miracidia could be recovered from those specimens containing eggs.

Gross pathological observations in the untreated controls showed enlargement of livers and spleens. Brownish discoloration and moderate spotting due to egg accumulation was also apparent in these organs. The gross appearance of lungs, and both small and large intestines was essentially normal except for the presence of petechial hemorrhages in the intestines of one animal (No. 88). By contrast, no gross abnormalities were observed in the livers, lungs and intestines of the treated and infected monkeys, although a moderate splenomegaly was noticeable in all of these animals.

TABLE XVIII

The prophylactic and curative effect of CIBA 32-644 Ba
in 25 monkeys exposed to 400 Schistosoma mansoni cercariae each

Group No.	Monkey No.	Dose (mg/kg)	Rx started (from day of exposure)	Results of necropsy: Number of live worms recovered				NEPFG*	
				Female	Male	Total	Percent	Maximum	Mean
XVIII	56	100 for 4 days	-1	1	20	21	5	0	0
	57			1	25	26	6	0	0
	58			0	4	4	1	0	0
XIX	59	50 b.i.d. for 6 days	56	3	19	22	6	15	2
	60			0	2	0	0.5	0	0
	61			0	0	0	0	2	0.2
	62			0	0	0	0	0	0
	63			0	0	0	0	0	0
	64			0	0	0	0	0	0
XX	65	100 for 6 days	56	6	31	37	9	1	0.1
	66			1	6	7	2	0	0
	67			1	6	7	2	0	0
	68			0	4	4	3	11	1
	69			0	2	2	0.5	0	0
	70			0	0	0	0	0	0
	71			0	0	0	0	0	0
XXI	72	--	--	49	56	105	26	31	18
	73	--	--	40	46	86	21	32	18
	74	--	--	17	62	79	20	39	21
	75	--	--	23	48	71	18	18	13
	76	--	--	21	47	68	17	30	20
	77	--	--	24	34	58	14	21	12
	78	--	--	29	44	73	18	35	16
XXII	79	100 for 6 days	--	--	--	--	--	--	--
	80		--	--	--	--	--	--	--

* Number of eggs per gram of feces from 2 weeks after completion of treatment to necropsy.

4. Oxygen Consumption of Heart and Liver Tissue of Mice Infected with Trypanosoma rhodesiense.

There is little information regarding the chemical contribution of trypanosomes to hosts. Accelerated weight gains observed in T. lewisi infected rats and T. duttoni infected mice suggested the possibility of enhanced host metabolism at a cellular level. Lincicome and Shepperson observed increased thiamine levels and increased oxygen consumption of T. lewisi infected rats fed a thiamine deficient diet.

Singer reported increased thiamine content of liver from rats infected with T. rhodesiense and suggested that the increased vitamin content probably reflected an attempt by the host to utilize or detoxify the metabolic products of the parasites. Increased oxygen consumption of mouse liver in T. evansi infection has been observed, but host tissue oxygen uptake has not been studied in T. rhodesiense infections.

Therefore, tests to measure the oxygen consumption of mouse heart and liver slices during the course of T. rhodesiense infections were carried out.

Sixty male albino mice weighing 18-21 grams were inoculated with 100 Trypanosoma rhodesiense cells to study the oxygen consumption of mouse liver and heart tissue during the course of trypanosome infection. Fifty-nine uninoculated mice were used as controls. Liver tissue slices of T. rhodesiense infected mice used 10, 20, 24, 46, 51 and 33% more oxygen than control tissues on days 2, 3, 4, 5, 6 and 7 after inoculation, respectively. Analysis by Student's "t" test showed that these increments were highly significant on days 3 to 7. There was no significant difference in the consumption of oxygen by heart slices.

A comparison of organ weights revealed that the livers of infected mice were significantly heavier than the livers of the controls on days 5 and 6. The hearts of the infected mice averaged 0.61% of the total body weight as compared to 0.49% in control mice. There was a significant difference in the heart weights of the two groups on day 4; otherwise, throughout the experiment control and infected hearts weighed about the same, 0.45-0.55% of body weight.

The results presented for liver sizes and liver respiration during the early stages of T. rhodesiense infections indicate that the metabolic state of the host remained essentially normal. However, as the parasitemia progressed, host reactions to the parasite were manifested in part by liver hypertrophy and increased liver respiration.

TABLE XIX

Oxygen Uptake of Liver Slices from Control and *Trypanosoma rhodesiense* Infected Mice

Mean QO_2 values ± S.E. of liver slices from -

Days	Control Mice	Infected Mice
1	3.2±0.1	3.0±0.2
2	3.1±0.1	3.4±0.2
3	3.1±0.1	3.7±0.1
4	2.9±0.1	3.6±0.1
5	2.9±0.1	4.3±0.1
6	3.4±0.1	5.3±0.2
7	3.4±0.3	4.5±0.2

TABLE XX

QO_2 Increments of Trypanosoma rhodesiense Infected Mouse Liver Over Uninfected Mouse Liver (with Statistical Significances)

Days	QO_2 μl	Increments %	T Values	Degrees of Freedom	Results of T Test
1	0.0	0.0	1.0	53	N. S. *
2	0.3	10	1.3	54	N. S.
3	0.6	20	3.3	55	S **
4	0.7	24	4.4	54	S
5	1.3	46	9.6	54	S
6	1.8	51	7.6	56	S
7	1.1	33	3.6	8	S

* N.S. = No Significance
** S. = Significant at 5% or lower

TABLE XXI

Oxygen Uptake of Heart Slices from Control and
Trypanosoma rhodesiense Infected Mice

Mean QO_2 values ± S.E. of heart slices from -

Days	Control Mice	Infected Mice
1	2.4±0.2	2.3±0.3
2	2.1±0.3	2.4±0.2
3	2.4±0.2	2.2±0.3
4	2.5±0.2	2.9±0.4
5	2.3±0.2	2.0±0.3
6	2.0±0.3	2.3±0.5
7	0.6±0.0	1.3±0.1

TABLE XXII

Mean Liver: Body Weight Ratios (in %) and Standard Errors (S. E.) of Control and Trypanosoma rhodesiense Infected Mice

Days	Control Mice	Infected Mice	Degrees of Freedom	T Values
1	6.0±0.1	6.0±0.1	15	1.13
2	6.6±0.3	6.7±0.3	15	0.23
3	6.1±0.3	6.6±0.2	15	1.50
4	6.4±0.2	6.3±0.3	16	0.15
5	5.9±0.1	7.0±0.3	15	3.71*
6	6.0±0.2	7.3±0.2	15	4.85*
7	7.0±0.0	7.2±0.3	1	0.24

* Significant at 5% level

TABLE XXIII

Mean Heart: Body Weight Ratios (in %) and Standard Errors (S.E.) of Control and Trypanosoma rhodesiense Infected Mice

Days	Control Mice	Infected Mice
1	0.56±0.03	0.54±0.02
2	0.52±0.02	0.51±0.02
3	0.51±0.02	0.53±0.01
4	0.50±0.01	0.62±0.02*
5	0.51±0.02	0.53±0.01
6	0.50±0.02	0.52±0.01
7	0.45±0.00	0.51±0.01

* Significant at 5% level

5. Serum chemical components in mice determined by the use of ultra-micro techniques

Mice are used extensively for microbiological and physiological studies in the laboratory. To date, however, there has been no detailed report of the normal values of the serum components in these animals. This is due perhaps to the fact that the relatively large volumes of serum required for many of the standard biochemical tests rendered these small experimental animals unsuitable for the task.

The purpose of this report is to provide data on the relative amounts of serum constituents in mice, to determine the accuracy with which such measurements may be made and to examine some of the variables which may influence these values.

Animals.

Young male and female mice of three different strains, BALBC, Cinnamon and Inbred Charles River (ICR), weighing between 12 and 28 grams were used throughout these studies. All mice received food and water ad libitum. Mice were fasted in wire bottom cages approximately 18 hours prior to blood withdrawal for biochemical analyses. Blood was obtained from the tail after it had been immersed in warm water (approximately 39°C.) for one to two minutes. After collection the blood was allowed to clot for approximately 4 minutes, centrifuged and the serum was separated immediately. Occasionally, when immediate testing was not practical, the serum was stored at 4°C until used (within 4 hours). Preliminary comparisons between blood obtained by this method and by exsanguination showed no difference in results.

Biochemical techniques.

Total Protein. A modified biuret method for total protein was used. The alkaline biuret reagent produces a violet color with proteins which is proportionate to the protein concentration. A 5ul sample of serum was added to 150ul biuret reagent and allowed to react for 30 minutes in the dark. Absorbance of each sample was read at 560 mu.

Phosphorus. An ultramicro adaptation of the phosphorus technique was used. The intensity of blue color developed by reducing the formed phosphomolybdic acid with aminonaphtholsulfonic acid is proportionate to the amount of inorganic phosphorus present in the serum. The sample was deproteinized by adding 20ul of serum to 150ul of 10% trichloroacetic acid. A 100 ul aliquot of the deproteinized sample was placed in a clean micro test tube and 20ul of 2.5% molybdic acid was added and mixed and allowed to stand at room temperature for 30 minutes. The absorbance of the sample was read at 650 mu.

Non Protein Nitrogen (NPN). The micro method for NPN based on the titration of excess hypobromite with sodium thiosulfate in the presence of starch was used. A 10ul serum sample was deproteinized with 40ul of 10% trichloroacetic acid, then a 20ul aliquot of this deproteinized sample and 100ul of hypobromite solution were mixed for exactly 1 minute in the micro-titrator. With constant mixing, 5 ul of 10% potassium iodide, 40ul of 18% hydrochloric acid, and a small drop of 1% starch solution were added and titrated with 0.2 N sodium thiosulfate until an end point was obtained.

Creatinine. A modification of the Folin and Wu technique was used. The concentration of creatinine was determined in an aliquot of deproteinized serum with alkaline picrate. A 20ul serum sample was deproteinized with 150ul of sodium tungstate-sulfuric acid mixture (equal parts of 3.2% sodium tungstate and o.2N sulfuric acid). From this deproteinized sample a 100ul aliquot was mixed with 50ul of alkaline picrate solution and allowed to stand for 20 minutes in the dark. The absorbance of each sample was read at 525 mu.

Glucose. An ultramicro adaptation of the coupled enzyme system utilizing glucose-oxidase-peroxidase and chromogen o-dianisidine was used. A serum sample of 10ul was deproteinized by adding 150ul of 0.08N sodium hydroxide and 40ul of 5% zinc sulfate. A 100ul aliquot of the deproteinized sample was placed in a clean micro test tube and 250ul of Glucostat (Worthington Biochemical Corp.) was added and allowed to react for exactly 10 minutes. At this time, 10ul of 5N hydrochloric acid was added to stop the reaction. After approximately 5-10 minutes the absorbance was read at 410 mu.

Glucose tolerance test. After sampling blood for an estimation of the initial fasting glucose level, 1 mg of glucose per gram of body weight was injected intravenously in the tail vein, extreme care being taken that none of the solution escaped into the adjacent tissues. Subsequent samples were taken at 15-minute intervals. The excess glucose was plotted against time and the percent removal rate per minute was calculated.

Chloride. A modification of the Schales and Schales technique was used. Serum was titrated with an acid solution of mercuric nitrate in the presence of s-diphenyl carbazone indicator. Each test consisted of 10ul of serum, 80ul of 0.03N nitric acid, and 80ul of indicator solution mixed in the above order and titrated with mercuric nitrate until the end point was obtained.

Alkaline Phosphatase. An ultramicro adaptation of the alkaline phosphatase method was used. Enzymatic hydrolysis liberates p-nitrophenol, a yellow salt that indicates the degree to which phosphates are split from the buffered p-nitrophenyl phosphate substrate. Five ul of serum were added to a micro test tube containing 50 ul of buffered substrate (Sigma 104 and 104-5) preheated at 37°C for 5 minutes. Subsequently 200ul

of 0.04N sodium hydroxide was added. Absorbance A was read at 410 mu; 5ul of concentrated hydrochloric acid were added to the cuvette, mixed well, and absorbance B was recorded. Values obtained for A gave a corrected reading for the alkaline phosphatase activity of the serum.

Glutamic Pyruvic Transaminase (SGP-T). An ultramicro adaptation of the serum glutamic pyruvic transaminase was used. The amount of pyruvate formed was determined colorimetrically by the formation of a "hydrazone" which is highly colored. A 19ul serum sample was added to 50 ul of GPT substrate (Sigma 505-51) which had been preheated in a water bath at 37°C for 5 minutes and the mixture was allowed to react for exactly 30 minutes. Fifty ul of dinitrophenylhydrazine were then added and allowed to react at room temperature for 20 minutes, after which 150 ul of 0.568N sodium hydroxide were added. Five minutes later the absorbance was read at 505 mu and the SGP-T values were obtained from the calibration curve.

Glutamic Oxalacetic Transaminase (SGO-T). An ultramicro adaptation of the serum glutamic oxalacetic transaminase was used. The amount of oxalacetate formed was determined colorimetrically by the formation of a "hydrazone". A 10ul serum sample was added to 50 ul of GOT substrate (Sigma 505-1) which had been preheated in a water bath at 37°C for 5 minutes and allowed to react for exactly 60 minutes. Subsequently, 50 ul of dinitrophenylhydrazine was added and allowed to react at room temperature for 20 minutes, after which 150ul of 0.568N sodium hydroxide was added and allowed to stand for 5 minutes. The absorbance was read at 505 mu and the SGO-T values were obtained from the calibration curve.

Electrophoresis. The cellulose acetate method of Scherr was used. A total of 3ul of serum distributed on a 2.5 x 12 cm cellulose acetate strip and separated at 200 volts for 90 minutes gave the best results. Strips were stained with Ponceau S and the grams percent of each fraction was calculated.

Bilirubin (Total)and(Direct). Both total and direct bilirubin were determined by a modification of the Malloy and Evelyn procedure based on the van den Bergh and Ehrlich reactions. The direct reaction was measured in an aqueous serum and the total reaction was measured in a 50% solution of methanol which on the addition of diazo reagent produced the pink-violet color of azobilirubin proportionate to the bilirubin concentration. A 20ul sample of the standard and/or unknown was used in the procedure according to Beckman Technical Bulletin No. 6076D. However, the standards were eliminated and a bilirubin standard serum was used as an unknown sample, and all values were calculated with this standard.

Sodium, Potassium, Calcium. These tests were performed with the Technicon Auto Analyser following standard procedures suggested by the manufacturer of this instrument.

Carbon dioxide CO_2. The CO_2 content was determined in 30 µl of plasma using the Natelson microgasometer and procedure as described in the Scientific Industries Instructions, Booklet No. 4. Blood was taken from the orbital sinus by glass capillary and allowed to run into a micro test tube. The tubes were capped except while samples were being removed.

Cephalin Flocculation Test. An ultramicro modification of the Hanger cephalin flocculation test was used with the antigen mixture described by Martinek. Ten microliters of serum were placed in a 6 x 50 mm glass test tube and 200 µl of the saline-cephalin-cholesterol mixture were added and mixed well. The tubes were allowed to stand at room temperature in the dark. Tests were read as described after 24 and 48 hours as neg. 1+, 2+, 3+ or 4+ reactions.

Bromsulphthalein retention (BSP). A modification of the Reinhold and Hutchinson procedure was used. A dose of 50 mgm/kgm of dye gave optimal results. All animals were bled before injection and 30 minutes afterwards. The procedure for measuring the amount of dye in the serum was as follows: a 20 µl aliquot of serum or plasma was placed in a test tube and 200 µl of glass distilled water were added. Then 20 µl of 0.5N sodium hydroxide were added and mixed well. Both samples were read against water at 560 mµ in the spectrocolorimeter. The absorbance of the control sample was subtracted from the absorbance of the 30-minute sample. The resulting value multiplied by a constant factor gave the percent retention of the dye.

Data Analysis. The range of normal values for each test was based on the mean, plus or minus the tolerance interval.

Equipment, Spectro-colorimeter: Beckman Spinco Model 151, with variable wave length selections from 400 to 650 mµ, direct reading meter and stationary cuvette, Microfuge: Beckman Spinco Model 152, with 20 micro test tubes which reaches a nominal speed of 15,000 rpm; Microtitrator: Beckman Spinco Model 153 with a delivering mechanism for an error not in excess of ± 0.01 µl; Micromixer; Small electric motor with heavy base, 1800-2000 rpm with a triangular shaped rubber of polyethylene block attached to shaft, which will give a vortex type mixing within the micro test tube; Micro Water Bath: Rotax model MTH/F designed to hold 52 micro test tubes and to control temperature at ±0.05°C; Micropipettes: Sanz pipette, hand drawn one piece polyethylene, calibrated to contain a desired volume; Electrophoresis: Shandon universal electrophoresis system using cellulose acetate.

a. Values obtained with the same serum specimen examined at different times.

This experiment was designed to determine the reproducibility of results with each biochemical test employed. Commercial control serum samples of different lots obtained from Hyland Laboratories were analysed in replicate. As indicated in Table XXIV, between 10 and 74 determinations were conducted at different times. Consistently similar results were obtained throughout this series of examinations. These were in agreement with the respective stated analyses. The mean and median of the various samples were identical or similar for each of the biochemical determinations described.

TABLE XXIV

Values obtained in replicate determinations of commercial control serum (human)

Tests	Units	No. of determinations	Mean	Median	Extreme values
Total protein	gms%	55	7.1	7.1	6.5 - 7.5
Phosphorus	mg%	24	4.0	4.0	3.6 - 4.4
Non protein nitrogen	mg%	24	22.7	22.0	20.0 - 25.9
Creatinine	mg%	10	1.2	1.2	0.9 - 1.3
Glucose	mg%	74	94.0	94.0	86 - 99
Chloride	mEq/L	14	91.5	91.0	88 - 97
Alkaline phosphatase	Units/ml	37	6.4	6.6	5.3 - 7.2
SGP-Transaminase	Units/ml	28	8	8	5 - 12
SGO-Transaminase	Units/ml	30	16	16	14 - 22

b. Values on duplicate serum samples.

This experiment was designed to determine the reproducibility of results obtained on duplicate serum samples taken a few minutes apart on the same animal. As indicated in Table XXV, the mean and median values of the two samples were either identical or very similar. When differences were observed between the two samples the values were almost always within two units of measurement.

TABLE XXV

Values obtained on duplicate serum samples from the same animal

Tests	Units	No. of mice	First sample		Second sample		Reproduceability			
			Mean	Median	Mean	Median	Identical	± 1 unit	± 2 units	± 3 or more units
Glucose	mg%	22	78	77	79	76	5	7	6	4
SGO-transaminase	units/ml	17	29	30	29	30	7	6	2	2
Total protein	gm%	16	6.4	6.5	6.4	6.5	5	8	3	0
Non protein nitrogen	mg%	20	40	40	40	40	8	9	3	0
Alkaline phosphatase	units/ml	11	5.2	5.5	5.3	5.5	2	4	5	0
Chloride	mEq/L	11	106.0	103.4	105.8	103.8	5	0	5	1
Phosphorus	mg%	10	9.1	9.3	9.2	9.5	2	4	2	2
Creatinine	mg%	32	1.7	1.6	1.7	1.6	14	15	2	1

TABLE XXVI

Biochemical determinations in normal mice

Tests	Units	No. of mice	Mean	Median	Standard deviation	Tolerance interval	Range*
Total protein	gm%	120	6.2	6.2	0.6	1.1	5.1 - 7.3
Phosphorus	mg%	104	9.2	9.1	1.9	3.6	5.6 - 12.8
Non protein nitrogen	mg%	80	39	37	6.5	12.0	26 - 52
Creatinine	mg%	54	1.1	1.1	0.2	0.5	0.6 - 1.6
Glucose	mg%	106	70	67	15.8	29.0	41 - 99
Chloride	mEq/L	54	112.2	112.2	4.9	9.9	102.2 - 122.0
Alkaline phosphatase	units/ml	72	4.4	4.3	1.2	2.4	2.0 - 6.8
SGP-transaminase	units/ml	78	23	21	8.6	16.0	7 - 39
SGO-transaminase	units/ml	86	73	74	15.6	30.0	43 - 103
Electrophoresis							
Albumin	gm%	63	3.4	3.3	0.5	0.9	2.5 - 4.3
Alpha 1	gm%	63	0.5	0.6	0.2	0.3	0.2 - 0.8
Alpha 2	gm%	63	0.8	0.8	0.2	0.3	0.5 - 1.1
Beta	gm%	63	1.5	1.4	0.3	0.6	0.9 - 2.1
Gamma	gm%	63	0.4	0.4	0.1	0.2	0.2 - 0.6
Bilirubin total	mg%	66	0.4	0.2	--	--	0.0 - 1.8
Bilirubin direct	mg%	65	0.15	0.12	--	--	0.0 - 0.4
Calcium	mg%	38	9.1	9.1	0.6	1.5	7.6 - 10.6
Sodium	mEq/L	32	149.8	150.2	2.8	7.1	142.7 - 156.9
Potassium	mEq/L	32	5.4	5.4	0.5	1.3	4.1 - 6.7
CO_2	mEq/L	52	21.9	20.7	3.4	8.3	13.6 - 30.2
Cephalin flocculation	neg. to 4+	48	--	--	--	--	24 hrs: neg. 48 hrs: neg.
Bromsulphalein	% retention	62	6.5	5.8	--	--	16.5 or less

* Mean ± Tolerance interval.

c. Biochemical determinations in normal mice.

Data on biochemical determinations conducted in 1101 mice of both sexes and various weights are given in Table XXVI. The mean, median, standard deviations, tolerance intervals and range were computed.

The results were also tabulated according to the strain of mice used (BALBC and ICR) and compared to determine whether or not strain differences occurred. No significant differences were observed in the values obtained between the two strains in the following determinations: total protein, phosphorus, non protein nitrogen, creatinine, alkaline phosphatase and serum electrophoresis. However, the mice of the ICR strain had much higher SGO-T levels (mean value 81 units and 59 units respectively). This difference was found to be highly significant (P= 0.001). The mice of the BALBC strain had a higher serum chloride level (115.9 mEq/L and 106.3 mEq/l respectively). This difference is significant with a P of 0.001.

d. Glucose Tolerance Test.

The consistent results obtained with the glucose determinations (Table XXIV and XXV) suggested the possibility of developing an intravenous glucose tolerance test for mice. When excess glucose was plotted against time at 15-minute intervals on a semi-logarithmic scale, a straight line relationship was obtained in most instances over a period of 45 minutes.

Since glucose tolerance is a function subject to many complex influences, some of the variables were studied. A first experiment was designed to determine whether the glucose removal rates (GRR) in mice were influenced by the sex of the animal. Glucose tolerance tests were conducted on 48 male and 51 female mice of the same strain (BALBC) and of similar weight. Nearly identical GRR were obtained in the two sexes (3.0 and 3.2 percent per minute, respectively.)

Another experiment was designed to determine whether significant differences in GRR were observed in 162 mice of different weights and strains. The animals of each strain were separated into four weight groups. The results (Table XXVII) indicate that the mice belong to two different categories with respect to their GRR. In the BALBC strain, high GRR were observed throughout these studies (2.8 percent per minute). Conversely, in the other 2 strains (ICR and Cinnamon) the GRR averaged 2.0 and 2.3 percent per minute. The difference between values observed with the BALBC strain and those in the two other strains is highly significant.

TABLE XXVII

Mean excess glucose removal rates (MEGRR)
in mice of different strains and weight

Strain	No. Mice	Weight in Grams		MEGRR
		Range	Mean	% per Min.
ICR	9	13.0 - 14.9	13.7	2.5
	24	15.0 - 19.9	18.7	2.0
	18	20.0 - 24.9	22.3	2.1
	15	25.0 - 27.8	26.3	1.7
	66	13.0 - 27.8	20.7	2.0
Cinnamon	9	12.0 - 14.9	12.8	2.6
	10	15.0 - 19.9	18.9	2.1
	14	20.0 - 24.9	21.5	2.1
	12	25.0 - 28.0	26.2	2.5
	45	12.0 - 28.0	20.4	2.3
BALBC	12	13.0 - 14.9	14.2	3.8
	22	15.0 - 19.9	17.4	2.9
	10	20.0 - 24.9	21.3	2.0
	7	25.0 - 26.5	25.6	2.0
	51	13.0 - 26.5	18.5	2.8

When mice of different weight groups were compared in all three strains, a tendency to increasing excess GRR was observed in lighter mice. Significant differences were obtained when mice of the three different strains were combined. However, these differences were not always conclusive, being often at the borderline of statistical significance. When mice of different weights were compared within each strain (Table XXVII) the only highly significant difference was observed in BALBC mice between the two groups weighing less than 20.0 grams (13.0 to 19.9 gms) and the larger groups (20.0 to 26.5 gms).

These results, in general, indicate that clinical biochemical observations in mice are consistent and in line with findings reported in considerably larger animals. It is of interest to point out that with respect to SGO-T, serum chloride levels and glucose tolerance tests, the physiological values obtained were different in different strains. This shows once again that genetic factors may be responsible for profound physiological modifications. The findings also indicate that some physiological values may be modified by age, body weight or both.

Understanding of the significance of the chemical constituents of blood and other body fluids in the diagnosis and treatment of disease has increased in recent years. It is obvious that reactions to degenerative, infectious and neoplastic disease states have their counterpart in somatic alterations which may find reflection in biochemical changes in the body fluids. Correlation of clinical observations with multiple quantitative determinations of the serum components may contribute to the differential diagnosis and to the prognosis of disease states. Recent methods for clinical biochemical determinations in very small samples of blood have made it feasible to utilize the mouse for such studies. However, accurate information on the normal physiology of the mouse is essential before studies can be conducted on some of the variables which may alter it. These studies provide some of the basic data necessary for a better utilization of the mouse as a test animal for physiological, biochemical and biological investigations and have shown in general the practicality and consistency of results possible with the ultra-micro techniques.

Summary and Conclusions:

1. Studies were conducted to determine whether extracts from egg and adult stages of schistosomes could be coated on cholesterol-lecithin crystals and be used for the serological diagnosis of schistosomiasis. The reactions obtained in the slide flocculation test with antigens from

eggs, cercariae and adult excretions and secretions were compared in artificially immunized and experimentally infected animals as well as in naturally infected humans to determine whether they stimulated or detected distinct humoral factors or whether they differed only in the relative proportions of common antigens. Attempts were made to isolate and define some of the soluble antigenic fractions in egg, cercaria and adult extracts to ascertain which are common to different stages; to characterize the fractions which are adsorbed onto cholesterol-lecithin crystals and to compare results obtained with the slide flocculation test and immunoelectrophoresis before and after homologous and heterologous serum absorption. Flocculation tests with adult worm excretion-secretion antigen appeared to be the most sensitive and specific. Each of the 3 antigenic extracts formed bands by immunoelectrophoresis when reacted against antisera from either artificially immunized or infected animals. Homologous and heterologous absorptions of sera against cercaria and egg extracts uniformly reduced their reactivity in the immunoelectrophoresis tests. Cercaria and egg antigens had more in common with each other than either had in common with adult excretion-secretion antigen.

2. Detailed parasitological, hematological, serological and biochemical observations on splenectomized chimpanzees infected with Schistosoma mansoni indicated that the patho-physiology of the infection in these animals was similar to that in man.

3. Studies of the prophylactic, suppressive and curative activity of selected compounds against Schistosoma mansoni in monkeys were completed. Antimony dimercapto succinic acid in olive oil, dicarbonic acid hydrazide and ciba 32,644-Ba had considerable activity, while an organo-phosphorus (Ronnel) did not.

4. The consumption of oxygen of heart and liver tissue of mice infected with Trypanosoma rhodesiense was determined and weight of these organs was compared with uninfected controls.

5. Determinations were made of the relative amount of some serum constituents in mice, the accuracy with which they can be made, and some of the variables which may influence these values. Data are provided on the following: total protein, phosphorus, non-protein nitrogen, creatinine, glucose, glucose tolerance, chloride, alkaline phosphatase, glutamic pyruvic transaminase, glutamic oxalacetic transaminase, electrophoresis, bilirubin, sodium, potassium, calcium,

carbon dioxide, cephalin flocculation and bromsulphthalein retention.

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RESEARCH AND TECHNOLOGY RESUME

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24. (U) Tech Objective - To define etiology of acute infectious diseases, and when known, to determine and evaluate factors influencing occurrence, distribution, severity and medical result of human virus infections. The emphasis is upon disease of military importance; the aim is to reduce disability.

25. (U) Approach - 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. Work is hampered by shortage of competent personnel, both professional and technical assistance.

26. (U) Progress (Jul 64 - Jun 65) - Physiological and psychological stress have been documented as important variables in production of recruit respiratory disease; Dengue viruses have been implicated in scattered epidemics of disease in southeast Asia. The nature of antiviral substances in respiratory secretions has been identified; the responsible immunoglobulin is gamma A. Cell culture systems for rapid recovery and identification of arboviruses have been developed. The natural history of certain temperate zone arboviruses has been further defined. The nature of antigenic variation among contemporary influenza A viruses has been described using more reproducible and sensitive virological tools.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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Project 3A014501B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 166, Viral infections of man

Investigators.

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Description.

To define the etiology and ecology of human virus infections, with particular reference to those occurring in military populations; to devise and evaluate means for precise diagnosis, control and prevention of disease. Studies have applied virological, immunological, epidemiological and physiological approaches to the understanding of disease caused by respiratory, arthropod-borne, and enteric viruses, and the factors influencing their transmission among man and other vertebrates, and their survival in nature.

Progress.

1. Isolation of Dengue Viruses in Tissue Culture from Patients During Epidemics in Puerto Rico and East Pakistan. Epidemic dengue fever occurred in Puerto Rico in the fall of 1963 and in Dacca, East Pakistan, in the late summer of 1964. Attempts by this and other competent laboratories to isolate the infecting viruses from the blood of acutely ill patients by conventional suckling mouse inoculation were generally unsuccessful. This failure to identify the etiology of the Puerto Rican epidemic led to attempts at virus isolation in African Green monkey kidney (GMK) cell monolayers using the challenge virus resistance (CVR) test. This method as described by Halstead proved successful, but with the Puerto Rican strains failed to provide the required reproducibility and accuracy for quantitation of infective virus and neutralizing antibody. As the test system was studied further modifications in cell cultures and techniques ultimately yielded a procedure which provides

the necessary accuracy. This report documents the recovery of dengue viruses from patients in the Puerto Rican and Pakistan epidemics, identifies them as closely related to the prototype 3 strain H-87, and describes the cell culture methods ultimately developed for recovery and identification of these viruses.

Blood was obtained from persons of both sexes, acutely ill with dengue fever in the cities of Fajardo, Naguabo, Humacao, and Yabucoa, in eastern Puerto Rico, during September-November 1963. These bloods were defibrinated by agitation with glass beads, rapidly frozen after defibrination, and stored at -70°C. College and medical students similarly ill in Dacca, East Pakistan, during August-September 1964 were also bled; these specimens were allowed to clot at ambient temperature, the serum was removed, frozen and stored as above. Defibrinated whole blood and serums were used for primary virus isolation attempts in this laboratory after their return in the frozen state.

Primary GMK cell monolayers and the diploid strain of BS-C-1 cells, 47th passage, were purchased from commercial sources. The continuous heteroploid line of BS-C-1 cells was obtained in approximately the 300th passage and propagated serially in this laboratory. Growth media used for the continuous BS-C-1 line was medium 199 (M199) containing 15% inactivated (56°C, 30 min.) fetal bovine serum (FBS), penicillin 100 units/ml and streptomycin 100 ug/ml. Eagles basal medium (BME) containing 2% inactivated chicken serum and antibiotics was used for maintenance of all cell types. Stationary tube cultures incubated at 35-36°C were used in all experiments.

a. Virus Isolation. The indirect interference technique described by Halstead was used with minor modifications. Serum or defibrinated blood was diluted 1:2, 1:20, and 1:100 in maintenance media and 0.1 ml amounts were added to 6 tissue culture tubes per dilution. Tubes were incubated and examined periodically for cytopathogenic effect (CPE). After 9-12 days' incubation 2 tubes from each dilution were challenged with approximately 1000 ID_{50} of polio II virus and examined for CPE at 24 and 48 hours after challenge. Tubes with less than 50% CPE at the time cell sheets in control tubes were completely destroyed, usually at 48 hours, were considered to have significant challenge virus resistance (CVR). In early experiments the unchallenged tubes were harvested at the time CVR was demonstrated, i.e. after 10 to 14 days of incubation. Experiments on dengue virus growth in BS-C-1 cells reported elsewhere indicated that maximal virus titers were reached by the fourth or fifth day, therefore in later experiments unchallenged tubes were harvested on the 6th day after inoculation. Seed virus from the tissue culture isolates was prepared by adding equal amounts of FBS to the media in the tubes, adding fine glass beads and agitating the tubes vigorously with a Vortex Jr. mixer to remove the cells from the glass and disrupt them. Virus seeds were clarified by centrifugation at 2000 rpm for 30 min. in a refrigerated centrifuge, shell frozen and stored at -70°C.

b. Reference Strains and Serologic Reagents. The following strains of dengue viruses, used for preparation of reference antigens and hyperimmune mouse ascitic fluid (HMAF), were obtained from Dr. William McD. Hammon: dengue type I, Hawaiian strain 127th mouse passage; dengue type II, New Guinea C, 29th mouse passage; dengue type III, H-87 strain 25th mouse passage; and dengue type IV, H-241 strain, 28th mouse passage. Polio type II virus was a strain isolated in this laboratory in 1956.

Hyperimmune mouse ascitic fluids to dengue types I through IV used for comparative serologic testing were prepared by the method described by Brandt. Complement fixation (CF) and hemagglutination-inhibition (HI) tests were performed by methods previously described. Preliminary typing of interfering agents recovered in tissue culture was done by a serum dilution neutralization test. Serial five-fold dilutions of HMAF were added to equal amounts of tissue culture media containing approximately 100 interfering doses (InD_{50}) of virus. Reagents were mixed in a 4°C water bath and 0.1 ml amounts were inoculated into tissue culture tubes without prior incubation. Cultures were incubated for 9 days and challenged with polio II virus. End points were determined by observing for CVR as described above. Representative strains of Puerto Rican and East Pakistan isolates were also tested by a constant serum, virus dilution neutralization test using a one-hour incubation at 25°C prior to inoculation of the tissue culture. Log neutralization indices were determined by the method of Reed and Muench.

c. Limitations of the Original CVR Test. Early experience using primary GMK and diploid BS-C-1 cells for isolation of Puerto Rican dengue strains revealed that detection of infected cell cultures by the CVR test was readily achieved. However, propagating newly recovered viruses to sufficient titer for identification presented several difficulties. Harvesting infected cells at 10 to 14 days after inoculation resulted in recovery of virus of low titer and titers did not increase with 8 to 10 serial passages. In addition, it was found that freezing and thawing of the cell sheet to release virus resulted in significant loss in titer and the harvested viruses proved to be relatively unstable when stored at -70°C in maintenance medium. Some strains were lost in passage due to harvesting after too long an incubation period and repeated freezing and thawing. Subsequent experiments have shown that maximal virus titers are attained from 3 to 6 days after inoculation depending upon the amount of virus in the inoculum. Careful attention to maintenance of an alkaline pH in the tissue culture medium was found to be necessary to prevent loss of virus. Incubation at 32°C or increasing the HCO_3 concentration of the media did not increase the virus yield.

Comparison of primary GMK cells and diploid BS-C-1 cells for primary isolation revealed that the GMK cells were significantly more sensitive (Table 1). Six positive serum specimens were tested

Table 1 Isolation of Dengue Viruses from Human Sera
by the Interference Method
Comparison of primary GMK and diploid (44th passage) BS-C-1 cells

Serum No.	Cell type	CVR(1) 1st pass.	CVR 2nd pass.	Highest serum (2) dilution positive
PR-22	GMK	+	+	1:100
	BS-C-1	0	+	1:2
PR-23	GMK	+	+	1:100
	BS-C-1	+	+	1:20
PR-25	GMK	+	+	1:20
	BS-C-1	0	+	1:2
PR-27	GMK	+	+	1:2
	BS-C-1	0	0	-
PR-30	GMK	+	+	1:20
	BS-C-1	+	+	1:2
PR-31	GMK	+	+	1:20
	BS-C-1	0	0	-

(1) Challenge virus resistance.
(2) Sera tested at 1:2, 1:20 and 1:100.

simultaneously in the two cell lines and in two cases virus was isolated only in GMK cells and not in BS-C-1 cells; in addition, GMK cells consistently gave positive results with higher dilutions of serum than the BS-C-1 cells. In two cases virus was recovered in diploid BS-C-1 cells only after a second passage.

The continuous (300+ passage) BS-C-1 cells, on the other hand, yield results comparable to GMK cells. They proved to be equally sensitive for isolation and comparable yields of virus were obtained (Table 2). This was true for dengue viruses from both Puerto Rico and Pakistan. The high passage BS-C-1 line, being free of contaminating simian agents and PPLO, was found to be the most useful of the three types for isolation and propagation of dengue viruses.

d. Virus Isolations from Defibrinated Blood and Sera. Thirty-eight blood specimens obtained from Puerto Rican patients in the first or second day of fever were tested for the presence of interfering agents. Twenty-one viruses were isolated and all were passed at least twice in tissue culture. Thirteen of the Puerto Rican isolates were tested by the serum dilution neutralization test and were found to be neutralized by dengue type III HMAF to a higher titer than by the other dengue

Table 2 Isolation of Dengue Viruses from Human Sera by the Interference Technique.
Virus growth in primary GMK and heteroploid (300+ passage) BS-C-1 cells

Serum[1] No.	Highest serum[2] dil. positive	Cell type	Day of harvest	Virus titer Log 10 InD_{50}/0.1 ml
PR-33	1:20	GMK	9	2.0
PR-37	1:100	GMK	12	3.5
PR-38	1:100	GMK	9	4.0
PR-38	1:100	BS-C-1	9	3.5
PR-44	$10^{4.0}$	GMK	9	4.0
PR-44	$10^{4.0}$	BS-C-1	9	4.5
Pak-14	1:100	GMK	10	2.0
Pak-17	1:100	GMK	10	3.0
Pak-20	1:100	GMK	10	3.0
Pak-22	1:100	GMK	10	2.0
Pak-36	1:20	GMK	10	1.5
Pak-18	1:100	GMK	8	3.5
Pak-18	1:100	BS-C-1	7	3.5
Pak-32	1:100	BS-C-1	8	3.5
Pak-16	1:100	BS-C-1	8	2.5
Pak-34	1:100	BS-C-1	7	3.5

(1) PR - Puerto Rican patients
Pak - East Pakistani patients

(2) Serum tested at 1:2, 1:20 and 1:100 except PR-44 which was titered to end point.

types although considerable cross reaction with dengue type I was found, especially with lower doses of virus. All Puerto Rican strains tested appeared to be very similar in growth and serologic characteristics.

Twenty-five acute phase serum specimens obtained from Pakistani patients were tested for the presence of interfering viruses and 18 agents isolated. Table 3 compares the results of virus isolation attempts and the results of HI tests on paired serum from this group. From 11 patients in whom a definite (four-fold or greater) serologic rise was found 10 viruses were isolated. Three patients with no serologic evidence of dengue infection had no virus present in the blood, and from the remaining 11 patients on whom paired sera could not be obtained 8 dengue viruses were isolated. All eighteen Pakistan isolates were tested by the serum dilution neutralization test and by this method they all appeared identical and could not be distinguished from the Puerto Rican isolates or dengue type III.

Table 3 Correlation of Virus Isolation and Serologic Response by HI Test(1) in 25 Pakistani Patients

Serologic status	No. tested	No. isolations
4-fold rise in HI titer	11	10
No change in HI titer	3	0
HI test not done	11	8

(1) Acute and six-week convalescent sera tested against 4 units dengue type III antigen.

Six of the twenty-one positive blood specimens from Puerto Rico and twelve of the eighteen positive specimens from East Pakistan were positive at a dilution of 1:100 indicating a virus titer of equal to or greater than $10^{3.5}$ InD_{50}/ml of blood. One specimen titered to the end point was found to contain $10^{5.0}$ InD_{50}/ml. In three instances virus was isolated from the 1:20 and 1:100 dilution of blood and could not be isolated from the 1:2 dilution.

e. Identification of Recovered Strains. Serum dilution neutralization tests using a constant virus dose were used for preliminary identification of the dengue strains isolated. Several variations in technique were tried before a satisfactory method, described below, was empirically arrived at. The time of incubation of virus and antiserum

prior to inoculation of tissue culture was found to be critical. Incubation at 37°C resulted in rapid loss of titer with the tissue culture dengue viruses and therefore proved to be unsatisfactory. The cross-reactivity of dengue strains due to shared antigens was enhanced by long incubation at 37° and 25°C and specificity was lost. Inoculation of tissue culture tubes immediately following mixing of the virus and antiserum gave lower serum neutralization titers but high specificity. The virus dose was also found to be a critical factor in obtaining specific neutralization reactions. Too much virus (greater than 100 InD_{50}) resulted in lack of complete neutralization at even the lowest serum dilution (1:5), and too little (less than 10 InD_{50}) resulted in cross reactions with loss of specificity. To obtain a satisfactory virus dose in a test it was necessary to use the virus at two or three different dilutions to be sure that one would fall into the critical range. Results of representative serum dilution neutralization tests are given in Table 4.

Table 4 Results of Serum Dilution Neutralization Tests on Four Representative Dengue Strains

Virus strain(1)	Virus dose Log_{10} InD_{50}	Neutralizing titer of anti-serum(2) type I (Haw.)	type II (N.G."C")	type III (H-87)	type IV (H-241)
PR-33	2.0	0(3)	0	0	0
	1.0	0	0	1:25	0
PR-38	2.0	0	0	1:5	0
	1.0	1:25	1:5	1:25	0
PR-41	2.0	0	0	1:25	0
Pak-18	2.5	0	0	1:5	0
	1.5	0	0	1:25	0
	0.5	1:25	1:5	1:125	1:5
Pak-16	2.5	0	0	0	0
	1.5	0	0	1:5	0
	0.5	1:25	1:25	1:25	1:5

(1) First tissue culture passage of indicated virus strain.
(2) Hyperimmune mouse ascitic fluid made to indicated reference strain.
(3) 0 = less than 1:5.

Virus dilution neutralization tests done in BS-C-1 cells were found to be useful for establishing the antigenic relationships between the agents isolated and dengue types I through IV. Two representative strains from each area were tested by this method. The results are summarized in

Table 5. Both the Pakistan and Puerto Rico strains were neutralized by dengue type III HMAF to approximately the same extent as the H-87 type III strain. Again, a significant cross reaction with dengue type I was seen as well as minimal cross reaction with dengue type II.

Table 5 Log Neutralization Indices of Reference Hyperimmune Mouse Ascitic Fluid Tested Against Puerto Rico, Pakistan, and Homologous Dengue Strains

	Dengue Viruses					
HMAF	type I (Hawaii)	type II (N.G."C")	type III (H-87)	type IV (H-241)	Puerto Rico	East Pakistan
type I	4.2	0.7	1.0	0.8	2.2	1.8
type II	<1.2	2.5	<1.0	0.8	1.5	1.5
type III	<1.2	0.0	3.0	1.2	>2.8	3.1
type IV	<1.2	0.5	<1.0	3.0	1.0	0.3

Complement fixation and hemagglutination-inhibition titers of the convalescent serum from the Pakistani patients reveal a pattern of immunologic response consistent with infection due to a type III dengue virus (Table 6). Most convalescent titers were higher when tested against dengue type III CF and HI antigens than the other types although in 2 cases the titer was 2-fold higher than to type I virus.

Table 6 Convalescent Serum[1] Hemagglutination-Inhibition and Complement Fixation Titers of Pakistani Patients From Whom Dengue Viruses Were Isolated

Patient No.	HI titer[2] vs indicated dengue antigen			CF titer[3] vs indicated dengue antigen			
	I	II	III	I	II	III	IV
Pak-13	640	320	640	32	32	64	0[4]
Pak-14	80	40	80	0	0	0	0
Pak-15	80	20	80	8	0	16	0
Pak-17	40	20	80	8	0	8	0
Pak-18	80	20	160	16	16	32	0
Pak-22	160	40	80	32	16	16	0
Pak-25	320	320	320	32	16	64	0
Pak-26	80	40	80	8	8	16	0
Pak-33	320	40	160	0	0	0	0
Fak-35	160	40	160	32	0	16	0

(1) Convalescent sera collected approximately 6 weeks after illness.
(2) Reciprocal titer vs 8 units of sucrose-acetone mouse brain antigen.
(3) Reciprocal titer vs 4 units of 20% mouse brain antigen.
(4) 0 = <8.

2. Mechanism of Challenge Virus Resistance Induced in BS-C-1 Cells by Dengue Virus. Dengue viruses often infect mammalian cell cultures without obvious cytopathogenic effect. It has been shown that such infected cultures resist superinfection with another virus. This challenge virus resistance (CVR), similar to that induced by rubella and other viruses has been found sensitive and efficient as an indicator for detection of dengue viruses in patients' blood, and offers a more workable host system for recovery and assay of dengue viruses than the suckling mouse, to which dengue viruses must be adapted by repeated intracerebral passage. The experiments here described show that the mechanism for CVR in BS-C-1 cells is based upon the elaboration by infected cells of an interferon-like substance. The characteristics of this interferon, its behavior in BS-C-1 cells, and the circumstances of its production are the subject of this report.

The dengue virus used was isolated from the serum of a patient during the Puerto Rico dengue epidemic in 1963. Original isolation was made in BS-C-1 tissue culture, and the third and fourth BS-C-1 passages were used in these experiments. This agent is representative of the 1963 Puerto Rican strains and is closely related antigenically to dengue type III. Polio II virus used was a strain isolated in 1956 in this laboratory.

The BS-C-1 line of continuous grivet monkey kidney cells was obtained in approximately the 300th passage and subcultured serially. Growth media used was medium 199 containing 15% inactivated (56°C, 30 min.) fetal bovine serum, penicillin 100 units/ml and streptomycin 100 ug/ml. Maintenance media was Eagles basal medium (BME) containing 2% inactivated chicken serum and antibiotics. BS-C-1 cells were maintained in 250 ml bottles and passaged at 4- to 6-day intervals. Tube cultures were used 4 to 5 days after planting at which time a confluent monolayer had formed. Tubes were maintained in stationary racks at 36°C. Maintenance media was changed every third or fourth day to maintain an alkaline pH.

Titrations of infective virus were made by inoculating 0.1 ml of serial ten-fold dilutions of virus in maintenance media into each of 4 to 6 BS-C-1 tube cultures per dilution. Cultures were incubated for 9 days with maintenance media changes made when necessary to maintain the pH. Approximately 1000 ID_{50} of polio virus were added on the ninth days and the tubes examined daily for the appearance of CPE. Less than 50% CPE at the time complete destruction of the cell sheets was seen in control tubes (usually 48 hours after inoculation of the polio virus) was taken to indicate challenge virus resistance (CVR). End points were calculated by the method of Reed and Muench.

For assay of interferons, virus in infected fluids was destroyed by adjusting the pH to 2 with HCl and holding at 4° for 18 hours. The pH of the fluids was returned to 7.2 - 7.4 with NaOH and serial two-fold dilutions made in maintenance media. One ml amounts of each dilution were added to BS-C-1 tubes after removal of the media. A minimum of four tubes per dilution was used. Tubes were incubated for 24 hours at 36°C, the fluids removed and replaced with maintenance media containing 1000 ID_{50} of polio II virus. Tubes were observed for CPE and the criteria described above were used to determine the end points of interferon activity.

Sephadex G-200 was used for gel filtration experiments. The Sephadex was washed and equilibrated with 0.15M phosphate buffered saline, pH 7.3, (PBS). A column 70 x 2 cm was packed with 10g of Sephadex under gravity flow. A two ml sample of tissue culture fluid was applied to the column and elutions made with PBS. Two ml fractions were collected and the protein concentrations were determined by the method of Lowry. Antibiotics were added to the eluates and 50% PBS was added to the fractions to be tested for virus content.

To investigate the temporal relationships between virus growth, challenge virus resistance (CVR) and the production of interferon in stationary tube cultures, approximately 100 tubes were inoculated with a single dose of dengue virus. Ten tubes were randomly selected daily; media and washed cell sheets were harvested separately for determination of infectious virus and interferon content. The media was aspirated and pooled. An aliquot to be used for virus titration was mixed with an equal amount of PBS, shell frozen and stored at -70°C. The remaining media was frozen and stored similarly. Cell sheets were washed with BME, and 1 ml of BME containing 50% PBS added to each tube. Cells were agitated vigorously with fine glass beads in a Vortex mixer. The suspensions were pooled, shell frozen and stored at -70°C. Materials were thawed and centrifuged at 2000 rpm in the cold prior to titration. In addition to harvesting cells and fluids, four randomly selected tubes were challenged with 1000 ID_{50} of polio virus on each day and observed for CVR. This experiment was repeated at several different inoculum doses.

a. Relationship between virus growth, CVR and interferon production. At 24 hours following inoculation with approximately 30 InD_{50}, dengue virus was not detectable in the cells or supernatant media (Table 7). After 48 hours of incubation intracellular virus was present at low titer ($10^{1.5}$ InD_{50}/ml) and increased in a linear fashion reaching peak levels of $10^{4.5}$ InD_{50}/ml on the fourth day. Extracellular virus was first detected at a titer of $10^{0.7}$/ml on the fourth day, at or near the time when intracellular virus titers reached their maximum, and rose slowly for the following four days. In a single experiment where pH of maintenance media fell into the acid range, extracellular virus was detectable only on the fourth and fifth days, and thereafter could not be detected. In this same experiment the intracellular virus titer diminished from a peak of 10^4 InD_{50} on day five to 10^2 InD_{50} by day ten. With an inoculum of 10^4 InD_{50} virus titers rose more rapidly and the maximum level of intracellular virus was reached on the third day with a peak titer of $10^{5.5}$ InD_{50}/ml.

Time of onset of partial CVR in the dengue infected cells was approximately 48 hours following infection with 10^4 to 10^6 InD_{50} of dengue virus (Table 7). At inoculum doses of less than 10^2 InD_{50}, onset of CVR was delayed to as late as the fifth day of incubation. CVR became complete one or two days after partial CVR was apparent. Partial CVR appeared at the time that maximal concentrations of intracellular virus were attained.

Interferon reached detectable levels in the media following the time maximal intracellular virus titers were reached and partial CVR was found. Maximal levels of interferon were observed after 6 to 8 days of incubation. In other experiments interferon titers of 1:8 to 1:64 were found when CVR became complete.

Table 7 Virus Growth and Interferon Production in Dengue Infected[1] BS-C-1 Cells

Day of Incubation[2]	Intracellular Virus Titer[3]	Extracellular Virus Titer	Interferon Titer	Challenge Virus Resistance
1	0	0	0	none
2	1.5	0	0	none
3	3.0	0	0	none
4	4.5	0.7	0	partial
5	4.3	1.3	0	partial
6	4.2	2.7	1:8	complete
7	4.0	2.7	1:8	complete
8	4.5	3.5	1:16	complete
9	4.0	3.5	1:16	complete
10	4.5	3.5	1:16	complete

(1) Inoculum 1.5 Log_{10} InD_{50}.
(2) 37°C.
(3) Log_{10} InD_{50}/ml.

b. Characteristics of dengue induced BS-C-1 interferon. The interfering substance produced by the dengue infected BS-C-1 cells was identified as an interferon by determining its stability at pH 2, sensitivity to digestion by trypsin, lack of inhibition by potent anti-dengue III serum, and sedimentation characteristics by ultracentrifugation. These physical and chemical characteristics are presented in Table 8. It meets the criteria established by Isaacs and other workers for interferon. Heating to 56°C for one hour destroyed over 90% of the interferon activity and 60°C for one hour resulted in complete loss of activity. This heat lability is similar to that reported for some mouse and human interferons.

Table 8 Properties of Interferon Produced by BS-C-1 Cells Following Dengue Virus Infection

Treatment	Interferon Titer untreated	treated
Heat (56°C, 1 hr.)	1:32	1:2
Heat (60°C, 1 hr.)	1:32	0
Trypsin digestion (0.5 mg/ml, 37°C, 1 hr.)	1:64	0
Ether treatment 20%, 4°C, 18 hr.	1:32	1:8
pH 2 (4°C, 24 hrs.)	1:64	1:64
pH 1 (4°C, 24 hrs.)	1:64	0
Ultracentrifugation (100,000 x g, 4 hr.)	1:64	0
Dengue III antiserum (4°C, 18 hr.)	1:64	1:64

c. Separation of infectious virus and interferon by gel filtration. Sephadex G-200 fractionation of infected tissue culture media without concentration was found to be unsatisfactory because the initial concentration of interferon was too low to allow satisfactory determination of interferon activity of the eluted fractions. Infectious dengue virus, however, was readily recoverable from the early fractions (26-32) associated with the high molecular weight substances. Ten-fold concentration of tissue culture fluids by lyophilization and rehydration to 1/10 the original volume increased the interferon titer to 1:512. When the concentrated material was fractionated a definite separation of the infectious virus from the interferon activity was obtained. Infectivity was again found only in the early fractions (26 to 32) and the interferon activity was detected in fractions 51 through 62 in the descending portion of the low molecular weight protein peak. Quantitative recovery of the interferon activity after fractionation could not be made by this method probably because of the adsorption of partially purified interferon onto glass surfaces.

3. Plaque Assay of Dengue Viruses. A reliable system for enumeration of individual infective units of dengue viruses using cell culture-plaque techniques is currently under development. Such a system would permit detailed antigenic analysis of dengue virus strains not currently possible,

and might provide an improved method for concomitant isolation and identification of dengue viruses from field collected specimens.

a. Reagents. Routine growth of the heteroploid line BS-C-1 cells (300+ passage) is described above. For plaquing, cells were grown to confluence in stoppered 2 oz prescription bottles or in 60 mm plastic culture dishes

Viruses used were the reference dengue strains types I - IV, PR-38 and Pak-18 described above. Terminal dilution purified dengue V (Th 36, 15th mouse passage) and dengue VI (Th Sman, 15th mouse passage) viruses were also used.

The basic overlay medium contained: 80% medium 199 without phenol red, 20% fetal bovine serum (heat inactivated), sodium bicarbonate (7.5% stock) 1 ml per 100 ml final volume, DEAE dextran 100 ugm per ml, 200 units penicillin per ml, 100 ugm streptomycin per ml, 100 ugm kanamycin per ml, 0.8% agar and neutral red (in second overlay only) at 1:30000 of the total volume of the first and second overlay.

b. Procedure. Virus diluted in M199 (pH 7.5 - 7.8) with 20% FBS was adsorbed for one hour at 37°C. Monolayers were overlaid with 5 ml of agar medium and incubated for one week at 36°C. A second 3 ml overlay containing neutral red was added and incubated overnight. Plaques were measured and counted, and cultures were held at room temperature (bottles) or returned to the CO_2 36°C incubator (culture dishes) and examined at 4-7 day intervals until the cell sheets died.

c. Plaque formation. Plaques were produced successfully in 2 oz bottles when the pH was changed from acid to alkaline at 48 hours and returned to neutrality by 1 week. Continuous incubation of the cells at an alkaline pH substantially reduced longevity and plaque clarity. Overlay medium containing 0.075% $NaHCO_3$ produced larger and clearer plaques than medium with no $NaHCO_3$ or $NaHCO_3$ at concentrations of 0.15%, 0.225%, 0.3% and 0.9%. Incubation at 30°C for 1 week and holding at room temperature following staining produced plaques, whereas incubation at 32°C or holding at 36°C, 32°C or 4°C after staining did not. Cells incubated at 32°C throughout developed foci of intensely staining aggregated cells rather than plaques. Monolayers incubated at 36°C often had clear plaques in the center of which foci of live aggregated cells appeared. These foci usually faded with holding at room temperature and often disappeared after one week. Cells held at a constant pH in the CO_2 incubator survived very well but failed to produce plaques.

In an attempt to assay the effect of cell density and age on plaque formation, a single lot of cells were grown in 2 oz bottles and plaqued for four consecutive days, beginning when the monolayers became confluent. All groups contained plaques with aggregated central foci. Plaque clarity

improved and central foci disappeared with holding at room temperature after staining. Only the most dense (oldest) monolayers produced plaques clear enough to count within 24 hours after staining (Table 9). Less dense monolayers appeared to have slightly larger plaques following clearing at room temperature.

Table 9 Size and Clarity of Dengue III (Pak-18) Plaques in Cells Grown 0-3 Days (Age) Beyond Formation of a Confluent Monolayer, 1-15 Days after Staining

	Days Post Staining		
Age	1	8	15
0	None	1.0 - 1.5 mm (clear)	1.5 - 2.5 mm (very clear)
1	1 mm (vague)	1.0 - 2.0 mm (vague to clear)	1.0 - 2.0 mm (very clear)
2	1.0 - 1.5 mm (fairly clear)	1.0 - 1.5 mm (clear)	1.0 - 1.5 mm (very clear)
3	1.0 - 1.5 mm (clear)	1.0 - 1.5 mm (clear)	1.5 - 2.0 mm (very clear)

Mouse adapted dengue I through VI viruses and dengue III tissue culture strains were plaqued utilizing 4-day-old cell sheets. Although the pH and cell survival was not optimal in this experiment, all types produced plaques of variable size and clarity.

Dengue virus replication in fluid medium BS-C-1 cell cultures is associated with production of an interferon. In an attempt to produce larger, more uniform and consistant plaques, monolayers were treated with actinomycin D, an inhibitor of interferon but not RNA virus synthesis. Cells were incubated at 36°C with 1, 0.75 and 0.5 ugm of actinomycin D per ml of medium for 24 hours prior to virus adsorption. The corresponding concentration of actinomycin D was included in the overlay medium of half the cells tested. Dengue III Pak-18 strain was plaqued both by the acid to alkaline shift procedure and by maintenance at a constant pH in the CO_2 incubator. The acid to alkaline monolayers produced variable results. Cells in the CO_2 incubator, however, developed highly uniform plaques in this and subsequent trials (Table 10). With cultures held in the CO_2 incubator, increasing concentrations of actinomycin D prior to virus adsorption resulted in smaller but more clearly defined plaques. When actinomycin D was incorporated into the overlay medium as well, the plaques were very small (0.5 mm) but extremely sharply defined immediately following staining.

Table 10 Plaque Size and Clarity Following 24-hour Cell Treatment with Actinomycin D Prior to Infection, and Pretreatment Plus Incorporation into the Overlay Medium. Incubation at 36°C at Constant Neutral pH

	Days Post Staining	
Actinomycin D	1	5
Pretreatment only at:		
1 ugm/ml	1.5 - 2.0 mm (vague)	1.5 - 3.0 mm (very clear)
0.75 ugm/ml	1.0 - 3.0 mm	3 - 4 (very clear)
0.5 ugm/ml	3 - 4 mm (vague)	3 - 4 (fairly clear)
Pretreat. + Overlay at:		
1 ugm/ml	Dead - Toxic	
0.75 ugm/ml	0.5 mm (very clear)	Dead - Toxic
0.5 ugm/ml	0.5 - 1 mm (very clear)	Dead - Toxic

Heparin in concentrations in excess of 200 ugm per ml has been reported to markedly increase the plaque size of some strains of EMC virus. In a preliminary trial, incorporation of heparin in the overlay medium in place of DEAE dextran significantly increased dengue plaque size with both the acid to alkaline shift procedure and with the constant pH of the CO_2 incubator (Table 11). Larger plaques were produced with heparin at 250 and 500 ugm per ml than with 750 and 1000 ugm per ml. In CO_2 incubated monolayers, plaque size was inversely proportional to clarity. Heparin treatment shortened cell survival; most monolayers survived only 7 - 9 days following staining.

The largest plaques were produced by a combination of 24-hour pretreatment of monolayers with 0.75 ugm per ml of actinomycin D and incorporation of 500 ugm per ml of heparin into the overlay medium. By day 12 very clear plaques 1-3 mm in size in the bottles containing 700 - 7000 plaques and 3-7 mm in size in bottles containing 7 - 70 plaques had formed. This same phenomenon of larger plaques at lower virus concentrations was also seen in the CO_2 incubated 500 ugm heparin treated cells.

Table 11 Dengue III (Pak-18) Plaque Size and Clarity at Varying Concentrations of Heparin at a Changing (acid to alkaline) and Constant (neutral) pH

pH	Heparin Concentration/ml	Plaque Size (Clarity)	Days Post Staining
Acid-Alk.	1000	0.5-2.0 mm (clear)	1
	750	2-3 mm (clear)	1
	500	0.5-5 mm (clear)	1
	250	1-4 mm (clear)	1
Constant	1000	0.5-1.5 mm (clear)	1
	750	1-2 mm (clear)	1
	500	1-5 mm (fairly clear)	1
	250	2-5 mm (vague)	1
	500*	1-7 mm (clear)	4

* Plus pretreatment with 0.75 ugm actinomycin D 24 hours prior to plaquing.

Attempts to differentiate dengue I and III virus by the Porterfield bead plaque neutralization test utilizing anti-dengue I through IV hyperimmune ascitic fluid were unsuccessful. Transitory zones of virus neutralization appeared around the anti-dengue I and III beads but were not unlike enough to differentiate the homologous and heterologous virus-antibody systems.

Attempts at dengue plaquing by a modification of the Cooper agar suspended cell system have been unsuccessful with both normal and actinomycin D pretreated BS-C-1 cells. A trial with these suspended cells and type II polio virus has shown that the cell concentration used with the dengue plaque attempts were adequate to support good plaque formation.

Attempts at plaquing dengue III virus in rabbit kidney, mouse embryo, and rhesus monkey kidney primary cells have been unsuccessful.

4. Ecology of Chincoteague-Assateague Arboviruses. This report continues description of studies of Group A and Bunyamwera group viruses encountered in the Chincoteague-Assateague areas commenced in 1961.

Earlier annual reports describe in detail the study areas, its fauna, and various arbovirus infections observed.

a. Identification of recovered viruses. Final identification of viruses recovered in 1961-1963 are currently in progress. Some viruses appear to be antigenically closely related to Cache Valley and Tensaw (Bunyamwera group) by the hemagglutination-inhibition (HI) test (Table 12) and neutralization tests (Table 13). Results of HI tests indicate a close relationship between M273 virus, the 1961 prototype, and Cache Valley (6V-633) virus. However, cross neutralization tests show that there is a closer relationship of M273 virus to Tensaw virus than to Cache Valley (6V-633) virus. Because reference antisera had neutralization indices higher than test virus titers, serum neutralization endpoints are being determined to further clarify these antigenic relationships.

Table 12 Cross Hemagglutination-Inhibition Tests With M273 Virus, Cache Valley BeAr 7272 Virus, Cache Valley 6V-633 Virus and Tensaw Virus

	Antigen							
	M273		CV 6V-633		CV BeAr 7272		Tensaw	
Antisera	2*	4*	2	4	2	4	2	4
M273	80	40	20	20	30	NT**	0	0
CV 6V-633	40	20	20	20	20	20	20	0
CV BeAr 7272	20	NT	0	0	40	NT	NT	NT
Tensaw	160	80	0	0	40	NT	320	160

* Units Antigen.
** NT = Not tested.

Table 13 Cross Neutralization Tests (log_{10} of neutralization index) With M273 Virus, Tensaw Virus, Cache Valley 6V-633 Virus and Cache Valley BeAr 7272 Virus

	Virus			
Antiserum	M273	Tensaw	CV 6V-633	CV BeAr 7272
M273	≥4.5	≥4.7		
	≥5.3		≥5.7	3.0
Tensaw	≥4.5	≥4.7		
CV 6V-633	3.5		6.5	
CV BeAr 7272	1.8			3.0

The antigenic relationship between three of the four viruses recovered in 1962 is shown in Table 14. Cross neutralization tests with the 1962 isolates are currently in progress. Preliminary tests indicate that the virus M1014 is identical with California encephalitis (CE) virus. This is the first isolate of CE virus reported from the State of Maryland.

Table 14 Cross Complement-Fixation Tests, 1962 Isolates

	Antigens			
Serum	M 784	M 875	M 1014	M 1035
M 784	128/128*	128/128	128/128	32/4
M 875	128/128	128/256	128/128	32/4
M 1014			256/128	256/4
M 1035			4/4	4/4

* Serum titer/antigen titer

During summer - fall 1964 twelve virus isolations were made from mosquitoes from the Pocomoke Cypress Swamp in Maryland and three from mosquitoes collected on Chincoteague and Assateague Islands, Virginia (Tables 15 and 16). Of the greatest significance is the isolation of eastern (EEE) and western (WEE) equine encephalitis viruses from Culiseta melanura mosquitoes collected in the Pocomoke Cypress Swamp. This is the first isolate of WEE virus from the State of Maryland.

b. Ecological studies. Isolation of EEE and WEE viruses from mosquitoes prompted a cooperative ecological study of vertebrates in the Cypress Swamp area with Dr. M. J. Collins, Jr. of the Department of Veterinary Science, University of Maryland. The objectives of the study were to determine whether vertebrate species in the Cypress Swamp are infected and, if so, to learn how they might be involved in the maintenance and possible dissemination of EEE or WEE virus. Preliminary studies determined relative numbers of the various vertebrate species present and how they might best be adequately sampled to determine their role in virus maintenance. Trapping efforts were carried out in the Swamp from September through February. A series of transect trap lines were established in various kinds of habitat. Aerial photographs (color 9" x 9" paired stereo transparencies) of the swamp were taken by Mr. Robert C. Heller and staff of the Forest Insects Laboratory, U. S. Forest Service, National Agricultural Research Center, Beltsville, Md. Photogrammetric vegetative analysis of the swamp was made. Relative

Table 15 Virus Isolations From Mosquitoes Collected During 1964

Species	Total specimens	No. pools tested	No. isolations
Pocomoke Cypress Swamp, Md.			
Aedes canadensis	375	9	0
Aedes sollicitans	500	8	0
Aedes taeniorhynchus	325	7	0
Aedes vexans	1025	26	0
Anopheles bradleyi-crucians	450	15	0
Culex salinarius	1525	31	0
Culiseta melanura	28,550	320	12
Uranotaenia sapphirina	75	3	0
Subtotal	32,825	401	12
Assateague & Chincoteague Islands, Va.			
Aedes cantator	25	1	0
Aedes sollicitans	5100	57	0
Aedes taeniorhynchus	4725	60	2
Aedes vexans	75	3	0
Anopheles bradleyi-crucians	75	3	0
Anopheles quadrimaculatus	400	16	1
Culex salinarius	5075	62	0
Subtotal	15,475	202	3
Total	48,300	603	15

Table 16 Source Identity and Location of 1964 Mosquito Isolates

Isolate	Identity	Date Collected	Mosquito Source	Location
M 2248	EEE[1]	1 Sep	Culiseta melanura	Swamp[3]
M 2249	EEE	1 Sep	" "	"
M 2587	WEE[2]	30 Sep	" "	"
M 2216	Unk.	10-14 Jul	" "	"
M 2278	Unk.	28 Jul	" "	"
M 2279	Unk.	28 Jul	" "	"
M 2281	Unk.	24 Jul	" "	"
M 2370	Unk.	13 Aug	" "	"
M 2420	Unk.	25 Aug	" "	"
M 2390	Unk.	13-18 Aug	" "	"
M 2467	Unk.	27 Aug	" "	"
M 2514	Unk.	10 Sep	" "	"
M 2410	Cache Valley-like	24 Aug	Aedes taeniorhynchus	Marsh[4]
M 2522	Cache Valley-like	27 Aug-10 Sep	Aedes taeniorhynchus	"
M 2525	Unk.	27 Aug-10 Sep	Anopheles quadramaculatus	"

(1) Eastern equine encephalomyelitis virus
(2) Western equine encephalomyelitis virus
(3) Cypress Swamp, Maryland
(4) Assateague, Virginia

trapping success was correlated with habitat types on the basis of vegetation. A sixty-acre gridded study area was established in the Swamp where trapping success was expected to be greatest. On this 60-acre study area a 1-chain (66 feet) interval grid was established.

Trapping on the 60-acre area began in March and is continuing. As in the preliminary trapping, animals are captured alive, weight, hind foot length, sex, and reproductive status recorded; blood drawn, the animals marked and released at the point of capture. HI antibody to EEE virus has been found in only one mouse, raccoon and turtle (Table 17). No HI antibody to WEE virus has been found. A base-line has been established so that during the 1965 mosquito season, increases in infection rates in several small and medium size animal populations can be detected.

Table 17 Hemagglutination-Inhibition Tests with Eastern Equine Encephalomyelitis Antigen and Sera from Wild Vertebrates Captured in the Pocomoke Cypress Swamp, Maryland

Binomial (Common Name)	No. Pos./No. Tested	Titer
Ageliaus phoeniceus (red-winged blackbird)	0/1	
Blarina brevicauda (short-tailed shrew)	0/3	
Cryptotis parva (least shrew)	0/1	
Chrysemys picta (painted turtle)	0/1	
Clemmys guttata (spotted turtle)	0/4	
Cyanocitta cristata (blue jay)	0/2	
Didelphis virginiana (opossum)	0/5	
Elaphne obsoleta (rat snake)	0/1	
Mephitis mephitis (striped skunk)	0/1	
Microtus pennsylvanicus (meadow vole)	0/3	
Mus. musculus (house mouse)	0/2	
Peromyscus leocopis (deer mouse)	1/126	1:80
Procyon lotor (raccoon)	1/3	1:20
Sciurius carolinensis (grey squirrel)	0/3	
Sorex cinereus (masked shrew)	0/6	
Terrapene carolina (E. box turtle)	1/1	1:80

c. Cache Valley-like virus in infections of cattle. Multiple isolations of Cache Valley-like viruses have been made from salt-marsh mosquitoes in 1961, 1963 and 1964. Preliminary serological surveys indicated a high attack rate among domestic ungulates in tidewater areas. This finding prompted a rather intensive serological survey of dairy cattle in Tidewater Maryland. Neutralization tests (BS-C-1 metabolic inhibition tests) were carried out on 1,181 cattle sera. HI tests were carried out on 281 of these same sera. The 1961 prototype Cache Valley-like virus, M273, was the test antigen for both tests. A high rate (85.2%) of neutralizing antibody and a lower rate (40%) of HI antibody was found (Table 18). Increasing prevalence of neutralizing antibody with age (Table 19) indicated a fairly constant rate of exposure, with half the animals two years of age or less being positive. These data suggest a continuing enzootic exposure of dairy cattle on the Eastern Shore rather than an epizootic infection which converted a large number of animals from serologically negative to

Table 18 Antibody to Cache Valley-like Viruses in Eastern Shore Cattle

	Serological Test	
	NT(1)	HI(2)
No. Tested	1181	281
% Positive(3)	85.2	40
Titer Range (Pos. only)	1.8 - ≥5.0(4)	20 - 64(5)
Geo. Mean Titer (Pos. only)	≥4.53(4)	40(5)

(1) Virus neutralization test.
(2) Hemagglutination-inhibition test.
(3) NT Pos = neutralization of 1.8 - 2.5 logs BS-C-1 cell LD_{50} virus. HI Pos - inhibition of 2 or 4 units of antigen by serum diluted ≥1:20.
(4) Log_{10}.
(5) Reciprocal.

positive in a comparatively short period of time. The discrepancy in the percentage of positive animals with the neutralization test versus the hemagglutination-inhibition test may be explained by the relative insensitivity of the HI test since animals having low neutralization indices have no detectable HI antibodies (Table 20).

As with any serologic survey, the specificity of the serologic reaction observed must be demonstrated. This was done with a pool of

Table 19 Eastern Shore Dairy Cattle
Prevalence of neutralizing antibody to M273 virus by age

Age(years)	No. Pos.*/No. Tested	% Pos.
2 or less	44/78	56
3	123/162	76
4	135/147	92
5	117/127	92
6	82/89	92
7	106/112	89
8	53/57	93
9	15/15	100
10 or over	46/46	100

* LNI ≥1.8

Table 20 Comparison of Log Neutralization Indices (LNI)
With the Range and Geometric Means of the Hemagglutination-Inhibition
(HI) Titer of Tidewater Maryland Dairy Cattle Sera Tested
Against M273 Virus

LNI Interval	No. of Sera	HI Titer	
		Range	Geo. Mean
≤2.0	3	≤8	≤8
2.1-3.0	1	-	≤15
3.1-4.0	4	8-34	19
4.1-5.0	1	-	8
>5.0	34	15-640	75

5 HI and neutralization test positive cattle sera by physiochemical means. Sera were subjected to starch block electrophoresis and fractions tested for virus neutralizing properties. These data (Table 21) show that

neutralizing properties of the serum were present in the gamma globulin fraction; this is interpreted as evidence of gamma globulin antibody resulting from natural infection of cows with M273 virus or a virus closely related to it.

Table 21 Zone Electrophoresis of 5 Pooled M273 Virus Neutralizing Cattle Sera. Comparison of Protein Concentration (Lowery Method), Virus Neutralizing Titer (Reciprocal) and Identification of the Gamma Globulin Region by Immunoelectrophoresis

Fraction No.	Protein Concentration	Neut. Titer	Gamma Globulin
2-6	0	0	-
7	0.17	0	-
9	0.56	3	+
11	0.89	12	+
13	1.04	12	+
15	1.01	6	+
17	1.11	12	+
19	0.80	6	+
21	0.43	Undil.	+
23	0.45	Undil.	+
25	0.52	0	-
27	0.63	0	-
29	0.32	0	-
31	1.13	0	-
33	1.14	0	-
35	0.08	0	-

The pathogenesis of M273 virus in cattle is unknown. An attempt was made to determine (1) if cattle could be infected with M273 virus and serve as source of virus for mosquitoes (2) if infection of cattle with M273 virus resulted in overt disease. Two groups of three 5-month-old calves housed in isolation facilities (Department of Veterinary Science, University of Maryland) were exposed to 13th suckling mouse passage virus. One group received 10^6 BS-C-1 cell LD_{50} of virus subcutaneously and the second group was given 100 BS-C-1 cell LD_{50} of virus intravenously. Blood, nasal swabs and rectal swabs were taken daily for 5 days. Aedes taeniorhynchus and Aedes aegypti mosquitoes were fed daily on each calf for 4 days. Temperatures and general condition of the calves were noted each day. M273 virus was not recovered from blood, nasal swabs, rectal swabs, or mosquitoes held 16-20 days post feeding. Temperatures of 101°F - 103°F were noted in animals having a shipping fever-like upper respiratory tract infection. Failure to isolate M273 virus from these animals suggests some other etiologic agent was responsible for the disease. Tests for antibody response to inoculated virus are as yet incomplete.

d. Transmission and infection of mosquitoes with Cache Valley-like viruses. Cache Valley-like viruses have been isolated five times from Aedes taeniorhynchus since 1961. It is not known if these isolations represent virus present in blood meals or infection of the mosquitoes. Laboratory experiments with Aedes taeniorhynchus and M273 are being cooperatively undertaken with Dr. P. H. Thompson of the Department of Entomology, University of Maryland. Objectives of this study are to determine (1) if infection of Aedes taeniorhynchus occurs, (2) the ID_{50} and minimum infectious virus dose, (3) the ability of this mosquito to transmit virus to 1-day-old suckling mice 15 days post feeding, and (4) the extrinsic incubation period. Aedes taeniorhynchus females have been fed on various dilutions of virus in defibrinated rabbit blood. Both a membrane feeding chamber (Annual Report 3A 0145 01B71 P 03035) and blood-soaked cotton were used. Fifteen days after the infected blood meal mosquitoes were fed upon 1-day-old suckling mice and then titurated and the suspension inoculated into BS-C-1 cells. Seven or eight mosquitoes ingesting 20 BS-C-1 cell LD_{50} of virus, the lowest concentration used, contained virus 15 days after the infectious blood meal. A 500-fold virus increase occurred in the one mosquito for which virus assay has been completed. Deaths occurred in mice bitten by mosquitoes which had ingested 20 to 2,000 BS-C-1 LD_{50} of virus. M273 virus isolation attempts from these mice are underway.

5. The Nature of Antiviral Antibodies. Physiochemical characterization of human and animal antibodies has been continued with the aim of identifying the biologically significant immunoglobulins, the kinetics of their production, and their behavior when in purified form.

a. Passively protecting antiviral antibody in guinea pigs. Having established the fact that guinea pigs develop neutralizing antibody associated with two major classes of gamma globulin following experimental arbovirus infection, studies were undertaken to investigate the passive protection afforded susceptible mice to challenge with RSSE virus by isolated gamma M- and gamma G-immunoglobulin fractions prepared from sera of RSSE immune guinea pigs. This virus-host system was used because RSSE is highly lethal for mice following extraneural inoculation.

Twenty susceptible 250 to 300g guinea pigs (Walter Reed strain) were infected by a single 0.3cc subcutaneous inoculation of approximately 10^6 mouse intracerebral (i.c.) LD_{50} of RSSE virus (Moscow, B-4 strain). Serum samples were obtained before inoculation and serially thereafter and neutralizing antibody titers determined on them. Preliminary analyses of these serum samples by sucrose density gradient ultracentrifugation were made to determine which possessed maximal quantities of neutralizing antibody of the gamma-M and gamma G-immunoglobulin type. Specimens obtained 11 days after infection were found to contain gamma M-immunoglobulin antibody exclusively; those at 29 days or later, exclusively gamma G-immunoglobulin antibody. Selected sera were subjected to zone electrophoresis and gel filtration with Sephadex G-200, neutralizing antibodies

to RSSE virus were measured on each fraction. Fractions containing N antibody were pooled and concentrated 4- to 5-fold by Carbowax. The concentrates were reassayed for N antibody, and the sedimentation properties of the active principle analyzed by partition cell in the Model E analytical ultracentrifuge and by sucrose density gradient ultracentrifugation.

Passive protection tests were performed on these concentrated pools as follows. Serial 4-fold dilutions of unheated pooled concentrates ranging from 1:2 to 1:1024 were prepared in 0.15 M NaCl; 0.1 ml of each dilution was inoculated subcutaneously into 10 mice. Four hours later, the mice were challenged intraperitoneally (i.p.) with 10 to 100 LD_{50} of RSSE virus. Titers were expressed as the highest dilution of serum which protected 50% of the mice against the virus.

The results of representative studies are given in Tables 22 and 23. Concentrated pools of fractions of 11-day sera obtained by both fractionation methods possessed neutralizing antibody in low titer (1:4 (#955, #958) to 1:8 (#956)). Neutralizing antibody was detected only in the lower compartment of the partition cell and in the bottom portion (fractions 1-4) of the sucrose gradient. No detectable protection was afforded mice by these pools.

Concentrated pools of fractions of 29- and 40-day sera possessed neutralizing antibody titering from 1:10 (#955) to 1:25 (#960). Neutralizing antibody was found in both upper and lower compartments of the partition cell and in the top of the sucrose gradient (fraction 6-8). These pools titered from 1:8 to 1:20 in the passive protection test protecting mice from fatal challenge infection. Even though the concentrated pools of fractions in both early and late sera contained comparable amounts of N antibody, passively protecting antibodies were associated with the gamma G-immunoglobulins found only in the late sera.

The data suggest that gamma M-immunoglobulin, shown to contain neutralizing antibody by a standard <u>in vitro</u> neutralization test, does not protect passively, whereas gamma G-immunoglobulin antibody, even in low titer, affords significant passive protection. These studies will be extended to other viral agents of military significance.

b. <u>Antibody induced in man by Yellow Fever vaccine</u>. An effort has been made to define mechanisms governing the sequential appearance of antiviral antibody in the human. One of the main objectives in this connection is to determine whether this sequential appearance of antibody is influenced by the nature of the virus; i.e. virulent, attenuated, or inactivated. The most productive approach to date in the human has been through the study of the antibody response following immunization with several types of viral vaccines. Immunization with the Yellow Fever vaccine has proven to be a most useful model for the study of the response of the human to a living attenuated viral vaccine.

Table 22 Antibody Activity of Concentrates
Obtained by Zone Electrophoresis of Serum

Animal No.	Days Post Infection	Neutralizing Antibody: Titer of Untreated Serum	Titer of Pooled Fractions	Titer of Partition Cell Compartments: Upper	Lower	Sucrose Density Gradient** Tube No. 1 2 3 4 5 6 7 8 9 10	Passively Protecting Antibody Titer*
956	11	1:16	1:8	0	1:8	0 0 + + 0 0 0 0 0 0	<1:2
955	11	1:10	1:4	0	1:5	0 0 + 0 0 0 0 0 0 0	<1:2
	29	1:1024	1:10	1:10	1:15	0 0 0 0 0 + + 0 0 0	1:8

* Measured against 3.2 to 50 LD_{50} of RSSE virus

** + = Presence of antibody
0 = Absence of antibody

Table 23 Antibody Activity of Concentrates
Obtained by Gel Filtration with Sephadex G-200

Animal No.	Days Post Infection	Neutralizing Antibody: Titer of Untreated Serum	Titer of Pooled Fractions	Titer of Partition Cell Compartments: Upper	Lower	Sucrose Density Gradient** Tube No. 1 2 3 4 5 6 7 8 9 10	Passively Protecting Antibody Titer*
958	11	1:64	1:4	0	1:4	+ 0 0 0 0 0 0 0 0 0	<1:2
960	29	1:50	1:25	1:16	1:128	0 0 0 0 0 + + + 0 0	1:8
955	40	1:500	1:16	1:8	1:32	0 0 0 0 0 + + 0 0 0	1:20

* Measured against 7.9 to 79 LD_{50} of RSSE virus

** + = Presence of antibody
0 = Absence of antibody

A group of six seronegative adult subjects were immunized with the 17D strain of Yellow Fever vaccine and bled at weekly intervals for a period of 16 weeks. Neutralizing antibody was determined on each serum specimen; those found to contain antibody were fractionated by sucrose density ultracentrifugation and Sephadex G-200 and antibody determined on the fractions.

Data obtained thus far are summarized in Table 24. The earliest detectable neutralizing activity was found at 14 days and was found in the lower portion of the gradient indicating it to be a 19S gamma M-immunoglobulin. Antibody of the gamma G type usually made its appearance by the 28th day. Of interest is the finding of 19S as well as 7S activity in sera obtained as late as 62 days following immunization. Although the significance of these observations is not established, they suggest that unlike the responses seen following other types of viral vaccines (see below) the sequential appearance of antibody in response to this form of attenuated vaccine leads to a more heterogeneous antibody response with persistence of macroglobulin antibody. These observations are being extended to include a comparison of the complement-fixing and hemagglutination inhibition antibody responses following immunization. The results of these studies will be compared with those obtained from the guinea pig studies.

Table 24 Results of Fractionation of Sera from Young Adults Immunized with Yellow Fever Vaccine

Subject	Interval after immunization (days)	Untreated Serum Titer	Antibody in Sucrose Density Gradient Tube No. 1	2	3	4	5	6	7	8	9	10
L.O.	14	1:125	0	0	±	0	0	0	0	0	0	0
	21	1:200	0	0	+	0	0	0	0	0	0	0
	28	1:625	0	+	+	+	+	+	+	0	0	0
	42	1:625	0	+	+	+	+	+	+	0	0	0
	62	1:625	0	±	+	+	+	+	+	0	0	0
J.S.	14	1:625	0	+	+	+	+	0	0	0	0	0
	21	1:625	0	+	+	+	+	0	0	0	0	0
	28	1:625	0	+	+	+	+	±	+	0	0	0
S.N.	14	1:25	0	+	0	0	0	0	0	0	0	0
	21	1:25	0	+	+	+	+	0	0	0	0	0
	28	1:1000	0	±	+	+	0	0	0	0	0	0
	42	1:800	0	0	+	+	0	+	+	+	0	0
	62	1:500	0	0	+	+	0	+	+	+	0	0

c. Antibodies formed following adenovirus infection of man. Over the past year a number of recruits at Fort Dix, New Jersey, with adenovirus respiratory disease have been studied in regard to the molecular varieties of neutralizing antibody formed in response to natural infection. All had received "Resprogen" vaccine on entry to the post. This vaccine contains adenovirus types 3 and 7 antigens.

Adenovirus type 4 infection was established in each patient by virus isolation and neutralizing antibody increase. Results of neutralizing antibody determinations on serum fractions are presented in Table 25. The results indicate an early macroglobulin antibody response followed by development of gamma G antibody within the 7-14 day. In many patients gamma M antibody persisted in the final sera obtained at 55-58 days.

Table 25 28 Adenovirus Type 4 Neutralizing Antibodies in Fractions of Sera from Recruits

Subject	Interval after onset of illness (Days)	Untreated Serum Titer	Antibody in Sucrose Density Gradient Tube No. 1	2	3	4	5	6	7	8	9	10
#208	3	5										
	11	20	0	0	+	+	±	+	0	0	0	0
	18	40	0	±	+	0	+	+	+	+	0	0
	25	80	0	+	+	+	+	+	+	0	0	0
	33	80	0	0	+	+	+	+	+	+	0	0
	39	40	0	0	+	+	+	+	+	+	0	0
	46	80	0	0	+	+	+	+	+	+	0	0
	53		0	0	+	+	+	+	+	+	0	0
#203	5	5										
	13	20	0	±	+	+	+	0	0	0	0	0
	20	20	0	0	+	+	+	+	+	0	0	0
	26	20	0	+	+	+	+	+	+	0	0	0
	35	20	0	±	+	+	+	+	+	0	0	0
	55		0	0	+	+	+	+	+	0	0	0
#207	7	5										
	22	20	0	0	±	+	0	+	+	0	0	0
	26	40	0	+	+	+	+	+	+	0	0	0
	51	40	0	0	+	+	+	+	+	+	0	0
	58	40	0	0	+	+	+	+	+	+	±	0
#103	1	5										
	8	20	0	0	0	0	0	0	±	0	0	0
	17	80	0	0	+	±	±	±	+	0	0	0
	24	80	0	+	+	+	0	+	+	0	0	0
#123	1	5										
	23	80	0	+	+	+	+	+	0	0	0	0
#138	1	20	0	0	+	+	+	+	+	0	0	0
	6	40	0	+	+	+	+	+	+	0	0	0
	9	160	0	+	+	+	+	+	+	0	0	0
	15	160	0	0	+	+	0	+	+	0	0	0
#146	4	5										
	7	20	0	0	0	±	+	+	+	0	0	0
	13	40	0	0	+	+	+	+	+	0	0	0
#147	7	5										
	12	20	0	0	0	0	+	+	+	+	0	0
	15	80	0	0	0	0	+	+	+	+	0	0
	55	80	0	0	0	0	0	+	+	+	0	0
#151	1	5										
	14	80	0	0	0	+	+	0	0	0	0	0
	21	160	0	+	±	0	0	0	0	0	0	0

6. Antibodies to P. tularensis in Nasal Secretions and Serum. Previous studies from this laboratory have identified the virus neutralizing antibody in respiratory secretions as a gamma A-immunoglobulin. These and other studies have suggested that this antibody, found also in other external secretions, is different from that of serum antibody and may provide the host an important local defense mechanism against otherwise invasive organisms. In order to assess the extent of this phenomenon as a defense factor to the airborne route of infection, these studies have been extended to bacterial systems. Persons recovering from tularemia or vaccinated against it contain in their sera antibody to the polysaccharide of P. tularensis. In the present studies, the physicochemical properties of these antibodies were examined in both the respiratory secretions and sera of a group of immunized and non-immunized volunteers challenged with viable P. tularensis, in order to assess: (1) whether local antibody can be detected in secretions following immunization or infection, (2) if such antibody appears, whether it differs in its physicochemical properties from serum antibody, (3) the role of local antibody in immunity to P. tularensis. The study was done in collaboration with the staff, Medical Unit, Fort Detrick, Maryland.

a. Experimental design and techniques. A group of 14 volunteers comprised of 8 individuals immunized percutaneously 3 months previously with an attenuated tularemia vaccine and 6 non-vaccinated controls were studied. One half of the group consisting of 4 vaccinates and 3 controls were exposed to an aerosol containing 2500 viable P. tularensis organisms; the second half similarly comprised, were exposed to an aerosol containing 25,000 viable organisms. Nasal secretions were collected and processed by methods described previously. Specimens were obtained daily for 3 days prior to challenge and daily for 5 days after challenge and at weekly intervals thereafter. The men were also bled prior to challenge and again at weekly intervals thereafter.

A polysaccharide fraction, extracted from Schu S4 strain organisms as described by Alexander, was used to sensitize sheep erythrocytes. Erythrocytes, maintained in Alsever's solution, were washed two times with 0.15 M saline and suspended at 5% concentration in 0.005% polysaccharide solution. The red blood cells were incubated at 37°C for 4 hours and then washed four times before they were diluted with saline to 0.5% concentration for use. Nasal secretions and sera were absorbed with unsensitized sheep red blood cells to remove non-specific agglutinins and were heated at 56°C for 30 min prior to testing. The test was performed in a microtiter using 0.05 ml of serum or nasal wash dilution and 0.05 ml of sensitized sheep erythrocytes. The test was allowed to stand overnight at 4°C and titers were recorded as the highest dilution of serum or nasal wash that produced an agglutination pattern in which all the erythrocytes remained as a uniform coating on the bottom of the cup after settling. Gel filtration with Sephadex G-200 of sera and nasal secretions and immunoelectrophoresis were performed by methods described previously.

b. Immunoelectrophoresis of secretions. Albumin, α-globulin and γA-globulin were found in all cases. γG-immunoglobulins, though present in most secretions, were found in highest concentration for 7 to 14 days following challenge, then fell to lower levels. The γM-immunoglobulins have not been detected in any of the secretions.

c. Relationship of hemagglutination titer in serum and nasal secretions. The results of hemagglutination titers in serum and nasal secretions are given in Tables 26 and 27. It will be observed that in each individual who had been immunized 3 months previously, antibody was found both in serum and nasal secretions. In the non-vaccinated controls, neither serum nor nasal antibody could be detected. Following challenge, the earliest detectable serum antibody was found on day 7, at which time nasal antibody first made its appearance.

d. Physicochemical studies of serum and nasal antibody. Selected specimens were subjected to fractionation by gel filtration with Sephadex G-200. The data thus far reveals that the serum antibody to P. tularensis is associated for the greater part with the 19S γM-immunoglobulins in all specimens so far studied which have included pre-challenge and post challenge specimens from both vaccinates and non-vaccinates. On the other hand, hemagglutinating antibody in the nasal secretions is associated only with the γA-immunoglobulins found in these secretions. Preliminary characterization of this sedimentation constant of this γA-immunoglobulin suggests it to be on the order of a 13S moiety.

e. Significance of observations. The present findings suggest that the tularensis hemagglutinating antibody in nasal secretions is associated with a γA-immunoglobulin with properties suggesting a larger molecule than that of serum γA-immunoglobulins which have been shown to be closer to 7S. These data are consistent with the estimates of colostral and parotid γA-immunoglobulins which have been shown by Tomasi to be slightly different immunochemically from serum A-immunoglobulin. Of interest is the finding of 19S antibody in the serum at the same time γA-globulin associated antibody is found in nasal secretions. Still unanswered at the present time is the origin of the γA-globulin antibody in secretions.

Table 26 Hemagglutination titers in serum and nasal washes (NW) in volunteers challenged with 2500 P. tularensis organisms

Day	Vaccinates								Non-vaccinates					
	Arnold		Bishop		Faber		Wisdom		Benfer		Fleming		Lance	
	Serum titer	NW titer	Serum titer	NW titer	Serum titer	NW titer	Serum titer	NW titer	Serum titer	NW titer	Serum titer	NW titer	Serum titer	NW titer
-4	1:256		1:8		1:512		1:1024		0		0		0	
-3		1:2		1:2		1:8		1:4		0		0		0
1		1:8		1:2		1:4 (dy 2)		1:16		0		0		0
3		1:8		1:2		1:4		1:16		0		0		1:2
7	1:128	1:8	1:8	1:4	1:256	1:8	1:512	1:8	0	1:2	1:2	0	1:2	1:4
14	1:64	1:8	1:128	1:2	1:512	1:2	1:512	1:4	1:256	1:16	1:32	1:2	1:32	1:8
21	1:256	1:8	1:1024	1:2	1:256	1:16	1:2048	1:2	1:1024	1:32	≥4096	1:128	1:512	1:32
42	1:128	1:4	≥4096		1:512		1:1024				≥512		1:128	

Table 27 Hemagglutination titers in serum and nasal washes (NW) in volunteers challenged with 25,000 *P. tularensis* organisms

Day	Vaccinates						Day		Non-Vaccinates					
	Foote		Bermingham		Wilson				Fernandez		Herd		Maxwell	
	Serum titer	NW titer	Serum titer	NW titer	Serum titer	NW titer	Serum titer	NW titer	Serum titer	NW titer	Serum titer	NW titer	Serum titer	NW titer
-4	1:512		1:1024		1:2048		1:256		0		0		0	
-3		1:2		1:16		1:8		1:32		0		0		0
1		1:2		1:8		1:8		1:16		0		0		0
3		1:2		1:16		1:16		1:16		0		0		0
7	1:512	1:2	1:128	1:16	1:1024	1:8	1:512	1:4	0	0	0	0	0	0
14	1:512	1:2	1:1024	1:16	1:1024	1:16	1:512	1:8	1:64	1:8	1:64	1:4	1:1024	1:64
21	1:512	1:2	1:512	1:32	1:4096	1:8	1:1024	1:4	1:128	1:8	1:512	1:16	≥4096	1:128
42	1:512	1:2	1:2048	1:8	1:2048	1:4	1:512	1:8	1:256	1:16	1:512	1:8	1:4096	1:16

7. Virologic and Physicochemical Studies of the Congenital Rubella Syndrome. Recently, with the development of techniques for the propagation of rubella virus in the laboratory, the virologic study of the congenital rubella syndrome has been possible. Following the 1964 epidemic of rubella, a number of cases of infants born to mothers exposed to rubella in the first trimester of pregnancy were seen on the Pediatric Service at Georgetown University Hospital. The main clinical manifestations in 7 infants who were studied included: (1) Purpura, (2) Cataracts, (3) Hepatosplenomegaly, (4) Congenital heart lesions, (5) Thrombocytopenia. Bone marrow aspirates revealed decreased numbers of megakarocytes and evidence of increased hemolysis.

The virologic and serologic data are summarized in Table 28. Rubella was isolated from the throat, urine, and stools of these infants during life, and in every case in which sera were available, neutralizing antibody was detectable. Of interest is the very high titer in case #7 Yal, the oldest child in the series.

Table 28 Virologic and Serologic Findings in Cases of Congenital Rubella Syndrome

Patient	Age	Source of virus isolation	Antibody Titer Baby	Maternal
1. Sol.	Newborn	Throat Autopsy	1:8	1:16
2. Brom.	Newborn	Urine Throat	1:4	1:16
3. Cass.	Newborn	Urine	1:2	-
4. Sch.	Newborn	Throat Rectal	1:4	1:4
5. Red.	8 weeks	Throat	1:4	1:16
6. Fle.	Newborn	Autopsy	-	1:32
7. Yal.	6 mos.	Throat	1:128	-

In addition, virologic data was obtained from 3 cases who expired in the newborn period (Table 29).

Table 29 Titer of Rubella Virus (IND 50/gm Tissue) in Congenital Rubella Syndrome

Tissue	#1 Sol.	#2 Brom.	#6 Fle.
Blood	0	0	
Liver	0	>5,000	0
Spleen	0	500	
Kidney	5,000	50,000	
Adrenal	200	0	
Pancreas	5,000	-	
Appendix	35,000	-	
Lymph node (mesenteric	>150,000	-	
para-aortic)	-	>25,000	0
Heart	0	0	
Pericardial fluid	0	0	
Lung	5,000	>5,000	
Thymus	>10,000	50,000	
Bone Marrow and Rib	1,650	>10,000	
Brain	5,000	>5,000	positive
Eye			
Lens	-	500	
Lens with cataract	>50,000	-	
Cornea and sclera		0	
Vitreous humour		500	

As can be seen from this table, virus was recovered in especially high concentrations in the lymphoreticular tissues and eye. Of unusual interest was the finding of virus in the cataract of infant #1 Sol. in very high concentration ($>10^4 IND_{50}/gm$).

Although antibody was detectable in the sera of the affected newborns, it was difficult to ascertain whether this was transmitted across the placenta or was being synthesized by the baby. It is known from other lines of evidence that the fetus and newborn produce predominantly a gamma M-macroglobulin antibody in response to immunization or infection, and that of the three immunoglobulins (gamma M, gamma G, and gamma A) the only one that is transmitted across the placenta is the gamma G-immunoglobulin. Therefore in an attempt to resolve the question of whether rubella neutralizing antibody detected in newborn sera was of fetal or maternal origin, the sera of affected infants was examined physicochemically.

Immunoelectrophoretic analysis of maternal and baby sera is summarized in Table 30. Each of the babies' sera had a prominent gamma M-immunoglobulin line; one infant (Brom.) also had a gamma A-immunoglobulin precipitin. The normal infant serum had only a gamma G precipitin line.

These data suggested that the infants were actively synthesizing their own antibody possibly as a result of intrauterine infection with rubella virus.

Table 30 Immunoelectrophoresis Analysis of Infants Sera with Congenital Rubella Syndrome

Baby		Immunoglobulin		
		Gamma M	Gamma A	Gamma G
Sol.	M	+	+	+
	B	+	0	+
Brom.	M	+	+	+
	B	+	+	+
Cass.	B	+	0	+
Sch.	M	+	+	+
	B	+	0	+
Red.	M	+	+	+
	B	+	0	+
Control	M	+	+	+
	B	0	0	+

M = maternal
B = baby
+ = presence of a precipitin line
0 = absence of a precipitin line

In order to determine whether neutralizing antibody in infants' sera was of 19S type, 3 such sera were fractionated by gel filtration. The data thus far show that the greater part of rubella antibody in infants' sera is of the 19S type; whereas, that in maternal sera is of the 7S variety.

These observations suggest that the infant is capable of producing his own antibody in response to intrauterine infection.

These interesting observations are being extended to include follow up studies of these infants to determine duration of excretion of virus and to determine the effect of this intrauterine infection on subsequent development of immunity in these infants.

8. Immune Responses of Mice to Influenza Virus Infection. Previous studies have demonstrated that specific antibody to a number of viruses is present in nasal secretions. In persons without respiratory infection, antibody activity is predominantly associated with the γA-immunoglobulin present. During acute respiratory infection, γG-immunoglobulin is usually found in nasal secretions in addition to the γA, and antibody activity is associated with both immunoglobulins. To study the role of this local antibody in resistance to respiratory infection, an animal model system was sought. Because of their known susceptibility to infection with influenza virus following respiratory inoculation, mice were used for a number of experimental procedures.

Preliminary experiments were made to determine the protein and immunoglobulin components of mouse respiratory secretions, and to determine the sequential appearance of influenzal antibodies in serum of mice following intranasal inoculation or aerosol administration of virus. These responses were compared with those following intraperitoneal inoculation of the same virus. The serum and respiratory tract antibody responses of infected mice were compared, and differences in the capacity of virulent and killed virus to evoke serum and respiratory antibody were sought. Finally, efforts were made to assess the role of respiratory tract antibody upon the nature of subsequent experimental infection.

a. Techniques. A method was devised for obtaining bronchial and respiratory secretions from mice. Mice (25 gm) were anesthetized intraperitoneally with pentobarbital, 2-4 mg, and pinned to a board. The trachea was exposed by dissection. A blunted #20 needle was then inserted into the trachea and secured by ligature. Normal saline, 0.8 ml, was injected into the trachea and lungs, and slowly withdrawn. The same material was injected again, and aspirated; approximately 0.5 ml of washings were recovered from each mouse. Washings from individual mice were pooled, centrifuged at low speed, and the presence or absence of a button of erythrocytes was recorded. Immediately after washing, the heart was exposed and blood obtained directly from the ventricle; 0.5 to 1.0 ml of whole blood was usually obtained. Lungs were removed by dissection and examined for gross evidence of pneumonic infiltrate.

Following low speed centrifugation pools of bronchial washes were stored frozen until use. Specimens were then defrosted, dialyzed overnight against distilled water in the cold; the volume and sulfosalicylic acid protein concentration of each pool was determined. Each pool was then shell frozen and lyophilized and rehydrated with distilled water or saline to a final concentration of 100-150 mg percent protein. Neutralization tests were performed on these concentrated specimens using 0.4 ml of heat inactivated (56°C for 30 min), concentrated bronchial wash and 0.1 ml of virus so that final dilution contained approximately 100 $TCID_{50}$ per 1/10 ml. Mixtures were incubated at room temperature

for one hour and inoculated in 0.1 ml volume into rhesus monkey kidney cell cultures. Cytopathic effects and hemadsorption inhibition were sought on the 3rd or 4th day. Concentrated washings were also subjected to immunoelectrophoresis.

Serum neutralizing antibody titers were determined using 100 $TCID_{50}$ of virus. Sucrose density ultracentrifugation was performed on serum specimens by techniques described previously. Following dialysis of the various fractions obtained by this technique, neutralizing antibody was determined using 0.3 ml of heat inactivated fraction and 0.1 ml (100 $TCID_{50}$) of virus.

b. Serum and bronchial antibodies. A 1963 strain of A_2 influenza (A_2DC/302/63) was administered by intranasal inoculation (0.05 ml of allantoic fluid HA titer = 1:160/0.5 ml; 16 units) to groups of 10 mice. In one experiment no deaths were recorded, in another about 10% of the mice died within 10 days. In the first experiment, performed in Bagg albinos (WRAIR) (Table 31), intranasal infection of mice induced the earliest serum antibody response in 8 days, but the small amount of antibody could not be detected in the sucrose fractions. By the 10th day the serum titer of neutralizing antibody was 1:20 - 1:40, and the antibody was primarily a 7S (γG) immunoglobulin by ultracentrifugation. By the 14th day the titer was $\geq$1:160 and primarily 7S antibody. When the experiment was repeated in Charles River mice, earliest antibody was detected on the 7th day, and this was found to be a macroglobulin. By the 10th day macroglobulin antibody was still noted, and 7S antibody had appeared. After this time only 7S antibody was detected. Mice administered the same virus by the intraperitoneal route (80 HA units) showed the earliest antibody on the 5th day, and macroglobulin activity was found between the 5th and the 10th day. Gamma G activity was first noted on the 10th day, and it alone was present between the 14th and 42nd day.

In Charles River mice, inoculated intraperitoneally with small doses (16-32 HA units) of the same egg adapted influenza virus (A_2DC/302/63), antibody was not detected until the 14th post inoculation day; this was primarily 7S antibody. With somewhat larger doses (80-160 HA units) 7S antibody was detected in serum 7 days post inoculation. 19S antibody was also found on the 7th and 14th days. With mouse adapted A_1 virus (CAM) higher serum titers were obtained than with egg adapted virus (Table 32). Mice so inoculated developed macroglobulin antibody by the 7th day, which sometimes persisted on the 15-day samples. Some 7S antibody was also present in 7- and 15-day samples, and after the 15th day, was the only species of antibody detectable. The dose of CAM virus used was more than 10-fold that of the A_2 strain.

Balb C mice similarly infected produced 4- to 8-fold less serum antibody than the other strains of mice independent of route of inoculation (Table 33); therefore, these serum antibodies were not characterized.

Table 31 Serum and Bronchial Antibodies Following Infection of Mice with Influenza Virus

Experiment	Specimen Date	Day	Neutralizing antibody to homologous virus: Whole serum	Sucrose Density Centrifugation Fraction 1	2	3	4	5	6	7	8	9	10	Concentrated Bronchial washes	Immunoglobulins in conc. Br. washes: Alb.	♂	A	G	RBC
A_2DC/302/63	7/17	3	<4											0	+	+	+	0	0
7/14/64	7/20	6	<4											0	+	+	+	±	0
0.05 I.N.	7/22	8	5											0	+	+	+	+	+
Bagg mice	7/24	10	20-40							+				+	+	+	+	±	+
16 HA units	7/28	14	≥160					+	+	+	+	+	+	+	+	+	+	+	+
	8/4	21	≥160					+	+	+	+	+	+	+	ND	ND	ND	ND	0
	8/27	44	≥64	----------	not done	----------								+	ND	ND	ND	ND	0
A_2DC/302/63	9/30	7	5			+								+	+	+	+	+	+
9/23/64	10/3	10	10-20		+	+		+	+	+				+	+	+	+	+	+
0.05 I.N.	10/7	14	40					+	+	+	+			+	+	+	+	+	+
CR mice	10/21	28	160					+	+	+	+	+	+	+	+	+	+	±	0
16 HA units	11/20	57	160				+	+	+	+	+	+	+	+	·	+	+	tr	0
A_2DC/302/63	9/30	7	<5																
9/23/64	10/7	14	10						+	+									
0.05 I.N.	10/22	29	40						+	+	+								
CR mice																			
16 HA units																			
9/23/64	9/30	7	5																
0.10 I.P.	10/7	14	40						+	+									
32 HA units	10/22	29	40					+	+	+									
1/22/65	1/29	7	40			+	+		+	+	+			tox	+	+	+	0	0
0.25 I.P.	2/1	10	20			+			+	+	+			tox	+	+	+	0	+
80 HA units	2/5	14	40						+	+	+			tox	+	+	+	0	+
	2/16	25	160						+	+	+	+	+	tox	+	+	+	0	0
	3/12	56	80	----------	not done	----------								+	+	+	+	0	0
9/23/64	9/30	7	10			+				+									
0.50 I.P.	10/7	14	80					+	+	+	+								
160 HA units	10/22	29	40						+	+	+	+							

Table 32 Serum and Bronchial Antibodies in Mice Following Infection with CAM Virus

Experiment	Specimen Date	Day	Neutralizing antibody to homologous virus: Whole serum	sucrose density centrifugation fraction 1	2	3	4	5	6	7	8	9	10	Concentrated Bronchial washes	Immunoglobulins in conc. Br. W.: Alb.	A	YA	G	RBC
CAM Flu (A_1)	1/4	0	<4											0					
12/28/64	1/4	7	40			+	+		+	+	+			+	+	+	+	tr	0
0.05 I.P.	1/12	15	160			+	+	+	+	+	+	+	+	+	+	+	+	0	0
256 HA units	1/25	28	160					+	+	+	+	+	+	+	+	+	+	0	+
	2/17	52	160					+	+	+	+	+	+	tox	+	+	+	tr	+
0.10 ml		7	160		+	+		+	+	+				0	+	+	+	tr	0
512 HA units		15	160					+	+	+	+			0	+	+	+	0	0
		28	320						+	+	+	+	+	+	+	+	+	0	+
		52	>320					+	+	+	+	+	+	+	QNS	----------			+
0.25 ml	(1/19)	7	80			+	+			+	+			0	±	±	+	tr	+
		15	160		+	+	+	+		+	+			+	+	+	+	tr	0
		28	>320					+	+	+	+	+	+	+	+	+	+	0	+
		52	>320					+	+	+	+			tox	+	+	+	tr	+

Table 33 Response of Balb C Mice to Experimental Influenza

			Homologous Neut. Antibody								
			Whole serum	Conc. Br. W.	Immunoglobulins in in Br. W.					Viable Virus in Br. W.
					Alb.	A	A	G	RBC	
A_2DC/302/63	6/22	3	<5	0						
0.25 I.P.	6/24	5	<5	0						
6/19/64	6/26	7	20	0	ND					ND
Balb C mice	6/29	10	5	0						
80 HA units	7/2	14	20	0						
	7/10	22	20-40	0						
0.05 I.N.	8/10	3	<5	0	+	+	+	0		10^4/0.1 ml
8/7/64	8/12	5	<5	0	+	+	+	+		10^4/0.1 ml
Balb C mice	8/14	7	<5	0	+	+	+	+		10^0/0.1 ml
16 HA units	8/17	10	5	+	+	+	+	+		0
	8/21	14	40	+	+	+	+	0		0
	8/28	21	40	+		+	+	+		0

Following intranasal administration of A_2DC/302/63 virus, bronchial secretions of these mice contained 10^4 EID_{50}/0.1 ml on the 3rd post. inoculation day. This same amount of virus was found on the 5th day; on the 7th day virus titers had declined to 10^0. On the 10th day and thereafter bronchial washes did not contain virus. Serum antibody titered less than 5 through the 7th day; by the 10th day serum antibody was detected at 1:5; on the 14th day, 1:40. In these mice, bronchial washings tested through the 7th day showed no neutralizing activity, whereas on the 10th, 14th and 21st days, when serum antibody was present and no virus was found in the bronchial wash, antibody activity was detected. In Balb C mice immunized by the intraperitoneal route with this A_2 virus, serum antibody was detected on the 7th day; no bronchial wash antibody was present through the 22nd day.

Proteins found in bronchial washes were identified by immunoelectrophoresis. In normal mice only albumin, an alpha globulin and γA globulin were found. During infection γG and other serum proteins were detected. These findings are essentially similar to previous findings in humans. Following I.N. infection antiviral activity in Br.W. appeared early and persisted over 42 days. Data concerning I.P. infections is inconclusive at present. The patterns of immunoglobulins in bronchial washings may be affected by the presence of traces of serum reflected by the presence of erythrocytes in the specimens. Currently, studies are underway to characterize the Br.W. antibodies. At the present writing the data suggest that local respiratory antibodies are produced by both intranasal and intraperitoneal immunization.

These observations suggest that the pattern of serum antibody, namely macroglobulin antibody early followed by the development of 7S antibody, is the same in mice with influenza as has been found with other viruses in other animal systems. Further, route of infection and virulence of virus do not seem to significantly affect the pattern of serum antibody seen.

9. Antigenic Composition of 1964-1965 Influenza A_2 Strains. The tendency of influenza A_2 virus to undergo antigenic variation is well documented. Since this shift apparently involves the loss of antigens, immunity to older strains may not protect effectively against infection with new mutants. Consequently, continual analysis of new influenza A_2 isolates has considerable epidemiological importance. As part of a continuing program to monitor contemporary influenza A_2 strains, those isolated in the latter part of 1964 were analyzed. Methods used were by hemagglutination-inhibition and rhesus monkey kidney tissue culture neutralization, as previously reported from this laboratory.

Viruses analyzed during the past year were as follows: A_2/Puerto Rico/1/64, A_2/Taiwan/1/64, A_2/D.C./301/64, A_2/Moscow/1019/65, and A_2/Leningrad/29/65. All strains were originally isolated from persons with clinical influenza in the designated localities.

a. Antigenic analysis. Hemagglutination-inhibition tests failed to demonstrate significant variation from the 1962 prototype A_2/Japan/170/62 for all but the Moscow strain (Table 34 and 35). This isolate shows closer relationship to the original A_2 strain appearing in 1957 (A_2/Formosa/313/57). Whether this represents a "back-mutation", hitherto unrecognized for influenza, or is an artefact by way of cross contamination of the original isolate cannot be ascertained.

A composite grid cross-neutralization chart is shown in Table 36. Here, figures represent the reciprocal of antibody titers of each sera measured against respective viruses. Again, there is little significant difference between 1963 strain (DC 302/63) and Puerto Rico 1, Taiwan/64 and DC 301/64.

On the other hand, Moscow/65 and Leningrad/65 isolates demonstrate a different reaction pattern. As with hemagglutination-inhibition the Moscow strain, after seven passages through rhesus kidney cultures, appears to be in an intermediate position between A_2/Formosa/313/57 and the contemporary strains. On the other hand Leningrad strain is antigenically similar to the other contemporary isolates of 1963 and 1964. The observed differences of greater heterologous inhibition by 1963 and 1964 antisera than homologous are possibly representative of the avidity of Leningrad strain for antibody.

The data indicate that 1964 influenza virus has remained relatively stable antigenically, with the exception of A_2/Moscow/65. As previously noted, this may be artefactual. Whether the strain analyzed here may in fact represent two antigenically distinct (i.e. 1957- and 1963-like viruses) is currently under investigation.

Table 34 Antigenic Analysis of Contemporary A_2 Influenza Viruses by HI Test

	ANTISERA			
ANTIGEN	Form/313/57	Jap/170/62	DC/303/63	PR/1/64
A_2/DC/301/63	200*	800	800	1600
A_2/Form/313/57	400	100	<50	50
A_2/Jap/170/62	200	1600	800	400
A_2/DC/303/63	100	800	800	800
A_2/PR/1/64	50	400	400	1600

* Reciprocals of HAI titers of antisera vs. indicated virus.

Table 35 Antigenic Analysis of Contemporary A_2 Influenza Strains by HI Test

	ANTISERA			
ANTIGENS	Form/313/57	Jap/170/63	DC/303/63	PR/1/64
A_2/Leningrad/29/65	<50*	50	<50	400
A_2/Moscow/1019/65	200	100	50	50
A_2/Form/313/57 E-5 Al Fld	400	<50	50	<50
A_2/Jap/170/62 E-7 Al Fld	100	3200	800	400
A_2/DC/303/63 E-8 Al Fld	100	800	1600	800
A_2/PR/1/64	50	400	400	1600

* Reciprocals of HAI titers of antisera vs. indicated virus

Table 36 Neutralizing Antibody Titers of 1964 Influenza Viruses VS Standard Antisera

	ANTIGEN							
ANTISERA	Jap/305/57	Jap/170/62	DC/302/63	PR/1/64	Taiwan/64	DC/301/65	Moscow/65	Leningrad/65
A_2Form/313/57	640*	10	20	10	40	40	640	20
A_2Jap/170/62	10	160	160	20	240	240	240	160
A_2DC/302/63	<5	20	80	20	160	240	80	320
A_2PR/1/64	<5	20	80	160	640	240	120	640
A_2Taiwan/64	<5	20	40	20	320	-	-	-

* Reciprocal of titer vs. 100-1000 TCD_{50} indicated viruses

10. The Immunogenicity of Contemporary Influenza A_2 Strains. With the continuation of antigenic shift among influenza A_2 strains, it has become apparent that the composition of influenza vaccines must be appropriately altered if they are to be at all effective. Thus, previous investigation has indicated significant variation of 1963 A_2 strains, from the prototype 1962 Japan/170/62 strains. Moreover, the contemporary antigenic structure has continued to drift away from that of the original 1957 A_2 strain. In order to assess the relative immunogenicity of 1964 candidate strains, the homologous immune response of humans to each of these contemporary strains and to Japan/170/62 was studied. In addition, a few selected individuals were studied for heterologous response elicited by each antigen.

Experimental monovalent vaccines were prepared commercially from influenza A_2 strains Japan/170/62, Sydney/2/64, Puerto Rico/1/64 and Taiwan/1/64. Each vaccine contained 200 CCA units/cc of candidate strains, and was formalinized.

One hundred twenty-five men of the 4th Training Battalion, Fort Gordon, Georgia, were divided into 5 groups of 25 men each, according to alphabetical order of last names. Groups received the following vaccine: Group A - Japan/170/62, Group B - Taiwan/1/64, Group C - Puerto Rico/1/64, Group D - Sydney/2/64, and Group E - no vaccine. Each subject was pre-bled, and received a single dose of 1.0 ml vaccine intramuscularly. Twenty-two days after receiving vaccine a post-immunization blood was collected. During the interim respiratory disease at the base was felt to be no higher than was usual for that season, and clinical influenza was not observed.

Paired sera were processed for hemagglutination-inhibiting antibody (HI) by periodate treatment and heat inactivation in the usual manner. Neutralizing antibody was assayed in primary rhesus monkey kidney cell cultures, end points being delineated by hemadsorption. In each group, five men showing the greatest rise in antibody and five showing the lowest or no change in antibody by HI test were checked for neutralizing antibody responses. Finally, two men in each group demonstrating the most significant rises to homologous HI antibody were checked for heterologous HI activity against the other three strains employed in the study. Antibody titers in Group E (control) were determined against Puerto Rico/1/64 virus.

a. Antibody responses. Pre- and post-immunization HI titers are shown in Table 37 for each group. The geometric mean titer (GMT) for each group is shown in the last column, and for the limitations imposed by group size, pre-bleed titers are comparable.

As an indication of response to immunization, Table 38 shows the magnitude of change between pre- and post-immunization sera. Here it may be seen that four men in Group E (control) demonstrated significant changes (four-fold or greater) in titers. All four were checked by

Table 37 Pre-(A) and Post-immunization (C) Serum HAI vs Homologous Strain

Group		Number of Men Demonstrating Each Titer										
		≤20	40	80	160	320	640	1280	2560	5120	10,240	GMT[1]
Jap/170/62	-A	1	1	7	11	-	2	2	1			173
	-C		1			2	11	7			4	1,090
Taiwan/64	-A	10	4	5	2	2	1					56
	-C			2	5	6	3	7	1			480
PR/64	-A		3	10	5	2	4					140
	-C			1	2	4	7	5	5			750
Sydney/64	-A		6	5	5	4	3	1				145
	-C			2	1	7	4	5	4	1		670
E	-A	2	5	10	3	2	2					93
	-C		1	11	5	5	2					148

1. GMT = geometric mean titer

Table 38 Number of Men Demonstrating Indicated Fold-rise In Homologous HAI Titers in Paired Sera

Group	2	0	2	4	8	16	32	64	128	G.M.
Japan/62	1	2	5	7	4	2	1	1	2	7.0
Taiwan/64		2	3	3	7	1	5	1	2	9.6
PR/64		2	7	3	6	2	2	2		5.9
Sydney/64		3	4	6	7		2	2		6.0
Control	2	12	6	2		1	1			1.5

neutralizing antibody and found to be significant. Thus, while clinically influenza was not prevalent in the population under study, serological confirmation of, presumably, natural infections was occurring and must be borne in mind during final interpretation. With very few exceptions, determination of neutralizing antibody by hemadsorption-inhibition parallelled the results of HI antibody, and will not be presented in detail. Suffice it to say that no man who failed to demonstrate a significant change in HI antibody showed the obverse by HAdI techniques.

Heterologous HI responses of two men from each group showing highly significant rises homologously are presented in Table 39. While this group is too small to warrant a definite conclusion, it would appear that certainly in those men tested there is essentially little difference between the degree of response to the homologous and heterologous influenza A_2 strains under study.

Immunization with monovalent influenza A_2 strains of contemporary origin demonstrate little variation in eliciting antibody as measured by hemagglutination- and hemadsorption-inhibition. Thus, pre-bleed and post-immunization titers, and magnitude of antibody rise were comparable in groups of men immunized with Japan/170/62, Taiwan/1/64, Puerto Rico/1/64 and Sydney/2/64.

Consequently, any of the three 1964 candidate A_2 influenza strains under study appear equivalent to Japan/170/62 in its immunogenic abilities. More sensitive studies have demonstrated subtle but persistent antigenic shifts of 1963 and subsequent 1964 strains. With the anticipation of further variations in the future, the rationale of incorporation of contemporary 1964 A_2 influenza strains in vaccines is supported by the present study.

Table 39 Heterologous HI Antibody in Vaccinated Subjects

Vaccine		Antibody Titer VS Jap/170/62	Taiwan	Puerto Rico	Sydney
Jap/170/62	- A	40	40	20	20
	- C	2560	640	1280	2560
	- A	20	20	20	20
	- C	1280	640	640	640
Taiwan/1/64	- A	80	20	80	80
	- C	2560	1280	2560	2560
	- A	80	40	40	80
	- C	5120	2560	2560	2560
P.R./1/64	- A	80	20	80	80
	- C	5120	5120	5120	2560
	- A	40	20	40	40
	- C	5120	2560	2560	2560
Sydney/1/64	- A	40	20	40	40
	- C	1280	640	1280	1280
	- A	80	40	40	80
	- C	2560	1280	1280	2560

Section 11 and Tables 40 through 43 are omitted from this report.

12. Pulmonary Function Following Pneumonia in Army Recruits. The project was described in detail in last year's report, but final results were not available at that writing. Briefly, the pulmonary function of recruits at Fort Dix, New Jersey, was assessed using a field laboratory established there. Normal values were determined on fifty-three recruits before beginning basic training and were repeated during the final week. Thirty-six recruits hospitalized with pneumonitis were tested within two weeks after hospitalization and at intervals thereafter for several weeks to as long as three months.

On the basis of virologic and bacteriologic study, four distinct groups were defined: (1) Adenovirus type 4 infection only - Nine patients with increasing antibody titers in whom six had the virus isolated. (2) Bacterial - Eight patients with pneumococcus recovered from throat or sputum, no adenovirus infection. (3) Mixed - Ten cases with adenovirus 4 infection and pneumococcus isolated. (4) No agent incriminated - Nine cases.

On clinical grounds there were few, if any, differences between the groups. Pulmonary infiltrates were not extensive in any patient, and no patient was cyanotic. Of interest, sore throat and chills

were encountered less frequently in those with bacterial infections, 38% and 25% respectively, than in the other groups (greater than 70%). Average duration of fever was shortest in the "unknown" group.

a. Pulmonary Function. Tests of vital capacity, one-second forced expiratory volume, mid expiration time, maximal mid expiratory flow, maximum voluntary ventilation (MVV) and carbon monoxide diffusion (DLCO) were normal when tested upon hospital admission and did not change during convalescence with the following exceptions: mean initial vital capacity in group 1 was significantly lower than the earlier control group but was, nevertheless, still within the normal range. MVV mean values were significantly lower than the controls in each pneumonia group initially and on several subsequent determinations. Significant increases in MVV during convalescence were not observed. Since this test involves a number of factors dependent upon patient cooperation and general state of health as well as pulmonary ventilation, it is not possible to relate these results of MVV to the pulmonary disease. DLCO results in all groups were normal, and no patient showed significant changes in sequential tests.

The results of this study are interpreted to mean that the various types of pulmonary infection in this group of recruits did not produce measurable changes in pulmonary function. It must be emphasized that all patients were mildly ill; more severe infections will have to be studied before this conclusion can be generalized. Thus, no evidence was found to suggest that acute interstitial pneumonia in recruits produces permanent lung damage.

13. Viral Chemotherapy. Studies were undertaken to determine the tiviral effect of two compounds reported to be effective against ertain viruses in cell culture systems. Amantadine HCl (Dupont) was tested against influenza and rubella; N-methyl isatin beta thiosemicarbazone (MIBT) was tested against vaccinia, adenovirus type 7 and a simian adenovirus.

a. Techniques. Amantadine was prepared as a stock solution 5000 micrograms/ml in saline and sterilized by filtration through a 450 mu Millipore filter. MIBT is quite insoluble; stock solutions were prepared by dissolving 94 mgs in 100 ml of 1 normal NaOH (4 millimolar) with vigorous stirring and mild warming or, in other experiments, by preparing 4 millimole solution in 0.1 normal NaOH and autoclaving at 10 pounds pressure for 10 minutes. Stock solutions were neutralized with HCl and diluted further with tissue culture fluids prior to use.

Three types of assays were utilized. Antiviral activity against a given virus was determined by 1) reduction in virus titer compared to controls, titers being based upon virus cytopathic effect, hemadsorption or interference, 2) titration of virus concentration after

a period of incubation in the presence of drug, 3) plaque reduction. Drug toxicity upon monolayer cell cultures was evaluated by microscopic examination of unstained cultures.

b. Amantadine and Influenza. Table 44 summarizes experiments with A_2/Jap/170/62 virus in rhesus monkey kidney cell cultures in which virus yields were determined by infectivity titration in cell cultures and by hemagglutinin titer (HA) of pools of 3-5 tubes. Amantadine was incorporated into the tissue culture medium (M199) 15 to 30 minutes prior to inoculation of virus and remained in the fluids for the 3-4 days allowed for virus growth.

Table 44 Effect of Amantadine on Influenza in Cell Culture

	Drug Concentration microgm/ml	Virus Inoculum (log 10 $TCID_{50}$) 3.7 yield infectivity	H.A.	1.7 yield infectivity	H.A.
Exp. 1	0	4.5	160	4.5	≥32
	10	4.5	80	0	<2
	25	3.75	40	0	<2
	50	3.5	10	0.33	<2
		3.5		1.5	
Exp 2	0	5.5	256	5.5	256
	25	5.5	32	2.5	32
	50	3.5	<2	0.25	<2
	100	2.5	<2	0	2

Drug in Exp. 1 was prepared from stock solution 2 weeks old;
In exp. 2 drug stock solution had been kept at 4° for 6 weeks.

These results indicate an inhibitory effect at all drug levels studied when the virus inoculum was small and inhibitory activity at higher drug concentration when virus inoculum was quite large. No drug toxicity to cells was apparent at any drug concentration used.

c. Amantadine and rubella. In preliminary "chessboard" types of titrations increasing concentrations of drug were incubated with serial half-log dilutions of rubella virus. No inhibitory effect was observed in continuous rabbit kidney cell culture (RK13) based upon cytopathic effects or in primary green monkey kidney cells (GMK) by interference with Echo 11 virus. A significant antiviral effect was observed when virus concentration was measured 6 days after inoculation of rubella virus in GMK cell cultures (Table 45). As with influenza virus the antiviral effect of amantadine is most apparent when virus inoculum is small.

Table 45 Effect of Amantadine Upon Rubella Virus

Amantadine Concentration	Virus Concentration Following Indicated Inoculum	
	2.6*	0.6
0	3.8	≥4.5
25 mcg/ml	3.7	3.5
50 mcg/ml	2.3	1.6

* Log 10 IND_{50}/0.1 ml.

d. Effect of N-methyl isatin beta thiosemicarbazone (MIBT) on rubella virus. In "chessboard" type of titrations MIBT has produced no delay or reduction in virus CPE in RK13 cell cultures or in interference in GMK cells. Titration of virus yields after 7 days' incubation in GMK cell cultures have shown minimal and probably insignificant inhibitory effects (Table 46).

Table 46 Effect of MIBT on Rubella Virus

	MIBT concentration	Virus yields following indicated inoculum (log 10 IND_{50})		
		0.6		Toxicity
Exp. 1	0	3.5		0
	1	3.8		0
	10	2.0		0
	25	2.4		0
	50	0.6		slight
		2.2	2.2	
Exp. 2	0	3.0	1.2	0
	20	2.8	0.8	0
	40	2.5	0.6	0
		1.5	0.5	
Exp. 3	0	2.5	2.2	0
	20	2.6	2.5	0
	40	2.5	1.6	0

In Experiments 1 and 2 MIBT stock solution was prepared in 1 N NaOH; in Exp. 3 stock was prepared in 0.1 N NaOH.

e. Effect of MIBT on vaccinia virus. In preliminary experiments in GMK cell cultures 10 micromolar concentration of MIBT (prepared from stock solution in 0.1 N NaOH) showed significant (>50%) plaque reduction of vaccinia virus. In another experiment in GMK tube cultures (Table 47) a decrease in virus yield was observed at 20 micromolar concentration although slight cellular toxicity was noted.

These results confirm previous information concerning the effect of MIBT on vaccinia virus and indicate the potency of our MIBT solutions used in studies with rubella and adenovirus.

f. Effect of MIBT on adenoviruses. Using a simian adenovirus isolated in this laboratory a slight inhibitory effect by MIBT was observed, although some evidence of cellular toxicity by the drug was noted with as little as 20 micromolar concentration (Table 47). A single experiment with a human type 7 adenovirus in Hep 2 cell cultures showed no antiviral effect by MIBT even though cell toxicity was marked (Table 47).

The results of this series of experiments with two antiviral compounds have been disappointing. Although amantadine produced partial inhibition of rubella virus proliferation in tissue culture when a small inoculum was used there was no discernible effect on experimental infection of monkeys. MIBT had no significant effect on rubella in tissue culture. Although the known inhibition of vaccinia by MIBT was reproduced, no effects upon a simian and a human adenovirus were discernible.

14. Effect of Amantadine HCl on Experimental Rubella Infection In Monkeys. Amantadine HCl has been reported to have significant antiviral effect against rubella virus in tissue culture and in ferrets. This report deals with the effect of Amantadine on experimental rubella infection in rhesus monkeys.

a. Techniques. Two- to three-kilo rhesus monkeys were used. Amantadine HCl (Dupont lot #5042-92) was given by stomach tube as a saline solution freshly prepared daily. Each drug-treated animal received 75 mgm/kgm/day in two divided doses for the duration of drug treatment. Animals were housed in individual cages. The M33 strain of rubella virus in third BS-C-1 passage was the challenge virus. Animals were inoculated either intravenously or intranasally under light pentobarbital anesthesia. Specimens for virus isolation were collected at two-day intervals. Throat, nasal, and rectal swabs were collected in Hank's BSS plus 1% BPA and antibiotics. Heparinized whole blood was collected from the femoral vein. One-half-ml amounts of each specimen were inoculated into each of three GMK monolayer tubes and held at room temperature for one hour. Tubes were drained and re-fed with standard maintenance media at five days, and challenged with 100 - 1000 $TCID_{50}$ E-11 at 10 days. Cells and supernatant cultures with no

Table 47 Effect of MIBT on Growth of Vaccinia and Two Adenoviruses

Virus and cell culture	Drug dose micro-moles per ml.	Virus titer/0.1 ml	Drug Toxicity/0.1 ml	Virus concentration following 300 $TCID_{50}$ inoculum (7 day pool) per 0.1 ml
Vaccinia GMK	0	5.5	0	3.3
	20	4.5	slight	0.7
	40	4.5	slight	0.3
Monkey Adenovirus	0	5.3	0	2.7*
	20	4.3	slight	1.3
	40	3.5	slight	1.3
Human Adenovirus Type 7 in Hep 2	0	1.3	0	1.2**
	20	2.5	marked	2.5
	40	2.5	marked	2.5

* 20 $TCID_{50}$ pool
** <10 $TCID_{50}$

detectable rubella virus were subpassaged once. All specimens were also tested for extraneous hemabsorbing viruses with 0.1% suspension of guinea pig red cells. All interfering agents were identified as rubella virus with specific rubella antisera. Monkey agents were grouped according to Hull on the basis of CPE produced in tissue culture. Monkey adenoviruses isolated were shown to fix complement in the presence of human adenovirus CF antibody. Rubella serum neutralizing antibody tests were done in continuous rabbit kidney.

b. Amantadine effect. Significant antiviral activity of the drug utilized in the experiments was shown against A_2 influenza virus and rubella virus (Tables 44 and 45, Section 13).

Two separate animal experiments were done. In the first experiment four animals received Amantadine 75 mgm/kgm/day via gastric tube on the day prior to, the day of, and for nine days after virus challenge. Four animals received virus alone. The animals were inoculated by either the intravenous or the intranasal route with between 1000 - 10000 $TCInD_{50}$ of virus. Infection by rubella virus was monitored by examination of throat swabs (TS), rectal swabs (RS) and blood obtained every other day from each exposed animal from the 2nd through the 21st day.

In the second experiment four animals received Amantadine 75 mgm/kgm/day on the day prior to, the day of, and two days following intravenous challenge with 100 - 1000 $TCInD_{50}$ of virus. Four animals received virus only. Specimens for isolation from the throat, nasopharynx and blood were taken at two-day intervals from day 5 to day 13. In both experiments, neutralizing antibody responses of inoculated animals were followed serially through a 60-day observation period. Table 48 summarizes the results of these experiments.

It is evident that Amantadine given in the dosage used here failed to inhibit infection of monkeys by rubella virus inoculated by either the intranasal or intravenous route. Further, no significant differences in numbers of monkeys circulating or shedding virus was observed between treated and untreated animals. The chronology of viremia or virus shedding was examined in the treated and untreated groups, to determine whether infection might have been modified by the drug. No significant differences were observed.

These two pilot experiments, however, are not directly comparable. The first, commenced in August 1964, was complicated by simultaneous natural subclinical infection of all test animals with simian adeno- and enteroviruses. While not significantly altering the course of rubella virus infection in the animals, these viruses did complicate the laboratory efforts at recovery of rubella virus. Monkeys in the second experiment were not so infected with adventitious viruses.

Table 48 Effect of Amantadine Upon Experimental Rubella Virus Infection in Monkeys

Exper.(1)	Drug Dose	Number Animals				
		Total	Route Inoc.(2)	Viremia	Virus Shedding	Infected(3)
1	75 mgm x 9	2	IN	0	0	2
		2	IV	2	2	2
	none	2	IN	0	1	2
		2	IV	2	2	2
2	75 mgm x 4	4	IV	1	4	4
	none	4	IV	1	4	4

(1) In the first experiment virus was sought in blood, stools and throat swabs every other day from the second to the 21st post inoculation day; in the second experiment, in blood, nasal swabs and throat swabs from the 5th through the 13th day.

(2) In Exp. 1, 10^3 - 10^4 IND_{50} were inoculated either intranasally or intravenously; in Exp. 2 the animals received 10^2 - 10^3 IND_{50} intravenously.

(3) Four-fold rise in neutralizing antibody response was considered indicative of infection.

c. Interpretation. There was no difference in the virus shedding pattern between drug and untreated groups in both experiments. The significance of the monkey viruses repeatedly isolated during the first experiment is not known. Fewer rubella virus isolations were made in the first experiment than in the second, but it seems unlikely that monkey agents played any role in altering the rubella infection. None of the agents were associated with gross evidence of any disease, and were in low titer in the stool as ıifested by their late appearance in second passage in tissue culture. bella virus was isolated on several occasions in the presence in the same specimen of a monkey agent.

All animals showed a typical pattern of neutralizing antibody development with the prompt appearance of antibody near the end of the second week after infection.

The mechanism of action of Amantadine is not completely defined, but it is felt that the drug blocks or slows penetration of host cells by the virus in tissue culture (Davies, Science 144:1620, 1964). In clinical studies with Amantadine in influenza volunteer experiments, pretreatment has been suggested to be more important than duration. The large doses and prolonged schedule of treatment with the drug in this experiment,

compared to human trials, would tend to rule out insufficient drug as a reason for our failure to demonstrate any modification of experimental rubella virus infection in monkeys.

The demonstration of the antiviral action of Amantadine against rubella in LLC-MK_2 tissue culture suggested that this drug might show promise in in vivo situations. The rhesus monkey has been demonstrated to develop a reproducible rubella infection with viremia, shedding of virus, and appearance of neutralizing antibody. The monkey has also been demonstrated to be at least as sensitive to rubella virus as tissue culture systems. In these two experiments the failure of Amantadine to prevent infection or modify either virus shedding or serological conversion does not confirm a previous report that Amantadine may modify experimental ferret infection with rubella virus.

15. Antibodies in Mouse Hyperimmune Ascitic Fluid (MHAF). Techniques that had been developed to obtain high-titered antibody to the arboviruses employing mouse ascitic fluid may be found in the previous annual report. Ascites was induced by sensitizing and provoking doses of Freund's complete adjuvant. The current report concerns some immuno- and physicochemical studies of serum and ascitic fluid from mice injected with adjuvant and either the Sandfly Fever viruses or Dengue II virus. The non-specific factors involved in the sero-diagnosis of these diseases have been defined. The electrophoretic behavior of normal mouse serum, immune mouse serum, and immune serum from mice injected with adjuvant are compared.

a. Non-specific factors in the hemagglutination-inhibition test. Zone electrophoresis was performed with pevikon-gel blocks 1 x 6 x 36 cm. Samples were applied 12 cm from the cathodal end and electrophoresed overnight at 4°C in 0.09 M, pH 8.6 barbital buffer. Electrophoresis was done with Dengue II ascitic fluid that had not been acetone extracted, a procedure required to remove non-specific inhibitors in arbovirus hemagglutination-inhibition (HI) tests. Protein and HI antibody determinations were performed on each 1 cm eluate. There were two areas of HI antibody activity (Table 49). The first was found between 9 and 16 cm from the cathode end which consisted of the gamma G-immunoglobulins, the second between 20 and 35 cm which consisted of the faster migrating alpha immunoglobulins and albumins. In between these two regions was a large peak containing the slower migrating beta immunoglobulins with little or no HI antibody activity. When these samples were tested with a non-related virus (EEE), only the area to the right of the beta globulin peak (fractions 21 to 35) inhibited. Heating the sample at 60°C for 30 minutes had no effect on this non-specificity. Acetone extraction of the fractions left only the gamma G-immunoglobulin region to react with the homologous virus, suggesting the presence of a lipid or lipoprotein in the alpha globulin-albumin region as the inhibitor. This situation was not found in the gamma G region; thus, once the gamma G-immunoglobulins are separated from other serum or ascitic fluid components, acetone extraction is not required.

Table 49 Hemagglutination Inhibition Antibody Titers of Untreated and Acetone Extracted Eluates From Zone Electrophoresis of Dengue II Immune Mouse Ascitic Fluid

Zone Fractions	Maximum 0.0 of peak, 700 mu	Peak HI antibody titer Untreated Deng II	EEE	Acetone Deng II	EEE	Globulin content Gamma	beta	alpha	album
9-16	0.40	64[a]	0	32	0	+	0	0	0
17-20	0.98	2	0	0	0	0	+	0	0
21-25	0.56	32	16	0	0	0	0	+	0
26-33	1.30	128	64	0	0	0	0	±	+

[a]Reciprocal of highest dilution of reagent effecting complete inhibition; 0 = <1:2

Table 50 Hemagglutination Inhibition Antibody Titers of Untreated and Acetone Extracted Eluates From Sephadex G-200 Gel Filtration of Dengue II Immune Mouse Ascitic Fluid

Sephadex Fractions	Maximum peak 0.0 700 mu	Peak HI antibody titer Untreated Deng II	EEE	Acetone Deng II	EEE	Globulin content Gamma M	Gamma G	albumin
38-44	0.46	128	128	0	0	+	0	0
48-66	0.76	32	2	8	0	0	+	0
68-80	1.20	4	0	0	0	0	0	+

Gel filtration with Sephadex G-200 provided additional information on the nature of the non-specific HI component in unextracted ascitic fluid (Table 50). The ascitic fluid proteins were separated into 3 peaks corresponding to the 19S, 7S and 4.5S components obtained by ultracentrifugation. The first peak contained the 19S gamma M immunoglobulins as well as large molecular weight components which reacted with both the homologous and non-related viruses. Acetone extraction eliminated the reaction with both viruses, as was found with the alpha globulin and albumin region after zone electrophoresis, suggesting that lipid associated globulins were responsible for the non-specific HI reaction. The second Sephadex peak contained the 7S gamma globulins which reacted with the specific virus. A weaker reaction with the heterologous virus terminated in the center of this peak. They may have been due to trailing of the 19S region into the 7S. After acetone extraction of the 7S region, HI antibodies were present only to the specific virus. There was no non-specific reaction with the unrelated virus in the third and largest of the Sephadex peaks containing the albumins. Therefore, the albumin region in zone electrophoresis that reacted non-specifically was probably the cause of some lipid-associated 19S rapidly migrating globulins or free lipids.

Sucrose density gradient ultracentrifugation demonstrated region of acetone extractable non-specific HI reactions (Table 51). Fractions 9 and 10 at the top of the tube contained the bulk of the acetone extractable activity (1:128). The characteristic flotation of lipid in a centrifugal field suggests that lipids are responsible for the non-specific activity found in the upper portions of the gradient. Immunoelectrophoresis located the alpha 2 macroglobulin in tube #3. Therefore, lipids or lipoproteins that migrated as alpha globulins and albumins in an electrical field, were excluded by Sephadex G-200 with the macroglobulins, but were separated from the macroglobulins by ultracentrifugal methods appear to be responsible for the non-specific HI reactions.

b. Complement-fixation reactions of mouse globulins. Zone electrophoresis of ascitic fluid heated at 60°C for 30 minutes displayed a broad peak containing alpha globulins and albumins. Apparently the heat treatment caused some coagulation of these proteins which also impeded their rate of migration when compared to an unheated aliquot electrophoresed in an adjoining trough. The greater part of the CF antibody was found in the gamma G region as discussed above. Some reaction was also seen in the beta globulin region in lower titer.

CF antibody determinations were done on unheated fractions from a sucrose density gradient ultracentrifugal run (Table 51). Tubes 2 and 3, corresponding to the 19S macroglobulin region, were anticomplementary when tested undiluted, and reacted with the test antigen and control antigen at 1:2. Heating eliminated all 19S CF activity. Fractions 5 to 7 containing the 7S globulins reacted only with the test antigen.

Similar results were obtained with unheated ascitic fluid from Sephadex G-200 fractions (Table 52). The 19S region reacted with all test controls and the 7S region reacted only with the test antigen. All 19S activity was again eliminated following the standard heat treatment for CF tests. Heat denaturation of this macroglobulin may have been responsible for the elimination of the anticomplementary activity and any CF antibody, if there is any. Alternatively, a heat labile cofactor required for 19S CF activity may have been destroyed. Since heating affected the surface area of the macroglobulin enough to alter its migration in an electrical field, the possibility of it fixing complement with a homologous antigen in the more complicated of the serological tests is probably minimal.

c. The effect of adjuvant on mouse serum electrophoretic patterns. Normal serum and immune serum from mice not injected with adjuvant produced essentially the same electrophoretic pattern and protein concentration within the pattern. The hemoglobin present in both samples migrated 5 cm from the point of application. Immune serum from mice injected with adjuvant demonstrated a larger amount of the globulins determined by protein concentration of the eluates. The rate of migration of the hemoglobin portion increased to 10 cm from the point of application. The adjuvant injections also caused a distinct alpha globulin peak that was not present in the ascitic fluid from the same mice or in normal and immune sera from mice not injected with adjuvant. Lipid and lipoprotein were shown to migrate with the alpha globulins above; the peak may represent the host response to the paraffin oil adjuvant.

The CF titers of immune serum were greater from mice injected with adjuvant suggesting that the heightened antibody levels may be correlated with the elevated globulin levels and not necessarily that the adjuvant effected a more efficient antibody synthesis. Virus injected without adjuvant gave the same pattern as normal serum indicating synthesis of antibody within the normal globulin levels.

Table 51 Hemagglutination-Inhibition and Complement-Fixation Antibody Titers of Sucrose Density Gradient Fractions

Sucrose fraction	protein O.D.@ 700 mu	HI antibody titer		CF antibody titer					
				unheated			heated		
		untreated	acetone	D-II	NMB	AC	D-II	NMB	AC
1	0.02	0*	0	0	0	0	0	0	0
2	0.17	0	0	2	U**	U	0	0	0
3	0.31	0	0	2	U	U	0	0	0
4	0.18	2	0	U	0	U	0	0	0
5	0.45	8	16	8	0	0	8	0	0
6	1.30	32	8	16	0	0	16	0	0
7	1.80	64	8	32	0	0	32	0	0
8	1.70	64	2	8	0	0	8	0	0
9	0.72	128	0	U	0	0	U	0	0
10	0.32	64	0	2	0	0	2	0	0

* 0 = 1:2
** U = undiluted

Table 52 Complement-Fixation Titers of Unheated and Heated Sephadex G-200

Sephadex fractions*	Complement-fixation titers					
	Unheated			heated		
	Deng II	NMB	AC	Deng II	NMB	AC
39-43	4	2	2	0	0	0
48-68	32	0	0	32	0	0
70-80	0	0	0			

* Protein concentration and globulin content similar to Table 50.

16. Clinical and Epidemiological Features of Coxsackie Group B Virus Infections. Since the report of their discovery in 1948, thirty distinct serotypes of Coxsackie viruses have been described. The group A Coxsackie viruses have been causally related to the syndromes of herpangina and aseptic meningitis. The group B viruses, six in number, have been found to cause epidemics of pleurodynia and aseptic meningitis and encephalomyocarditis of infants. In addition, many reports have implicated the group B Coxsackie viruses in some cases of acute benign pericarditis, myocarditis, polio-like infection and orchitis. In order to provide some assistance in clinical diagnosis the experience gained in the study of 180 laboratory-confirmed cases of Coxsackie group B virus infections (1955-1960) has been reviewed.

The significant epidemiologic features of these cases were their summer-fall distribution and the predominance of a single serotype in a given year. In this series the following clinical syndromes were observed: aseptic meningitis (64%), pleurodynia (21%), abdominal distress (4%), mouth lesions (3%), myocarditis (4%), pericarditis (3%), myalgia (4%). Exanthems, orchitis and nonspecific febrile illness were each diagnosed in less than 2% of patients. Age appeared to play a determining role in the type of clinical syndrome produced by the group B Coxsackie viruses. Five infants with myocarditis went on to a fatal conclusion, but the two adult patients with myocardial involvement had mild courses. Five patients with pericarditis were all adult males. Although abdominal pain occurred most often in children under 10 years of age, pleurodynia was diagnosed infrequently in this age group.

Clinical diagnosis of group B Coxsackie infection can be made with a high degree of success in the following circumstances; 1) epidemics of pleurodynia, 2) aseptic meningitis, orchitis, pericarditis, exanthem, abdominal pain, etc., occurring in patients exposed to a person with pleurodynia, 3) neonatal myocarditis. In temperate climates Coxsackie B infections are rare except during the summer and fall months.

17. Central Nervous System (CNS) Syndromes of Viral Etiology. For 22 years etiologic investigation of CNS infections of presumed viral origin have been made by this department. More than half of the 2137 patients have been investigated in the past 10 years by techniques which ordinarily yield specific etiologic diagnoses in most instances. The clinical syndromes have varied but little over this interval. On the other hand, the causative agents, and the frequencies of specific diagnoses appear to be changing. To determine the extent of this change, an analysis of the etiology of disease in 569 patients, ill between January 1958 and December 1963, was made; these data were then compared with those obtained earlier for the interval 1953-1957.

Three syndromes were commonly seen. Aseptic meningitis accounted for the majority (374/569) of clinical diagnoses; 107 patients had paralytic disease, and 88, encephalitis. Specific etiologic diagnoses were

obtained for 211 of the 374 patients with aseptic meningitis. Of the 211, 162 were associated with enterovirus infections. Group B Coxsackie viruses (71) were more commonly implicated than any other agent or family of agents; Echo viruses were next most commonly implicated (55) followed by mumps (28), Coxsackie group A and polioviruses (18 each). Lymphocytic choriomeningitis, herpes simplex, arboviruses and leptospires account for the remainder of aseptic meningitis. Forty-nine of 88 patients with encephalitis had specific diagnoses. However, no single agent was responsible for a majority of cases. Each of the above agents except LCM virus was implicated. Finally, polioviruses were associated with the majority (66 of 68) patients with paralytic disease of proved etiology. This association of etiologic agents with the various syndromes is essentially similar to that observed for the earlier 5-year interval.

The analysis showed, however, that the frequency with which specific agents were encountered 1958-1963 varied from earlier years. The diagnoses of mumps and lymphocytic choriomeningitis were made significantly less frequently (50%). Herpes simplex and leptospires continued to account for about 4% of diagnoses. Enterovirus infections remained essentially the same (43% of diagnoses). However, polioviruses were less frequently encountered, and group A Coxsackie viruses more frequently than in the past.

No significant change in the seasonal patterns of prevalence of specific pathogens was observed. Enterovirus and arbovirus infections continued to show a summer peak prevalence, mumps virus, in the spring, and LCM in the winter.

Summary and Conclusions.

1. The challenge virus resistance (CVR) test for detection of dengue viruses in cell cultures, as described by Halstead, was modified and successfully used to recover strains of dengue viruses from Puerto Rico (1963) and East Pakistan (1964). Strains from both places were identified uniformly as type 3 or closely related viruses. Significant factors influencing the reproducibility of the test system are described, and the techniques for strain identification are described.

2. CVR, induced in BS-C-1 cells by dengue viruses, was found to be associated with an interferon-like substance. Investigations of rates of virus growth, induction of CVR and of production of this interferon showed that complete CVR was attained only after peak infection of cells occurred, and was concomitant with maximal production of cell free interferon.

3. A system for inducing plaque formation in dextran overlayed BS-C-1 cells by dengue viruses is under study. Factors which influence production of plaques are complicated, and relationships between those currently known to be important are described.

4. Arboviruses recovered from several species of salt and fresh water marsh mosquitoes between 1961 and 1964 include Cache Valley-like agents, California encephalitis virus, Eastern Equine and Western Equine viruses. The mosquito species involved, the sites and times of their collection are described. Preliminary results of a year-long investigation of the small animal inhabitants of a fresh water swamp as hosts for EEE virus are described. The occurrence and specificity of antibody to Cache Valley viruses in dairy cattle of the Eastern Shore of Maryland is presented.

5. Studies of the physico-chemical nature of antiviral antibody have been extended from the arboviruses to other human pathogens. The investigations continue to show that in vitro neutralizing activity is associated with 3 immunoglobulins, γA, γM and γG. However, passively protecting activity produced in mice and assayed in guinea pigs appears to reside solely in the γG immunoglobulins.

6. A immunoglobulins were found to be the only hemagglutination inhibiting antibody to P. tularensis in human nasal secretions. Simultaneously-studied circulating antibodies were on the other hand found to be primarily γM immunoglobulins.

7. Investigations of the congenital rubella syndrome were continued. These showed that the organs of newborn infants who succumb contain large quantities of rubella virus without apparent direct association with observed pathology save for the lens, which was uniformly found to be infected. The antibodies produced by surviving infants are primarily γM immunoglobulins, whereas those in the mother simultaneously are γG immunoglobulins.

8. The nature of circulating and respiratory antibodies in mice infected or immunized with influenza virus was studied. Preliminary experiments show again that respiratory antibody activity is associated primarily with γA immunoglobulins, whereas circulating antibody activity resides in the γM and γG immunoglobulin fractions.

9. All recently recovered strains of Influenza A_2 viruses appear to be closely related to one another, except for a single strain tested from Moscow in 1965; this latter strain is more closely related to classical A_2 strains than the contemporary variants. Evidence for continued antigenic drift away from the 1962-1963 strains is presented.

10. Immunogenicity of several contemporary A_2 influenza viruses as monovalent formolized vaccine was compared in man. Only minimal differences in potency were observed between contemporary strains and the vaccine strain A_2/Japan/170/62 virus.

11. Section 11 was omitted from this report; see Project 3A013001A91C, Task 01, Work Unit 020, Effects of Physiological and Psychological Stress upon Infection and Disease, Annual Report.

12. Pulmonary function in recruits with interstitial pneumonia of adenovirus origin was not found to be significantly altered during disease and convalescence.

13. The antiviral effects of Amantadine HCl and N-methyl isatin beta thiosemicarbazone (MIBT) were assayed against several viruses in cell culture systems. Amantadine was shown to suppress propagation of both influenza and rubella virus when the challenge dose of virus was small (50 doses or less); drug effect against higher concentrations of virus was less obvious. MIBT showed no inhibitory effect for rubella or adenoviruses, although it was effective against vaccinia.

14. Amantadine HCl was administered to rhesus monkeys to evaluate its antirubella activity in vivo. Given in maximal dosage to monkeys during the incubation period, it had no discernable effect upon infection patterns of viremia or excretion of rubella virus.

15. Antiviral substances in mouse hyperimmune ascitic fluids (MHAF) were characterized. Non-specific arbovirus hemagglutinin inhibitors were, like those of serum, found to be lipoproteins; specific CF antibodies were found to be associated with both γM and γG globulins. Only the latter was heat stable.

16. Patients with Coxsackie virus group B infections seen during the past 5 years were reviewed to determine the spectrum of associated illness, and then seasonal distribution. Clinical diagnoses of infection with these viruses can be inferred only during epidemics of pleurodynia in cases of aseptic meningitis or orchitis occurring in persons with known exposure, and in neonatal myocarditis.

17. Patterns of non-bacterial central nervous system infections seen between 1958 and 1964 were compared with those studied 1953-1957. Significant differences in the frequencies of association with specific viruses were observed for the two intervals. No significant difference in syndromes or their seasonal distribution was found.

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RESEARCH AND TECHNOLOGY RESUME

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21. TECHNOLOGY UTILIZATION	22. COORDINATION
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23. KEYWORDS Rickettsia; epidemiology; ecology; vectors and reservoirs; vaccines; diagnosis, laboratory; serology

24. (U) Tech Objective - Principal aims include (1) development of a vaccine for protection against scrub typhus. (2) Evaluation of the effectiveness of Q fever vaccines. (3) Definition of the biotic and abiotic factors affecting perpetuation, distribution and dispersion of tick-borne diseases to provide means of assaying the risk of infection of man in enzootic foci, and (4) Study of the occurrence and distribution of rickettsial diseases in S.E. Asia.

25. (U) Approach - (1) A potent polyvalent killed scrub typhus vaccine in which representative antigenic types have been incorporated is to be developed. (2) Serologic methods are evaluated for suitability in detecting immune state in vaccinees, and their applicability for assaying vaccine potency. (3) Quantitative data is collected coincidently on the population dynamics and interaction among tick vectors, vertebrate hosts and R. rickettsiae. (4) Seroepidemiological surveys of man and animals, and recovery of rickettsiae from suspected vectors and hosts are undertaken in S.E. Asian countries.

26. (U) Progress (Jul 64 - Jun 65) - (1) Virtually all newly isolated strains of R. tsutsugamushi studied to date share antigens with one or more of three prototype strains. (2) Q fever complement fixation tests have been perfected and agglutination tests are being evaluated. (3) Data provided by the statistically designed investigations indicate the relative importance of the various components of the infection cycle in the survival of R. rickettsii in nature. (4) Scrub typhus, Q fever, tick typhus and murine typhus are endemic and widely distriouted throughout continental Thailand.
For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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Project 3A014501B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 167, Rickettsial infections

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Description.

An antigenic analysis of strains of Rickettsia tsutsugamushi recovered from wild animals, chiggers and patients in Thailand was undertaken to determine the existence of possible relationships between the antigenic composition of the strain, geographic locality, species of rodent host and severity of human disease. Ecological studies of Rocky Mountain spotted fever are in progress to assess the biotic and abiotic components of the infection cycle which are important to the maintenance and dispersion of Rickettsia rickettsii in an enzootic area. Q fever serological tests are being developed and evaluated for use in studies of the serological response of man following vaccination. Methods are under development to obtain purified suspensions of scrub typhus rickettsiae for use in immunological studies of human disease and experimental infections.

Progress.

Antigenic Analysis of Strains of R. tsutsugamushi Recovered in Thailand.

During the period November 1962 through November 1963, collaborative efforts of the Thai and U.S. components of the SEATO Medical Research Laboratory in Bangkok, resulted in the recovery of 103 strains of Rickettsia tsutsugamushi. The definitive identification of all strains was based upon the results of immunity-challenge tests. Scrub typhus was considered present in an isolation line when infection of mice with the unknown agent provided at least 2.5 logs protection against subsequent challenge with the lethal Karp strain.

The geographic origin of the 88 strains obtained from animals, 11 from chiggers and 4 from patients, is summarized in Table I. In the Northern Hills and Valleys portion of the Continental Highlands, isolations were made from small wild animals and from Leptotrombidium chiggers infesting them, as well as from 4 Thai farmers who had mild nondescript febrile illnesses. Scrub typhus rickettsiae were recovered from animals trapped in the Central Valley, the Southeast Coast, and the Khorat Plateau, where, in addition, strains were isolated from chiggers. Field studies during the period covered by this report were limited to the 4 physiographic provinces of Continental Thailand and were not undertaken in the Peninsular region.

TABLE I

ORIGIN OF RICKETTSIA TSUTSUGAMUSHI STRAINS RECOVERED IN THAILAND

Geographic Location		No. of Strains Isolated From		
Physiographic Province	Location District	Animals	Chiggers	Patients
Northern Hills and Valleys	Huay Mae Sanam	1		
	Chieng Rai	3		
	Sanpantong	6		3
	Sarapi		1	1
Khorat Plateau	Khon Kaen-Loey	1	1	
	Chong Mek	54	9	
Southeast Coast	Pong Nam Ron	2		
Central Valley	Prakanong	2		
	Samudprakan	5		
	Smudsakorn	3		
	Nakornnayok	11		
Totals		88	11	4

Shishido and his coworkers (Jap. J. Med. Sci. and Biol. 17:59-72, 1964) reported recently on an apparent relationship between serological types of Rickettsia tsutsugamushi and certain epidemiological features of scrub typhus in Japan. These investigators showed that it has been possible to classify all strains recovered in Japan whether from man, mites or rodents, into one of three antigenic categories. Classification is based upon the complement-fixing reactivity of serum from infected guinea pigs in tests with purified suspensions of the prototype Karp, Kato and Gilliam strains. The guinea pigs were inoculated intracerebrally with

spleen homogenates prepared from mice infected with the strains under study. Although the newly recovered strains varied in degree of cross-reactivity with the three prototype strains, they were classified according to their major antigenic component. Thus far their results suggest relationships exist between the antigenic type of R. tsutsugamushi, the type of disease produced in man, the species of vector mites, and the geographic locality of naturally infected wild rodents. For example, Kato-like strains produced the classic type of summer scrub typhus which was transmitted by Leptotrombidium akamushi in the Niigata Prefecture. One of the milder winter varieties of scrub typhus seen in the Izu Islands, on Mt. Fuji and in the Kanagawa district was caused by Karp-like strains transmitted by Leptotrombidium pallida. The results of the present study failed to show similar relationships with the Thai strains of R. tsutsugamushi.

An antigenic analysis of 81 of the strains of scrub typhus recovered in Thailand was undertaken by members of the Department of Rickettsial Diseases and Captain Vichai Sangkasuvana of the Thai Component, SEATO Lab, during the period of his training assignment at WRAIR. This report presents the results of the study of the 72 strains which were successfully re-established at WRAIR.

Groups of 3 guinea pigs were inoculated intracerebrally with a spleen suspension from white mice infected with each of the Thai strains and each was bled 4 weeks later. Guinea pigs were similarly inoculated with the Karp, Kato and Gilliam strains. Complement fixation tests were carried out on serum from surviving guinea pigs, using as antigens 2-4 units of partially purified suspensions of the three prototype strains.

Serum from guinea pigs infected with the Karp, Kato and Gilliam strains fixed complement only in the presence of the homologous antigen. On the other hand, considerable variation in the titer and the type of antibody response was seen among the guinea pigs infected with the same Thai strain. In many instances it was not possible to place a strain into a single prototype category on the basis of the highest antibody titer since the predominant antibody response in animals inoculated with the same strain was often to different prototype antigens. Furthermore, all of the guinea pigs receiving the same inoculum did not regularly develop detectable complement-fixing antibodies; one or two animals in certain groups were negative in tests with all three antigens.

For the purpose of this report, the strains were tentatively classified under 8 categories. Table II presents the results of the antigenic analysis of the Thai scrub typhus strains and relates them to their geographic origin. The sorting was done on the basis of the types of antibodies detected in the group as a whole without considering the relative titers or the responses of individual guinea pigs.

TABLE II

GEOGRAPHIC DISTRIBUTION AND ANTIGENIC CLASSIFICATION OF 72 STRAINS OF SCRUB TYPHUS RECOVERED IN THAILAND

ANTIGENIC CATEGORY (CF)	PHYSIOGRAPHIC REGION				
	NORTHERN HILLS AND VALLEYS	KHORAT PLATEAU	SOUTHEAST COAST	CENTRAL VALLEY	TOTAL
Karp	1	11		3	15
Kato	1	3		2	6
Gilliam		8			8
Karp Kato	1	3			4
Karp Gilliam	3	3	1		7
Kato Gilliam		1	1	3	5
Karp Kato Gilliam	3	14		6	23
Ungrouped		3		1	4
TOTAL	9	46	2	15	72

Antibody responses to only one of the three prototype strains were produced by 29 (40%) of the Thai agents. Antibodies detected by two of the prototype strains were evoked by 16 (22%) of the agents and antibodies to all three antigens were found in the sera from guinea pigs inoculated with 32% of the Thai strains. It is not known at this time whether the multiplicity of serological responses in the combined categories represents diversified antigenic composition of pure strains or the recovery of a mixture of strains originally. The following account will signify why isolation of a mixture of strains should be expected. One strain recovered from a rodent trapped at Chong Mek in the Khorat Plateau was classified polytypic. The guinea pigs inoculated with the agent developed Karp, Kato and Gilliam antibodies. The rat was heavily infested with chiggers and two pools were prepared from these ectoparasites. A Karp-like strain was recovered from one pool while a Gilliam-like strain was isolated from the other.

Included in the ungrouped category are the 4 strains which thus far have shown no antigenic relationship to any of the 3 prototype strains. A more extensive study of the ungrouped strains is currently in progress to confirm these findings and to characterize the degree of antigenic heterogeneity among these agents. If additional antigenic types are defined, representative prototypes will be selected and the existence of possible relationships to agents currently included in the various Karp, Kato and Gilliam categories will be determined.

Inspection of the physiographic distribution of the strains included in the different antigenic categories does not reveal any relationship between geographic locality and antigenic type. The strains from the Khorat Plateau were recovered from rodents and chiggers collected in Chong Mek. The distribution of the strains among all 8 antigenic categories indicates the existence of multiple antigenic types in a limited area. The four strains isolated from patients are included under the Northern Hills and Valleys region. Two of these were placed in the Karp-Gilliam category. Another has been classified as a Karp-Kato type and the last as a pure Kato type. Thus, this limited experience does not support the existence of a relationship between antigenic type, severity of disease and geographic localization.

The source of the 11 pools of chiggers from which scrub typhus rickettsiae were recovered is presented in Table III.

Infected chiggers were collected from 7 Rattus rattus (6 strains), 2 Tupaia glis (2 strains) and 1 Bandicoota indica (1 strain). R. tsutsugamushi was isolated from 7 of 21 (33.3%) pools of chiggers infesting scrub typhus-infected rodents, but in only 4 of 108 (3.7%) pools from negative animals. Antigenic analyses were carried out on 6 strains: 2 were classified in the Karp category, 3 as Gilliam-like, and the last in the combined Karp-Gilliam group. It is not possible to comment on the association of antigenic types and species of chiggers at this time, since information on the composition of the positive pools is not yet available.

The relationship between the mouse virulence of the Thai strains and their antigenic classification is presented in Table IV. Most of the agents in the pure Karp and pure Kato categories, and the combined Karp-Kato group manifested 4+ virulence. All mice receiving 0.2 ml of a 10-20% mouse spleen suspension of these strains died by the 14th post-inoculation day. Within the other antigenic categories, the agents varied in their ability to kill mice. Inapparent infections were produced by 11 of the 72 scrub typhus strains. Their presence in mice could be detected only by the results of immunity-challenge tests. Analysis of the data did not reveal any association between mouse virulence, antigenic classification and geographic locality.

TABLE III

RECOVERY OF R. TSUTSUGAMUSHI FROM CHIGGERS
COLLECTED IN THAILAND, NOV 1962-NOV 1963

CATEGORY OF POOLS[+]	COLLECTION REGION NORTHERN HILLS AND VALLEYS	KHORAT PLATEAU	SOUTHEAST COAST	CENTRAL VALLEY	TOTAL
Total Number Inoculated	46	76	0	7	129
Number From Infected Animals	8	11	-	2	21
Positive Pools From Infected Animals	1	6[++]	-	0	7
Positive Pools From Negative Animals	0	4	-	0	4
Total Number Positive Pools	1	10	-	0	11

[+] Pools varied in composition from 80 to 500 chiggers with the majority containing 150 to 250.

[++] Two pools prepared from chiggers collected from a single animal were both infected.

TABLE IV

MOUSE VIRULENCE OF THAI SCRUB TYPHUS STRAINS

ANTIGENIC CLASSIFICATION (CF)	MOUSE VIRULENCE*				TOTAL
	4+	3+	2+	1+	
Karp	11	1	2	1	15
Kato	5		1		6
Gilliam	2	1	3	2	8
Karp Kato	3	1			4
Karp Gilliam	2		4	1	7
Kato Gilliam	1		1	3	5
Karp Kato Gilliam	14		8	1	23
Ungrouped			1	3	4
TOTAL	38	3	20	11	72

* 0.2 ml of 10-20% mouse spleen suspension intraperitoneally.
  4+ All animals died by 14th post-inoculation day.
  3+ All animals died between 14th and 28th post-inoculation days.
  2+ Some animals died during 28 day observation period.
  1+ No animals died; inapparent infection.

It should be pointed out here that there was no relationship between mouse virulence and the severity of the disease produced in man. Three of the strains recovered from Thai patients had 4+ virulence while the fourth had 1+ virulence. Similar variation in the mouse virulence of strains isolated from cases of scrub typhus in Malaya has been reported.

TABLE V

RECOVERY OF R. TSUTSUGAMUSHI FROM SMALL WILD ANIMALS TRAPPED IN THAILAND, NOV 1962-NOV 1963

SPECIES	TRAPPING REGION								TOTAL	
	NORTHERN HILLS AND VALLEYS		KHORAT PLATEAU		SOUTHEAST COAST		CENTRAL VALLEY			
	Pos.Pools* / No.Animals	%	Pos.Pools / No.Animals	%	Pos.Pools / No.Animals	%	Pos.Pools / No.Animals	%	Pos.Pools / No.Animals	%
R. rajah	0/6	0	23/149	15.4	0/17	0	0/9	0	23/181	12.7
T. glis	1/34	2.9	11/85	12.9	1/2	50.0	6/56	10.7	19/177	10.7
R. rattus	2/69	2.9	13/122	10.7	0/24	0	15/119	12.6	30/334	9.0
R. berdmorei	0/1	0	3/14	21.4	1/5	20.0	--		4/20	20.0
M. berdmorei	0/9	0	5/31	16.1	0/27	0	--		5/67	7.5
B. indica	6/35	17.1	0/3	0	0/11	0	0/38	0	6/87	6.9
R. exulans	1/42	2.4	0/5	0	0/2	0	0/3	0	1/52	1.9
R. niviventer	0/2	0	0/5	0	--		0/4	0	0/11	0
Miscellaneous	0/14	0	0/7	0	--		0/5	0	0/26	0
TOTAL	10/212	4.7	55/421	13.1	2/88	2.3	21/234	8.9	88/955	9.2

* Positive pools / Number animals captured

TABLE VI

ANTIGENIC CLASSIFICATION OF STRAINS OF SCRUB TYPHUS RECOVERED FROM SMALL WILD ANIMALS IN THAILAND (NOV 62-NOV 63)

ANTIGENIC CATEGORY (CF)	ANIMAL SPECIES							TOTAL
	R. rattus	R. rajah	T. glis	M. berdmorei	R. berdmorei	B. indica	R. exulans	
Karp	8	4				1		13
Kato	2	2	1					5
Gilliam	1	3	1					5
Karp Kato		3						3
Karp Gilliam		2		1	1			4
Kato Gilliam	2		2		1			5
Karp Kato Gilliam	12	6	3	1			1	22
Ungrouped	1	1	1	1				4
TOTAL	26	21	8	3	2	1	1	62

TABLE VII

MONTHLY INDICES OF R. TSUTSUGAMUSHI INFECTION IN SMALL WILD ANIMALS IN THAILAND, NOV 1962-NOV 1963

MONTH	TRAPPING REGION: NORTHERN HILLS AND VALLEYS Pos.Pools* / No.Animals	%	KHORAT PLATEAU Pos.Pools / No.Animals	%	SOUTHEAST COAST Pos.Pools / No.Animals	%	CENTRAL VALLEY Pos.Pools / No.Animals	%	TOTAL Pos.Pools / No.Animals	%
NOVEMBER	1/36	2.8	1/75	1.3					2/111	1.8
DECEMBER	3/65	4.6	1/23	4.3					4/88	4.5
JANUARY	1/47	2.1							1/47	2.1
FEBRUARY					2/57	3.5			2/57	3.5
MARCH							0/30	0	0/30	0.0
APRIL					0/31	0			0/31	0.0
MAY	0/28	0	16/157	10.1			2/60	3.3	18/245	7.3
JUNE										
JULY	5/31	16.1					4/12	33.3	9/43	20.9
AUGUST			24/87	27.5			3/18	16.6	27/105	25.7
SEPTEMBER	0/5	0					12/114	10.5	12/119	10.1
OCTOBER										
NOVEMBER			13/79	16.5					13/79	16.5
TOTAL	10/212	4.7	55/421	13.1	2/88	2.3	21/234	8.9	88/955	9.2

* Positive pools / Number animals captured. Pools contained tissues from 2-3 animals of the same species. Isolation attempts on individual members of infected pools were not made.

Among the 4 genera of animals from which scrub typhus rickettsiae have been isolated are included, Tupaia, Menetes, Bandicoota and 4 species of Rattus (see Table V). The total numbers of strains recovered from the different species is more a reflection of the degree of success in trapping the animal than an indication of specific host relationships. The rate of isolation of R. tsutsugamushi from Rattus rattus, Tupaia glis and Rattus rajah, which were the species captured most frequently, ranged only from 9 to 12.7%.

The results of antigenic characterizations undertaken on 62 of the 88 strains recovered from wild animals are presented in Table VI. A random distribution of the various antigenic categories among the different animal species is evident. No relationship was found between the species of rodents, the antigenic category of the strains and the physiographic region in which the animal was trapped.

Trapping was not carried out at monthly intervals throughout the year in the areas studied. However, accumulation of the available data summarized in Table VII reveals a suggestive trend in the rate of recovery of R. tsutsugamushi from small mammals in the country as a whole. In general, variation in the monthly indices of scrub typhus infection corresponded with climatic changes influenced by the monsoons. Infection rates during the rainy season which extends from May through October ranged from 7 to 26 percent, while much lower rates were observed during the dry season. Although no specific information is available, it is expected that the rates of infestation of the vertebrate hosts with vector species of Trombiculid mites would follow the same pattern.

In review, an antigenic analysis of 72 strains of R. tsutsugamushi isolated from man, chiggers and rodents in Thailand is being undertaken. On the basis of the complement-fixing antibody response evoked in guinea pigs by these agents, 94% of the Thai strains show antigenic relationships with the Karp, Kato and Gilliam strains. Further studies of the 4 strains which could not be classified in this manner are in progress. The results, thus far, fail to show any relationship between the antigenic category of the strain, physiographic region, species of rodent host, and severity of human disease in Thailand. Thus, the association between antigenic category and epidemiology of scrub typhus in Japan reported by workers in that country does not seem to exist in Thailand.

Factors Influencing the Maintenance, Distribution and Dispersion of Rickettsia rickettsii in Nature.

In the previous annual report (Annual Report 1963-1964, pp 248-255) a description was given of the experimental design used to obtain quantitative data coincidently on the populations of adult and subadult vector ticks, their vertebrate hosts, and Rickettsia rickettsii in a known endemic area. This approach also provided information on the interaction that occurs among the animal, arthropod and microbial components of the

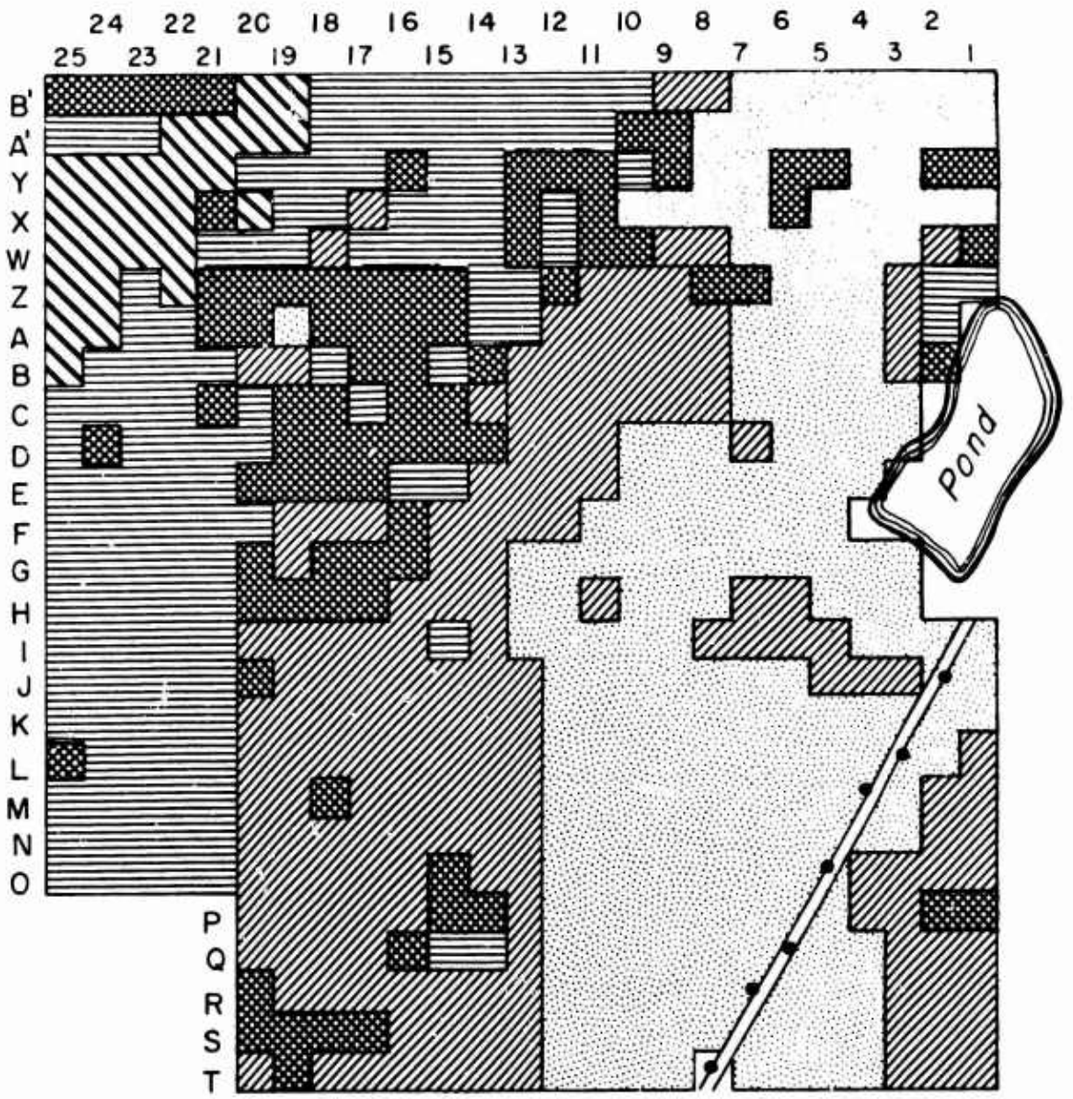
infectious cycle and reveals how they are influenced by environmental conditions. These ecologic studies of Rocky Mountain spotted fever were initiated in March 1963 by members of the Departments of Rickettsial Diseases and Biostatistics, WRAIR, The Department of Biology, Old Dominion College, Norfolk, and the Virginia State Department of Health.

This present report will be limited to a preliminary assessment of the importance of certain factors in the perpetuation of spotted fever rickettsiae. The role of small mammals as hosts for subadult forms of Dermacentor variabilis and the influence of vegetative habitat type on the distribution and density of ticks and small mammals will be evaluated. Although the report in general will cover the period March 1963 through December 1964, certain aspects will be incomplete since some of the laboratory studies are still in progress and a portion of the data requires further analysis. However, the results that are presented will document the value of this investigative approach in providing knowledge about the ecology of tick-borne enzootic diseases that could not be obtained heretofore.

The study area which is located in Montpelier, Virginia, is schematically represented in Figure 1. The distribution of vegetation classified by dominant types, density and height in each of 0.1 acre plots is shown. The original study area was 40 acres but early in 1964 it was increased to encompass 60 acres in order to include more deciduous tall trees as well as a stand of evergreen trees. These are two classes of woody species commonly found in the Piedmont physiographic province. It may be recalled that the Piedmont province of Virginia has a significantly higher rate of occurrence of Rocky Mountain spotted fever than the other physiographic regions. Whenever a comparison between 2 years is made, the results obtained in 1964 have been adjusted to the 40 acre area used in 1963.

The vegetation of the 60 acre study area consists of second and third growth deciduous forest, a small area of evergreen forest and old fields. The deciduous forest which occupies 67% of the area has been classified into 3 types with respect to height of dominant species; viz. low, less than 10 feet, medium, 10 to 25 feet and tall, more than 25 feet. Oak and hickory species are predominant in the deciduous forest, especially in the tall deciduous category. In addition to these trees, other hardwoods commonly found were beech, sweet gum, black gum and tulip poplar. Occasionally, pignut, birch, white ash, sycamore, aspen, willow and elm were also found. In the portions classified medium deciduous were included the above species and ironwood, flowering dogwood, red maple, red-bud, American holly, black cherry, black locus, red cedar, sassafras, alder and greenbrier. The low deciduous category included reproductions of the aforementioned species and the following ground cover species: Japanese honeysuckle, poison ivy, trumpet vine, Virginia creeper, grape vine, blackberry, sumac, coral berry, Christmas fern and other ferns, and ground cedar. The low deciduous portion of

Figure 1



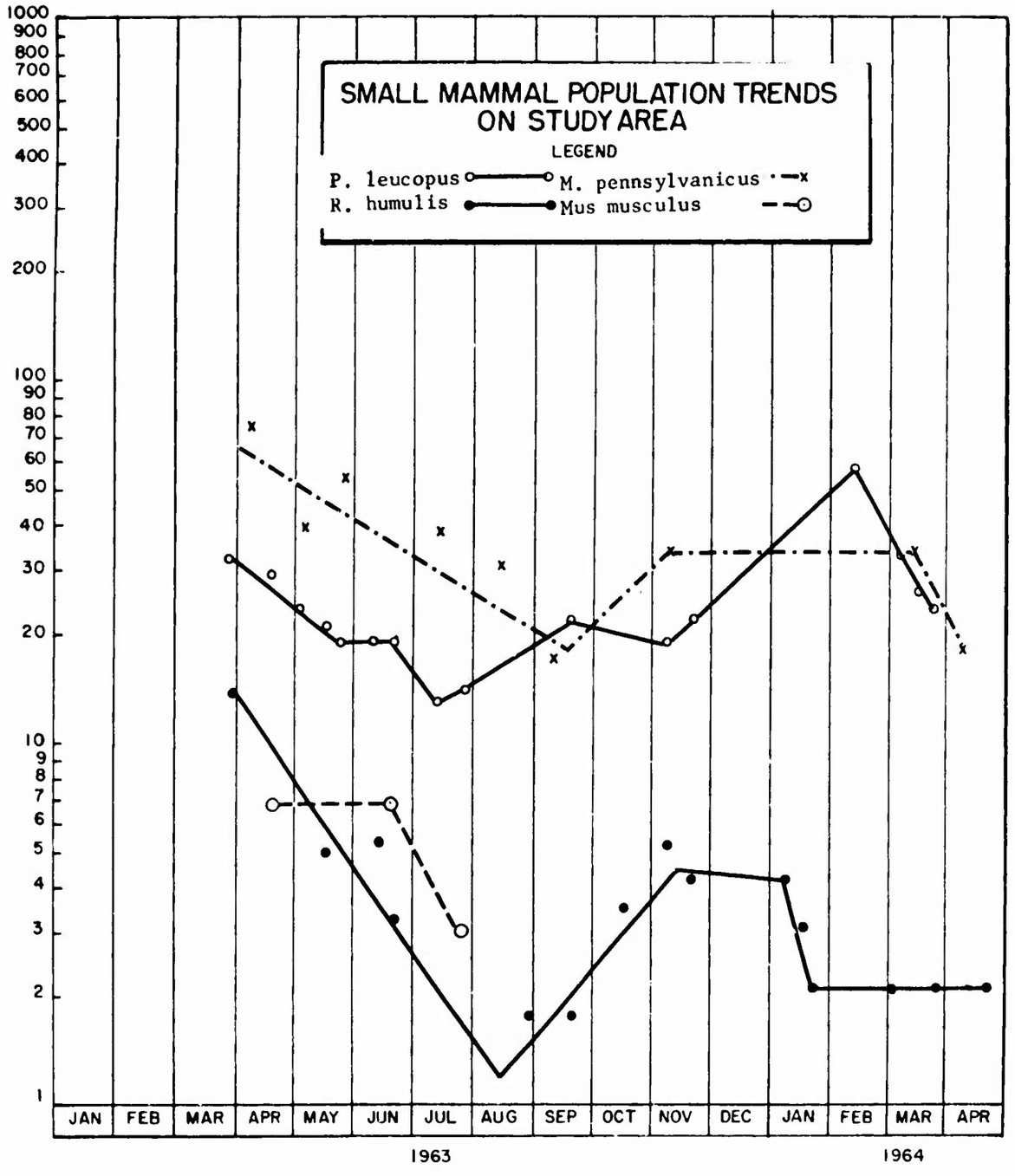
Legend:

- Deciduous, Low
- Deciduous, Medium
- Deciduous, Tall
- Grasses and Herbs
- Evergreens
- Plot Not in Study

Distribution of Vegetation on Study Area by Physiognomic Classification

Montpelier, Virginia 1964

Figure 2


SMALL MAMMAL POPULATION TRENDS
ON STUDY AREA
LEGEND
P. leucopus
M. pennsylvanicus
R. humulis
Mus musculus
1000
900
800
700
600
500
400
300
200
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
JAN
FEB
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
NOV
DEC
JAN
FEB
MAR
APR
1963
1964

the forest borders the fields and represents the advancing woody species. The significance of this vegatative habitat in the distribution of adult and subadult ticks, the wild animal hosts of the ticks and the spotted fever rickettsiae in the study area will be described below. The evergreen forest contained yellow pine, Virginia and loblolly pines with extensive invasions of deciduous reproduction. The old fields, which constituted the remaining 33% of the study area were covered with grasses and a variety of herbs. These fields have lain fallow for 5 to 10 years. Brome grass is the dominant domestic grass with timothy and orchard grass and red clover occurring in low density. Wild grasses were also present.

During 1963 and 1964, 14 different species of mammals were trapped in the study area. Only two species were trapped with sufficient frequency to permit estimation of their populations by mark-recapture methods; the meadow vole, Microtus pennsylvanicus, and the white-footed mouse, Peromyscus leucopus (see Figure 2). The populations of the harvest mouse, Reithrodontomys humulis, and the house mouse, Mus musculus also represented in the same illustration, were calculated by ratio estimates. The values indicated by the curves represent the estimated numbers of animals in a 40-acre area which is the usual way of presenting population density data. Analyses are in progress which will indicate the density of the different animal species present in their preferred vegetative habitats. This latter method of population estimates should provide a more meaningful evaluation of the importance of the small mammals in the survival of R. rickettsii.

The results of complement fixation tests for spotted fever antibodies performed on sera collected from the wild animals trapped in the study area during 1963 and 1964 are presented in Table VIII. Serological evidence of Rocky Mountain spotted fever infection was found in only 4 of the species examined in 1963. Spotted fever complement-fixing antibodies were found in 49% of the Peromyscus and 7% of the Microtus in 1963. In 1964, although more animals were trapped and examined, the rate of infection in the Peromyscus decreased to 27% while that in Microtus remained essentially unchanged. Marked differences were found also in the rate of infection of the harvest mouse, Reithrodontomys. In 1963, 1/3 of the animals tested had complement-fixing antibodies while in 1964 no positive reactors were found. The only other species of small mammal shown to be infected in 1964 was Pitymys, the pine vole. Infection of two species of medium-sized mammals, the cottontail rabbit, Sylvilagus, and the racoon, Procyon, was found for the first time during 1964.

TABLE VIII

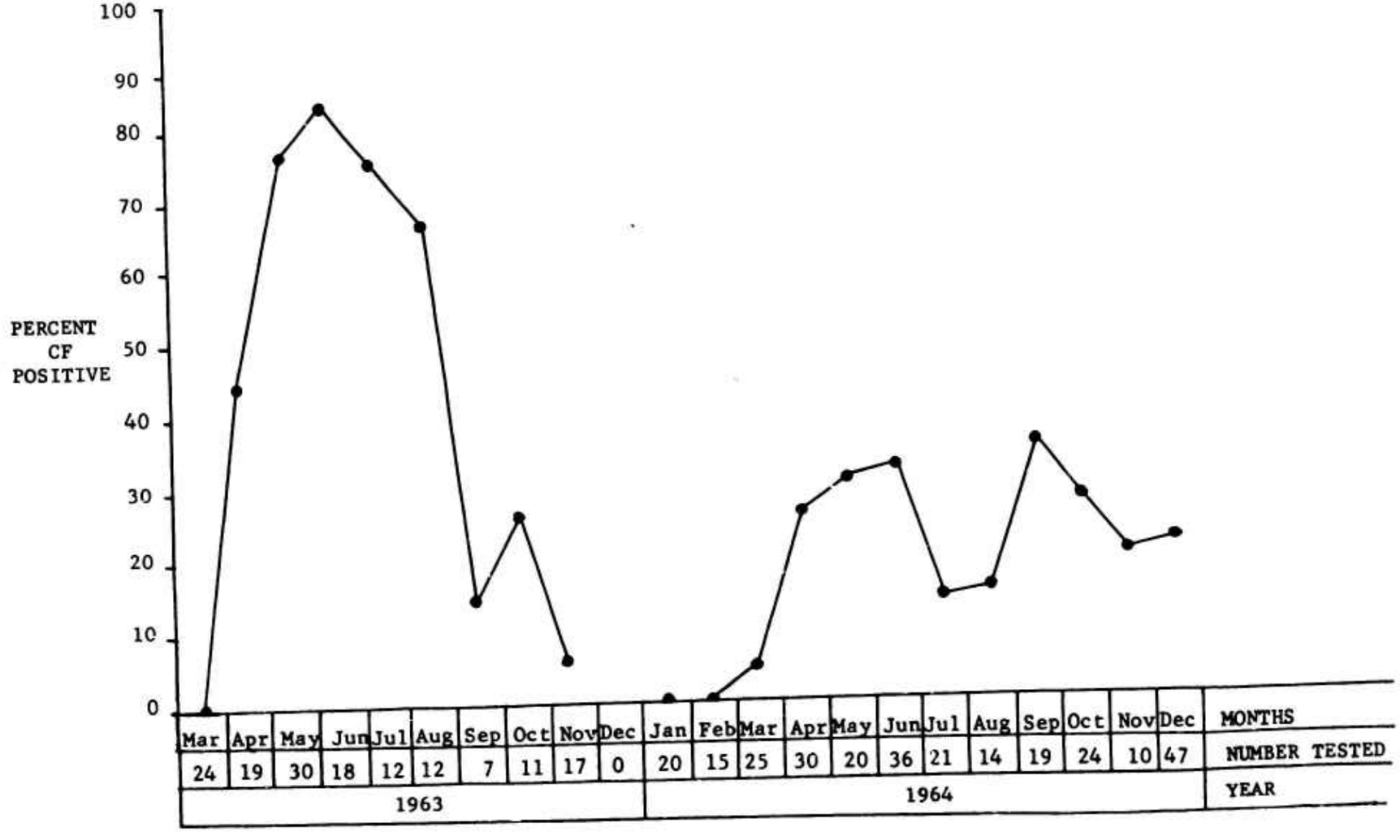
SEROLOGICAL STATUS OF SMALL ANIMALS TRAPPED IN MONTPELIER STUDY AREA, MARCH 1963-DECEMBER 1964

Species	Animals with Spotted Fever CF Antibodies 1963 Number Tested	1963 Number Positive	1963 Percent Positive	1964 Number Tested	1964 Number Positive	1964 Percent Positive
Peromyscus	94	46	49	142	38	27
Microtus	56	4	7	73	4	6
Reithrodontomys	15	5	33	46	0	--
Blarina	16	2	13	3	0	--
Pitymys	2	0	--	5	1	20
Sylvilagus	2	0	--	4	2	50
Procyon	1	0	--	11	2	18
Glaucomys	5	0	--	17	0	--
Didelphis	5	0	--	7	0	--
Mus	5	0	--	6	0	--
Tamias	4	0	--	4	0	--
Mephitis	--	--	--	3	0	--
Sorex	1	0	--	0	--	--
Totals	206	57	28	321	47	15

The course of Rocky Mountain spotted fever infection in the Peromyscus population during 1964 was different from that observed in 1963. The curve depicted in Figure 3 indicates the percent of animals trapped each month having detectable spotted fever complement-fixing antibodies. Positive reactors were first detected in March 1964, a month earlier than in 1963. However, the maximum rate obtained in September 1964 was 37% as compared to 83% in June of the previous year. In 1963, no positive reactors were encountered after November, whereas in November and December of 1964, 20 to 21% of the animals still had demonstrable antibodies.

Figure 3

ROCKY MOUNTAIN SPOTTED FEVER INFECTION OF PEROMYSCUS IN MONTPELIER STUDY AREA



The earlier occurrence of seropositive Peromyscus in 1964 is in accord with the earlier appearance of D. variabilis larvae on the small mammals in late January (see Figure 4). Infestation of animals in 1963 (Annual Progress Report 1963-1964, Fig 3, p 252) was not detected until March. The bimodal character of the infestation rate curve for the larvae was apparent in both years. The rates, however, were higher in 1964 and larvae were found on animals later in the year. Essentially the same observations apply to the infestation rates of the nymphs in 1964. The second peak of nymphal activity present in 1964 was not seen in 1963. Nymphal forms were not found on animals after August of 1963, while in 1964 they were encountered in October and again in December. The higher rates and longer persistence of infestation in 1964 appear to be due to differences in climatic conditions between the two years. Frequent occurrence of overcast days and rainy weather in 1964 provided a high humidity which is favorable to the survival of subadult forms. In contrast, almost drought or near drought conditions existed throughout most of the late spring and summer of 1963.

Although the host range of subadult D. variabilis included 7 species of small mammals trapped in the Montpelier study area, 94 to 97% of the larvae and nymphs were found on Peromyscus and Microtus. Examination of the frequency of occurrence of the immature stages on these two animal species revealed that nymphs have a marked predilection for feeding on Microtus, but will frequently infest Peromyscus. Larvae, on the other hand, appear to prefer Peromyscus, but also will feed readily on Microtus.

The population indices of adult D. variabilis during their periods of activity in 1963 and 1964 are compared in Figure 5. The trends over the 2 years were generally similar with the following important differences. Adult activity in 1964 began almost 3 weeks later than in 1963 and ended slightly earlier. Peak activity occurred slightly later in 1964 and the maximum number of active ticks found in 1964 was 1½ times that of 1963. The average number of active ticks per acre in 1963 was 1,400 and in 1964, 2,225.

Statistical analyses carried out on the distribution of the adult ticks in the different vegetative habitats in 1963 show the greatest adult tick density was present in the low deciduous forest. In Table IX it can be seen that ticks were almost twice as abundant in this habitat as in the fields covered with the grasses and herbs, and relatively infrequent in the other woody types.

The distribution of the adult ticks is determined by the distribution of the immature stages. This in turn is related to the vertebrate host density and suitable conditions for tick survival in the different vegetative types. As might be expected, the distribution of the subadult ticks on the two principal animal hosts generally corresponded to that of the adult ticks. Table X shows the average number of immature ticks occurring on Peromyscus and Microtus in each of the

Figure 4

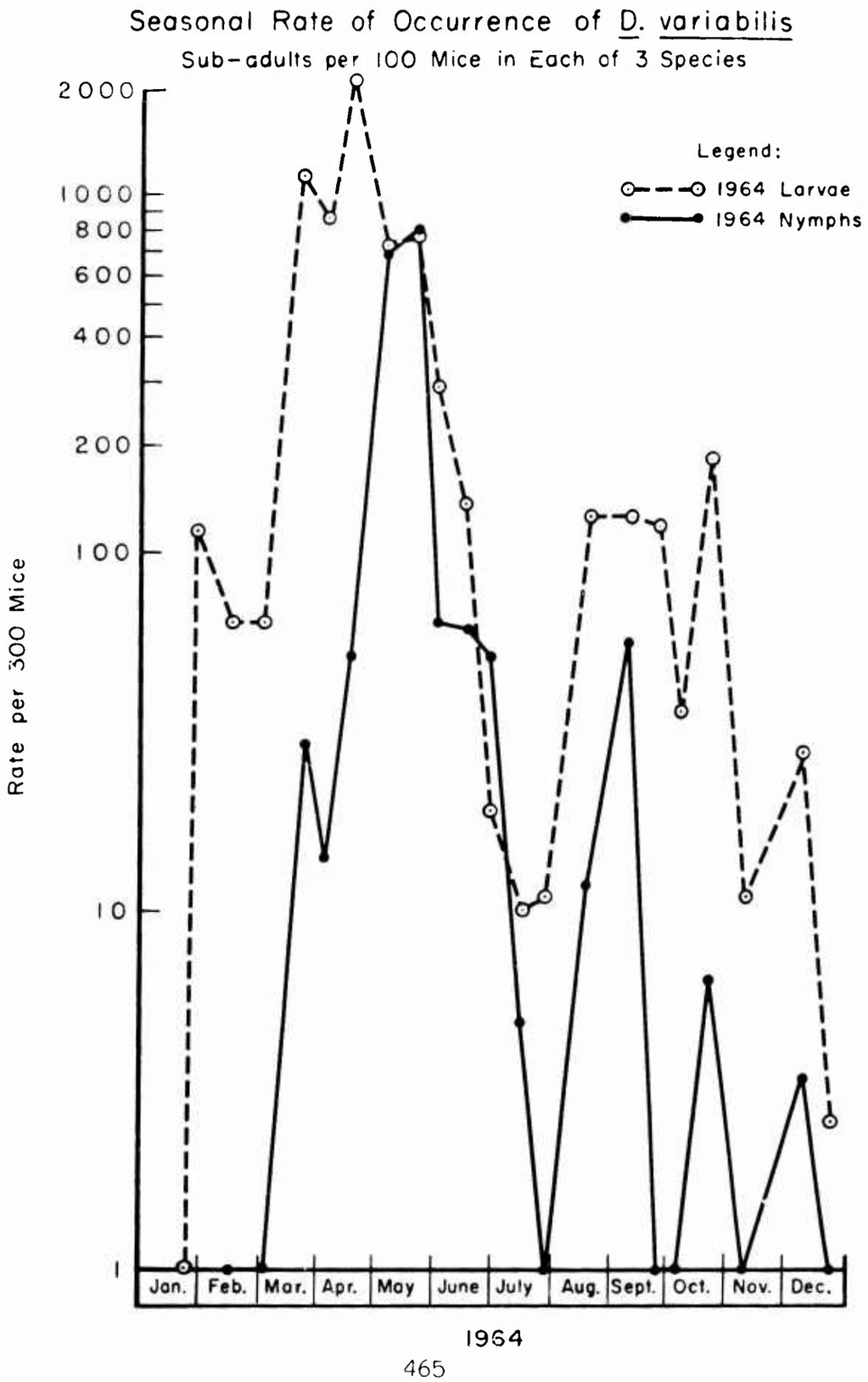
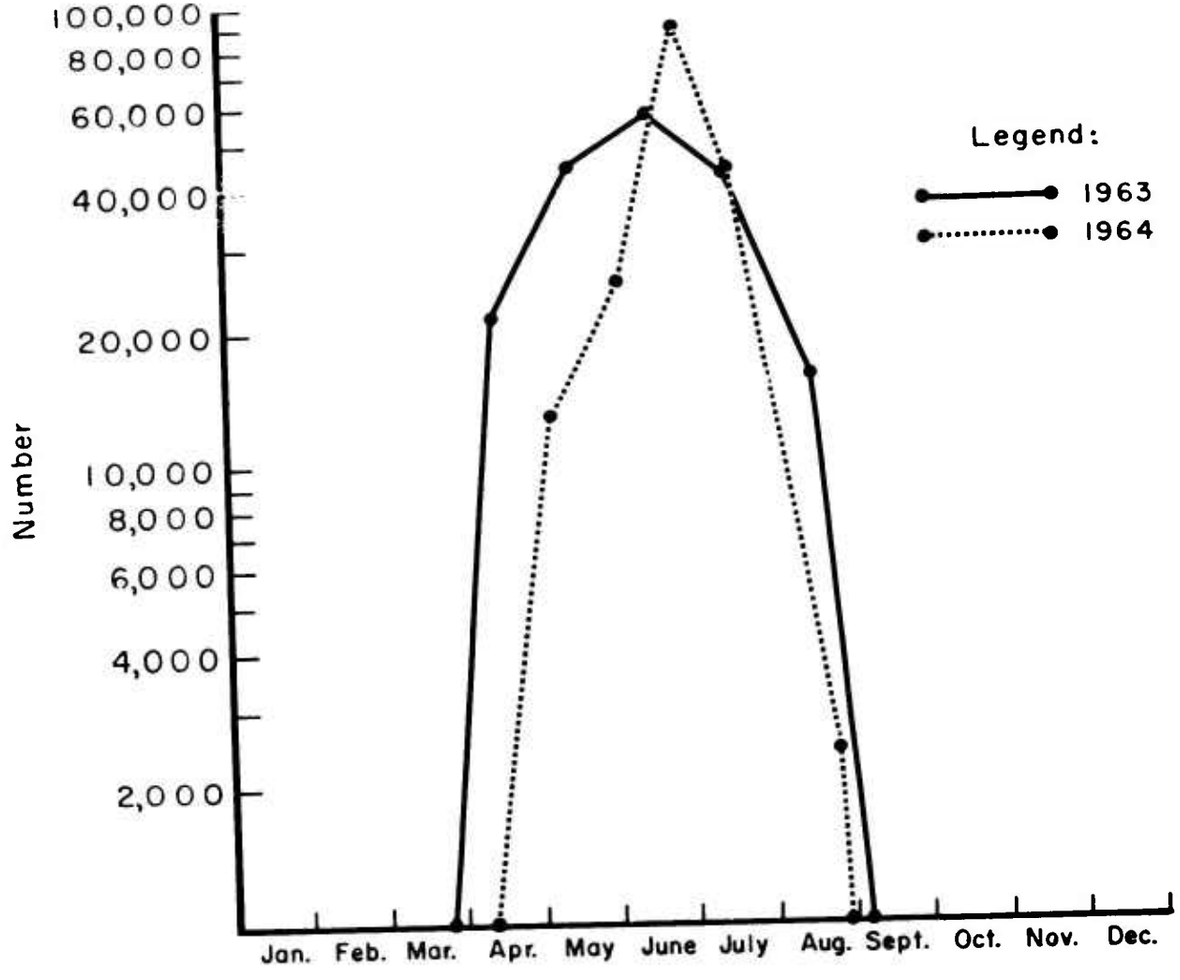

Seasonal Rate of Occurrence of D. variabilis
Sub-adults per 100 Mice in Each of 3 Species
Legend:
1964 Larvae
1964 Nymphs
Rate per 300 Mice
2000
1000
800
600
400
200
100
10
1
Jan.
Feb.
Mar.
Apr.
May
June
July
Aug.
Sept.
Oct.
Nov.
Dec.
1964

Figure 5

Population Indices of Total Number of Adult *D. variabilis* by Mark and Recapture

Montpelier Study Area, 1963-64



vegetative types. A statistically significant greater density was present in the low deciduous vegetative habitat. The tall deciduous category was excluded from consideration because of the infrequent trapping of tick-infested small mammals in that habitat. Irrespective of the vertebrate host, the highest density was found in the low deciduous portions of the forest. The fields with the grasses and herbs habitat ranked second and the medium deciduous forest last. This ranking of vegetative types with respect to density of immature forms, parallels that presented earlier for adult D. variabilis. Considerably higher infestation rates were found on Peromyscus in all the habitats.

Although 6 species of ticks have been recovered from small and medium-sized animals trapped in the study area, and by flagging, R. rickettsii have been found only in D. variabilis. Other species examined and found negative thus far include Amblyomma americanum, Haemaphysalis leporis-palustris, Ixodes dentatus, Ixodes cookei and Ixodes texanus. The rate of Rocky Mountain spotted fever infection of the 3 stages of D. variabilis collected in 1963 and during the first half of 1964 is presented in Table XI. Laboratory studies on the ticks collected during the latter half of 1964 are still in progress. The infection rates in the adults and nymphal forms were somewhat lower in 1964 than were found in 1963, but a final analysis must await the completion of last year's investigations.

An assessment of the importance of certain factors in the survival of spotted fever rickettsiae in an endemic area can be made on the basis of the information presented above. Included in the area under study are 5 major vegetative habitats. The grasses and herbs category of the fields comprises 1/3 of the area. The woods with representatives of 3 deciduous and 1 evergreen category make up the remaining 2/3. The low deciduous species border the fields and encroaches upon them. Adult D. variabilis are distributed in all of the vegetative types but the greatest density occurs in the low deciduous forest habitat. Of the 14 species of mammals known to be present in the area, Microtus pennsylvanicus and Peromyscus leucopus have the highest populations and are the most important hosts for subadult ticks. Microtus is found principally in the fields, and Peromyscus in woods, but some overlap of these species occurs in portions of the low deciduous forest which borders on the fields. Although Peromyscus infested with subadult D. variabilis have been found in all the vegetative habitats and Microtus in all except the stand of evergreen trees, significantly higher rates of infestation occurred on both animals in the low deciduous forest. In all habitats Peromyscus is more heavily infested than Microtus. The distribution of animals known to be infected with Rocky Mountain spotted fever generally corresponds to the distribution of tick-infested animals. Peromyscus sustained the highest rates of infection and infection occurs most frequently in the low deciduous forest. Adult ticks infected with spotted fever rickettsiae were collected on flags principally in the low deciduous forest. Animals carrying infected immature forms were also

trapped principally in this same habitat.

Thus, with respect to enzootic Rocky Mountain spotted fever in this endemic area, this simple correlative review indicates that low deciduous woody vegetation represents the high risk habitat and the Peromyscus is the most important small vertebrate host in this ecological circumstance. The low deciduous habitat constitutes a substantial area of the eastern United States. Recent logging, pulp and other commercial tree-cutting operations, as well as the tendency to allow farm land to lie fallow for long periods, favor the creation and maintenance of such habitats. The ecological relationships presented may have epidemiological significance in explaining the distribution of cases of Rocky Mountain spotted fever.

TABLE IX

ESTIMATED POPULATIONS OF ACTIVE ADULT D. VARIABILIS IN THE VEGETATIVE TYPES IN MONTPELIER STUDY AREA, 1963

Vegetative Type	Acres	Average Active Population Indices
Deciduous low	16.8	25,370
Grasses and herbs	17.1	14,540
Deciduous medium	4.8	2,500
Deciduous tall	1.3	490
Totals	40.0	42,900

TABLE X

SUBADULT D. VARIABILIS INFESTATION OF SMALL MAMMALS BY VEGETATIVE TYPE, MONTPELIER 1963

Species	Number of Larvae and Nymphs Per Animal Vegetative Types Deciduous Low	Medium	Grasses and Herbs
Peromyscus	7.100	3.667	4.708
Microtus	3.690	2.647	3.304

TABLE XI

INFECTION OF D. VARIABILIS WITH SPOTTED FEVER GROUP RICKETTSIAE IN MONTPELIER STUDY AREA

Collection Period	Developmental Stage	Number Tested	Number Positive	Percent Positive
1 Apr - 15 Oct 63	Adult	611	30	4.9
	Nymph	163	6	3.7
	Larvae	62	1	1.6
15 Jan - 15 Jul 64	Adult	403	6	1.5
	Nymph	101	2	2.0
	Larvae	1058	25	2.4

Q Fever Vaccine Study.

The Department of Rickettsial Diseases is participating with other governmental agencies and academic institutions in a collaborative project sponsored by the Commission on Rickettsial Diseases to evaluate the relative efficacy of Q fever phase I and phase II vaccines. The purpose of the studies undertaken by this Department was to evaluate various tests to determine which serologic response in man would best define the existence of a satisfactory level of immunity following vaccination. In addition, the serological procedures would be evaluated for their applicability for vaccine potency assays in animals and for sero-epidemiological investigations. Initially, sera collected from recipients of the vaccines and corresponding control subjects were to be tested for CF antibodies and agglutinating antibodies if a reliable test could be found for the latter reaction. This report presents the results of efforts to prepare and standardize reagents necessary for the characterization of the serologic response of volunteers included in the project.

Table XII presents a list of the strains of Coxiella burnetii used for the production of antigens, passage history of each strain and intended use in the vaccine trial. Formalinized yolk sac homogenates corresponding to the same passage level of the Henzerling and Nine Mile strains used respectively for preparation of vaccine and challenge material, as well as low and high-egg passage Ohio strains were provided by the Department of Biologics Research, WRAIR. The designation of early egg passage material as phase I and later egg passages as phase II was made advisedly since both phases were undoubtedly present with the possible exception of Nine Mile phase II which is presumably pure. The phase description of the antigens, therefore, refers to the predominant component. Purified suspensions of Q fever rickettsiae were prepared by Ormsbee's method (J. Immunol. 88:100-108, 1962) which employs differential centrifugation in M/1 KCl and ether extraction. Characterization of the antigens with respect to nitrogen content, yolk sac tissue content and rickettsial complement-fixing antigen titer has not yet been completed. It had been planned to determine the relative concentration of the phase I and phase II organisms in the different preparations using density gradient centrifugation (Hoyer, B.H. et al., Nature, 197:573-574, 1963) but this did not prove to be feasible (vide infra).

In order to standardize the different Q fever antigens and define their relative homologous and heterologous reactivity, groups of 10 guinea pigs were immunized with each of the CF antigens. Each animal received intraperitoneally a single dose containing approximately 200 μg of the killed purified suspensions. Sera were collected on the 15th post-inoculation day when only phase II CF antibodies would be expected to be present, and again on the 50th day when both phase I and II antibodies are usually demonstrable. The results of CF tests with 4 units of the phase I and II complement-fixing antigens and the 15th and 50th day sera from animals receiving the corresponding homologous antigens are summarized in Tables XIII, XIV and XV.

TABLE XII

STRAINS OF COXIELLA BURNETII TO BE USED IN VACCINE EVALUATION TRIALS

STRAIN	PHASE*	PASSAGE HISTORY**	USE
Henzerling	I	GP-1-EP-5	Phase I Vaccine
Henzerling	II	EP-23	Phase II Vaccine
Nine Mile	I	GP-306-EP-5	Phase I Challenge Seed
Nine Mile	II	EP-88	Phase II Challenge Seed
Ohio	I	EP-5	Control Antigen for Serologic Testing Only
Ohio	II	EP-29	

* Since all of the materials listed are mixtures of Phase I and Phase II organisms with the exception of Nine Mile II which is presumably pure, the phase designated indicated the predominant component.

** The number following GP signifies the number of successive passage in guinea pigs and those following EP, the number of passages in embryonated eggs.

TABLE XIII

SEROLOGICAL RESPONSE OF GUINEA PIGS INOCULATED INTRAPERITONEALLY WITH A SINGLE DOSE OF HENZERLING Q FEVER ANTIGEN

GUINEA PIG SERA		CF ANTIBODY TITERS					
		HENZERLING I GP-1-EP-5 (4 units)			HENZERLING II EP-23 (4 units)		
Inoculum	Day of Bleeding	Range	Mean	Conversion*	Range	Mean	Conversion
Henzerling I (149 µg)	15	<5	<5	0/10	640-1280	1200	10/10
	50	40-160	52.8	10/10	20-320	171	10/10
Henzerling II (179 µg)	15	<5	<5	0/10	80-640	278	10/10
	50	<5-20	3.7	6/9	20-640	85.7	9/9

* Number of guinea pigs developing complement-fixing antibodies / Number of guinea pigs inoculated.

TABLE XIV

SEROLOGICAL RESPONSE OF GUINEA PIGS INOCULATED INTRAPERITONEALLY WITH A SINGLE DOSE OF NINE MILE Q FEVER ANTIGEN

GUINEA PIG SERA		CF ANTIBODY TITERS TEST ANTIGENS					
		Nine Mile I (GP-306-EP-5) 4 units			Nine Mile II (EP-88) 4 units		
Inoculum	Day of Bleeding	Range	Mear	Conversion*	Range	Mean	Conversion
Nine Mile I (147 μg)	15	<5-40	25.8	9/10	<5-320	110	9/10
	50	<5-160	16.3	7/9	<5-40	8.6	6/9
Nine Mile II (228 μg)	15	<5	<5	0/10	10-80	37.3	10/10
	50	<5	<5	0/10	<5-20	2.4	6/10

* Number of guinea pigs developing complement-fixing antibodies / Number of guinea pigs inoculated.

TABLE XV

SEROLOGICAL RESPONSE OF GUINEA PIGS INOCULATED INTRAPERITONEALLY WITH A SINGLE DOSE OF OHIO Q FEVER ANTIGEN

GUINEA PIG SERA		CF ANTIBODY TITERS					
		OHIO I EP-5 (4 units)			OHIO II EP-29 (4 units)		
Inoculum	Day of Bleeding	Range	Mean	Conversion*	Range	Mean	Conversion
Ohio I (163 µg)	15	<5-20	10.1	8/10	<5	<5	0/10
	50	<5-80	33.4	8/9	<5-40	16.7	8/9
Ohio II (203 µg)	15	<5-40	5.6	6/10	<5	<5	0/10
	50	40-320	117	9/9	40-160	80	9/9

* Number of guinea pigs developing complement-fixing antibodies / Number of guinea pigs inoculated.

Guinea pigs inoculated with Henzerling phase I developed higher antibody titers than animals receiving the phase II suspensions (see Table XIII). As expected, the 15-day sera obtained from animal in both groups did not fix complement in the presence of the phase I antigen. The reactivity between the phase I antigen and the 50-day sera from guinea pigs immunized with the Henzerling phase II suspension undoubtedly is due to the antibody response to the phase I content of the inoculum.

The reactivity demonstrated between the 15-day sera from the Nine Mile phase I immunized guinea pigs and the Nine Mile phase I antigen shown in Table XIV can not be readily explained at this time. Two possible explanations can be offered. The fixation of complement may be due to reaction between the phase II component of the Nine Mile phase I antigen and phase II antibody in the sera; or may be attributed to presence of phase I antibody in the early specimens. Neither reason is wholly satisfactory and other serological tests are in progress to resolve the problem. Animals immunized with the phase II antigen did not develop demonstrable phase I antibody which is in accordance with the presumed purity of the Nine Mile phase II strain.

The results of the tests between the Ohio antigens and immune sera were completely unexpected. Indeed, the data presented in Table XV reveal that the strain labeled Ohio II (EP-29) had the serologic and immunizing characteristics of phase I material and the strain designated phase I behaved as phase II. Records of the processing of the various reagents after receipt of seed material were carefully reviewed. At no time were both antigens handled in the laboratory at WRAIR on the same day, thereby obviating an inadvertent mixup of the strains here. Efforts are continuing to explain the peculiar results and to have available reagents of known passage history which give expected reactivity.

In general the results of the homologous tests indicate that the phase I antigens were more potent antibody producing substances than the same strains containing organisms principally in phase II. Grid-type complement fixation tests to evaluate the heterologous reactivity between the antigens and immune sera are in progress and cannot be reported at this time. When homologous and heterologous reactivity of the reagents has been completely characterized, the antigens will be used to test serum specimens collected from volunteers in the vaccine trial.

In the course of initial studies with a CF antigen prepared from Nine Mile I (GP-306-EP-5), results were obtained which suggested that the 5th egg passage (EP) of the Nine Mile strain had a significant phase II component. Table XVI summarizes the results of tests with this antigen and compares it with the reactivity of a similar antigen prepared from the 4th egg passage, kindly supplied by Dr. R. Ormsbee, Rocky Mountain Laboratory. The Nine Mile I EP-4 antigen reacted only with the immune serum containing phase I and phase II antibodies and not with sera having only phase II antibody. These results are comparable to the reactivity of the Henzerling I (GP-1-EP-5) antigen which is known to be predominantly

in phase I with a very minor component of phase II organisms. The 5th egg passage Nine Mile antigen, however, did fix complement with the phase II antibody signifying the presence of a phase II component in the antigen. The Nine Mile II (Lederle) antigen, a commercial product which is wholly in phase II, reacted with all three immune sera as expected.

TABLE XVI

COMPARISON OF SEROLOGICAL REACTIVITY OF NINE MILE PHASE I EP-4 and EP-5

ANTIGENS	ANTISERA PHASE I AND II Q43-RSA-111 (CALIFORNIA)*	PHASE II GP POOL A HENZERLING II (14 & 21 DAY)	PHASE II GP POOL T-1 HENZERLING II (21 & 28 DAY)
Henzerling I (GP-1-EP-5)	16/512**	<8/<8	<8/<40
Nine Mile I* (GP-306-EP-4)	32/512	<8/<4	<8/<20
Nine Mile I (GP-306-EP-5)	4/512	4/16	8/160
Nine Mile II (Lederle)	160/512	160/32	160/320

* Supplied by Dr. R. Ormsbee, Rocky Mountain Laboratory.
** Antigen titer / Serum titer.

Since the 5th egg passage of the Nine Mile strain was to be used as the seed suspension for infectious challenge in the vaccine trial, it was imperative that the initial findings be unequivocally documented. Attempts were made to separate the two phases of Q fever rickettsiae employing a method of density gradient centrifugation reported first by Hoyer et al. (Nature, 197:573-574, 1963). This technique had been used successfully by Ormsbee et al. (Am. J. Hyg., 79:154-162, 1964) to characterize the phase composition of three strains of Coxiella burnetii used in early vaccine studies by Smadel, Benenson and Tigertt. Briefly the procedure was to suspend a purified suspension of the rickettsiae in a solution of cesium chloride (specific gravity 1.3) and centrifuge the mixture at 100,000 xg for 16 hours. These conditions resulted in the establishment of a density gradient ranging approximately from 1.2 to 1.4 in the tube. The phase I rickettsiae localized toward the bottom of the gradient where the density was about 1.3 and the phase II organisms were found at the surface.

Preliminary trials showed that it was not possible to reproduce completely the results of other workers employing the conditions stipulated by them. Even when the duration of centrifugation was extended to as long as 47 hours, the majority of the phase II organisms sedimented into the depth of the gradient where they were indistinguishable from the phase I rickettsiae. However, a portion of the phase II rickettsiae were found on the surface of the gradient. Table XVII presents the results of attempts to characterize the phase composition of Henzerling I (GP-1-EP-5) and Henzerling II (EP-23). The purified suspensions of these strains were prepared from bottles of dried killed vaccine prepared by the Department of Biologics Research, WRAIR. The complement-fixing activity of the reconstituted whole vaccines and of the fractions recovered from cesium chloride gradients is given. The total volume of the vaccine processed and the final volume in which the fractions of the gradient were suspended are presented to provide an index of the relative amount of antigenic material in the materials tested. It can be seen in Table XVII that separation of the two phases of the Henzerling I (GP-1-EP-5) vaccine occurred as expected. Although the whole vaccine reacted only with the phase I-II immune sera, a small amount of phase II antigen was recovered from the surface of the gradient. This fraction fixed complement in the presence of phase II serum. The phase I rickettsiae sedimented toward the bottom of the gradient. Density gradient centrifugation of the Henzerling II (EP-23) vaccine, however, resulted in the distribution of the phase II antigenic activity between the top and bottom zones. This unpredicted sedimentation of the phase II component illustrates the problem encountered and makes the technique impractical for estimation of the relative concentration of the two phases in a given preparation.

TABLE XVII

COMPLEMENT FIXING ACTIVITY OF Q FEVER ANTIGENS AND FRACTIONS RECOVERED FROM CESIUM CHLORIDE GRADIENTS

STRAIN	ANTIGENS TYPE	VOLUME(ML)	TITER WITH 4 UNITS ANTISERA PHASE I AND II (Q43-RSA-111)	PHASE II (GP POOL A)
HENZERLING I GP-1-EP-5 (LOT 1 VACCINE)	WHOLE	36	2	0
	GRADIENT SURFACE	1	1	1
	GRADIENT BOTTOM ZONE	1	≥64	<4*
HENZERLING II EP-23 (DP-5)	WHOLE	36	4	2
	GRADIENT SURFACE	1	4	4
	GRADIENT BOTTOM ZONE	1	≥64	≥64

* 1:2 dilution anticomplementary

Since some portion of the phase II component did move to the surface of the gradient, the procedure was useful in demonstrating the presence of a phase II fraction in the 5th egg passage Nine Mile challenge seed. The results of cesium chloride density gradient centrifugation of Nine Mile I, EP-4 and EP-5 and Nine Mile II, EP-88 is given in Table XVIII. No phase II component was found in the 4th egg passage of Nine Mile I, but was demonstrable in material collected at the surface of the gradient containing the 5th egg passage. In addition, a zone midway between the surface and the bottom zone similarly had phase II CF activity. The phase II rickettsiae of the Nine Mile II, EP-88 antigen again were distributed at the surface of the gradient, as well as in the bottom zone.

TABLE XVIII

COMPLEMENT FIXING ACTIVITY OF Q FEVER ANTIGENS AND THEIR FRACTIONS RECOVERED FROM CESIUM CHLORIDE GRADIENTS

STRAIN	ANTIGENS TYPE	VOLUME (ML)	TITER WITH 4 UNITS ANTISERA PHASE I AND II (Q43-RSA-111)	PHASE II (GP POOL A)
NINE MILE I* GP-306-EP-4 (RSA-139)	WHOLE	1.0	32	<8
	GRADIENT SURFACE	4.0	0	0
	GRADIENT BOTTOM ZONE	4.0	4	0
NINE MILE I GP-306-EP-5 (SEED)	WHOLE	2.8	64	16
	GRADIENT SURFACE	1.0	4	4
	GRADIENT MID ZONE	0.5	2	2
	GRADIENT BOTTOM ZONE	1.0	32	0
NINE MILE II EP-88 (SEED)	WHOLE	3.5	16	16
	GRADIENT SURFACE	1	8	8
	GRADIENT BOTTOM ZONE	0.5	16	16

* Supplied by Dr. R. Ormsbee, Rocky Mountain Laboratory.

Although the cesium chloride density gradient centrifugation did not give the precise separations hoped for, it did provide the means for unequicoval demonstration of the partial conversion of the Nine Mile strain from phase I to phase II in the course of a single egg passage.

Investigations to evaluate the sensitivity and specificity of the agglutination-resuspension (AR) test for Q fever antibodies (Ormsbee, R., J. Immun. 29:159-166, 1964) are in progress. Not enough experience has been gained as yet to permit complete reporting at this time. The initial studies indicate that the degree of purity of the rickettsial suspensions markedly influences the stability and sensitivity of the antigen. Although the molar salt-ether extraction method can provide highly purified antigens, the number of replications of the different steps of the process necessary to obtain the same degree of purity in different preparations varies, making the procedure impractical for use in large-scale production. The AR test requires that serial dilutions of the serum under examination be made in 25% normal bovine serum in 0.15 M NaCl. To date, extensive CF testing of numerous bovine sera has shown all of them to fix complement with one or more of a variety of Q fever antigens prepared from different strains. It has not been possible to substitute appropriate concentrations of crystalline bovine albumen or bovine plasma Fraction V for the "normal" serum in the diluent and get the enhancement of sensitivity seen with the 25% bovine serum diluent. These observations suggest that the increased sensitivity attributed to the diluent is due to low levels of antibody in the bovine serum diluent which are not in sufficient concentration to agglutinate the suspension. The antigen sensitized in this manner would be more readily agglutinated in the presence of minimal amounts of antibody in the sera under test. Efforts are being directed toward confirmation of these preliminary observations as well as to the search for other more acceptable and controllable means of increasing the sensitivity of the agglutination reaction.

Preparation of Scrub Typhus Antigens.

Numerous methods have been developed which provide highly purified suspensions of typhus group, spotted fever group and Q fever rickettsiae. However, these procedures cannot be satisfactorily applied to the purification of Rickettsia tsutsugamushi owing to the extreme fragility and lability of the organism. Suspensions of scrub typhus rickettsiae, free of contaminating yolk sac tissue, are needed for use as antigens in complement fixation and other serological tests. A sensitive and specific complement fixation test could be used for (1) the laboratory diagnosis of human infections, (2) as a sero-epidemiological tool for the recognition of recent and past infections in human populations and detection of enzootic foci and (3) for characterization of the immune response of animals following experimental infections. In addition, highly purified suspensions are a necessary prerequisite for investigations concerned with development of a vaccine for protection of man against disease.

The method developed initially by Yamamoto, T. (Jap. J. Exp. Med., 28:329-336, 1958) and recently modified by other Japanese workers, which employs extraction of infected yolk sac suspensions with a cation-exchange resin (Amberlite), bovine serum albumin precipitation and serial differential centrifugation, has been studied intensively in this laboratory over the past 2 years. Although it has been possible to obtain suspensions of intact scrub typhus rickettsiae relatively free of yolk sac tissue, these antigens have undesirable properties which make them unsatisfactory for certain studies. Suspensions of the Gilliam, Karp and Kato strains purified by the Amberlite method were used successfully as complement-fixing antigens in tests with sera from guinea pigs inoculated intracerebrally with spleen homogenates from mice infected with scrub typhus strains recovered in Thailand. The results of the antigenic analysis of the Thai strains is described elsewhere in this report.

Problems encountered with the Amberlite purified antigens were attributed to anticomplementary activity, residual yolk sac components and differences in the physical properties of the Gilliam, Karp and Kato strains. The antigens were markedly anticomplementary (AC) when suspended in the sucrose-phosphate buffer diluent recommended by the Japanese workers. The AC activity was reduced somewhat by suspending the rickettsiae in triethanolamine buffered saline (TBS). The residual yolk sac components contributed to some of the AC activity and caused non-specific reactivity with certain human sera. In addition, the host tissue in the antigens fixed complement in the presence of sera from guinea pigs and mice containing yolk sac antibodies resulting from infection or immunization with crude suspensions of rickettsiae prepared from infected yolk sacs. Attempts were made with the Amberlite antigens to characterize the serological response of volunteers included in the Malayan chloramphenicol field trials who had first and second infections following natural exposure and experimental inoculation with known strains. Although fourfold or greater increases in CF titer were demonstrated in the majority of the cases with one or more of the antigens, considerable non-specific reactivity was encountered in the 1:10 to 1:40 dilutions of acute phase and several normal control sera. Since it was not possible to determine from the results the time of appearance and persistence of antibodies, further testing was postponed until more satisfactory antigens could be produced.

During the year other methods of purification were investigated. A modification of the high molar salt procedure of Ormsbee (J. Immunol. 88: 100-108, 1962) was evaluated. Gilliam antigens could be prepared, which had less AC activity and lower yolk sac content than the corresponding Amberlite preparation, by centrifuging infected yolk sac suspensions in first 2 molar and then 1 molar KCl. However, the high molar salt method did not provide better Karp and Kato antigens. Although initial yolk sac suspensions of the Karp and Kato strains were comparable to the Gilliam suspensions with respect to infectivity titer and numbers of organisms visible in stained smears, the antigens obtained by the Amberlite and KCl processes regularly had lower rickettsial antigen content. Centrifugation

of the 3 strains in sucrose gradients showed the Karp and Kato organisms to be less dense. Increasing the centrifugal force or the duration of centrifugation in the purification procedures succeeded only in reducing the rickettsial antigen content and increased the yolk sac content and AC activity.

Substitution of 1 molar $MgCl_2$ or potassium acetate in the purification procedure showed the potassium acetate to be less deleterious to the Karp and Kato rickettsiae. The complement-fixing reactivity of a Gilliam, Karp and Kato antigen prepared with potassium acetate is compared with antigens prepared by the Amberlite method in Table XIX. Each antigen was titered against (1) 4 units of the homologous guinea pig antiserum, (2) 32-64 units of heterologous guinea pig antiserum, (3) 4 units of anti-yolk sac rabbit serum, (4) a 1:10 dilution of a normal human serum previously shown to react non-specifically with yolk sac antigens, and (5) tested for its anticomplementary activity. It should be noted that the results presented are <u>not</u> representative of the antigens usually obtained by the two purification methods. These antigens were selected because the AC activity, although present, did not obscure the results.

TABLE XIX

COMPARISON OF THE COMPLEMENT-FIXING REACTIVITY OF SELECTED SCRUB TYPHUS ANTIGENS

Sera	Antigen Titer[+]							
	Gilliam		Karp		Kato		Normal	
	AMB[++]	KAc*	AMB	KAc	AMB	KAc	AMB	KAc
Homologous Guinea pig (4 units)	40	80	40	80	40	40	<10	<10
Heterologous Guinea pig (32-64 units)	<20	10	<20	10	<20	<10	<10	<10
Anti Yolk Sac Rabbit (4 units)	320	160	320	320	160	320	320	160
Normal Human** (1:10 dilution)	20	10	>20	20	20	10	20	20

[+] Expressed as reciprocal of dilution of antigen showing 50% or less hemolysis in CF-52 test. All antigens are free of significant anti-complementary activity in the dilutions reported.

[++] Antigens purified by the Amberlite method.

* Antigens purified by the potassium acetate method.

** Serum chosen because of non-specific reactivity with yolk sac antigens.

The specific homologous rickettsial antigen content of the antigens shown are comparable. The potassium acetate method is slightly better than the Amberlite procedure since it is not as lengthy a procedure, and generally, higher specific homologous antigen titers are obtained without an increase in heterologous reactivity. However, the non-specific reactivity with normal human serum and the anticomplementary activity are not significantly reduced. Usually the potassium acetate antigens are less anticomplementary but it is rare to prepare an antigen which is sufficiently devoid of AC activity to permit the use of 4 to 8 units of antigen in a test. Antigens prepared by both methods are turbid and a heavy precipitate forms upon standing at 4 C. Removal of the precipitate by filtration or centrifugation or extraction with diethyl ether removes specific and non-specific reactivity, as well as the AC activity.

Recent efforts have been directed toward the characterization of the residual egg tissue components in the antigens. Fractionation of the components by magnesium sulfate precipitation techniques (Bernards and Cook, Biochem. Biophys. Acta, 44:86-96, 1960) suggest that they are a complex mixture of lipids and lipoproteins. Attempts to remove extraneous material in the antigens with techniques developed by colloidal and lipid chemists are promising but too preliminary to permit reporting at this time.

Summary and Conclusions.

The results, thus far, of an antigenic analysis of 72 strains of R. tsutsugamushi isolated from man, chiggers and rodents in Thailand show that 94% of the strains are antigenically related to one or more of the prototype Karp, Kato and Gilliam strains. No evident association was found between the antigenic category of the strain, geographic region, rodent host or severity of disease.

The ecological studies of Rocky Mountain spotted fever, in progress in a known enzootic area in Virginia, have provided quantitative data which will permit the assessment of the relative importance of various factors concerned with the maintenance and dispersion of R. rickettsii in nature. A preliminary analysis ranks Peromyscus leucopus and Microtus pennsylvanicus more important than 5 other animal species which serve as hosts for the subadult forms of the vector Dermacentor variabilis. Of the 5 major vegetative types present in the study area, the low deciduous forest and the grasses and herbs habitats are the sites of greatest density of adult D. variabilis and greatest interaction of vertebrate host, vector tick and spotted fever rickettsiae.

Antigens comprised of purified suspensions of the Nine Mile, Henzerling and Ohio strains of Q fever, predominantly in phase I and phase II, have been prepared. Standardization of the complement-fixing reactivity of the 6 antigens with homologous immune guinea pig sera has been completed. Characterization of heterologous reactivity with the animal sera and testing of serum specimens from volunteers in the vaccine trial

will be undertaken with these antigens shortly. Agglutination tests are still being evaluated.

Methods currently available for the purification of R. tsutsugamushi are not wholly satisfactory because residual yolk sac contaminants and anticomplementary activity do not permit testing of human sera with these antigens. Although an alternative procedure has been developed which reduced somewhat these undesirable properties, better methods are required and are being investigated.

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RESEARCH AND TECHNOLOGY RESUME

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19 GOV'T LAB INSTALLATION ACTIVITY
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24 (U) Tech Objective - 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 chronic disease, atypical pneumoniae and sulfa-resistant meningococcal infections.

25 (U) Approach - Development of bacteriological techniques (holding media, genetic homology, serological, bacteriophage and bacteriocine typing systems, antibiotic sensitivity tests, and cultural methods) for recovery and study of bacteria, bacterial L forms and Mycoplasma; collection of sera, case histroies, clinical materials, etc. from cases of above disease entities. Field studies on prophylactic regiments, spread and persistence of meningococci in recruit populations.

26 (U) Progress (Jul 64 - Jun 65) - Relationships of Mycoplasmataceae to Reiter's syndrome, demonstration of genetic and taxonomic relationship between bacterial parent and L form, and genetic unrelatedness of M. pneumoniae to Streptococcus MG. Isolation and propagation of bacteriophages from N. meningitidis, studies of meningocines, field evaluation of sulfa prophylaxis, demonstration of L forms of meningococci and transformation from sensitive to resistance of N. meningitidis in vitro. Results tend to explain development of drug resistant populations and provide approaches for study of pathogenesis and epidemiology.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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Task 01, Communicable Diseases and Immunology

Work Unit 168, Bacterial diseases

Investigators.

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Description.

Investigations are related to studies on the etiological, pathogenetic, physiological, immunological, ecological and laboratory diagnostic aspects of diseases of microbial origin which are current or potential problems to the military forces. Current emphasis is on the study of such diseases as rheumatic fever, Reiter's syndrome, plague, sulfa-resistant meningococcal infections and acute gastroenteritis, as well as basic studies on the physiology, physiochemistry and biology of the microorganisms (bacteria, Mycoplasmataceae, transitional forms and L forms of bacteria) associated with these diseases.

Progress.

1. Significance of Mycoplasma (PPLO), Bacterial L Forms and Transitional Forms in Human Disease.

   a. Phylogenetic Relationships between L Forms or PPLO and Bacteria.

It has been suggested that the Mycoplasmataceae (PPLO) are L-forms of bacteria, because of the reported similarities between the mycoplasmata and L-forms. Since certain Mycoplasma species have been shown to be the etiologic agents in a variety of diseases of man and animals, it is important to ascertain whether or not these agents are L-forms of bacteria. In the past, methods used to test for such a relationship have essayed transformation and reversion of a Mycoplasma species to a suspected bacterial parent. These studies, though highly suggestive, have failed to present conclusive evidence that such a relationship exists.

To circumvent the pitfalls of the previously used methods, we have studied this problem in conjunction with Dr. M. Rogul of the Division of Veterinary Medicine and Dr. S. Falkow of the Department of Bacterial Immunology using physiochemical tests to quantitate the genetic relationship between organisms. With these tests Proteus mirabilis 9 and its stable L-form, L 9, were found to have DNA indistinguishable in composition and nucleotide base sequence. Using this model system as the prototype of the genetic relationship between a bacterium and its stable L-form, certain mycoplasma-bacterial pairs have been tested to see if their relationship is the same as that demonstrated in the model system, thus determining if the mycoplasma is, in fact, the L-form of the bacterium.

Since Mycoplasma gallinarum, Mycoplasma gallisepticum and Haemophilus gallinarum have all been implicated in chronic respiratory disease of chickens, and evidence of the reversion of M. gallinarum to H. gallinarum has been presented (McKay & Truscott, Ann. N. Y. Acad. Sci., 1960, 79, 465), the genetic relationships among these organisms were studied. The molar base ratios of the DNA from the organisms expressed as % GC were H. gallinarum, 41.9% GC; M. gallisepticum, 32.7% GC; and M. gallinarum, 28.1% GC. As might be expected with this diversity of DNA composition, there was no detectable homology of nucleotide base sequence between M. gallinarum or M. gallisepticum and H. gallinarum. Thus these organisms appear completely unrelated.

The next mycoplasma-bacterial pair tested was Mycoplasma pneumoniae (the Eaton agent) and Streptococcus MG. The suggestion that these agents are related as L-form to parent bacterium has been made (Marmion & Hers, Amer. Rev. Res. Dis., Supplem., 1963, 88, 198) because of the rise in agglutinin titers to Streptococcus MG which occurs in about 25% of cases of atypical pneumonia caused by M. pneumoniae. Although the DNA base compositions of the two organisms were identical (39.3% GC), there was no evidence of any nucleotide base sequence homology between them. It was concluded that the Eaton agent is not the L-form of Streptococcus MG.

Further studies are in progress to determine if the Eaton agent is the L-form of any other of the bacteria having approximately the same DNA base composition and to determine the extent of any relatedness of M. pneumoniae to other members of the family Mycoplasmataceae. Such information is critical in determining if the members of this family have close phylogenetic relationships or if they are unrelated organisms taxonomically grouped only because of the consequences of absence of the cell wall.

b. The L form of Neisseria meningitidis.

Recent studies have indicated that organisms infecting man may persist as L-forms undetected by ordinary cultural techniques during a

course of antibiotic therapy, only to reappear in characteristic bacterial form following cessation of therapy. Since such a phenomenon might be operative during prophylaxis or therapy for N. meningitidis infections, efforts have been directed toward the production and propagation of L forms of meningococci. Preliminary studies with the parent organisms were performed to determine (1) sulfadiazine and penicillin sensitivity, (2) duration of survival in isotonic saline and broth, and (3) growth curves using both stationary incubation and continuous shaking at 6500 R.P.H. Subsequently, in vitro and in vivo systems were employed in an effort to produce and propagate L-forms of N. meningitidis. Attempts to recover L forms from the peritoneal exudate and heart blood of mice following intraperitoneal injections of log phase growth of one meningococcus strain were unsuccessful. The penicillin and sulfadiazine gradient plate technique has been the in vitro system employed to date.

Organisms used in these studies were both recent isolates and older laboratory strains originally recovered from either carriers or patients with clinical disease. Meningococci of different groups (A, B, and C) and of varying resistances (0.015 to 4 mg%) were tested. L-forms were produced from six of these strains, five of group B and one of Group A. One of the L-forms has been propagated through six passages. This is the first known instance of propagation of L-forms of N. meningitidis. Following reversion of these L-forms to the bacterial form grouping and fermentation characteristics were identical to those of the respective parent. Sulfadiazine sensitivity patterns were also comparable for paired reverted and parent meningococci.

Studies are now in progress to define the optimum growth conditions for propagation of stable L-forms. Current protocols are designed to determine (1) whether parent strains which easily produce L forms in vitro can do so in the experimental animal, (2) the in vivo site of production of L-forms and (3) biochemical and serologic methods for rapid species identification of the L-form. This information will then be applied to define the role of L-forms of meningococci in the carrier state during prophylaxis, chronic meningococcemia and in recurrent meningococcal meningitis.

c. Mechanisms of Sulfadiazine Resistance in Neisseria meningitidis.

Experiments concerning the nature and mechanism of sulfadiazine resistance in N. meningitidis are under way in this laboratory. In preliminary studies using purified DNA from N. meningitidis, strains 7-II and VF, with levels of sulfa resistance of 10 mg% and 1 mg% respectively, a sensitive strain (± .01 mg%) has been transformed so that it is resistant to levels of sulfa ranging from 0.5 mg% to 1.7 mg%. The findings indicate that sulfa resistance is a genetic and not just a phenotypic change. The studies currently in progress are designed to yield information concerning those factors influencing the frequency of

this genetic change. It is hoped that the results of these studies will be helpful in decreasing the frequency of mutation to drug resistance and in dealing with these strains when they arise.

d. Reiter's Syndrome.

Synovial fluid and blood specimens from a patient with Reiter's syndrome were cultured for PPLO and transitional forms of bacteria. A patient who was undergoing a first attack and who exhibited the classical triad of symptoms was cultured within 12 hours after the onset of arthritis and within 24 hours after the start of tetracycline therapy. The joint fluid yielded colonies which upon microscopic examination resembled those of a Mycoplasma. There was no morphologic evidence during eight weeks of cultivation and passage to suggest that the agent is the transitional form or L form of a bacterium, although this possibility still exists. The agent grows very slowly and sparsely and to date has not been maintained beyond the third passage on culture media. However, there are sufficient frozen stocks of the joint fluid, original and first passage cultures to continue intensive efforts to cultivate and maintain this very interesting isolate.

e. Type Culture Collection of PPLO and L-Forms.

WRAIR in collaboration with the American Type Culture Collection has increased the number of reference strains in the collection of Mycoplasmataceae and L-forms to 43. Acquisitions now include six strains of the paired parent bacterial or reverted bacterial form of the reference L-form strains. Dr. Louis Dienes is presently submitting his valuable collection of L-forms with their paired bacterial forms to WRAIR for inclusion. Nine other investigators in various parts of the world have submitted strains to WRAIR for processing and placement in the ATCC. Increasing use of the collection is being made, and the demand for reference cultures has necessitated the replenishment of seed stock of eight strains (approximately 250 lyophilized vials). Our records show a fairly constant demand for 20 of the better known Mycoplasma species out of the 31 species in the collection.

Viability tests have been carried out on 28 lyophilized strains which have been stored for one to four and one-half years, and all have yielded good growth. Although the lyophilization methods employed by the ATCC are satisfactory for most Mycoplasma species, they are not dependable for strains of M. pneumoniae (Eaton agent) nor for L-forms of streptococci or corynebacteria. M. pneumoniae has been preserved in serum broth frozen in liquid nitrogen, while the streptococcal and corynebacterial L-forms have been preserved as colonies on agar blocks frozen at -60°C. The ATCC plans to investigate other methods for preserving these fastidious strains.

Antisera to PPLO and L-form strains in the collection are now being prepared so that identity of the strains can be verified over the years. Tests are being employed to determine the stability of the biochemical characteristics of the strains after various periods of storage. The wide variety of media used for cultivation of the strains is constantly under investigation in an effort to limit the types of media to two or three basic formulae which will be suitable for all strains of the collection.

f. Biology of Mycoplasma and L-Form Species.

Studies have been resumed on growth curves, particle size and morphology of Mycoplasma and L-form strains (for methods and earlier findings see WRAIR Annual Progress Report, 1963). Colony counts of the filterable elements at three-hour intervals during the growth period of two Mycoplasma species and two L-forms are comparable to results obtained in the earlier study, indicating stability of these characteristics for a given strain. Furthermore, the morphology of these strains has remained characteristic for each species, i.e., small, spherical to oval cells for Mycoplasma laidlawii and the L-form of Gaffkya tetragena, and beaded, filamentous forms for Mycoplasma arthritidis.

Antibiotic sensitivity of Mycoplasma and L-form strains is being tested at various times during the growth of the organisms. Twenty-four-hour-old cultures of two rodent strains, Mycoplasma arthritidis and the L-form of Streptobacillus moniliformis, were tested against penicillin, lincocin, chloramphenicol, tetracycline, chlortetracycline, oxytetracycline, erythromycin and streptomycin. The L-form was considerably more sensitive to most of the drugs than was Mycoplasma arthritidis. The L-form was most sensitive to chlortetracycline and lincocin and the Mycoplasma to tetracycline and lincocin There are wide differences of opinion concerning the antibiotic sensitivity of L-forms and very little experimental evidence to support these opinions. Basic information is required on the sensitivity of the large body stage compared with the small granular stage, actively multiplying stage compared with resting stage, and on L-forms (all of which stain gram negative) derived from gram positive bacteria compared with those derived from gram negative bacteria. This work is designed to lead in time to a comparison of in vivo produced L-forms and in vitro produced L-forms which may differ markedly in their antibiotic sensitivity. This information may be applicable in treating patients whose disease is complicated or protracted by the presence of L-forms.

2. Diagnostic Methodology.

a. Field Trial of a Transport Medium for Shipment of Clinical Specimens.

Arrangements were made with D. C. General Hospital for a clinical trial on the survival of Shigella and Salmonella in a new transport medium designed for the shipment of specimens to a central laboratory (Cary and Blair, J. Bact., 1964, 88:96-98). All fecal specimens were collected in duplicate on alkaline swabs, placed in the transport medium and immediately taken to the D. C. General Hospital laboratory where one set was immediately cultured for the presence of Salmonella or Shigella, using MacConkeys agar, S-S agar and selenite F broth.

The duplicate specimen was transported to WRAIR and cultured as above. Time interval from collection to initial culture varied from one to three days. All specimens containing Salmonella or Shigella were stored at room temperature and subcultured at varying time intervals to determine maximum survival time.

The 22 enteric pathogens isolated from 162 specimens included strains of Shigella sonnei I and II, Shigella flexneri 5, Salmonella typhimurium, Salmonella saint-paul, Salmonella enteriditis, and Salmonella manhattan, and there were no differences in recoveries between the collaborating laboratories. Original specimens containing less than six colonies of Shigella on initial isolation were negative after two weeks' storage. Those with at least six colonies initially (Shigella sonnei I) were recoverable after 12 days' storage but not after 19. In specimens containing a moderate number, S. sonnei I was recovered and agglutinable after 49 days at room temperature. All subcultures were negative after 56 days.

In two specimens containing S. saint-paul from which six colonies were isolated initially, there was very little drop in the relative number over a storage period of 32 days. One of these specimens also contained large numbers of Pseudomonas aeruginosa which did not prevent recovery of the Salmonella until the specimen had been stored for 45 days. In a specimen containing Proteus, as well as S. enteritidis, the Salmonella was still recoverable at 53 days. S. enteritidis from another specimen was isolated up to a maximum of 45 days.

Two specimens submitted contained a portion of bowel and minced bowel from the same patient. Shigella sonnei I was isolated from both specimens. A Proteus sp. did not interfere with recovery of the Shigella strain for 34 days.

No rough variants or loss of specificity in the somatic antigen was noted for any of the Salmonella or Shigella isolates recovered from this holding medium.

The results of this field trial, as well as a much more extensive one conducted in Southeast Asia, (Gaines, et al., Am. J. of Trop. Med. & Hyg., 1965, 14:136), clearly demonstrate that this medium is capable of maintaining the viability of enteric pathogens in clinical and survey fecal material for sufficient lengths of time to permit field studies in remote areas with a minimum expenditure of personnel and equipment. Previous studies have demonstrated the value of this media for transport of N. meningitidis in nasopharyngeal swabs (Ann. Report, WRAIR, 1963-1964), and recent reports from Viet Nam indicate its value as a transport and holding media for Pasteurella pestis.

b. Improved Technic for H Antigen Phase Change in the Salmonella.

Most strains of Salmonella are diphasic, containing two distinct flagellar antigens designated as phase 1 and 2 according to the Kauffman scheme. Demonstration of both antigens is required for species identification. In many cases, one phase is dominant, suppressing the production of the other phase. For a number of years, this laboratory has used a phase tube for the suppression of one phase consisting of a serological test tube from which the bottom has been cut out and a rubber cork inserted. The tube is sterilized and approximately 5 ml. of semi-solid motility agar added containing .1 ml of the homologous H antiserum. After the serum-agar mixture has hardened, the agar is inoculated at the surface by just puncturing the agar with a loopful from a broth culture of the organism under study. After 18 hrs. incubation, the homologous phase organism will have been immobilized at the inoculation site while the alternate phase will have grown through the agar. The stopper at the bottom of the tube is removed and a tube of trypticase soy broth is inoculated with a loopful of the agar. After overnight incubation the culture is tested for the other H phase antigen. For the identification of monophasic strains, this procedure requires an additional 48 hrs.

In 1961 Harvey and Price (Mon. Bull. of the Min. of Health and Pub. Health Lab. Ser., 1961, 20:11-13) described a phase tube employing a Pasteur pipette filled to the juncture of the stem and barrel with a 15% nutrient agar containing the appropriate H antiserum. The tube is inoculated with a dense suspension of a Salmonella culture at the tip of the stem, a small amount of air sucked up, and the tip sealed in a flame. The Pasteur pipette is then placed in a test tube and incubated overnight. After incubation, the culture growth is removed from the top of the barrel and used for H slide agglutination. Since all H antisera available to the U. S. Armed Forces is prepared for tube agglutination and requires a broth culture for antigen, a modification of the Harvey and Price procedure was investigated. In this laboratory, a Pasteur pipette (240 mm) with part of the barrel cut off is placed in a 150 mm X 20 mm screw cap tube and sterilized. Motility agar containing appropriate antiserum is allowed to harden and then sucked up in the Pasteur pipette, almost to the top of the barrel. Ten ml. of trypticase soy broth is

added to the test tube containing the pipette. The serum agar mixture is inoculated with a loopful from a broth culture of the Salmonella to be identified. After overnight incubation, the alternate phase strain has grown through the pipette into the broth. This broth culture is then used for the serological identification of the second flagellar phase.

This simplified technic has been successfully used for approximately six months in our laboratory. Its full potential would be realized in laboratories involved in the examination of food stuffs and epidemiologic investigations of salmonellosis in human enteric disease where large numbers of Salmonella must be identified. The commonly used specific phase antisera-agar mixtures can be prepared in bulk, preserved under refrigeration, and used with a minimum expenditure of time, materials and space.

c. Bacteriophage Typing.

(1) Staphylococcal Phage.

Propagation and preservation of large quantities of stock staphylococcal bacteriophage is subject to many technical difficulties, such as, inadequate initial titers, loss of activity and variations in specificity. Serial passage often provides satisfactory titers but may also give unwanted cross reacting patterns with other staphylococcal strains, thus numerous batches of phage with satisfactory specificity must be discarded due to unsatisfactory titers. A simple method for concentration would be a useful laboratory tool.

A simple, economical collodion bag protein filter, recently marketed by S & S, was evaluated as a potential method for phage concentration. The apparatus consists of a small (15 ml.) collodion bag attached to the inside of a vacuum flask through a ground glass connecting tube, the bag suspended in liquid (in this case deionized distilled water) to the level of the group joint connection. The flask is attached through a loop to a vacuum source of approximately 400 mm of Hg.

One hundred ml. of stock phage 42D was concentrated to a final volume of 5 ml. by overnight filtration in the walk-in refrigerator. The phage titer was increased (10^9 to 10^{10}) with no apparent loss is viability. Since phage 42D does not cross react with any other staphylococcal propagating strains, no alteration in specificity could be noted. Currently other phages (6, 52A and 3A) are under study for effect of the method on specificity.

(2) Meningococcal Phage.

In 1953 Stone, et al. (Bacteriol. Proc., 1953, 39) described the isolation of a bacteriophage active against a chromogenic strain of Neisseria inhabitating the nasopharynx. The activity of this phage was

described by the same authors in a later publication (J. Bact., 1956, 71: 516-520). They tested the host range using a number of strains of Neisseria perflava, N. subflava, N. flavescens, N. catarrhalis, and one strain of N. meningitidis. None of the strains were sensitive. At the 1965 meetings of the Am. Soc. for Microbiol., Phelps and Kellogg described the isolation of three distinct phages from throat washings which were active against a strain of N. perflava.

In our laboratory, since the inception of the study on N. meningitidis infections in military recruits, considerable experimental work has been carried on to determine whether strains of N. meningitidis carry bacteriophage and the feasibility of using phage typing as a tool for definitive classification of the strains isolated. Several recognized procedures for the demonstration and production of bacteriophage have been studied. Of 13 strains of N. meningitidis examined to date bacteriophage active against a number of these strains were demonstrated from eight. The four most active, showing semi-confluent lysis or a 2+ (50-100 plaques) reaction, have been propagated.

Several lots of these four phages (designated P1, P2, P3 and P4) have been propagated to titers in the range of 10^6 particles per ml. Preliminary typing on a sampling of case and carrier strains is summarized in Table I. Efforts are currently in progress to increase the phage titers to more useful ranges (10^9-10^{12}) and to provide sufficient stock phage to permit the typing of preserved N. meningitidis strains from recent field studies. Once the technics for phage isolation, propagation, and preservation have been adequately developed, efforts will be made to increase the numbers of Neisseria phage stocks and thus increase the range and specificity of the procedure.

3. Gastroenteritis Studies.

a. Ultra-violet Induced Colicines.

Escherichia coli are known not only to be active producers of colicines but to also possess the potential for colicine production in the form of pro-colicines (colicinogeny) which are activated under conditions similar to those which cause induction of bacteriophage from lysogenic bacteria (Ryans, et al, Biochem. Biophys. Acta, 1955, 18:131). What additive effect the potential for colicine production might have on the fluctuations of intestinal E. coli flora has never been investigated. Therefore, to complete our study on the effect of colicines in intestinal flora as previously reported (Branche, et al., Proc. Soc. Exptl. Biol. & Med., 1963, 114:198-201), it was decided to induce colicine production by ultra-violet irradiation from the same strains used in the previous study. A total of 247 E. coli strains from the previous study were induced by ultraviolet light. Biochemically typical E. coli were typed

TABLE I

Strain No.	Source	Phage Type	Ser. Gr.	Sulfadiazine MIC*
II-1	Carrier	P2/P4/P3	Gr. B.	.025
V-2	Carrier	P2/P4/P3/P1	Boshard	.01
33II	Carrier	P4/P1		
66VI	Case	Nontypable	Gr. B	.05
17II	Carrier	P3		
9 Misc.	Case	Nontypable	Gr. C	.05
4-IV	Case	P1	Gr. B	3.0
21-II	Carrier	P4/P1		
20-II	Carrier	P1		
Harkins	Carrier	P4/P3	Gr. B	.025
50Eur	Case	P2	Gr. C	.2

*Minimum inhibitory concentration in mgm%

by the method of Edwards and Ewing using antisera for 12 enteropathogenic and 25 non-enteropathogenic (serotypes 01-025) types. Eighteen isolates which did not belong to any of these serotypes were used to produce antisera in order to identify all similar serotypes. A total of 234 biochemically typical E. coli strains collected at random from routine laboratory specimens at the Clinical Center, National Institutes of Health, were used as indicator strains.

For determination of colicinogeny, a sufficient quantity of an 18 hr. broth culture was transferred to 10 ml of fresh trypticase soy broth to approximate a #1 MacFarland tube. Five ml of this suspension were placed in a petrie dish 11 inches from an ultra violet light source (2537 A) in an inoculating hood. Each culture was irradiated for 60 seconds, incubated further at 37°C. for 3½ hrs., refrigerated overnight and then incubated at 56°C. for 20 min., to allow for maximum cell lysis and subsequent release of colicine. After centrifugation each of 247 E. coli producers was tested against 234 E. coli indicator strains. Lawns of the indicator strains were prepared by swabbing trypticase soy agar plates with 18 hr. TBS cultures. Lysates were applied to lawns with a multiple syringe bacteriophage applicator (Zierdt, et al, Am. J. Clin. Path., 1960, 33:233-237). Results were tabulated after overnight incubation at 37°C. In order to differentiate between colicine and phage zones each zone was picked with an inoculating needle and streaked on an appropriate lawn. Colicines produced a broad zone of inhibition extending along the initial streak whereas phages produced characteristic pinpoint holes which gradually diminished in number as they became more dilute.

A total of 159 (64.4%) of the 247 E. coli strains were normal colicine producers. Seventy-eight (31.6%) were colicinogenic, i.e. produced colicines by ultra-violet induction. Seventeen of the 78 colicinogenic strains were not normal colicine producers. Twenty-three of the E. coli produced induced colicines which were ative against the parent strains. This unusual finding was in contradiction to the statement of Adams (Bacteriophages, Interscience Pub., Inc., N. Y., 1959) that bacteria are "resistant to the colicines they produce", however, Ryan, et al. (1955) reported on an E. coli strain that produced colicine in response to ultra-violet irradiation which acted against the parent strain and moreover had specific activity like that of the parent strain. In the present study, this activity occurred chiefly in those persons with the least stable flora, though what role this phenomenon played in the stability of E. coli in the intestine is not known.

Although there was great variation among the E. coli isolated from the six subjects under study it was apparent that those individuals having the least stable E. coli flora and a wider variety of serotypes had the greater number of strains which were unable to produce colicine except by induction. All inducible E. coli except one were recovered from two subjects who also carried the greatest variety of serotypes.

Conversely, Subject A of this study had the most stable flora and 97% of the E. coli isolates produced both normal and induced colicine. These data provide evidence that total colicine activity, i.e. normal and induced, of some E. coli strains is associated with their ability to maintain themselves in the human intestine.

As a corollary to this study, it was thought that the inhibition patterns of colicines from E. coli strains might serve as an epidemiologic tag in outbreaks involving a relatively specific strain. As can be seen in Table II, the majority of strains from one individual produced colicines. Strain "X" of Subject A, which was that individual's dominant strain for five of six months provided the most extensive data. When a strain producing colicines differed in its pattern of inhibition by the addition or loss of three or more it was considered a different strain. On this basis, 14 colicine patterns appeared for the single E. coli serotype "X" over a five-month period. In this instance the colicine patterns would appear to have been of little use for epidemiologic purposes.

b. Microtiter Hemagglutination Technique for Shigella Antibodies.

Although the microtiter system has been employed extensively for hemagglutination (HA), little use has been made of it in the field of enteric bacteriology. In the present study which is an outgrowth of a previous one (Ann. Reports, WRAIR, 1963-1964), an adaptation of the microtiter method was tested in parallel with tube hemagglutination method previously described by Young, et al. (Bact. Proc., 1964) to determine the validity of applying such a microtitration method to establishing Shigella flexneri 1a and Shigella flexneri 4b HA titer levels in adult human sera.

The standard components of the microtiter system for HA tests were employed in the procedure. It should be noted that only conical bottom (V) wells were utilized. The preparation of the lipopolysaccharid extracts has been described in detail in previous Annual Reports. Because Sh. flexneri 1a possesses cross reactivity to a high level with all Sh. flexneri antisera except 4b, the 1a antigen was used to test normal sera as a broad indicator of prior contact with Sh. flexneri serotypes or their antigens. On the other hand, Sh. flexneri 4b which reacts weakly with only two other strains, namely Sh. flexneri 2a and 4a, was used as an indicator of prior infection with this more specific antigen.

Essentially the microtiter HA method is the same as the tube HA test except that the microtiter uses much smaller quantities of reagents and a modified buffer, 0.1% bovine serum albumen in tri-ethanolamine buffered saline. The bovine serum albumen served as a protein carrier to enhance the adherence of the red cell pattern formation. A 10% suspension of fresh human O Rh negative cells preserved in Alsevers was washed and added to an equal amount of polysaccharid antigen which had already been diluted to its optimum reactivity capacity as determined by

TABLE II

Colicine Activity of Strain "X" Recovered from Subject A*

Month	Total no. Strains	Indicator Strains																			
		Ø	B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
July	6	+	+			+	-1	+	+	+	-2	+	+	-1	+	+					
	1	+	+			+	+	+	+				+		+						
	1				+																
Aug	5	+	+			+	+	+	-2	+	+	+	+	+	+	+	+	+	-2		
	3	+	+			+	+	+	+	-2		+	+		+		+	+			
	1	+	+			+	+	+	+			+	+	+	+		+		+		
	1	+	+			+	+	+		+		+	+	+		+	+			+	
Sept	1	+	+			+	+	+		+	+			+						+	+
	2	-1	-1			+	+		+	+	+	+	+				+			+	
	2	+	+			+	+					+								+	
	1	+	+			+	+		+			+	+		+		+			+	
Oct	10	+	+			+	+	+		+	+			+2						-1	-5
Nov	4	+	+			+	+	+		+	+			-1						+	+1
	2	+	+			+	-1														

*All of these strains are the same E. coli serotype.

Note: Numbers preceded by a minus sign represent the number of "X" strains which showed loss of activity for a particular indicator strain; numbers preceded by a plus sign indicate a gain of such activity.

standardization. After two hrs. adsorption the then sensitized red cells were washed and resuspended to a 1% suspension.

Normal adult sera were obtained from the state laboratories of the District of Columbia, Illinois, California, Massachusetts, Texas and Mexico. These sera were inactivated at 56°C. for one-half hour prior to use. Microtiter hemagglutination tests employed equal quantities (0.025 ml) of all reagents. First a standard amount of carrier buffer was added to each well. Secondly, two-fold serial dilutions were made of the test sera. Then one drop (0.025) of sensitized cells was added to each well with a dropper pipette. The plate was rotated gently to ensure thorough mixing, covered with a lucite plate and incubated at 37°C. for one hour. After this incubation, the plate was refrigerated overnight.

Microtiter hemagglutination levels were read by pattern formation only. Positive tests presented several patterns. (1) Cells were so strongly agglutinated that the tightly bound sheet of cells appeared to fold. (2) Cells appeared to cling to the curvature of the well bottom and the sheet thus formed irregular edges. (3) Cells were partially spread over the bottom of the well with complete clumping and were ragged in appearance. A plus-minus reaction showed a button of unagglutinated cells which was surrounded by a zone of very small clumps of agglutinated cells. The negative test had the appearance of a button formed by unagglutinated cells which could be checked by tilting the plate and observing the appearance of a "tear drop" pattern. The end point of the titration was the last dilution showing complete agglutination.

The microtiter system results in titers comparable to those obtained with the standard tube test, not varying by more than one tube. A total of 374 sera (Table III) were tested and 338 (90.5%) of the samples gave identical results in both tube and microtiter HA tests. Seventy-nine of the samples had titers of less than eight, while others ranged in titer from 8 to 1024. Thirty-six sera that gave different titer levels were re-examined by both systems (Table III).

The microtiter system gave higher titers than the standard tube test, i.e. 24 samples of the 36 samples in disagreement showed an increase of a two-fold dilution in the microtiter system while only 12 showed an increase in the tube test. The sensitivity and reproducibility, together with the obvious technical advantages it affords, makes this procedure an extrerely useful laboratory tool in support of epidemiologic and ecologic investigations.

TABLE III

Comparison of Titers of Microtiter and Tube Hemagglutinations

State	Total No. of Sera Tested	Identical Titers in both Tests	Tube HA		Microtiter HA	
			2-fold increase	4-fold increase	2-fold increase	4-fold increase
Texas	12	10	0	0	2	0
Mexico	12	10	0	0	2	0
D. C.	200	183	11	0	6	0
Illinois	100	91	0	0	9	0
Mass.	25	22	1	0	2	0
Calif.	25	22	0	0	3	0

Total No. of Sera Tested - 374.

4. Plague

We have suggested the use of serological procedures in plague surveys and the possibility of delineating plague foci both geographically and chronologically (Ann. Report, WRAIR, 1964) by these technics. The following experiments were carried out to validate some of the field data that have been collected.

Two groups of 40 white female mice, $3\frac{1}{2}$ to $4\frac{1}{2}$ weeks old, were selected. One group of 40 mice received 40 ug of $FeSO_4$ in 0.5 ml of saline while the other group received 0.5 ml of saline without $FeSO_4$. All inoculations were performed by the intraperitoneal route. Each group of 40 mice was then subdivided into groups of eight mice each and inoculated with log dilutions of an iron dependent strain of Pasteurella pestis grown on blood agar base for 12 hours. The organisms were harvested in saline and the inoculum calibrated spectrophotometrically. Plate counts of the inoculum indicated that the mice received 8.10^1 to 8.10^5 organisms in log increments. The mice were observed daily for 21 days. Mice were bled on alternate days so that four mice from each group were bled daily to determine the presence or absence of septicemia. All mice dying during the 21-day observation period were autopsied and examined for evidence of P. pestis infection. At the end of 21 days all surviving mice were bled and complement fixation and hemagglutination tests carried out on the sera.

Table IV indicates the lethality of the strain in the presence of Fe^{++} demonstrating the iron dependent nature of the strain. Table V indicates the numbers of mice found to be septicemic in the presence and absence of iron. It is obvious that had these animals been examined for septicemia at more frequent intervals, the group of mice receiving organisms plus $FeSO_4$ would no doubt have demonstrated a considerably higher rate of septicemia. Table VI indicates the serological response of the surviving mice at 21 days post challenge. In no instance did any of these animals demonstrate hemagglutinating antibodies.

Previous field observations have suggested that CF antibody appears in infected animals prior to hemagglutinating antibody and has a relatively shorter half life. Unfortunately, later sera specimens could not be obtained to follow the hemagglutinating antibody but the early appearance of the complement fixing antibody with lack of hemagglutinating antibody is consistant with prior field observations. Experiments are planned to demonstrate this phenomenon in laboratory animals with respect to variations observed in wild rodents.

TABLE IV

Lethal Effects of Avirulent P. pestis
Challenged in Laboratory Mice

No. of Organisms	+ Fe	- Fe
8.10^5	5/8	1/8
8.10^4	2/8	0/8
8.10^3	4/8	0/8
8.10^2	1/8	0/8
8.10^1	2/8	0/8

TABLE V

Occurrence of Septicemia in Mice Challenged
with Avirulent P. pestis

No. of Organisms	+ Fe	- Fe
8.10^5	5/8	0/8
8.10^4	5/8	0/8
8.10^3	5/8	0/8
8.10^2	5/8	0/8
8.10^1	3/8	0/8

TABLE VI

Mice Demonstrating Complement Fixing Antibodies following Challenge with Avirulent P. pestis

No. of Organisms	+ Fe	- Fe
8.10^5	2/3	4/8
8.10^4	6/6	5/8
8.10^3	1/3	1/8
8.10^2	5/7	0/8
8.10^1	1/6	0/8

5. Meningococcal meningitis.

a. Epidemiological Investigations.

Following the recognition at Fort Ord that sulfonamide resistant strains of Neisseria meningitidis were responsible for the ineffectiveness of sulfa prophylaxis in the control of meningococcal meningitis on that post, attention was directed to other recruit training centers where resistant strains were less prevalent. Two questions were apparent: (1) would a population of sulfa-resistant strains emerge at any post where prophylaxis was used? and (2) could this be avoided by modifications of the prophylactic dose of sulfa? Drug resistance in bacteria has been shown to be a genetically transferred character. For several drugs the genotype responsible for resistance has been demonstrated to arise spontaneously in the absence of the drug. Although this has not been demonstrated for all drugs in the case of N. meningitidis, it is generally assumed to be the usual mechanism of appearance of drug resistance in bacteria. Despite continued spontaneous development of resistant organisms, strains sensitive to sulfa predominate, indicating that resistance does not give any survival advantage except when sulfa is present in their environment.

Penicillin resistance in meningococci appears in a stepwise fashion; first to a low level and then to successively higher levels. Mutation to the second level occurs only in the population having attained the first level. Thus the probability of each successive step occurring is presumably a function of the size of the population having achieved the previous step. If the same genetic mechanism operating for penicillin operates for sulfonamides, then a rapidly rising concentration of sulfa would theoretically be less favorable for the development of a resistant population than a slowly rising concentration.

As a first step toward answering these questions, attempts were made to follow groups of meningococcal carriers before and after sulfa prophylaxis. During postwide prophylaxis at Fort Knox in February 1964, two groups of trainees were cultured during and after sulfa administration. Carrier rates in a group having only sensitive strains fell to 30 per cent. After interviews with recruits revealed that many failed to take their tablets, the persistence of sensitive strains was attributed to incomplete administration of the sulfa tablets. When postwide sulfa prophylaxis was repeated in April, the Department of Bacteriology, WRAIR, surveyed samples from several companies in which sulfa administration was carefully supervised. Unfortunately, information regarding the number of sulfa resistant strains prior to sulfa administration is not available. Three of these sample groups were again surveyed two weeks after sulfa was begun. The results are shown in Table VII. The rapid appearance of sensitive strains within such a short time suggested that prophylaxis merely suppressed even sulfa-sensitive strains. Use of a dose of sulfa

TABLE VII

Neisseria meningitidis Carrier Rates

Fort Knox, April-1964

Company	Sulfa Regimen	Carrier Rate before Sulfa	Total Carrier Rate 14 Days after Sulfa	Carrier Rate for Resistant Strains 14 Days after Sulfa
B-14-4	2 gm every 12 hrs X 4	58% of 150	28%	9%
E-14-4	2 gm every 24 hrs X 2	52% of 140	32%	19%
F-14-4	1 gm every 12 hrs X 4	15% of 150	15%	9%
Combined Total		41% of 440	25%	12%

which merely suppresses sensitive strains provides a circumstance that permits existing organisms with even low resistance to become the predominant organism. It thus appeared that the standard regimen for sulfa prophylaxis may have promoted the emergence of a resistant population. This was compatible with the observation that companies in training cycles receiving sulfa prophylaxis twice showed a higher percent of strains resistant than companies receiving only the second prophylaxis.

One of the test companies was given twice the standard dose and although the carrier rate returned as quickly in this company as the others, fewer strains were resistant than in the other two companies. This suggested that increasing the dose of sulfa may reduce the likelihood of resistant strains emerging.

Thus the following questions were formulated:

1. Does the standard prophylactic dose select resistant organisms from carriers of apparently sensitive strains?

2. If so, would a higher dose of sulfa avoid that occurrence?

3. Does sulfa prophylaxis select more highly resistant mutants from a population of resistant organisms?

With these considerations in mind the current study was organized into three test groups:

1. One group receiving standard prophylaxis, 1 gm sulfadiazine every 12 hours for a total of 4 gms.

2. A second group receiving 2 mg sulfadiazine every 12 hours for 4 doses plus 2 gm of gantrisin with the first dose for a total of 10 gms.

3. A third group receiving no sulfa served as an index of possible variations in carrier rate due to season or climate.

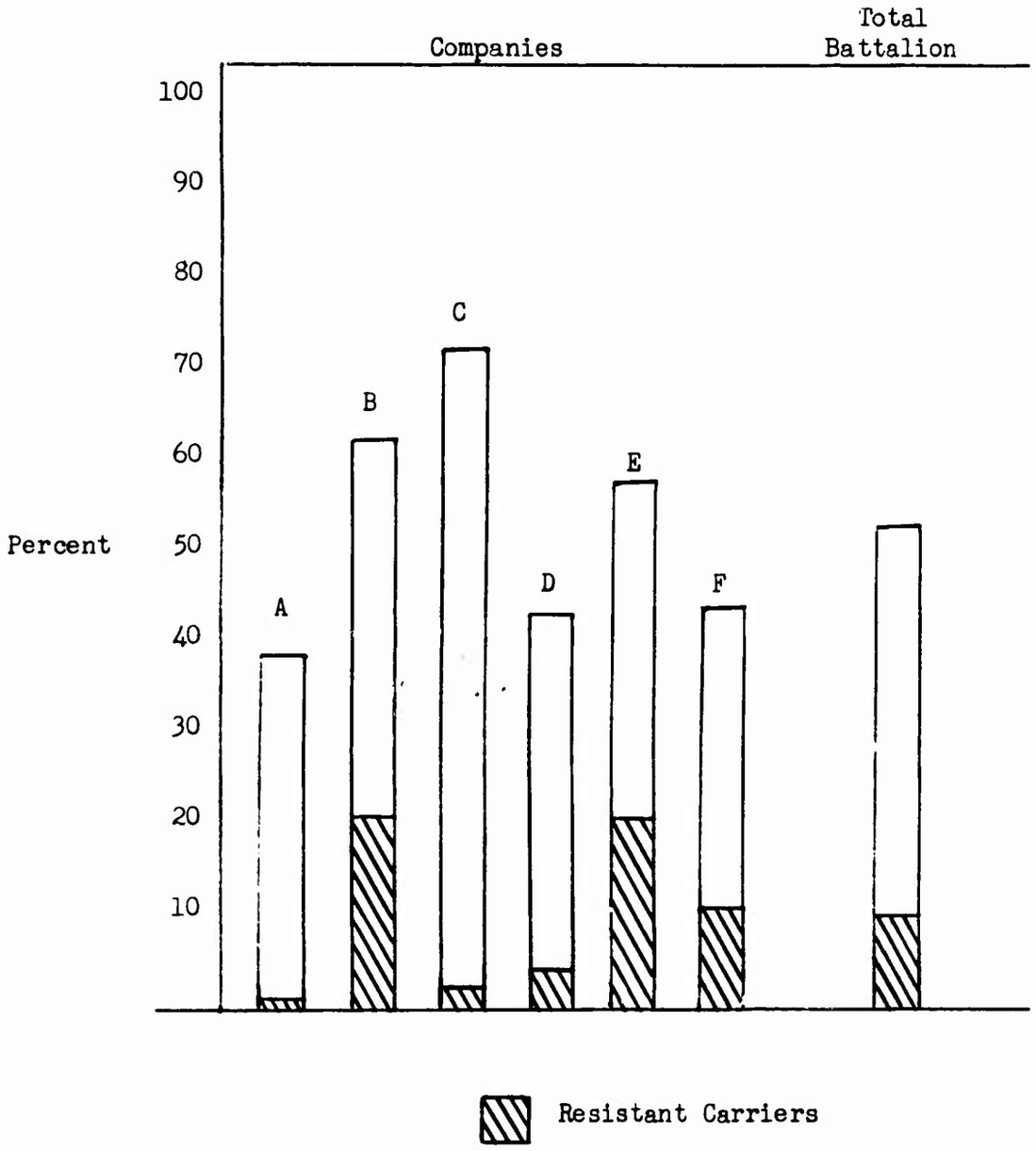
These same treatments would be repeated to see if the response was similar. Selection of a higher dose for comparison with the standard prophylaxis was a balance between the need to make the difference in dose as great as possible in order to obtain a detectable difference in effect and the need to allow a considerable margin of safety.

Six companies of a basic combat training battalion (Fort Knox) in the fourth week of training were surveyed twice (see Graph I). Although these six companies were in the same week of training and, in fact, had

GRAPH I

MENINGOCOCCAL CARRIER RATES

10th Battalion - 3rd Training Brigade



been formed with troops arriving at the post within a few days period, considerable variation was found in carrier rates, 39 to 73 per cent. However, the variation in carrier rates of resistant strains was even greater, 1 to 21 per cent. Clearly any survey drawn from a single company will not necessarily reflect the carrier rate in all recruits in the same week of training.

Companies A, C, and D with the lowest number of resistant strains already present were chosen for this study. These companies were isolated from the rest of the post and from each other by restricting them to their own company area. Although the cadre were not isolated they were given the same sulfa regimen as their recruits and followed with nasopharyngeal cultures. Occasional contacts outside the company area were necessary for personnel actions, but these were minimized within the limits of practicability. Sick call was conducted in each company area. PX, barber shop, service clubs, and movie theaters were made available to one company at a time during hours when no other post personnel were permitted to attend. Wives and parents of recruits were permitted to visit but recruits could not leave their company area except as required for training or as a supervised group visit to the PX, etc.

Although a uniform dose is administered in postwide prophylaxis, the concentration of sulfa attained in the blood of different individuals varies widely due to irregularities in absorption from the gut, differences in body size, large variations in the rate of detoxification and excretion, and unknown factors governing the transport of sulfa from the blood to the nasopharynx.

To obtain an estimate of the variation in blood levels achieved by the two sulfa regiments, eight serial blood samples were obtained from ten men on each regimen during the 72 hours after the dose was begun (see Table VIII). The larger dose utilizing gantrisin produced peak levels within four hours and maintained approximately twice the blood concentration maintained by the standard sulfa regimen. The difference between the concentrations of sulfa in the nasopharynx corresponding to these serum concentrations is unknown.

Cultures were obtained as the recruits were enroute to meals. Sulfa was given just before meals and the administration was supervised by WRAIR personnel with the assistance of the cadre of each company. Carrier and resistance status was determined at the time of the first dose of sulfa and again 60 hours later when most but not all of the total dose would have been eliminated. Thereafter observations were repeated at intervals ranging from 36 to 96 hours.

The course of the over-all carrier rate in the three groups is shown in Tables IX-XII. The carrier rate in the control company showed an almost steady increase throughout the study period. In contrast both

TABLE VIII

Serum Concentration of Active Sulfa Expressed as mg. Sulfadiazine per 100 ml.

Company		Hours after Sulfa							
		0	2	6	12	24	36	48	72
C-10 3*	Mean	0.5	2.9	2.7	2.3	2.9	3.2	3.7**	1.2
4 gms sulfa	S.E.($\bar{x}$)	0.1	0.3	0.2	0.2	0.2	0.2	0.2	0.1
	Range	0.1-1.1	1.7-4.0	2.0-3.5	1.6-3.4	2.0-3.7	2.7-4.3	2.2-4.5	0.7-1.7
D-10-3*	Mean	0.7	10.0	10.2	8.5	5.6	6.8	7.4	2.6
10 gms sulfa	S.E.($\bar{x}$)	0.1	0.9	0.4	0.6	0.5	0.4	0.3	0.3
	Range	0.2-1.2	5.0-12.8	3.2-11.8	5.2-12.7	3.4-8.4	5.0-8.7	6.5-9.7	1.3-4.6

* 10 men from each company volunteered to give these samples.

** 9 samples available for this determination only.

TABLE IX

Carrier Status at Time of First Sulfa Administration

Company	Treatment	Number Cultured	Number Carrying *N. meningitidis*	Percent of Company Carrying *N. meningitidis*	Number Carrying Resistant Strains	Percent of Company Carrying Resistant Strains
A	Control	235	101	43	3	1
C	4 gms Sulfa	225	140	78	8	4
D	10 gms Sulfa	238	176	59	13	6

TABLE X

Carrier Rates after First Sulfa Expressed as a Percent of the Carrier Rate before Sulfa

Treatment	Initial Carrier Rate(%)		Days after Sulfa					
			0	2	4	7	10	14
Control	43	Sens.	98	93	93	105	108	125
		Res.	2	5	5	4	4	5
		Total	100	98	98	109	112	130
4 gms Sulfa	78	Sens.	95	1	0	5	5	4
		Res.	5	5	8	12	13	15
		Total	100	6	8	17	18	19
10 gms Sulfa	59	Sens.	90	0	0	0	2	5
		Res.	10	8	12	19	32	58
		Total	100	8	12	19	34	63

TABLE XI

Carrier Status at Time of Second Sulfa Administration

Company	Treatment	Number Cultured	Number Carrying *N. meningitidis*	Percent of Company Carrying *N. meningitidis*	Number Carrying Resistant Strains	Percent of Company Carrying Resistant Strains
A	Control	233	131	56	6	3
C	4 gms Sulfa	212	38	18	26	12
D	10 gms Sulfa	232	85	37	79	34

TABLE XII

Carrier Rates after Second Sulfa Expressed as a Percent of the Carrier Rate before Sulfa

Treatment	Initial Carrier Rate(%)		Days after Sulfa				
			0	2	4	6	8
Control	56	Sens.	96	99			108
		Res.	4	5			5
		Total	100	104			113
4 gms Sulfa	18	Sens.	21	0	0	0	5
		Res.	79	72	83	117	139
		Total	100	72	83	117	144
10 gms Sulfa	37	Sens.	8	0	0	0	0
		Res.	92	114	132	143	154
		Total	100	114	132	143	154

treatment regimens appeared to be equally effective in reducing the carrier rate and both left the resistant strains unaffected. Both companies showed a similar slight increase in sensitive strains during the next two weeks. The carrier rate for resistant strains rose more rapidly in the group on 10 gm sulfa than the group on 4 gm sulfa, but these strains appeared in recruits who were not carriers before sulfa. Thus there were not resistant strains emerging from a carrier of apparently sensitive organisms. This difference in the rate of spread of resistant strains is discussed later. Repetition of the treatment produced comparable results with respect to the few sensitive strains now present.

Longitudinal study of meningococci in nasopharyngeal carriers to determine whether treatment is promoting the emergence of resistant strains is complicated by observational limitations. Due to the transient nature of the carrier state, one can never be certain whether a difference between organisms recovered in two successive observations represents a change in the organism or replacement by another organism acquired from another carrier. This uncertainty can be reduced but not eliminated by limiting opportunity for person-to-person spread. For this reason contacts outside the study group were reduced to the maximum extent possible. To minimize changes in carrier status between observation and treatment, carrier status was defined by the cultures taken at the time sulfa was administered. To minimize possible erroneous identification of an acquired organism as an altered organism, observation of the effect of sulfa should be made as soon as possible. However, if sulfa selects a resistant mutant from the population of organisms in a carrier, the population of organisms may for a time be small. Since the reliability of the technique in the recovery of organisms presumably decreases when few organisms are present, a follow-up observation immediately after sulfa may fail to recover organisms that are actually present. Since the amount of time necessary for an emerging strain to establish itself is unknown, observation was extended for ten days after which any changes in carrier status could with confidence be attributed to person-to-person spread. This technique could only hope to detect an effect of large magnitude.

Within ten days after sulfa, 11 sensitive strains had reappeared in the original 167 carriers in C-10-3, the standard dose group. Two recruits who carried sensitive strains had been unable to take sulfa because of allergy. Five of the strains returning after sulfa were in recruits having contact with these two recruits by virtue of being in the same platoon. The other six individuals whose sensitive strains apparently returned had no known contact with sensitive carriers. Only four sensitive strains reappeared in the 126 original carriers in D-10-3, the high dose group. Three of these had a known contact with a sensitive carrier in their platoon.

Subsequent cultures from individuals who initially carried sensitive organisms were examined for evidence regarding selection of resistant mutants in apparently sensitive carriers (see Table XIII).

TABLE XIII

Appearance of Sulfa-Resistant Strains of Neisseria meningitidis after Sulfa among Carriers of Apparently Sensitive Strains prior to Sulfa

Treatment	Population (Sensitive Carriers before Sulfa)	Days after Sulfa			
		2	4	7	10
Control (A-10-3)	98	1	1	1	1
4 gm Sulfa (C-10-3)	167	2	5	13	15
10 gm Sulfa (D-10-3)	126	0	2	4	11

The two groups appear to be comparable with respect to the number of candidates. Since the time required for a surviving resistant mutant to establish itself is unknown, data for the first ten days is shown. Some or all of these resistant strains that appeared may have been acquired from a resistant carrier. If consideration is limited to candidates who could not have acquired infection from another member of their platoon, no candidates remain in the high dose company and only two remain in the low dose company. Since there were in addition carriers of resistant strains among the cadre of both groups, the possibility of acquired infection cannot be ruled out. Phage typing of these candidates' organisms is proposed to determine whether more specific strain identification is compatible with selection of a mutant from the pre-existing population. Strains are being maintained for this purpose.

Daily rosters of those attending sick-call were kept. From these rosters a record of prescriptions for antibiotics, usually penicillin or tetracycline, was obtained. Comparison of these with the results of throat cultures during the period of the prescription showed that although some individuals lost their organism while on the antibiotic, others did not. Antibiotics received at sick-call apparently accounted for some of the temporary failures to recover organisms from a carrier but affected few carriers in either of the companies receiving sulfa.

One difference between the two treatment groups was evident; the carrier rate for resistant strains rose more rapidly in the high dose company than in the standard dose company. This is most probably another example of the variation in spread of infection characteristically seen in different companies. Several aspects of the particular instance in question support this interpretation. Since platoons behave to some degree independently as to the spread of organisms, it is justifiable to examine them separately. If this is done (Table XIV) one platoon (5th platoon, C-10-3) receiving the standard dose of sulfa is seen to show just as rapid spread of infection as the company receiving the high dose. Moreover, the standard dose company as a whole shows an equally rapid spread of infection somewhat later in time. Finally, this difference in rate of spread of infection is not manifest until a week after the last of the sulfa has been excreted. If sulfa potentiates the transmission of resistant strains of N. meningitidis, the effect would probably be more evident at the time sulfa was present than after it had been eliminated.

Resistant strains recovered from the same individual before and after sulfa administration were examined for changes in the level of sulfa resistance. This investigation revealed that the population of resistant organisms in the nasopharynx of a single carrier was actually a composite of organisms showing different levels of sulfa resistance. The relative numbers of organisms at each level cannot be determined accurately, but it appears that following exposure to either sulfa regimen, the population shifts in favor of organisms with higher levels of resistance. In one individual in which sensitive organisms and

TABLE XIV

Number of Resistant Strains of N. meningitidis
in Each Culture Period
(By Platoon)

Company C-10-3 (4 gm Sulfa)

Platoon	Days after Sulfa										
	0	2.5	4.5	7.5	10.5	13.5	15.5	2.5	4.0	6.5	8.0
	First Sulfa							Second Sulfa			
1	0	0	0	1	1	1	1	1	3	4	5
2	0	0	1	1	1	3	3	3	3	5	5
3	4	4	4	5	4	4	4	4	6	10	13
4	0	0	0	0	0	2	2	1	1	3	7
5	4	6	8	14	16	17	19	20	21	25	27

Company D-10-3 (10 gm Sulfa)

Platoon	Days after Sulfa										
	0	2.5	4.5	7.5	10	14	15.5	2.5	4.5	6.5	8.5
	First Sulfa							Second Sulfa			
1	3	3	4	5	9	16	14	18	20	21	23
2	9	7	10	13	16	25	23	24	28	28	29
3	1	1	1	1	2	10	9	17	19	22	25
4	0	0	1	3	9	20	24	27	32	34	37
5	0	0	0	4	8	10	11	14	19	22	22

organisms resistant to 1 mg percent sulfa were equal in number, the population shifted to all resistant organisms after the first sulfa administration. Most of the resultant population had a resistance level less than 5 mg percent with a few showing higher levels. Following the second sulfa administration most of the organisms present were resistant to more than 5 mg percent and some were resistant to greater than 15 mg percent.

The above field trials have demonstrated that: (1) Standard sulfa prophylaxis (4 gms) in a population in nearly complete isolation apparently eliminated sensitive strains of N. meningitidis from 93 percent to 96 percent of carriers which is considered equal to the 97 to 99 percent effectiveness achieved by increasing the dose to 10 gm. (2) Certain of the resistant strains appearing after a standard prophylactic regimen may represent selection of a resistant mutant within a carrier of an apparently sensitive strain, but this was not avoided by the use of a larger dose of sulfa. (3) In a situation where one has a nearly perfectly isolated population, a high transmission rate for meningococci such as is found at recruit training centers, and only a few carriers of sulfa-resistant meningococci, then religiously administered sulfa prophylaxis which eliminates all sensitive strains of meningococci can within four weeks result in a high carrier rate of sulfa-resistant strains such as was found at Fort Ord in 1963.

b. Laboratory Investigations.

(1) Recovery of Neisseria meningitidis from Nasopharyngeal Specimens.

The selective medium of Thayer and Martin (Pub. Hlth. Rpts. 79:49, 1964), designed for the recovery of pathogenic Neisseria sp from clinical materials containing mixed bacterial flora, and a modification of this medium containing sulfadiazine (WRAIR Ann. Rpt. 1963-1964) has been extensively used during the past year in support of epidemiologic investigations, field trials of prophylactic antimicrobial agents, and in the maintenance of a surveillance program on the nasopharyngeal infection rates with N. meningitidis at the major recruit training centers. Using these two media, it has been shown that a laboratory crew of four men could process up to 750 nasopharyngeal specimens per day and provide accurate information on the total meningococcal carrier status and the percentage of sulfa resistant carriers present.

Ristocetin (Spontin), the gram positive microorganism inhibitor used in this medium, was removed from the market by Abbott in November of 1964. While sufficient supplies were on hand in our own laboratory and the six Army Area laboratories to maintain the various investigative programs for six months to a year, it was evident that a suitable replacement would have to be found. Lincomycin hydrochloride

monohydrate (Lincomycin) recently released for clinical use appeared to have the stability and antimicrobial properties required.

One hundred randomly selected strains of N. meningitidis were tested for sensitivity to Lincomycin incorporated in Mueller-Hinton medium at concentrations up to 20 mcg per ml. All strains grew well at concentrations up to 15 mcg/ml with some diminution in colony size at 20 mcg/ml. It was then incorporated into Mueller-Hinton medium containing 25 units/ml of Polymyxin B in the same range of concentrations. The results were essentially the same as for Lincomycin alone. Maximum growth occurred in the range of 1 to 6 mcg/ml. There was some slight reduction in colony size at concentrations of 7 to 9 mcg/ml. In the range of 10 to 14 mcg/ml., the colonies were as large as in the 1-6 mcg/ml range with some diminution in size above this concentration. There were no apparent differences in the 100 meningococcal strains tested, either in their ability to grow or in their resistance levels to sulfadiazine.

Nasopharyngeal swabs from seven laboratory personnel in the Department of Bacteriology, WRAIR, were cultured on Lincomycin (6 mcg/ml)-Polymyxin B (25 units/ml) Mueller-Hinton medium as compared to the standard Ristocetin (10 mcg/ml)- Polymyxin B (25 units/ml) Mueller-Hinton medium. There was no difference in the recovery of meningococci (2 positives) nor in the amount of contaminating flora present.

In a recent study of Navy enlisted personnel at Andrews Air Force Base, done in collaboration with personnel of the Naval Medical Research Institute (NMRI) at Bethesda, 189 nasopharyngeal specimens cultured on Lincomycin-Polymyxin B (L-P) and Ristocetin-Polymyxin B (T-M) media with and without sulfadiazine (0.1 mgm %) were compared. All swabs were collected in dilution broth for standardization of the inocula. The results are shown in Table XV.

TABLE XV

Meningococcal Distribution on Antibiotic Selection Media

	No. of individuals pos. for meningococci	
	Ristocetin-Poly B	Lincomycin-Poly B
With Sulfa	24	20
Without Sulfa	53	79

As can be seen from these results, the Lincomycin-Polymyxin B (L-P) medium compared very favorably with the T-M medium and in this trial would appear to be superior. On the medium containing no sulfa, the L-P medium was positive in every instance that a meningococci was found by either method. In those specimens containing numerous meningococci (60) the T-M medium demonstrated 46 (76%), while in those showing from 1 to 16 colonies (19), the T-M medium recovered 7 (37%). There were 27 instances in which meningococci were recovered from the sulfa containing media. From the more concentrated specimens (18) the L-P medium was positive in 16 (89%) and the T-M medium in 17 (94%). In those specimens containing few meningococci (9) the L-P medium was positive in 4 (44%) and the T-M medium in 7 (78%).

If one considers only those specimens containing sufficient meningococci to minimize the chance of missed inocula, the L-P media would appear to give a higher total recovery rate with comparable results for the detection of sulfa resistant strains. As with the previously noted findings on T-M medium, Lincomycin had no apparent effect on the sensitivity of meningococci to sulfadiazine.

It should be noted that these comparisons were carried out in two laboratories. NMRI incubated the T-M and L-P (with sulfa) in a CO_2 incubation (8% CO_2) at 100% humidity. WRAIR incubated the L-P and T-M (with sulfa) plates in candle jars. Either method is reportedly satisfactory for the growth of meningococci. However, in view of the discrepancies noted, further studies on techniques of incubation with respect to these media are contemplated.

Evaluations are currently in progress in conjunction with the Second, Fifth and Sixth Army Area Medical Laboratories.

(2) Concomitant Bacterial Flora of the Nasopharynx in Relation to the Meningococcal Carrier Status.

In a study of Neisseria meningitidis carriers, a new transport medium (Cary and Blair, J. Bact., 1964, 88:96-98) was also evaluated for the collection of nasopharyngeal culture specimens (Ann. Report, WRAIR, 1963-64). During a study on the effect of storage temperatures on viability of N. meningitidis, a throat swab from a normal individual (negative for Neisseria) was mixed with the Hampton strain and placed in a transport vial for 3 days at 26°C. All subcultures were negative for meningococci. Since previous trials with the Hampton strain had yielded positive cultures after 3 days' storage, the question of the effect of the total flora on the carrier state was raised. Throat swabs were taken on the normal individual and mixed with the Hampton strain. Duplicate swabs were taken from Hampton, a constant carrier. All swabs were placed in transport medium, stored at 26°C, and subcultured after 3 days storage. N. meningitidis was recovered from the Hampton swabs, while all subcultures made from the normal throat swabs mixed with the Hampton strain

were negative. The aerobic bacterial flora of this normal individual was then isolated and the predominating organisms tested against the Hampton meningococcus in the following manner. Agar plates flooded with a suspension of the Hampton strain were cross streaked with these organisms and the plates incubated at 35°C for 24 and 48 hrs. Inhibition of Neisseria was observed around a streak of Corynebacterium xerosis, which was the predominating organism recovered from the original throat swab. This strain when tested with a number of other strains of N. meningitidis exhibited the same zone of inhibition, indicating a broad range of activity against the meningococci.

In a cooperative URI study with the Virus Department at Fort Dix, notations on the total aerobic flora were made at weekly intervals from 48 recruits. The predominating organisms from each weekly specimen were tentatively classified and preserved by lyophilization. During the nine-week study, two recruits who were negative for N. meningitidis on 22 March 65 had been positive carriers for the previous three weeks. The predominating organism in the specimen collected on 22 March from one recruit was a Corynebacterium sp., while in previous specimens the predominating organisms had been alpha hemolytic Streptococci and Pneumococci. Earlier specimens from the other recruit had been predominantly alpha hemolytic Streptococci and Neisseria, while on 22 March his specimen was negative for Neisseria and predominantly a Corynebacterium sp. and Staphylococcus epidermidis.

The Todd-Hewitt broths used on 22 March for the collection of throat swabs on these recruits were centrifuged and the filtrates added to medium flooded with strains of N. meningitidis isolated from carriers at Fort Dix. After incubation at 35°C for 24 and 48 hrs, growth inhibition was observed. The clear zones observed at 24 hrs increased in size at 48 hrs. Similar plates inoculated with N. meningitidis were made and streaked at right angles with the strains of Corynebacterium and S. epidermidis isolated from the negative specimens. After 24 and 48 hrs. incubation, a clear zone around the growth of the strains of Corynebacterium and Staphylococcus was present.

(3) Occurrence of a "New" Serological Group of Meningococci.

It has long been noted that a number of the gram negative diplococci recovered from cases of meningitis and from nasopharyngeal specimens, while morphologically, culturally and biochemically indistinguishable from N. meningitidis could not be classified in the four recognized serological groups (A,B,C,&D). With the advent of the current sulfa resistant meningococcal problem, and the increased laboratory and epidemiological investigations, the numbers of nontypable isolates being recovered are increasing in scope and complexity.

In August of 1963, a gram negative diplococcus was recovered from the blood of a patient with meningitis at the U. S. Army Hospital, Fort Ord, which has resisted all attempts at serological classification with available antisera. The strain was identified by cultural, morphologic, and biochemical criteria as N. meningitidis, nontypable, sensitive to sulfadiazine.

Agglutinating antisera (Group Bo) prepared by inoculating this nontypable strain into rabbits, appears to be quite specific, not reacting in slide agglutination tests with any meningococci of the four known groups. Whether or not this strain will eventually prove to be representative of a new serological group remains to be established. This antiserum has proved to be of value in several surveillance studies, especially in the recent field trial of sulfadiazine at Fort Knox, by providing a means of quick serological identification of meningococcal strains not reacting with A, B, C, or D typing sera. As many as 80% of strains recovered by the various Army Area laboratories have on occasion been reported as nontypable (NT) meningococci. The large majority of those NT strains submitted to this laboratory have been classified by this new serum. To date approximately 600 strains studied have been classified as Group Bo.

In addition to the nasopharyngeal meningococci classified as Group Bo, two additional cases (one at Fort Knox and one at Fort Polk, including one death) have been so classified. The collection of N. meningitidis cultures from cases of meningitis and meningococcemia contain six additional NT strains which are being tested with this sera.

We have recently acquired three N. meningitidis strains designated X, Y and Z by K. W. Saterus of the University of Amsterdam, Amsterdam, The Netherlands. Strains X and Y were recovered from both cases and carriers while strain Z had been recovered from carriers only. Our Group Bo antiserum in preliminary tests with slide agglutination gives agglutination with strain Y but not X or Z. Antisera to these strains are not available but are currently being prepared.

All isolates of the Bo Group have been sensitive to sulfadiazine with the exception of a series of strains recovered during the February sulfa prophylaxis study reported above. Whether or not these resistant strains were created during the prophylaxis trial is not known.

(4) In vivo Trials of Potential Prophylactic Antimicrobial Agents.

(a) Nalidixic Acid - Thirty freshly isolated strains of N. meningitidis of varying resistance to sulfadiazine were tested for sensitivity to Nalidixic Acid (Winthrop Laboratories). The technique used was the same as that used for testing sensitivity to sulfadiazine. All strains tested were sensitive to 0.75 µg/ml. of the drug and resistant to 0.5 µg/ml. The results were in agreement with those reported by Winthrop Laboratories as bacteriostatic levels for N. meningitidis.

With the uniformly high *in vitro* susceptibility of these meningococcal strains to Nalidixic Acid an *in vivo* trial was proposed. Two adult males on the staff of the Department of Bacteriology, WRAIR, who have been nasopharyngeallyinfected with sulfadiazine resistant meningococci for seven and nine months respectively, were treated with Nalidixic Acid in attempts to eliminate the infections. Sixty mg/Kg were administered daily for four days. Nasopharyngeal cultures were obtained twice daily for three days preceding treatment, during treatment and two days following therapy. These cultures, in every instance, demonstrated *N. meningitidis*, Group B resistant. It was concluded that Nalidixic Acid was of no value as a prophylactic agent for *N. meningitidis* infections.

(b) Pyrimethamine plus Sulfadiazine - Pyrimethamine was examined for its possible effectiveness as a potentiator of sulfadiazine since it has been reported that in malarial therapy there is a synergistic action between sulfadiazine and pyrimethamine. Two volunteer carriers (see above) infected with sulfadiazine resistant meningococci were treated daily with 50 mg of Pyrimethamine plus 2 gms of sulfadiazine for three days. At this time Pyrimethamine was discontinued and sulfadiazine was continued for another two days. Nasopharyngeal cultures were taken daily during this regimen and for three days following completion of this regimen. At no time was a culture obtained that was free of meningococci. Since the dosage of Pyrimethamine approached the maximum usable levels, the results obviated further consideration of this drug combination as a possible prophylactic regimen.

(5) Characteristics of *N. meningitidis* Case Strains.

Since the recognition of sulfadiazine resistant meningococci in the Spring of 1963, and the inability to control meningococcal disease by use of prophylactic measures, all meningococci recovered from cases occurring in the Army have been forwarded to WRAIR for study and preservation. To date 472 isolates have been classified, examined for resistance to sulfadiazine and preserved for current and future investigators, both military and civilian. The breakdown of these isolates is as follows: 7 Group A (1 resistant), 401 Group B (250 resistant), 51 Group C (4 resistant), 3 Group Bo (see above - all sensitive), and 10 non-groupable isolates (1 resistant). Since there is some current speculation that the present rise in meningococcal disease may be the forerunner of a more serious national epidemic caused by Group A meningococci it is interesting to note that of the 7 Group A strains characterized to date, 6 were isolated from U. S. personnel or dependents stationed in the European theatre.

Group B meningococci have been responsible for the majority of the sulfadiazine resistant strains encountered. Of 256 resistant strains recovered from clinical disease, 250 or 98% have been identified

as Group B N. meningitidis. Table XVI shows a breakdown of meningitis cases from which Group B N. meningitidis have been isolated. The data is presented to show differences in the sulfadiazine sensitivities of meningococci recovered with respect to geographic training area and time. The time interval 1 May 1963 to 1 July 1964 covers that period during which all recruit posts had used sulfadiazine as a mass prophylactic agent on one or more occasions. During the period 1 July 1964 to 1 June 1965 mass prophylaxis was not used on any recruit training post, except at Fort Ord (Sixth Army Area), where mass prophylaxis was used just prior to Christmas 1964. (However, this post had stopped all BCT input in September 1964.) It is interesting to note that during the period in which prophylaxis was given 74% of the strains were resistant, while in the second period 51% were resistant. The change in ratio of resistant versus sensitive Group B case strains is even more obvious when one looks at the Fort Ord data. Here the change is from 76% resistant isolates in the first period to 23% of strains resistant in the second period. The one major difference between Fort Ord and the other five recruit training areas is that no sulfa prophylaxis had been given since June of 1963. In the light of past prophylactic and epidemiological studies (see above and WRAIR Ann. Rpt., 1963-1964) and the apparent extended carrier status of Group B carriers, it will be interesting to note whether or not this reversal in the ratio of resistant to sensitive Group B strains continues in the absence of sulfadiazine prophylaxis at other posts.

(6) N. meningitidis in Tissue Culture.

It has previously been reported (Ann. Rpt., WRAIR, 1963-1964) that sulfadiazine resistant Group B meningococci would grow in antibiotic free rhesus monkey kidney tissue cultures (RMKTC), and that these meningococci appeared in the rhesus monkey kidney cell cytoplasm in numbers directly proportional to the incubation time. However, using the basic experimental system described previously (Ann. Rpt., WRAIR, p. 305, 1963-1964) the following tentative observations have been made:

(a) Inoculation of RMKTC with acetone killed Group B meningococci failed to present microscopic evidence of an intracellular tissue phase.

(b) Group B rabbit antiserum (1-10 final dilution) added to RMKTC followed by addition of viable Group B meningococci, prevented the intracellular appearance of the meningococci in the tissue cell cytoplasm.

(c) It was shown that streptomycin at a concentration of 1.0 mcgm/ml. would kill extracellular meningococci, while viable intracellular organisms could be recovered from RMKTC cells following removal of the streptomycin.

TABLE XVI

Group B Case Strains

Time Period	Sulfa Sens.	Army Area							Total
		I	II	III	IV	V	VI	Europe	
1 May 1963 to 1 July 1964	Sens.	0	5	8	3	7	10	19	52
	Res.	21	9	16	38	19	31	13	147
1 July 1964 to 1 June 1965	Sens.	4	5	11	6	19	31	23	99
	Res.	5	16	16	20	18	9	19	103

These limited experiments provide evidence that (1) N. meningitidis invades the RMKTC cells and are not phagocytized, (2) an experimental system is now available to demonstrate whether or not the bacterium multiplies within the tissue cell, and (3) presents a possible in vivo system for study of virulence, and a method for determining protective antibodies in patient serum or body fluids.

Summary and Conclusions.

1. Physiochemical tests have demonstrated a phylogenetic relationship between Proteus mirabilis and its stable L-form but have failed to demonstrate a similar relationship between certain Mycoplasma species and their hypothetical bacterial parents. No homology of nucleotide base sequence was found between M. pneumoniae (Eaton agent) and Streptococcus MG nor between M. gallinarum or M. gallisepticum and Haemophilus gallinarum. Comparisons are also being made between Mycoplasma and L-form species on the basis of particle size, morphology, growth curve patterns and antibiotic sensitivities.

The L-forms of several strains of Neisseria meningitidis have been derived in vitro and one of these L-forms has been propagated through six passages. This is the first reported instance of propagation of the L-form of N. meningitidis.

An agent morphologically resembling a mycoplasma has been isolated from the synovial fluid of a patient with Reiter's syndrome.

2. Field evaluation of a transport medium for collection and shipment of clinical specimens is presented. This medium has been shown to be useful for the preservation of clinical specimens containing enteric pathogens, N. meningitidis or P. pestis.

A simplified, rapid technique for inducing H antigen phase change in Salmonella sp. is described.

The isolation and propagation of N. meningitidis bacteriophage and its potential value for definitive identification of meningococcal strains from cases and carriers is shown.

3. Induced colicine production and its possible implications in intestinal flora changes are reported. A microtiter hemagglutination technique is described and compared with the standard tube method for determination of antibodies to Shigella sp.

4. The enhancement of antibody production by an iron dependent avirulent P. pestis strain in the presence of Ferrous ion is described. The early appearance of complement fixing antibody is demonstrated in laboratory mice without concomitant hemagglutinating antibody.

5. A carefully controlled field evaluation of two different sulfa prophylactic regimens (4 gms vs 10 gms), showed that there was no difference in their ability to eliminate sensitive strains of N. meningitidis from an isolated recruit population. It also showed that sulfa prophylaxis given to a recruit population containing relatively few carriers (2-4%)of sulfa-resistant meningococci can within four weeks result in a high carrier rate of resistant strains.

Preliminary field trials of a new antibiotic (Lincomycin) indicate that this drug is equal to or better than ristocetin as a component of Thayer-Martin medium for the isolation of N. meningitidis from nasopharyngeal swabs.

It has been shown that certain strains of bacteria commonly occurring in the throat and nasopharynx are inhibitory in vitro to the meningococcus.

A N. meningitidis strain not serologically identifiable within the currently recognized groups (A,B,C,&D) has been recovered from three cases of meningitis occurring in Army recruits. Antisera to this strain have proven of value in characterizing many previously non-groupable strains recovered during the course of carrier studies.

In vivo trials of Nalidixic Acid and a Pyrimethamine-sulfadiazine combination as possible prophylactic agents for the control of meningitis outbreaks were unsuccessful.

Growth of N. meningitidis in rhesus monkey kidney tissue cultures and its implications as a possible in vivo system for study of virulence and protective antibody is discussed.

An analysis of the N. meningitidis case strains characterized during the past two years is presented.

Publications.

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Gaines, Sidney, Haque, Shamin Ul, Paniom, Wichiyan, Duangmani, Chiraphun, Cary, Sylvia G. and Blair, Eugene B.: A Field Trial of a New Transport Medium for Collection of Feces for Bacteriologic Examination. Am. J. of Trop. Med. & Hyg., V14, 136-140, Jan. 1965.

Branche, William C., Jr., Young, Viola Mae, Houston, Felicenne M. and Koontz, Lucille W.: Characterization of Prototype Virus ECHO-32. Soc. for Exp. Biol. & Med., V118, 186-190, 1965.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6444	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9 LEVEL OF RESUME
01 07 65	A. NEW	U RPT U WRK	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61145011 3A014501B71Q 01 169

10b PRIOR NUMBER/CODE: None

11. TITLE: (U) Mycotic infections (09:H0)

12. SCIENTIFIC OR TECH. AREA	13 START DATE	14. CRIT. COMPL DATE	15. FUNDING AGENCY
010100 Microbiology	08 55	NA	OTHER | DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. NUMBER / b. TYPE / c. AMOUNT / d. DATE	18 RESOURCES EST.	a PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	2	33
		CURRENT FY 66	2	35

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21. TECHNOLOGY UTILIZATION: Microbiology

22. COORDINATION: NA

23. KEYWORDS: Fungi; Mycoses; histoplasma; coccidioides; blastomyces; immunology; skin tests

24. (U) Tech Objective - Improve serodiagnostic techniques to provide more rapid and accurate diagnostic aids for systemic mycotic diseases. Investigate the ecology and epidemiology of mycotic agents of importance to the military.

25. (U) Approach - Evaluation of fungal antigens currently employed in complement fixation tests to better determine the specificity of these tests and the areas (stages of disease, multiple infection, etc.) requiring improved antigen or a fresh technical approach. Immunized animals are being investigated as laboratory models for determining influence of previous exposure to a mycotic agent on serology during subsequent infection (mycotic or bacterial).

26. (U) Progress (Jul 64 - Jun 65) - Preliminary attempts indicate that histoplasmin can be combined with a cholesterol-lecithin mixture to make a satisfactory slide flocculation antigen. Optimal concentrations of antigen and carrier have been determined and extensive comparative testing with the Latex Agglutination test must be conducted. If a slide flocculation test is developed, results can be obtained within one hour versus the overnight incubation required with the present test. Immunized monkeys (histoplasma, blastomyces, coccidioides) are being examined over an eight month period by complement-fixation, agglutination, flocculation, and agar gel diffusion tests, and this animal appears to be an excellent model for future antigenic studies of the mycoses.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY	28	29. OSD CODE	30 BUDGET CODE
[ ] a. COMSEC OR COMSEC RELATED [X] b. NOT RELATED		BR	1

31 MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands)	36
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A014501B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 169, Mycotic infections

Investigator.
Principal: Lt Col Robert L. Taylor, MSC

Description:

Investigations are related to the improvement of serological techniques as rapid and accurate diagnostic aids for mycotic diseases (histoplasmosis, North American blastomycosis, and Coccidioidomycosis), and the ecology and epidemiology of mycotic agents of importance to the military.

1. Slide flocculation test for detection of early cases of systemic mycoses.

The serological techniques utilized as diagnostic aids for the systemic mycoses include a complement-fixation test (CFT) employing four antigens, a latex agglutination test (LAT) using histoplasmin as the sensitizing antigen, precipitin tests and more recently agar gel diffusion tests have been used on an experimental basis.

In general, the latex agglutination test is positive early in the course of mycotic diseases, frequently before a titer can be demonstrated with the CFT, and reverts to negative as the titer begins to develop in the CFT. For these reasons both the CFT and the LAT are routinely performed on all sera submitted for fungus serology. The LAT, although a simple test to perform, requires approximately 24 hours to complete. For this reason an equally simple test requiring less than one hour to complete was investigated.

Preliminary investigations have been completed which prove that histoplasmin can be incorporated into a cholesterol-lecithin complex and serve as an antigen for use in a slide flocculation test. A commercial histoplasmin (CL-521, Parke, Davis & Co.) was used as antigen. Various concentrations of antigen, cholesterol (Pfanstiehl Labs, Inc.) and lecithin (Sylvania Chemical Co.) were combined and compared using several known positive human sera. The best combination of reagents employed proved to be antigen prepared in the following manner. A stock solution of 1% cholesterol and 0.2% lecithin was prepared in absolute ethanol. Six-tenths (0.6) ml. of histoplasmin was transferred to a 30 ml. round glass-stoppered bottle. The bottom of the bottle was held against the table top and rotated rapidly, while 0.4 ml. of the cholesterol-lecithin mixture was added drop by drop. Rotation of the bottle was continued for 10 seconds after the last drop was added. The stopper was inserted

and the bottle shaken vigorously for 1 minute. Then 1.0 ml. of 0.85% NaCl solution was added rapidly and the bottle again shaken for 1 minute. The sides of the bottle were allowed to drain for about 30 seconds and the emulsion was transferred to a 13mm by 75mm test tube using a sharp tipped pipette. The test tube was centrifuged at 1000 X G for 15 minutes. The supernate was decanted and the tube held in an inverted position while the sides of the tube were wiped dry with cotton tipped applicator sticks. The volume was restored to 2.0 ml. with 0.85% NaCl. When not in use, the antigen was stored at 3 - 6°C. Antigens have been stored for 10 days without loss of sensitivity; however, maximum storage time has yet to be determined.

The qualitative slide flocculation tests are performed on 0.05 ml. of heat inactivated serum (56°C for 30 minutes) placed in one well of a Boerner glass slide. One drop of antigen emulsion is delivered to each serum from a 1 ml. tuberculin syringe fitted with a 23 gage needle. The slides are then rotated at 180 RPM on a rotary shaker for 4 minutes and the results read immediately under the low power of a compound microscope.

Quantitative comparative tests using the standard LAF and the slide flocculation test are in progress using 190 sera obtained by serial bleedings from 15 monkeys. Five monkeys each were immunized with killed cells of Histoplasma capsulatum, Blastomyces dermatitidis and Coccidioides immitis and bled periodically over a ten-month period. Preliminary results of this comparison are included in Section #4 and when complete they should indicate the relative merits of the slide flocculation test and the necessity for further evaluation with human serum.

2. Microsporum gypseum in soils of Thailand.

In the summer of 1964, 140 top-soil samples were collected under the direction of Dr. Howard E. Noyes from 70 of the 71 provinces in Thailand.

These specimens were collected for bacteriologic examination; however, representative portions were also processed for recovery of pathogenic fungi. Two procedures were used (1) an 0.85% NaCl flotation technique followed by intraperitoneal injection of the supernate into mice and (2) a sterilized human hair-baiting technique specifically utilized for the isolation of keratinophilic fungi.

Subsequent cultures of livers and spleens of mice sacrificed three weeks following inoculation with the soil supernates failed to show the presence of pathogenic fungi in these soils.

M. gypseum was recovered from 39.3% of the hair-baited specimens, providing the first demonstration of this dermatophyte in the soils of Thailand.

3. Histoplasmin and Coccidioidin sensitivity in Honduras, C. A.

Studies on histoplasmin sensitivity among the residents of Honduras have been in progress since 1962. These studies are being conducted in collaboration with Dr. Rigoberto Alvaredo L., Director of the Tuberculosis Division in the Office of the Director of Public Health. A total of 58,014 histoplasmin skin tests have been completed during this period (10,884 in 1962; 14,853 in 1963 and 32,277 in 1964). This survey includes inhabitants from every geographic locale in the Republic and will provide extensive data on the prevalence of histoplasmin skin sensitivity in this area of the world.

A compilation of results since 1963 by Departamento is shown in Table I. The results indicate a high endemicity for histoplasmosis throughout this Central American country.

An ancillary study using coccidioidin was also conducted in 1964. Inhabitants in areas selected for the arid climate were tested and the results presented in Table II. Further study is required to properly evaluate the results obtained with coccidioidin especially in the area of Ojojona where 7.4% of the inhabitants appear to be hypersensitive.

4. Serologic response in monkeys immunized with Histoplasma capsulatum, Blastomyces dermatitidis and Coccidioides immitis.

The serodiagnosis of systemic mycoses has always been plagued by the difficulty of interpreting results obtained with the serologic tests commonly employed. Frequently serum obtained from cases of known etiology will react with multiple heterologous antigens in the complement-fixation test and occasionally to a higher titer than with the homologous antigen. Inability to obtain complete and accurate histories makes evaluation of antigen specificity and interpretation of results even more difficult.

In an attempt to determine the amount of cross reactions (or reactions due to common antigenic components) 15 rhesus monkeys were immunized with the three most commonly involved agents causing systemic mycotic disease in the United States (H. capsulatum, B. dermatitidis, C. immitis), the purpose of the experiment being to follow the serology of these monkeys with all of the currently available serologic techniques until the animals revert to a non-reactive status and then to determine the effect of subsequent heterologous fungous antigens (Aspergillus, Cryptococcus neoformans, etc.) in these previously sensitized animals.

Serologic results obtained with human serum indicate that individuals hypersensitive to H. capsulatum, due to a previous exposure, may have complement-fixing antibodies demonstrable with histoplasma and or blastomyces antigens during a subsequent infection due to Aspergillus

TABLE I

Results of Histoplasmin Skin Test Survey in Honduras, C.A.

(1962-1964)

Departamentos	Tests Completed	Percentage Positive Reactions
Francisco Morazan	19,698	50.6
Cortes	220	44.1
Choluteca	21,238	31.7
Valle	4,805	25.0
Compayagua	548	26.3
La Paz	8,739	26.2
Santa Barbara	707	60.7
Clancho	2,059	39.5
Total	58,014	37.4

TABLE II

Results of Coccidioidin Skin Tests in Honduras, C.A.

(1964)

Localidades	Tests Completed	Percentage Positive Reactions
La Venta, Nueva Armenia Sabanagrande	4,896	0.6
Cofradia	527	0.9
Ojojona	1,004	7.4
Santa Ana	776	0.8
Santa Lucia	849	1.1
Penitenciaria Central	1,301	1.5
Total	9,353	1.5

or Cryptococcus neoformans. Verification of such a phenomenon in humans is difficult; therefore, the monkey was selected as a model in which to demonstrate the influence of heterologous fungal antigens on the serodiagnostic tests routinely employed.

A total of 15 rhesus monkeys, three groups of five monkeys each, were immunized with formalin killed whole cells of H. capsulatum, B. dermatitidis and C. immitis incorporated into incomplete Freund's adjuvant. One ml. of the antigen-adjuvant mixture was inoculated under the right scapula of the monkey. All monkeys were pre-bled and skin tested with histoplasmin, blastomycin and coccidioidin prior to immunization. The monkeys were bled periodically (12 times) during the next nine months and all serum subjected to complement-fixation tests using four antigens, latex agglutination tests, agar gel diffusion tests and an experimental slide flocculation test.

The serologic results obtained from the 15 monkeys closely parallel those found in proven cases of human coccidioidomycosis, histoplasmosis and blastomycosis.

All five monkeys immunized with C. immitis had complement-fixation titers with histoplasma and blastomyces antigens which present the same interpretive problems as seen in human cases. The agar gel diffusion test, using 10 X histoplasmin as antigen, was negative with all 65 sera in contrast to the sera from monkeys immunized with H. capsulatum. Latex agglutination and slide flocculation tests were negative or low titer throughout.

The five monkeys immunized with H. capsulatum all showed complement-fixation titers with both histoplasma and blastomyces antigens, but the homologous histoplasma titer was always equal to or exceeded that obtained with the blastomyces antigen. Precipitin lines were demonstrated in the agar gel diffusion test only with the anti-histoplasma monkey serum and proved to be identical with the lines obtained in human histoplasmosis. Latex agglutination and slide flocculation titers were highest among the serum from monkeys immunized with H. capsulatum and the two tests appear to be comparable.

The five monkeys immunized with B. dermatitidis all showed complement-fixation titers with both histoplasma and blastomyces antigens but like the monkeys immunized with H. capsulatum the homologous antigen always equalled or exceeded that obtained with the heterologous antigen. No precipitin lines were obtained in the agar gel diffusion test using histoplasmin as antigen. Latex agglutination and slide flocculation tests were not helpful in differentiating the sera of monkeys immunized with either H. capsulatum or B. dermatitidis.

After nine months the complement-fixation titers are slowly diminishing while the agar gel diffusion, latex agglutination and slide

flocculation tests have all reverted to non-reactive. When the complement-fixing antibodies are no longer demonstrable the monkeys will be subjected to heterologous antigens, bled periodically and the serum studied using the same tests outlined above.

The immunized monkey appears to be a good model in which to study the serology of mycotic diseases. Monkeys subjected to an aerosol to establish an active pulmonary infection, and subsequently administered chemotherapy should provide an ideal model for future investigation.

Summary and Conclusions.

Preliminary studies with monkey sera indicate that a simple rapid slide flocculation test may give results equivalent to those obtained with the more time consuming latex agglutination test. Additional evaluation using human sera is needed before the value of the slide flocculation test can be determined in the serodiagnosis of systemic mycotic disease.

Data obtained from 58,014 histoplasmin skin tests applied to residents of Honduras show histoplasmosis to be highly endemic in that Central American Republic. Results obtained from 9,353 coccidioidin skin tests indicates the status of coccidioidomycosis in Honduras will require additional investigation.

Microsporum gypseum was recovered from 39.3% of 140 soil samples representative of all areas of Thailand.

Fifteen rhesus monkeys, three groups of five each, were immunized with formalin killed H. capsulatum, B. dermatitidis and C. immitis. One pre-bleeding and 12 periodic bleedings during the past nine months have been studied using the complement-fixation test (four antigens), the latex agglutination test, a slide flocculation test and agar gel diffusion tests. The results indicate the monkey is a good model for studying the serology of mycotic disease.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6445	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT	SECURITY BRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 65	A. NEW	U	U	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61145011 3A014501B71Q 01 170

10b. PRIOR NUMBER CODE: None

11. TITLE: (U) Pathogenesis of enteric disease (09)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
010100 Microbiology	05 59	NA	OTHER | DA

16. PROCURE. METHOD	17. CONTRACT GRANT a. NUMBER b. TYPE c. DATE d. AMOUNT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	4	201
		CURRENT FY 66	4	220

19. GOV'T LAB INSTALLATION/ACTIVITY
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21. TECHNOLOGY UTILIZATION	22. COORDINATION
Public Health	NA

23. KEYWORDS: Diarrhea; dysentery; bacillary; cholera; immunity; immunization

24. (U) Tech Objective - To find improved procedures to control diarrheal disease. Present work involves the testing of oral vaccines against bacillary dysentery. If successful vaccines will be of value to the military and to certain civilian populations.

25. (U) Approach - Attenuated dysentery strains are being developed. They are being evaluated for safety in several systems and are being tested for potency in monkeys.

(U) Progress (Jul 64 - Jun 65) - Two kinds of attenuated dysentery strains have been found. One cannot penetrate the intestinal epithelium and is completely avirulent. The other is a Shigella - E. coli hybrid; it penetrates the epithelium and causes a mild inflammatory reaction, but no detectable signs of disease. Both strains protect monkeys against homologous challenge. Fewer doses of the hybrid strain are required to confer protection.

26. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
a. COMSEC OR COMSEC RELATED ( ) b. NOT RELATED (X)		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A014501B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 170, Pathogenesis of enteric disease

Investigators.

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Description.

The pathogenesis of enteric disease is studied to elucidate the mechanisms by which enteric pathogens produce symptoms. By understanding the disease process, improved procedures for prevention and treatment of diarrheal diseases will become evident.

Progress.

1. Our previous studies have demonstrated that the sequence of events which occur in a classical dysentery infection involve penetration of the intestinal epithelial cell by the dysentery bacillus, entrance of the organism into the lamina propria where it multiplies and causes an intestinal ulcer. Mutants of virulent strains of Shigellae which lack the capacity to penetrate the epithelial cell cause neither diarrheal symptoms nor intestinal lesions when fed in large doses to monkeys. The animals react no differently from those fed E. coli. Five doses (5×10^{10} viable cells/dose) of an avirulent mutant strain of S. flexneri 2a employed as an oral vaccine protected monkeys against the disease when they were subsequently challenged with a virulent strain of the same serotype.

An avirulent mutant strain of S. flexneri 1b and an avirulent mutant strain S. sonnei I have been tested for safety and potency. Neither strain causes death or intestinal lesions when fed to starved guinea pigs; neither produces keratoconjunctivitis; neither penetrates HeLa cells; neither evokes symptoms of diarrhea when fed to monkeys. Both of these strains confer protection against homologous challenge when used as oral vaccine in monkeys. The results of the protection tests are summarized in Table I.

Table 1

Diarrheal Symptoms in Monkeys Immunized Orally with 5 Doses of Avirulent Mutant Strains of Shigellae and Challenged with Virulent Strains of Shigellae of the Homologous Serotype

Exp. No.	Treatment*	Vaccine Strain	Symptoms** / Total	P
M45a	Vaccine	S. flexneri 1b strain 2381	1/14	.03
	Control		6/14	
M47	Vaccine	S. sonnei I strain 9774	4/26	.003
	Control		14/25	

* Vaccines were fed at intervals of 3 days and the animals were challenged 10 days after the last vaccine dose.

** Symptoms consisted of either a severe propulsive diarrhea or classical dysentery with blood in the diarrheal stool.

2. We previously demonstrated that hybrid strains obtained by mating a recipient virulent S. flexneri 2a strain with a donor Hfr E. coli strain, lost their ability to cause a fatal enteric infection in starved guinea pigs providing the rhamnose-xylose region of the E. coli chromosome was incorporated into the Shigella genome. The reason for this apparent loss of virulence was studied using one such hybrid strain. The strain retained the characteristics of being able to produce keratoconjunctivitis and to penetrate HeLa cells. While it failed to cause death when fed to starved guinea pigs, it did retain the ability to penetrate the intestinal epithelium, reach the lamina propria, and produce an inflammatory reaction. However, this reaction subsided and the intestinal tract returned to normal within 96 hours. Using viable count procedures and the fluorescent antibody technique we demonstrated that the hybrid strain was unable to multiply to any extent and maintain itself in the lamina propria. When the hybrid strain was fed in large doses to monkeys it was capable of causing mild intestinal inflammation. However, the animals exhibited no rise in temperature and no signs of diarrheal disease.

This hybrid strain has been used as an oral vaccine to protect monkeys against experimental oral challenge. In the first experiment 5 doses were employed. In this study the abiiity of 5 doses of acetone killed and dried (AKD) cells to protect was also assessed. The results of this study are summarized in Table 2 where it is evident that the hybrid strain conferred a significant degree of protection on the test animals. On the other hand evidence of protection was not obtained in the group of animals which received the AKD cells as an oral vaccine.

Three experiments were conducted to determine if fewer than 5 doses of the hybrid strain could protect the animals against experimental infection. In experiment M35, 2 doses of the hybrid strain conferred protection; in experiment M34, 1 dose sufficed; and in experiment M37, we demonstrated that there was no significant difference in groups of animals receiving 1 or 2 doses of the hybrid strain. The results of these experiments are summarized in Table 3.

Because of logistical necessity we have challenged our animals 10 days after the last dose of vaccine was fed. However, one experiment has been completed in which the animals were challenged one month after the last vaccine dose was administered. A significant degree of protection was observed. The results of this experiment are summarized in Table 4.

Table 2

Diarrheal Symptoms in Monkeys Immunized Orally with either One or Two Doses of an Attenuated E. Coli K-12 - S. flexneri 2a Hybrid Strain and Challenged with Virulent S. flexneri 2a.

Group	Treatment*	Symptoms**	P
1	5 doses vaccine	0/8***	group 1 vs.group 3 p = .02
2	5 doses AKD cells (30 mg/dose)	3/8	
3	Control	5/9	

*Vaccines were fed at intervals of 3 days and the animals were challenged 10 days after the last vaccine dose.

** Symptoms consisted of either a severe propulsive diarrhea or classical dysentery with blood in the diarrheal stool.

*** $\frac{\text{Number with symptoms}}{\text{Total}}$; 2 of the 5 animals with symptoms in the control group died.

Table 3

Diarrheal Symptoms in Monkeys Immunized Orally with Either One or Two Doses of an Attenuated E. coli K-12 - S. flexneri 2a Hybrid Strain and Challenged with Virulent S. flexneri 2a

Exp.	Group	Treatment*	Symptoms** / Total	
M35	1	2 doses vaccine	1/14	p = .0003
	2	control	13/18	
M34	1	1 dose vaccine	1/20	p = .02
	2	control	7/19	
M37	1	1 dose vaccine	1/15	group 1 vs. group 2 p = .6
	2	2 doses vaccine	0/10	group 1 + 2 vs. group 3 p = .0003
	3	control	12/24	

* The two doses of vaccine were fed at an interval of 3 days and animals were challenged 10 days after the last vaccine dose was fed.

** Symptoms consisted of either a severe propulsive diarrheal or classical bacillary dysentery with blood in the diarrheal stool.

Table 4

Symptoms of Monkeys Challenged with Virulent S. flexneri 2a One Month after Receiving an E. coli - S. flexneri Hybrid Strain as an Oral Vaccine

Treatment	Symptoms / Total	P
2 doses vaccine	2*/17	< .01
control	8**/15	

* Animals in the vaccine group had symptoms of diarrhea.

**Animals in the control group had symptoms of dysentery.

3. Two experiments were performed in which animals which received either the avirulent mutant or the hybrid strain of S. flexneri 2a as oral vaccines were challenged with heterologous serotypes. In each experiment there was no evidence of protection against the heterologous challenge. Table 5 summarizes the results of these studies.

4. We are currently conducting studies on the mechanism of the protective action of oral immunization against experimental bacillary dysentery in monkeys. Coproantibody (antibody in the intestinal lumen) has been implicated as a possible protective factor in enteric diseases. Preliminary attempts to detect coproantibody in monkeys immunized after administration of five oral doses of the live mutant vaccine or two oral doses of the hybrid vaccine were unsuccessful. Fecal specimens for study of possible coproantibody were collected by inducing diarrhea in the animals with oral administration of magnesium sulfate. The fact that coproantibody could not be detected, although the animals were protected against challenge with the virulent parent strain, was attributed to the high dilution of stool samples.

A modification of the method of processing stool samples was used in the next experiment. This was accomplished by mixing the supernatant samples of stool specimens, obtained as above, with dry sephadex G-25. By this method, samples were concentrated to approximately 10% of the original volume. In this experiment one group of four monkeys was immunized by three subcutaneous injections of formalinized S. flexneri 2a vaccine four days apart. Another group was immunized with two oral doses of the living attenuated hybrid vaccine. Serum and stool samples were collected before and five days after immunization. The following day the animals were sacrificed, tissues were collected for histological, and immunological examination. For immunological tests minced fragments of washed spleen and mesenteric lymph nodes were cultured in vitro and the presence of antibody in the supernatant culture fluids determined by agglutination of latex particles sensitized with homologous antigen (S. flexneri 2a endotoxin). The serum and concentrated fecal samples were assayed for antibody by the hemagglutination method.

The results are presented in Table 6. All of the monkeys had preimmunization serum antibodies against S. flexneri 2a. Both groups exhibited some rise in serum antibody titers with higher increases occurring in the group immunized by its parenteral route. Unfortunately, most of the animals given the oral vaccine appeared to have significant coproantibody titers against S. flexneri 2a prior to immunization. The reason for

Table 5

Diarrheal Symptoms in Monkeys Immunized Orally with Either the Mutant or the Hybrid Vaccine Strains and Subsequently Challenged with S. flexneri Strains of the Homologous or Heterologous Serotypes

Exp. No.	Treatment*	Challenge	Symptoms	P
	Mutant Vaccine	S. flexneri 2a	0/5	
M25	Control	S. flexneri 2a	2/6	
	Mutant Vaccine	S. flexneri	2/5	
	Control	S. flexneri 2b	4/6***	
	Hybrid Vaccine	S. flexneri 2a	0/14	p = 0.57
	Control	S. flexneri 2a	4/15	
	Hybrid Vaccine	S. flexneri 6	5/14	p = [illegible].2
	Control	S. flexneri 6	6/15	
Pool of M25 + M41	Vaccine	Homologous (S. flexneri 2a)	0/19	p = 0.02
	Control		6/21	
	Vaccine	Heterologous (S. flexneri 2a or 6)		p = [illegible].2

* Five doses of the mutant or two doses of the hybrid strain were fed at intervals of 3 days. Animals were challenged 10 days after the last vaccine dose.

** Symptoms consisted of either a severe propulsive diarrhea or classical dysentery with blood in the diarrheal stool.

*** Three of these animals died.

Table 6

Exp M-45
Serological Titrations of Serum, Fecal Samples, and Culture Fluids from Monkeys Immunized with Oral and Parenteral Vaccines

Vaccine Route and Monkey #	Serum		Fecal Sample		Autopsy[1] fecal sample		Culture fluids day					
	Pre[2]	Post[3]	Pre	Post	Ileum	Cecum	Spl[4]	LN[5]	Spl	LN	Spl	LN
Sub Q												
4	160[6]	320	1	1	4	0[7]	0	0	0	0	-[8]	-
7	160	640	0	0	4	0	4	4	2	0	0	0
11	40	2560	1	-	8	1	2	1	2	0	0	0
15	40	2560	0	4	8	0	0	0	0	0	-	-
Oral												
25	160	320	64	32	0	1	1	4	0	4	-	-
26	160	320	16	32	16	1	4	2	1	1	-	-
31	160	320	256	32	32	0	0	0	0	0	-	-
36	160	320	64	4	-	4	1	2	0	0	-	-

(1) Specimens collected from indicated regions at time of sacrifice; (2) pre = pre-immunization; (3) post = post immunization; (4) spl = spleen; (5) LN = mesenteric lymph nodes; (6) numbers are the reciprocal of the final dilution of samples giving positive reaction; (7) 0 = negative; (8) - = no sample.

Note: Serum and fecal samples were assayed by hemagglutination, culture fluids were assayed by latex particle agglutination.

the high pre-immunization coproantibody titers remains unclear just as the post-immunization decrease in coproantibody titer is not understood. The persistence of agglutinins in the in vitro cultures probably represents a real continued production of antibody by lymphoid tissue fragments.

Fluorescent antibody studies of the tissues collected at autopsy are in progress to determine if any antigen remains in the tissue of animals and to see if antibody forming cells can be detected in the various samples removed at sacrifice. In conjunction with these studies we have explored the use of other methods for finding antibody-forming cells in the tissues of animals immunized by various methods. The purpose of these studies is to determine the most practical and sensitive systems for studying antibody formation after oral immunization procedures. To this end we have used a modified Jerne technique for detection of antibody forming cells in animals immunized with Shigella vaccines. This method is based on the passive hemolysis by complement of shigella antigen-coated sheep red cells which have been sensitized by the release of antibody from suspensions of lymphoid cells. The reaction mixture is mixed in a soft-agar overlay and positive reactions are noted as clear circular plaques representing hemolysis of sensitized red cells.

A number of experiments were conducted to find the optimum conditions for the demonstration of antibody-forming cells by this method. Although mice produce good hemagglutinin responses to single injections of shigella antigens, passive hemolysis of sensitized antigen-coated sheep red cells by addition of guinea pig or mouse complement was consistently negative. Single injections of antigen into rabbits consistently produced good hemagglutinin titers and large numbers of antibody-forming cells (200 plaques/10^7 spleen cells at four days post-immunization) in spleen and mesenteric lymph nodes were detected by the Jerne technique. Guinea pigs produce serum hemagglutinins and antibody-forming cells are detectable, but only after 2-3 injections of the Shigella vaccine. The results show that the system can be used to survey various tissues from one animal. The advantage of the method is that the results can be obtained in a few hours. Experiments are planned to utilize this technique with monkeys immunized with oral vaccines.

5. Studies were conducted in which avirulent mutant strains of S. flexneri were mated with an Hfr strain of E. coli. Recombinants were selected on xylose. It was considered that by incorporating the E. coli-

xylose-rhamnose region into already avirulent mutant strains of S. flexneri that this would add another margin of safety to prospective vaccine strains. However, unexpected results were obtained with a single clone of an avirulent strain of S. flexneri 5. This isolate did not produce keratoconjunctivitis, did not penetrate HeLa cells, and did not produce a fatal infection or intestinal lesions in starved guinea pigs. The Hfr donor strain of E. coli was also completely avirulent in all of these models. However, after recombination, S. flexneri 5-E. coli hybrids which had incorporated the maltose region of the E. coli chromosome into the S. flexneri genome had regained the capacity to penetrate epithelial cells. Such hybrids had regained all the attributes of virulent dysentery bacilli.

6. The ability of tissue homogenates made from various organs of the guinea pig to inactivate bacterial endotoxin in vitro was studied using the chick embryo bioassay for endotoxin. Kidney, liver and spleen were found to possess the greatest activity, and were approximately equal in this regard. Cardiac muscle showed an intermediate degree of activity, while skeletal muscle and blood were much less active. Other studies were designed to elucidate the role of the spleen in the response of guinea pigs to endotoxin. When otherwise normal animals were subjected to splenectomy and later challenged with endotoxin, they proved to be no more susceptible to its lethal action than operated controls. When splenectomized animals were given a sublethal dose of CCl_4 and then challenged with endotoxin, they showed no more susceptibility to endotoxin than normal animals treated with CCl_4. These experiments failed to provide evidence that the spleen participates in any important way in the response of either normal guinea pigs or those with liver damage to injection of bacterial endotoxin.

7. Acute liver injury was produced in guinea pigs with three chemically unrelated hepatotoxins: CCl_4, allyl alcohol and DL-ethionine. The effects of these agents on liver morphology, susceptibility of animals to E. coli endotoxin, endotoxin-inactivating ability of tissue homogenates, and substrate oxidation by liver mitochondria were studied. CCl_4 markedly reduced oxidation of all substrates studied except succinate, impaired the ability of liver homogenates to detoxify endotoxin in vitro and increased the susceptibility of animals to the lethal effect of endotoxin by 150-fold. Allyl alcohol produced a severe morphological lesion of the liver but did not impair fatty acid oxidation by mitochondria, diminish endotoxin detoxification by liver homogenates, or greatly enhance susceptibility of the

animals to endotoxin. Ethionine showed an effect intermediate between the other two agents. These findings are consistent with the hypothesis that the liver performs an important function in the detoxification of endotoxin by the oxidation of fatty acid residues in the endotoxin molecule.

8. The pathogenesis of the fatal illness caused by penicillin in the guinea pig has been investigated. A standard intramuscular injection of 50,000 units of aqueous penicillin G was used in female Hartley strain guinea pigs weighing between 300 and 400 grams. In the first 12 hours after penicillin the total number of culturable organisms (predominantly gram-positive bacteria) in the cecum falls to less than 1% of the pretreatment level. Between 24 and 48 hours a ten million-fold increase in the number of coliform bacteria in the cecum occurs. An even greater increase in the number of anaerobic gram-negative rods is also seen in some animals. These changes in the intestinal flora are accompanied by a severe ascites, moderate ileitis and acute regional lymphadenitis. A high incidence of bacteremia due to coliform organisms is found in animals that become ill. This syndrome can be prevented by the administration of non-absorbable antibiotics effective against coliform bacteria. We believe that this disease is an example of bacterial superinfection induced by alterations in the microbial flora of the intestine, and may prove to be a useful experimental model for the further study of this phenomenon.

Summary and Conclusions.

1. Avirulent mutant strains of Shigella flexneri 1b and Shigella sonnei I have been isolated and characterized. Both of these strains when used as oral vaccines protect monkeys against the symptoms of bacillary dysentery following experimental challenge with the homologous serotype. Approximately 5 doses of vaccine are required to confer protection.

2. An attenuated hybrid strain of S. flexneri 2a has been obtained by mating with E. coli. A single dose of this hybrid protects monkeys against symptoms following homologous challenge. The resistance induced by this hybrid strain is still evident one month after the last vaccine dose was administered.

3. Evidence has been obtained which indicate that there is a degree of specificity involved in the resistance induced by either the mutant or the hybrid vaccines.

4. Attempts to demonstrate a consistent antibody response to the orally administered dysentery vaccines have so far been inconclusive.

5. Experiments have demonstrated that a virulent strain of Shigella can be obtained by mating an avirulent Shigella strain with an avirulent strain of E. coli.

6. Homogenates of guinea pig kidney, liver and spleen are capable of inactivating bacterial endotoxin in vitro. Heart, skeletal muscle, lung and blood contain much less activity. Removal of the spleen did not increase the susceptibility of guinea pigs to endotoxin.

7. Study of the effects of CCl_4, allyl alcohol and ethionine on the susceptibility of guinea pigs to endotoxin revealed a correlation between the effect of the hepatotoxin on mitochondrial oxidation of fatty acid substrates and its effect on the susceptibility of the animal to endotoxin.

8. Penicillin in small doses produces a lethal syndrome in the guinea pig. This was shown to be due to suppression of the normal gram-positive intestinal flora, overgrowth of coliform organisms, severe enteritis and bacteria.

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RESEARCH AND TECHNOLOGY RESUME

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23. KEYWORDS: Enteritis; cholera, dysentery; intestinal disease, parasitic; salmonella infections; typhoid; intestinal secretions; intestinal microorganisms

24. (U) Tech Objective - The pathology and pathogenesis of various conditions of the gastro-intestinal tract of man and experimental animals is studied by multidisciplinary approaches with emphasis on morphology. These investigations are considered essential parameters for a comprehension and scientifically based therapy of diarrheal diseases.

25. (U) Approach - Principally morphologic, including light, fluorescent and electron microscopic examinations. Kinetic studies using tritium-labelled thymidine and histochemical investigations are also employed.

26. (U) Progress (Jul 64 - Jun 65) - The height of microvilli of guinea pig ileum diminishes within hours following peroral challenge with virulent S. flexneri 2a. The mechanism by which non-motile but virulent Shigella organisms penetrate the intact intestinal epithelial lining was elucidated in part. The sequence of morphologic alterations of the intestinal mucosa incident to an oral challenge by a Shigella hybrid was described. In collaborative studies with the Pakistan-SEATO Cholera Research Laboratory and the Univ. of Maryland Pakistan Medical Research Center, autopsy and biopsy specimens from patients in West and East Pakistan were studied. The guinea pig model was modified to give a more severe, toxic cholera infection in order to better evaluate the role of endotoxin. Oral administration of staphylococcic toxin in the monkey resulted in acute and subacute gastritis and enteritis. The influence of anti-nerve growth factor on intestinal epithelial renewal was investigated. It was noted that immunologic and pharmacologic blockade of the sympathetic system greatly retarded cell turnover in the jejunum of rats. A heretofore not recorded protozoal enteritis due to a species of Cryptosporidium was described in the guinea pig.
For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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Project 3A014501B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 171, Histopathologic manifestations of diarrheal disease

Investigators.

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Description.

We endeavor to study morphologic manifestations of the reaction of the gut and certain other target organs to various enteric toxic and bacterial challenges in order to elucidate pathogenic mechanisms of diarrheal diseases. This entails both clinical and experimental research of a basic as well as an applied nature. We are using predominantly, but not exclusively, a morphologic approach which includes light microscopic, histochemical, fluorescent antibody and electron microscopic studies. Certain projects have been undertaken in collaboration with the Department of Applied Immunology, WRAIR.

Progress.

The principal investigator acts as consultant in pathology to the Pakistan-Seato Cholera Research Laboratory, Dacca, Pakistan and the Pakistan Medical Research Center, Lahore, Pakistan, and collaborates also with Professor K. Kobari, Tokyo Municipal Komagome Hospital, Tokyo, Japan, on problems of enteric diseases. Series of human small bowel biopsies and autopsy specimens received from Southeast and East Asia have been processed and evaluated here and have helped to direct the experimental research program.

Non-specific enteritis. a) Gastritis and enteritis in healthy West Pakistani men. A study of gastric and small intestinal biopsy specimens from 50 apparently healthy Pakistani men revealed a varying degree of non-specific enteritis in all subjects and a high incidence of antral and superficial gastritis. This study was done in conjunction with P. K. Russell, M. A. Aziz, N. Ahmad and E. J. Gangarosa.
b) Jejunal changes in patients with cholera and other intestinal disorders from East Pakistan. Seventy-eight biopsy specimens have been examined. Analysis of the findings in these specimens is underway.

Cholera. Our original conclusion that a choleraic moiety of Vibrio cholerae and not the whole organism penetrates an anatomically intact epithelial barrier has been amply confirmed in clinical cases as well as in experimental models. The epithelial lining is preserved in cholera of the classic vibrio, El Tor and the non-vibrio types. In addition, electron microscopic studies have shown that in experimental cholera in the guinea pig the intestinal microcirculation is very early involved at a time when there are only equivocal changes in the intestinal epithelial lining. A study of vascular permeability to macromolecules (plasma proteins) in experimental cholera is in progress. The role of the major intestinal glands is being assessed by histologic and biochemical methods. A special effort is expanded to define the role, if any, of cholera endotoxin in the pathogenesis of the disease. Although no positive correlation has been established as yet, we noted focal degenerative lesions suggestive of endotoxin effect in the myocardium, liver, pancreas and renal tubules of guinea pigs subjected to a fulminating vibrio cholerae infection. Several similar lesions have been found in human autopsy material but the presence of concurrent diseases in these patients makes interpretation difficult. Such lesions have not been noted in guinea pigs infected without standard challenge. The pattern of serum gamma globulin responses in patients with acute cholera (Ogawa type), vaccinees and controls was studied in sera of residents of Japan and Thailand by immuno-electrophoresis, and by determination of agglutinating titers and cholera toxicity neutralizing capability. The greatest immunoresponsiveness occurred between 10 and 28 days following onset of disease or vaccination, accompanied by a rise of 19S-antibody titers. The globulin pattern of Thai subjects suggested the presence of antigenic factors leading to active antibody stimulation prior to a cholera outbreak.

Shigellosis. Several aspects of the histopathologic manifestations of experimental shigellosis in the guinea pig were studied. We ascertained that in contrast to cholera virulent, whole shigella organisms penetrate the intact intestinal epithelial lining early after challenge. Their presence in the tunica propria is associated with a generalized vascular as well as an exudative, inflammatory cellular response, which progresses with time. We consider the disturbance of the microcirculation a key mechanism in the pathogenesis of the experimental and probably also of the human disease. Mucosal ulceration, so characteristic of bacillary dysentery, is not due to a direct action of shigella toxin as stated in the textbooks; but occurs, according to our observations, as a consequence of the disturbance of gut microcirculation compounded

by the direct effect of intracellular shigella organisms on individual epithelial cells. Sequential electron microscopic studies elucidated the evolution of intracellular responses of intestinal epithelial cells to invasion by shigella organisms; both degenerative and progressive changes were noted including evidence of phagocytosis of lymphocytes, multi-structured dense bodies and bacteria. The transport of the non-motile shigella organisms through the epithelial cell is probably brought about by a system of rapidly forming and reforming vesicular structures related to the endoplasmic reticulum and Golgi apparatus. In another study the evolution of the intestinal lesion caused by enteric challenge with the non-virulent hybrid strain of E. coli-S. flexneri was compared with that caused by the virulent shigella parent strain. Using the starved guinea pig as the experimental model the latter causes a progressive, acute ulcerative enteritis while the hybrid strain is only able to produce an attenuated lesion, an enteritis which ameliorates with time resulting in the complete restitution of the anatomical integrity of the mucosa. Additional experiments are in progress to study the morphologic responses of the bowel of rhesus monkeys and starved guinea pigs to various shigella hybrids, to enteric shigella vaccines and to subsequent challenge with virulent shigella organisms.

Salmonellosis. Evaluation of findings in chimpanzees experimentally infected with S. typhi is continuing. Intestinal biopsies of human volunteers experimentally infected with S. typhi are being studied. Experiments have been initiated to investigate the histopathologic reaction of normal and starved guinea pigs to enteric challenge by strains of Salmonellae.

Staphylococcal enteritis. The time sequence of gastric and small intestinal lesions produced by sublethal doses of purified staphylococcal enterotoxin have been studied in rhesus monkeys. The gastric and intestinal lesions reach a peak in 6 to 8 hours and regress by 48 to 72 hours. In additional experiments chronic gastritis and mild enteritis was produced by daily doses of staphylococcal enterotoxin.

Viral enteritis. Studies of the response of the intestinal mucosa to the lethal intestinal virus of infant mice (LIVIM) are being continued.

Protozoal enteritis. A unique infection of the small intestinal mucosa by an as yet unidentified species of Cryptosporidium has been described. This organism is situated in the brush border

and causes a severe enteritis but no overt signs of illness. The morphologic and histochemical manifestations of this infection have been described; further studies are in progress.

Histopathologic manifestations of starvation. The study of the effect of starvation of 4 to 5 days on the morphology and mucosal enzymes of the guinea pig small intestine has been continued. It is being extended to a study of the changes in cell renewal and the composition of mucous secretions in the small intestine, as well as to a study of the changes in fat, glycogen content and in the distribution of certain enzymes in the liver. There are indications that the rate of cell renewal is reduced in the small intestine; there is a reduction in the number of cellular elements of the lamina propria, except the polymorphic neutrophils, and a reduction in the activity of the Brunner glands. Some enzymes appear to show a light reduction in activity, while the activity of the acid phosphatase of the macrophages increases. In the liver, starvation produces a massive fatty degeneration, a decrease in the stores of glycogen especially perilobularly, and a decrease in the glucose-6 phosphatase which is very pronounced centrolobularly. The activity of the other liver enzymes studied changes less dramatically.

Autonomic nervous system of the gut. The role of the autonomic nervous system in diarrheal states was investigated in a series of morphologic studies concerned with the normal anatomy in conventionally raised and germfree rodents. The capacity of the system to adapt to changes in size of the area of innervation was established. The effect of endotoxin on abdominal sympathetic ganglia was studied in guinea pigs. Intraganglionic injection of endotoxin (and ethanol) did not produce diarrhea. It is our conclusion that morphologic changes of the abdominal sympathetic ganglia do not have a causal relationship to diarrhea. In a related study in guinea pigs we noted that pathologic changes in abdominal sympathetic ganglia follow a bout of fulminating experimental diarrhea after a lag phase of 72 hours, suggestive of an ascending axonal degeneration which are initiated by hypoxic lesions of intramural ganglia during the acute phase of the diarrhea. Partial destruction of abdominal sympathetic ganglia brought about by immunologic means (injection of bovine anti-serum to the Nerve Growth Factor) or pharmacologic blockade with reserpine does not result in massive diarrhea but has a profound showing effect on the kinetics of intestinal epithelial cell renewal.

Summary and Conclusions.

Histopathologic manifestations of the following conditions were studied:

a) Cholera. The epithelial lining of the intestinal mucosa is preserved in experimental cholera of guinea pigs, even in the most fulminating forms, as well as in human cases of the classic vibrio, El Tor and non-vibrio types. This confirms and extends our original observation. The intestinal manifestations of a V. cholera infection can be reduplicated in the infant rabbit model with cell-free vibrio extracts but not with purified cholera endotoxin. The choleraic moiety causing the diarrhea alters the microcirculation of the intestinal mucosa at a time when the epithelial lining is minimally affected. Serologic studies on human sera have been completed. The role of the major and accessory intestinal glands and the role of endotoxin in cholera is being studied.

b) Shigellosis. The impact of bacterial invasion on the intestinal mucosa was studied in several experimental models employing strains of varying virulence. Virulent dysentery bacilli penetrate the intact epithelial lining and reach the tunica propria within a matter of hours. The non-motile organism is passively transported through the epithelial cell presumably by cytoplasmic organelles related to the endoplasmic reticulum and taking the form of membrane-enclosed vesicles resembling phagosomes. In addition, epithelial cells were seen to phagocytize lymphocytes and cell fragments. The exudative, vascular and cellular inflammatory reaction in the tunica appears early and precedes generalized alterations of villus epithelial cells. Mucosal ulceration is a secondary phenomenon. The epithelial lining cells initially show concurrent degenerative and progressive changes.

c) Non-specific enteritis. The occurrence of this entity was observed in a significant number of subjects from both East and West Pakistan, a finding which greatly enhances the significance of similar findings reported by us in a group of Thai people.

d) Salmonellosis. Chimpanzees respond to a repeat challenge with S. typhi with a slightly greater tendency to specific typhoid granuloma formation than seen on first challenge. Evaluation of the material is continuing. Human volunteers respond to a peroral typhoid challenge with a diffuse enteritis which is characterized by occasional focal granulomatous lesions which were observed three days after challenge at the end of the first half of the incubation

period, suggestive of an important role of cell-bound antibodies in this experimental setting. Follow-up biopsy in these volunteers showed no anatomic lesions. Additional studies of the pathogenesis of salmonellosis are being carried out.

e) Staphylococcal enterotoxin. The mechanism by which this agent is provoking diarrhea is being studied. A histologic study of gastric and enteric manifestations is being completed.

f) Viral enteritis. The intestinal manifestations of the LIVIM virus infection were studied in mice. This infection represents an important new model for study of the response of the intestinal mucosa to injury. The studies are being continued.

g) Protozoal enteritis. We discovered a new entity, a small bowel infection by Cryptosporidia, in guinea pigs. This organism causes a non-specific enteritis which may greatly interfere with experimental studies of other enteric infections. Further work is in progress.

h) Starvation. A study in depth of the effect of starvation on the guinea pig is being pursued because starvation is a prerequisite for the successful take of experimental cholera and shigella infection in this species.

i) Autonomic nervous system of the gut. The anatomic organization of this system, its adaptability to change in size of area of innervation and its role in diarrheal states have been investigated. It was found that damage to the abdominal sympathetic ganglia does not cause diarrhea, that severe diarrhea may result in ganglion cell damage via an ascending axonal degeneration and that immunologic or pharmacologic blockade results in a marked deceleration of intestinal epithelial cell turnover.

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23. KEYWORDS Zoonoses; animals, laboratory; antibody formation; genetics; immunity; epidemiology; leptospira; mycoplasma; animal viruses

24. (U) Technical Objective - To define zoonoses and study infectious diseases of animals that bear on human diseases of potential military significance, and diseases of lab animals used in mil med research. Current studies are: characterization of leptospiras for improvement of diagnostic tests; prevalence of lepto in populations to define areas of infection hazards; genetic relatedness of L; PPLO, and bacterial agents to resolve sources of PPLO; vaccines for WEE to improve current prophylactic measures; etiology of acute respiratory disease in procured dogs to reduce disease rates; etiology of fatal respiratory infections in monkeys to assess infection hazards; transfer of antibody-formation capabilities in animals by genetic means.

(U) Approach - Conventional epidemiol and microbiol technics for study of lepto and lab animal diseases. Immunogenicity and pathogenicity studies on attenuated WEE vaccine are conducted on mice, guinea pigs and ponies. Genetic tools, e.g., determination of G-C base ratios, DNA homologies, and nucleic acid extracts are used in determination of microbial relationships and in antibody (Ab) synthesis studies.

(U) Progress (Jul 64 - Jun 65) - High prevalence of lepto Ab found in C.Z. troops. New lepto types disclosed in U.S. cattle. Saprophytic and parasitic lepto differed in G-C base ratios. New genetic tools had potential applicability for finding bacterial sources of PPLO. An attenuated WEE vaccine with high antigenicity but slight neurotropic properties merits further study. The course and multiple etiology of respiratory disease in purchased dogs were analogous to that in recruits. Known as well as new viral agents were disclosed in dogs and monkeys. Monkey B virus isolated from sick monkeys with no oral lesions. Nucleic acid can induce synthesis of immunoglobulins in a heterologous species.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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Project 3A014501B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 172, Zoonotic diseases

Investigators.
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Description.

The purpose of this task is to study diseases of animals that have or may have a bearing on human infections of potential military significance. Studies encompass: basic biological characteristics of infectious agents, their mode and course of infection; diagnosis, treatment, and control measures; the nature of immunity. Current studies relate to leptospirosis, melioidosis, Western Equine Encephalitis, viral infections of laboratory animals, genetic relatedness of PPLO to bacteria, and induction of antibody synthesis in animals through nucleic acids.

Progress.

1. Leptospirosis.

a. Studies on outbreak of leptospirosis at Fort Kobbe. Studies on an outbreak of leptospirosis in troops at Fort Kobbe, Canal Zone were reported in previous Annual Reports (1963-1964). In reviewing the microscopic agglutination (MA) test data on antibody prevalence in 167 non-hospitalized soldiers in companies involved in this outbreak, it was deemed advisable to reexamine the sera with genus-specific tests. The sera comprised paired samples obtained approximately 1 and 2 months after the outbreak. The presence in Panama of serological types that did not cross-react with MA screening antigens was suspected. Both the hemolytic test (HL) and CF test (Patoc antigen) were used. In previous MA tests, significant reactions were found in 7 soldiers. HL antibodies were disclosed in 5 of these 7 individuals. In addition, significant HL antibodies with titers of 1:160 or greater were found in 21 (12.5%) of the soldiers in whom agglutinins were not demonstrated. Equivocal reactions (titers of 1:40) in HL tests were seen in sera from 34 (20.2%) other soldiers, also, without demonstrable agglutinins. The percentage of low-titer reactions was well above the expected range of non-specific

reactions (e.g. 5%). These antibodies could not be related to the recent disease episode in the companies; the reactions probably reflected, for the most part, previous exposure to leptospirosis. The CF test employing a biflexa antigen is a relatively new procedure. The principal reason for its use in this series was to gather comparative data on its sensitivity and specificity. Positive reactions (titers of 1:2 or 1:4) were obtained on sera of all proved cases of leptospirosis and on 5 of 7 non-hospitalized soldiers with agglutinin titers. The negative CF and HL findings in this group of 7 did not coincide. Satisfactory CF tests were made on 157 soldiers (negative MA tests) of which 113 (72%) were positive. A comparison of CF and HL reactions in sera from 54 non-hospitalized soldiers with positive HL reactions in either or both serum samples is summarized in Table 1.

Table 1. Distribution of Hemolytic (HL) and Complement Fixation (CF) Test Reactions in 95 Sera from 54 HL Positive Soldiers

HL	CF* Neg.	CF* Pos.
Neg.	5	7
1:40	21	34
1:160	18	4
1:640	2	4

* CF test conducted on undiluted sera only.

The correlation of CF results with that of HL tests was poor. Of 28 significant HL reactions (1:160 or >) only 8 were CF positive. The significance of CF reactions requires further study. The CF reactions could not be attributed to non-specific phenomena. Control tests conducted with sera from 50 WRAIR personnel were all negative. In previous tests on 100 normal sera from U. S. and subsequent tests on 149 sera from SF troops, the percentages of positive reactions were 1 and 6 per cent, respectively.

Details of the Ft. Kobbe study are in 2 papers projected for publication in the November 1965 or January 1966 issue of the American Journal of Tropical Medicine and Hygiene.

b. Viability of leptospiras in dog kidney tissue cultures. During this period, a final experiment was completed on the viability of leptospiras in kidney tissue cultures prepared from experimentally infected dogs. The experiment incorporated technics now used in the commercial preparation of an attenuated measles vaccine. Details of the study will be presented in an article -- "Use of antibiotics in the preparation of canine kidney tissue culture vaccines to eliminate leptospiral infection hazards" by Ellison et al.-- to be published in the July 1965 issue of Applied Microbiology. A summary of the paper is presented in this report.

The potential leptospiral infection hazard in the use of vaccines prepared from canine kidney monolayer cultures was studied. Cell cultures were prepared from kidneys of dogs experimentally infected with Leptospira serotype canicola. Viable leptospires were found in kidney cell suspensions at the time of seeding, surviving trypsinization either at room temperature for approximately 2 hr or overnight at 4°C even in the presence of antibiotics. In tissue cultures maintained without antibiotics, leptospires were cultured up to the time of involution of cells at 25 to 34 days of incubation. Cytopathogenic effects of leptospiras on cultured kidney cells were not noted, neither was growth of leptospires remarkable. Generally the leptospire culture titer decreased to 10^{-4} or 10^{-5} from the 4th hr or 1st day of incubation to 10^{-1} or negative by the 30th or 34th day of incubation. The addition of either a combination of penicillin (100 units ml) plus streptomycin (100 ug ml) or polymyxin B (50 units per ml) plus dihydrostreptomycin (100 ug ml) to seeding cell suspensions resulted in the elimination of viable leptospires by the 4th hr of incubation. From cell cultures treated with neomycin (100 ug/ml) or chloramphenicol (100 ug/ml), leptospires were recovered, respectively, after 24 and 48 hr, but not thereafter. It was apparent that antibiotics, particularly the combination of polymyxin B and dihydrostreptomycin could be effectively used to eliminate leptospires in tissue culture. Other antibiotics with known antileptospiral activities probably would be effective also. If antibiotics are not used in canine kidney tissue culture employed for viral vaccine preparations, rigid testing for the presence of leptospires in donor dogs and tissue-culture vaccine is indicated.

c. Serological studies. Serological tests were conducted on cultures or serum or both, submitted to the WHO/FAO Leptospirosis Reference Laboratory of the Division of Veterinary Medicine, WRAIR, from California, Illinois, Canada, Republic of El Salvador, Nicaragua and Equador.

Culture typing tests on leptospiras isolated from dairy cattle in both California and Illinois have affirmed the presence of grippotyphosa infections. In both sections the infections have been associated with disease. The outbreaks in cattle in California occurred in the Arnheim area near Los Angeles. In addition to grippotyphosa, infections with canicola and icterohaemorrhagiae were also present in the same or adjacent herds. A mixed infection in a cow with grippotyphosa and icterohaemorrhagiae was demonstrated bacteriologically. One strain ("R") isolated from the urine of a sick calf in California was identified to be a biflexa type - semaranga (Patoc) on the basis of serological as well as cultural properties. Serological tests were conducted on 62 cattle in Arnheim herds with episodes of disease. Thirty-one had titers

in the order of 1:100 to 1:400; another 28 cattle had titers of 1:25. Reactions were obtained against one or more antigens, primarily borincana, wolffi, pomona, canicola, "R" and hyos. The relative paucity of high titer reactions in herds with recent history of disease episodes and from which isolates were obtained was surprising, and was reminiscent of analogous findings in cattle in Thailand.

The outbreaks in Illinois occurred in the southern portion of the state. In addition to grippotyphosa, serotype hardjo, a leptospira which could not be related serologically to known pathogenic types was isolated from cattle. The strain had growth characteristics of biflexa strains (e.g., resistance to bacteriostatic action of Cu^{++} and 8-azoguanine). The isolation of so-called saprophytic (e.g., biflexa) types in cattle coupled with recent and past reports of their purported presence in man and animals poses new questions on the potential infectiousness of biflexa leptospiras. Experiments are projected to determine conditions that would allow infections with Leptospira biflexa.

Strains from a white-tailed deer, a groundhog and 3 skunks in Canada at Ontario Veterinary College were identified to be pomona serotypes.

Preliminary culture typing tests were completed on 66 of 152 leptospiral isolates obtained from various species of wild and domestic mammals in Nicaragua by a team of workers from the University of Pennsylvania. The distribution of strains by serogroup was as follows: canicola - 32, pyrogenes - 9, hyos - 15, javanica - 2, grippotyphosa - 2, djasiman - 1 and hebdomadis - 1. Four others could not be related to any known serogroups.

Micro-agglutination tests were conducted on sera from 110 cattle from Equador. These animals were in herds with a history of abortion and mastitis. Significant agglutinins (1:100 to 1:400) were found in 56 (51%) of the animals. Titers were primarily elicited against hebdomadis group serotypes (borincana and wolffi) and pomona, and less frequently against autumnalis, butembo and bataviae. A large percentage of the animals had titers for Brucella. Therefore, the disease episodes could not be associated to leptospirosis solely.

No agglutinins were found in 82 bovine sera (impregnated and dried on filter paper) from the Republic of El Salvador.

d. Genetic characteristics of leptospirosis. Genetic studies were initiated to clarify possible taxonomic differences between and within the pathogenic and so-called saprophytic leptospiras. An ancillary objective was to develop methods for the study of basic genetic

mechanisms that may have a bearing in the development and use of prophylactic agents.

Initially, attention was directed to the study of physico-chemical characteristics of 13 pathogenic strains, including homologous as well as heterologous serotypes, and 2 related and unrelated serological types of saprophytes.

For the preparation of DNA extracts, leptospiras were grown in Stuart's medium containing 10^{-3}M NH_4Cl and 5 ug/ml thiamine. Organisms were harvested after 4-to 7-days' incubation, during the logarithmic growth phase. Cells were collected by centrifugation in a Serval RC-2 centrifuge at 16,300 g for 25 minutes. Four liters of culture generally yielded 1.5 to 1.75g (wet weight) of leptospiras. Centrifuged cells were resuspended in a solution of saline citrate and disrupted by treatment with 1% sodium dodecyl sulfate (SLS) at 65°C for 5 minutes. The resulting highly viscous solution was cooled to 4°C and DNA was purified according to the method of Marmur and by the use of cold phenol extraction methods. In some cases, deproteinization was aided by the use of 3 mg/ml of pronase in the presence of 1% SLS for 1-2 hours at 37°C. Ribonucleic acids (RNA) were removed by treatment of preparations with RNAase. Yield of DNA was generally in the order of 1.5 mg/g of wet cells. Thermal denaturation temperature of DNA was determined spectrophotometrically by use of a Beckman DU spectrophotometer specially adapted with a Gilford Model 2000 system to measure optical densities automatically at selected increments of temperature per unit of time. The relationship of thermal denaturation to per cent guanine-cytosine (GC = guanine + cytosine/adenine + thymine + guanine + cytosine) was determined for test conditions by reference to a standard curve derived from measurements of melting profiles (Tm) of DNA from 11 bacterial species with chemically determined base ratios.

DNA was purified from two separate batches of each culture. Two different methods of extraction produced no detectible difference in the physical-chemical properties of DNA. The determination of % G+C by thermal denaturation is a compilation of at least four determinations on each of the duplicate preparations, except for canicola for which one preparation was available. As shown in Table 2, the pathogenic leptospiras with the exception of javanica had similar G+C base compositions which were remarkably different from those of the 3 saprophytic strains. The G+C ratios of the latter were essentially identical. The divergent findings with javanica have been attributed to the presence of a slow-growing contaminating bacteria in the media. The culture of this strain is being purified and additional tests are planned. The findings were consistent with other criteria for the taxonomic separation of pathogenic and saprophytic leptospiras and provides evidence that they are genetically distinct. DNA from 5 of the strains studied were also examined in the analytical ultracentrifuge by Mr. R. V. Citerella and the % G+C was determined from the buoyant density in a cesium chloride gradient. The discrepancies in the Tm profile and $CsCl_2$ gradient findings on % G+C ratios were surprising and may reflect the presence of

minor purine and pyrimidine bases in leptospiral DNA. The presence of 5-methyl cytosine, hydroxymethyl cytosine, and glucosylated pyrimidines has been established in the T-even phages and in DNA from higher organisms, and in such cases, buoyant density determinations cannot be correlated precisely with G+C base compositions. No bacterial DNA examined to date contains these unusual bases in sufficient quantity to alter the buoyant density appreciably. Thus, the possibility of unusual bases occurring in leptospiral DNA poses interesting questions on their phylogenetic origin. Attempts are now being made to characterize leptospiral DNA by chemical analysis.

Table 2. Guanine+Cytosine (G+C) base composition of deoxyribonucleic acids of pathogenic and saprophytic leptospiras

Species	Strain	% G+C determined by melting profile	CsCl gradient
pathogens	australis (Ballico)	36.7 ± .18*	
	hebdomadis (JIH)	36.0 ± .17	
	pomona (Pomona)	36.0 ± .28	33.6 ± .3**
	pomona (CB)	36.2 ± .24	
	bataviae (V.T.)	36.7 ± .14	
	ictero. (RGA)	36.2 ± .16	33.3†
	ictero. (M20)	36.0 ± .16	33.3†
	australis (Aki A)	35.6 ± .32	
	canicola (H.U.)	36.7†	
	javanica (V.B. 46)	40.5 ± .17	
saprophytes	andaman (Correo)	39.2 ± .21	
	Sao Paulo	39.0 ± .32	35.6 ± .3
	patoc	39.0 ± .23	35.7 ± .6

* Standard error of the mean.
** Average deviation.
† Determination on one sample only.

2. Genetic relatedness between bacteria, bacterial L-forms and Mycoplasma sp.

The only universally accepted criterion differentiating stable L-forms from species of Mycoplasma (PPLO) is the knowledge that the L-form was previously derived from a bacterium, whereas, the origin of the PPLO is, as yet, unresolved. Otherwise, these two groups are indistinguishable in their morphological and cultural characteristics.

Heretofore, attempts to relate species of mycoplasma with bacteria by using conventional taxonomic criteria have been unsuccessful.

In recent years technics and information have become available which have further aided in relating and distinguishing various groups of organisms. It is well known that most organisms which are capable of genetic interchange also have similar molar per cent base ratios (expressed as G+C ratio) in their deoxyribonucleic acids (DNA). The converse of this statement is not necessarily true and only serves as a predictive hypothesis in aiding systematic classification. In addition, the agar column hybridization technic (McCarthy and Bolton, Proc. Natl. Acad. Sci. U. S. 50: 156, 1963) has made possible a method for determining the ability of denatured nucleic acid fragments to bind and form specific duplexes with complementary nucleotide sequences of long, single stranded, nucleic acids immobilized in agar. The comparison of binding abilities of DNA fragments to DNA strands from homologous and heterologous organisms has then been interpreted in terms of complementary nucleotide sequence and genetic homology.

It was then assumed that, if PPLO are naturally evolved L-forms of bacteria, their base composition and duplex formation of DNA's might have the same or similar relationships as a bacterium and its artificially produced stable L-form.

In the present study we have used the G+C ratio (as determined by thermal denaturation) and per cent DNA duplex formation to predict and determine the relative homology between a bacterium, Proteus mirabilis, strain 9, and its stable L-form P. mirabilis, L-9. The same rationale was used to determine whether there was any genetic homology between Hemophilus gallinarum and Mycoplasma gallinarum or M. gallisepticum. This study was made in collaboration with Drs. Z. A. McGee and R. G. Wittler of the Department of Bacteriology, and Dr. Stanley Falkow of the Department of Bacterial Immunology.

The pertinent data of this study are listed in Table 3. The first series of experiments were performed with P. mirabilis, strain 9 and its stable L-form to determine whether any significant difference occurs in G+C content or duplex formation of DNA between a bacterium and its stable morphological variant (L-form). It is obvious that these two bacterial forms are indistinguishable by the methods used. The close quantitative base composition of the two morphological forms is in good agreement with the findings that the DNA of Sphaerophorus necrophorus and its "large body" form, both have G+C contents of 31% (Dowell, Soper and Hill, J. Bact. 88: 1805, 1964). Panos, (BBRC 19: 62, 1965) was also unable to find any alteration in the molar base ratios of DNA from a group A streptococcus or its stable L-form. It then follows that if PPLO are stable L-forms of bacteria, the estimation of base composition and nucleic acid homology could aid in their identification. The first PPLO-bacterial series of experiments were

performed with H. gallinarum, M. gallisepticum and M. gallinarum. These organisms were chosen for study because they are all inhabitants of chickens, have been implicated in fowl coryza, have similar sacchrolytic properties and evidence has been presented concerning the reversion and transformation of avian PPLO to H. gallinarum. The extreme difference in DNA G+C content between the mycoplasma species and H. gallinarum, which is approximately 9% for H. gallisepticum and 13.8% difference for M. gallinarum, suggests that there is little, if any, genetic homology between these PPLO and the bacterium.

Previous investigators have demonstrated that the degree of genetic compatibility and molecular hybridization in solution became less with increasing differences in the DNA base composition of heterologous organisms. Sueoka, (J. Mol. Biol. 3: 31, 1961) has even concluded that since the DNA molecules of any specific microorganism are unimodal in distribution and have an average G+C content which is narrow in range, a difference of 10% G+C is predictive of few DNA molecules in common between two bacterial species. Although this is essentially true, it has been demonstrated that the per cent base composition can vary somewhat in small areas of the DNA molecules of a bacterial strain (Guild, J. Mol. Biol. 6: 214, 1963) and denatured nucleic acid fragments of organisms with slightly different G+C ratios can anneal with portions of nucleic acids immobilized in agar (McCarthy and Bolton, 1963). To determine if any regions of nucleic acid homology existed between the bacterium and PPLO, the agar technic was also utilized. As illustrated in Table 3, no genetic homology could be demonstrated between H. gallinarum and the two PPLO.

Another pair of organisms which has often been suspected of being genetically related is Streptococcus MG and M. pneumoniae (the Bru strain of the Eaton agent). Subsequent experiments in our laboratories (McGee et al., In press, 1965) have demonstrated that the DNA of both strains had an almost identical composition. However, although the nucleic acids of each organism were capable of specific duplex formation in homologous reactions, there was no specific duplex formation in heterologous preparations. Thus, even though there was a similarity in gross base composition, there was no genetic homology between Streptococcus MG and M. pneumoniae.

To date, we have been unable to provide any evidence of genetic homology between PPLO and bacteria. None the less, this does not preclude the possibility of such a relationship. Further hybridization attempts between a species of Mycoplasma and a variety of bacteria with base compositions may yet expose a genetic relatedness.

Table 3. Guanine + cytosine per cent composition and duplex formation of DNA

Radioactive labeled DNA	DNA trapped in agar		% labeled DNA bound	% DNA bound relative to bact. form	% G+C of DNA (from Tm)*
20 ug P. mirabilis-9 C^{14}	200 ug P. mirabilis-9		77.6	100	
			78.1	100	
		AV	77.8	100	39.5 ± .2
"	200 ug L-form of P. mirabilis-9		69.4	89.2	
			83.6	107.7	
			83.3	107.0	
		AV	78.8	101.3	39.7 ± .3
	Agar blank		2.4	(3.1)	
			3.6	(4.7)	
20 ug H. gallinarum H^3	200 ug H. gallinarum		41.0	100	
			38.8	100	
		AV	39.9	100	41.9 ± .2
	200 ug M. gallisepticum		1.4	3.5	
			3.6	9.1	
		AV	2.5	6.3	32.7 ± .1
	200 ug M. gallinarum		2.2	5.6	
			1.4	3.6	
		AV	1.8	4.6	28.1 ± .1
	Agar blank		1.5	(3.6)	
			2.7	(6.6)	

* Mean derived from at least three determinations.

3. Viral Zoonoses.

a. Inactivated Western Equine Encephalitis (WEE) vaccine. The evaluation of Western Encephalitis vaccine, produced and partially purified at WRAIR in 1963 (WRAIR Annual Progress Report, FY 64) has continued. The vaccine has been supplied to a number of institutions to be used for the protection of their personnel at risk to exposure. The vaccine was administered in three doses of 0.5 ml each, given subcutaneously on days 0, 7, and 21. All of the individuals received vaccine within a 4-month period.

Pre-and post-immunization serum specimens were collected, the post-immunization specimen was taken 14 days after the 3rd dose of vaccine. The sera were submitted to this Department to be examined for mouse neutralizing antibody (WRAIR Annual Progress Report, FY 64). Where the vaccine was administered, the results obtained are shown in Table 4.

Table 4. Response of human beings to an inactivated Western Encephalitis killed vaccine

Test group	No. Vaccinated	No. of reactions* Positive	Equivocal	Negative
Univ. of Oklahoma	6	6	0	0
Natl. Animal Dis. Lab.	10	2	4	4
N. Y. State Health Dept.	20	8	2	10
Univ. of Kansas	10	0	0	10
Univ. of California	20	12	1	7
Univ. of Alberta	13	6	0	7
Fort Detrick	24	1	0	23
Univ. of Saskatchewan	24	13	1	10

* Criteria for reactions: positive - difference of 1.2 logs or greater in paired sera; equivocal - from .2 to 1.2 log difference; negative - less than 0.2 log difference.

Serologic overlap in cases of vaccination with Eastern, Western or Venezuelan encephalitis viruses has been reported by many workers (Smithburn, J. Imm., 72: No. 5, 376-388; Hearn & Rainey, J. Imm., 90: No. 5, 720-724; Theiler & Casals, Amer. J. of Trop. Med. & Hyg. 7: No. 6, 585-594). The group at Fort Detrick had previously received Venezuelan Encephalitis (VE) tissue culture vaccine at varying times up to 2 years before the WE vaccine was administered. No indication was found that prior vaccination with VE contributed to the development of WEE antibody.

As reported in WRAIR Annual Progress Report, FY 64, sufficient vaccine to immunize approximately 1,000 individuals was furnished the Provincial Health Department of the Province of Saskatchewan, Canada. This area had experienced large outbreaks of WEE in both the animal and human populations in 1941, 1952 and 1963.

The first two groups immunized consisted of 106 poultry ranchers and their families residing in a part of the province where the disease occurred each year, and a group of 64 employees of the Health Department Laboratory and the Departments of Agriculture and Health, all living in the city of

Regina. Many of these employees had worked with the virus, both in the laboratory and in the field. The Provincial Epidemiologist of the Department of Health selected sera from each group for initial testing which he felt would give maximum information, both as to previous exposure and response to the current immunization program.

Paired serum samples from 36 of the poultry ranchers (Group 1) and 28 of the Provincial employees (Group 2) were tested. The results are shown in Table 5.

Table 5. Response of human beings in an enzootic area of Western Encephalitis (WE) to a WE killed vaccine

Group	No. Vaccinated	No. of reactions*		
		Positive	Equivocal	Negative
I Poultry workers (total)	36	11	4	21
Positive pre-immunization titers	8	5		3**
Negative pre-immunization titers	28	6	4	18
II Provincial employees†	28	1	1	26

* Criteria for reactions: positive - difference of 1.2 logs or greater in paired sera; equivocal - from .2 to 1.2 log difference; negative - less than 0.2 log difference.

** Positive but no rise in titer following immunization.

† All preimmunization sera negative.

The 36 individuals in Group 1 consisted of 30 who were 19 years old or younger and 6 adults. All of the adults lived in rural areas of the Province during the 3 pandemics and all 6 had pre-immunization antibody. Group 2 was composed of individuals ranging in age from 19 to 55. The failure of this group to respond to the vaccine is not readily explained. Previous studies have not indicated any difference in the response of different age-groups.

Two other groups in the Province were immunized. Group 3 was composed of residents of the area where the 1963 outbreak occurred and Group 4 comprised a large number of individuals who received three doses of 0.1 ml of the vaccine intradermally. Unfortunately, sera from these two groups have not been received for testing.

In addition, 13 of the paired specimens have been examined by the bead neutralization (BN) and the hemagglutination-inhibition (HI) tests. If the difference in the mouse LD_{50} titers of the sera was greater than 1.0 logs, both the BN and the HI tests detected antibody; if the difference in titer was 1.0 logs or less, both the BN and the HI tests were negative. Both these tests also detected pre-immunization antibody.

A new lot of WE vaccine was produced and partially purified by the Department of Biologics Research, Division of Communicable Disease and Immunology. However, due to the present restrictions on the use of experimental vaccines, only very small amounts of this new lot have been issued. No sera from individuals receiving it have been available for testing.

b. Evaluation of an attenuated Western Equine Encephalitis (WEE) vaccine. The inoculation of ponies and burros with Johnson's attenuated clone 15 virus is described in the previous annual report. These preliminary studies provided evidence that the attenuated virus had little neurotropic potentiality and produced significant levels of neutralizing antibody. Further studies were made of the response of ponies to the attenuated virus, clone 15, and to field strains of WEE virus. The latter studies were conducted for purposes of comparison and to select a suitable challenge virus. In addition, the parental virus, B628 and clone 15 viruses were inoculated into mice and guinea pigs to compare the pathogenicity and immunizing capacity of each virus.

Titrations of clone 15 virus in weanling mice (WM) showed that the virus was not lethal by either the intracerebral (IC) or intramuscular (IM) routes. The (log_{10}) LD 50 titer in each instance was less than 2.0. In contrast, the parental virus was pathogenic by both routes; the LD 50 titers by the IC and IM routes were 5.5 and 2.9, respectively. Control IC titrations of these viruses in suckling mice (SM) indicated that the clone 15 virus was at least 100 times less pathogenic than the parental virus. Approximately 600 SM IC LD 50 were present in the WM IC LD 50 of parental virus, and more than 60,000 SM IC LD 50 of clone 15 virus was not lethal for WM by the IC route.

Clone 15 virus protected mice against IC challenge with the B11 strain of virulent WEE virus. The IC route of immunization was more efficient in protecting mice as 60 SM IC LD 50 were contained in the immunizing dose 50 (ID 50), whereas, approximately 1000 SM IC LD 50 were required for the IM ID 50. Comparison of the parental and clone 15 viruses' immunizing capacity by the IM route, indicated that clone 15 was a more efficient antigen since 100 times less virus was required for protection.

Titrations of clone 15 and parental viruses were made in 250-400 gm female guinea pigs by the IC and subcutaneous (SC) routes. The temperatures of the animals receiving the greatest dose of virus was recorded for 12 days post inoculation. Each guinea pig inoculated by the IC route and 4 of 5 animals inoculated by the SC route with parental strain developed a febrile reaction ($\geq$ 104.0°F). The animals inoculated by the IC route had an average of 3.6 days of fever, while those inoculated by the SC route had an average of 1.2 days of fever. In contrast, a shorter period of fever, average duration of 1.2 days, developed in 4 of 5 animals inoculated by the IC route with clone 15 virus and no febrile reaction followed SC inoculation. Only 2 deaths occurred in 150 guinea pigs inoculated with either parental or clone 15 viruses. These animals were inoculated by the SC route with 6.8 and 4.8 SM IC LD 50 of the clone 15 virus. Clone 15 virus was recovered from the brain of the former but not the latter animal. The deaths in the guinea pigs were probably due to an intercurrent infection as gross signs of pneumonia were noted in the former animal and signs of an enteric infection in the latter animal.

The guinea pigs were challenged on day 21 with 300 guinea pig IC LD 50 of a virulent WEE virus, strain B 11. Following IC or SC inoculation with parental virus and IC inoculation of clone 15 virus, 10-30 SM IC LD 50 were contained in the ID 50. However, by the SC route, approximately 3,000 SMIC LD 50 of the clone 15 virus were present in the ID 50. These experiments provided evidence of the low pathogenicity and high immunogenicity of clone 15 virus.

To select a suitable WEE virus to challenge previously vaccinated ponies, the pathogenicity of 4 field strains of WEE virus was tested. Two strains originated from California, B628 and BFS1703, one from Canada (sparrow 265) and one from Florida, B64. Weanling Shetland-type ponies, free of WEE and EEE virus antibody were injected by either IC or SC routes. Plasma specimens were taken for 5 days post inoculation for virus isolation attempts, temperatures were taken daily for 14 days and the ponies were observed critically for clinical signs of disease for at least 14 days after inoculation. Blood for serological tests was taken at 7-day intervals for the first month, at 14-day intervals for the second month, and at 28-day intervals, thereafter.

To detect viremia, the plasma specimens were inoculated into 1-2 day-old suckling mice (0.01 ml IC), into "wet" chicks (0.1 ml SC), and onto chick embryo tissue culture monolayers (0.1 ml in 0.5 volumes). For each pony, one or more positive viremia isolates were identified as WEE-virus with specific hyperimmune serum, (Porterfield, J. S., Bull. WHO, 1960, 22: 373).

Ponies infected with the B628 and Canadian strains developed a similar pattern of infection. Following IC inoculation, 4 to 5 days of viremia occurred during the first 5 days. Elevated temperatures were noticed usually at the end of the viremic period and lasted 2 to 3 days.

Central nervous system disease symptoms developed after the onset of fever. The animals inoculated subcutaneously with these viruses had an inapparent infection with a 2-4 day period of viremia. The ponies inoculated with the Florida virus by either the IC or SC routes showed no clinical signs of disease. The BFS 1703 virus produced 4 to 5 days of viremia in the animals with a higher level of circulating virus. Two animals inoculated by the IC route had a febrile reaction beginning on the first or second day and persisting to the fifth day. Signs of central nervous system disease began on the fourth or fifth day and progressed to the seventh day. The animals were moribund on the seventh day and killed. WE virus was recovered from the brains of both animals, from the lungs and spleen of one pony and from the cervical cord of the other pony. The pony inoculated by the SC route developed a fever on the sixth day which persisted to the eighth day and signs of central nervous system disease were present from day 8 through day 12. On the basis of these findings, the BFS 1703 virus was selected for challenge of vaccinated ponies.

To determine the pathogenicity of clone 15 virus, two ponies were inoculated by the IC route. In contrast to the response of ponies to the parental B 628 virus, neither of these ponies developed viremia or exhibited signs of encephalitis. One pony had an elevated temperature on days 2, 3, and 12. This rise was unexplained except for an upper respiratory disease then occurring in this and other uninoculated animals. For pathology and virus studies, one pony was killed on day 8 and the other on day 14. For purpose of comparison, two ponies inoculated by the IC route with the parental virus were killed on the same days post inoculation. Gross pathological changes were observed only in the brain tissues of ponies inoculated with the parental virus. They were characterized by excessive venous congestion, mild meningeal edema, and a slight excess of cerebrospinal fluid in the lateral ventricles. Histologically, microscopic evidence of encephalitis was noted in all 4 ponies. The B 628 virus produced severe encephalitic changes; evidenced by gliosis, perivascular cuffing, and neuronophagia. In contrast, the clone 15 virus produced mild encephalitic changes. Virus isolations were attempted from the cerebrum, sections of spinal cord, submaxillary gland, trachea, lungs, kidney, spleen, and pancreas. Virus was recovered only from the cerebrum of 1 pony. This animal was inoculated with B 628 eight days previously, and was exhibiting signs of central nervous system disease. The results of these clinical, pathological, and virus studies indicate that clone 15 virus is markedly attenuated for ponies when inoculated directly into the brain.

Further studies were made of the response of ponies to S.C. inoculation of clone 15 virus. Approximately 5 million SMIC LD 50 were injected into each of 6 ponies and the animals were examined for viremia

and signs of disease as described above. No signs of disease or elevated temperature were noted in any of these animals. However, a minimal viremia (e.g., 1-5 plaque-forming units of virus/0.1 ml of plasma) was observed in 5 of 6 animals. In three instances the viremia occurred for 1 day and in the remaining animals 2 and 3 days. Tests for neutralizing antibody were conducted in SM injected by the intraperitoneal route. Serial 10-fold dilutions of the B11 strain of WE virus were mixed with an equal volume of heat inactivated serum and incubated for 1 hour at 37°C. For purposes of comparison, commercial inactivated WEE vaccine (Cutter) was inoculated into 4 ponies. These animals received two 1 ml doses of vaccine on day 0 and 10. Two of 6 ponies inoculated with clone 15 virus and 2 of 4 ponies receiving commercial vaccine developed a log neutralization index (LNI) of 1.6 or greater on the seventh day. All the ponies had significant levels of antibody on day 14 which persisted to day 56 or 84 when the animals were challenged. The neutralizing antibody response to one dose of clone 15 virus was comparable to 2 doses of the commercial vaccine.

Evaluation of the challenge experiments of vaccinated ponies is in progress. These experiments provide further evidence of the suitability of clone 15 virus as an attenuated vaccine for equine species.

c. Isolation of Eastern Equine Encephalomyelitis (EEE) virus from pheasants. In the summer of 1964, an outbreak of disease in pheasants occurred at Brindle Lake, Fort Dix, New Jersey. Following an on-site investigation by Lt Col S. Vivona, MC, and Maj J. Scanlon, MSC, this department received 7 frozen pheasants for virus studies. Gross examination of birds' tissues did not reveal any significant signs of pathology. From each bird, tissue suspensions were prepared and inoculated into suckling mice, one-half-day-old chicks, and embryonated eggs. From 3 birds, transmissible agents were obtained. Each isolate was identified as EEE by neutralization test.

Eastern equine encephalitis virus has been previously isolated from pheasants in New Jersey (Sussman, O., et al., Ann. N.Y. Acad. Sci., 70: 328, 1958). Although inactivated EEE vaccine is recommended to control this disease, the vaccine has not completely protected pheasants. The present findings confirm Sussman's observations and point out the need to develop potent EE vaccines.

The situation at Fort Dix illustrates the potential hazard which may exist at military installations which maintain concentrations of game birds, i.e., pheasants and chukars, and the necessity of adequately vaccinating the birds before possible exposure.

d. Virus studies of laboratory primates. In cooperation with the Department of Laboratory Animals, studies are being conducted on the etiology of respiratory diseases in newly procured laboratory primates. From the lungs of 4 rhesus monkeys which died of respiratory disease, transmissible agents have been recovered. Pathological examination of the animal tissues was made by Capt T. Magnani, MC, and Capt C. Biggers, MC. Pneumonia, with giant cells consistent with measles virus, was noted in two animals. From the lungs of one of these animals, B-virus was recovered. In addition, B-virus was recovered from the lungs of another animal which showed pathological evidence of pulmonary emphysema. Respiratory syncytial virus was recovered from the lungs of the third monkey which showed pathological evidence of lobar pneumonia. An unknown virus was recovered from the lungs of a fourth monkey. This virus produced an"enterovirus-like" cytopathogenic effect in rhesus monkey kidney cells. This agent was resistant to ether, chloroform and 5-iodo-2-deoxyuridine. Attempts to serologically identify the agent with antiserum against the human enteroviruses -- poliovirus I, II, & III, echo virus types 1 through 28, and coxsackie A-9 and B-1 thru B-5 -- were unsuccessful (tests conducted by Mr. Parker, Virus Dept, CD&I, WRAIR).

In order to ascertain the importance of each of these agents and other known pathogenic agents of monkeys, a serological screening program has begun. Approximately 10% of incoming monkeys are bled on the day of arrival, and on days 28 and 42 of the conditioning period. These studies are in progress.

The isolation of B-virus, of great potential hazard to laboratory personnel, made it desirable to determine whether the B-virus was spreading in the area where the new animals were quarantined. For this purpose neutralization tests versus B-virus were conducted. Neutralization tests were conducted by mixing 4-fold unheated serum dilutions with approximately 100 tissue culture doses of B-virus. The mixture was incubated for 1 hour at room temperature and inoculated into rabbit kidney cell cultures. A known positive serum and virus titration was included in each test for control purposes.

On the day of arrival, the sera from 7 of 26 monkeys contained neutralizing activity against B-virus (Herpesvirus simiae). In general the titers were low, 3 at 1:4, and 4 at 1:16. During quarantine these titers either decreased or remained unchanged. In only one instance of the 24 paired serum samples tested was the possible development of neutralizing activity noted. The serum titer of this animal was less than 1:4 on arrival and the titer was 1:4 at the completion of quarantine. These data suggest that B-virus is not actively spreading in the colony at this time.

The presence of neutralizing activity in the sera of approximately 30% of incoming monkeys indicates past contact of these animals with B-virus. As herpes viruses may persist in a latent or carrier state, caution in handling monkeys must be stressed, and in this regard, it is particularly noteworthy that B-virus was isolated from monkeys without oral lesions.

4. Respiratory diseases in newly procured dogs at the Walter Reed Army Institute of Research animal colony.

In cooperation with the Department of Laboratory Animals, studies have continued to define the etiology of respiratory disease in newly procured dogs. Approximately 70% of the dogs become ill with respiratory disease during the three weeks of quarantine after procurement. Many of the sick animals develop a protracted pneumonia. The mortality rate among sick animals is approximately 20%. A bacterial etiological agent could not be demonstrated. This, coupled with the consistent failure to reduce morbidity rates by antibiotic treatment directed attention to study of viral agents. The isolation and characterization of several distinct viruses from dogs with respiratory disease, as well as serological findings in test animals are described.

a. Isolation studies. Specimens for virus isolations were collected from a group of 75 dogs which were employed in a trial of an inactivated adenovirus vaccine (Ann. Report 1963-1964).

Throat specimens from sick dogs and suspensions of post-mortem tissues were treated with antibiotics and centrifuged to remove bacteria. The specimens were inoculated into primary dog kidney (DK) cell cultures, primary cercopithecus kidney (CMK) cell cultures, Hep-2 cells, and embryonated hen's eggs. The cell cultures were examined for the presence of cytopathic effects (CPE) and for the presence of hemadsorption (H) after the addition of guinea pig erythrocytes. At least one subculture at 7-14 days post-inoculation was carried out before considering a specimen negative. Suitable controls were included in each cell culture isolation test system to rule out the presence of adventitious agents.

The isolated agents were tentatively placed in three groups by the characteristic type of CPE or by the presence of H. The first group of isolates caused an adenovirus type of CPE, the second a "herpes-like" focal CPE, and the third group of agents were detected by the H technique.

Prototype isolates were selected and purified by three passages at terminal dilutions. Antisera against these isolates were produced in rabbits. The "herpes-like" isolate and the H isolates were identified

in neutralization tests. The adenovirus isolates were typed by an hemagglutination-inhibition (HI) test by use of antisera prepared against infectious canine hepatitis (ICH) virus and Ditchfield's canine adenovirus, Toronto strain. The latter virus was reported to be serologically distinct from ICH virus (Canad. Vet. J. 3: 238, 1962).

The canine adenoviruses and the "herpes-like" viruses were recovered only in primary DK cells. The H agent was recovered in both CMK and DK cells. Isolation attempts in Hep-2 cells and in embryonated eggs were unsuccessful. From specimens obtained on the first day of illness, 16 transmissible agents were recovered and identified from throat specimens of 13 of 38 dogs tested. The 16 isolates included 7 Toronto-like adenoviruses, 3 ICH adenoviruses, 5 H viruses and 1 "herpes-like" virus. From 3 dogs, both the Toronto-like canine adenoviruses and the H agent were recovered. Virus recovery attempts from the post-mortem tissue suspensions of 10 dogs are summarized in Table 6. From 9 dogs which died of respiratory disease, 30 transmissible agents were recovered. In 5 instances, the same agents, ICH, Toronto-like adenovirus, and the H agent were recovered from both the acute throat specimen and from the post-mortem tissues after an interval of several weeks. Each of the agents, with the exception of the ICH virus, was recovered from the lungs of one or more dead dogs. ICH virus was recovered from 2 tonsils and from 1 trachea. Multiple isolations were obtained from the post-mortem tissue of 5 of 10 dogs. From 4 dogs, 2 agents were recovered; the fifth yielded 3 agents. For most of the dogs, the same agent was isolated from more than 1 tissue and from 2 dogs, the viruses were recovered from tissues other than that of the respiratory tract. It is of interest to note that 3 of the isolated agents were present in high titer, i.e. 10^5 tissue culture doses of virus per gram of lung. These agents were a Toronto-like canine adenovirus recovered from one dog (#C955) and both a herpes-like and H agent were recovered from a second dog (#D004).

Table 6. Agents isolated from dogs with fatal respiratory disease

Dog No.	Day of Onset*	Acute specimen Agents isolated**	Day died	Agents isolated from the indicated tissue: Tonsil	Trachea	Bronchi	Lungs	Liver	Spleen	Kidney	Pathological findings†
C955	6	ICH‡	30	ICH	ICH		Toronto‡				Acute broncho-pneumonia
C958	8	Toronto	33	Herpes	(Herpes	Herpes)**	Hem‡				Lobar pneumonia
C960	13	Toronto	33	Toronto			Toronto				Lobar pneumonia
C982	9	ICH	69		Hem	Hem	Hem				Acute suppurative bronchopneumonia
C990	6		63		Hem						Acute suppurative bronchopneumonia
D001	14		16								Acute lobar pneumonia
D004	13	Toronto,Hem	49	Hem-Herpes	(Herpes-Hem)**		Hem-Herpes‡				Pneumonitis with giant cells
D007	5	Toronto*	51	ICH			Hem				Acute suppurative bronchopneumonia, tonsillitis
D016	5	Hem	37		(Hem	Hem	Hem)**		Hem	Hem	Lobar pneumonia acute bronchitis
D033	5		39	Herpes Hem	(Herpes	Herpes	Herpes)**	Hem	Herpes	Hem‡ ICH	Focal pneumonitis, giant cells present

* Day 0 equals day of arrival
** ( ) Pooled tissue suspension
† Pathological examination was made by Capt D. Biggers, MC, WRAIR
‡ Agent reisolated

b. Serological studies. Serological tests were conducted to determine the incidence of infection with each of the isolated agents, canine distemper virus (CDV) and reovirus. Neutralization tests were conducted in eggs for CDV, in primary DK for Herpes-like agent, and in Maden dog kidney for H agent. Hemagglutination-inhibition tests were employed for the adenoviruses and for reoviruses. Confirmatory neutralization tests for the adenoviruses and reoviruses are in progress.

Sera of 3 groups of dogs were studied. First the 75 dogs employed in the adenovirus vaccine trial; second, a group of 73 dogs which received the attenuated ICH-CDV vaccine, and a third group of 21 dogs which were not vaccinated against either ICH or CDV. The dogs which received the attenuated CDV-ICH vaccine represented an approximate 10% sample of incoming dogs over an 8 months' period. Each animal was bled on the day of arrival and at the completion of the 21-day period of quarantine and/or 2 to 3 weeks post onset of respiratory disease. The professional staff of the Department of Laboratory Animals examined the animal daily for signs of respiratory disease.

Canine distemper virus neutralizing antibody is known to be long lasting and Gillespie has found that animals having a neutralizing serum titer of 1:100 are immune to CDV (Ann. N.Y. Acad. Sci., 101: 540, 1962). Therefore, a preliminary CDV neutralization test at this serum dilution was conducted on the serum specimens obtained on day of arrival. Table 7 summarizes these results and subsequent illness in the 3 groups of dogs. Approximately half of the dogs were serologically immune to CDV. During quarantine, the 3 groups of dogs had roughly similar incidences of respiratory disease (approximately 70%) and mortality (approximately 20%). Fifty to 70% of the sero-positive animals developed respiratory disease. Canine distemper virus antibody studies on serum samples before and after illness of 9 seropositive dogs showed no change in titer, thus supporting the concept that animals having a titer of 1:100 are not reinfected. Dogs which were CDV seropositive on arrival and well during quarantine had levels of CDV neutralizing antibody comparable to CDV seropositive animals which became ill. These findings pointed to the probable presence of etiologic agents of respiratory disease other than CDV.

Most dogs (37/43) which were CDV seronegative on arrival, and which were not vaccinated against CDV, developed a significant rise in antibody titer. More than 90% of these seronegative dogs (40/43) became ill, as compared to 50% of the seropositive animals (25/53). The vaccination of the dogs with attenuated CDV vaccine did not essentially change the incidence of respiratory disease during quarantine for the CDV seronegative animals.

Table 7. Canine distemper virus antibody status of newly arrived dogs and subsequent respiratory illness

Vaccine study	Observation during quarantine	Presence of CDV antibody No. positive*	No. negative	Total
Live ICH-CDV	Respiratory illness	26 (1)**	32 (10)	58 (11)
	None	8	7 (1)†	15 (1)
		Total 34 (1)	39 (11)	73 (12)
Inactivated adenovirus vaccine trial	Respiratory illness	20 (1)	33 (9)	53 (10)
	None	19	3	22
		Total 39 (1)	36 (9)	75 (10)
None	Respiratory illness	5	7 (1)	12 (1)
	None	9	-	9
		Total 14	7 (1)	21 (1)

* A 1:100 serum dilution neutralized 100 EID_{50} of CDV virus
** Number in parenthesis subsequently died
† Death not due to respiratory disease

Serological studies of animals which died of respiratory disease revealed that 21 of 23 animals were seronegative on arrival. On the basis of chance, approximately equal numbers of dogs with and without antibody would have died. Although other agents (Table 6) have been isolated from the dead dogs, the role of CDV as the cause of respiratory disease appears to be very important. Evidence that at least half of the non-vaccinated animals were infected with CDV was provided by histopathological examination findings in 2 dead animals and by demonstration of rises in antibody titer in 6 of 11 animals, although in 4 instances the antibody rises were small. In contrast, nearly all the seronegative unvaccinated dogs that survived respiratory disease developed high levels of CDV neutralizing antibody. Furthermore, the duration of illness and pneumonia in CDV seronegative dogs was 30 and 23 days, whereas, that of CDV seropositive animals was 10 and 3 days respectively.

Determination of the incidence of adenovirus infection in the dogs was complicated by the use of the attenuated ICH vaccine and inactivated adenovirus vaccines in the adenovirus vaccine trial. However, a group of 38 dogs were not vaccinated and HI tests were conducted with ICH and the Toronto-like viruses. The HI test was chosen because of simplicity and specificity of reaction. Further studies with the neutralization test are in progress. Three dogs had 4-fold or greater antibody rises to ICH virus only, and 2 animals were overtly ill. Five dogs had rises to only the Toronto virus and 3 were clinically ill. Nine additional dogs had a rise to both viruses and 6 had clinically apparent infections. Although nearly half the 38 dogs had an adenovirus infection, the incidence of infection was the same in the sick and well animals. Twenty-five of these 38 dogs were clinically ill. The rise in titer to both agents may represent a double infection or a heterotypic antibody response following exposure to a serologically related agent.

Neutralization tests on serum specimens obtained on the day of arrival revealed a 3% (4/149) prevalence of antibody against the canine herpes virus. During the quarantine period, 15 dogs developed a rise in antibody titer. The overall incidence of infection in sick and well animals was roughly the same, i. e. 11% (11/106) in sick dogs and 10% (4/43) in well dogs. There was no evidence from these data that the canine herpes virus is readily transmitted from animal to animal.

Sera of 149 dogs were tested for neutralizing antibody against the hemadsorption (H) agent. On the day of arrival, 4 dogs (3%) were found to have neutralizing antibody. At the end of their quarantine, 106/139 or 76% of the surviving dogs had acquired antibody. Thus, it was apparent that the H agent is spread rapidly and transmitted among dogs. The occurrence of infection in sick dogs 72/98 or 73% was essentially the same as well dogs 31/41 or 83%.

Lou and Wenner (Am. J. Hyg. 77: 293-304, 1963) have isolated reovirus type I from a dog with respiratory disease and they found that one-third of dogs had antibody to reovirus type I. Therefore, the sera of 86 dogs were tested for HI antibody against reovirus type I. Forty-seven or 55% of the animals had antibody titers on the day of arrival. During quarantine 12 dogs had a 4-fold rise in HI titer. The incidence of infection in sick and well dogs was essentially the same, i.e. 9/64 (13%) rises in sick dogs and 3/22 (14%) in well dogs. Although no isolations with reoviruses have been made, there was serological evidence of their presence in the dogs.

c. Identification of a canine herpesvirus. The recovery of a transmissible, filterable agent from primary dog kidney tissue culture (DKTC) in which cytopathic effects (CPE) appeared spontaneously was reported in the Annual Report of 1964. Moreover, agents which produced similar CPE were repeatedly recovered from tissue cultures prepared from kidneys of puppies obtained at the WRAIR dog colony and from puppies which had died of pneumonia. The prototype agent, designated S4-63, was initially believed to be a PPLO, since it was possible to isolate a PPLO in artificial medium. During the course of this study, Carmichael et al. (Proc. Soc. Exp. Biol. & Med. 117: 826, 1964) recovered a serologically related agent, which was reported to be a PPLO, from a fatal septicemic disease of puppies.

An agent, designated D004 which produced a similar CPE to that of S4-63 was recovered from the lungs of a dog which had died of a respiratory infection. In addition to D004, a PPLO and an adenovirus were also isolated from the lungs of the same dog. Hyperimmune serum was prepared in rabbits against D004, and using serum neutralization tests in DKTC, D004 was found to be indistinguishable from the cytopathic S4-63 propagated in dog kidney cells. The inability of the isolated PPLO to produce the cytopathic changes in DKTC posed questions on the possible presence of a second agent.

Monolayers of dog kidney cells on coverslips in Leighton tubes were infected with D004 and S4-63 propagated in DKTC and were examined by acridine orange fluorescence microscopy. With this method DNA-containing compounds fluoresce green and RNA-containing compounds fluoresce red. Large, green, intranuclear inclusions similar to those produced by the herpesviruses were observed in the infected cells. Examinations of these preparations provided no evidence of the presence of mycoplasma. Infected cells stained with either hematoxylin and eosin or May-Grunwald Giemsa contained type A intranuclear inclusions; also, in these examinations, organisms suggestive of mycoplasma were not observed.

D004 multiplied to high titer and produced cytopathic changes in dog kidney cells. Attempts to subculture the agent in artificial medium from dog kidney tissue culture were unsuccessful, although a PPLO could be readily isolated from the original tissue suspension.

The sensitivity of the agent to various antibiotics was determined by the incorporation of kanamycin, tylosin and tetracyline into the nutrient fluid of infected cell cultures. Replication of the agent was not affected by concentrations of antibiotics which were not toxic to the host cells.

The biological, chemical and physical properties of the agent, viz., a DNA core, ether sensitive, 150-200 mu in size, heat sensitive, and type A intranuclear inclusions, are consistent with those of the herpesviruses. On the basis of cross-neutralization tests, the canine herpesvirus was serologically unrelated to pseudorabies, B virus and herpes simplex.

It was apparent from the experimental findings that S4-63 may have contained two organisms, a virus and a PPLO; however, the cytopathic changes produced in dog kidney tissue culture could only be associated with the herpes virus. The cytopathic agent is apparently a new member of the herpesvirus group. Carmichael (personal communication), on the basis of further studies, has concluded that the agent originally reported to be a PPLO is a herpesvirus.

d. Identification of myxovirus SV-5 in dogs. The hemadsorbing isolate, C-958, used as a prototype strain was obtained from the lung of a dog that had died with respiratory disease. The isolate was purified by three successive passages of terminal culture dilutions in primary dog kidney cell cultures. Antiserum was prepared in rabbits using antigen grown in BS-C-1 tissue cultures. The virus was completely inactivated by treatment with chloroform for 10 minutes. The size was estimated to be 140 mu by use of filters of various porosity sizes. The titer of virus was not decreased by the presence of 5-iodo-2-deoxyuridine in the medium, providing evidence of an RNA-type virus. Virus prepared in 2% fetal bovine serum-BME (Engles basal medium) in Maden dog kidney cultures did not lose titer after 6 hours at 37°C, room temperature, and 7 days at 4°C. There was a 3 log loss in titer after 3 months at 4°C, a 4 log loss after 10 minutes at 56°C; after 30 minutes at 56°C, the virus was completely inactivated (6.5 log loss in titer). It had been stable at -70°C for 8 months. The virus can be propagated in a wide range of cell cultures including chick embryo fibroblast, primary and serial dog, bovine, rhesus and African green monkey kidney and primary hamster and human kidney. Rabbit kidney cell culture did not support growth. Specific cytopathic effects have been observed only in primary bovine kidney where a "syncytial-like" cell is formed. Infected cultures of primary and Maden dog kidney, serial monkey and bovine kidney stained with hemotoxylin and eosin showed eosinophilic cytoplasmic inclusions. The virus can also be propagated in the amniotic fluid of embryonated eggs. Dog, guinea pig, rooster and human type O red blood cells are agglutinated by infected amniotic fluid at 37°C, room, and 4°C temperatures. These characteristics of the agent are consistent with those of the myxovirus group.

Neutralization tests were performed by incubating equal volumes of inactivated serum dilutions and virus containing 100 tissue culture dose 50 (TCD 50) at room temperature for 1 hour and inoculating Maden dog kidney tissue cultures. Neutralization was determined on day 6 by inhibition of hemadsorption of guinea pig red blood cells. Hemagglutination-inhibition (HI) tests were performed by incubating equal volumes of inactivated kaolin extracted serum dilutions and 4 units of antigen (amniotic fluid) at room temperature for 1 hour. A 0.25% suspension of human type O red blood cells was then added and allowed to settle for 2 hours at room temperature. The close antigenic relationship between the hemadsorbing agent and SV-5 and the absence of any significant cross-reaction with mumps virus are shown in Table 8. Tests to deny or affirm this relationship of the agent with mumps were included in view of the reported presence of mumps antibody in dogs by Morris et al., (Cornell Vet. 46: 525, 1956).

Table 8. Antigenic relations of a hemadsorbing virus (C-958) isolated from dogs and SV-5 and mumps virus

Test for antibody	Serum	Reciprocal of titer with antigen C-958	SV-5	Mumps
Neutralizing	anti-C-958 (rabbit)	64	64	4
	dog C-966*	256	64	4
	anti SV-5 (rabbit)	1024	256	not done
	anti SV-5 (guinea pig)	256	256	not done
	anti-mump (rooster)	4	4	256
	anti-mump (guinea pig)	4	not done	2560
Hemagglutination inhibition	anti-C-958 (rabbit)	640	not done	20
	dog C-974*	80	not done	10

* Cross reactions in 2 of 106 dogs that developed high antibody titers for hemadsorbing (H) virus. H virus was isolated from throat of dog C-974 during an acute illness.

Neutralizing antibody to the hemadsorbing agent was present in human globulin, canine globulin (Pittman-Moore) and in 5 of 10 sera from personnel at Foresc Glen; one of the last sera had a neutralization titer of 1:256 and an HI titer of 1:640, but no neutralization and an HI titer of 1:20 against mumps. The hemadsorbing agent was not neutralized by specific antisera to paraflu 1 (HA-2 and Sendai strains),

paraflu 2 (Greer), paraflu 3, influenza A, influenza B, canine distemper, canine adeno (ICH and Toronto), rabies or canine herpes viruses. The demonstration of rises in antibody titers in dogs provided additional evidence that the virus originated from the dogs and was not a laboratory contaminant. Although many species of animals are known to be infected with SV-5 (Hsiung, G. D. et al., J. Immunol. 94: 67, 1965) to our knowledge there has been no report on the recovery of SV-5 from dogs.

e. Serologic and pathogenicity studies on canine adenoviruses. The adenovirus isolates were identified on the basis of characteristic CPE in canine kidney cell cultures, serological, neutralization, and hemagglutination-inhibition tests with standard anti-ICH-sera. Three representative isolates (strains 13-64, C-955L and F-346) were selected for further serological study. They were compared with 4 type strains of canine adenoviruses -- 3 infectious canine hepatitis (ICH) strains -- Utrecht, Cornell No. 1, PM vaccine -- and canine adenovirus strain -- Toronto A26-61. The last strain was isolated by Ditchfield (Canad. Vet. J. 3: 238, 1962) and was reported to be serologically related but distinct from ICH virus.

Rabbits and mice were used for production of antibodies against the selected viruses by use of a conventional multiple inoculation regimen. In hyperimmunized mice, Freund's complete adjuvant was inoculated intraperitoneally to produce ascitic fluid. Both antisera from rabbit and hyperimmune mouse ascitic fluids were used for serological studies. The serological procedures used were hemagglutination-inhibition (HI) conducted with human type O erythrocytes, neutralization (NA) in dog kidney tissue culture (DKTC) and complement fixation (CF) with DKTC antigens.

A summary of cross HI, CF, and NA reactions of 3 representative isolates and 4 type strains of canine adenoviruses using mouse ascitic fluids are summarized in Table 9. Consistent findings were derived in all three procedures.

It was apparent that the canine adenoviruses comprise 2 distinctly interrelated serological types; the ICH viruses form one type and the Toronto virus the second type. The representative isolates were distributed between the two types, viz., strain 13-64 with the ICH types and strain C-955L and F-346 with the Toronto type. Differences between the two types were more marked in HI and NA tests than in CF tests. Nonreciprocal cross reactions between anti-Toronto strain fluids and ICH antigens were particularly evident in HI and NA tests. The higher degree of cross reactions in CF tests was not remarkable and probably reflects the activity of a "group-specific" CF antigen characteristically seen in CF tests with adenoviruses.

Table 9. Characteristic serological cross-reactions between representative adenovirus strains, 13-64, C-955L and F-346, and type strains of infectious canine hepatitis (ICH) virus (Utrecht, Cornell 1 vaccine) and Toronto virus

Test	Hyperimmune ascitic fluid*	Reciprocal of titers with antigen ICH Utrecht	WRAIR 13-64	Toronto	WRAIR C-955L
Hemagglutination inhibition	Utrecht	320	320	160	80
	13-64	160	160	40	40
	Toronto	10	10	320	320
	C-955L	10	10	320	320
Complement fixation	Utrecht	128	128	64	64
	13-64	128	128	64	64
	Toronto	8	8	64	64
	C-955L	4	16	64	64
Neutralizing antibody	Utrecht	320	320	20	20
	13-64	80	80	20	20
	Toronto	20	20	320	320
	C-955L	20	20	320	320

* Reactions against Cornell #1 and PM ICH vaccine strains were essentially identical to ICH Utrecht. F-346 gave same reactions as that of C-955L, except that in CF test, cross-reaction of hyperimmune F-346 fluid vs. ICH strains was 2-to 4-fold greater than C-955L anti-ascitic fluid.

The hyperimmune rabbit sera were found to be anti-complementary and hence unsatisfactory for CF tests. The HI findings with rabbit antisera were the same as those obtained with mouse ascitic fluids. Serum neutralization tests with rabbit sera are in progress.

Using rabbit antisera in a HI procedure, it was possible to type the other 13 adenovirus isolates obtained from dogs during the course of this study. Five were found to be typical ICH viruses; the other 8 were identified to be Toronto-type adenoviruses. Monovalent modified live ICH vaccines obtained from 4 different companies were typed and identified as ICH viruses, as was the Lederle virulent ICH type strain of American Type Culture Collection.

The serological findings extend observations of Ditchfield on the occurrence of two distinct canine adenoviruses.

In view of the paucity of information on the significance of the Toronto adenovirus in canine disease, the pathogenicity of this virus was studied and compared with that of ICH virus. The C-955L representative of the Toronto type was selected for study because it was found in high titer (e.g. approximately 10^5TCID$_{50}$ per gram) in lungs of a dog that died of respiratory disease. The ICH strain selected was Cornell No. 1. Studies were conducted in 13-to 14-week-old puppies which were carefully selected and raised to ensure absence of infection and serum antibodies for adenoviruses. Two groups of 4 dogs each were respectively inoculated with approximately 400,000 TCID$_{50}$ (tissue culture infection dose) of C-955L and Cornell #1 ICH virus. The groups were segregated in separate rooms. One control puppy was placed with each group for observation of contact infection. During course of study, a second control dog was placed in same room with dogs infected with ICH virus.

Within each group, two dogs were inoculated intraperitoneally and two were exposed by both the oro-naso-intratracheal and intraocular routes. Multiple routes of exposure were used in view of the absence of information on the mode of natural infections. The intraocular route had been previously used to incite and study ICH infections in dogs, particularly to elicit corneal opacity (Ann. N. Y. Acad. Sci., 101: 498, 1962). Throat and rectal swabs for virus culture were taken prior to inoculation and daily for 7 days after inoculation, every other day during the second week and weekly thereafter for 8 weeks. Plasma for viremia studies was obtained on alternate days of the first week post-infection. Blood for serological examinations was collected at appropriate intervals.

All four dogs experimentally infected with ICH died with acute hepatitis within 4 to 7 days postinfection. The major clinical pathological findings were: 1) fever,which appeared generally 36 hours after infection and which persisted during course of disease; 2) leukopenia, which coincided with time of fever; 3) depression; 4) blanched mucous membranes; 5) generalized lymphadenopathy; 6) anorexia; 7) hematochezia; 8) increased tone in abdominal muscles; and 9) hepatomegaly. Diffuse corneal opacity and marked conjunctival edema occurred in two dogs that had received the intraocular injection. There were no signs of respiratory disease in any of the ICH infected dogs except for slight concentration of parenchymal lung sounds observed by auscultation in one dog inoculated by multiple route. To date ICH virus was recovered from plasma, throat, and rectal samples. The initial control dog placed in contact with ICH group developed

a mild clinical case of ICH 9 days after time of experimental infections. A second dog placed in contact with the first control dog 20 days after appearance of disease in the latter, similarly developed a mild infection. In both instances ICH virus was recovered from throat and rectal swabs; only one of the two cases of ICH contracted infections from plasma. The identity of isolated ICH viruses was established by serological tests.

In marked contrast to the disease in puppies provoked by ICH, experimental infections with the Toronto-like adenovirus agents were mild. Fever of 1 to 3 days' duration appeared approximately 48 hours after infection in 3 of 4 dogs. Leucopenia was seen in all 4 dogs. Mild to moderate depression and transient blanching of mucous membranes were the only other additional signs observed in two dogs inoculated intraperitoneally. Diffuse corneal opacity and moderate conjunctival edema appeared in the eye that received the intraocular injection in both dogs. One of the latter dogs had a slight mucoid nasal discharge on the sixth day; the other developed signs of lower respiratory disease characterized by a harsh, dry, barking cough of a paroxysmal nature that would terminate in gagging. The cough started on day 3 and persisted to day 10 postinfection. Auscultation of right lung gave impression of some consolidation during days 5 through 8. A mucoid nasal discharge was present on days 6 and 7. Similar respiratory signs developed in the control dog on the 9th day of contact, and persisted for 7 days. No other signs, e.g. fever or leukopenia, were seen in this dog. The Toronto-like adenovirus was isolated from throat and rectal swabs but not from plasma of all 5 dogs in this group. Virus was detectable in throat and rectal samplings from days 2 to 9 postinoculation.

All animals in the respective groups, including contact controls, developed specific antibody titers. Serological tests for canine distemper virus were negative. A summary of HI, NA and CF test findings is given in Table 10. Animals in the respective group developed maximum titers to challenge virus types. The cross-reactions were similar to those observed in hyperimmunized animals. The reactions of dogs infected by contact exposure were essentially the same as the experimentally infected dogs. Table 10 also summarizes the serological response of 4 additional dogs subsequently given a commercial attenuated ICH vaccine. These animals were vaccinated for projected cross-protection studies. The reactions in vaccinated dogs were of same type as that of ICH infected animals, except that titers were lower.

Evidence to date points to marked differences between the two serologically distinct canine adenoviruses in their clinical manifestations in dogs. The Toronto-like adenovirus did not produce classic ICH disease signs but elicited an inapparent infection or a mild lower respiratory disease. Additional pathogenicity studies to extend preliminary findings are planned. The possible relationships of Toronto-like adenovirus to other known adenovirus types will be studied also.

Table 10. Serological response of puppies infected with Infectious Canine Hepatitis (ICH) and Toronto-like Canine Adenovirus (C-955L)*

Infected with	Reciprocal of titer with antigen in specific test					
	Hemagglutination		Complement-fixation		Neutralization	
	ICH	Toronto	ICH	Toronto	ICH	Toronto
ICH**	80-320	10-20	64-128	64	5120	320-1280
C-955L	20-40	80-640	8-16	32-64	320	1280
Vaccine (ICH)	80-320	10	8-32	4-32	80-320	20-320

* Sera taken 28-35 days postinfection or postinoculation.
** Response of 2 cases of contact infections

5. Nucleic acid induction of specific antibody synthesis.

Previous studies by various workers to determine the role of nucleic acids (NA) in the synthesis of immunoglobulins have been somewhat equivocal. Generally, in these experiments, NA from lymphoid tissue of antigenically sensitized animals were given to unimmunized neonates or immunologically impotent members of the same species. The induction of immune globulin synthesis in recipients by occult antigen in NA preparations could not be ruled out. To circumvent this limitation, a more sensitive and specific technique was studied to detect the production of foreign globulins in the sera of recipients.

NA from hyperimmunized animals were inoculated into immunologically incompetent recipients of a different species. A hemagglutination inhibition (HI) test was employed to detect specifically the presence of nucleic acid donor (NAD) globulins in the recipient sera. An increase in the NAD globulins for one week or more after NA inoculation provided indications that NA were responsible for protein synthesis in a heterologous species. In the conduct of the HI test, a constant amount of recipient serum was added to serial two-fold dilutions of anti-NAD globulin; 24 hours later, sheep red blood cells (RBC) tagged with NAD globulins were added and mixtures were incubated. Thus, a reduction in HI titer provided evidence of the presence of NAD globulins in recipient sera. All antisera to NAD globulins were prepared in the species serving as recipient. Sheep cells

were formalinized and treated with tannic acid and the appropriate globulin test sera and antisera were adsorbed with formalinized normal sheep RBC prior to test. Tests were conducted initially in Kahn tubes. Subsequently, the microtiter technique was utilized.

Initial tests were conducted to determine the relative sensitivity and specificity of HI tests employing RBC coated with gamma and beta, gamma 2, or gamma 1M mouse globulins with rat anti-mouse sera, and known amounts of the respective mouse globulins added to a 1 in 20 dilution of normal rat serum used to simulate recipient's sera. Conventional techniques were used to isolate the globulin components. Gamma-2 globulin coated cells were more sensitive and specific in their HI reactions than gamma and beta globulin coated cells. In comparing the use of gamma-2 and gamma 1M coated cells, the hemagglutination titers of both cell preparations (e.g., 1:20480) were the same. The gamma-2 globulins (added to normal rat sera) did appear to be somewhat more effective than gamma 1M in the inhibition of HA for either cell preparation.

An experiment was designed to determine if NA from rats hyperimmunized to bovine serum albumin (BSA) could induce synthesis of rat globulin in rabbits. To enhance acceptance of foreign NA in recipient hosts, the various test NA preparations were incubated initially with splenic cells from normal rabbits prior to inoculations. The test preparations were native or inactivated (by nucleases) DNA and RNA, employed singly or in combinations with BSA. The BSA was incorporated with the inoculum to anticipate its possible requirement for inducing protein synthesis in the recipient host.

Rats that provided the NA were immunized by a series of weekly inoculations over a 2-month period. Standard phenol and sodium lauryl sulfate extracting methods were used to separate NA from spleens of immunized rats. DNA and RNA were precipitated by treatment with ethanol. Contaminating RNA in the extracted DNA preparation was removed by isopropanol extraction according to method of Marmur. The separated NA were suspended in a buffered physiological salt solution (SBS). A portion of each suspension was treated with its respective nuclease. Spleen cells from normal rabbits were obtained by raking with a stainless steel wire mesh, washed in SBS and resuspended to a concentration of 24.5 X 10^6 cell/ml. Native or inactivated DNA (3.8 mg), RNA (6.4 mg) or combinations thereof were added to 5.0 ml portions of spleen cell suspension, the osmolarity was adjusted to the equivalence of 0.22 M NaCl by addition of sucrose, the mixture was incubated at 37°C for 40 min, centrifuged, and resuspended in 5 ml SBS containing 0.2 mg BSA. This mixture was incubated 10 min at 37°C and 20 min at 25°C and then used to inoculate rabbits i.p. Six rabbits were inoculated as follows: 1) DNA, RNA, BSA; 2) DNA (inactivated), RNA (inactivated), BSA; 3) RNA,

BSA; 4) RNA (inactivated) DNA, BSA; 5) DNA, BSA; 6) DNA (inactivated). RNA, BSA. All but the last rabbit were given 550 R X-irradiation 24 hours prior to inoculation. The results of HI tests are summarized in Table 11. Only the first rabbit had a notable difference in HI titer. This was detected 6 days after NA inoculation but wasn't demonstrable at 3 or 8 days postinoculation. Both NA appeared to be necessary to induce globulin synthesis. Cells coated with ammonium sulfate precipitated rat gamma and beta globulins were used in this series of tests. The lower sensitivity of this system may not have allowed detection of relatively small amounts of globulin in the other rabbit sera.

Rabbits were X-irradiated to inhibit immunological response to proteins, characteristic of the rat donor. The necessity to irradiate recipients and to pretreat donor NA with normal recipient spleen cells were considered. A second experiment was conducted with 4 non-irradiated rabbits employing similar NA extracts used in the previous tests. No antigen was incorporated into the inoculum in view of new reports on the presence of antigen (e.g., BSA) in phenol extracts of NA. Rabbits were inoculated i.v. as follows: 1 rabbit with 17 mg DNA and 16 mg RNA, both incubated with their respective nucleases, 2 rabbits with 17 mg DNA and 16 mg RNA, and the fourth rabbit with 8.5 mg DNA and 8 mg RNA. All of the sera were tested for BSA antibodies using a HA procedure and found to be negative. There was no demonstrable rat globulin found in HI tests conducted on serial rabbit serum samples obtained from 2 to 13 days postinoculation. (The preinoculation sera were depleted after HA testing). The absence of demonstrable rat globulin may have reflected the need for tolerant recipients, the presence of added antigen in the NA preparation, or the need for prior incubation of NA with normal rabbit spleen cells, or combinations thereof. A third experiment was conducted with variations in antigen (BSA), NA content of inoculum and pretreatment with normal rabbit spleen cells. For this purpose NA from spleens of rats hyperimmunized with BSA were extracted as previously described. Three rabbit recipients were X-irradiated with 500 R 24 hours prior to inoculation and given the following inocula respectively: 1 - DNA - 2.3 mg, RNA 1.1 mg and BSA - 5 mg by the i.v. route; 2 - DNA and RNA in comparable doses but incubated with normal spleen cells prior to i.p. inoculation; 3 - RNA alone with the spleen cells by i.p. route. Only the first rabbit inoculated with both NA and BSA had a significant progressive increase in HI from 1:51,200 at day 2 to 1:6400 at day 4 to 1:1600 at day 8 post-inoculation. At this time, it appeared that X-irradiation of recipient and the introduction of both DNA and RNA in the presence of antigen (BSA) were necessary to incite the best production of donor globulins in recipient rabbits. There appeared to be no advantage in the pretreatment of NA with normal spleen cells from recipient species.

Table 11. HI titer of sera from rabbit recipients of rat splenic nucleic acids*

Rabbit No. and inoculum**	No. of days post NA inoc.	Reciprocal of HI titer
1. RNA, DNA, BSA	preinoculation sera	12800
	3 days post	25600
	6 " "	200
	9 " "	25600
2. RNA - RNased	preinoculation sera	12800
DNA - DNased	3 days post	6400
BSA	6 " "	3200
	9 " "	12800
3. RNA, BSA	preinoculation sera	1600
	3 days post	800
	6 " "	800
	9 " "	1600
4. RNA - RNased	preinoculation sera	800
DNA, BSA	3 days post	800
	6 " "	400
	9 " "	1600
5. DNA, BSA	preinoculation sera	25600
	3 days post	"
	6 " "	"
	9 " "	"
6. RNA, BSA	preinoculation sera	25600
DNA - DNased	3 days post	"
only one not	6 " "	"
X-irradiated	9 " "	"

* Cells coated with ammonium sulfate precipitated globulins. The rabbit antisera were prepared against ammonium sulfate precipitated rat globulins as well as whole rat serum. Serology: Recipient rabbit sera was incubated for 24 hours with rabbit anti-rat antisera before adding rat globulin tagged cells. Inhibition titers were recorded 24 hours later.

** All rabbits received normal rabbit spleen cells which had previously been incubated with their nucleic acid preparations. All but rabbit 6 were X-irradiated 24 hrs. prior to inoculation.

Further studies were conducted in a mouse donor rat NA recipient system using NIH Buffalo inbred rats for the following reasons: economy of required experimental materials, availability of larger numbers of test animals, diminution of genetic variation in recipient animals. More specific RNA extraction methods were used in these series. Spleen cells from mice hyperimmunized with BSA were fractionated according to the method of Fisher and Harris (Proc. Roy. Soc., Series B, 156: 521, 1962) to effect separation of nuclear and cytoplasmic subunits. NA were extracted in the presence of bentonite and polyvinyl sulfate to minimize RNase activity. The phenol extraction and isopropanol methods for isolating DNA and RNA were used. In view of recent evidence that messenger RNA is isolated more efficiently at high temperature (65°C), the method of Lang was also employed (Life Sciences 3: 161-167, 1964). Determined amounts of NA were incubated in vitro with 150 X 10^6 spleen cells from normal Buffalo rats. BSA was added as in previous experiments. Following incubation, the BSA-NA-spleen cell mixtures were placed in diffusion chambers which were then sealed and placed in the peritoneal cavity of rats, X-irradiated 500R, 24 hours previously. The chambers were used to prevent possible cellular antagonism between rat recipient cells and those cells previously incubated with mouse NA. The porosity of the chambers allowed free diffusion of proteins. Initial tests were done on 3 rats, two of which were given Nuclear RNA-2.6 mg (isopropanol extract), DNA - 1.18 mg and BSA - 0.15 mg; the third was given the same preparations except that RNA had been extracted at 65°C.

Test HI findings are summarized in Table 12. Two rats had a maximum increase in NAD globulin at 7 days postinoculation. The third rat died on the 3rd day. Only chamber fluids from the last were available for test and were found to contain HA inhibiting activity. There was no apparent difference in response attributable to two different preparations of RNA used. Tests for anti-BSA activity using HA technique were negative. Thus the anti-BSA specificity of the synthesized proteins in recipients could not be affirmed. Nor could it be dismissed because of limitations in the sensitivity of the HA techniques. The procedure used on the 3rd rat that died was repeated in two additional rats. Mouse protein was detected in one rat only at 5 days postinoculation, both in the serum and chamber fluids employing RBC tagged with gamma-2 mouse globulins in HI tests. The diffusion chamber of the second (negative) rat was grossly contaminated with bacteria when examined on the 5th day.

In view of the probable necessity for recipients to be tolerant of the NAD globulins and the fact that sublethal irradiation induced tolerance is only maintained in this state for approximately 1-to 2-weeks, another method of inducing tolerance was desired. Rat foetuses within 1 week of parturition were used for this purpose. Individuals in six litters were inoculated i.p. as follows:

Litters 1 and 2 - DNA (0.131 mg), cytoplasmic RNA (0.25 mg)
3 - DNA (0.262 mg)
4 - Nuclear RNA, 65°C extract (0.5 mg)
5 - Cytoplasmic RNA (0.5 mg)
6 - DNA (0.5 mg)

Table 12. HI titer of sera from rat recipients of mouse splenic nucleic acids with normal spleen cells in diffusion chambers*

Rat No. and inoculum**	No. of days post NA inoc.	Reciprocal of HI titer
#5 Nuclear RNA (Isopropanol Ext.) DNA, BSA	preinoc. bleeding	6400
	5 days post	1600
	7 " "	800
	12 " "	6400
	12 " " (Chamber fluids)	12800
#10 "	preinoc. bleeding	6400
	5 days post	1600
	7 " "	400
	12 " "	1600
	12 " " (Chamber fluids)	3200
#9 Nuclear RNA (65°C ext.) DNA, BSA	preinoc. bleeding	12800
	3 days postinoc. (Chamber fluids)	800

* Serology performed with sheep RBC coated with mouse gamma globulins obtained by ammonium sulfate fractionation. Rat antisera prepared with ammonium sulfate, methyl alcohol and DEAE fractionated mouse globulins. Recipient rat sera were incubated with rat anti-mouse globulin antisera. Twenty-four hours later, sheep RBC coated with ammonium sulfate extracted globulins were added and the amount of inhibition was recorded.

** All rats received normal spleen cells.

The accuracy of injection and retention of inocula could not be ascertained. Approximately 50 per cent of the foetuses died. About 2 weeks after birth, each neonate was given the antigen (BSA) which was used to immunize the NA donor. One (No. 4) of the six litters treated appeared to have NAD globulin. These animals had been given nuclear RNA only. This was noted at 14 days but not at 7 days after antigenic stimulation. Its time of detection was 33 days following

NA inoculation. The dilution of the recipient sera used in HI tests was 1:100 rather than the usually employed 1:20 dilution. The use of this high serum dilution, coupled with the 26-day span between NA inoculation and assay may have precluded detection of positive reactions in other litters.

The difficulties and variables encountered in using fetal animals led to the subsequent use of neonatal rats. Animals in each of two litters were inoculated i.p. within 24 hours after birth with the following materials: 1) DNA - 0.92 mg, RNA (nuclear, 65°C extraction - 1.4 mg and BSA - 0.4 mg; 2) DNA and associated RNA - 0.23 mg. Two weeks later blood samples were obtained from animals in each litter and pooled. Animals were inoculated with BSA and egg albumin and bled 8 days later. High HI titers were obtained in both groups on the 2-week post NA inoculation serum samples but not on serum samples obtained 8 days later. The exposure to antigen at the time of the first bleeding did not appear to enhance production of more mouse protein.

In an attempt to produce greater tolerance to mouse proteins, a group of neonatal animals were treated with various NA in the presence of concentrated mouse serum and another group with just the serum. On the 8th and 9th weeks after inoculation, the sera from both groups of rats were assayed for the presence of mouse globulins and were found to be similar. No anti-mouse globulin was demonstrable in the sera from either group of animals when tested with HA procedure using RBC sensitized with mouse globulins. Thus, it would seem that tolerance had been induced to mouse globulins. There was no evidence of protein-synthesis in the recipients of NA and mouse sera.

Summary and Conclusions:

1. Serological studies on 167 unaffected troops in companies at Ft. Kobbe, Canal Zone, in which an outbreak of leptospirosis occurred, provided evidence of previous exposure to leptospirosis in a relatively high percentage of soldiers. With a genus-specific hemolytic test (HL), approximately 12.5% of the soldiers had significant antibodies. Another 20% had questionable reactions. A very high percentage of positive reactions, 72%, were elicited with a newly proposed (Sturdza) genus specific CF test. The correlation of CF and HL test findings was poor. The significance of the CF reactions is not known.

The potential leptospiral infection hazards in the use of canine kidney tissue cultures for vaccine production was demonstrated. A combination of polymyxin B and dihydrostreptomycin now used in the commercial production of attenuated measles vaccine, effectively eliminated leptospirosis from kidney tissue cultures prepared from experimentally infected dogs.

Tests conducted on sera or cultures referred to this laboratory provided evidence of occurrence of canicola, icterohaemorrhagiae, and grippotyphosa infection in cattle in California. In episodes of disease in cattle in Illinois, grippotyphosa and hardjo were isolated; in both states isolates were also derived from cattle with growth properties of "saprophytic (biflexa) leptospires." The grippotyphosa infection in cattle has high potential public health significance. The potential infectivity of biflexa strains for man and animals merits reexamination. The presence of pomona in deer, groundhogs and skunks in Canada was affirmed. Culture typing tests on leptospiral strains isolated from wildlife in Nicaragua have established the occurrence of multiple leptospiral types in this country. It was also apparent that new serological entities occur there which are unrelated to known serotypes. The existence of such Nicaraguan types, their possible occurrence in other parts of the world, directs attention to limitations in current microscopic agglutination technics for serological diagnosis of broad antigenic spectrum covered by proposed multivalent vaccines. Evidence of the occurrence and high prevalence of leptospirosis in cattle in Equador was obtained. Infections with hebdomadis and pomona serotypes are predominant, but other types are also present. There was no evidence of leptospiral infection in dried sera submitted from El Salvador.

The guanine-cytosine base composition (%GC) of deoxyribonucleic acids (DNA) of 13 pathogenic and 3 "saprophytic" strains of leptospira were studied. The % GC within the two groups were relatively uniform, between groups there was a remarkable difference. Findings support the subdivision of two species by other criteria. A consistent difference between the % GC of DNA as determined by the methods of thermal denaturation and buoyant density in $CsCl_2$ was observed. These may reflect the presence of minor purine and pyrimidine bases.

2. The molar per cent of guanine + cytosine (G+C) in the DNA of Proteus mirabilis, strain 9 and its stable L-form were determined by thermal denaturation and found to be approximately 39.5% G+C. The DNA homologies of this bacterium and its L-form were estimated by the agar column technique and were equivalent in their abilities to anneal and form specific duplexes. The next series of comparisons were performed between two mycoplasma species and their often suggested bacterial parent. The G+C ratios of Mycoplasma gallisepticum (32.7%), Mycoplasma gallinarum (28.1%) and Hemophilus gallinarum (41.9%) varied to a high degree. In the homologous system, the denatured DNA of H. gallinarum trapped in agar, bound approximately 40% of its sheared, denatured and H^3 labeled DNA. In comparison, the nucleic acids of M. gallinarum and M. gallisepticum were incapable of binding the labeled DNA of H. gallinarum. There was evidence that genetic mating techniques have potential usefulness in determining the parental bacterial sources of PPLO.

3. The evaluation of the response of human beings to WE chick embryo vaccine has continued. The percentage of individuals responding has been extremely variable from group to group. In the Province of Saskatchewan, Canada, there are indications that a high percentage of individuals residing and/or working in rural areas of the Province have pre-immunization antibody, presumably resulting from sub-clinical or unrecognized infections with the virus during one of the large epidemics that have occurred there.

In comparison to field strains of WEE virus, the clone 15 virus was markedly attenuated for mice, guinea pigs, and ponies. Clone 15 virus protected mice and guinea pigs against an intracerebral challenge with virulent virus. Following subcutaneous inoculation of ponies with clone 15 virus, a barely detectable viremia occurred in 5 of 6 ponies, and each animal developed significant neutralizing antibody by day 14. On the basis of these findings, the potential use of clone 15 virus as an attenuated vaccine strain deserves further attention.

Eastern equine encephalitis virus was isolated from pheasants at a military installation. The use of vaccines to control disease in pheasants has not been satisfactory. These observations point out the need to develop more potent vaccines.

Herpes simiae (B-virus), respiratory syncytial virus, and an unknown simian enterovirus have been recovered from the lungs of monkeys dying of respiratory disease. Serological studies on paired sera of monkeys obtained at the beginning and end of quarantine indicated that 30% of the monkeys had antibody to B-virus. There was no evidence of the spread of infection in the colony. The isolation of B-virus from animals without oral lesions and the presence of antibody in one-third of the animals, emphasizes the need for proper precautions in handling monkeys.

4. Studies of the etiology of respiratory disease of dogs indicated the presence of at least 6 agents in the conditioning colony. The agents included canine distemper virus (CDV), infectious canine hepatitis (ICH), a serologically distinct canine adenovirus, a canine herpes virus, a hemadsorption agent (SV5) and reovirus. The CDV appeared to be the most important in producing severe illness and death. For the remaining agents, the incidence of infection was essentially the same in the sick and well dogs.

The canine herpes-like virus was found to be chloroform sensitive and IUDR sensitive and approximately 150-200 mu in size. These properties place the virus in the herpes group. Serological tests showed that the virus was distinct from herpes simplex, B-virus, and pseudo-rabies.

The isolation of a herpesvirus from fatal respiratory infections of puppies and from tissue cultures of dog kidney cells merits further study. The virus may be an important etiological agent of respiratory disease in dogs. Whether the virus has the ability to infect man remains to be determined. Since the virus was first recognized in tissue cultures which had undergone spontaneous degeneration, the problem of viral contamination of cell cultures used in the production of vaccines is further emphasized. These findings point up the need for further studies of the herpesvirus group.

The hemadsorption agent obtained from dogs was found to be ether-sensitive, IUDR resistant, and incapable of passing through a 100 mu millipore membrane filter. These properties placed the virus in the myxovirus group. Serological studies showed that the virus was identical with SV5, a member of the parainfluenza group. This is the first reported recovery of SV5 from dogs.

The 2 canine adenoviruses, ICH and Toronto, are markedly different in their clinical manifestations in dogs. The ICH virus elicited classical signs of ICH, whereas, the Toronto-like virus produced either an inapparent infection or a lower respiratory disease. From dogs infected with the Toronto-like virus, isolation of virus from the throats and feces were obtained for 4 to 7 days postinoculation, indicating a long period of communicability. Neutralization and hemagglutination-inhibition tests on the sera of dogs revealed significant differences from dogs infected with ICH. Immunological studies of ICH and Toronto-like canine adenoviruses indicated a significant difference between these 2 groups. Within groups, strains were serologically homogenous. The hemagglutination-inhibition test employing ICH and Toronto-like antisera was a simple and efficient means of typing isolates. The relationship of the Toronto virus to other adeno-viruses and its potential infectivity for man merits further study.

5. A number of the variables involved in the NA induction of protein synthesis in a foreign host were explored. Because of the broad scope of the experiments, it was impossible to do the repetitive work necessary for statistical analyses. However, some general conclusions can be derived.

In these experiments, the induction of apparent NAD protein in X-irradiated recipients was best enhanced by preparations containing the DNA and RNA from hyperimmunized NA donors and the specific antigens. No definite evidence was obtained for the superiority of any extraction method or cell fraction source of RNA. The temporary state of tolerance obtained with X-irradiation or the use of immunologically immature animals may have been responsible for the ability to detect the transient presence of foreign globulin.

The apparent presence of NA donor globulin in sera of heterologous species is evidence for the capability of NA to function between species. In no case was antibody specificity for BSA demonstrated in the recipient sera. Partial infidelity of code transcription may be responsible for this lack of detectable specificity.

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24. (U) Tech Objective - This work unit is concerned with the development of manufacturing methods for the production of new effective vaccines and for the modification of existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.

25. (U) Approach - 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.

26. (U) Progress (Jul 64 - Jun 65) - Laboratory and field studies have been carried out on a variety of new and modified biological products: 1. A practical method for the large scale production of freeze-dried formalin-inactivated cholera vaccine was developed. This product is currently being evaluated in a controlled field trial by the World Health Organization. 2. An inactivated vaccine for WEE, produced by growth of the virus in chick embryo cell cultures, has proved to be effective in the protection of guinea pigs and mice. 3. A live attenuated vaccine for Q fever was prepared and is being evaluated, along with inactivated phase 1 and phase 2 Q fever vaccines. 4. Laboratory studies have demonstrated the feasibility of a combined vaccine consisting of tetanus toxoid, diphtheria toxoid and acetone-killed and dried typhoid organisms. 5. Long term efficacy and stability studies have continued on the following experimental biologicals: acetone-killed and dried typhoid vaccine, freeze-dried EEE vaccine, and freeze-dried RSSE vaccine. 6. Developmental studies leading to the preparation of a freeze-dried live attenuated plague vaccine have been initiated.
For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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Project 3A014501B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 173, Development of biological products

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Description. This work unit is concerned with the development of manufacturing methods for the production of new effective vaccines and for the modification of existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.

Progress.

1. Typhoid

a. Surveillance of the viability of the freeze-dried Salmonella typhosa, Ty2 strain, seed culture, prepared by the Department of Biologics Research, WRAIR, in 1960, was continued during this period. This seed material was prepared at the request of the World Health Organization for use in the production of typhoid vaccines for current and future human field studies. A summary of the results of the titrations of the number of viable organisms, as determined by plate counts on samples stored at various temperatures over a period of 4 years, is as follows:

Surveillance of Freeze-Dried Typhoid Seed Culture

	Temperature of Storage			
Time	4° C	22° C	37° C	45° C
12 mos.	1.89×10^9	1.12×10^8	$>3.00 \times 10^5$	$<10^1$
25 mos.	1.15×10^9	4.17×10^7	2.30×10^4	$<10^1$
37 mos.	7.17×10^8	8.20×10^6	$<10^1$	
45 mos.	8.50×10^8	1.47×10^7		

The results of this stability study indicated that the viability of the seed culture remained satisfactory after storage in the dried state at 4°C and at 22°C for 4 years.

b. Since the Commission on Immunization of the Armed Forces Epidemiological Board, at its 1964 meeting, had expressed an interest in a mixed vaccine consisting of acetone-killed and dried (AKD) typhoid organisms and tetanus and diphtheria toxoids, a laboratory investigation of the compatibility of these antigens was initiated. For these experiments

the combined product was obtained by rehydrating the AKD typhoid vaccine with tetanus and diphtheria toxoids (alum precipitated, for adult use). For each component antigen, the standard potency test, as outlined in the NIH minimum requirements for that product, was performed. The results are as follows:

Compatibility of AKD Typhoid Vaccine with Tetanus and Diphtheria Toxoids

	Assay for Potency					
	Typhoid	Diphtheria		Tetanus		
Preparation	Mouse ED_{50}(ml)	A.U.*/ml GP serum		A.U.*/ml GP serum		
AKD Typhoid Vaccine	0.0069	-		-		
AKD Typhoid Vaccine and Tetanus-Diphtheria Toxoids	0.0060	>1.0	<2.0	>6.4	<12.5	
Tetanus-Diphtheria Toxoids	-	>1.0	<2.0	>6.4	<12.5	

*Antitoxin units

The results indicated that the combination of the AKD typhoid organisms with the tetanus and diphtheria toxoids had no effect on the potencies of any of the components.

2. Q Fever

a. Under the auspices of the Commission of Rickettsial Diseases of the Armed Forces Epidemiological Board, a cooperative investigation is in progress to determine the relative efficacy for man of the phase 1 and phase 2 Q fever vaccines prepared by the Department of Biologics Research, WRAIR (annual report, 1964). During this year, this laboratory was requested to provide 1) a supply of antigen for use in laboratory studies on animal and human sera, and 2) phase 1 and phase 2 rickettsial slurries for use as challenge materials in these investigations.

b. Approximately 2000 7-day old embryonated eggs were inoculated with the Henzerling strain, phase 1 (EP-4). At the appropriate time the yolk sac membranes were harvested and were processed to a 30% suspension by the method described by Ormsbee for the preparation of antigen for the agglutination-resuspension test (Jour. Immunol., 1962, 88:100). Portions of this material were distributed to other laboratories for experimental studies to determine the most suitable serological test. The bulk, approximately 8 liters, is being held pending the outcome of these experimental studies, since the method employed for further processing of the antigens will depend on the test selected.

c. Approximately 40 bottles, each containing 25 ml of a 50% suspension of yolk sac membranes infected with the 9-Mile Strain in phase 1 (GP-306/EP-4), and an equal number of bottles containing a suspension of membranes infected with the 9-Mile Strain in phase 2 (EP-87), were prepared for use in human challenge experiments. For each strain approximately 600 7-day old embryonated eggs were inoculated via yolk sac with

GUINEA PIG RESPONSES TO C. burnetii SLURRY
9 Mile Strain - Phase 1 (GP-306/EP-5)

Response		Dilns: (1 ml. I.P.) 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	
FEVER													
Infected GPs* / Total GPs	Test 1	10/10	10/10	7/10	10/10	10/10	5/10	7/10	6/10	4/10	4/10	0/10	ID_{50}=8.6
	Test 2	10/10	10/10	10/10	10/10	10/10	10/10	8/10	4/10	6/10	1/10	0/10	ID_{50}=8.4
Ave. No. of Fever Days	Test 1	5.8	4.1	3.5	5.4	5.5	2.6	2.6	1.7	1.4	1.1	0.0	
	Test 2	5.9	6.3	6.3	5.6	5.6	3.8	3.1	1.1	1.7	0.2	0.0	
CF RESPONSE (on Day 28)													
Positive / Total	Test 1 (vs. Phase 2 antigen**)									9/10	8/10	0/10	ID_{50}=10.3
	Test 2 (vs. Phase 2 antigen**)								10/10	5/5	9/10	1/10	ID_{50}=10.5
CF RESPONSE (on Day 42)													
Positive / Total	Test 2 (vs. Phase 1 antigen***)								0/8	0/9			ID_{50}=<8.0
	(vs. Phase 2 antigen**)								8/8	9/9	6/7	1/8	ID_{50}=10.5
DEATH													
No. Dead / Total	Test 1	6/10	7/10	2/10	2/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	LD_{50}=2.3
	Test 2	10/10	8/10	2/10	2/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	LD_{50}=2.6

* 104°F for 2 or more days
** Lederle Diagnostic Antigen - 9 Mile Strain
*** Honeerling strain, prepared at WRAIR

GUINEA PIG RESPONSES TO C. burnetii SLURRY
9 Mile Strain - Phase 2 (EP-88)

Response		Dilns: (1 ml. I.P.) 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	
FEVER													
Infected GPs*	Test 1	10/10	8/10	9/10	9/10	2/10	0/10	0/10	0/10	0/10	0/10	0/10	ID_{50}=4.6
Total GPs	Test 2	10/10	10/10	10/10	10/10	8/10	0/10	0/10	1/10	0/10	0/10	0/10	ID_{50}=5.4
Ave. No. of	Test 1	4.9	3.7	4.6	3.1	0.5	0.0	0.0	0.0	0.0	0.0	0.0	
Fever Days	Test 2	6.3	4.4	4.5	4.0	2.6	0.0	0.0	0.2	0.0	0.0	0.0	
CF RESPONSE (on Day 28)													
Positive	Test 1	(vs. Phase 2 antigen**)								10/10	2/10	0/10	ID_{50}=9.6
Total	Test 2	(vs. Phase 2 antigen**)							10/10	7/10	4/10	2/10	ID_{50}=9.8
CF RESPONSE (on Day 42)													
Positive	Test 2	(vs. Phase 1 antigen***)						0/7	0/7				ID_{50}=<7.0
Total		(vs. Phase 2 antigen**)						6/7	7/7	7/8	3/8	1/9	ID_{50}= 9.8
DEATH													
No. Dead	Test 1	6/10	2/10	2/10	0/10	0/10	1/10	1/10	0/10	0/10	0/10	1/10	LD_{50}=1.4
Total	Test 2	5/10	2/10	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	LD_{50}=1.3

* 104°F for 2 or more days
** Lederle Diagnostic Antigen, 9 Mile Strain
*** Henzerling strain, prepared at WRAIR

0.2 ml of the appropriate dilution of the seed material which was provided by Dr. Richard A. Ormsbee of the Rocky Mountain Laboratory. At the appropriate time, the yolk sac membranes from surviving embryos were harvested in groups of 25. Each group was blendorized to a 50% suspension in Snyder I buffer. Samples were removed from each suspension for sterility tests, and the suspensions were stored at -70°C until completion of the tests. Those suspensions showing no evidence of contamination were thawed and pooled. After removal of samples for bulk sterility tests, the pooled suspensions were dispensed in 25 ml volumes into 100 ml serum bottles. In addition 2 ml volumes were dispensed into 10 ml serum bottles for laboratory studies. Bottles were sealed with butyl rubber stoppers covered with aluminum seals. Random bottles were selected for the final container sterility tests and the remainder were frozen and stored at -70°C.

To compare the infectivity of the phase 1 (GP-306/EP-5) and phase 2 (EP-88) challenge strains, Fort Detrick Hartley strain guinea pigs (350-450 grams) were inoculated intraperitoneally with 1.0 of 10^{-1} through 10^{-11} dilutions (10 guinea pigs per dilution) of the rickettsial slurries. Rectal temperatures were taken for 2 days after the last day of fever, or for 21 days if no fever was recorded. The guinea pigs at the indicated dilutions were held and were bled on the 28th and 42nd days post-inoculation to obtain sera for complement-fixation (CF) tests. Summaries of the guinea pig responses are given in the large tables on the preceding pages.

From the CF responses, it appeared that the two strains had approximately the same level of infectivity for the guinea pig. However, the febrile and lethal responses showed that the phase 2 strain was considerably less virulent than the phase 1 strain. These results, therefore, suggested that the phase 2 strain may serve as a live, attenuated vaccine for the immunization of man against Q fever.

d. Laboratory studies on the use of genetron-113 (trifluorotrichloroethane) as a substitute for ether in the processing of Q fever vaccine were interrupted during this period but will be resumed when time and facilities permit.

3. Russian Spring-Summer Encephalitis

Studies on RSSE vaccine were limited during this period to a continuation of the surveillance of the stability of the freeze-dried mouse-brain vaccine prepared in 1959. The results, summarized in the following table, indicated that after 6 years' storage at 4°C the protective index, as measured in mice, was still at an acceptable level.

Stability of Freeze-Dried RSSE Vaccine Stored at 4°C

Lot No.	Period of Storage (yrs.)	Protective Index (logs)
18	0	4.9
	1	4.8
	2	5.0
	3	4.6
	6	4.0

4. Eastern Equine Encephalomyelitis

During this period, studies on EEE were concerned with evaluating the immunological response of animals to both the whole chick embryo vaccine and the cell culture vaccine. In addition to the standard guinea pig protection and mouse neutralization tests, both of which give *in vivo* indices of the vaccine's ability to elicit neutralizing antibody, more critical methods are being studied in order to determine the time of appearance, duration, and quantity of the specific antibody globulins elicited by vaccination.

Groups of guinea pigs were injected with 0.5 ml of vaccine subcutaneously on days 0 and 8. Sera were collected at pre-determined intervals (days 3, 8, 11, 15 and 21) and were fractionated in a sucrose density gradient in order to separate and collect the specific 19S and 7S antibody globulins.

The 7S fractions are currently being examined for complement-fixing and hemagglutination-inhibiting antibodies. From the limited results obtained to date it appeared that antibody in the 7S fraction was detected earlier by the hemagglutination-inhibition technique than by the complement-fixation test. It also appeared that the cell culture vaccine used produced a slightly higher and more consistent response than the whole embryo vaccine employed.

The virus neutralizing antibody content of these 7S fractions, as measured by both the *in vivo* mouse test and the *in vitro* tissue culture test, is currently being determined. In addition, measurement of the antibody content of the 19S fractions by the same techniques as are being used for the 7S fractions, is in progress.

5. Western Equine Encephalomyelitis

a. During this period a new lot of freeze-dried purified WEE vaccine (chick embryo origin) for human use was prepared by the method of Randall, Mills and Engel (J. Immunol. 1947, 55:41). Since laboratory tests indicated that the potency of the previous lot of this vaccine had dropped significantly after 1 year's storage at 4°C, a study was initiated with the objective of improving the stability of this product. Human serum albumin (HSA) was added to a portion of the final fluid vaccine to yield a final concentration of 2% HSA. The vaccine plus HSA was

dispensed into bottles and freeze-dried along with the standard vaccine. Bottles of the vaccine with and without HSA were placed at -20°C and +4°C for stability studies. Periodically samples will be removed from storage and tested for potency.

b. During the past year studies on the development of freeze-dried WEE vaccine prepared in chick embryo fibroblast tissue culture were completed. Experimental studies were directed toward determining the optimum conditions for the various phases of the vaccine production, such as 1) relationship between the multiplicity of infection and virus yield, 2) relationship between length of incubation and virus yield, 3) the inter-relationship of formalin concentration, temperature and time on inactivation of the virus, 4) evaluation of methods of clarification, and 5) the effect of freezing and drying on potency.

On the basis of the results obtained in these experimental studies a standard procedure was evolved for the preparation of a freeze-dried cell culture WEE vaccine. Chick embryo cell monolayers in 32-ounce prescription bottles were overlayed with 39 ml of Minimum Essential Medium (Eagle) and then seeded with 1 ml of a 10^{-3} dilution of chick embryo suspension containing WEE virus strain B-11. Infected monolayers were incubated at 35°C for 18 hours, at which time the virus yields were of the order of $10^{6.5}$ to $10^{7.5}$ mouse IC LD_{50}s per 0.03 ml. After low speed centrifugation of the harvest fluids, the virus was inactivated with 0.05% formalin at 22°C. After 72 hours' inactivation the material was filtered through a Millipore filter (HA porosity), and the filtrate was then held at 22°C for 5 additional days. The formalinized pools were not infectious for mice after 2 days but the inactivation period was continued for 6 additional days as a safety factor. The product was then stored at 4°C until completion of safety and sterility tests. The formalin was neutralized with 10% sodium bisulfite solution immediately before the vaccine was dispensed into vaccine bottles and freeze-dried. A comparison between 3 lots of freeze-dried cell culture vaccine and a lot of freeze-dried whole chick embryo vaccine is given in the following table:

Comparison of Cell Culture and Whole Chick Embryo WEE Vaccines

			Potency for Guinea Pigs	
Vaccine	Nitrogen (mg/ml)	ED_{50} (ml)	95% C.L.	Relative Potency
Whole embryo #7	0.219	0.0441	(0.0191-0.1001)	1.0
Cell culture #14	0.102	0.0077	(0.0038-0.0159)	5.7
#15	0.092	0.0125	(0.0071-0.0222)	3.5
#16	0.092	0.0239	(0.0096-0.0497)	1.8

These results demonstrated that the cell culture vaccines, although lower in nitrogen content, were as effective in protecting guinea pigs as the whole chick embryo vaccine.

c. Experimental studies are in progress to develop a mouse po-

tency test for WEE vaccine to replace the current guinea pig assay method. Preliminary results suggested that the mouse test, which has many advantages over the cumbersome guinea pig test, is feasible.

6. Cholera

a. Surveillance of the viability of freeze-dried Inaba (NIH 35A-3) and Ogawa (NIH 41) seed cultures, prepared in September 1962 (annual report, 1963), was continued. The results of periodic titrations of the number of viable organisms in samples stored at different temperatures are as follows:

Surveillance of Freeze-Dried Cholera Seed Cultures

	Temperature of Storage					
	-20°C		+4°C		+22°C	
Time	Inaba	Ogawa	Inaba	Ogawa	Inaba	Ogawa
0 mo.	7.6×10^8	2.6×10^9	-	-	-	-
7 mo.	1.4×10^8	2.9×10^8	8.9×10^7	3.0×10^8	2.0×10^2	3.1×10^3
12 mo.	1.5×10^8	8.9×10^8	7.0×10^6	3.7×10^7	1.7×10^2	8.2×10^2
21 mo.	1.3×10^8	2.1×10^8	1.8×10^6	1.4×10^7	2.5×10^3	2.9×10^3
33 mo.	1.7×10^8	2.1×10^8	6.5×10^6	7.5×10^6	$<10^1$	8.7×10^2

These results augmented the previously reported results (annual report, 1964). After approximately 3 years' storage at -20°C and at +4°C, the cholera cultures were still satisfactory for use as seed material for vaccine production. For long-term storage, however, -20°C is recommended as the temperature of choice.

b. Preliminary results of the 1964 WHO cholera vaccine field trial, conducted in the Calcutta area of India, indicated that a single 1 ml dose of the freeze-dried formalin-inactivated cholera vaccine prepared by the Department of Biologics Research, WRAIR, in 1963 (annual report, 1963) reduced the case rate approximately 50% as compared to a placebo. The freeze-dried vaccine proved to be either as effective or more effective than the fluid cholera vaccines, prepared by various methods at other laboratories, used in the trial.

c. As a consequence of the 1964 field trial, the WHO, in cooperation with the Indian Council of Medical Research, has planned a larger field study of cholera vaccines in the Calcutta area for 1965. For this study the WHO has requested 30,000 doses of a new lot of freeze-dried formalin-inactivated cholera vaccine, containing about 3 times the antigenic content of the previous lot in an attempt to improve the efficacy of the vaccine. This new lot has been prepared and has been shipped to WHO representatives in India for use.

7. Plague

In March 1965 studies were initiated on the development of a freeze-

dried live attenuated plague vaccine, prepared with the EV76 strain of Pasteurella pestis obtained from the Pasteur Institute, Saigon, Viet Nam. These studies were indicated because of the instability of the fluid EV76 vaccine currently prepared in Southeast Asia. In the fluid state, this vaccine must be used within 10 days from the time of manufacture, thus providing insufficient time for the control tests which should be carried out on this product.

Experimental studies were conducted to provide information on the most satisfactory methods for growing and processing the EV76 strain of the plague bacillus to yield a stable potent immunizing agent. Some of the parameters investigated included 1) growth and assay media, 2) incubation period, 3) harvesting medium, and 4) freeze-drying conditions.

On the basis of the results obtained in these experimental studies a production procedure was evolved, as follows: Kolle flasks containing double veal infusion agar were inoculated with 2 ml of a 16-hour veal infusion broth seed culture and were incubated at 28°C for 24 hours. The growth on the surface of the agar was harvested in 10 ml of a sucrose-phosphate-glutamate solution containing 2.5% human serum albumin. The harvest suspension was immediately filled into 6 ml vaccine vials, 1 ml per vial, frozen and dried. Viable counts were performed before and after drying. With this method the fluid harvests averaged 3×10^9 viable organisms per ml, and the dried products averaged 1×10^9 viable organisms per ml.

An experimental lot of 250 bottles of dried live plague vaccine was prepared employing the procedure described above. Sample vials were placed at several temperatures for stability studies. Periodically samples are being removed from storage for determination of the number of viable organisms. The results thus far are as follows:

Surveillance of Freeze-Dried EV76 Plague Vaccine

	Temperature of Storage				
Time	-20°C	+4°C	+28°C	+37°C	+45°C
0	1.6×10^9	-	-	-	-
1 week	1.2×10^9	1.4×10^9	3.0×10^8	1.0×10^8	4.0×10^5
2 weeks	1.1×10^9	-	1.4×10^8	2.3×10^7	9.9×10^3
3 weeks	-	-	1.1×10^8	4.1×10^6	6.0×10^1
4 weeks	1.6×10^9	1.7×10^9	7.3×10^7	3.5×10^6	-
8 weeks	2.0×10^9	5.0×10^8	7.5×10^5	2.0×10^5	$<10^1$

The results of these limited studies indicated that the temperature of choice for long-term storage is -20°C. The protective potency for guinea pigs and mice of the stored vaccine is currently under investigation. Preliminary tests showed that the freshly prepared dried vaccine, diluted to contain 1×10^6 viable organisms, protected guinea pigs and mice against challenge with a virulent strain of P. pestis.

Summary and Conclusions.

1. Surveillance of the stability of the freeze-dried typhoid seed culture prepared for the World Health Organization in 1960 was continued. The viability of cultures stored in the dried state at 4°C and 22°C for 4 years was satisfactory. Laboratory studies indicated that the combination of acetone-killed and dried typhoid vaccine with tetanus and diphtheria toxoids had no effect on the potencies of any of the components.

2. A supply of phase 1 and phase 2 Q fever rickettsial slurries was prepared and characterized in guinea pigs. These preparations will be used as challenge materials for field studies to determine the relative efficacy for man of the killed phase 1 and phase 2 Q fever vaccines. A stock supply of antigen was also prepared for use in serological tests in connection with this study.

3. Surveillance of the stability of a lot of freeze-dried RSSE vaccine indicated that this product still gave an acceptable level of protection, as measured in mice, after storage at 4°C for 6 years.

4. Studies on Eastern Equine Encephalomyelitis were concerned with evaluating the immunological response of animals to both the whole chick embryo vaccine and the cell culture vaccine. In addition to the standard guinea pig protection and mouse neutralization tests, various serological methods are being employed on serum fractions obtained by sucrose density gradient techniques.

5. A new lot of Western Equine Encephalomyelitis vaccine (chick embryo origin) was made available for human use. Studies on the production of a freeze-dried WEE vaccine prepared in chick embryo fibroblast tissue culture were completed. Several experimental lots of this vaccine were prepared and when tested in guinea pigs compared favorably with the whole chick embryo vaccine.

6. Surveillance of the viability of the freeze-dried cholera seed cultures prepared for the World Health Organization in 1962 was continued. The results indicated that, for long-term storage, -20°C is the temperature of choice. A new lot of freeze-dried cholera vaccine, containing three times the antigenic content of the previous lot, was prepared and has been shipped to WHO representatives in India for use in a second field trial.

7. Studies were initiated on the development of a freeze-dried live attenuated plague vaccine. A production method has been developed which consistently yields fluid harvests containing 3×10^9 viable organisms per ml and a dried product containing 1×10^9 viable organisms per ml. The freeze-dried product is currently being studied for stability and for protective potency in guinea pigs and mice.

Publications.

Benenson, A. S., Grogan, E. W., Lowenthal, J. P., Berman, S., Abrams, A., and Altieri, P. L. Preparation of dried acetone-inactivated and heat-phenol-inactivated typhoid vaccine. Bull. Wld. Hlth. Org., 1964, 30:635-646.

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23. KEYWORDS Antibody; antigens; complement; complement fixation tests; fluorescent antibody technics; parasitic diseases, serodiagnosis; serology; treponemal infections

24. (U) Tech Objective - Method development and improvement for serorecognition of microbial infections. Procedures evaluated for diagnostic ability, guide for therapy and relationship to course of disease. Serological procedures urgently required in diseases where causitive agent is difficult to demonstrate.

25. (U) Approach - Technics such as CF and FA are utilized in conjuntion with antigen purification methods (e.g. physico-chemical fractionation). Also, new serological technics are developed, e.g. soluble antigen FA (SAFA). Technical problems include, at times, limited availability of organism and its separation from host tissues. Improved technology in one case often facilitates research even in unrelated areas.

26. (U) Progresss (Jul 64 - Jun 65) - SAFA, a major advance in technic development, permits objective selection of antigen and mechanical reading. Excellent results obtained in S. mansoni and T. cruzi infections. SAFA also holds promise in other diseases and possibly may replace TPI test. Separation of malaria parasites from rbcs without detriment to antigen is a difficult problem. However, advances are being made by differential filtration of lysed rbcs and by gradient separation of parasitized rbcs before lysis. Exoantigens of living T. cruzi and S. mansoni show high specificity and sensitivity in CF tests. The antigens are chemically less complex than somatic antigens and require minimum purification. Value of exoantigens as guides to therapy and for following course of infection is being studied. These examples of technic development and antigen improvements have direct application to serodiagnosis.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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Project 3A014501B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 174, Sero-recognition of microbial infections

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Description.

This task is concerned with the mechanisms and patterns of the immune response. In vitro and in vivo methods are used to study host response to antigens. In vitro studies involve the development, improvement, and evaluation of procedures for detection of host antibodies. The studies also entail isolation, purification, and identification of antigens by chemical and serological methods. In vivo studies include (1) ability of antigens to stimulate serologically detectable antibodies and (2) production of specific antisera by infection and/or injection of known antigens for identification and characterization of experimental antigens or antigen fractions. Antigens which show a high level of serological sensitivity and specificity are evaluated for immunogenicity.

Progress.

1. Development of a soluble antigen fluorescent antibody (SAFA) technic for serodiagnosis of infectious diseases. Indirect fluorescent antibody (FA) tests employing whole or fragmented organisms as antigen have been used advantageously in the serodiagnosis of numerous infectious diseases. In these procedures, the organism serves as a natural matrix for the functional antigen(s) residing on the surface of the organism under the conditions of the test. Antibody reacting with the functional antigen is detected by exposure to fluorescein-tagged antiglobulin and subsequent visualization with a compound microscope under ultraviolet illumination.

In the current studies, a practical soluble antigen fluorescent antibody (SAFA) technic was developed for the serodiagnosis of schistosomiasis and American trypanosomiasis. Emphasis was placed on methods which would: 1) permit objective selection of the antigen to be employed; 2) allow objective mechanical reading of the test results; and 3) compensate for nonspecific fluorescence encountered in the test system.

Initial experiments indicated that the choice of matrix for the test was of considerable importance. After study of many substances, cellulose acetate filter paper (circles 1/4" dia., 0.45μ porosity) was selected for the matrix. In early studies, schistosome antigen was fixed to the matrix with 95% ethanol. However, th. step was not only unnecessary, but was detrimental to the system; simple air drying of the antigen-saturated discs proved to be satisfactor· even after several months of storage. Nonspecific adherence of test rum to the matrix originally presented some difficulties, but this s. sequently was overcome by the addition of 1% bovine serum albumin (BSA) the system. These findings led to the development of an acceptable s andard technic. Briefly, the system involves the use of a test disc (antigen diluted in 1% BSA) and a control disc (1% BSA alone). Both discs are incubated in the test serum. The remainder of the procedure is essentially that of the conventional fluorescent antibody technic. Results of each test are read mechanically with a fluorometer which is adjusted to zero with the control disc and the reading representative of specific reactivity then obtained with the test disc.

Initial studies were performed with a somatic protein antigen obtained from the adult stage of Schistosoma mansoni. Sera from patients with schistosomiasis and a variety of other infectious diseases were studied, as well as sera from healthy individuals. All sera from the group of 87 cases of schistosomiasis were reactive. The 62 normal sera were uniformly nonreactive as were 37 sera from patients with diseases other than schistosomiasis.

It was of interest to determine whether antigens other than somatic protein of S. mansoni would adhere to the artificial matrix, and to determine whether the SAFA technic would yield comparable results in other diseases. Attention therefore was directed to Chagas' disease where somatic protein, somatic carbohydrate and exo (presumably glycoprotein) antigens were available. Preliminary studies indicated that all three of these antigens adhered equally well to the matrix, and that SAFA tests could be performed without essential change from the method previously developed for schistosomiasis. However, only the somatic protein and exo antigens of T. cruzi were critically evaluated; the somatic carbohydrate was not included because it had been shown to be both less sensitive and less specific than the other antigens in complement fixation tests.

The results obtained with the two T. cruzi antigens proved to be as impressive as those previously noted in schistosomiasis. Of the 82 Chagas' disease sera tested, 78 showed a reaction with each antigen. All sera from the 54 healthy persons were nonreactive. In the category of diseases other than T. cruzi infection, a total of 69 sera were tested. All were nonreactive in tests with the exoantigen and 68 were nonreactive with the somatic protein antigen; the single serum that reacted with the protein was from a patient with leishmaniasis.

Development and evaluation of SAFA technics for other diseases currently are in progress. Calf thymus nucleoprotein (CTNP) antigen as a diagnostic aid for lupus erythematosus is being investigated. The CTNP antigen currently used in the CF procedure is particulate and therefore presents an interesting challenge for the SAFA technic. In preliminary studies, considerable difficulty was encountered in obtaining reproducible results and efforts were made to improve and solubilize the antigen. Freeze-thaw and shaking, in conjunction with treatment with dilute KOH,were employed in efforts to render the antigen more soluble. The preparation yielding the most consistent results was obtained by prolonged shaking followed by the addition of 0.03N KOH immediately before use. The CTNP studies will be continued along these lines and will include evaluation of sera from patients with various immunologic disorders.

The SAFA technic also is being evaluated in syphilis with a soluble antigen obtained from the Nichols' strain of pathogenic Treponema pallidum. Early results indicate that the technic may be feasible. The first studies were performed with a desoxycholate-fractionated antigen prepared according to the method employed for previous complement fixing (TPCF) antigens, thus providing a point of reference. In the SAFA technic with treponemal antigen, it was shown that reagin was not responsible for the observed reactions with syphilitic sera. Attempts are being made to ascertain whether antibody detected by the Treponema pallidum immobilization (TPI) test and/or the fluorescent treponemal antibody (FTA) test is involved in the SAFA reaction, or whether a distinct antibody is involved. In addition, various other methods of obtaining the soluble antigen are being investigated as rapidly as possible, the limitation being the limited availability of treponemes sufficient for antigen fractionation.

Future plans for the SAFA technic include investigation of soluble antigen systems in a wide variety of infectious diseases. Tuberculosis and mycotic infections will receive early attention. In addition, studies will be continued on improvement of the basic methods of the technic itself.

2. Changes in the osmotic fragility of sheep erythrocytes after the addition of hemolysin and various components of complement. It has been observed that spontaneous lysis of sheep erythrocytes increases after sensitization with high concentrations of hemolysin and subsequent treatment with certain components of complement (C'). Since sensitized sheep cells are an essential reagent in the complement fixation procedure, the recogniton and understanding of factors which influence stability of the cells are of obvious importance in the design and control of CF test procedures. Moreover, these findings also may have an important bearing on other serologic tests such as HA and HAI which utilize erythrocytes sensitized with substances other than hemolytic antibody. Studies therefore were initiated to investigate the changes in osmotic fragility of sheep erythrocytes resulting from sensitization with rabbit antibody (hemolysin) and successive addition of various C' components. The quantitative technic of Shields and Allen (Department of Hematology, WRAIR) was employed in determining osmotic fragility curves ("osmograms") of the erythrocytes of nine healthy sheep to provide a normal base line. The osmotic fragility curves of washed, nonsensitized red cells then were

compared with osmograms obtained with optimally sensitized cells treated with various C' components (i.e. C'1; C'1,4; C'1,4,2; C'1,4,2,3c,3b,3f; and C'1,4,2,3c,3b,3f,3a). In addition, differences between the fragility of cells sensitized with 19S gamma-m globulin (γm) and 7S gamma-G globulin (γG) were studied.

Early results revealed that sensitization with hemolysin significantly increased the osmotic fragility of sheep erythrocytes. Moreover, it appeared that sensitization with pure 7S antibody induced changes in osmotic fragility greater than those related to sensitization with commercially prepared Forssman antibody,the latter primarily being 19S gamma globulin. It is noteworthy, however, that the osmotic fragility of the sensitized cells reverted to the pre-sensitization level when the first C' component (C'1) was added. Moreover, the stability of the cells was further increased with each successive addition of subsequent C' components until the reaction with C'3a occurred; the latter caused a slight increase in the osmotic fragility of the cells. Since these investigations were initiated only recently, the data obtained thus far are incomplete. Nevertheless, the findings indicate that further studies along these lines should be worthwhile.

3. The effect of certain therapeutic agents on complement (C') activity. A number of drugs have been reported to inhibit in vitro activity of serum C' and thus appear to represent a potential source of error in serologic test procedures. Among these therapeutic agents, acetylsalicylic acid (ASA) is of interest because of its wide general use and its beneficial effects in rheumatic fever. Since it has been suggested that the capacity of ASA to inhibit C' activity might explain its therapeutic effects in some immuno-pathologic disorders, the present studies were made to investigate the effects of ASA on the hemolytic activity of human complement (HuC') and guinea pig complement (GpC').

In initial experiments it was observed that addition of 40 mg% ASA to well buffered hemolytic systems enhanced rather than decreased HuC' activity. On the other hand, this concentration of the drug was markedly inhibitory when weakly buffered diluent was employed in the C' assay. However, when the weakly buffered diluent containing ASA (pH 4.9) was readjusted to pH 6.8, HuC' activity returned to normal levels. In view of the obvious influence of pH on the hemolytic activity of C', it appears that the observed enhancement of HuC' activity that followed addition of ASA to a well buffered system was due to a slight reduction of pH to a level more optimal for immune hemolysis (i.e. reducing the pH of the system from 7.2 to 6.8). It is noteworthy that similar results were obtained in parallel experiments with GpC'.

In an effort to study the effects of ASA on HuC' under conditions more nearly comparable to those obtaining in vivo, C' activity was measured in a system employing heat inactivated human serum rather than buffered saline as reagent diluent. Addition of ASA to this system did not alter the C' activity. Furthermore, ASA administered by gavage to 7 adult guinea pigs in doses sufficient to produce serum salicylate levels as high as 85 mg%, failed to influence GpC' activity. Since ASA apparently is capable of inhibiting C' only at pH levels considerably below those compatible with life, it seems unlikely that the therapeutic effects of ASA in diseases are associated with in vivo suppression of C' activity. In addition, it has been shown that ASA in therapeutic concentrations is incapable of altering the pH of well-buffered systems sufficiently to cause nonspecific suppression of C' activity and thus interfere with serologic tests.

In addition to ASA, it has been reported that cortisone also may suppress C' activity. Preliminary studies in this laboratory, however, indicate that cortisone does not reduce C' titers in vitro, and there is some evidence suggesting that the drug may even enhance in vivo C' levels. Further investigations on the in vitro and in vivo effects of cortisone on GpC' will be conducted.

4. The effect of storage on sheep erythrocytes used in complement studies. During investigations on the total hemolytic complement (C') titers of humans with certain connective tissue diseases, it was observed that there were marked differences in the degree of immune lysis obtained with a given sample of C' when tested simultaneously with various lots of sheep erythrocytes. In addition, it was noted that the amount of immune hemolysis obtained with an individual lot of sheep cells progressively changed as the cells were stored for various periods in modified Alsever's solution. Since sheep erythrocytes are an essential component in a variety of serologic and immunologic test procedures, it was deemed worthwhile to investigate the effects of storage on such factors as immune hemolysis, osmotic fragility and susceptibility to spontaneous lysis.

Nine randomly selected sheep were used in these studies. In each case, whole blood was collected aseptically in an equal volume of modified Alsever's solution and stored at 3°C. On the day of testing, erythrocytes were washed, standardized and sensitized by the method of Kent and Fife (Am. J. Trop. Med. & Hyg., 12:103, 1963). Sera from 47 healthy human males, age 18-25, were pooled, divided into aliquants, and stored at -60°C to provide a constant source of human complement (HuC'). The same lots of HuC', hemolysin and reagent diluent (triethanolamine-buffered saline) were used throughout the investigation.

The degree of immune hemolysis obtained with erythrocytes from the various sheep was determined by the method of Hook and Muschel (Proc. Soc. Exper. Biol. & Med., 117:292, 1964). Osmotic fragility of the red cells was determined by the quantitative technic of Shields and Allen (Department of Hematology, WRAIR) and by the multiple tube dilution method of Dacie. Spontaneous lysis also was evaluated in each instance by centrifuging the sensitized cells at 1000 rcf for 5 minutes and observing the degree of hemolysis in the supernate.

Results of these studies revealed that the amount of immune hemolysis obtained with the various lots of sheep erythrocytes increased with each week of storage. Moreover, the most significant increase occurred during the first 3 weeks. The osmotic fragility of the sheep cells also increased with storage and the maximum change was observed during the third and fourth week. Furthermore, the increase of spontaneous lysis paralleled that of the osmotic fragility. In view of these findings, it is apparent that sheep erythrocytes stored for more than 3 weeks in Alsever's solution may be unsatisfactory for use in serologic tests and that caution should be exercised in employing cells beyond this period.

Further studies on the storage of sheep erythrocytes appear to be in order and investigations of red blood cell preservatives other than modified Alsever's solution have been initiated. In addition, the feasibility of storing sheep erythrocytes in the frozen state will be investigated.

5. The influence of X-irradiation on the mechanism and pattern of the immune response. Studies were performed to determine whether rabbits produced antibodies against their own tissue as a consequence of exposure to ionizing radiation. Contrary to reports in the literature which suggested that autoantibodies might appear as a result of radiation damage of tissue, no evidence was obtained to indicate that new antigenic characteristics appeared in saline extracts of rabbit testes obtained 2-, 10- or 42-days after exposure to 2000 r. In fact, when isologous and autologous antigens were titrated, 10- and 42-day post-2000 r antigens were found to be serologically less reactive and less anticomplementary than antigens from 2-day post-2000 r or non-irradiated testes. Irradiated tissue antigens contained more lipid but less protein than non-irradiated controls.

Naturally occurring antibody against rabbit testes, Salmonella typhi and beef heart cardiolipin antigens showed no large alterations in titer following 500 r whole body radiation of the donor rabbits. However, a significant increase in natural hemolytic antibody was seen in these animals 35-120 days post-irradiation. No important changes were observed in normal antibody levels of rabbits given 2000 r to the testes only. Although chemical and immunological differences were observed between irradiated and non-irradiated testes antigens, radiation-altered tissue did not appear to be immunologically "foreign" to its host. It was concluded that radiation damage either to the testes or to the entire body of rabbits probably did not induce autoantibody formation. Investigations relating to autoimmunity in irradiated animals were terminated during the period covered by this report.

6 . "Cell-bound" antibodies in microbial infection. Phagocytic cells harvested from the peritoneal cavities of immune mice were shown to possess the capacity to passively transfer immunity against mouse typhoid. These findings corroborated the observations of Rowley et al., (Aust. J. Exp. Biol. Med. Sci., 42:237, 1964) and extended the previous investigations by including additional strains of Salmonella typhimurium. Experiments to clarify the role of "O" antigen (endotoxin) in protection also were initiated.

To study the protective effect of "O" antigen, mice were given single or multiple injections of endotoxin extracted by the Boivin procedure from avirulent S. typhimurium. In addition, 0.45 μ or 100 mμ porosity diffusion chambers containing living, avirulent S. typhimurium cells were implanted in the peritoneal cavities of another group of mice. All animals were then challenged 14 days later with approximately 1000 lethal doses of a virulent strain of S. typhimurium and their survival compared with controls protected with living, avirulent cells and similarly challenged. Results of preliminary experiments indicated that protection against subsequent challenge was afforded by the endotoxin alone, and in some cases by the diffusible material from avirulent cells, although to a lesser degree than the highly efficient protection elicited by injection of living bacteria. However, it was observed that the "O" antigen showed relatively low activity as a complement fixing antigen in tests against antibody contained in extracts of peritoneal exudates from Salmonella infected mice. Efforts are being made to learn whether the protection afforded by endotoxin in these experiments is serologically specific or is due simply to non-specific pharmacological action of the Salmonella endotoxin.

The results obtained seemed to support the concept that a "carrier state" induced by injection of living avirulent bacteria was highly efficient in protection against subsequent challenge with virulent strains of S. typhimurium, and that endotoxin or diffusible material from bacterial cells may play a role in this protection. Further studies are being made along the lines indicated.

7. Anticomplementary substances in serums and antigens. A problem sometimes encountered with diagnostic complement fixation tests is the tendency of certain antigens and antisera to nonspecifically inhibit complement activity and thus alter sensitivity of the test. Anticomplementary activity may result from a variety of factors such as: 1) The presence of aggregated gamma globulin; 2) Chelation by compounds which bind Ca++ and Mg++; or 3) Traces of antibody present in the guinea pig serum serving as a source of complement and which may form immune complexes with antigenic groups present on molecules of test antigen or antiserum. Recent findings obtained with human and animal sera have indicated that anticomplementary activity may also result from natural antibodies in the test serum forming immune complexes with antigens present in the guinea pig complement. A study has been undertaken with the aim of eliminating the latter reactions so that certain "problem" sera may be satisfactorily tested in the complement fixation procedure.

Preliminary results have shown a correlation between the time of appearance of natural complement fixing antibodies in sera from young rabbits and the time when anticomplementary activity of these sera becomes demonstrable. Fractionation of rabbit sera has revealed that natural antibodies against a variety of microbial and tissue antigens commonly are associated with the gamma-m type of globulin and that sera with this fraction removed often have reduced anticomplementary activity while retaining immune antibody of the gamma-G variety. Further investigations are being made to determine the feasibility of applying a simple fractionation procedure to human and animal sera to eliminate this type of anticomplementary effect. The overall problem of anticomplementary substances in sera and in microbial antigens is also being studied.

8. Mass cultivation of Trypanosoma cruzi in a chemically defined medium. In a previous report (Tarrant, et al., Am. J. Parasitol., 51: 277, 1965), qualitative and quantitative chemical analyses of the T. cruzi exoantigen revealed the presence of both carbohydrate and protein (possibly glycoprotein) in all fractions exhibiting a significant degree of serologic activity. However, Kjeldahl nitrogen values greatly exceeded the amounts that would have been anticipated from the protein concentrations indicated by the quantitative Lowry procedure. Since peptone was a major component of the culture medium, it appeared that this medium constituent could be responsible for the unexpectedly high

nitrogen content of exoantigens. It was thus apparent that only a general appraisal of the chemical nature of the T. cruzi exoantigens was possible. The obvious solution to this problem would be the cultivation of the organisms on a chemically defined medium. The present report deals with preliminary studies along these lines.

The previously employed mass-culture technic (Fife and Kent, Am. J. Trop. Med. & Hyg., 9:512, 1960) was followed except for the substitution of Locke's solution for peptone in the medium. Thus, cellulose sacs containing Locke's solution were suspended in a blood coagulum-Locke's solution mixture and seeded with T. cruzi from a stock culture. After incubation at 23°C for 35 days, one ml of the sac contents was transferred to a second culture sac assembly and incubated as before. The procedure was repeated once again, constituting the third serial passage in the peptone-free medium. Although the growth in this passage was somewhat less than that obtained in the standard medium, it was considered adequate for the production of exoantigen for serological and chemical studies.

The third-passage culture was centrifuged to remove the trypanosomes and then was dialyzed to remove excess salts and other dialyzable components. The dialysant, designated unfractionated exoantigen, was lyophilized and stored in the dried state. Preliminary studies showed that exoantigen prepared in this manner reacted well in complement fixation tests with homologous antiserum, and apparently contained the specific antigen components present in the earlier preparations, but without the peptone contaminant. Various qualitative and quantitative chemical analyses now are being employed in an effort to more precisely characterize the serologically active component(s) of the T. cruzi exoantigen.

9. Studies on the immunogenic properties of Trypanosoma cruzi exoantigens. Various antigen preparations have been used as vaccines in attempts to immunize experimental animals against T. cruzi infection. For the most part, the vaccines have consisted of T. cruzi culture forms disrupted by physical methods, or fractions isolated from the organism by physico-chemical methods. Investigators employing antigenic material obtained from either of these methods used alone or in combination have reported that the antigens generally are ineffective as immunizing agents (Muniz & de Freitas, Brasil-Medico, 43:337, 1946; Kagan & Norman, J. Inf. Dis., 108-109, 213, 1961; Cox, Am. J. Parasitol., 50:11, 1964).

Tarrant et al., (Am. J. Parasitol., 51:277, 1965) showed that the exoantigens of T. cruzi were excellent complement fixing (CF) antigens and yielded highly specific serodiagnostic tests for Chagas' disease.

Furthermore, when injected intravenously into rabbits, relatively small amounts of the exoantigen stimulated rapid production of circulating antibodies, even when administered without an adjuvant. Within 21 days, all animals developed CF titers of 32 or greater. This was in contrast to the relatively low antibody response observed in animals immunized with somatic antigens of T. cruzi.

The present studies were undertaken to determine whether the exoantigen used as a vaccine would protect mice against challenge with virulent T. cruzi (Tulahuen strain), and if so, whether the degree of protection could be correlated with the circulating antibody titer. The exoantigen employed in these preliminary studies was the pH 4.6 soluble fraction used in the previous serological evaluations.

To investigate the ability of the T. cruzi exoantigen to stimulate production of circulating and/or protective antibodies in mice, eighty randomly selected adult mice were divided into two groups. One group (40) was inoculated with the pH 4.6 soluble fraction (vaccine), and the other received culture medium control. Each mouse received four intraperitoneal inoculations of 0.5 ml exoantigen or control medium without adjuvant at four-day intervals. At day 21, each mouse was challenged with 2×10^4 virulent trypanosomes. Complement fixation tests were performed on representative sera from immunized, control and normal animals. In sera collected at the time of challenge (21 days post inoculation), circulating antibodies were demonstrated in mice receiving the vaccine. Antibody titers ranged from 8-16 in the immunized group whereas no detectable CF antibodies were demonstrated in sera from the medium control or normal mice. Nevertheless, even though circulating antibodies were present in the immunized animals, no appreciable protection against virulent T. cruzi could be demonstrated. These results were in contrast to earlier experiments with smaller groups of animals wherein some degree of protection was indicated.

In view of these divergent findings, consideration is being given to the possible advantages of using more concentrated antigen for the vaccine, to the selection of other routes of inoculation, and to extending the time between immunization and challenge. The feasibility of incorporating metabolizable adjuvants with the exoantigen also is being investigated.

10. Studies on the exoantigens of Leishmania brasiliensis, L. donovani, and L. tropica. For years, investigators have devoted considerable effort to the development and improvement of serologic tests for leishmaniasis. Nevertheless, progress has been slow and still leaves much to be desired. For example, the best antigens currently available for serodiagnosis of various leishmanial infections

are derived from certain acid-fast bacilli, and not from the etiologic agents themselves; somatic antigens extracted from cultured Leishmaniae generally react poorly in conventional serologic tests. The employment of antigens from various species of Mycobacterium, however, has introduced problems of reactivity also in cases of tuberculosis and leprosy, thereby seriously limiting the usefulness of the procedure as a diagnostic aid for leishmaniasis. Thus, the need for a more satisfactory antigen is apparent. In view of the promising results recently obtained in this Department with exoantigens of Trypanosoma cruzi, consideration was given to the possibility that exoantigens of Leishmaniae might similarly improve serologic tests for leishmaniasis. The present report summarizes preliminary studies along these lines.

The mass-culture technic used for production of T. cruzi exoantigens was employed for the cultivation of various species of Leishmania. Excellent growth was obtained and the exoantigens were harvested and processed by the methods previously described for T. cruzi exoantigens. In initial studies, the unfractionated and pH 4.6 soluble fractions of the various Leishmania exoantigens reacted well in complement fixation tests with homologous antisera from experimentally infected animals. Moreover, the antigens exhibited no anticomplementary activity. The specificity and sensitivity of the exoantigens currently are being critically evaluated in CF tests with human sera. In addition, studies to characterize the serologically active antigen components by various qualitative and quantitative chemical methods are being undertaken as sufficient material becomes available.

11. Studies on the exoantigens of Schistosoma mansoni cercariae. Preliminary investigations on the serological properties and general chemical nature of S. mansoni cercarial exoantigens were summarized in an earlier report (Annual Progress Report, 1964). These studies showed that the exoantigen exhibited physico-chemical properties of a carbohydrate rather than a protein, even though a small amount of protein appeared to be present. In view of these findings and supporting data of quantitative chemical analyses, it was suggested that the antigen primarily was a polysaccharide or possibly a glycoprotein. More recent studies have indicated that protein plays little if any role in the serologic activity of the antigen since chloroform-gel fractionation effected a two-fold reduction of the already low protein value, but had no effect whatsoever on the reactivity of the antigen with homologous antiserum. It therefore seemed unlikely that protein per se was an essential component of the antigen, and it is suggested that the trace amounts of protein apparently remaining in the fractionated antigen might be due either to the presence of firmly bound peptide units in the polysaccharide, or to linkage of the sugar units of the polysaccharide through aromatic amino acids (e.g. phenylalanine, tyrosine or tryptophane) which give strong reactions with the phenol reagent of

the Lowry procedure. The latter particularly would be consistent with earlier observations which showed that considerable linkage of the antigen molecule was through amino groupings, and these could be those of conjugating amino acids.

The character of the carbohydrate components of the exoantigen also has been further elucidated. Paper chromatograms of the antigen revealed the presence of two reducing components, one with a Rf corresponding to that of glucose and/or galactose, and the other to glucosamine and/or galactosamine (Annual Progress Report, 1964). Recent assays using the specific enzymes glucose oxidase and galactose oxidase revealed that glucose and/or glucosamine were present only in trace amounts if at all. On the other hand, galactosamine was demonstrated in quantities sufficient to account for all of the hexosamine detected by the Elson-Morgan procedure. Other carbohydrates also appeared to be present in the exoantigen, but their identity has not been established at this time.

In the earlier preliminary serological evaluation of the exoantigen, specificity appeared to be excellent; sera from individuals with parasitic diseases other than schistosomiasis or trichinosis did not react with the exoantigen. However, subsequent evaluations employing larger numbers of sera from other disease categories revealed significant cross reactivity with syphilitic sera. Efforts to remove the components responsible for these overt reactions, however, have been unsuccessful thus far. Physico-chemical methods (chloroform-gel fractionation and treatment with anhydrous ether) which had been effective in removing similar components from T. cruzi and adult schistosome somatic antigens, failed to reduce the reactivity of the cercarial exoantigen with syphilitic sera. It appeared therefore that the undesirable components were firmly bound to the antigen complex and that more rigorous fractionation methods must be employed.

In comparative tests with additional sera from individuals residing in a highly endemic schistosomiasis area, the exoantigen exhibited patterns of reactivity that differed from those of the somatic antigen. This was in accordance with observations noted in the preceding report and further supported the postulate that the exo and somatic antigens do not react with precisely the same antibodies, and that the relative concentrations of these antibodies may vary significantly during the course of infection and among different individuals. In view of these findings, consideration was given to the possibility that the exoantigen might be superior to the somatic antigen for appraising the effectiveness of therapy. Studies along these lines, using both human and simian sera, are being initiated. In addition, it is proposed to investigate the immunogenicity of the exoantigen and attempt to relate the findings to host resistance.

Summary and Conclusions.

1. The development of a soluble antigen fluorescent antibody (SAFA) technic for serodiagnosis of schistosomiasis and American trypanosomiasis has overcome certain major deficiencies inherent in conventional FA procedures using intact microorganisms as antigen. The SAFA procedure allows objective selection of the antigen to be employed, provides for mechanical reading of test results, and compensates for nonspecific fluorescence which may be encountered in the test serum. In addition, factors such as excessive chyle, hemoglobin or anticomplementary activity which may on occasion render sera unsatisfactory for testing in other serologic procedures apparently have no adverse effect on the SAFA test. Excellent results have been obtained with all antigens studied thus far. These have included: protein antigens from adult S. mansoni; protein, carbohydrate and glycoprotein antigen from T. cruzi; nucleoprotein antigens from calf thymus; and a protein antigen from virulent T. pallidum. In each instance, the specificity and sensitivity of the SAFA procedure was at least as good as that manifested in standard serologic tests. Studies to determine the applicability of the SAFA procedure for serodiagnosis of other infectious diseases are in progress.

2. The osmotic fragility of sheep erythrocytes significantly increased after sensitization with hemolysin. It was further shown that sensitization with pure 7S antibody induced significantly greater changes in fragility than did sensitization with 19S gamma globulin. Treatment with various components of complement (C'), however, reversed the pattern of fragility. Thus, addition of C'1 caused reversion of the osmotic fragility to the presensitization level. Moreover, successive addition of subsequent C' components further increased the stability of the cells until the reaction with C'3a occurred. The latter effected a slight increase in osmotic fragility. Further studies along these lines are in progress.

3. Studies were conducted to determine whether the therapeutic effects of acetylsalicylic acid (ASA) in certain immuno-pathologic disorders were due to in vivo inactivation of C'. The findings showed that this could not be the mechanism involved; ASA inhibited C' activity only at pH levels far below those compatible with life. It was also apparent that ASA in therapeutic concentrations would not interfere with diagnostic complement fixation tests provided that an adequately buffered system was employed.

4. The stability of sheep erythrocytes preserved in modified Alsever's solution was found to vary with the length of storage. Susceptibility to immune lysis, osmotic fragility and tendency to undergo spontaneous lysis all increased with storage. Moreover, most lots of cells became unsuitable for serologic tests after three weeks of storage. Efforts are being made to develop more satisfactory methods for preserving erythrocytes for use in serologic tests.

5. Studies to determine whether rabbits produced antibodies against their own tissues as a result of exposure to ionizing radiation were concluded. Some chemical and immunological differences between irradiated and non-irradiated tissue antigens were noted. However, radiation-altered tissue did not appear to be immunologically "foreign" to the host. Thus, it seemed unlikely that autoantibody formation was induced by either partial or whole body irradiation.

6. Phagocytic cells were shown to possess the capacity to passively transfer immunity in mouse typhoid. Moreover, the "O" antigen (endotoxin) appeared to play some role in host resistance to infection. Although immunization with avirulent, living Salmonella afforded the greatest protection to challenge with virulent strains, the "O" antigen, and to some extent diffusible material from living cells, elicited an immune response that provided a significant degree of protection. Efforts are being made to determine whether the protection induced by endotoxin is serologically specific or is due simply to nonspecific pharmacological action of the product.

7. A variety of factors are known to cause anticomplementary (AC) activity in complement fixation tests. In addition to the presence of aggregated gamma globulin, chelation of Mg++ and Ca++, etc., recent studies have shown that certain natural antibodies in test sera which form immune complexes with antigens present in the guinea pig complement also can cause AC activity. Further experiments revealed that natural antibodies often are associated with gamma-M globulin and that removal of this fraction significantly reduced AC activity without interfering with the reactivity of the gamma-G immune antibody. Efforts are being made to develop a simple fractionation procedure to eliminate this type of AC effect.

8. Precise chemical analyses of T. cruzi exoantigens have not been possible because of contamination with culture medium components, principally peptone. Therefore, efforts to develop a chemically defined culture medium were undertaken. Preliminary findings indicated that substitution of Locke's solution for peptone in the standard medium supported growth of the trypanosomes for at least three serial passages. Although the growth on this medium was somewhat less than that obtained with the original culture technic, sufficient exoantigen was produced to allow chemical and serological evaluation of the product. Exoantigens obtained in this manner reacted well in CF tests with homologous sera and apparently contained the antigenic components present in the earlier preparations. Qualitative and quantitative chemical analyses now are being conducted to more precisely characterize the exoantigen.

9. Earlier studies revealed that relatively small amounts of T. cruzi exoantigen produced a rapid antibody response in rabbits even when administered without an adjuvant. In view of the unusual immunologic capacity of the exoantigen, studies were conducted to evaluate the immunogenic properties of the product and investigate its potential as a vaccine. In preliminary experiments with a relatively small number of mice, immunization with exoantigen significantly increased the survival time following challenge with a virulent strain of T. cruzi. However, subsequent experiments using a larger number of animals failed to show protection against challenge. Factors responsible for these divergent findings are not apparent at the present time. Efforts are being made to resolve the problem by employing a more concentrated antigen, by using other routes of inoculation, by extending the time between inoculation and challenge, and by incorporating metabolizable adjuvants with the inoculum.

10. In view of the significant improvement of serodiagnostic tests achieved with use of T. cruzi exoantigens, investigations were conducted to determine whether exoantigens also could be obtained from various Leishmaniae. It was observed that exoantigens were produced when Leishmaniae were cultivated in the manner previously employed for mass-culture of T. cruzi. These antigens reacted well in CF tests with homologous antibody developed in experimentally infected animals and now are being evaluated in tests with sera from human cases of leishmaniasis.

11. Studies on the chemical and serological properties of S. mansoni cercarial exoantigens were continued. Although small amounts of protein apparently remained in the fractionated exoantigen, evidence thus far indicates that protein plays little if any role in the serologic activity of the product. Carbohydrate analyses using specific enzymes revealed that galactosamine was the principal sugar present in the antigen and that the product was essentially free from glucose and glucosamine. Comprehensive serological evaluations with non-schistosomiasis sera revealed cross reactions only with sera from cases of trichinosis and syphilis. Efforts to remove the factors responsible for the latter reactions by treating the antigen with lipid solvents were unsuccessful and indicate the necessity for employing more rigorous fractionation methods. Continued studies on sera from individuals residing in a schistosomiasis endemic area corroborated earlier findings and provide further evidence that the exoantigen and somatic antigen do not detect precisely the same antibodies. The potential value of the exoantigen for following the course of therapy is being investigated.

Publications.

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PROJECT 3A014501B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 01
Surgery

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6450	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 65	A. NEW	U RPT U WRK	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61145011 3A014501B71R 01 090

10b PRIOR NUMBER/CODE: None

11. TITLE: (U) Blood and blood disorders (09)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002600 Biology; 008800 Life Support; 003500 Clinical Medicine	05 58	NA	OTHER | DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	c. TYPE NA	PRIOR FY 65	9	122
		CURRENT FY 66	9	135

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21. TECHNOLOGY UTILIZATION: Hematology

22. COORDINATION: NA

23. KEYWORDS: Blood; hemoglobin; iron; absorption; excretion

24. (U) Tech Objective - To improve the Army Transfusion Service by increasing the availability of blood for the treatment of casualties in the isolated task force.

(U) Approach - A "Walking Universal Blood Donor" program was proposed and methods for increasing its effectiveness were investigated. If an accelerated rate of hemoglobin production could be sustained after repeated phlebotomy, increased quantities of blood
25. would be available for transfusion. Since depletion of body-iron is the limiting factor, mechanisms regulating iron kinetics were investigated.

(U) Progress (Jul 64 - Jun 65) - A hypothetical model to explain the regulation of iron absorption was proposed. Evidence was accumulated to show that gain or loss of body-iron was regulated in a rate-limited manner by the iron content of intestinal cells and that this, in turn, was controlled through plasma iron turnover. Loss of body iron from the skin and gut were quantified and shown to be selective. Changes in the absorption of other metals were observed in various states of iron repletion.
26. Studies of the absorption of porphyrin iron were initiated to investigate factors regulating the absorption of dietary iron.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A014501B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 090, Blood and blood disorders

Investigators.

Principal: Colonel William H. Crosby, MC

Associate: Major Marcel E. Conrad, MC; Simeon Pollack, M.D.; Capt Richard M. Kaufman, MC; Capt Lewis R. Weintraub, MC; Capt David A. Sears, MC; Capt James N. George, MC; Capt Marvin Adner, MC; Capt Stanley Cortell, MC; J. -H. Huser, M.D.; Egmond Rieber, M.D.; Mr. James W. Eichelberger; Harvey J. Weiss, M.D.; and Johannes Blom, M.D.

Description.

A continuing study to improve the quality and safety of the Army's blood transfusion service and investigation of functions and disorders of blood and blood forming organs.

Progress.

A universal blood donor program was proposed which would identify soldiers in the troop population who could be used for emergency, life saving transfusion of casualties in areas and situations where banked blood was unavailable. Implementation of this program would provide a source of blood for transfusion of casualties in the isolated task force. It is estimated that one-third of U. S. soldiers have blood which could be used in a universal walking donor program. Thus, fresh, whole blood could be made available by proper identification of universal donors and by providing containers for collection and tubing through which blood can be transfused.

Studies have been continued to increase the quantity of blood which can be obtained from donors. A blood donor program which would support the isolated task force could be more effective if lack of iron in the body were not a limiting factor in the formation of hemoglobin. By studying the mechanisms regulating absorption and loss of iron, means were sought to increase the production of blood in normal donors. Intestinal mechanisms regulating the quantity of body iron were studied and showed to be a rate limited active transport system which was mediated through the duodenal mucosal cells. Factors regulating the mucosal uptake of iron from the gut lumen and mucosal transfer of iron into the body were studied. Loss of iron from the body was shown to occur from the gut and skin in a limited but selective manner. Food was shown to decrease the absorption of iron salts while the absorption of porphyrin iron was relatively unaffected.

A program to study the red blood cell-parasite relationship in malaria was continued. Glucose-6-phosphate deficiency is believed to protect humans from malarial infestation. This deficiency was simulated with drugs reducing red blood cell glutathione levels. Phynyl-hydrazine and alloxan were shown to suppress parasitemia and prolong the lifespan of mice infected with Plasmodium berghei. These observations suggest that antimalarial drugs may produce their effect by changes in the metabolism of the host red blood cells rather than by producing direct damage to the parasite. Mechanisms of in vitro oxidative hemolysis of red blood cells were studied. Damage to the red cell membrane with oxidative reagents was markedly reduced in solutions of albumin; partial protection was observed by the addition of dextran, sucrose or mannitol to solutions. The effect of primaquine upon red blood cells was shown to require energy from glucose metabolism through the pentose phosphate shunt. The hemolytic effect of acetylphenyl hydrazine, hydroquinone, menadrone sodium bisulfite and 6-naphthol was not enhanced by glucose alone.

Study of the anemia caused by teflon cardiac prosthetic implants showed that hemolysis was related to activity and cardiac output. The anemia was complicated by excessive loss of iron in the urine of patients when the rate of hemolysis was significant and exceeded the heme binding capacity of plasma.

The increased experimental use of monkeys and primates has increased the requirement for normal laboratory values in these animals. Studies of the hematologic values in a number of species were carried out under normal and experimental conditions of stress, sedation and nutritional deficiency. The importance of antigenic properties of red blood cells and platelets was investigated in rhesus monkeys with venous homografts and in animals receiving mismatched blood transfusions.

Studies of the immunoglobulins in human blood were initiated to simplify current techniques and provide specific antisera for immunofluorescent studies. Lipid binding was observed in human gamma globulins; its importance in lipid metabolism is being investigated.

Summary and Conclusions.

1. A walking universal blood donor program was proposed for use by troops in isolated areas.

2. Mechanisms controlling the regulation of iron metabolism were studied and delineated the role of the intestine in iron kinetics.

3. Drugs producing oxidative hemolysis of red blood cells reduce malarial infestation in animals. Their mechanism of action was investigated.

4. Teflon grafts produce a mechanical hemolytic anemia which is complicated by excessive loss of iron in the urine.

5. Normal hematological values have been established in a number of species of monkeys and primates. Homograft reactions and transfusion reactions are being studied in these animals.

6. Studies of immunoglobulins have been initiated to improve techniques and provide materials for immunofluorescent work in autoimmune disorders.

Publications.

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PROJECT 3A014501B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 02
Internal Medicine

RESEARCH AND TECHNOLOGY RESUME

1.	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6451	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT	6. SECURITY WRK	7 REGRADING	8. RELEASE LIMITATION	9 LEVEL OF RESUME
01 07 65	A. NEW	U	U	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE
61145011 3A014501B71R 02 085

10b PRIOR NUMBER CODE
None

11. TITLE
(U) Vascular components of cardiorespiratory disease (09:21)

12. SCIENTIFIC OR TECH. AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
021900 Physiology; 002400 Bioengineering; 002300 Biochemistry	08 63	NA	OTHER DA

16 PROCURE. METHOD	17. CONTRACT/GRANT b. NUMBER c. TYPE	d. AMOUNT	18 RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	NA		PRIOR FY 65	11	246
			CURRENT FY 66	11	270

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TEL 202-576-5121 TYPE DA

21. TECHNOLOGY UTILIZATION
Medicine

22. COORDINATION
NA

23. KEYWORDS Cardiovascular system; circulation; heart; blood; coronary vessels; myocardium; oxygen

24. (U) Tech Objective - Development of a standardized biological preparation in which appropriate apparatus can be implanted chronically 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.

25. (U) Approach - Control of regional blood flow and metabolism has been studied in the unanesthetized resting and/or active dog via chronically implanted electromagnetic flowmeters (our design) and special tubes in aorta and coronary sinus for blood pressure and blood sampling. Left coronary flow and myocardial oxygen usage have been obtained in semibasal state. Additional experiments have evaluated radiographic method for determining coronary flow in humans, and extended previous findings of effects on the coronary circulation and systemic dynamics of exercise, excitement, intra and extracardiac reflexes, blood transfusions, and compensation of blood pH during hypovolemic shock. The controls of the splenic circulation have been outlined. The effect of a controlled (paced) heart on systemic and coronary energetics has been explored.

26. (U) Progress (Jul 64 - Jun 65) - Technical problems have been largely resolved. Stroke coronary flow and stroke cardiac output increase considerably with exercise and excitement though heart rate is generally dominant in promoting minute blood flow. Paced hearts respond well with exercise and excitement with large increases in flow per heart beat. Preliminary experiments in paced and unpaced hearts indicate existence of alpha and beta receptors in the coronary circulation.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
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31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A014501B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 085, Vascular components of cardiorespiratory disease

Investigators.
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Associate: Eric C. Elliot, M.D.; Darrell W. Haas, Ph.D.;
Edward M. Khouri; Maj Arthur S. Leon, MC;
Capt Colin M. Bloor, MC; Capt Bertram Pitt, MC.

Description.

The general plan of study is to obtain a broad perspective of the central and local control of regional blood flow and metabolism of the unanesthetized dog exposed to the normal, abnormal, and pathological stresses of every day life.

Progress.

A standardized dog preparation has been developed with chronic implantation of regional blood flow transducers and special intravessel tubes which permits the appropriate hemodynamic and metabolic studies of the heart and other organs in the unanesthetized state for at least three months.

1. Development of instruments and methods for cardiovascular research.

a. The pneumatic occlusive cuff originally designed for obtaining mechanical flow zero while using the electromagnetic flowmeter has been further miniaturized and simplified. New techniques of fabrication have been developed resulting in a stronger and more durable cuff.

b. The signal/noise ratio of the electromagnetic flowmeter has been greatly improved. A molding technique has been developed for encapsulation of the electromagnetic flowmeter transducers and is presently being used. This new method permits the use of better plastics, not suitable for hand potting, resulting in a significant improvement of transducer quality, uniformity, and functional life.

c. Indwelling fine vinyl tubes have been successfully implanted in the coronary circumflex artery. These tubes have been kept patent up to two months, during which time phasic arterial pressures have been followed. There is hope this technique can be used as an adjunct in the study of the development of collateral circulation as reflected by alteration in the peripheral coronary pressure. The technique has also been used in conjunction with flow probes on the coronary circumflex to study the effects of intracoronary drug administration on coronary flow and cardiac dynamics. Both the above applications are still in a preliminary stage but they offer promise.

2. Hemodynamics of heart block. Work has progressed in the study of coronary blood flow in the unanesthetized dog at varying heart rates, and the effect of various interventions at fixed heart rates. Three dogs have been studied one to ten weeks after implantation of coronary flow transducers. The effects of varying ventricular rate before and after beta adrenergic blockade have been studied, as have the effects of excitement and exercise at fixed heart rates. Coronary blood flow has been studied at fixed ventricular rate under the influence of epinephrine, isoproterenol, and norepinephrine before and after alpha and beta adrenergic blockade. Studies have also been performed at fixed rates during hypoxia and hyperoxia. During the course of these studies, a cove-shaped coronary flow wave, which, in dogs with normal sinus rhythm occurs in late diastole, was found to be related to atrial contraction. In dogs with heart block at slow ventricular rates, two or more of these cove-shaped waves occur in each diastole, corresponding to atrial contraction. It is thought that vibrations of the heart related to atrial contraction lead to the production of this wave by causing oscillations in a fluid filled system.

3. Myocardial reactive hyperemia. The views of Szentivanyi et al., that elimination of sympathetic coronary artery constrictor tone abolishes reactive hyperemia and leads to a state of coronary artery rigidity resembling angina pectoris, were investigated. Coronary artery flow was studied in four anesthetized open-chest animals and in two unanesthetized animals. Peak coronary blood flow during reactive hyperemia was 50-750% greater than control flows. Adrenergic blockade of the sympathetic coronary artery constrictor fibers with i.v. dibenzyline 5-15 mg/kg, as well as intracoronary hexamethonium 5-25 mg, intracoronary dinitrephenol 5 mg, and intracoronary dibenamine failed to eliminate reactive hyperemia. There was, however, a decrease in the peak coronary blood flow during reactive hyperemia as compared to that obtained prior to blockade. The reactive hyperemia could, however, be restored to near control values by raising aortic pressure in the open-chest dog by aortic constriction, and in the unanesthetized dog with angiotensin. On the basis of these observations, it is concluded that diminution of sympathetic coronary artery constrictor tone does not lead to a state of coronary artery rigidity, and, therefore, it is highly unlikely that this is the mechanism responsible for angina pectoris.

4. The genetic determination of coronary artery patterns. Heredity is generally considered to be an important factor in coronary artery disease. The possibility exists that the inheritance of certain coronary patterns may make an individual more susceptible or resistant to coronary artery disease. With this possibility in mind, this study was undertaken to determine the mode of inheritance of coronary artery patterns in an animal suitable for experimental breeding, the rat. Coronary artery patterns were first observed in randomly selected male rats from three unrelated inbred strains, by means of either gross dissections or vinylite plastic injections. The coronary artery pattern on each side was found to be one of two types: 1) a single main trunk arising from a single ostium in its sinus of

Valsalva, 2) two main trunks arising from separate ostia in the respective sinus of Valsalva. The incidence of the type 2 pattern of the left and right coronary arteries was observed to be significantly different between the three strains. Thus far, after examining more than 65 litters of rats (about 700 newborn) and the parents, it appears that the coronary artery patterns are subject to polygenetic determinism. Currently, the breeding program is designed to determine more specifically the mode of genetic inheritance of the type 2 pattern, and to produce a strain with a high incidence of this pattern in order to compare the vulnerability to experimental coronary artery disease of rats with this phenotype with rats of other coronary artery patterns.

5. Factors affecting extracoronary collateral circulation. Very little is known regarding factors affecting extracoronary collateral circulation. The effects of prolonged exercise (regular swimming sessions) on the cross-sectional areas of these vessels were determined in rats. It was found that exercise sufficient to produce cardiac hypertrophy resulted in a substantial increase in the cross-sectional areas. Follow-up studies are underway to determine whether these vessels regress in size following cessation of exercise. If this is so, additional studies will be undertaken to find the minimal amount of exercise necessary to maintain the enlargement. Experimental occlusions of coronary arteries in rats were found to produce a more marked increase in cross-sectional areas of the extracoronary vessels than did exercise. To test the hypothesis that hypoxia is an important factor in the enlargement of these vessels, rats are being subjected to hypoxic hypoxia (periodic breathing of 10% oxygen), anemic hypoxia (iron deficiency state), and cellular hypoxia (chronic administration of sublethal doses of cyanide). It is also planned to test the effects on these vessels of various cardiac-stimulating drugs, catecholamine induced myocardial necrosis, and other factors. Use will be made of stimuli, which prove effective, to test the functional significance of the extracoronary collateral circulation in rats and dogs. The rats will be subjected to experimental coronary artery occlusions, and the mortality rate and size of infarcts will be compared to control animals. In dogs, it is planned to isolate the aortic segment from which these vessels originate, by means of an intra-aortic perfusion tube, making it possible to control flow and pressure in this segment. The coronary sinus drainage will be collected and measured with an electromagnetic flowmeter. It will then be possible, using a constant infusion dye dilution method, to measure the extracoronary collateral blood flow and determine its hemodynamic factors and also drugs affecting it. The ultimate test of the efficiency of this collateral circulation will be to place the entire burden of cardiac blood supply on this source.

6. Cardiac metabolism. The biochemistry section is engaged in setting up a program for the study of energy metabolism in heart sarcosomes. The long-range objective of this program is to learn more about the mechanism by which ADP is phosphorylated to ATP during the transfer of electron equivalents from substrate to molecular oxygen. These studies are being carried out in "normal" systems before beginning examination of these reactions in "abnormal" systems (i.e. tissue from

hearts subjected to stress, drugs or disease). Some of the approaches which are being used are: 1) energetics of reversed electron transfer (i.e. the reduction of pyridine nucleotide by succinate or cytochrome c, the reduction of fumarate or cytochrome b by cytochrome c and other related reactions); 2) the effect of "site-specific" inhibitors of oxidative phosphorylation on both the forward and backward electron transfer; 3) the role of the energy-linked transhydrogenase reaction in pyridine nucleotide oxidation-reduction; 4) the role of some reported "high-energy" intermediates in oxidative phosphorylation--such as the alkaline-stable NAD^+ reported by various workers in heart sarcosomes. Techniques for other types of experiments related to these studies are being developed. Many of these experiments are being carried out on subsarcosomal particles prepared by both chemical (e.g. digitonin treatment) and physical (e.g. ultrasonic disintegration) methods. In addition, other means are being investigated for obtaining subparticles which carry out some, but not all, of the functions of intact sarcosomes. Thus far, most of the work has been with sarcosomes derived from bovine heart and rat heart.

7. Other studies. A number of additional studies in the unanesthetized dog have been either completed or temporarily suspended. Studies being written-up for publication, or in press, include the splenic circulation and reflex control of the coronary circulation, and are also concerned with the effects on the systemic and coronary circulation of exercise, excitement, blood transfusion, anesthesia and hemorrhagic shock. Evaluation of methods for estimating coronary blood flow in man has been temporarily suspended.

Summary and Conclusions.

The control of regional blood flow and metabolism has been studied in the unanesthetized resting and/or active dog by means of chronically implanted electromagnetic flowmeters (our own design), and special tubes in the aorta, coronary sinus and coronary arteries for blood pressure, injection and blood sampling. Left coronary flow and myocardial oxygen usage have been obtained in the resting state. Additional experiments have extended previous findings of the effects on systemic and coronary dynamics of reactive hyperemia, heart block, exercise, excitement, coronary reflexes, blood transfusion, anesthesia and hemorrhagic shock. Preliminary experimentation is underway concerning the genetic determination of coronary artery patterns, the factors affecting the extracoronary collateral circulation, and the energy metabolism of the heart at the level of the sarcosomes under different stress states.

Publications.

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RESEARCH AND TECHNOLOGY RESUME	1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
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4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
01 07 65	A. NEW	U RPT U WRK	NA	9R	A. WORK UNIT

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61145011 3A014501B71R 02 086	NONE

11. TITLE

(U) Gastrointestinal disease (09)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
012900 Physiology; 003500 Clinical Medicine	08 63	NA	OTHER / DA

16 PROCURE. METHOD	17 CONTRACT GRANT a DATE / b NUMBER / c TYPE / d AMOUNT	18 RESOURCES EST	a PROFESSIONAL MAN YEARS	b FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	4	126
		CURRENT FY 66	4	136

19 GOV'T LAB INSTALLATION ACTIVITY	20 PERFORMING ORGANIZATION
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RESP. INDIV Rosenberger, Lt Col E.A.	INVESTIGATORS PRINCIPAL Scott, Jr. Col N.N.
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	TEL 202-576-3530 TYPE DA

21 TECHNOLOGY UTILIZATION	22 COORDINATION
Medicine	NA

23. KEYWORDS Intestine, small; diarrhea; liver extracts; intestinal secretions; enteritis; intrinsic factor; microcirculation

24. (U) Tech Objective - a. Determination of the incidence of jejunitis and malabsorption among Southeast Asia returnees. b. Investigation of cause of diarrhea due to toxins, hypertonic solutions and dietary inadequacy. c. Elucidation of the frequency of disaccharidase deficiency among asymptomatic individuals and causal relationship to diarrhea diseases. d. Characterization of electrophoretic pattern of liver cells and cell subfractions in normal and hepatic disease states. e. Elucidation of cause of
25. postgastrectomy anemia (PGA). f. Measurement of intrinsic factor.

(U) Approach - a. Clinical survey Vietnam returnees using absorptive tests, peroral intestinal biopsy, histochemical enzyme analysis and microscopic appearance of stomach and jejunal mucosa. b. Utilizing same tests to study effects of toxins, drug deficiencies and isocaloric low protein diets on gastrointestinal functions of animals. c. Disaccharidase activity measured in human gastrointestinal diseases. d. Analysis of electrophoretic patterns of liver cell subfractions in disease. e. Whole body radioiron analysis. f. Electrophoretic radioactive analysis.

26. (U) Progress (Jul 64 - Jun 65) - a. Ten percent of Vietnam returnees found with jejunitis. b. Niacin and protein deficiency produce gut lesions despite normal caloric intake. c. Disaccharidase deficiency occurs in about eight percent of asymptomatic individuals and is common in sprue, viral enteritis and viral hepatitis. d. Definite electrophoretic patterns found in liver disease-technic being applied to human studies. e. Cause of PGA is iron deficiency due to rapid intestinal transit. f. Technic being utilized clinically.
For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29 OSD CODE	30. BUDGET CODE
a. COMSEC OR COMSEC RELATED [X] b. NOT RELATED		BR	1

31 MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST FUNDS (In thousands)	36.
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A014501B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task No. 02, Internal Medicine

Work Unit 086, Gastrointestinal disease

Investigators.

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Associate: Lt Col Thomas W. Sheehy, MC
Major Dorsey T. Mahin, MC
Tatsuo Hase, M.D.
Pearl R. Anderson, PhD

Description.

Current investigations include studies dealing with:

1. Enzyme activity in normal and diarrheal states.

2. Post-gastrectomy anemia.

3. The separation by electrophoresis of soluble intracellular proteins in liver cells and intestinal epithelium.

4. Quantitative analysis of vitamin B-12 binding property of gastric juice.

5. The incidence of intestinal disease in Viet-Namese returnees (in conjunction with Surgeon, U.S. Army Special Warfare Center).

6. The vascular network of the normal gastrointestinal tract and associated organs and the effects of various drugs and toxins on the vascular integrity of these organs.

7. Technics for determining the rate of cell turnover in various bodily organs.

Progress:

I. Intestinal Enzymes--A. Histochemical Studies: Nine enzymes were studied histochemically in gastric, jejunal and rectal mucosa taken from normal adults. These enzymes were selected to reflect the oxidative, glycolytic and nucleoprotein metabolism of the gastrointestinal tract. The glycolytic and oxidative enzymes of man and the rat are located primarily in the villous epithelium and within the brush border area of these cells. Those enzymes indicative of cellular regeneration were located mainly within the crypt epithelium. Current studies indicate many of these enzymes are disturbed by toxic agents, anti mitotic drugs and certain diseases before microscopic changes become apparant.

Two manuscripts, one dealing with the histochemistry of the normal stomach and one with the normal human small intestine are in press.

B. Quantitative Enzyme Studies. Disaccharidase (i.e. the sugar-splitting enzymes-lactase, maltase and sucrase) activity was measured in specimens of gastric, duodenal and jejunal mucosa taken from 50 healthy, normal adults and 50 patients with different types of gastrointestinal diseases. Little disaccharidase activity was found in normal gastric mucosa and enzyme activity found in the duodenal mucosa was significantly less than the activity found in the jejunal mucosa. About 50 percent of normal individuals have hypolactasia but these individuals are not lactase deficient for they retain their ability to split lactose and quickly regenerate the enzyme when it is needed. A secondary deficiency of disaccharidase activity was found in most patients with tropical and non-tropical sprue, and in a significant number of patients with active ulcerative colitis, acute viral hepatitis and other enteric diseases. These undoubtedly contribute to the diarrhea associated with these diseases.

A quick semi-quantitative, chromatographic technic was set up for identification of urine sugars. A study was initiated to evaluate the relationship of fecal disaccharidase activity to sloughing of the intestinal epithelium. This approach appears promising. The contribution of sloughed cells versus enteric bacteria to fecal disaccharidase activity is now being evaluated. A significant amount of fecal disaccharidase activity appears to be contributed by the sloughed epithelium. Studies have also been undertaken to determine the normal dipeptidase activity of the small bowel mucosa and to evaluate the effects of certain intestinal lesions on these enzymes.

Two papers concerned with this topic are in press.

C. Deficiency States and the Gastrointestinal Tract. Protein depletion and severe niacin depletion caused significant changes in the histologic appearance, enzyme activity and epithelial cell turnover of the gut in certain animals. A mucosal lesion was found in the small bowel of two patients with pellagra and the lesion improved with niacin therapy. Studies of intestinal function and intestinal morphology continue in humans with deficiencies.

One paper in press.

D. Viruses: Lesions of the intestinal mucosa have been found in patients ill with infectious hepatitis, adenovirus type 4, a non-specific enteritis and rubeola. The electron-microscopic appearance of the mucosa in adenovirus infection, rubella and other viral diseases affecting the gut is under study.

II. Post-gastrectomy Anemia--The cause of iron deficiency anemia was investigated in patients with partial gastrectomy. Many patients with

a Billroth II type partial gastrectomy showed a "drop-through" phenomenon when their ability to retain orally administered radioactive iron was monitored by placing the patient in a human whole body counter (HUMCO). Many of these individuals lost from 85 to 98 percent of the tracer iron in their stools within 24 to 36 hours of its ingestion whereas control individuals retained a significant amount of radioactive iron at this time. In contrast, when patients with pancreatic disease were given the same dose of isotopic carrier iron they retained much larger amounts of iron than normal individuals. The cause of the "drop-through" phenomenon, the ability of partially gastrectomized individuals to retain hemoglobin iron and the effect of pancreatic extracts on iron absorption in pancreatic disease are now under study

One manuscript is in press.

III. Intracellular Protein Studies--A technic was devised for the separation and characterization of soluble intracellular proteins and the method has been published. This technic was also used to conduct a comparative study of the soluble protein patterns of intra and extracellular rat liver fluid. The initial results of this study suggest different protein systems participate in the maintenance of colloidal osmotic pressure across the hepatic cell membrane. These systems may serve to protect the hepatic cells against sudden quantitative changes in the protein composition of the extracellular fluid caused by conditions such as acute dehydration, hemorrhage, shock, etc. This investigation has been extended to study the hepatic cell subfractions including cell nuclei, mitochondria, microsomes and cytoplasmic sap. Preliminary data suggests the microsomal fraction may be a key site for serum protein synthesis. Similar studies of the soluble protein pattern of the intestinal mucosa have been initiated.

IV. Vitamin B-12 Binding--An attempt has been made to detect the cellular location of vitamin B-12 binding in rat gastric mucosa using tritiated vitamin B-12 and radioautographic technics. A simple, quick and inexpensive electrophoretic method was devised for quantitatively assaying the vitamin B-12 binding capacity of human and rat gastric juice. This method provides a clear-cut separation of bound and free Co^{57}B-12 added to samples of gastric juice. Preliminary observations indicate the Co^{57}B-12 binding pattern of normal gastric juice is readily distinguishable from that of patients with pernicious anemia. Histamine stimulation increases vitamin B-12 binding capacity of normal gastric juice. The study concerned with the uptake and bodily distribution of Co^{60}vitamin B-12 by normal and gastrectomized rats has been completed. Gastrectomized rats lose body radioactivity due to Co^{60}B-12 more rapidly than normal animals.

Three papers on this subject have been submitted for publication.

V. Malabsorption Problems--One hundred-eight soldiers were selected at random from members of Special Forces units that had returned from six months duty in Viet-Nam and these individuals were screened for evidence

of impaired intestinal absorption. Such evidence was found in approximately 6.5 percent of the group. A small bowel lesion was found in these individuals and it had many of the microscopic features of the sprue lesion. The incidence of malabsorption and other enteric diseases will be evaluated in returnees who have lived in Viet-Nam for one year. A compilation of data concerned with two previous surveys showed the annual incidence of tropical sprue among continental Americans residing temporarily in Puerto Rico was about 8 percent. This incidence has a tendency to rise among newcomers in relation to time in an endemic area. The mean time of diagnosis for 37 continental Americans who acquired tropical sprue in Puerto Rico was 15 months. A relationship was found between early diagnosis of tropical sprue in North Americans and their therapeutic response to folic acid.

VI. Microcirculation Studies--Silicon rubber perfusion of the arterial and venous circulation of normal animals was carried out to permit a detailed study of the microcirculation of the skin, liver, kidney, stomach and intestines. Tissue clearing with the aid of glycerine allows visualization of the blood vessels of an organ in its natural state and without the surrounding cell structures obscuring a vessel's course or connections. This technic is particularly adaptable to a study of the microcirculation of the liver, stomach, kidney and intestina in abnormal conditions. This was shown in a study in which the microcirculation and histopathologic changes were followed in rats made cirrhotic by repeated injections of a carbon tetrachloride-petroleum-ether mixture. Weekly perfusion and histologic studies showed an intimate relationship between derangement of the hepatic microcirculation and initiation of fibrous tissue proliferation and the subsequent development of hepatic arterio-venous shunts. Prolonged alcohol ingestion in adequately fed rats showed a similar effect. The effects of drugs, toxins, etc., on vascular integrity of the stomach, small bowel and kidney and the relationship of these vascular changes to cellular damage is also being studied. The mechanism of gastric ulcer formation and healing is under study.

One paper is in press.

VII. Cell Regeneration and Turnover Time--Studies of cell regeneration in man have been prevented by lack of a safe or suitable technic. Several attempts were made to devise new technics to accomplish this but these were not adaptable to man. Recently, however, a method was devised in which carbon-tagged thymidine was used for tissue analysis in a manner similar to the technic used to determine the age of archeological specimens. This involved combustion of a sample of labeled tissue to carbon dioxide, subsequent resynthesis of this gas to benzene and finally counting of the benzene's radioactivity in a liquid scintillation counter. Combustion and benzene synthesis were accelerated by use of a special instrument provided for trial by the Packard Instrument Company. The method devised makes it possible to count extremely small amounts of radioactivity. As little as 1 uuc or 2 dpm could be detected in as much as 10 grams of

carbon. A preliminary experiment was designed to test the effectiveness of the method by studying C-14 tagged glucose metabolism in samples of whole blood. After initial incubation, the red cells, the supernatant plasma and the evolved CO_2 were processed separately. Each was combusted with 10 g of anhydrous glucose which served as a carbon carrier. The activity recovered in the three separate fractions accounted for almost 100 percent of the initial radioactivity in each experiment. This method may constitute a breakthrough in adapting low level counting technics to biological investigations of man.

As a result of problems encountered in liquid scintillation counting with this method and a lack of exact data pertinent to many aspects of liquid scintillation counting, a series of studies was undertaken to determine solvent efficiency at various solute concentrations, the extent and significance of oxygen quenching, the rate of gas exchange into and out of scintillation solutions and the effect of sample temperature on counting efficiency.

Summary and Conclusions:

1. The normal histochemical pattern of the mucosa of the stomach, small intestine and rectum was determined. Now, the effects of various diseases on these patterns are being scrutinized. Disaccharidase activity in the bowel was found depleted quantitatively in generalized malabsorption syndromes and in certain acute enteric infections. Protein depletion was found to cause both histologic and enzymatic changes in the gut mucosa. Electron-microscopic studies of human intestinal mucosa taken during various enterovirus infections are underway.

2. Patients with a Billroth II type partial gastrectomy fail to retain ingested inorganic iron normally. These patients lose 85 to 98 percent of an orally ingested dose of radio iron within 24 to 36 hours. In contrast, patients with chronic pancreatitis were found to retain larger than normal amounts of ingested iron. The causes for these variations in human absorption of inorganic iron are under study.

3. The soluble proteins of liver cells i.e. nuclei, mitochondria, microsomes and cytoplasm, were characterized electrophoretically. These electrophoretic protein patterns were seriously distorted in the liver cells of animals exposed to toxic substances. If the human liver cell's protein patterns can be characterized, similarly, this technic may serve as a useful diagnostic and perhaps prognostic tool for studying liver disease.

4. Starch-agar gel electrophoretic analysis in combination with an isotopic tracer (Co^{57} B-12) was used to develop a simple means for assaying the vitamin B-12 binding capacity of human gastric juice. The vitamin B-12 binding capacity of gastric juice taken from patients with pernicious anemia is low or abnormal. This method provides a useful diag-

nostic tool for detecting loss of intrinsic factor in patients with pernicious anemia or in patients with a partial gastrectomy.

5. Tropical malabsorption was found in 6.5 per cent of a large group of returnees from Southeast Asia. The annual incidence of this disease among North Americans living temporarily in Puerto Rico was found to be 8 per cent. Usually the disease became clinically apparent in Americans after 6 to 12 months' residency in Puerto Rico. A relationship was found between early diagnosis of sprue and therapeutic response to folic acid.

6. A silicon rubber perfusion technic used in correlation with histologic study of perfused tissue proved to be a very useful tool for studying normal animal microcirculation. This method was also used to study the development and pathophysiologic changes which occurred as a result of vascular injury to organs of animals given toxic substances.

7. A benzene-synthesis method for carbon dating was developed and adapted to biological study. This appears to be a sensitive technic for biological investigation with wide potential applications. The influence of several variables upon the counting efficiency of a liquid scintillation counting system was investigated. A method was found to remove oxygen from samples, thereby increasing the counting efficiency of this type system.

Publications.

Hase, T. and Mahin, D. T.: Electrophoretic and immunoelectrophoretic characterization of soluble cellular proteins in rat liver cell sap. J. Immun. 94: 191-196, 1965.

Sheehy, T. W. and Floch, M. H.: The Small Intestine: Its Functions and Diseases. Hoeber Medical Division, Harper and Row, New York., 1964.

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The department has eleven papers in press.

RESEARCH AND TECHNOLOGY RESUME

2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
	DA 0A6453	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT / RMK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
01 07 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a CURRENT NUMBER/CODE: 61145011 3A014501B71R 02 087

10b PRIOR NUMBER CODE: None

11 TITLE: (U) Military nursing (09)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
003500 Clinical Medicine	03 58	NA	OTHER / DA

16 PROCURE METHOD	17 CONTRACT/GRANT a. NUMBER / b. TYPE / c. DATE / d. AMOUNT	18 RESOURCES EST	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	6	10
		CURRENT FY 66	6	10

19 GOV'T LAB/INSTALLATION ACTIVITY
NAME: Headquarters
ADDRESS: U.S. Army Medical Res & Dev Command
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RESP. INDIV: Rosenberger, Lt. Col. E.A.
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20 PERFORMING ORGANIZATION
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Washington, D. C. 20012
INVESTIGATORS PRINCIPAL ASSOCIATE: Verhonick, Lt. Col. P.J.
Ginsberg, Maj. M.K., McCarthy, Maj. R.
TEL: 202-576-2191 TYPE: DA

21 TECHNOLOGY UTILIZATION: Nursing Practice, Nursing Education

22 COORDINATION: NA

23 KEYWORDS: Military nursing; decubitus ulcer; isolator; asepsis; monitoring, blood pressure determinations; decision-making; symptoms; prevention; health survey

24 (U) Tech Objective - To develop rationale underlying military nursing practice by identifying & testing principles of nursing in military setting; identify nursing role & functions in continuity of care to military patient & family; develop & improve standards of nrsg care; gain information regarding nrsg judgments, observations and actions in terms of symptoms; & develop new concepts of nrsg care.

25 (U) Approach - Compare effectiveness of nrsg measures for decubitus ulcer care; identify nursing's role in continuity of care thru analysis of questionnaire survey findings & test hypothesis in pt care w/ a view toward nursing's role in prevention of illness; develop theoretical framewk for nrsg decision - making based on survey of nurse observat. & actions; test acuity of observat. of cardinal signs with automatic patient monitoring device; survey health status of Army females; continue testing asepsis & description of nrsg care & pt response during confinement in plastic isolator. Factors tending to decelerate work: physical separation of Dept. staff & inadequate lab & clinical facilities

26 (U) Progress (Jul 64 - Jun 65) - Collection & analysis data continue for decubitus ulcer care & continuity; observer-error of B/P determinations found contrary to published reports & standardization of procedure nullifies observer-error; collaborative health status survey of Army females w/historical review; plastic isolator tested in clinical setting for asepsis & feasibility; survey nrsg judgments completed at 2 Natl Nrsg. Conventions.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY	28	29 OSD CODE	30 BUDGET CODE
a. COMSEC OR COMSEC RELATED [X] b. NOT RELATED		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) CY+1	36

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A014501B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 087, Military Nursing

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Description.

Research conducted by the Department of Nursing is designed to systematically examine the practice of nursing. Nursing investigations are directed toward assessing the need for redefining the concept of Military Nursing and ultimately to revise and improve practice within the mission of the Army Medical Service. A series of nursing investigations aiming to document the rationale of military nursing practice are being carried on through description, identification, and testing of principles underlying nursing care. Testing and description of various measures of nursing care of patients with decubitus ulcers is on-going. The identification of nursing's contribution toward continuity of care of military patients is being continued. An extensive survey has been completed to describe the responses of nurses with varying educational and experiential backgrounds to a series of filmed sequences depicting common patient situations. The nursing care of patients within isolator systems, designed to ascertain the feasibility of using a closed system for care of patients requiring isolation; to describe the actual nursing care of patients confined within the enclosure; and to test, modify and adapt the equipment as indicated has been completed. An evaluation of the reproducibility of indirect blood pressure measurements using a standardized procedure with multiple observers is being continued. Recommendations for the development of three items of equipment for use in the field have been made. A design for a folding field hospital bed constructed of tubular anodized aluminum was submitted for manufacture and testing. Innovations in field clothing for female personnel and design of a molded plastic external urinary catheter for application by female personnel, facilitating use of the latrine in the field have been completed.

Progress.

a. Decubitus Ulcer Care - Nursing measures for the care of patients with decubitus ulcers and pressure areas is being

continued. The limitation of available clinical facilities precludes accumulation of extensive patient data. Applications of various measures for a small number of patients on those wards attached to the Walter Reed Army Institute of Research have been tested for effectiveness in rate of healing, comfort to the patient and simplicity of use. The ultimate aim of this research is to isolate the principles of physiology and pathology in order to furnish the professional nurse with guides upon which to base a nursing judgment in the care of patients with decubitus ulcers and pressure areas. Two guides for decubitus ulcer care have been developed to date.

b. Continuity of Nursing Care - Analysis of the survey of Army Health Nurses, nurse administrators, and hospital care nurses is continuing. Perceptions relating to concepts of continuity of care in the Army and continuity of nursing care generally are being tabulated and compared. Appraisal of the functions of nursing related to patient care in the hospital, the clinic, and the home is being made. The analysis of questionnaire responses reveals differences in perceptions of continuity of care but all respondents focus attention on concern for excellence of patient care. The need for transmitting information concerning the nursing needs of patients, as well as information on the medical plan of care, is emphasized as the patient is discharged from the hospital to return clinic visits or home care. Comparisons between the three groups of nurse respondents are being made and will eventually be tested in the patient setting.

c. Nursing Care of Patients Confined Within Isolator Systems - A closed system of isolation has been explored by members of the Department of Nursing. The particular model which was tested in the past year is composed of three main parts: a clear plastic tent-like inclosure enveloping a motorized hospital bed; a filtration ventilation unit; and an ultraviolet equipped dual-chambered pass-through lock. The aereation unit and the lock are housed in a console which serves as the footboard of the bed. The tent is sealed to the console and to the headboard. A pair of arm-length gauntlets, built in each side of the plastic tent may be used to provide direct care to the patient. An attendant, with arms in gauntlets, may move from the head to the foot of the bed because of the accordion-like construction of

the tent inclosure. The unit, with its necessary modifications of care, has been called the "RES-System" which stands for "Regulated Environment for Safety-System." The acronym, implying rest, was deliberately chosen for its psychological effect.

In the past year, it has been shown that this closed system of isolation is superior for use for reverse isolation (protecting the patient from his environment) but has no capability for conventional isolation (protecting the environment from a patient with a contagious disease.) A procedure manual has been prepared which will assist physicians and nurses in providing care for patients within the system. A movie has been made which pictorially describes basic nursing procedures. Extensive bacteriological tests have been made and are being reported in the open literature. Two clinical trials have been completed which showed the effectiveness of the procedure manual and the possibility of caring for patients.

It is recommended that extensive use be made of this equipment by physicians in their treatment of patients in order to ascertain its wider feasibility for medical therapy.

d. Nursing Judgment Study - A technical exhibit, entitled "Nursing Research" was designed with data collecting devices to conduct an extensive survey. The exhibit was shown at a National nursing convention to obtain the opinions of convention attendees to filmed sequences of patient situations. Thirteen hundred nurses participated in the survey by citing their observations of a randomly selected filmed patient situation and listed what nursing actions they would take to correct the situation. Analysis of the responses of the 1300 nurses responses has begun by developing a code to categorize comments and seek patterns of similarity or difference in response between various types of nurses. The analysis of responses to one sequence has been completed.

e. Reproducibility of Blood Pressure Measurements - A pilot phase using six observers and six subjects has been accomplished. Because a trend in reduction of observer error was suggested, the use of 36 different observers recording blood pressure readings has been completed to date. The total number of observers to be used in the sample will be 60. The findings to date show that when control and standardization of the procedural steps used in taking blood pressure are exercised, there is a reduction in observer error, regardless of the experience, preparation, or skill level of the observer.

The significant factor in the reduction of observer error appears to be controlling the rate of deflation of the blood pressure cuff. The evidence also suggests that individuals with a hearing loss of 20% or greater, create an 18% error in diastolic readings.

f. Development of Equipment - The suggested model for an Army field hospital bed to be made of anodized tubular aluminum has been considered in designing a field bed for use in the "MUST." Modifications of the original design submitted by the Department of Nursing were made prior to evaluation at the Medical Equipment Research and Development Laboratory.

As a result of concern expressed by the Research Assistant, Obstetrical-Gynecological Service, Walter Reed General Hospital, for inconvenience of going to the latrine in the field by female personnel, two developmental projects were undertaken. A pair of female fatigue trousers have been modified by placing the standard zipper in the crotch seam to facilitate going to the latrine in the field. The zipper is sewn in the crotch seam in such a way as to be invisible when closed and makes an almond-shaped passage when opened.

An external urinary catheter was made from a heat moldable plastic which becomes rigid when cooled. The catheter, designed for application to the meatus, has a spout for easy flow of urine.

Preliminary trials showed both items to be effective in solving the problem of inconvenience of going to the latrine in the field by female personnel. Both the modified fatigue trousers and the plastic catheter have been given to the Research Assistant, Obstetrical-Gynecological Service for more extensive trial and evaluation.

Summary and Conclusions

The five researches being conducted by the investigators in the Department of Nursing relate directly or indirectly to the improvement of patient care. Four on-going projects relating to decubitus ulcer care, continuity of nursing care in the Army Medical Service, a survey of nursing observations and actions, the reproducibility of blood pressure measurements have been described. The final testing phase of nursing care of patients within isolator systems has been completed after a three-year period. Recommendations for prescription of RES-System care by physicians for patients requiring reverse

isolation are made. Suggestions for trial and evaluation of three equipment items designed and developed for field use have been submitted. Six publications have appeared in the professional literature during the report period.

Publications.

Ginsberg, Miriam K. and La Conte, Maria L. "Reverse Isolation." The American Journal of Nursing, 64: 88-90, September 1964.

McCarthy, Rosemary T. "Vomiting." Nursing Forum, 3: 48-59 No. 1, 1964.

Verhonick, Phyllis J. "Natural Science Basis for Nursing Research, Education and Practice," in The Continuing Search for Meaning. American Nurses Association, June 1964, 15-18.

Lewis, Betty J. and Gunn, Ira P. "Tracheostomy, O_2 Administration, and Expiratory Air Flow Resistance." Nursing Research, 13: 301-308, Fall 1964.

Ginsberg, Miriam K. and Yoder, Ann E. "The Effectiveness of Some Traditional Methods in Oral Hygiene Nursing Care." Journal of Periodontology, 35: 513-518, November-December, 1964.

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PROJECT 3A014501B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 03
Psychiatry

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6454	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 65	A. NEW	U RPT U WRK	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61145011 3A014501B71R 03 025

10b. PRIOR NUMBER CODE: None

11. TITLE: (U) Analysis of behavior and of mediating mechanisms: Experimental psychological factors (09)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT COMPL DATE	15. FUNDING AGENCY
013400 Psychology (indiv. & group behavior)	08 63	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	9	170
		CURRENT FY 66	9	185

19. GOV'T LAB/INSTALLATION/ACTIVITY
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TEL: 202-576-2483 TYPE: DA

21. TECHNOLOGY UTILIZATION: NA

22. COORDINATION: NA

23. KEYWORDS: Animal behavior; avoidance; conditioning; motivation; operant behavior; performance decrement; reinforcement learning; stress; time perception

24. (U) Tech Objective - 1) To develop and analyze complex behavioral repertoires and the study of basic variables affecting behavior. 2) To develop behavioral methods for producing stress and fatigue. 3) To investigate the effects of stress-inducing procedures on complex behavioral repertoires.

25. (U) Approach - The approach emphasizes the application of modern behavioral technology and the techniques of the experimental analysis of operant behavior.

26. (U) Progress (Jul 64 - Jun 65) - A number of complex repertoires have been studied. Timing behavior, repeated acquisition of new behavioral chains and of new stimulus discriminations, adjusting avoidance behavior, vigilance, matching-to-sample behavior, and basic social interactions among primates have received experimental study. The production of stress and fatigue has focused upon prolonged sessions of avoidance conditioning where the animal must work continuously for extended periods of time in order to avoid an electric shock. The effects have been analyzed both in terms of decrement in behavioral performance and in terms of changes in patterns of hormonal secretions. Other stress procedures under investigation are the conditioned emotional response technique and the direct punishment of behavior. Behavioral effects of stress have been evaluated in terms of timing behavior, disruption of learned performances, lowering of rate of behavioral output, and interference with ability to acquire new discriminations and new behavior.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSI CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A014501B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 03, Psychiatry

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

Investigators.
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Associate: Bernard Beer, M.S.; Lt Col Joseph V. Brady, MSC; Joseph F. Dardano, Ph.D.; William Hodos, Ph.D.; 1st Lt Solomon S. Steiner, MSC; Gerald Tolliver, B.A.

Description.

The program can be described under two classes of projects: 1) The experimental analysis of basic behavioral variables and the development of complex behavioral repertoires. 2) Stress-inducing procedures and their effects on complex behavioral repertoires. The first class of projects includes studies on the repeated acquisition of new behavioral chains, timing behavior as affected by amount of reinforcement, and a series of social interaction experiments dealing with the inter-reinforcement process, competition, and social behaviors among chimpanzees. The second class of projects is concerned not only with the control and disruption of complex behavior by aversive stress techniques but also with the physiological and endocrinological consequences of such stresses. The experimental work includes studies of extended avoidance sessions, prolonged vigilance, punishment of responding, psychotomimetic drugs, and the disruption of complex repertoires of matching behavior and of discrimination acquisition by aversive stress.

Progress.

I. The Experimental Analysis of Basic Behavioral Variables and the Development of Complex Behavioral Repertoires

A procedure has been developed for studying the repeated relearning of new behavioral repertoires. Monkeys were required to learn repeatedly a new four-member behavioral chain in a chamber containing four groups of three levers. During each daily session the monkey had to acquire a new four response chain by pressing in sequence the correct lever from each group. After a stable pattern of relearning was established, the number of incorrect responses declined to a steady state. Then this steady state served as the dependent variable to study the two major sources of incorrect responses in this procedure. One source has been identified as error responses which have become superstitiously chained with the correct responses. For example, if the reinforced (correct) sequence of levers was 1-4-8-12, the monkey might repeatedly press the sequence 2-1-4-8-12--an unnecessarily long sequence but one

which is reinforced. It was found that larger work requirements (larger fixed ratios) reduced the probability of superstitious chains. Even more effective in reducing superstitious chaining was a time-out stimulus (a stimulus marking a period of non-reinforcement) following errors. When an equivalent time period (no stimulus) was substituted for the time-out stimulus, the errors due to superstitious chaining were not reduced. Thus, the stimulus was necessary to coerce immediate cessation of responding and to provide not only a mild punishment for errors but also to insure that a delay between error responses and a reinforcement systematically occurred. The second major source of errors in this situation is the initial learning period at the beginning of each session. Without special procedures, the monkeys must learn the unfamiliar response sequence by trial and error. A stimulus fading procedure has been devised to guide the monkey over this trial and error period. At the beginning of the session, a light clearly indicated the correct lever. Then, as the session proceeded, the light was gradually faded out so that the monkey could not depend on it for a cue and was required to discriminate only the correct levers. The early results indicate that stimulus fading was only moderately effective in reducing overall errors. As long as the stimulus was clear, the monkey responded appropriately. However, when the stimulus was faded so that it was non-discriminable, the monkey was likely to make errors at this point. He apparently had learned only to press the correct levers indicated by the stimulus, but had not thereby learned the correct levers themselves. Whether this result is general or whether other variables can modify it, is a subject for further investigation.

Timing behavior is often studied in a free operant situation by making reinforcement contingent on the temporal spacing of responses. This schedule of differential reinforcement of low response rates (DRL) characteristically generates an interresponse time (IRT) distribution in which the modal IRT's approximate the minimum time required for reinforcement. The present study investigated the effects of various sizes of reinforcement on DRL behavior. One, two, or four food pellets were delivered when a rat spaced its responses longer than 18 sec. The subject was required first to press Key A, then after 18 sec. or longer to press Key B. "Efficiency" ratios (reinforced A-B responses/ total A-B responses) were computed. For all animals there existed a negative linear relationship between size of reinforcement and "efficiency" ratio. Modal IRT's for the largest reinforcement peaked earlier while IRT's for the smallest reward were longest. Measures of variability indicated greater variance for the smallest reinforcement and lowest variance for the larger reinforcement size.

A technique was explored for studying a basic social interaction--the inter-reinforcement process. Two pairs of monkeys served as subjects in adjoining but separate lever-pressing chambers. After extensive pretraining, monkey A of each pair pressed his lever to feed monkey B a food pellet, and monkey B pressed to feed monkey A. One problem of this study was to determine if the social interaction

could be maintained. When alternation of reinforcement was coerced, the monkeys worked rapidly and provided a daily food ration for each other reliably. However, with a free responding procedure which imposed no restrictions, the social behavior deteriorated to very low levels; the final consequence would have been starvation had the experimenter not intervened. The social interaction was then reinstated by retraining with the alternation-of-reinforcement procedure. When the free responding was again permitted, one monkey usually came to do most of the work while the other monkey came to do most of the eating. This condition remained stable for long periods of time. The variables maintaining this apparent "altruistic" behavior by the worker monkey are under investigation.

The development and study of complex and social behaviors in the chimpanzee have been realized in an experimental situation that utilizes three chimpanzees in a laboratory colony environment. These animals are required to work on a spaced responding (DRL) schedule ranging 15 seconds to 25 minutes for their daily diets in individual work chambers. Under certain conditions the animals are allowed to leave their work chambers to have access to a play area. Under other conditions the animals can work to obtain access not only to the play area but also to direct interaction with another chimp. A comparison of the two conditions can be made in terms of the frequency of social interaction, the work requirements the animals will withstand to obtain the social condition versus the non-social play condition, and the schedule requirements and response efficiency in obtaining their food reinforcements. The establishment of the latter as a behavioral baseline has resulted in some interesting findings. The use of DRL 15 seconds FR 100, DRL 3 minutes FR 100, and DRL 15 minutes FR 100 has led to the manipulation of variables which appear to play a critical role in the animal's timing efficiency. Upon changing the DRL requirements from 15 seconds to 15 minutes the animal's work efficiency drops below 20% and remains there for more than a month. Various attempts to increase this efficiency met with failure. Increasing the FR in steps of 200, 800, and finally to 1600, have resulted in dramatic increases in efficiency. We are now in the process of lowering the FR requirement to find out if the behavior is reversible or if the manipulations have led to permanent changes in the animal's performances.

A social situation is being studied where rats were trained to compete with each other for food on a fixed ratio (FR) schedule. Two rats placed side by side in separate plastic chambers are required to press a lever twenty times (FR 20) in the presence of auditory stimuli in order to obtain a food pellet. The animal that finishes his FR requirement first receives the food and terminates the auditory signal. Only one rat receives reinforcement on each trial. The FR requirement is made one increment smaller for the unreinforced rat on the following trial. The FR is thus changed on each trial to handicap the animal receiving reinforcement. A titration point is soon reached

whereby each rat is reinforced on alternate trials. This point remains reliably constant from session to session. The titration point seems to be sensitive to motivational changes such as food deprivation level. Other motivational variables, primarily involving stress and fatigue, will be superimposed and tested on this baseline of social behavior.

II. Stress-inducing Procedures and the Effects on Complex Behavioral Repertoires

Once effective procedures for training complex behaviors have been developed and after an understanding of the behaviors has been achieved through basic studies, the behavioral repertoires can be used as baselines for assessing the effects of stress-producing procedures. This section of the report will deal with various methodologies for producing stress and with the behavioral and physiological consequences of such stress.

A basic stress-producing technique has been used for a number of years in this laboratory and has been found to be consistently useful in the production of certain physiological changes. This technique is a non-discriminated avoidance situation in which the monkeys must make at least one lever response every twenty seconds to avoid a mildly painful foot shock. Animals exposed to this requirement for prolonged periods of time, or repeated two-hour weekly sessions, demonstrate substantial endocrinological changes during and after such sessions. The behavior is characterized by an increasing rate of lever responding which levels off and then declines slightly, with a final rate of about 33 responses per minute. The shock frequency quickly reaches a low value of less than one shock per hour and remains at this rate throughout the following sessions. An initial finding of interest suggests that a 24-hour avoidance session is as effective as a 72-hour session in terms of its physiological consequences. That is, it produces similar increases in the output of adrenal and thyroid hormones, as well as that of insulin.

In addition to this basic procedure, two recently developed techniques are being employed to study in more detail behavioral changes occurring concomitantly with physiological alterations. The first is a procedure which requires a dark-adapted rhesus monkey to be vigilant to the unpredictable presentation of a very dim stimulus light. When the light is presented, the animal has twenty seconds in which to make five lever responses. If the animal should fail to make the required number of responses in the limited amount of time or if the animal should respond when the light is off, the animal receives a mildly painful foot shock. Using this as the basic procedure, the intensity of the stimulus light is then made to adjust automatically with the efficiency of the animal's performance. When the animal responds successfully to the stimulus, the next presentation will be one step lower in intensity. After a number of successful trials the stimulus intensity will approach the animal's

absolute threshold, at which point the animal will fail to respond to the light. This failure will not only produce a foot shock but will cause the next stimulus presentation to be one step brighter. The major dependent variable then becomes a measure of the animal's threshold, while the independent variable of current interest is the interstimulus interval. We have found the threshold to be dependent upon this interstimulus interval. Average intervals of 3, 5, and 10 minutes have produced reliable changes in the threshold values up to as many as four intensity steps. With longer intervals the monkey is unable to respond to the lower intensities. In addition, it has been found that exposure to this procedure continuously for 42 hours produces little if any change in the threshold values. This surprising finding has been replicated on two occasions.

The second procedure requires a monkey to perform a memory problem typically called "match-to-sample." The animal must respond to a sample stimulus and then remember it through a delay time, which is interposed between the sample stimulus and the presentation of the match stimuli. A correct response is defined as a response to the match stimulus identical to the sample. A correct response increases the delay period of the next problem by 10 seconds, while a failure to match the sample stimulus decreases the delay of the next problem by 10 seconds. The reinforcement for a correct response is food and water. The apparatus is so arranged that the monkey can perform this task with his nose, while being able to operate a hand lever to avoid foot shocks. With this procedure we are able to study the interaction between these two requirements both behaviorally and physiologically. Initial observations have indicated that a monkey can in fact perform both the matching-to-sample and Sidman avoidance concurrently. We have seen little if any change in the former during and following sessions lasting up to 24 hours.

The experimental analysis of behavioral stress of the conditioned "fear" or "anxiety" type in relationship to psychophysiological changes has been undertaken in a series of experiments with rhesus monkeys. The initial studies in this series were concerned with a systematic replication of the previously reported attenuating effect of a series of electroconvulsive shocks (ECS) upon such a conditioned emotional response (CER) in laboratory rats. Results obtained thus far have replicated this basic effect of ECS upon the CER with the monkey and have confirmed the transient nature of this effect. Subsequent studies, however, have been less successful in confirming the protective effect of "prophylactic" ECS treatments in preventing the reappearance of the CER 20 days after completion of an intensive ECS treatment series. Similarly, these studies with the rhesus monkey have provided only a partial replication of the previously reported reduction in the effectiveness of delayed ECS in attenuating the CER. Significantly, however, it has been possible to record cardiovascular changes associated with the development and maintenance of the CER and ECS treatments and to demonstrate that the reductions in heart rate and blood pressure

which accompany the behavioral changes observed during conditioning of the "fear" response can be markedly attenuated as a result of the ECS treatments.

Punishment of behavior by aversive stimuli is another stress-producing procedure which has received experimental attention. One investigation analyzed the effects of punishment on fixed-ratio behavior. The purpose was to determine whether different parts of the fixed ratio (FR) performance are differentially sensitive to punishment. After a stable FR 60 performance was established for several pigeons, one of three punishment contingencies was introduced: punishment of the first 20 responses of the ratio, or the middle 20, or the final 20. The punishing stimulus was electric shock administered through permanently implanted electrodes. Several intensities of shock were employed. Higher intensities were introduced after a stable performance developed under the preceding lower shock intensities. It appears that the effect of punishment on FR performance depends on the locus of the punishment. That is, a given intensity of shock applied to the first third of the ratio produced a greater response suppression than shock applied to the last third of the ratio. In addition, responding prior to the punished responses were also markedly suppressed. The basis of this differential effect seems to lie in the chained nature of responses comprising fixed ratio performance and the acquisition of a conditioned aversive property by non-shocked responses which precede the shocked responses.

The adjusting avoidance procedure, a technique previously used for inducing stress and fatigue, has received further study. With this procedure each avoidance response accumulates a constant amount of shock-free time, and stimuli indicate the amount of shock-free time remaining. The effect of shock intensity upon the behavioral performance was studied. As the intensity was reduced, the animal subjects permitted the shock to approach more closely and normally maintained a smaller amount of shock-free time. In another study the distribution of shock-free time was modified by shocking the animal both for pausing too long and for responding too much. The animals adjusted appropriately to the contingencies by responding at intermediate rates and by maintaining intermediate amounts of shock-free time.

The paced avoidance procedure was used as a technique for simultaneously producing stress and for analyzing the effects upon timing behavior. This procedure required that the monkey respond during a brief time-interval in order to avoid an electric shock. If he responded too soon, he was shocked, or if he waited too long he was shocked. Thus, a fine temporal discrimination was required. Stress was imposed upon the monkey by requiring him to maintain his performance over a five-day period. The performance on the temporal discrimination was accurately maintained over three days, but after four days the performance gradually deteriorated. The effect of the fatigue

stress was to increase the number of shocks received and to decrease the accuracy of the temporal discrimination.

An evaluation of the behavioral effects of the psychotomimetic drug Ditran (JB 329) has been undertaken. Rats were trained in the presence of one visual stimulus to avoid foot shock, and in the presence of another visual stimulus to work for a water reward. The water-rewarded trials and the avoidance trials were separated by five minutes of time out (TO). After administration of the drug the water-rewarded responses declined markedly while avoidance responses remained unchanged. The TO responses were dramatically increased. Separate measures of general activity showed striking elevations.

The following study demonstrated the disruption of a complex behavioral repertoire (the repeated acquisition of new stimulus discriminations) by an emotionalizing punishment. Monkeys were trained to press a center lever in order to produce and to change stimuli, such as a triangle, a circle, etc. On a given session, one of these stimuli sets the occasion for reinforcement; that is, when this stimulus is present, a response on a second lever produces a food reinforcement. The monkey's task, then, was to find the correct stimulus and to indicate it by pressing the food lever. Since the correct stimulus is different for every session, the monkey is required to develope a flexible, learning-how-to-learn repertoire. A set of experiments investigated the effects on this repertoire of two ways of punishing incorrect discriminations. In one case, a response on the food lever to an incorrect stimulus was mildly punished by a brief time out followed by a large work requirement (fixed ratio) to reinstate the next stimulus. Even though the size of the work requirement was made very large, the behavioral performance was not appreciably changed. However, when the incorrect responses were followed by a time out and in turn by an aversive electric shock, the performance was adversely affected. The monkeys responded on the center lever to produce hundreds of stimuli before pressing the food lever. When they did press the food lever, an incorrect stimulus was often present. In other words, the monkeys repeatedly viewed the alternative stimuli and could not come to a decision; and when they did make the decision, it was likely to be wrong. Thus, in contrast to the mild consequences of a large work requirement, punishment by strong electric shock had a disruptive effect on the discriminative performance and strengthened inappropriate behavior.

Summary and Conclusions.

The progress reported above may be summarized under the two aspects of the program.

1) The experimental analysis of basic behavioral variables and the development of complex behavioral repertoires. The complex behaviors investigated in this report period include the repeated acquisition of new behavioral chains, timing behavior, and complex social behavior in

primates. These studies have not only furnished basic scientific information but they have also provided the groundwork for future research on the disruption and deterioration of complex performances by stress.

2) The development of stress-producing methods and the study of how stress affects physiological systems and complex behavioral performances. To broaden the generality of the findings, several stress producing methods have been studied. These methods include extended avoidance sessions, emotional conditioning procedures, prolonged vigilance, psychotomimetic drugs, and the punishment of behavior. The consequences of such stress-producing procedures have been assessed by complex behavioral repertoires previously developed in the other section of the program. These repertoires include ongoing performance on schedules of reinforcement, discrimination of threshold stimuli, matching-to-sample behavior, timing behavior, avoidance, and reversal of stimulus discriminations.

Publications.

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RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA OA6455	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT	6 SECURITY WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
01 07 65	A. NEW	U	U	NA	QR	A. WORK UNIT

10a CURRENT NUMBER/CODE: 61145011 3A014501B71R 03 026

10b PRIOR NUMBER CODE: None

11 TITLE: (U) Analysis of behavior and of mediating mechanisms: Psychophysical and electrophysiological data correlation (09:21)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15. FUNDING AGENCY
012900 Physiology; 013400 Psychology (individual and group behavior)	08 63	NA	OTHER, DA

16 PROCURE METHOD	17 CONTRACT GRANT a. NUMBER	b. TYPE	c. DATE	d. AMOUNT	18 RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE		NA			PRIOR FY 65	6	120
					CURRENT FY 66	6	130

19 GOV'T LAB/INSTALLATION/ACTIVITY
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Biersdorf, W.R., Ph.D.
TEL: 202-576-5260 TYPE: DA

21 TECHNOLOGY UTILIZATION: NA

22 COORDINATION: NA

23 KEYWORDS: Sensation; vision; visual sensitivity; visual discrimination; neurophysiology; electrophysiology; electroretinography; electroencephalography; physiological correlates

24. (U) Tech Objective - This research is concerned with correlations between sensory physiology and sensory psychology. Particular attention is being paid to physiological mechanisms which mediate color vision, and dark adaptation, to the effects of attention upon response sensitivity, and to relations between retinal structure and response sensitivity. Results provide basic information regarding the functioning of the visual system and provide new techniques for recognizing various visual and other sensory disorders.

25. (U) Approach - Psychophysical procedures and a variety of electrophysiological procedures are used in this work. These include microelectrode and gross electrode recording electroencephalography, electroretinography, and the applications of computers to data analysis.

26. (U) Progress (July 64 - June 65) - Investigation of dark adaptation as a test of flash wave test length has been completed. Stimulus factors which control the transmission of impulses to the visual cortex have been and continue to be investigated. Changes which occur in the sensory systems during their early development have been investigated. Optical factors which determine the quality and nature of tne retinal image have been studied.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY a. COMSEC OR COMSEC RELATED		NOT RELATED	28	29 OSD CODE	30 BUDGET CODE
	X			BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST. FUNDS (In thousands) CY+1	36

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Project 3A014501B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 03, Psychiatry

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

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Description: The main purpose of this work unit is to investigate relations between stimuli, concomitant neurophysical functioning and response behavior. The principal methods used are those of psychophysics and behavior, electrical physiology, and physiological optics. Principal concern is with the visual system. Emphasis is placed upon sensory discrimination, sensory coding, information processing, central integrating networks, and development. The work is divided into three categories: 1) studies of sensory and receptor mechanisms, 2) relations between peripheral and central processes, and 3) correlative psychophysical and behavioral studies.

Progress:

I. Studies of Sensory and Receptor Mechanisms:

1) Temperature effects on spectral sensitivity: The intent of this experiment is to measure the activation energy of rhodopsin in situ in order to estimate the contribution of receptors to the noise level of the visual system. The method is to measure the spectral sensitivity of the electroretinogram as a function of the temperature of the retina. The spectral sensitivity of the electroretinogram is taken to be that of the visual pigment. Effort during the past year has been directed towards obtaining a suitable preparation. The frog eye with its anterior segment and lens removed has proven to be a good one, giving responses of normal waveform and stable sensitivity for as long as 7½ hours. 2) The effects of an artificial pupil and changes in accommodation on retinal image size: The state of accommodation and the target distance influence the size of the retinal image. In this experiment a haploscope was used to present a test and a comparison target at different distances from the two eyes. The subject was required to match their size. The subject viewed the test target through a fine artificial pupil with one of his eyes. The comparison target was viewed directly with the other eye. A coincidence optometer measured the accommodative level of the eyes. Quantitative data were obtained relating difference in image size between the two eyes to their state of accommodation. When the subject was accommodated closer than the target distance, the artificial pupil reduced the retinal image size. However, when the subject was

accommodated to a further distance than the target distance, the retinal image size was enlarged. The magnitude of the change depended partly on the distance of the artificial pupil from the eye, and amounted to 25% or more. Control runs indicated that the effect was not due to convergence, pupil size, or choice of reference point at the eye. The quantitative data provide a means of calculating the optical constants of the eye during accommodation. 3) Spectral sensitivity systems of primitive eyes: Basic mechanisms of vision were studied in both the compound lateral eye and the median ocelli of Limulus polyphemus. The lateral eye apparently contains a single spectral mechanism while the median ocellus contains two spectral mechanisms. These characteristics afford the opportunity to study fundamental mechanisms of their vision in some detail. The electroretinograms of both eyes were recorded using stimuli whose energy and wavelength were systematically varied. Amplitude vs energy functions were measured at a variety of wavelengths spaced throughout the spectrum. Finally, the spectral sensitivities of the two kinds of eye were determined from these functions. The data showed that the sensitivity of the lateral eye has a single maximum in the green part of the spectrum which agrees quantitatively with a photopigment absorption curve obtained by Hubbard and Wald (1959) when a correction for corneal optical density is made. The data also agrees with the average spectral sensitivity curve obtained from optic fibers recordings by Graham and Hartline (1935) and with ERG data by Wald and Krainin (1963). The ERG spectral sensitivity curve could not be altered in shape by chromatic adaptation at 400 mu and at 600 mu. The spectral sensitivity curve of the ocellus showed two maxima, one in the green part of the spectrum (similar to the lateral eye) and the other in the near ultra-violet. Similar curves were obtained from excised ocelli, thus indicating that the ultra-violet sensitivity was not dependent on the presence of the cornea. The independence of the two spectral maxima was studied by chromatic adaptation. Long wavelength adaptation depressed longwave length sensitivity. Possibility of a dual response mechanism as well as that of a dual photopigment mechanism is being tested by analyzing the features of the response waveform to see how they change with intensity at different wavelengths. 4) Spectral sensitivity of the human electroretinogram during prolonged dark adaptation: An experiment has been completed on the changes in sensitivity of the human electroretinogram during the first 30 minutes of dark adaptation following an intense white pre-exposure. The shapes of the curves relating electroretinal sensitivity and electroretinal peak latency to time in the dark both indicate breaks or discontinuities similar to the 'rod-cone break' occurring in psychophysical dark adaptation. These breaks indicate transitions from x-wave function to b-wave function in the case of the positive waves. The spectral sensitivity changes from a dual peaked curve representing a scotopic plus red process functioning before the break to an increasingly pure scotopic functioning after the break. The form of the dark adaptation curve depends upon the color of the test flash with which it is measured. The results provide evidence against a simple stray light interpretation for the red shoulder seen in b-wave spectral sensitivity curves and point to the participation of cones prior to the break in the dark adaptation function. 5) Measurement of incremental thresholds with the human electroretinogram: Work is

continuing on the derivation of incremental thresholds for the human electroretinogram by the criterional technique. Graded levels of light adaptation proceeding upwards from dark adaptation are being used. An experiment has been completed in which both test and adaptation fields were white and subtended 52° of visual angle. One striking feature of the results was a plateau of sensitivity which extended 3 log units above the absolute visual threshold. The luminance of the adaptation stimulus did not begin to affect the sensitivity of the b-wave until it was 3 log units above the absolute visual threshold. The b-wave followed Weber's law for adaptation luminances above the plateau. Each addition of a log unit of adaptation luminance lowered the sensitivity of the b-wave by one log unit. Work is proceeding on a further experiment using full-field adaptation to see whether the plateau is caused by stray light. Preliminary results indicate that full-field adaptation does not eliminate the plateau. An even longer plateau occurs for the a-wave, indicating it is even less affected by light adaptation than the b-wave. 6) Development of the electroretinogram in the neonate: An investigation has been conducted to determine whether an electroretinogram can be detected in newborn infants using computer techniques of data analysis. Satisfactory routine methods for recording from babies have not been available previously. Thus, a new type contact lens electrode and a speculum have been developed for recording electroretinogram from unanesthetized, sleeping subjects. With this device a small electroretinogram can be obtained from babies. It has a much smaller amplitude and a somewhat longer latency than that produced by adults under similar recording conditions. In other respects the response components of babies are the same as those of adults. The results suggest that infants possess rudimentary photopic and scotopic sensitivity. The electroretinogram of the infant is now being compared with its evoked occipital responses.

II. Recording directly comparing central and peripheral processes:

1) Development of instrumentation: The Packard Bell general purpose computer continued to be used for the analysis of on-line data from many of the experiments of this work unit. Because of scheduling problems associated with an increasing workload, however, much of the data now is stored on magnetic tape and played into the computer later as time becomes available. Punched tape is also used for this purpose. The computer is not generally available for on-line programming of experimental procedures. Instead, it is used to prepare punched tapes which actually do control experimental sequences. A tape reader, directly connected to the apparatus for several different experiments, permits the administration of designs which have been worked out by the computer in advance. Thus, limited computer control is possible, and scheduling problems are reduced. 2) Effects of the retinal location of the stimulus upon the sensitivity of the human electroretinogram and occipital response: Experiments in which the spacial sensitivity of the visual system is mapped by spotting stimuli at different locations on the retina at selected distances from the fovea have continued. The sensitivities of simultaneous psychophysical measures, retinal responses and

occipital responses have been measured by a method involving moving stimuli. This method reduces errors introduced by stray light and scatter within the eye. Responses triggered by the foveal cones can now be detected using white stimuli. Previously, this could be accomplished only with orange stimulation. The occipital response is much more sensitive to the position of the stimulus on the retina than is the electroretinogram. This result may be due to the manner in which the optic radiations project to the cortex in combination with the position of the recording electrodes on the scalp. 3) Study of electrical changes in peripheral nerve injury: Experiments have been carried out to investigate the changes which occur in evoked cortical responses and in electrical potentials picked up from the forearm of patients who have suffered nerve injury to either of their hands. The effect of recurrent light pressure stimulation of the injured hand is compared with the effect of stimulating the uninjured hand. Computer methods are used to isolate the resulting tactual responses. A marked dimunition of response is noticed when one compares stimulation of the injured with the uninjured hand. 4) Neural mechanisms in a vertebrate visual system: Research was continued on the visual system of intact, immobilized Rana catesbiana. The research has been concerned with the relations between neural activity and spectral as well as luminosity mechanisms with emphasis on the time course of neural-electrical responses to the onset and cessation of light stimuli. The optic nerve discharge and the electroretinogram (ERG) are recorded simultaneously with light flashes of long duration in order to isolate the on- and off-responses. The energy and wavelengths of the light stimuli are systematically varied to permit analysis of their effects. A microelectrode apparatus has been set up to obtain some data from retinal ganglion cells. Computer programs have been developed for measuring various aspects of the optic nerve and retinal responses. These measures include peak amplitude, peak latencies, total area of the response, and location of various waves and ripples. The data suggest a close relation between the amplitude and total area measures. Latency measures relate to these in a more complex way. Both the optic nerve and ERG waveforms vary with the wavelength of light and thus support the hypothesis that the time course of neural activity might serve as a neural code for color and hue discrimination. Methods of specifying these waveform differences quantitatively are being explored. The spectral sensitivity of on- and off-responses of the ERG and optic nerve are being determined and compared. These curves then are directly compared with behavioral spectral sensitivity data which is being gathered in connection with this project. 5) Electrical brain responses following saccadic eye movements: The visual system responds vigorously to changes in stimulation, but response tends to diminish in the absence of stimulus variation. Stabilized image experiments have shown that the movement of stimulus images over the retina is required for maintenance of vision and that the saccadic eye movements, which displace the image suddenly, are of particular importance. This result suggests the hypothesis which was tested here, namely, that bursts of activity occur in the visual nervous system following saccades. Electrical responses were recorded through scalp electrodes while the subject fixated targets of fixed luminance. Concurrent eye movements were recorded photoelectrically. The occurrence of saccades was detected automatically by electronically

differentiating the eye movement signals. An average response computer, connected to the scalp electrodes, was triggered on the occurrence of saccades. In this manner the average waveform following 50 saccades was obtained. A regular response was found to occur following saccades. Both the latency and the amplitude of the responses were found to be functions of the luminance of the fixation target. Thus, it was demonstrated that saccadic eye movements do elicit visual responses. 6) Evoked response activity in the infant: Study of the development of electrical responses of the human brain to light and sound stimuli has been continued. During the past year emphasis has been placed upon the responses of the neonate. Electrical data are stored on magnetic tape for later analysis using a general purpose digital computer programmed to average responses to repetitive stimulation, or they are analyzed "on line" using a CAT electronic averager. Evoked responses to lights and sounds are seen in the early newborn period. These show a characteristic wave form and latency which change with age. Study of the relationship of stimulus intensity to response in the sleeping newborn has shown that the amplitude of response is directly dependent on stimulus intensity. There is a shorter latency of response in lighter sleep. Children with several types of neurologic and sensory abnormality are being studied. The technique appears to have value as an aid to diagnosis. For example, the evoked responses to clicks, tones and flashes of 22 infants with prenatal rubella syndrome have been examined. 12 of these infants have diminished or absent responses to sound. It is hoped that, with early diagnois of auditory defect, rehabilitation can be started early and improve the child's chances for normal speech and language development. 7) Evoked responses to meaningful stimuli: A procedure of potential value for understanding cognitive processes was utilized in further studies evoked responses. Human subjects solved problems that required perceiving numerical visual stimuli presented in sequence. Other stimuli, irrelevant to the problem, were a regular part of the stimulus sequence. Evoked responses to each of the stimuli were averaged from scalp derivations. The general finding has been that the relevant stimuli produce larger evoked responses than the irrelevant stimuli. Controls showed that the effect did not depend on relative energy in the stimuli, changes in pupil size or accommodation, monocular viewing, eye movements, or amount of alpha EEG activity. A further test of the interpretation that the enhancement of the evoked responses is due to the "meaningfulness" of the stimuli was made. For this purpose a sequence of number and letter stimuli were used. On some runs the numbers were task-relevant and the letters task-irrelevant, and on other runs the relevance was reversed. For each run the numbers (1-6) and letters (A-F) were randomized. The responses to a stimulus tended to be larger when it was task-relevant than when it was task-irrelevant. The data also showed two other trends. One trend was for the first response in the sequence to be larger than later ones. The other trend was for the responses to numbers to be larger than those to letters. Since the numbers were generally wider than the letters, this finding suggested that the stimulus shapes also influence the evoked responses. This was tested in a second experiment in which four stimuli were selected so that relevant and irrelevant stimuli each had a thin and thick shape. For this purpose the relevant stimuli were 1 and 2 and the irrelevant stimuli were

- and +. In addition a blank was used as a large area irrelevant stimulus. Both relevant stimuli evoked larger responses than the irrelevant stimuli. The response to 2 was larger than the response to 1. The results of both experiments support the interpretation that evoked responses are larger when the stimuli are relevant to the task and, in addition, that evoked responses are larger for stimuli with more complex shapes. As might be expected of responses from the central nervous system, their amplitude is modulated by a variety of influences. Appropriate physical parameters of the stimulus, such as light energy (Chapman & Bragdon, 1964) and shape, affect the evoked responses. In addition, the relevance of the stimuli to the subject's behavior modulates the evoked responses. The technique of presenting a sequence of stimuli and separately averaging the electrical responses to each stimulus in the sequence facilitates the investigation of these effects.

III. Psychophysical and behavioral experiments:

1) Test for visual inhibition during involuntary saccadic eye movements: Several authors have suggested that vision is inhibited during saccadic eye movements. The early writers on the subject were impressed by the fact that no blur is perceived even though the image moves rapidly over the retina. More recently both Volkman and Latour both have produced data demonstrating an elevation of thresholds during voluntary eye movements. Experiments have been conducted in this laboratory to determine whether a similar effect occurs during involuntary saccades. The procedure involved the delivery of test flashes which were contingent on the occurrence of saccades. A photoelectric eye-movement recorder was used to detect the occurrence of saccades. Test flashes were presented automatically either during a saccade or after a fixed delay . Two visual functions were investigated: absolute threshold and vernier acuity. In neither case were the thresholds higher (or lower) during saccades than at various times after them. Thus, there is no evidence of a "blanking out" during involuntary saccades. 2) Determination of fundamental response functions for the color naming of small monochromatic stimuli: The minimum possible condition for seeing a flash in the fovea is that one neural unit be activated. The neural unit might be as small as a single cone or might be a ganglion cell summating the activity of a number of cones. It might even be a unit higher in the neural system. The doctrine of specific energy of nerves holds that whatever the unit is, it has a specific experiential quality associated with it. Thus, if one unit is stimulated at a time, the experience generated will be determined by which unit is stimulated. It was shown that during the past year when stimuli are close to visual threshold, with a probability of seeing approaching zero and when stimuli are of small angular extent, reports of seeing are due to the activation of single units. Under these conditions the reported color name of repeated test flashes of the same test wavelength is not the same. This variation in reported color, when investigated as a function of wavelength and intensity, provides information about the spectral sensitivities of the various classes of receptor units. If the units are homogeneous with respect to the type of receptor (red, green, etc.) then the relative frequencies of color reports may be used to deduce the spectral sensitivity of the fundamental units. Evidence from

color naming experiments supports the assumption of homogeneity. A simple method for separately evaluating the spectral sensitivity of the different classes of fundamental units has been developed. To date two sensitivity curves have been determined. One curve is for a red mechanism and the second for a green mechanism. 3) Behavioral measures of vision in the turtle: The aim of this task is to develop several techniques for the investigation of basic behavioral processes in turtles and to apply these methods to the study of sensory mechanisms. To this end techniques for measuring and controlling food and air intake and for a withdrawal response have been developed and used to study response habituation, conditioned emotional response, respiratory regulation, visual processes, and the relation between stimulus intensity and response amplitude. i) A method for obtaining the luminosity function of the turtle eye by flicker photometry has been devised and improved. This technique was recently extended to the study of color processes. ii) Previously devised techniques for studying free-operant behavior, where the reward is access to air, were used to investigate several problems. (a) Respiratory regulation was found to differ among several species. Chrysemys picta picta showed an intermittent pattern, e.g. work for air for five minutes, rest for five minutes; whereas Pseudemys scripta elegans and Terrapina carolina showed a more continuous performance. In a further experiment, where air exposure time was varied, evidence was obtained that the regulatory mechanism, at least in Chrysemys, is learned. (b) A technique for obtaining stable discrimination functions between visual stimuli differing in brightness or hue has been developed. Successive, rather than simultaneous, presentation of the discriminanda proved to be better, and this technique is currently being used to study differential susceptibility to temperature change of the color processes. (c) Evidence was obtained that turtles differ from other organisms in the conditioned emotional response situation. Following training to press a blue panel for air, the color of the panel was periodically and briefly shifted to red. Mild shocks were given at the end of the red period. Under certain conditions a facilitation, rather than a suppression of responding which is the usual case in other animals, was observed. An investigation of the conditions under which this facilitation occurs is under way. iii) Several investigations of the conditions affecting neck withdrawal were started. Pilot studies indicated that reliable withdrawal could be obtained by passing a mild current across the body of a submerged turtle whose head was extended outside of his shell by means of a string attached to his beak. The string also permitted measurement of the amount of withdrawal. The amount of response was found to be directly related to shock intensity, and it decreased very gradually with successive shock administrations. Further, withdrawal could be obtained with a lesser shock if light was beamed upon the turtle's head -- the brighter the light, the stronger the withdrawal. This "light effect" was also found to be a function of the position of the light. Studies are in progress to determine whether the action of the light is mediated by a sensory interaction mechanism, a light-induced increase in arousal, or a vigilance-anxiety process. 4) Color mechanisms in the visual system of the frog: Behavioral studies of the visual system of Rana catesbiana were conducted in relation to the electrical recording data obtained from the same species. The behavioral studies were

organized around two basic questions: i) does this species have color vision in the sense of wavelength discrimination when brightness is controlled and ii) what spectral sensitivity curves does this species exhibit behaviorally. The technique involved a forced-choice, paired-comparisons preference procedure with test lights of controlled wavelength and energy. Four related experiments were performed. In the first experiment with lights of equal energy, a preference for short-wavelengths was found. The preference was heightened in a second experiment when lights of "equal brightness" were used, i.e., lights equated on the basis of the bullfrogs' electroretinal spectral sensitivity. Higher energies were preferred for 460 mu and 540 mu, but not for 620 mu, in a third experiment in which the choice was between stimuli of different energies of the same wavelength. In a fourth experiment, the preference for 460 mu over 620 mu could not be reversed by varying the energy of each over a range of 10,000-fold. These four experiments indicate that bullfrogs can discriminate between wavelengths as well as between energies of light stimuli. That the blue-sensitive preference exhibited by the bullfrogs' behavior may be related to a blue-sensitive mechanism in its retina shown is by three other kinds of data; 1) the green-rods absorb strongly in the short wavelengths (Denton & Wyllie, 1955); ii) selective bleaching experiments using frog retina extracts have uncovered a pigment difference spectrum (Dartnall, 1957) which agrees with iii) spectral sensitivity curves from some ganglion cells in frog retina (Chapman, 1961; Donner & Reuter, 1962). Quantitative comparisons with these mechanisms requires spectral sensitivity data rather than spectral response data. The behavioral preference technique is being modified to produce such spectral sensitivity data.

SUMMARY AND CONCLUSIONS:

This work unit has been concerned with sensory processes, behavior and underlying physiologic mechanisms. The work may be divided into three categories: 1) studies of sensory mechanisms, per se, 2) studies of relations between peripheral and central processes and 3) correlative psychophysical behavioral experiments.

In the study of peripheral mechanisms, it was found that the size of the image on the retina depends upon the accommodation of the eye with a small artificial pupil. A quantitative treatment of the relations between these variables has been worked out. Electrical studies have made data available describing the course of dark adaptation in the human ERG. Another study has dealt with the incremental threshold of the ERG as a function of light adaptation level. The ocellus of the horseshoe crab has been reported to have two visual pigments. Some of their properties have been ascertained. Experiments concerned with the effects of temperature upon the visual pigments are being initiated. In another series of studies the development of vision is being investigated. Evidence for both photopic and scotopic activity has been found in the newborn human infant.

In the second group of experiments new devices and methods based upon computer technology have been developed for the administration of experimental procedures and for the analyses of the resulting data. The manner in which visual responses are propagated to the cortex and distributed over occipital scalp has been investigated with attention to the position

of stimuli in the visual field. Techniques for following recovery of sensitivity accompanying nerve regeneration in peripheral hand injury are being examined. The development of auditory and visual function in the central nervous system of infants is being investigated. Some of the methods may provide a means of detecting neural damage in newborn infants whose mothers were stricken with german measles during pregnancy. An electrophysiological study is providing information relative to color coding in the optic nerve. It also has been shown that information does not pass up the optical nerve continuously but in bursts. The bursts are paced by eye movements. Study has been made of the other complex factors which influence evoked potentials. The electrical response of the central nervous system depends not only upon the physical characteristics of the stimulus but also upon the significance of the stimulus to the subject and the behavior it elicited.

In the behavioral group of experiments color naming data provided information pertaining to the basic color processes which contribute to vision. It was demonstrated that visual threshold is maintained constant during involuntary eye movements and is not suppressed even though the observer is unaware of the blur which movements produce across the retina. Improved psychophysical methods have been developed for studying the vision of animals. These include both learned or conditional techniques, and unlearned withdrawal and preference methods.

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RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
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4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT / WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
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19 GOV'T LAB INSTALLATION ACTIVITY
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21 TECHNOLOGY UTILIZATION	22. COORDINATION
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23. KEYWORDS Sleep; evoked potential; electroencephalography; language; learning; psychological tests; operant behavior

24. (U) Tech Objective - Ascertainment of: Markovity properties of EEG stages of sleep; behavior during sleep; relation between evoked potentials and behavior; information requirements for decision making; muscle action potentials in paralyzed limbs and amputees. Formulation of: the process by which grammar is learned; miniature syntactic systems to study thinking and concept formation. Assessment of: pattern of psychological stress in the basic training cycle; psychological factors determining host
25. resistance to URIs in recruits; incidence of successful completion of obligated tour of duty by soldiers seen at MHCS during basic training. Development of operant programming techniques in mother-child interaction and in the reinforcing properties of verbal behavior.

(U) Approach - Sleep, evoked potential and muscle action studies are performed in the laboratory utilizing standard electrophysiological techniques. Data for linguistic studies obtained from co-operation of local school systems and "pools" of adult military subjects. Data on basic training cycle collected in URI field study utilizing
26. traditional psychometric instruments, repeated measures of affective arousal state, sociometric devices and leadership judgments. U.S. Army Records Center furnishes documents on MHCS soldiers and Data Processing, WRAIR, provides keypunch and computer support. Operant methods with human subjects require further laboratory development.

(U) Progress (Jul 64 - Jun 65) - Sleep and decision making studies terminated. Other studies in various stages of progress.
For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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Project 3A014501B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 03, Psychiatry

Work Unit 027, Analysis of behavior and of mediating mechanisms: Measurement of performance and decrement of performance

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Description.

Broadly conceived, the purpose of this work unit is to study the performance of the human organism under a continuum of conditions ranging from laboratory manipulation of the independent variable (e.g., sleep deprivation, incentive, information load, word-order exposure) to empirical documentation of the dependent variable in settings of natural or intrinsic occurrence (e.g., muscle action potential in paretic patients, military performance in a select sub-population of soldiers, affective arousal in basic training and implementation of eugenics laws in the United States). The wide spectrum of human behavior thusly studied leads to a variety of research content, design, methodology, instrumentation and technique. The body of this report discusses separately and in some detail each particular research inroad made toward the goal of describing, cataloging and characterizing human performance.

Progress.

1. Studies of Human Performance Utilizing Electrophysiological Techniques. Studies in this section, conducted within the confines of the laboratory, share in common an attempt to bridge the gap between physiological processes and overt behavior. The work primarily employs measures of ongoing central nervous system functioning and relates these to behaviors which, either by taking strategic advantage of an occurring behavior such as sleep or by direct induction of a behavioral orientation such as vigilance, can be experimentally manipulated. In the past and to some degree still, there has been particular emphasis on defining the parameters of performance decrement. There is ordinarily a modest number of subjects used in an experiment; however, what is lost in possible generality of results is more than compensated by the amount of experimental control possible.

a. Behavior During Sleep. A long-term project on behavior during sleep was completed with a closer analysis of data that were collected over a three-year period. Previous progress reports contain a detailed description of the procedure. Briefly, the aim of the present analysis was to determine whether human subjects could respond to controlled auditory stimuli while asleep, whether they could respond discriminatively to one of two tonal stimuli, and whether the addition of negative reinforcement would improve performance. Each of six subjects had slept in a laboratory enclosure for six to eight nights during which continuous EEG recordings were taken and stimuli were automatically presented. The evocation of the waking alpha rhythm by the stimuli was used as a criterion of transient awakening.

Upon analysis, using the information statistic proposed by Kullback, Kupperman and Ku, which is essentially a chi-square analog of analysis of variance, the performance data revealed several interesting points: (1) The number of correct responses was a declining function of sleep "depth." (2) Responding was not more probable when the stimulus produced evidence of transient awakening, except for the rapid eye movement stage (REM) where responding was significantly, $p < .05$, more probable when the alpha rhythm was evoked by the stimulus. (3) Responding increased when reinforcement was added, and this effect was greater when the stimulus did not induce the alpha rhythm. (4) Stage of sleep had a significant effect on the relation between reinforcement and responding for each subject. Reinforcement had its greatest effect, generally, during Stages 2 and REM. (4) Reinforcement improved discriminative responding, but only for stimuli that did not evoke the alpha rhythm.

As has been found repeatedly, performance improves when reinforcement is used. Suprisingly, however, the effect of reinforcement here was consistently greater when there was no evidence of transient awakening. The criterion of transient awakening may have been too stringent; however, in any event, the results indicate that a finer-grained analysis of sleep is needed to determine just how it differs from waking. The effect of reinforcement suggests that variables which effectively alter behavior during the waking state operate also during sleep. It appears that sleep is not a totally different state from waking.

b. Markovity Properties of the EEG Stages of Sleep. During the course of a normal night's sleep, the EEG appears to show consistently different patterns in frequency and amplitude, as if the sleeper were cycling through several stages of sleep. This periodicity has often been noted, but no quantitative investigations of this apparent regularity have as yet been reported. This investigation was conducted to determine whether these stage sequences can be characterized as simple Markov processes of order one or as a more complicated Markov process of order two or more. That is, does the occurrence of a particular stage depend only on which stage preceded it or does it depend on more remote ones as well?

Continuous EEG's from eight volunteer subjects who slept 4 nights in the laboratory were recorded. An analysis of the transitional probabilities of the changes from one stage to another was carried out using the information statistics proposed by Kullback, Kupperman and Ku. The analysis revealed a strong dependence between any stage and the just preceding one. Further, among those EEG stages which occur frequently during the night a two stage dependence was observed. This analysis also showed that the subjects could be classified into two groups: A restless group who frequently shifted to a waking stage and a non-arousal group who showed the more-or-less classic sleeping EEG pattern. Within both groups the patterns of stage sequences were homogeneous among the subjects.

The transition from one EEG stage to another is an orderly process. There is a strong dependence between the occurrence of any stage and the preceding one, in some cases the preceding two. Individuals differ somewhat in their patterns of EEG stage transitions and further work is called for to explore the implications of this for waking performance.

c. <u>The Relation of Cortical Evoked Potentials to Behavior</u>. The primary aim of this endeavor is to use the evoked cortical potentials, recorded from the scalps of humans, in order to clarify two opposing views of the source of performance decrements in vigilance tasks. It has been observed in this and other laboratories that the components of cortical evoked potentials generally decline in amplitude when the stimulus evoking the potential is ignored; whereas the evoked potential changes form when the subject falls asleep. Now, two suggestions as to why signals in vigilance tasks are missed with increasing frequency as the test progresses are one, the subject becomes increasingly distracted from the stimuli he is to observe, and two, the monotonous nature of the task lowers arousal. Thus, the evoked potentials produced by the stimuli that the subject must observe would seem to be ideal for determining which of these possibilities is the source of performance decrement; although, at the outset it was not known whether the evoked potential would indicate changes in arousal during waking.

In the previous year five enlisted volunteers listened to clicks of moderate intensity and were instructed to close a switch whenever they detected a slight decrease in the loudness of one of the clicks. During the two hours of the test, 16 such "critical signals" were presented, and the average evoked potential to the 50 clicks preceding each was recorded. In the past year three undergraduate subjects were added to the subject pool. In seven of the eight subjects the evoked potentials to stimuli immediately preceding the critical stimulus indicated a lower level of arousal for missed than for detected signals. A further study is under way in which a monetary incentive for successful detections has been added. Incentive is known to improve performance. The question is whether it produces a corresponding change in the evoked potential, i.e., toward greater arousal.

Preliminary results suggest it does. Further analysis of this data will be made to determine the relations of incentive and evoked potentials to pulse rate and reaction time. An additional investigation of the relationships between evoked potentials, response time and incentive is being carried out in which the subject is required to respond as quickly as possible to randomly occurring clicks, whose evoked cortical potentials are recorded. In one setting this is done for a series of 50 reactions; in another setting the subject responds continuously for 30 minutes. In each case the effects of adding incentive are observed on the reaction speed to the clicks and the associated evoked potentials of the clicks.

Judging from the cortical evoked potentials, the decline in performance in the present vigilance tasks is the result of lowered arousal. When incentive is used, performance improves, and the cortical evoked potential shows an increase in arousal. In addition, these results indicate that the evoked potential is sensitive to changes in arousal during waking and suggest that the effect of adding incentives, at least in this situation, is to increase arousal.

d. Electromyographic Studies. Electromyography provides a means toward detecting very small changes in action potential in muscle systems. One study made use of this electrophysiologic technique to compare muscle action potentials in a paralyzed limb with those in the contralateral ("normal") limb in the case of sleeping hemiplegic patients. EEG, EMG and eye movements were recorded by using surface electrodes in the conventional manner. The recordings were obtained at night during spontaneous sleep. In contrast to the normal muscle, which was electrically silent for the most part, the paretic muscle showed intermittent electrical activity which seemed to increase in amplitude during the REM stage of sleep. A final report of this study is in preparation.

Another study used additional physiological measures as dependent variables. Two behavioral measures, speed and accuracy of performance on a serial choice reaction-time task, were collected along with pulse rate, respiration rate, muscle action potential, skin conductance and pulse amplitude on 12 normal subjects. The study was designed to investigate the relationship of these various response measures to the experimental manipulation of two independent variables: incentive and task complexity. Pulse rate and respiration rate were observed to vary with incentive level; skin conductance and pulse amplitude varied with task complexity. Co-variance of the muscle action measures with any of the other variables could not be detected. A report is being written for publication.

A display depicting the uses of electromyography in physical therapy was prepared for a scientific exhibit sponsored by the U. S. Army Medical Service Corps. The exhibit illustrates the value of electromyography for diagnostic, instructional and research purposes. Diagrams of the neurone motor unit and the schematic changes

in the muscle membrane upon excitation demonstrate the anatomic and physiologic basis of the action potential. Another part of the exhibit represents the characteristics of the action current of the individual motor unit in normal as contrasted with pathological conditions. The recording instruments and accessory equipment, including an integration unit developed at WRAIR, are displayed.

2. Human Decision-Making. Decision-making is a crucial aspect of human performance. One systematic investigation was conducted in this area. The brief description presented is an abstract of a Ph.D. dissertation, now on file at the University of Pennsylvania.

a. Effects of Preference on Information Requirements for Decisions. In everyday life one finds examples of individuals apparently requiring more information to make a decision when the information goes against the decision they should like to make than when it is in favor of their preferences. For example, it was found in an opinion poll some years ago that the more cigarettes a person smoked daily, the less likely he was to consider proved the claim that cigarette smoking led to lung cancer. Eliminating such tendencies is desirable not only because the costs of gathering information might be increased and/or a delay might be dangerous, but also, because the individual might make the decision he prefers simply because he feels the undesirable alternative is not bolstered by sufficient information. This experiment represented an attempt to test whether such tendencies would occur under controlled laboratory conditions and to study some of the conditions which might influence them.

Thirty-eight undergraduates were required to decide whether several large packs of marked and blank cards contained a predominance of marked or blank cards. In some cases it was highly desirable to the subject for the pack about which he was required to make a decision to contain a predominance of marked cards; in other cases this was highly undesirable. The subjects were permitted to sample cards from the packs, and the number of cards sampled was used as an index of the amount of information they required. The difficulty of making a correct decision and the incentive for decision correctness were also manipulated. In general, more cards were sampled before a subject made a decision when the information he obtained from sampling indicated the pack had an undesirable constitution than when the information indicated the pack had the desirable constitution. Increasing the incentive for correctness of decision, which one might suppose would suppress irrational decision-making processes, had no effect on this tendency. However, increasing the difficulty of reaching a correct decision enhanced the effect for men but not for women.

The results indicate that information requirements for decisions are affected by the preferences of the subjects as to the decision they should like to reach. However, further experimentation, utilizing other information measures and a wider range of variables, is needed before firm conclusions can be reached as to the nature of this tendency or the variables controlling it.

3. The Acquisition of Linguistic Structure: Verbal Behavior in Human Performance. The work in this section comprises a series of experimental studies designed to ascertain the processes by which grammar is learned. Methodologically the research in this area is noteworthy in that it utilizes rigorous experimental techniques in the investigation of models developed on purely formal grounds to account for linguistic data. Both this section and the section which follows on concept formation illustrate the use of traditional experimental methods and controls to test hypotheses about the relationships between complex human variables.

The ultimate concern of the psycholinguistic effort continues to revolve around the appropriateness of various models in explaining "what is learned" when human beings are exposed to strings of verbal units. The position-learning theory was developed in previous work here. A second closely related theory draws heavily on the concept of mediated association as well as on the thinking of conventional learning theory. An empirical test has been developed which contrasts the two theories. Certain models derived from work on the formal properties of generative grammars, in particular, the phrase-structure and finite state models, are also being explored empirically.

a. Experiment I. This is an experiment in which evidence was obtained to show that adult subjects exposed to a patterned set of semantically empty strings learn positional regularities. The form of these regularities suggests that a formally adequate grammatical description in terms of a finite state grammar does not capture the nature of the patterns the subject has actually learned. The latter are best interpreted by a phrase-structure grammar or a finite automaton containing a push-down storage mechanism. A method for exploring grammar acquisition, viz., the "verbal reconstructive memory" technique, is outlined in the published report of this experiment.

b. Experiment II. This is an experiment connected with the problem of word-class formation. It explores the relation between the learning of temporal positions of elements and the four-stage stimulus-equivalence mediation paradigm. Subjects listened to letter pairs having the structures AB and CD, where A, B, C, and D denote lists of letters. They were then required to recall the pairs after various amounts of exposure. Results indicated learning of the positions of letters, but not learning of a distinction between the categories A and C, or B and D. That is, no evidence for "mediation" was found. A report on this experiment has been published.

c. Experiment III. Further study on the learning of letter pairs having the structure AB, CD, as described above, is in process. This work replicates and extends the previous work, and results so far confirm the interpretation that mediated stimulus-equivalence does not occur under the conditions employed. These results suggest a limitation on the scope of the stimulus-equivalence paradigm, and suggest a set

of conditions under which distinctions between word-classes tend not to be learned. A report on this work is in preparation.

d. Experiment IV. This is an experiment which explores the learning of the positions of words, where position is defined relative to "function" word markers. The learning of positional relations like "second-after x" are not easily accounted for in terms of traditional association theories, and suggest a phrase-structure, or "push-down storage," model for the relations learned, rather than either a finite-state model, or a traditional "chain-of-association" model. A report of this experiment is in preparation.

e. Experiment V. This is an experiment exploring the learning of grammatical structure from a verbal environment which contains ungrammatical as well as well-formed sentences. Using nonsense material, a subset of the sentences generated by a miniature grammar are read onto tape; randomly interspersed among these are some ungrammatical sentences, having various degrees of statistical approximation to the grammatical sentences. Subjects listen to the tape without being given information as to which sentences are well-formed. After listening, they are given a number of tests to determine how much of the grammatical structure they have learned. The results indicate that for the relatively low percentage of ungrammatical sentences used, the presence of ungrammatical sentences on the tape hardly interferes at all with the learning of the patterned regularities in the construction of the well-formed sentences. The results argue strongly against current theories of grammar acquisition in which the learner is viewed as computing differences between grammatical and ungrammatical sentences.

f. Experiment VI. Analysis has continued of data previously gathered in a field study of the development of English structure in a small sample of children under three years of age. Some evaluation criteria for grammars have been developed and tested which are appropriate to a field situation where metalinguistic judgments (e.g. of grammaticality) are not obtainable from informants. One such criterion was discussed in a paper delivered at the 1965 meetings of the Linguistic Circle of New York. This paper also reported evidence as to the nature of the first true "sentence" structures of English-speaking children, and suggested a reason why young children's speech has the "telegraphic" style noted by many workers in the area.

In general, the series of experiments in the psycholinguistic area contribute additional support to the theory of position learning. At the same time, the work draws important distinctions between the claims of the theory and those of rival formulations proposed on the one hand by more traditional learning theorists and on the other by proponents of generative grammar whose motivations stem from the formal study of language.

4. Concept Formation: Cognitive Aspects of Human Performance. This set of investigations concerns itself with basic conceptual processes. The ultimate goal is to describe developmentally those aspects of cognition which lead to what is usually called "abstract" thinking. The development has been explored of some basic physical concepts underlying measurement and of concepts of order, similarity and logical class. Almost without exception, young children have been used as subjects. This has been made possible by the willing co-operation of nursery schools in the metropolitan area.

a. Size and Shape. Work has focused on the development of the distinction between "real" size and phenomenal size. Illusory size changes were created by a lens or by standard visual illusions. In one experiment an illusory shape was created by immersion of rods in water. Children's ability to understand the difference between such questions as "Which looks bigger?" and "Which is really bigger?" was studied in a variety of circumstances. The results indicate that at around five years of age a majority of children distinguish real from phenomenal properties of objects. The work thus suggests a reformulation of Piaget's conception of the development of "conservations." The experiments here are reported in two articles in press.

b. Transitivity. A published study describes the development of a grasp of the transitivity property of length. Preschool children's ability to infer A>C after having been shown A>B and B>C was explored in circumstances when it was not possible to tell whether or not A>C by visual inspection.

c. Order. An experiment has been completed wherein children were required to select from two or more sequences of colors (e.g., red-green-yellow vs red-yellow-green vs green-red-yellow) the order that was the same as a sample sequence. Stimuli in a variety of arrangements and lengths were presented. It was found that total inversions (e.g., red-blue-green vs green-blue-red) were not more difficult than other arrangements--a finding contrary to what is suggested in the literature.

d. Similarity. In one experiment subjects were first taught to respond to an identical pair of figures and to avoid a non-identical pair. They were then tested on generalization problems. These involved stimulus-dimensions not used in the initial learning and problems in which the positive pair of figures were similar but not identical. A group of three-year-olds and a group of 18-month-olds have been run. The results indicate that both groups of children generalize very widely indeed. Even the 18-month-old has an extremely general concept of resemblance. The data also suggested that the difficulty in detecting similarities is a function of the number of stimulus dimensions which the subject has to scan in order to detect the similarity. Age differences are probably differences in ability to scan a number of dimensions rather than differences in the degree of generality of the concept of similarity.

An extension of this very problem into the animal laboratory has been accomplished at George Washington University by a student under the direction of Dr. Braine. Monkeys had more difficulty than 18-month-old children with generalization problems that involved the introduction of stimulus dimensions not used in the initial learning. Other kinds of generalization problems, including some which 18-month-olds find relatively difficult, were readily solved by the macaques.

Another experiment explores developmental changes in the concept of similarity after three years of age. In a procedure similar to that used with the young toddlers, children aged three to six years were first taught to respond to a pair of identical or similar objects and to avoid a pair which were different. In a series of generalization problems, the subjects were confronted with a pair of objects similar in appearance but not in function (e.g., a green grape and a green marble) and a pair similar in function but not in appearance (e.g., a grape and a banana). They were then asked which were "more the same." The main group of subjects were reinforced for choosing the pair that were similar in function; other subjects were reinforced for choosing the pair similar in appearance; and a third group were not differentially reinforced. The results indicate that the proportions of subjects in each age-group who learn to respond consistently to the functional similarity are the same as the proportions of subjects distinguishing real from phenomenal attributes found in the previous work.

The overall results in the area of concept formation add further to the definition of the cognitive processes that develop in the young child. In particular, they suggest that a close connection exists between the development of the "conservations" and the development of a concept of "type" or "class" which is based on something more than mere phenomenal resemblance. All these relatively complex and fundamental concepts appear to develop at a surprisingly early age.

5. Human Performance In Military Settings. Several studies assess human functioning as it occurs embedded in the military milieu. The methodology typically used in these studies relates one dependent variable with another (i.e., correlates response with response). Or, alternatively, a dependent variable may be singled out for close scrutiny and a strictly rational assessment made of the independent variable thought to be operative. The immediate product of this particular methodological bias is usually a statement involving the degree of relationship between two events previously not noted to be related. The more enduring product, however, is the development of a construct, or idea, which, when placed in juxtaposition with other constructs, can point the direction toward future field studies or can suggest the content of controlled laboratory research.

a. A Follow-up Study of Mental Hygiene Clients. This study attempts to ascertain the military outcome of a large number of soldiers who, at some point in their basic training process, were served by the

Mental Hygiene Consultation Service (MHCS) located at one of the large U. S. Army Infantry Training Centers. It makes use of the so-called "follow-up" method of investigation. Subjects studied are the entire population of first- and second-eight weeks trainees utilizing the services of the Fort Ord MHCS during calendar years 1962 and 1963. A total of approximately 1400 trainees were served by the Fort Ord MHCS during this two-year period. Of these 1400 men roughly 50% were obligated for a three-year tour of duty (RA), 29% had been inducted for a two-year tour (US) and 21% were called to active duty for six months only (RFA).

Pre-follow-up data were abstracted from each soldier's Fort Ord MHCS clinical file. Examples of pre-follow-up indices recorded are the following: Soldier's unit at time of referral; date initially served by MHCS; referral source; disposition and recommendation made by MHCS; number of field consultations required to service the soldier-problem; number of psychiatric consultations required.

The follow-up data themselves are made accessible by the Commanding Officer, United States Army Administration Center, Office of the Adjutant General, St. Louis, Missouri. After sufficient time has elapsed to enable each soldier to have completed his initial, obligated tour of active duty, four documents from the soldier's service record file are searched, reproduced and made available to the investigators by staff at the U. S. Army Administration Center. Information from the service documents (DD Form 214, DA Form 20, DA Form 24 and DA Form 26) is abstracted, coded and key-punched on standard IBM cards for data analysis. The Department of Data Processing, WRAIR, provides the key-punch and computer support.

From the follow-up documents a determination can be made as to whether or not each soldier successfully completed his obligated tour of duty; or, if he failed somewhere along the way, the specific exit route from the Army is coded. In addition to the length of service successfully completed and the regulation governing the soldier's separation from active duty, numerous other indices are recorded from the follow-up documents. Among these: Traditional demographic data; GT score; CI score; conduct and efficiency ratings; number of assignments; degree and kind of MOS training; number of lengthy hospitalizations; occurrence of overseas duty; station from which discharged; reductions in rank; and several military delinquency indicators.

The entire sample will not have "matured" for follow-up purposes until 1 January 1967. However, procurement of follow-up data on portions of the sample began in October 1964. At the time of this writing complete data on 239 RFA cases have been coded and key-punched. From the US group, data on 212 cases have been recorded. Preliminary data analysis has begun on 207 US soldiers. An early finding is that of this group of 207 US soldiers, who required the attention of an MHCS sometime during basic training, only 86 (i.e., 41.5%) went on to complete fully their obligated tour of duty in spite of the fact that

146, or 70.5%, of this group of 207 men were returned to duty without separation recommendations from the Fort Ord MHCS.

In addition to the 1400 MHCS cases to be studied it is necessary to obtain a control group of non-MHCS soldiers for comparative purposes. The selection and procurement of follow-up data on a matched control group is again made possible by the co-operation and assistance of the Commanding Officer, U. S. Army Administration Center. The four follow-up documents on the initial portion of the US sample have been requested.

No firm conclusions can be drawn at this early date. It is expected that the study will result in a statement of the "success-failure" incidence of a select group of deviant soldiers. Differences within this MHCS group of soldiers between the "successes" and "failures" can be searched and isolated on military performance indices and other individual-difference variables. Whether or not so-called "situational" variables weigh as heavily as personality variables in the determination of successful outcome can be evaluated.

b. Measurement of Achievement Motivation in Army Security Agency Foreign Language Candidates. A report of this study, under the above title, appears in the current (1965) volume of Educational and Psychological Measurement. The investigation was carried out at the Defense Language Institute, West Coast Branch, Presidio of Monterey, California. Three achievement scales from Gough's California Psychological Inventory were administered to 290 soldier-students prior to their engagement in full-time study at the Institute. Results were interpreted as suggesting that achievement motivation can be inferred as a distinct construct apart from aptitude or intelligence and is to be given consideration in the prediction of academic success in the case of foreign language pursuit.

c. Fort Dix Interdisciplinary Respiratory Disease Study. Investigators from this Work Unit participated in an interdisciplinary research carried out at Fort Dix in the early months of 1965. The details of (a) a study which charts affect arousal levels in a platoon of basic trainees as it moves through the basic training cycle and (b) a study which concerns itself with a quest for psychological correlates of host resistance to respiratory disease are reported elsewhere. (See Project 3A013001A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH; TASK 01; Work Unit 020, Effects of Physiological and Psychological Stress Upon Infection and Disease.)

6. The Effect of Formalized Social Structures upon Human Performance. This endeavor employs a variety of information-gathering methods in its attempt to make explicit the ramifications of institutionalized, legalized and codified procedures upon the behavior of individuals and groups. Historical reviews, literature surveys, court proceeding, public statutes, military regulations, press periodicals, written correspondence and face-to-face interviews are the principal data sources.

a. The Implementation of Sterilization Laws in the United States. The first study is near completion and is fully described in a manuscript which will be distributed for public consumption by a private publishing house. The volume systematically assesses the present function of state eugenic sterilization laws in the United States in terms of due process and equal protection standards. In particular, it studies the role of the medical administrator in determining the enforcement or non-enforcement of these laws; it traces the historical process whereby a given cluster of scientific thought (viz., human genetics and eugenics) influences public policy in the form of new legislation, repeal of existing laws, enforcement of statutes and judicial decision; it makes known the ensuing obstacles which vitiate against the introduction of sound scientific discoveries into the body politic. The reaction to, implementation of and public concern with sterilization laws provide the model from which the more general thesis is developed.

b. Military Procedures Governing the Management of Homosexuality. A second work is in the formulation stage. It will utilize similar methodology to study the area of homosexuality in the U. S. Armed Forces. Congressional and Department of Defense concern with the military management of this problem provide impetus for an historical-administrative analysis of the pertinent formal directives and informal policies. Another opportunity will thusly be provided to appraise the general thesis having to do with the effects of a body of scientific or quasi-scientific knowledge upon legislation, administration, policy making and ultimately upon target individuals and groups.

7. Application of Operant Conditioning Methods to Human Performance Problems. A research program which uses operant conditioning techniques toward the goal of achieving substantial and stable changes in human behavior is in its beginning stages. To embark upon this course of action has required the addition of personnel trained in this particular methodological orientation as well as the modification of laboratory space and equipment. Illustrative of long-range research studies planned for the operant program are the following: Effects of conditioned fear on behavior; environmental conditions controlling aggressive behavior; effects of punishment on performance; the role of "superstitious conditioning" in the control of maladaptive behavior; the stimulus generalization of conditioned fear.

a. Mediating Processes in the Suppressive Effects of Punishment. As an opening wedge into this vast area, a preliminary procedure has begun. The experiment in progress concerns a comparison of the effects of immediate punishment versus delayed punishment. Lever pressing is the ongoing behavior and electric shock is the punishment. It is generally realized that immediate punishment suppresses ongoing behavior more effectively than delayed punishment. The purpose of the experiment is to examine the underlying behavioral processes responsible for this difference in effect.

One possibility to be explored is that during the immediate punishment condition subjects make covert "escape" responses which compete with the ongoing positively reinforced behavior. On the other hand during delayed punishment subjects may not make covert "escape" responses. Covert escape responses can be procedurally transformed into overt escape responses by allowing the subject to release a switch thereby terminating punishment. Thus the hypothesis that absence of a competing response during delayed punishment is responsible for the maintenance of a higher response rate can be experimentally tested. Looked at in another way, the experimental procedure translates conditioned fear or "anxiety" into an observable event, namely, the presence or absence of competing escape responses.

Subjects have been tested on the basic conditions of immediate versus delayed punishment. Difficulty has been encountered in obtaining stable baseline response rates so that it has become necessary to focus work upon developing different reinforcement magnitudes, reinforcement schedules and various pacing stimuli. Testing of the central hypothesis of the experiment must await the outgrowth of a reliable procedure for stabilizing the base response rate.

Summary and Conclusions.

Human performance tolerates description from a variety of vantage points. When in the laboratory, where the independent variable can be precisely excised from the complex world of massively interacting variables and placed under the discretion of the experimenter, it is possible to state functional relationships between the "determiner" and the "determined." We have seen, for example, that the auditory discrimination performance of a sleeping human subject is a function of his EEG stage of sleep; that reinforcement, even under sleep, influences discrimination behavior; that an EEG sleep stage is within limits predictable, given the knowledge of just-prior stages; that performance decrement in vigilance tasks is at least grossly reflected in cortically evoked responses; that at least in some situations preferences influence the information requirements of the decision-maker; that, in the psycholinguistic laboratory studies, the empirical data gathered conform better to certain theoretical models than to others; that children at a remarkably young age are already learning to bypass the universe of phenomenological illusion and beginning to go directly to more "abstract" relationships between objects.

Studies in the areas of sleep, performance decrement, electrophysiology, psycholinguistics and concept formation have been strongly emphasized in this work unit for a considerable length of time. A step-by-step progression of the direction and findings from these researches can be acquired from prior annual progress reports and from resultant publications. Besides a continuing interest in the development of these formerly programmed research areas, the current

year brought with it the addition of personnel new to the work unit and, consequent with this, the commencement of further endeavors in the expanse of human performance.

A new program of laboratory effort which holds considerable future promise is the one devoted to study of the maintenance and control of human behavior by operant conditioning methods. At the other end of the experimental control vs field study continuum is the recent launching of the study of formal social structures and their effects upon past and present human events. Still a third area to be gotten underway this year is the attempt to assess "indigenous" human functioning by the more or less traditional psychometric methods coupled with repeated measures design and longitudinal study.

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Task 01
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12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
005100 Documentation & Info Tech; 007800 Hyg & san; 007000 Geography	07 63	NA	OTHER / DA

16 PROCURE METHOD	17 CONTRACT GRANT a DATE / b NUMBER / c TYPE / d AMOUNT	18 RESOURCES EST	a PROFESSIONAL MAN YEARS	b FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	11	143
		CURRENT FY 66	11	155

19 GOV'T LAB INSTALLATION ACTIVITY
NAME: Headquarters
ADDRESS: U.S. Army Medical Res & Dev Command
Washington, D. C. 20315
RESP INDIV: Donnelly, Lt. Col. H.J.
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20 PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012
INVESTIGATORS PRINCIPAL ASSOCIATE: Gauld, R. L., M.D.; Bowditch, S.H., M.D.
TEL: 202-576-3553 TYPE: DA

21 TECHNOLOGY UTILIZATION: NA

22 COORDINATION: NA

23 KEYWORDS: Inf Dis; Dis of Animals; Dis of Nutr & Metab; Pub Hlth; Epid; San Eng; Geography; climate; prev med; animal kingdom - vertebrates & invertebrates.

24 (U) Tech Objective - Health Data Reports are prepared for the use of Army Medical Service Officers and contain unclassified information regarding the health and sanitary conditions likely to be encountered in foreign countries to whi h they are deployed.

25 (U) Approach - Health Data Reports are unclassified reports of health and sanitary conditions in foreign ountries for the use of Army Medical Service Officers. They describe the geography, climate, religion, living conditions, animals and plants of medical importance, water supply, methods of waste and sewage disposal, diseases present, medical facilities, etc., of each country reported on.

26 (U) Progress (Jul 64 - Jun 65) - To the end of FY 1965 28 Health Data studies had been completed. The last 3 of these were published during the past year: Indonesia, India and North Vietnam. In addition, reports on the following countries are in varying stages moving toward completion: Venezuela, Congo, Panama (revision), Senegal, Mozambique, Burma and Upper Volta.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY	28	29 OSD CODE	30 BUDGET CODE
a COMSEC OR COMSEC RELATED X / b NOT RELATED		AR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
1439d (21)	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) CY / FY	36

DD FORM 1 AUG 64 1498 — Items 1 to 36 identical to NASA Form 1122. REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A025601A806, Military Preventive Medicine

Task 01, Military Preventive Medicine

Work Unit 030, Global Health Data

Investigators.
Principal: Ross L. Gauld, M.D.
Associate: Sarah H. Bowditch, M.D.

Description.

Health Data Reports are prepared for the use of Army Medical Service Officers and contain unclassified information regarding the health and sanitary conditions likely to be encountered in foreign countries to which they are deployed. They describe the geography, climate, religion, animals and plants of medical importance, water supplies, methods of waste and sewage disposal, diseases present, medical facilities, etc., of each country reported on.

Progress.

To the end of FY 1965, 28 countries have been completed. The last three, Indonesia, India and North Viet-Nam, were published during the past year. In addition, the following reports are completed and are waiting final typing: Mozambique, Algeria, Congo (Leopoldville) and Senegal. Cuba is in the process of being revised and is almost completed. Others in varying stages are Venezuela, Colombia, Burma, and the Upper Volta.

PROJECT 3A025601A811
MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 01
Military Medical Research Program S. E. Asia

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6506	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 62156011 3A025601A811 01 108

10b. PRIOR NUMBER/CODE: NONE

11. TITLE: (U) Study of malaria and antimalarial therapy (09)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002300 Biochemistry 012600 Pharmacology 016800 Toxicology	07 64	NA	OTHER | DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	3	100
		CURRENT FY 66	5	150

19. GOV'T LAB/INSTALLATION/ACTIVITY
NAME: Headquarters
ADDRESS: U.S. Army Res & Dev Command
Washington, D. C. 20315
RESP. INDIV.: Meroney, Col W. H.
TEL: 202-0X 61485

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012
INVESTIGATORS PRINCIPAL ASSOCIATE: Knoblock, Lt Col E. C.
Siu, P. M. Ph.D.
TEL: 202-576-3528 TYPE: DA

21. TECHNOLOGY UTILIZATION: Biochemistry; pharmacology.

22. COORDINATION: NA

23. KEYWORDS: Chloroquine; malaria control; malaria; plasmodium; carbon dioxide; metabolism; enzymes; drug resistance.

24. (U) Tech Objective - The basic biochemical requirements for plasmodia will be studied to provide additional information regarding the ability of the malarial parasite to resist chloroquine therapy.

25. (U) Approach - Plasmodium berghei will be used as a test system to establish biochemical characteristics at the enzyme level which will then be used as a means of observing the effects of antimalarial drugs on the malarial parasite.

26. (U) Progress (Jul 64 - Jun 65) - Biochemical reactions of P. berghei have indicated the formation of oxaloacetate by fixation of carbon dioxide to phosphoenolpyruvate. This reaction serves to link the glycolytic pathway and the tricarboxylic acid cycle in organisms. The enzyme catalyzing the reaction has been shown to be sensitive to chloroquine. A study of in vivo degradation of chloroquine in the rat shows two degradation products with approximately one-half of the drug being excreted unchanged. A synthesis of suspected biodegradation products is currently in progress to provide the necessary reference material for identifications.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR CCMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
AR 705-5	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A025601A811, MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 01, Military Medical Research Program S. E. Asia

Work Unit 108, **Study of Malaria and Antimalarial Therapy**

Investigators.
Principal: Lt Col Edward C. Knoblock, MSC
Capt Peter K. Iber, MSC
Patrick M. L. Siu, PhD
Associate: Betty J. Boone, MS
Sp 6 Albert F. Lingle
Sp 5 Michael J. Smith

Description: The basic biochemical requirements for plasmodia will be studied to provide additional information regarding the ability of the malarial parasite to resist chloroquine therapy.

Plasmodium berghei will be used as a test system to establish biochemical characteristics at the enzyme level which will then be used as a means of observing the effects of antimalarial drugs on the malarial parasite. Biochemical reactions of P. berghei have indicated the formation of oxaloacetate by fixation of carbon dioxide to phosphoenolpyruvate. This reaction serves to link the glycolytic pathway and the tricarboxylic acid cycle in organisms. The enzyme catalyzing the reaction has been shown to be sensitive to chloroquine. A study of in vivo degradation of chloroquine in the rat shows two degradation products with approximately one half of the drug being excreted unchanged. A synthesis of suspected biodegredation products is currently in progress to provide the necessary reference material for identifications.

Progress:

Study of Malaria-

a. Fixation of Carbon Dioxide in Plasmodia. Biochemical reactions of plasmodia are being studied in order to learn more about the intermediary metabolism of these parasites. The formation of oxaloacetate by the fixation of carbon dioxide to phosphoenolpyruvate has been the principal study to date since this reaction serves as a link between the glycolytic pathway and the tricarboxylic acid cycle in organisms.

The Plasmodium berghei is cultured in mice and the malarial parasites are harvested from the infected red blood cells of the mice weekly. The infected red blood cells are lysed by ten volumes of cold distilled water and the plasmodia are removed from the lysate by centrifugation at 0°, resuspended and disrupted by sonication. The CO_2

fixation enzyme is then isolated from the crude preparation by the various procedures of enzyme purification.

The CO_2 fixation enzyme is being further purified by various techniques and the properties of the enzyme and the kinetics of the reaction will be investigated. It has been demonstrated that the enzyme is also inhibited by chloroquine. Attempts will be made to elucidate the mechanism of action of chloroquine and similar compounds on the fixation of CO_2 in the presence of a highly purified or homogeneous enzyme. Indications have been obtained to suggest that this enzyme can be stabilized. If this is true, then the enzyme could be purified much further for the question of the stability of the enzyme has been a serious obstacle in its purification.

b. Study of Degradation of Antimalarials in vivo. This investigation is designed to elucidate the mechanism of biochemical degradation of synthetic antimalarial drugs with hope that these data will facilitate design of a new clinical assay method for antimalarial drugs and design and synthesis of new organic compounds for use as antimalarials against the resistant strains of Plasmodium falciparum based on the mechanism of biodegradation and products produced from this process.

Examination of the distribution of chloroquine between RBC, serum and urine and a complete haemotological examination of homogeneous community of Thais who are undergoing antimalarial therapy is currently under way. The examination of the biodegradation products in urine will be used to determine the degradation scheme. This field trip will extend through the summer of 1965.

A phosphorimetric method for the clinical determination of antimalarials without interference from biodegradation products has been designed and put into use. This method allows the determination of chloroquine in the region of 0.004 to 100.0 micrograms per milliliter of body fluid. The method may be used for whole blood as well as serum and urine.

Preliminary experiments with the rat show that when chloroquine diphosphate is given intraperitoneally, two degradation products are formed in the detoxification process with approximately 50% of the drug being excreted unchanged.

C^{14} chloroquine free base labeled in the 3 position of the quinoline ring has been obtained and collaborative experiments have been initiated with the Division of Veterinary Medicine to measure RBC and serum levels in chimpanzees by radioactive counting after dosing the chimpanzee per os. Initial results show that the levels curve is more complex than originally anticipated.

Synthesis of all suspected biodegradation products and the resolution of the optical antipods of chloroquine has begun. Four possible products of metabolism have been prepared and their structure verified by IR and elemental analysis. An initial separation of the d and l forms of chloroquine has been performed. An initial design has been made for three new antimalarial drugs and the synthesis of the first is well on its way to completion.

Summary and Conclusions: The CO_2 fixation enzyme in Plasmodium berghei has been isolated and partially purified. The rate of oxaloacetate formation in the presence of radioactive CO_2 is shown to be inhibited by chloroquine. Further purification of the enzyme is being made so that studies can be made on the properties of the enzyme, the kinetics of the reaction it catalyzes, and the mechanism of action of chloroquine on the enzyme.

Publications: None

RESEARCH AND TECHNOLOGY RESUME	1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6458	CSCRD-103

4. DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT	WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
01 07 65	A. NEW	U	U	NA	QR NF	A. WORK UNIT

10a CURRENT NUMBER/CODE: 62156011 3A025601A811 01 113

10b PRIOR NUMBER CODE: None

11. TITLE: (U) Military medical research program, SEASIA (Thailand) (TH)

12. SCIENTIFIC OR TECH. AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
003500 Clinical medicine	08 63	NA	OTHER I DA

16 PROCURE. METHOD	17. CONTRACT GRANT a DATE / b. NUMBER / c TYPE / d AMOUNT	18 RESOURCES EST	a PROFESSIONAL MAN-YEARS	b FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	32	1335
		CURRENT FY 66	32	1400

19. GOV'T LAB INSTALLATION: ACTIVITY
NAME: Headquarters
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Washington, D. C. 20315
RESP. INDIV: Meroney, Col W. H.
TEL: 202-OX 61485

20 PERFORMING ORGANIZATION
NAME:
ADDRESS: Walter Reed Army Institute of Research
Washington, D. C. 20012
INVESTIGATORS PRINCIPAL ASSOCIATE: Tigertt, Col W. D.
TEL: 202-576-3551 TYPE: DA

21 TECHNOLOGY UTILIZATION	22 COORDINATION
Medicine	NA

23. KEYWORDS: Malaria; diarrhea; venereal disease; scrub typhus.

24. (U) Tech Objective - To conduct the studies required to improve medical capabilities to support limited war ground combat in Southeast Asia.

25. (U) Approach - A balanced laboratory staff is maintained in Bangkok augmented, as necessary, by TDY personnel.

26. (U) Progress (Jul 64 - Jun 65) - A major concern has been with drug-refractory Plasmodium falciparum malaria. Studies have also been performed on liver flukes, venereal disease, diarrhea, and rickettsial diseases.

For technical reports, see Annual Progress Report, SEATO Medical Research Laboratory, Clinical Research Center, Bangkok, Thailand, 15 April 1965.

27. COMMUNICATIONS SECURITY	28.	29 OSD CODE	30 BUDGET CODE
[ ] a. COMSEC OR COMSEC RELATED [X] b. NOT RELATED		AR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
AR 705-5	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands)	36.
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6459	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 65	A. NEW	U / U	NA	QR NF	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 62156011 3A025601A811 01 117

10b. PRIOR NUMBER/CODE: None

11. TITLE: (U) Military medical research program, SEASIA (Vietnam) (VS)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
010100 Microbiology	08 63	NA	OTHER / DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	4	50
		CURRENT FY 66	4	150

19. GOV'T LAB/INSTALLATION/ACTIVITY
NAME: Headquarters
ADDRESS: U. S. Army Medical Res & Dev Command
Washington, D. C. 20315
RESP. INDIV: Meroney, Col W. H.
TEL: 202-0X 61485

20. PERFORMING ORGANIZATION
NAME
ADDRESS: Walter Reed Army Institute of Research
Washington, D. C. 20012
INVESTIGATORS PRINCIPAL ASSOCIATE: Tigertt, Col W. D.
TEL: 202-576-3551 TYPE: DA

21. TECHNOLOGY UTILIZATION: Medicine

22. COORDINATION: NA

23. KEYWORDS: Plague

24. (U) Tech Objective - To conduct the studies required to improve medical capabilities to support limited war ground combat in Southeast Asia.

25. (U) Approach - A joint study with the Pasteur Institute of Vietnam.

26. (U) Progress (Jul 64 - Jun 65) - Primary interest has been in studies of plague in Vietnam including diagnosis, distribution, method of transmission and drug response.

For technical reports, see **Annual Progress Report, USA Medical Research Team (WRAIR) Vietnam and Institute Pasteur of Vietnam, 1 Oct 1964 - 31 Aug 1965.**

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		AR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
AR 705-5	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6462	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 62156011 3A025601A811 01 118

10b. PRIOR NUMBER/CODE: None

11. TITLE:
(U) Military medical research program, SEASIA (WRAIR) (Zoonoses) (09)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
010100 Microbiology; 005900 Environmental biology	08 63	NA	OTHER / DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	c. TYPE: NA	PRIOR FY 65	9	375
		CURRENT FY 66	9	375

19. GOV'T LAB/INSTALLATION/ACTIVITY
NAME
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20. PERFORMING ORGANIZATION
NAME
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Washington, D. C. 20012
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Alexander, A. D. Ph.D.
TEL: 202-576-3518 TYPE: DA

21. TECHNOLOGY UTILIZATION: NA

22. COORDINATION: NA

23. KEYWORDS: Zoonoses; leptospirosis; melioidosis

24. (U) Tech Objective - To define zoonoses that have potential military significance, to determine prevalence, sources and modes of infection, and to devise measures for diagnosis, prevention and control. Studies are coordinated with field units, emphasizing leptospirosis and melioidosis and include: typing of lepto. isolates obtained in epidemiol. investigations; development of a multivalent lepto. vaccine for S. E. Asia; development of cultural and serol. diagnostic technics for melioidosis; surveillance of these diseases in U. S. soldiers.

25. (U) Approach - Conventional cultural, and serological technics are used in epidemiol. studies for lepto. Hamsters and monkeys are used to evaluate lepto. vaccine. Major problems: Unpredictable course of lepto. infection in experimental animals; paucity of sera from documented cases of melioidosis for evaluation of serological tests.

26. (U) Progress (Jul 64 - Jun 65) - Approximately 1630 lepto. isolates from soil and water in Malaya were classified into 27 serotypes. Similar multiplicity of leptospiral types in Thailand and N. Borneo was not apparent from cultural studies. Comparative serologic studies point to the potential value of HL and HA tests for lepto. and melioidosis, respectively, for epidemiological surveys. Improved cultural technics have aided in location of environmental foci of melioidosis in Malaya. The sensitivity of cultural methods has been enhanced further by use of millipore filter technics. Significant lepto. antibodies were found in 2 of 75 U. S. soldiers; 6 others has questionable reactions. The development of more potent leptospiral vaccine, free of foreign protein is now in progress. The virulence of leptospiral strains is being enhanced for vaccine evaluation studies.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		AR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
AR 705-5	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

ANNUAL PROGRESS REPORT

Project 3A025601A811, MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 01, Military Medical Research Program S. E. Asia

Work Unit 118, Mil med rsch prog, SEASIA (WRAIR--zoonoses)

Investigators.
Principal: Col Robert H. Yager, VC (retired)
Associate: Lt Col Thomas G. Murnane, VC; Aaron D. Alexander, Ph.D.; Capt Arnold R. Smith, VC; LaRue B. Evans, B.S.; Mary Baker, B.A.; Arthur Dorsey, M.S.; Albert Warner, Jr.

Description.

Major objectives are to evaluate the real or potential military significance of selected zoonoses--leptospirosis and melioidosis, to characterize etiological agents, to define epidemiological factors and to establish methods of diagnosis, treatment, and control.

Progress.

1. Leptospirosis.

Studies were continued on the serological characterization of leptospiral isolates obtained during the course of an epidemiological study on ecological factors that govern the infectiousness of environmental foci of endemic leptospirosis in Malaysia (See Annual Progress Reports, 1962-1965, USAMRU, Kuala Lumpur and WRAIR). The employed technique for culture typing of strains was similar to that given in previous reports (Alexander, et al., Am. J. Trop. Med. & Hyg., 4:492, 1955; 6:871, 1957) with minor modifications. Strains were screened initially for cross-agglutination reactions with a battery of eight anti-leptospira rabbit sera as follows: autumnalis, alexi, medanensis, javanica, bataviae, pooled australis-grippotyphosa-djasiman, patane, mankarso. On the basis of observed cross-reactions, strains are tested with additional antisera to determine further antigenic relationships. These tests served to identify strains by group. Representative strains of different patterns of cross-agglutination were then selected for comparative serologic studies with type strains.

To date, 1651 strains have been submitted; 228 were received since the time of the last report. Except for 60 isolates from North Borneo, strains were recovered from the Malay peninsula. One hundred and two strains were lost because cultures were contaminated or non-viable. Preliminary culture typing tests were completed on all but 52 strains. Moreover, comparative serological studies were completed on 76 representative types distributed in 10 of 13 recognized serological groups of leptospiras. A summary of culture typing findings on 1489 isolates from the peninsula and 60 isolates from North Borneo are shown in Tables 1 and 2, respectively.

TABLE 1

Classification of 1489 Leptospiral Strains from the Malay Peninsula

Group	Serotype	Type Strain	No.	%*
icterohaemorrhagiae (298 str. = 20.7%)	mankarso	54i	260	18.1
	smithii	75-373	28	1.9
	birkini	M-33	9	0.6
	undetermined	M-563	1	- **
australis	australis	M-85	280	19.5
canicola (194 str. = 13.5%)	jonsis	M-23	96	6.7
	schuffneri	M-9	75	5.2
	sumneri	M-51	12	0.8
	malaya	M-302	11	0.8
bataviae (175 str. = 12.2%)	undetermined	20-96	138	9.6
	bataviae	267	37	2.6
pyrogenes (152 str. = 10.6%)	zanoni	55-271	99	6.9
	biggis	113-561	37	2.6
	hamptoni	29	15	1.0
	abramis	1440	1	- **
hebdomadis (122 str. = 8.5%)	wolffi	H-61	107	7.4
	ricardi	256	14	1.0
	undetermined	193-972	1	- **
autumnalis (120 str. = 8.4%)	bangkinang	Blythe	40	2.8
	rachmat	13KKB	49	3.4
	gurungi	M-12	8	0.6
	undetermined	M-826	12	0.8
	undetermined	M-7	11	0.8
grippotyphosa	grippotyphosa	H-65	65	4.5
javanica (27 str. = 1.9%)	undetermined	33-SB	25	1.7
	coxus	M-807	2	0.1
pomona	pomona	M-514	3	0.2
undetermined	undetermined	267-1348	1	- **
not typed			52	

* Per cent of 1437 strains typed
** Less than 0.1%

TABLE 2

Classification of 60 Leptospiral Strains from North Borneo

Group	Serotype	Type Strain	No.
bataviae (40 str.)	undetermined	20-96	35
	bataviae	267	5
autumnalis (15 str.)	bangkinang	Blythe	11
	gurungi	M-359	2
	undetermined	M-826	1
	undetermined	M-7	1
icterohaemorrhagiae	mankarso	54i	5

The more definitive comparative tests have served to reduce the number of representative types in Malaya from 76 to 28, in North Borneo from 10 to 7. The types found in North Borneo were also present in Malaya. Twenty-two of the representative types were identified on the basis of comparative cross-agglutination tests with recognized serotypes. The remaining six representative types may be new serological entities. Additional cross-agglutinin-adsorption studies with type strains are projected for these strains.

Superficially, the high percentage of strains in the icterohaemorrhagiae (20.7%) and australis (19.5%) groups in Malaya contrasted markedly with the relative frequency of human infections with these types (e.g., approximately 5% each) disclosed in an analysis of 245 cases (Alexander, et al., Am. J. Trop. Med. & Hyg., 6:871, 1957). The correlation and analysis of culture typing findings and epidemiological data now in progress may provide clues on relative infection hazards with various serotypes. In the limited survey in North Borneo, two-thirds of the strains were found to belong to the bataviae group; one-fourth were autumnalis types and the remainder were serotype mankarso.

Additional data were collected bearing on the sensitivity of hamster inoculation methods for detecting leptospiras in Malayan streams and soils (see Annual Reports 1963, 1964). The demonstration of leptospiras by this technique is contingent on the recovery of organisms from dead or moribund experimental animals. Microscopic-agglutination tests with 18 screening antigens were performed on sera from 178 hamsters comprising various groups of animals that survived following inoculation of tissues or fluids from rats, wild pigs, human beings with leptospirosis, and of water from ricefields, mining pools, etc. Partial or complete agglutination reactions at serum dilution of 1:25 were obtained in hamsters as follows: biflexa (patoc) - 7, javanica - 3, djasiman and hyos - 1, celledoni and javanica - 1, djasiman - 1, celledoni - 1. "Non-specific" reactions versus autumnalis (strain Akiyami A) noted in previous tests on experimental and normal

animals (see previous Annual Report) were not seen, probably because a different autumnalis strain--Fort Bragg--was used. The celledoni, javanica, djasiman and hyos reactions were probably significant. Two of the four reactors with these antigens were inoculated with specimens that presumably contained leptospiras--one with urine from a proved carrier rat, the second with culture from a patient. A third hamster inoculated with urine from another proved carrier rat elicited titers against the biflexa antigen--patoc. Whether the patoc titer in this case was significant is questionable. There is no information on non-specific patoc reactions in hamster sera. Particularly noteworthy was the absence of agglutinins in 10 hamsters that were inoculated with known positive or probably positive material. These findings again raise questions as to whether serological tests on hamsters could be used as an indicator of the occurrence of infections. Previously, in this laboratory, no detectible agglutinins (at 1:25 serum dilution) were found in a large proportion of hamsters that survived severe experimental infections with serotypes pomona, canicola, or sejroe. On the other hand, in Malaya, the USAMRU group provoked good agglutinin responses in experimentally infected hamsters.

In the 1964 Annual Report, it was noted that a high prevalence (53%) of leptospiral antibodies was disclosed in sera from 190 indigenes in South Viet Nam (Phu Ban) at USAMRU, Malaya, with the hemolytic test (HL). This high prevalence was not affirmed by microscopic agglutination (MA) tests done at the WRAIR. Most of the HL positives were low titer reactions (e.g., 1:40) within the range of "non-specific" reactions. At the WRAIR and elsewhere, non-specific HL reactions in normal sera or sera from non-leptospirosis cases rarely exceeded 5%. To resolve the significance of these reactions, the sensitivity and specificity of HL procedures at the WRAIR and USAMRU were compared. The activity of the same lot of HL antigen was titrated with a standard antiserum at both laboratories. The WRAIR technique provided a slightly more sensitive test. Comparisons were also made on paired sera from 47 persons, of which, 27 had HL antibody conversions. The HL test findings in the two laboratories were similar. At the WRAIR, the sera were also tested for agglutinins with 18 screening antigens (MA test). MA positive test findings were obtained in 21 of the 27 patients with positive HL tests. All patients negative by HL tests were also negative with MA tests. These findings were not surprising and were reminiscent of previous HL and MA test comparisons. Further comparisons were made with 100 normal sera or sera from non-leptospirosis cases derived from the United States. "Non-specific" HL reactions with U. S. sera were 12%, 9% at 1:40 and 3% at 1:100 serum dilutions. Reactions in the Phu Ban sera at comparable titer levels were 25% and 19%, respectively. Findings to date provide indications that most of the HL findings in the Phu Ban sera were specific; moreover, that the HL test has potential usefulness as an epidemiological tool for population surveys.

Previous attempts to evaluate the efficacy of a multivalent (five serotypes) vaccine in hamsters provided equivocal results employing a rigid criterion, (See Annual Report 1964). It was deemed advisable to continue studies in a larger animal which would permit more detailed clinical, bacteriological, and serological study. Rhesus monkeys, readily available from other investigators at the WRAIR, were selected for study. The animals had been used for Shigella vaccine trials.

Initial efforts were made to produce a predictable course of disease with each of three strains used in the vaccine, 13KKB--an autumnalis serotype, 198--a hebdomadis serotype, and GP58--a grippotyphosa serotype. These strains were highly virulent for hamsters. Hamster-virulent cultures of each of the strains were inoculated subcutaneously (sc) into two monkeys using 0.5 and 1.0 ml doses, respectively. The concentration of organisms in the cultures was approximately 2×10^8 per ml. Monkeys were examined daily for fever and other signs of disease. Blood samples for hematology and serologic tests were taken at four-day intervals. Samples were also taken on alternate days during the first two weeks post-infection for cultural studies. No clinical illness was seen in test animals. All, however, developed titers and leptospiras were recovered from three of six test animals, two of which were infected with the hebdomadis strain, one with the autumnalis strain. The only signs of disease were a reversal of lymphocytic-neutrophil ratio and an enhanced sedimentation rate.

Additional attempts to incite frank disease in monkeys were made with the most virulent (for hamsters) strain--13KKB. In these experiments, the inocula consisted of 4 to 12 ml of culture or 4 to 6 ml of 30% suspension of infected hamster liver tissue, with hamster lethal doses ranging from 8×10^5 to 2×10^6. Various routes of infection were tried. Although leptospiremia could be demonstrated and high titer antibodies were provoked, there was no remarkable or consistent pattern of clinical disease other than an elevated sedimentation rate which generally appeared in three days, reaching maximum levels in the order of 20 to 40 mm per hour at four to five days and persisting to the tenth day post-infection. Reversal of lymphocyte-neutrophil ratio was also evident in these groups of infected monkeys. Attempts to produce frank disease in a splenectomized monkey were unsuccessful. More virulent strains were sought among recently isolated Malayan leptospiras for further infectivity studies. A bataviae (strain 1415) and an autumnalis (strain 1456) were selected for this purpose. Cultures of each of the strains with hamster LD50 titers of 10^{-4} to 10^5 per ml were given intraperitoneally (ip) to rhesus monkeys in a 30 ml dose. Three animals were inoculated with each strain, three others with a combination of the two cultures. The mixture was tried in view of the reported enhancement of pathogenicity of strains in experimental multiple infection. Findings in monkeys were similar to those in previous experiments except for one animal that developed jaundice of three days' duration. Infectivity studies in monkeys have been discontinued. Current efforts are being made to enhance the virulence of other serotypes for projected vaccine evaluation studies. Attempts are being made to grow vaccine strains in a medium containing human albumin in lieu of rabbit serum to develop a more potent vaccine. Good growth has been obtained in initial passages of vaccine strains in the experimental medium.

A review of serological survey data on livestock from the SEATO laboratory, Thailand, raised questions on the sensitivity and specificity of their microscopic-agglutination tests. Particularly noteworthy was, firstly, the relatively low prevalence rate of leptospiral antibodies in survey animals with a high incidence of infections; secondly, the predominantly low titer reactions in animals in infected herds, also in known carrier animals seen in abattoirs. Duplicate serological tests were, therefore, conducted at the WRAIR on 140 positive bovine sera. Except for 10 sera, the results at the WRAIR served to validate the SEATO laboratory findings. It was apparent from tests conducted at the WRAIR that the strain of pomona previously sent to SEATO for use as an antigen was not as sensitive as local or other strains for detecting leptospiral agglutinins in cattle. The substitution of another pomona strain in the screening antigens has been recommended.

The source and preliminary culture typing findings on 106 leptospiral isolates submitted from the SEATO laboratory are summarized in Table 3. Forty-eight were submitted during the period of this report (See Annual Report 1964, 1965, SEATO Laboratory, for additional information on isolates).

TABLE 3

Source and Culture Typing Findings on 106 Leptospiral Isolates from Thailand

Source	Number Isolated	No. Typed	Group Classification				
			hyos	canicola	pomona	autumnalis	javanica
cattle	4	1			1		
swine	46	46		1	45		
rodents	38	9					9
buffalo	1	1				1	
dog	8	0					
water	5	0					
unknown	3	2	1		1		

In a previous serological survey of leptospirosis in SF troops in Viet Nam, no agglutinins were detected in sera from 79 soldiers (see Annual Report, 1964). During the period of this report, paired sera from a second group of 75 SF soldiers (FUO cases) were tested. In addition to microscopic agglutination (MA) tests, the hemolytic (HL) and CF (Patoc antigen) tests were used. Significant agglutinin reactions were obtained in two soldiers. In one case, titers (1:100) were stationary in early and late sera; in the other soldier, there was a conversion from negative to positive (1:400 titer). Serum from the latter soldier also had high HL antibodies and was positive with the CF test. No other reactions with the HL test were elicited. Two other soldiers had partial or questionable agglutinins in their second serum specimens. CF positive reactions were obtained in the first serum samples from these soldiers. Positive CF reactions were also seen in sera from six other soldiers; three of the reactions were in early sera, the remainder in late sera. The significance of the CF reactions is not known. The data afforded evidence of one unequivocal case of leptospirosis in 75 CF soldiers, attributable to exposure in Viet Nam.

2. Melioidosis.

Collaborative studies on melioidosis were continued with the USAMRU in Malaya. At USAMRU, attention was initially directed to the location of natural foci of infection and to the recognition of cases among FUO cases. Attempts to locate cases have been unproductive; however, efforts to locate natural foci of melioidosis organisms were remarkably successful. To date, 359 strains suspected to be Pseudomonas pseudomallei were isolated from various Malayan soils and waters and submitted to the WRAIR for verification of identities. The use of selective media developed at the WRAIR contributed significantly to detection of melioidosis organisms in soil and water. Two hundred and seventy-nine were affirmed to be P. pseudomallei. The isolates for the most part were obtained through hamster inoculation techniques. The limiting concentration of organisms in samples for their detection by animal inoculation techniques is 1-10 per ml.

Attempts were made to develop a simpler, more sensitive direct cultural technique applicable in the field by the use of a modified Millipore filter technique for the bacteriological analysis of water. A selective medium, previously developed, was utilized (brain heart infusion agar, pH 6.8, containing 3% glycerol, 20 U/ml penicillin, 200 U/ml polymyxin, and 400 ug/ml of cycloheximide, and crystal violet in final concentration of 1:200,000). Tests were conducted with soil and H_2O specimens obtained from streams, ponds, and swamps in the Washington area. Soil samples were mixed with nine parts of H_2O, filtered through coarse paper filter to remove debris and coarse particles, and filtrates were used for bacteriological studies. Antibiotics (20 U penicillin, 50 U polymyxin and 400 µg cyclohexamide per ml) were added to 50 ml portions of test samples which in turn were used for serial dilutions of a known concentration of P. pseudomallei. Each portion was then passed through a millipore membrane filter (porosity 0.3 micron). The filter pad was placed on an agar plate containing the selective media. Cultures were incubated at 37° C for 48 - 72 hours. It was found that as few as one to ten P. pseudomallei organisms could be readily discerned and recovered from 50 ml water or soil samples containing approximately 5 x 10^5 contaminating microorganisms. Higher concentrations of contaminating bacteria served to decrease the sensitivity of the millipore filter cultural technique for detecting melioidosis organisms. The applicability of the in vitro technique is now being tested in Malaysia.

Additional information was obtained on the sensitivity and specificity of a hemagglutination test (HA) for melioidosis. It was previously found that the HA test for melioidosis was markedly more sensitive than the complement fixation (CF) test (Nigg) for detection of antibodies in human sera. In a survey of 91 Malaysian engineers, 34 were positive with the HA test in contrast to 7 positives found with the CF test. Tests conducted on 130 human sera from the U. S. yielded 5 low-titer HA reactions (1:5). Furthermore, only one reaction was obtained in HA tests conducted on paired sera from 70 SF troops. The few "non-specific" reactions in the latter two series provided evidence that the HA test reactions, particularly at dilution levels of 1:10 and greater, were specific. The CF findings in 70 SF troops yielded no significant positive reactions.

To simplify the test for melioidosis with the HA antigen, the feasibility of using latex particles in lieu of erythrocytes as a matrix for the reaction is being investigated. The HA antigen is readily adsorbed onto latex particles. Optimum concentration of HA antigen for sensitizing latex particles was established. In preliminary tests with anti-melioidosis sera, the latex agglutination test was slightly less sensitive than the HA test. Differences in titers were two-to four-fold.

Summary and Conclusions.

1. Leptospirosis.

Serological characterization studies were continued on Malaysian leptospiral strains isolated during epidemiological studies of factors that govern the infectiousness of environmental foci of endemic leptospirosis. Culture typing tests have been completed on 1437 strains from Malaya and 60 strains isolated in North Borneo. More extensive comparative serological tests have reduced the number of representative types in Malaya from 76 to 28, in North Borneo from 10 to 7. The types found in North Borneo were also present in Malaya. Twenty-two of the representative types have been definitively identified; the remainder may be new serological entities. These types were distributed among 10 of the 13 known groups of leptospiras. The distribution of isolates from the Malay Peninsula by serogroup was as follows: icterohaemorrhagiae - 20.7%, australis - 19.5%, canicola - 13.5%, bataviae - 12.2%, pyrogenes - 10.6%, hebdomadis - 8.5%, autumnalis - 8.4%, grippotyphosa - 4.5%, javanica - 1.9%, pomona - 0.2%. The percentages of icterohaemorrhagiae and australis types were relatively high compared to distribution of infection with this type previously seen in troops. In North Borneo, bataviae types comprised two-thirds of the isolates, one-fourth were autumnalis types, the remainder were serotype mankarso.

Additional serological data collected on hamsters used in Malaya for isolation of cultures posed questions on the accuracy of serological procedures to determine infections in hamsters in view of negative serological test findings in hamsters that were at high risk, or, that were known to have received infectious material. Comparative hemolytic test (HL) findings on selected sera from cases and normal persons served to validate the significance of low titer (1:40) HL reactions and pointed up the potential usefulness of the HL test as an epidemiological tool for population surveys.

Attempts were made to incite a predictable leptospiral disease in rhesus monkeys with different serotype strains used in a projected multivalent vaccine. The purpose was to obtain a suitable larger test animal for evaluation of the vaccine. Monkeys could be infected, but failed to show frank disease signs even when inoculated with massive doses of highly hamster-virulent cultures or tissue suspensions. Further efforts to develop a test system in monkeys were discontinued. Another species (e.g., dog) will be examined as a suitable test animal for vaccine evaluations. Efforts are continuing to enhance virulence of different leptospiral serotypes for projected vaccine evaluation studies and to develop a more potent vaccine by use of a medium containing human albumin in place of rabbit serum.

Duplicate serological tests at the WRAIR served to affirm unusual findings of an extensive serological survey of livestock conducted at the SEATO laboratory in Bangkok, viz., predominant low titer agglutinins in infected herds and livestock carriers. The use of a more sensitive pomona antigen was recommended. Culture typing tests were completed on 59 of 106 leptospiral strains isolated in Thailand. All but 1 of 47 isolates from cattle and swine were pomona; the exception was a canicola type from swine, a strain of autumnalis was isolated from a buffalo; 9 rodent strains were in the javanica group. An additional serotype isolated was hyos (host not specified).

Serological tests for leptospirosis were conducted on a second group of paired sera from 75 SF troops. Significant rise in agglutinins was found in one soldier. A second soldier had significant stationary titers. Questionable CF or agglutinin reactions were elicited in eight other soldiers.

2. Melioidosis.

To date, 279 of 359 strains isolated from various soils and waters in Malaysia were affirmed to be Pseudomonas pseudomallei. Further efforts were made to improve cultural and serological techniques. A relatively simple direct cultural technique for isolation of organisms from soil and water was developed to increase sensitivity of detection methods. The technique is a modification of Millipore filter technique for bacteriological examination of water and is now being tested by USAMRU in Kuala Lumpur. A hemagglutination (HA) technique for serological diagnosis was evaluated further and was found to be markedly more sensitive than currently used CF tests. The specificity of the test was good; when tested with 130 human sera from the United States, only 5 low-titer "non-specific" reactions were obtained. In the second series of sera from the SF troops, no CF or HA antibodies to melioidosis were found. The HA procedure is being simplified by use of latex particles in lieu of erythrocytes as the matrix for the agglutination reaction. Studies on the latex agglutination test are now in progress.

Publications.

Baker, H. J., Baker, M. F., Alexander, A. D., and Yager, R. H. Distribution of pathogenic leptospiras in Malayan jungles. Proc. 64th Annual Meeting of the Am. Soc. Microbiol., Washington, D. C., May 1964, p 145.

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24. (U) Tech Objective - The aim of research in malaria is to provide knowledge which can be used ultimately to develop new means of treating the infection caused by drug-resistant strains of the parasite.

25. (U) Approach - Through planning and experimentation base lines are determined and laboratory models are developed which can be used by scientists in other disciplines.

26. (U) Progress (Jul 64 - Jun 65) - Splenectomized chimpanzees were shown to be susceptible to Plasmodium falciparum and the course of the infection in this host is being studied to determine if this system will serve as a model for refined chemotherapeutic studies. Studies on the physiological pathology of P. berghei in mice were completed and quantitative determination of base lines for various rates of infection were continued. A survey of the susceptibility of North and South American rodents was concluded, and the study on the effect of ambient temperature on the infection in mice was begun.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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ANNUAL PROGRESS REPORT

Project 3A025601A811 MILITARY MEDICAL RESEARCH PROGRAM, S.E. ASIA

Task 01, Military Medical Research Program S.E. Asia

Work Unit 120, Mil med rsch prog, SEASIA (WRAIR-Malaria)

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Description.

Malaria is a disease of considerable military importance especially in South East Asia. The object of this task is to obtain needed information on the pathogenesis, physiology, and immunology of malaria.

Progress.

1. The natural history of Plasmodium berghei infection in mice.

Although numerous studies have been conducted in mice injected with P. berghei, little information was available on some quantitative relationships of this infection. Therefore, studies were undertaken to provide some of the lacking basic information on blood induced infections with primary emphasis on size of inoculum, parasitemia levels, routes of inoculation, numbers of erythrocytes, effects of ambient temperature on the viability of the parasites, etc.

a. Parasitic densities from inoculation to death. Table I summarizes results of an experiment characterizing the course of standard P. berghei infections produced by intravenous injection of ICR strain mice with 10^7 parasitized red blood cells. Such infections were uniformly fatal. In this case all 20 experimental mice died within 8 days after infection. Another indication of the fulminating nature of infection is shown by the rapidly progressive parasitemia; on the second day of infection only 6% of the RBC were parasitized, but by the 5-6th day over 80% were parasitized. Although percent parasitemias increased up to the times of death the largest number of parasitized

RBC/cu mm were noted on the 4th day of infection and by the 5-6th days this numbe · began to decrease. This drop in total numbers of circulating parasites is due to the marked decrease in total numbers of circulating red blood cells. This anemia began on the 3rd day of infection, at which time infected animals had on the average of 1.5 million RBC/cu mm less than the controls. The anemia became progressively more marked

TABLE I

The course of Plasmodium berghei infections produced by intravenous injection of 10^7 parasitized RBC into 20-25 gm ICR mice

	Day after intravenous infection with 10^7 parasitized RBC						
	1	2	3	4	5	6	7
No. RBC/cu mm (x 10^6)							
Normal mice	9.5	8.8	9.3	8.7	8.6	9.7	
Exper. mice	9.2	8.8	7.8	4.7	3.9	1.9	
Normal - Exper.	-0.3	-0.0	-1.5	-4.0	-4.7	-7.8	
% RBC infected		6	30	72	80	83	
No. infected RBC/cu mm(x 10^6)		0.5	2.3	3.4	3.1	1.6	
% pluriparasitized RBC	2	6	10	25	47		
No. parasites / infected RBC*							
1	+*	+	+	+	+		
2	+	+	+	+	+		
3		+	+	+	+		
4				+	+		
5					+		
Deaths	0	0	0	0	0	65%	30%

* +means that at least half of the mice had some RBC parasitized with the indicated number of organisms/RBC

until by the 6th day infected mice had less than 2 million RBC/cu mm, a loss of almost 8 million RBC/cu mm. As the total number of RBC decreased, more and more of the infected RBC had multiple infections and by day 6 approximately half of the infected RBC had more than one parasite. This tendency toward pluriparasitism is accentuated by the progressive increase in the average number of parasites per RBC. Additional work has shown that when blood from day-old infections was used as an inoculum for experimental animals the latter underwent an infection significantly different from that in other animals injected with inoculum prepared from donor mice infected for only 3 days. Accordingly, all subsequent work has been standardized using inocula prepared from mice infected for only 3-4 days.

b. Route of inoculations. Additional work was done to evaluate the reproducibility of infections produced by intravenous injections of parasites and to compare such infections with others produced by intraperitoneal injections of parasites. In each of 12 different experiments infected blood was collected at room temperature from donors infected for 4 days; a standard inoculum, 10^7 parasitized RBC/in 0.2 ml was prepared and injected within one hour of collection (Table II). Animals injected intravenously died on the average 7.3 days after infection as compared with an average of 7.8 days for mice injected intraperitoneally. There was some indication in individual experiments that survival times were more variable in mice injected intraperitoneally and that this may have accounted at least in part for the significant increase in survival time over that for mice injected intravenously. In a separate series of 7 experiments infected blood was drawn and kept at 4°C until injected. In this case average survival times for both intravenous and intraperitoneal infections were reduced by approximately one day, but again mice infected intravenously died sooner than mice infected intraperitoneally. Evidence for greater variability (as shown here by the range of means) in survival times of intraperitoneally infected mice was more marked than in the previous series.

Two additional experiments were done to compare infections produced by intravenous vs. intraperitoneal routes of inoculation. In these experiments mice were injected with graded inocula ranging from 10^3 to 10^7 parasitized red blood cells and the results from replicate experiments were pooled (Table III). These results tended to confirm those made in the previous table, i.e., intravenous infections were less variable and produced death more rapidly than intraperitoneal infections. A comparison of average survival times for the two treat-

TABLE II

A comparison of mean survival times for groups of mice injected intravenously vs. intraperitoneally with 10^7 parasitized red blood cells

Inoculum collected at	No. of Experiments	Mean survival time in days (range) for mice injected	
		Intravenously	Intraperitoneally
Room temperature	12	7.33 (6.5-9.1)	7.80 (6.1-9.0)
4° C	7	6.23 (5.6-6.6)	6.93 (6.2-8.2)

TABLE III

Comparison of i.p vs. i.v. infections produced by injections of inocula ranging from 10^3 to 10^7 parasitized red blood cells per cu. mm.

Size of inoculum (No. parasitized RBC)	Average day of death* (range) for mice injected	
	Intravenously	Intraperitoneally
10^7	6.05 (5-8)	7.15 (6-10)
10^6	7.10 (6-9)	8.85 (7-12)
10^5	8.05 (7-10)	9.40 (8-12)
10^4	8.80 (8-10)	11.00 (8-14)
10^3	9.55 (9-11)	11.11 (10-14)

* Individual means calculated for 20 mice

ments shows that for any given inoculum mice injected intraperitoneally died on the average of from 1.1 to 1.7 days later than mice injected intravenously but that this delay was not apparently related to inoculum size. Individual mice injected intravenously died from 6-11 days after infection, the exact time depending in part on the size of the inoculum, but for any given dosage all mice died within a 2-3 day period. On the other hand, mortalities in the intraperitoneally groups were more variable; for any given inoculum individual mice died over a period of 4-6 days. Data on parasitemias reached 5-10%, they increased very rapidly and averaged 70-80% within a few days. The rate of this increase for mice injected intravenously was relatively constant regardless of the size of the inoculum (10^7 to 10^3); on the other hand, the beginning of this increase was progressively delayed as the inoculum size decreased, approximately a one-day delay for each one log decrease in inoculum size. For an inoculum of any given size, parasitemias in intravenous infections began their rapid increase approximately a day before those in mice injected intraperitoneally, and there may have been a tendency for more variability in individual parasitemias in the latter group.

c. Viability of parasites in inoculum. A number of experiments were done to evaluate the effect of storage on the viability of parasites in infected blood. Inocula were prepared in concentrations of 10^7 parasitized red blood cells per 0.2 ml and stored at either room temperature or refrigerator temperature for 1 to 6 hours. Inocula prepared and injected immediately, i.e., without storage, killed control animals within the expected 6-8 days. On the other hand, Table IV shows that incubation of inocula at either room or refrigerator temperatures had an adverse effect on the viability of parasites as reflected by the increase in mean survival time beyond that of the controls. This loss of viability became progressively more pronounced with prolonged storage of parasites, but there is a definite indication that lowered storage temperature tended to minimize this effect.

d. Ambient temperature. The effect of ambient temperatures on the mortality of infected animals was studied in four experiments.

Three groups of 15 mice each of the same strain and weight were maintained at the following ambient temperatures: 17°C, 23°C, and 34°C. After two days at the listed constant temperatures all the animals were injected intraperitoneally with one million parasitized red blood cells each. Blood smears were taken for parasite counts

TABLE IV

Loss of viability of P. berghei inoculum association with prolonged pre-Injection survival time of test mice over that of control mice*

Inoculum Stored at	Prolonged survival time in mice infected with inoculum stored for			
	1 hour	2 hours	4 hours	6 hours
Room temperature	0.25 days	0.85 days	1.80 days	1.50 days
4° C	0.15 days	0.55 days	0.82 days	1.00 days

* Control mice died 6-8 days after infection

TABLE V

The influence of ambient temperature on survival time of mice infected with Plasmodium berghei

Ambient Temperature	Average survival time (in days) of mice infected with 1×10^6 parasitized RBC's				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Average
34°C	7.25	7.25	7.00	7.25	7.25
23°C	9.00	8.00	7.50	8.25	8.25
17°C	10.50	10.00	8.50	9.75	9.75

at regular intervals during the course of infection and the time of death was recorded. In four experiments the average survival time at 34°C, 23°C, and 17°C was 7.25, 8.25 and 9.75 days, respectively. (Table V)

Since appreciable differences in mortality were observed consistently at different temperatures, attempts will be made to determine what factors produce these changes.

2. The range of host susceptibility to Plasmodium berghei infection.

Work has been done to further define the range of host susceptibility to Plasmodium berghei in an attempt to find a susceptible host 1) of a sufficient size so that relatively large amounts of parasitic material could be harvested and 2) in which viable gametocytes would be produced in numbers permitting experiments on natural transmission of the infection by mosquito vectors.

Several animals for each of 9 North American and South American rodent species were inoculated with large numbers of mouse red blood cells infected with P. berghei. Repeated examinations (both thick blood smears and mouse subinoculation) failed to show any evidence of infection in the following South American rodents: capybara, agouti, nutria, paca and chinchilla and three other species of rodent, guinea pig (intact and splenectomized), woodchuck and prairie dog. Intact and splenectomized rabbits were similarly resistant to infection. On the other hand, muskrats were readily infected with P. berghei.

Muskrat infections with P. berghei were characterized by rapidly progressive parasitemias and death within 2 weeks (Table VI). Terminally, parasitemias reached 24-75% with polyparasitism common but reticulocytosis was not prominent. Muskrats also produced large numbers of gametocytes which were infective for both Anopheles quadrimaculatus and A. stephensi mosquitoes. A single attempt to infect muskrats by injection of macerated, infected mosquitoes was not successful, but this is being repeated.

TABLE VI

P. berghei in the Muskrat

Number	Pre-patent Period (Days)	% Parasit.	Survival time (Days)
1	4	72	10
2	3	38	8
3	3	68	9
4	1	25	5
5	ND	33	9
6	4	76	12

Attempts were also made to infect intact and splenectomized Rhesus monkeys with large numbers of P. berghei-infected mouse red blood cells (Table VII). Although the two intact monkeys showed no microscopically discernible parasitemia, one of these was demonstrated by mouse sub-inoculation to be subpatent for at least 3 weeks after injection of infected blood. Splenectomized monkeys were more susceptible to infection, this is indicated by the fact that all four of animals employed were patent for 7 to 15 days after prepatent periods ranging from 6 to 10 days. Peak parasitemias for these animals ranged from 0.02 to 0.06%. Two of these animals were examined for chronicity of infection and were shown by mouse subinoculation to be subpatent for at least 7 weeks, i.e., approximately 4 weeks after parasites were no longer visible in the blood. However, only one of these animals had a recrudescent parasitemia.

P. berghei was passed in splenectomized monkeys to determine whether or not such treatment would yield a parasite strain better adapted to this host. With continuous intravenous passage in monkeys (Table VIII) there was a tendency toward lengthening of patent periods as well as toward an increase in peak parasitemias. After peak parasitemias had been achieved, parasite numbers diminished to the point where the organism could no longer be demonstrated microscopically. However most of these same monkeys later underwent parasitic recrudescences.

3. The pathophysiology of Plasmodium berghei infection in mice.

In spite of the enormous amount of literature available on malaria, very limited information exists on the physiological changes produced in the host by the malarial parasites.

Recent investigations with ultramicro biochemical techniques provided information on the amounts of some serum constituents in uninfected mice. These techniques can be used to great advantage in obtaining estimates of serum components in mice with parasitic infections.

Therefore studies were set up to determine some of the detectable biochemical changes occuring in mice following infection with P. berghei.

Young male albino mice, Inbred Charles River (ICR) strain,

TABLE VII

Plasmodium berghei infections in Rhesus monkeys injected with 10^9 parasitized mouse red blood cells

Course of infections as determined by										
Microscopy			Mouse subinoculation							
Prepatent Period (days)	Patent Period (days)	% Parasitemia at peak	Monkey blood positive on days							
			1	6	12	20	43	65	92
Intact Monkeys										
-	-	-	+	-	-	-	-	-	-
-	-	-	+	+	+	+	-	-	-
Splenectomized Monkeys										
7	8	0.02	+	+	+	+	+	-	-
10	7	0.03	+	+	+	+	+	-	-
6	15	0.05							
7	10*	0.06							

* This animal recrudesced on day 27.

TABLE VIII

Continuous passage of P. berghei in splenectomized monkeys

Number	Patency (Initial)	% Parasit.	Recr.
1	15	.02	+
2	11	.03	+
3	7	.08	+
4	7	.02	+
5	9	.02	+
6	8	.08	+
7	12	.12	-
8	12	.20	-
9	13	.20	+
10	12	.03	+
11	11	.46	-
12	21	.10	+
13	13	.23	+
14	10	.27	-
15	14	ND	?

weighing between 20 and 24 grams at the beginning of each experiment were used. The experimental mice were injected intraperitoneally with approximately ten million parasitized red blood cells (RBC's) harvested from the same strain of mice seven days after inoculation with approximately ten million parasitized RBC's. Parasitized cell counts were done by a direct nile blue sulfate hemocytometer method described by Hillyer and Diggs. Sodium citrate was used as a diluent and anticoagulant. Control mice were inoculated with an amount of sodium citrate diluent and uninfected erythrocytes equivalent to that which was used in the experimental animals. Parasitemia levels were determined on the fourth day following inoculation by counting the number of parasitized cells in 200 RBC's on a thin film, and an RBC count. The percentage of parasitized cells was multiplied by the RBC count giving an approximate level of parasitized cells per cubic millimeter of blood.

Throughout these studies, mice having on the fourth day of infection two million or more parasitized red blood cells per cubic millimeter of blood were called "heavily" infected; while those with less than two million parasitized cells were considered "moderately" infected.

When less than 60 microliters of blood were needed, the mice were tested prior to infection and again 2, 4 and 6 days following infection (Tables IX and X). In order to reduce the effect which loss of blood might have on the course of infection and/or on the results of subsequent biochemical tests, only 2 or 3 tests were performed in each experiment and no more than 60 microliters of blood were withdrawn from the animals at any one time. For analyses which required more than 60 microliters of blood, the animals were bled only once, either prior to infection or at 2, 4 or 6 days after infection (Table XI).

The individual biochemical values on serum from fasting mice before and after infection with P. berghei have been summarized in Tables IX, X and XI.

Total protein - No marked changes in the amount of total protein occurred in the 67 mice during the first four days following infection. A reduction in total protein was observed on the sixth day in all groups; the reduction being greatest in the mice with heavy infections.

TABLE IX

Serum values in mice bled prior to and again 2, 4 and 6 days following infection with *P. berghei*

Test	Parasitemia ($X10^6/mm^3$)	No. of Mice	Mean Values (Days)			
			0	+2	+4	+6
Total Protein, gm%	>2.0	20	6.5	6.7	6.9	4.9
	<2.0	18	6.4	6.7	6.9	5.5
	Contr.	29	6.0	6.2	6.5	5.5
Non Protein Nitrogen, mg%	>2.0	20	38	41	40	57
	<2.0	12	40	40	37	46
	Contr.	9	37	38	36	32
SGP Transaminase, units	>2.0	24	27	46	72	112
	<2.0	8	30	58	47	80
	Contr.	9	27	27	33	23
SGO Transaminase, units	>2.0	21	81	121	150	210
	<2.0	7	75	123	119	167
	Contr.	10	81	75	85	61
Glucose, mg%	>2.0	14	63	81	22	65
	<2.0	85	68	72	65	50
	Contr.	27	71	78	80	154
Alkaline Phosphatase, units	>2.0	25	4.2	3.7	3.8	2.1
	<2.0	7	3.2	2.7	2.8	1.6
	Contr.	10	4.5	3.8	3.8	3.0
Phosphorus, mg%	>2.0	12	9.9	9.4	9.3	7.9
	<2.0	5	10.0	8.8	9.1	8.8
	Contr.	4	10.5	10.0	9.4	9.5

TABLE X

Serum electrophoretic values in mice bled prior to and again 2, 4 and 6 days following infection with P. berghei

Test	Parasitemia ($X10^6/mm^3$)	No. of Mice	Mean Values (Days)			
			0	+2	+4	+6
Albumin, gm%	≥2.0	19	3.4	3.6	3.7	2.6
	<2.0	16	3.5	3.4	3.6	2.5
	Contr.	9	3.8	3.9	4.0	3.4
$Alpha_1$, gm%	≥2.0	19	0.6	0.6	0.6	0.4
	<2.0	16	0.6	0.6	0.6	0.5
	Contr.	9	0.6	0.5	0.5	0.4
$Alpha_2$, gm%	≥2.0	19	0.8	0.7	0.8	0.5
	<2.0	16	0.7	0.8	0.8	0.5
	Contr.	9	0.8	0.7	0.8	0.6
Beta, gm%	≥2.0	19	1.4	1.5	1.6	1.2
	<2.0	16	1.4	1.5	1.6	1.2
	Contr.	9	1.4	1.2	1.1	1.0
Gamma, gm%	≥2.0	19	0.4	0.4	0.3	0.3
	<2.0	16	0.3	0.4	0.4	0.3
	Contr.	9	0.3	0.5	0.3	0.3

TABLE XI

Serum values in uninfected mice and in mice sacrificed 2, 4 or 6 days after infection with *P. berghei* (>$2X10^6/mm^3$)

Test	Total No. of Mice	Mean Values (Days)			
		0	+2	+4	+6
Bromsulphthalein, % Retention	81	5.2	5.8	11.0	35.5
Bilirubin (Direct), mg%	91	0.17	0.11	0.23	0.28
Bilirubin (Total), mg%	46	0.6	ND	ND	0.8
Calcium, mg%	41	9.5	ND	ND	9.6
Creatinine, mg%	81	1.1	ND	ND	1.1
Carbon Dioxide, mEq/L	43	18.9	ND	ND	21.2
Chloride, mEq/L	83	112.1	ND	ND	111.0
Sodium, mEq/L	45	150.7	ND	ND	147.7
Potassium, mEq/L	45	5.3	ND	ND	5.8
		Positive over Negative (Days)			
Cephalin Flocculation 1+ to 4+	72	0/15	0/15	6/9	27/0

ND - Not Done

Non-protein nitrogen - No deviations from the normal range were observed in the 41 mice during the first four days following infection. On the sixth day, however, there was a marked increase in NPN in the infected animals. This increase was greater in mice which had been heavily infected than in the mice with a relatively lighter infection.

Serum transaminases - As observed in the 60 infected mice used for the SGP-T and SGO-T determinations, dramatic increases in both transaminases were observed as early as 2 days following infection. Increasingly significant differences between infected mice and their normal controls were observed up to the end of the experiment.

Glucose - A total of 126 mice was used in five experiments. Markedly lower fasting glucose levels were observed in the heavily infected mice 4 days following infection. Mice with lighter infections had serum glucose values which were somewhat intermediate. Six days following infection essentially normal values were observed in the infected mice. This contrasted vividly with significantly higher fasting glucose levels which were observed in the controls. The results of the five experiments showed basically identical trends.

Alkaline phosphatase - A moderate reduction in alkaline phosphatase was observed in the 32 infected mice 6 days following infection.

Phosphorus - Observations obtained in all of the 21 mice used were always within the normal range, regardless of infection.

Electrophoretic values - A decrease in albumin values was observed in the 35 infected mice as early as 2 days following infection. This difference increased with time and reached its maximum at the end of the experiment, six days after infection. A small decrease in albumin levels was observed 6 days following infection in the 9 uninfected controls. Nonetheless, there was a marked difference between infected and uninfected animals. No alteration in the globulins was observed between infected and uninfected mice.

Bromsulphthalein (BSP) - In the 51 infected mice used in the BSP retention tests, marked increases were observed as early as 4 days following infection. This increase progressed with infection and at the end of the experiment, 6 days after infection, an average increase of retention of nearly 700 percent was observed.

Bilirubin (direct and total) - A moderate average increase in direct bilirubin values was observed in the 32 mice with P. berghei 6 days following infection. Although some increase was also observed as early as 4 days after infection, the values in most of these animals at that time were still within the normal range. No consistent changes in the total serum bilirubin values were detected.

Calcium, Creatinine, Carbon Dioxide, Chloride, Sodium and Potassium - A total of 338 mice was used in these experiments. Observations obtained in all of the mice 6 days following infection were within the normal range in spite of the very high parasitemias present.

Cephalin flocculation - Whereas all of the mice had negative values prior to and 2 days following infection, positive cephalin flocculation reactions were registered in 6 of 15 infected mice 4 days after infection and in all of 27 mice 6 days after infection.

These studies indicate that ultramicro biochemical techniques can be used to great advantage in mice infected with P. berghei to gain some insight on the changes produced in the serum constituents by malarial infection. It is obvious that the interactions of the malarial parasites and their hosts have their counterpart in somatic alterations which may find reflection in biochemical changes in the body fluids. Therefore, correlation of clinical observations with multiple quantitative determinations of the serum components may contribute to the diagnosis and prognosis of this infection, and may even permit an early assessment of the effect of various chemotherapeutic agents.

The reduction in serum protein which could be accounted for entirely by the reduction in albumin confirms observations of previous workers. More recently, other workers have confirmed the decrease in albumin levels in the active stages of human malaria infection and the restoration of normal levels after successful therapy. Albumin is formed slowly and its synthesis is disturbed in liver dysfunction. The lower values of albumin in malaria may thus be associated with the hepatic damage in the disease.

Our studies revealed a dramatic drop in fasting serum glucose levels in the infected animals as early as 4 days following infection. However, by the sixth day of infection, most of these animals had recovered and essentially normal values were observed. The unexpected results prompted us to repeat these experiments five times, with similar results.

A disturbance of the glycogenetic function of the liver was observed in malaria by Ruge. The literature on the blood sugar in malaria was reviewed by Sinton and Kehar and by Fulton and Maegraith. Both increases and decreases in blood sugar may occur in human malaria, depending on the stage of the disease in which the blood is examined and on the severity of the infection. In human infections of P. vivax and P. falciparum, a rise in serum glucose levels during fever was observed. In a case of falciparum malaria values up to 206 mg percent were reported. The rise of the blood sugar during the paroxysm was interpreted as being due to an increased glycolysis brought about by the increased metabolism of fever, damage to the hepatic cells and/or increased activity of the adrenals resulting in an increase in circulating adrenalin. Hypoglycemia has been observed by Fulton in P. knowlesi infections in monkeys. He found that the lowest blood sugar levels were present in those animals with the lowest glycogen stores in the liver. Marvin and Rigdon reported a severe terminal hypoglycemia in ducks infected with P. lophurae and suggested that this was due to the impaired liver function. Glucose is an important factor in the metabolism of plasmodia and mouse erythrocytes infected with P. berghei were found to utilize considerably more glucose than did the normal cells.

In our experiments dramatic changes in the serum transaminases were observed among the infected mice. Both the SGO-T and SGP-T levels increased very markedly following infection. Further observations have indicated that these changes are already noticeable 1 day following infection. Although the significance of these changes is not yet fully understood, they are probably related to the erythrocyte destruction, anoxia and liver damage occurring in acute malaria. These findings may have some value in the early diagnosis of infection and for an early assessment of chemotherapeutic results in drug screening.

The decrease in alkaline phosphatase values observed in malarious mice 6 days following infection cannot be fully interpreted in the light of our present knowledge.

The increase in direct bilirubin values observed in our experiments is not surprising in view of the indications of disturbance of liver function observed in our mice in several other tests and because of several reports that considerable jaundice may develop in human malaria. Kingsbury and Ross found increases in the average concentration of serum bilirubin in P. vivax, P. malariae and P. falciparum infections. Apparently the hyperbilirubinemia of malaria is not always related to the degree of parasitemia or to the degree of anemia

existing at the time of examination. Although some authors regard the hyperbilirubinemia of malaria as the result of abnormally high blood destruction, many others regard it as evidence of hepatic insufficiency. The interpretation of results of humans, however, has been complicated by the fact that most observations were drawn from malaria induced in syphilitics, where the latent or active syphilis may have given rise to the changes which malaria alone might not have otherwise produced. Furthermore, since most of the patients were treated with anti-malarial drugs relatively early in the disease, it is not surprising that differences of opinion exist as to the possible influence of therapy in producing or accentu ating some of the changed hepatic functions.

Consistent and marked increases in the bromsulphthalein retention were observed in the infected mice as early as 4 days following infection, indicating a derangement of the excretory functions of the liver. This had been observed injust a few of over 1,100 cases of all types of human malaria reviewed by Kern and Norris.

Indications of hepatic damage were confirmed by our observations of the deviations in the cephalin flocculation test. In a study of 10 patients infected with P. vivax and P. falciparum, Mirsky et al discovered that at some time during the disease this test became positive. Since some of the patients had been treated with anti-malarial drugs, one could not exclude entirely the possibility that the reaction was affected by the drugs themselves. However, since the flocculation tests returned to normal after treatment, the authors ascribed the changes primarily to the malaria.

The present investigations show clearly that during the acute phase of P. berghei infection, deviations in some liver function tests can be demonstrated. Although the full assessment of these results in terms of liver damage and the determination of whether such disturbances are of a transitory character must await further experimentation, there is ample evidence to conclude the P. berghei blood induced infection in mice brings about an early inhibition of certain important liver functions. Although the results of these studies leave little doubt that with infection the liver has become actively involved, they do not reveal how such involvement occurs, whether the changes are merely functional with no obvious anatomical basis and whether restoration of normal liver function takes place after the termination of infection. In addition, further detailed and well controlled studies are needed to determine whether the observed changes are produced by the direct effect of the malaria parasite or by the secondary

effects of infection such as anoxia and hemolysis.

4. Antigenic analysis of Plasmodium berghei

Some knowledge of the immunochemical nature of P. berghei is necessary for the design of specific and sensitive diagnostic tests. Furthermore, the production of a protective vaccine can be facilitated by knowledge of the antigen or antigens which stimulate the host's protective immune mechanisms. Antigenic similarities between host and parasite may help to understand host-parasite interaction, and may also afford a pathogenetic basis for some of the "autoimmune" reactions which have been postulated.

Previous work from this department has shown that soluble extracts of P. berghei from mice contained a complex mixture of antigens, some of host origin and others from the parasite. In fact, the strongest antigen in these extracts was one which was similar or identical to host hemoglobin. Therefore, further immunochemical studies of the parasite required separation of the parasite-specific antigens.

Ion exchange chromatography was employed to separate and purify the different antigenic components. Infected mouse cells were lysed in cold CO_2 saturated distilled water. The residue of parasites (with some red cell stroma and degenerated leucocytenuclei) was extracted with cold veronal acetate buffer, pH 8.2, in a Ten Broeck tissue homogenizer. The supernatant fluid was lyophylized and used as soluble antigen.

Antiserum was obtained from adult rabbits immunized by weekly intramuscular injection of rehydrated antigen obtained from either mice or rats and complete Freund's adjuvant.

Most of the experiments have been with columns of diethylamino ethyl cellulose, 33cm x 2.5cm, using phosphate buffer, pH 8.0. Ionic gradients were produced with a 7 or 9 chambered variable gradient device ("Varigrad"). Fractions were collected by volume, 200 drops each (approx. 12). Protein content was measured by UV absorption at 200 mgm in a Beckman DU spectrophotometer.

The bulk of the reddish-brown color of the antigenic extract could be separated from the rest of the protein by using a buffer of 0.005M phosphate and o.05M NaCl. After 250 ml of this

solution was passed through the column, a continuous linear gradient was used, with a starting buffer of 0.005M phosphate and o.07M NaCl, and a limiting buffer of 0.0125M phosphate and 1.0M NaCl. After 350 ml of this gradient, the column was washed with 150 ml of 3M NaCl. The pH at all times was maintained between 7.5 and 8.0 Aside from the colored material most of the protein was eluted in a varying number of peaks, usually three. The first peak was slightly colored, and probably contained some additional hemoglobin. The other peaks had no detectable color. The combined optical density of these three peaks was usually considerably less than that of the initial colored one. The yield in all of the fractions was approximately 60% of the starting optical density at 280 mμ. A black residue remained at the top of the column which could not be removed from the cellulose even when the cellulose was washed in a beaker with 3M NaCl. The nature of this material is still undefined.

The immunologic characteristics of the fractionated mouse antigens were studied by micro-double diffusion in gel using rabbit anti-rat P. berghei antiserum. This heterospecific system was utilized in the hope that there would be little or no cross reaction between rat and mouse tissue, and that only the parasite antigens would be precipitated. This appeared to be a valid assumption, at least for the hemoglobin, insofar as there were usually no bands in the fractions with the most intense color.

The unchromatographed antigenic extract usually contained 4 or 5 poorly separated but apparently distinct bands; this is probably a minimum number of antigenically distinct specificities. A maximum of only 2 weak bands could be recovered from the chromatographed fractions even after pooling and concentrating as much as 10-fold by volume. These bands were usually in the first peak of the linear gradient and were usually eluted at 0.2 to 0.5M NaCl; the exact molarity varied in different runs. These antigens had a positive mobility in immunoelectrophoresis at pH 8.2, were completely denatured by heating to 100 degrees C. Preliminary experiments suggested that they were destroyed by trypsin but were not ribonuclease or deoxyribonuclease. Attempts to further characterize these antigens and to evaluate their diagnostic and/or functional significance are in progress. In addition to these bands, occasionally another fraction has been noted in the region of the most intense red color. Whether this is merely an artifact, a weak reaction with hemoglobin, or one of the parasite antigens which is eluted at a lower ionic strength, has not yet been

ascertained. There appeared to be also a single band in the whole antigen extract which was not destroyed by heating. However, this antigen has not yet been recovered from the column.

In addition to gel diffusion studies, other assay systems have been tried. Complement fixation has been unsatisifactory because of marked anti-complementary effects. These effects may be due to the antigen itself, as they tended to occur in fractions where the antigen was thought to be. Immune adherence was used on several occasions, but sometimes there was adherence in the absence of antiserum. Tanned cell hemagglutination tests did not permit reproducible results, probably in large measure because the low protein concentration in the fractions was insufficient to coat the cells.

There are several explanations for the small recovery of antigenic material. Since cellulose has a fairly high non-specific absorbing capacity, it probably accounts for much of the loss. It is also possible that the antigens may become denatured by interaction with the cellulose, and therefore cannot be detected even though they are eluted. Furthermore, the very marked dilution (70- to 100-fold) may diminish the amount of antigen below the level at which it can be detected by the relatively insensitive technique of double diffusion.

Better recovery of antigen is being attempted by using a different anion exchange medium, such as diethylaminoethyl sephadex, which does not have as much non-specific absorption. It is also possible that a cation exchange resin, with elution at an acidic pH, will be more effective.

5. Chloroquine resistant Plasmodium falciparum in the chimpanzee

The increased incidence of chloroquine resistant Plasmodium falciparum strains has emphasized the desirability of having a susceptible primate for use in studies of this parasite. Chimpanzees were selected as test animals to determine (1) their susceptibility, (2) the character of the infection and (3) their suitability as a possible chemotherapeutic model.

In a preliminary experiment chimpanzees less than three years of age were utilized. Four methods of infections were attempted using: (1) a saline sporozoite suspension obtained from the salivary glands of mosquitos fed on patients with chloroquine-resistant

P. falciparum: (2) freshly drawn blood from the same or similarly infected patients: (3) direct feeding by infected mosquitoes on the selected experimental subjects followed by inoculation of a sporozoite suspension obtained after dissection of the same mosquitoes, and (4) freshly drawn blood from an infected chimpanzee.

The sporozoite inoculation failed to produce infection in either an intact or splenectomized animal. Failure in the case of the splenectomized chimpanzee may possibly be attributed to a time lag between dissection of the mosquitoes and inoculation.

Infected human blood failed to produce a parasitemia in the intact chimpanzee. Smears from this animal also remained negative following splenectomy performed four months after inoculation. However, infected human blood did produce a parasitemia in the splenectomized animal.

Combined feeding by infected mosquitoes followed by inoculation of sporozoites was attempted at various times in relation to splenectomy. One animal was splenectomized seven days prior to inoculation and the others, 7 and 100 days post inoculation respectively. All three became infected. The appearance of parasites was directly related to the time of splenectomy. The parasite burden was very low in the animal on which splenectomy was delayed 100 days.

One splenectomized animal was inoculated with blood from an infected chimpanzee and subsequently became positive for P. falciparum.

This work confirmed Bray's findings that the splenectomized chimpanzee will support the growth of both the pre-erythrocytic and erythrocytic phases of P. falciparum. The duration of the acute phase of the infection lasted an average of 19 days (onset of parasites to 1 parasite/100 WBC). Maximum parasitemias obtained ranged from 10 to 204 thousand parasitized cells per cmm. Four of the five animals successfully infected remained positive with low level parasitemias for a minimum of three months. Infections that recrudesced never exceeded 3000 parasitized cells per cmm. All stages of the parasite except mature gametocytes were found and readily identified. Clinically there was no evidence of chills or alteration in activity or appetite. Transient low-grade fever and decreased hematocrit was evident only in the most severely parasitized animals. (Table XII)

Nine young splenectomized chimpanzees were inoculated with human

TABLE XII

The natural history of infection in chimpanzees inoculated with chloroquine resistant Plasmodium falciparum

Animal Number	6	8	6	8	2	9	11	4
Splenectomized (day before or after inoculation)	-	-30	+120	-65	-7	+7	+100	-30
Inoculum (see key)	a	a	b	b	c	c	c	d
Day of Patency [1]	-	-	-	5	12	11	114	7
Day of maximum parasitemia [1]	-	-	-	14	19	17	*	8
Maximum parasitemia (1000 parasites/cmm. blood)	-	-	-	120	10	119	-	204
End of acute stage [1]	-	-	-	26	26	29	-	28
Length of acute stage (days from onset to < 1 parasite/100 WBC)	-	-	-	21	14	18	-	21

* This animal never showed more than an occasional parasite.

a 0.6 ml sporozoite suspension of P. falciparum obtained from the salivary glands of infected mosquitoes intravenously.

b. 3.0 ml of infected human blood containing 18,500 trophozoites per cmm. intravenously.

c. 39 to 43 infected mosquitoes allowed to feed on the animal, then sporozoites collected in a saline suspension and injected intravenously.

d. 2.0 ml of blood from chimpanzee number 9 injected intravenously; 119,000 trophozoites/cmm.

1. After inoculation.

blood containing approximately 20 million cells parasitized with Malayan (Camp.) Plasmodium falciparum. These animals had been treated earlier with chloroquine and primaquine to eliminate natural simian malaria. All nine had patent infections between day two and eight after inoculation. Three animals served as untreated controls while three were treated with injectable chloroquine hydrochloride (3.3 mg per pound daily for three days) and the remaining three were treated orally with quinine sulfate (13.3 mg per pound daily for seven days). Qualitative determinations for serum drug levels indicated that therapeutic levels of both drugs were present during treatment.

In the controls the acute phase averaged 17 days in length (day of onset of parasites to <1 parasite/100 WBC). Maximum parasitemias ranged from 11,000 to 400,000 parasitized cells per cmm. Two chimpanzees had a mild recrudescence during the following 15 days while there were only occasional parasites observed in the third during the next 90 days.

Treatment of the chloroquine groups was initiated during the ascending period of parasitemia. Maximum parasite levels were obtained within 24 hours thereafter and ranged from 4,500 to 13,500 parasitized cells per cmm. The acute phase of the infection was shortened to an average of 11 days. All three animals demonstrated recrudescences of a substantial nature during the next 15 days ranging from 2,500 to 19,000 parasitized cells per cmm.

Treatment of the quinine group also was initiated during the ascending period of parasitemia. Maximum parasite levels were obtained within 48 hours and ranged from 2,500 to 550,000 parasitized cells per cmm. The average length of the acute phase was 14.3 days. Two animals exhibited a mild recrudescence within 15 days while the third remained negative for 45 days after which an occasional parasite was identified.

Clinically, no chills were observed in any chimps and only the three animals with the highest parasite population demonstrated a mild temperature elevation and a significant leucocytosis. All animals had a slight decrease in hematocrit shortly after onset of infection which was transient in nature. Other fluctuations in hematocrit were noted following individual recrudescences.

These studies revealed that chimpanzees are very susceptible to a blood-induced infection of P. falciparum. Relatively high parasitemias

can be obtained (up to 10%) but are transient in nature. Recrudescences do occur but they are usually mild. Even at the highest level of parasitemia the chimpanzee shows little clinical effect of the infection. In the dose, form, dosage schedule and route administered, chloroquine appeared to shorten the length and probably limited the severity of the acute phase of the infection but did not produce a complete parasitological cure. Quinine in the dose, form, dosage schedule and route administered had no apparent significant effect on the course of the infection as compared to the controls. (Table XIII)

To the group of seven chimps still positive forty days after inoculation another chimp still showing P. falciparum from an earlier experiment was added. Two groups of four animals were formed, one treated with quinine sulfate orally (33.3 mg per pound for seven days) and the second with injectable chloroquine hydrochloride (10 mg per pound on a standard 40%, 20%, 20%, 20% schedule).

At the time of treatment all but one animal was showing parasites at a level less than 500/cm. After completion of treatment with chloroquine three of the four chimpanzees were positive on 3 or more blood samples during the next 30 days while one was found to be negative and remained negative for over 90 days. Of the four animals treated with the increased dosage of quinine all became negative and remained negative more than 90 days.

The larger dose of quinine appeared to have been an effective chemotherapeutic agent while the chloroquine continued to be inadequate. (Table XIV)

Current studies include quantitative determination of serum chloroquine levels following chloroquine administration, results of reinfection using frozen infected chimp blood and fresh infected human blood as an inoculum, and the effect on the parasite level following transfusion of infected animals with normal human blood of the same blood type.

Summary and Conclusions.

1. Some of the quantitative base lines that are necessary to study adequately infections with Plasmodium berghei in mice were determined.

The inoculum for routine passages of the parasites was standardized. Intravenous and intraperitoneal inoculations were compared, and

TABLE XIII

The effect of treatment with chloroquine and quinine on chimpanzees injected with a chloroquine resistant strain of Plasmodium falciparum

Drug	Control			Clhoroquine HCL			Quinine Sulfate		
Animal Number	6	12	18	2	21	23	14	19	22
Day of Patentcy*	6	7	2	8	6	6	8	6	2
Day of Treatment*	-	-	-	15	12	12	15	12	12
Day of Maximum Parasitemia*	18	18	14	16	13	12	17	13	13
Maximum Parasitemia (1000 parasites/cmm blood)	11	400	210	11.1	4.7	13	26	550	2.7
End of Acute Stage*	21	24	21	18	18	17	21	21	17
Length of Acute Stage (days from onset to 1 parasite/100WBC)	15	17	19	10	12	11	13	15	15
Maximum Second Parasitemia (1000 parasites/cmm)	1.9	45	-	14	19	2.8	2.6	.30	-

* After inoculation

Quinine dosage - 13.3 mg/pound body weight daily for seven days, orally.

Chloroquine dosage - 3.3 mg/pound body weight daily for three days, intramuscularly.

TABLE XIV

Effect of retreatment with chloroquine and quinine in chimpanzees infected with a chloroquine resistant strain of Plasmodium falciparum

	Drug							
	Chloroquine HCL				Quinine Sulfate			
Animal Number	2	6	19	23	12	14	15	21
Parasitemia on day of treatment (parasites/cmm.)	500	500	500	500	20,000	500	500	500
Day of clearance (after initial treatment)	4	2	5	6	2	4	2	5
Positive during next 90 days	yes	no	yes	yes	no	no	no	no

Quinine dosage - 33.3 mg/pound body weight daily for seven days, orally.
Chloroquine dosage - 10 mg/pound body weight divided; 40% loading dose, 20% 6 hours later, 20% 24 hours after initial dose, 20% 48 hours after initial dose, intramuscularly.

the effects of time and temperature on the viability of parasites were determined. Infected mice kept at low ambient temperature lived significantly longer than those kept at room temperatures or above.

2. The course of P. berghei infections was studied in muskrats and splenectomized monkeys. Seven species of North and South American rodent were refractory to infection.

3. Using microtechniques changes in serum transaminase, glucose, alkaline phosphatase, bromsulphthalein retention and cephalin flocculation were found in sera of mice infected with P. berghei.

4. Attempts were made to fractionate P. berghei antigens by ion exchange chromatography and assay systems such as gel diffusion, complement fixation and immune adherence have been tried.

5. Preliminary studies revealed that splenectomized chimpanzees are very susceptible to blood-induced infection with P. falciparum. Parasitemias up to 10 percent were obtained but the animals showed little clinical effect of the infection.

Publications.

Diggs, Carter L. Immunodiffusion studies of Plasmodium berghei. J. Parasit. 50 (Suppl.): 17, 1964.

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Hillyer, George V. and Diggs, C. L. Determination of Plasmodium berghei density for inoculum preparation. J. Parasit. 50 (Suppl.): 49, 1964.

PROJECT 3A025601A816
MILITARY MEDICAL MATERIEL

Task 01
Military Medical Materiel

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
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19. GOV'T LAB/INSTALLATION/ACTIVITY
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21. TECHNOLOGY UTILIZATION: Clinical chemistry, toxicology

22. COORDINATION: NA

23. KEYWORDS: Instrument; equipment & supplies; chemistry, analytical; spectrophotometry; spectrum analysis; toxicology; water purifications; gas chromatography

24. (U) Tech Objective - To develop equipment & procedures for the clinical chemistry lab applicable to the support of the army in the field with reduced logistical requirements. Improved specificity & reduction in analysis time are desirable for toxicology.

25. (U) Approach - The evaluation of procedures & equipment by statistical analysis & modifications of published methods to simplify vet maintain acceptable levels of performance. Equipment developments to allow use in remote areas has continued & improved techniques for toxicological examination were developed.

26. (U) Progress (Jul 64 - Jun 65) - Thirteen clinical chemistry procedures for ultramicro samples have been adapted from the literature or developed in this lab. These are presently considered applicable for use under field conditions. Statistical analyses demonstrate that the micro procedures compare very favorably with their macro counterpart while greatly relieving logistical burden. Selected procedures cover the bulk of the work expected to be required for field use. Final prototypes of the pH meter, titrator and centrifuge have been delivered. The spectrophotometer awaits completion. Expansion of toxicology effort includes both gas chromatography for more rapid identifications & a digital readout ultraviolet spectrophotometer for recording reference spectra of potentially toxic substances & drugs. These systems markedly decrease the time required to make a toxicology report available & improve the specificity of analyses.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A025601A816, MILITARY MEDICAL MATERIEL

Task 01, Military Medical Materiel

Work Unit 205, Military Medical Materiel

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Description:

The purpose of this task was to investigate items of laboratory equipment which appear to enhance the capabilities for laboratory support of the U.S. Army and to investigate new analytical programs which may improve diagnostic potential and possibly reduce logistical burden for laboratory support. A series of investigations have been continued or initiated in this regard.

Progress:

Equipment Development:

a. Spectrophotometer. A final version of a field spectrophotometer has been designed as a result of field testing of the previous prototype. The spectrophotometer design includes potential for use in the field for approximately 30 hours of laboratory time with a very minimum of glassware or ancillary equipment. The unit is entirely self-contained and capable of transport over rough terrain. No cuvettes are required for

analytical measurements and the range of wave length selection is between 365 to 700 mu. Batteries are rechargeable from a variety of power sources and in the event power is available, the instrument may be operated on line voltage. All circuit components make maximum use of solid-state electronics to cut power requirements and to reduce maintenance. A laboratory desk to transport the entire microlaboratory assembly is currently being constructed. When finished, the entire assembly to accomplish 20 biochemistry determinations will weigh approximately 130 pounds and will be self-sufficient for approximately 30 days for field investigations. Requirements for design have included minimum setup time and maximum weight reduction while retaining optimum analytical capability.

b. Preparation of Pyrogen-Free Water. Work has continued on mechanical improvements of the rotary vapor compression still for preparation of USP quality water under remote conditions. The compressor remains the weakest component in the system; however, a developmental program has been initiated which will overcome the difficulties associated with hydrocarbon contamination of effluent and with mechanical failure. The system continues to produce in 24 hours approximately 250 liters of pyrogen-free, sterile water, which meets the specifications of USP for water for injection. Electrical energy requirements approximate 750 watts per hour; scaling and cleaning problems are minimal. Plumbing connections do not require positive pressure water source and are as simple as possible. With delivery of the compressor the unit will be field tested.

Evaluation and Modification of Ultramicrochemistry Procedures and Equipment:

a. Procedures. The use of ultramicro techniques in clinical chemistry holds great promise for application in military medicine. The relative compactness of equipment as well as the tremendously decreased requirement for supplementary glassware suggests that this system may have great application in mobile laboratories. The procedures currently utilized, however, are not necessarily applicable and/or usable for this use. Some of the equipment is also not satisfactory. The intent of this investigation was to derive and evaluate a group of basic procedures which would be required or considered desirable in a field medical laboratory and to determine whether equipment modifications would be necessary in the performance of these procedures under field conditions.

The following ultramicro procedures for blood constituents have been investigated:

(1) Glucose: Two methods have been demonstrated as suitable. The glucose oxidase method has high sensitivity and specificity and is performed on trichloracetic acid extracts of blood or serum. The orthotoluidine procedure is the simpler of the two from the standpoint of reagent preparation but is not quite so sensitive. Its specificity has not been investigated. This method also utilized trichloracetic acid extracts of blood or serum. It was determined, by using both methods, that glucose values of trichloracetic-acid-blood mixtures did not vary during a testing period of 10 days at room temperature.

(2) Bilirubin: The widely used Malloy and Evelyn technique proved to be lacking in sensitivity for near normal values of bilirubin. A procedure utilizing an antipyrene-urea-methanol coupling reagent was developed which demonstrated much greater sensitivity and has a high degree of reproducibility. With this procedure, even normal amounts of direct reacting bilirubin can also be estimated ultramicrochemically.

(3) Urea Nitrogen: The Barthelot reaction involving indole information with urea nitrogen as used by many pediatric ultramicroclinical laboratories proved sensitive but had a low degree of reproducibility. A method using direct nesslerization of urea nitrogen was more satisfactory, but reproducibility was unsatisfactory at low urea concentrations. A third method utilizing diacetylmonoxime-gluconolactone direct coupling to urea has been claimed to give a stable colored complex. This method is currently being investigated.

(4) Cholesterol: A procedure derived from published methods utilizing a sulfosalicylic acid-acetic anhydride reagent has been found to be very simple, sensitive, and highly reproducible. This is a direct test on serum with no previous protein precipitation being required. The presence of high concentration of bilirubin in the serum tends to make cholesterol values slightly higher than they should be with this method.

(5) Calcium: Two titrimetric methods have been demonstrated as satisfactory. The more sensitive ultraviolet endpoint is obtained utilizing a high density ultraviolet lamp with calcein as the indicator. A less sensitive endpoint was obtained utilizing calred as the indicator. The second method does not require an ultraviolet lamp;

however, the second method was considered as more suitable for field laboratory use.

(6) Cholinesterase: An extremely sensitive procedure with thiocholine as substrate has been developed from a newly published method. The procedure is simple and reproducible and its sensitivity is sufficient to consider its use in studying cholinesterase activity in unconcentrated spinal fluid.

(7) Uric Acid: The procedure requiring urea-cyanide as reagent for the quantitation of uric acid gave high sensitivity but the low degree of reproducibility obviated its use in the ultramicro system. A more dependable method utilizing alkaline phosphotungstic acid is currently being used and is considered as an acceptable procedure although its sensitivity is somewhat less than optimal.

(8) The following procedures which were basically scaled down from their macro counterparts to ultramicro ranges with minor modifications, are considered to be satisfactory for routine use: chloride, creatinine, alkaline phosphatase, acid phosphatase, carotene, bromosulfaphthalein liver function test, phosphorus, total protein, glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, tactic dehydrogenase, and leucine amino peptidase.

b. Equipment: Several conclusions were reached as far as the ultramicro equipment was concerned.

(1) Flame Photometer: Since the only generally accepted procedures for sodium and potassium currently available require the use of flame photometer, the investigation of flame photometric equipment satisfactory for use with the ultramicro system was initiated. Most flame photometers cannot perform adequately on dilutions containing 10 to 20 microliters of serum either because of the lack of sensitivity of the instrument or because of the rapid loss of the small sample into the flame. A flame photometer was found, however (manufactured by National Instrument Laboratories), that permitted at least duplicate determinations for both sodium and potassium on 10 microliters of serum diluted to 4.0 ml. The instrument demonstrated a high degree of sensitivity and gave reproducible results.

(2) Temp-Blok Unit: The possible substitution of a dry incubator for the cumbersome water bath was investigated. The most satisfactory substitute was the "Temp-Blok" unit (manufactured by

Lab-Line Instruments) for which a special aluminum block was prepared which snugly held a maximum of thirty micro tubes. Upon testing at 37° C. over eight-hour periods with temperature readings every half hour, the unit had an average deviation of ±0.4 C. and a standard deviation of ±0.5° C. This incubator is considered to be usable in field performance of ultramicro analysis.

(3) Gasometric Equipment: Due to the inherent fragility of gasometric equipment, the use of such an apparatus under field conditions was considered undesirable. The substitution of an accurate titrimetric bicarbonate procedure for the standard carbon dioxide capacity is currently being investigated. The most satisfactory ultramicro procedure was found to be one in which plasma proteins have been removed prior to titration by methanol. This technique is still being evaluated.

(4) Spectro Colorimeter: The basic pieces of equipment utilized in this study are those supplied with the Beckman-Spinco ultramicro system. The centrifuge and titrator have been found to be quite satisfactory. The tube mixer, however, was inadequate and a substitute item was built here. The spectro colorimeter was found to have undesirable features and is considered to be a weak point in the analytical system.

Tryptic-Like Esterase Activity of Normal Human Serum and Plasma:

The utilization of benzoyl arginine ethyl ester (BAEE) as substrate to measure tryptic-like activity of rat plasma has been previously reported (Megel, H., Strauss, R., Ho, R., and Beiler, M., Arch. Biochem. Biophys. 108, 193 (1964). A sensitive method for human serum and plasma BAEE esterase activity is proposed utilizing the known absorption difference of BAEE and its esterolytic product, benzoyl arginine, at 253 mm.

Since several enzymes normally present in blood and part of the coagulation-fibrinolytic system (thrombokinase, thrombin and plasmin) are trypsin-like enzymes, the effect of two mechanistically different anticoagulents (oxalate and heparin) as well as clotting was investigated using this assay and utilizing three different reaction mixture pH's. The effect of various agents, inhibitors and heat inactivation was also studied.

The procedure as devised was performed with 0.1 ml. serum or plasma in reaction mixtures containing 0.2 mgs./ml. BAEE .1-1 Cl. The

reaction mixture total volume was 3.2 ml. and assays were performed at 37° C. The mixture was buffered with Tris (0.1 N). The assay was accomplished with the Model 2000 Multiple Sample Absorbance Indicator and Recorder (Gilford Instruments Laboratories, Inc.), equipped with an auxiliary offset control.

Twelve normal bloods were collected and the following BAEE esterase activities were obtained at varying pH:

Average and Range of BAEE Esterase Activities in Twelve Samples
(in μMoles/min/1000 ml.)

Reaction Mixture pH	Serum	Plasma Heparinated	Plasma (Oxalated)
7	320 (209-493)	359 (156-696)	130 (78-211)
8	365 (255-571)	206 (95-435)	171 (106-213)
9	323 (236-492)	315 (128-685)	120 (70-162)

The samples consistently showed peak activity at pH 8 for serum and oxalated plasma while heparinized plasma had its lowest activity at this pH.

When the same assays were performed with calcium added to reaction mixtures, oxalated samples showed tremendous activity rise due to the formation of thrombokinase and thrombin. The heparinized sample activity was invariably inhibited at pH 9 but not significantly at other pH values. Serum activity was just slightly affected with varying pH. Oxalate had little effect when added to reaction mixtures with the exception of heparinized samples which were consistently increased at pH 9. When heparin was added to reaction mixtures, the activities of serum and plasma (heparinized and oxalated) showed distinctly different responses with both inhibition and activation occurring.

When soybean trypsin inhibitor was added to reaction mixtures, the heparinized sample activities were markedly depressed while serum activities were only slightly affected. Oxalated sample activities showed an intermediate inhibited response. Ovomucoid trypsin inhibitor inhibited all serum and oxalated sample activities approximately to

the same extent but heparinated samples consistently showed an increase in activity at pH 8. Heat inactivation (52° C. for 30 minutes) showed greatest effect on the activities of the heparinated samples.

The tremendous variation of response in serum and plasmas in which clotting was inhibited by anticoagulants by two different agents indicates that tryptic-like activity in these samples is intimately involved with the coagulation system and possibly the fibrinolytic system. Not only must the enzymes contributing to this activity be considered as different, but also the various inhibitor effects must be taken into account. Further studies into this problem are being planned with the intent of elucidating the mechanisms which caused the results obtained in this study.

Study of Proteins and Isozymes:

a. Microzone Electrophoresis Unit (MEP) for Neurological Disorders. An evaluation has been made of the accuracy of the microzone unit in resolving protein fractions. The values have been compared to the published percentages for normal sera protein fractions as determined by the more complex procedures of free boundary, paper and agar gel electrophoresis. Use of the microzone cellulose acetate technique in resolving the proteins in cerebrospinal fluids for neurological diagnoses is under study.

b. Study of Proteins and Isozymes in Health and Disease. The introduction of gels (agar, starch, and acrylamide) in the field of protein analysis and the discovery of multiple molecular forms of enzymes, known as isozymes, stimulated the development of new techniques for the determination of proteins and isozymes. These techniques are rapid, inexpensive, provide greater resolution of proteins than previous methods, require very small amounts of samples and demand greater accuracy. Their application in the determination of proteins and isozymes in various tissues and fluids has brought forth new information and they hold great promise for further progress in medical research.

Methods include: Agar-gel Electrophoresis, Disc Electrophoresis, Enzymoelectrophoresis and Immunoelectrophoresis.

For the agar-gel electrophoretic technique the apparatus consists of a plexiglass box with attached platinum electrodes (the cost about $25 compared to $25,000 for the original moving boundary electrophoretic apparatus), a microscope slide as a cell (the cost 1.5¢ compared to $15,000

for the original moving boundary) and a conventional power supply. This apparatus was made locally. The method is simple, rapid, and highly reproducible. Serum proteins are resolved into six bands including albumin, $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, and γ globulins. In its present form a skillful technician is required, and application to routine clinical analysis of serum proteins is limited by this factor.

Acrylamide gel was introduced as a molecular sieve technique with high resolution of serum proteins. Excellent resolution of serum proteins has been achieved, with more than 20 bands being observed on a single gel.

Fifty serum samples collected from normal individuals and from patients with various diseases have been analyzed by agar-gel and disc electrophoresis. Characteristic patterns for various diseases have been obtained by agar-gel electrophoresis which may serve as an aid for the differential diagnosis of disease. The evaluation of the disc electrophoretic analysis has been more difficult due to the large number of protein bands, many of which are still undefined. Adequate instrumentation for quantitative determination is a second problem. This technique allows differentiation of macroglobulinemia from multiple myeloma; thus the long and complicated procedure of ultracentrifugation may be avoided.

Study to determine changes of serum proteins and enzymes under different storage conditions, including time and temperature, was made using Biuret for total protein, ultracentrifugation for sedimentation coefficients, and Agar-gel electrophoresis for fractionation of proteins. Enzymoelectrophoresis was employed for the determination of LDH-isozymes. Normal and pathological serum samples were maintained in the refrigerator, deep-freeze and liquid nitrogen and analyzed every week for six weeks. The results showed that proteins undergo structural changes under different conditions of storage with largest changes in a few pathological specimens. The total LDH decreased with storage and the decrease was due to the disappearance of the thermolabile LDH-isozymes 4 and 5. Continued storage in dry ice or liquid nitrogen appeared best for minimizing changes over a 30-day period.

Enzyme-electrophoresis, as used for the determination of lactic dehydrogenase isozymes, is based on agar-gel electrophoresis for the separation of proteins, coupled with a histochemical procedure for the development of individual zones. Considerable effort has been

devoted to establishing the conditions required for the development of isozyme zones in normal human serum on a clear background for densitometric analysis. The technique has been worked out to give five clear LDH-isozyme zones in human serum with good reproducibility in the hands of a skilled technician.

Each tissue and organ has its own characteristic pattern of isozymes. Organ injury can result in the release of LDH into the blood where it may be detected. Application of this method to the analysis of human serum revealed five isozymes which have been designated 1, 2, 3, 4, and 5. One is the rapidly migrating toward the anode and 5 the slow migrating toward the cathode. LDH-isozyme determinations in extracts from human heart, lung, and liver, showed that LDH-isozymes 1, 3, and 5 predominate in each organ respectively. LDH-isozyme determination in serum samples from patients with heart, lung, and liver diseases showed characteristic isozyme changes reflecting the origin of injury thus the potential for diagnostic assistance is demonstrated.

The technique of LDH-isozyme determination has been applied in the determination of isozymes in tissue culture samples received in our laboratory from the Department of Cell Physiology. Only one isozyme was demonstrated in these cells. Since most animal tissues and cells contain five LDH-isozymes, this finding is of particular importance. Our results have been incorporated in the project of the above department: 3A014501B71P, 08, 075.

Immunoelectrophoresis is also based on agar-gel electrophoresis for the separation of proteins, followed by immunodiffusion against antisera for the identification and determination of individual proteins. This technique is currently being used for the study of immunoglobulins.

Immunoelectrophoretic analysis of serum samples reveals over 20 precipitin arcs. At the present time the study of the three immunoglobulins, IgG, IgA and IgM, is continuing in order to determine which fraction is elevated in the hypergammaglobulinemia cases shown by agar-gel electrophoresis.

Collaboration with the Germ-Free laboratory has been fruitful. The method of agar-gel electrophoresis has been applied for the study of serum protein in samples from a germ-free monkey. Significant results have been obtained and are incorporated in the project. 3A014501B71P, 04, 017.

The high degree of resolution and sensitivity of the electrophoretic methods exceeded the capacity of the commercial instruments used for quantitative analysis by densitometry. Considerable time has been devoted in screening various densitometers. The Joyce Lobel Chromoscan has been selected and it is presently standardized for the quantitative determination of protein fractions and isozyme bonds.

Serum Lipoprotein Separation and Characterization:

Work was continued on methods for the separation and quantitation of serum lipoproteins and the lipids contained therein using thin-layer chromatography (TLC) for the separation and eventual quantitation of the lipids contained in serum lipoproteins.

Two solvent systems were found that separate the serum lipids into four classes when applied successively.

Petroleum ether , ether, and glacial acetic acid (90:10:1), resolves free and esterified cholesterol, and triglycerides. The phospholipids remain at the origin.

Chloroform, methanol, and water (65:35:4) resolves the phospholipids. Other lipids move with the solvent front. If the plates are developed in the second solvent system for 5 cm, then removed and developed in the first system for 10 cm, the 4 lipid classes are completely resolved.

Methods of detection included:

Rhodamine G which was found to be a very good agent for locating the lipids, but it is not quantitative. Use of a 50% sulfuric acid spray followed by heating on a hot plate or in an oven produces charred spots which can be quantitated with a scanner that measures by reflection.

Thin layer chromatography is now being further applied to study the lipid components in cobalt and calcium heparin complexes of lipoproteins.

Additional investigation involves the chemical stability of serum lipoproteins in the hope that such studies will be applicable to atherogenesis. A method is being developed whereby the easily extracted cholesterol is determined.

When serum is shaken with ether, only a small amount of lipid is extracted; however, it was found that the amount of lipid varies from serum to serum and is not a function of the ount of total lipid. Thin layer chromatography techniques demonstrate that this lipid contains cholesterol and triglycerides but no phospholipids. This easily extracted lipid may be a measure of the st ity of the lipoproteins. Further work will be directed in this area.

Applications of Electroanalytical Chemi try to Biochemical Analyses:

a. A Sensitive Amperometric Endpoint Detection System for Microcoulometric Titrations. For maximum sensitivity of coulometric titrations, a system which will detect endpoints in titrations to 10^{-8} N or less is desirable. Since many coulometric titrations reported in the literature employ amperometry for detection of the endpoint, a sensitive amperometric system was investigated. A method was developed in which 9 ng. of arsenic (III) (2.5×10^{-10} eq.) in 35 ml. (7×10^{-9} N were titrated with generated bromine. The electrolyte is pretitrated to sufficient excess of bromine to yield a linear increase in amperometric current. The bromine is in excess of the sample size. The sample is then added and the decrease in current is measured. The titration time is calculated from the slope of the current-time curve.

b. Chromium Detection.

(1) Polarography: The importance of chromium as an important micronutrient has prompted investigation for a satisfactory method of determination of chromium in biological samples. Existing polarographic methods for the determination of the various chromium species were investigated to determine if trace amounts could be detected. It was found that with conventional polarography a lower limit of 10^{-6} M Cr could be detected. This is not sufficiently sensitive for biological materials.

The polarography of chromium at the hanging mercury drop electrode (HMDE) was investigated. Various solvents were tried to establish a sensitive detection method for chromium. Chromium (III), in aqueous and/or acetonitrile solutions, gave a sensitive forward peak, the height of which was proportional to the concentration. The limit of detection was extended to 10^{-7} M, thus making it useful for some biological materials, but additional sensitivity was still desirable.

(2) Coulometric Method: Ultramicrocoulometric titration, as described above, of chromium (VI) was successfully accomplished with electrogenerated iron (II) using a platinum indicating electrode at +0.92 V. vs. the S.C.E. The range of 800 to 4 nanograms of chromium in 30 ml. were determined with an average error of 3.7%.

(3) Atomic Absorption Methods: In recent years atomic absorption spectroscopy has become an increasingly important tool in trace metal analysis. Conditions were studied for the determination of trace amounts of chromium by atomic absorption spectroscopy. Solution matrix, flame composition, and extraction procedures were the variables studied. A detection limit of 0.006 p.p.m. of chromium was attained with an air-hydrogen flame.

The above method was applied to the determination of chromium in biological materials. An ashing mixture of 3:1:1 nitric-sulfuric-perchloric acid was used. The material was oxidized with potassium permanganate and extracted into methyl-isobutyl ketone. It was then aspirated into the flame and the absorption reading taken. A comprehensive study of chromium in various tissues and serums was done. An average serum level of 0.025 p.p.m. was observed with pooled human serum.

c. Coulometric Generation of Molybdenum (V). Molybdenum (V) was generated at a platinum cathode from 0.7 M molybdenum (VI) in 4 M sulfuric acid. A current efficiency of 99.9% was attained. A limiting current density of 0.05 ma./cm^2/mM was found. The formal potential of the Mo (VI) - Mo (V) couple in 4 M sulfuric acid was determined to be ca. 0.55 volt vs. N.H.E. Chromium (VI) solutions were titrated over a wide range of sample size and generating current. Amerometric titration curves were interpreted from current-voltage curves. Titrations could be performed in the presence of oxygen at the 1 μeq. level. The effect of nitrate, perchlorate, orthophosphate, and chloride ions on the titration was determined. Nitrate was the only anion found to interfere. The intermediate reduction potential of molybdenum (V) makes this a potentially selective reducing agent.

d. Coulometric Calibration of Micropipettes. Since coulometric titrations require no standardization, these methods should be ideally suited for calibration of microvolumetric apparatus. One stock standard solution could be used for all volumes to be calibrated without dilutions accompanied by volumetric errors; the generating current would merely have to be changed to an appropriate value to keep the titration

time at a convenient value and to allow for the maximum precision desired in measurements. A procedure was developed in which primary standard potassium dichromate (1.0000 N) is titrated with coulometrically generated molybdenum (V). Pipettes from 0.5 ul. to 200 ul. can be calibrated with a precision and accuracy of about 0.2%. This titration can be performed automatically by recording the amperometric current and can be done in the presence of air.

e. A Rapid Colorimetric Determination of Microgram Amounts of Selenium in Soils and Biological Samples Using Millipore Filters. A method was developed for the determination of selenium in biological samples or soil samples, following acid digestion or distillation as the tetrabromide, respectively. The selenium is reduced to the element by ascorbic acid. The element is filtered and collected on a white millipore filter. The intensity of the coloration of the filter is used as a measure of the selenium content. The range of the method is from 0.2 μg. selenium to greater than 0.1 mg. selenium. Complete analysis time for a single biological sample is about 60 minutes while that for a soil sample is less than 2 hours. Control of variables is not critical.

f. Anodic Stripping Voltammetry of Biological samples. An investigation was undertaken to determine the possibility of applying anodic stripping voltammetry directly to several different biological substances following dry ashing and subsequent solution of the ashed sample in purified water. In this way, no supporting electrolyte would be needed and impurities from the electrolyte would be eliminated. Measurements were made of pH and resistance of solution and linearity was established between concentration of metal ion and stripping peak height for several tissues, including liver, skin, blood, muscle, urine, brain, lung, and kidney. A standard addition method is recommended to obtain qualitative and quantitative results.

Biogenic Amines:

Analyses of pressor substances are being done to provide diagnostic assistance to service hospitals whose facilities are not adequate for the performance of these complicated techniques. Additionally collaborative studies with other investigators are being carried out in various types of trauma. New analyses and modifications of old analyses are carried out as time permits.

Some 50 samples of plasma were analyzed for catecholamines in suspected cases of pheochromocytoma from army and air force hospitals. One confirmed case was found with high catecholamine levels. This was received from Andrews A.F.B.

Some five plasma serotonin analyses were done for WRGH. One patient with a carcinoid tumor of the rectum was found to have extremely high plasma levels of serotonin.

A modification of a method for the fluorometric determination of phenylalanine by McCaman and Robins (J. Lab. Clin. Med. 59: 885 (1962)) was set up and taught to the class on "Methods of Instrumental Analysis." This is a quantitative method and will become of widespread importance as more and more states pass mandatory laws to test all newborn babies for possible phenylketonuria. The fluorometric method described above will be needed to test quantitatively the blood of suspicious cases picked up by any screening procedure and to follow up cases after they are put on the special diets needed.

The collaborative effort this year was the study of the incidence of peptic ulceration after portocaval shunts in cirrhotic patients ranges from 10 to 30 percent. It has been theorized that this may be caused by histamine-rich portal blood reaching the systemic circulation without passing through the liver. Preliminary results, so far, in dogs with various types of shunts show that as compared to control dogs, the plasma histamine values increase much more in small intestinal and transposition shunts, whereas no elevation occurs after a pancreatic-duodenal venous or gastric venous shunt.

Improved Toxicologic Analysis:

Efforts to consolidate or systematize an approach to Analytical Toxicology by applications of spectroscopy and gas chromatography depend upon the efficient utilization of data acquired by these techniques. With the expansion of the applications of these techniques to Analytical Toxicology, the data amassed has become considerable, and a way to expedite the use of this information is of prime concern. Consequently, attention is focused to data processing methods and computer programming. The current trend toward computerization has prompted the modification of a newly acquired spectrophotometer to include digital readout accessory apparatus. In addition to facilitating data input for the computer, the accessory apparatus increases the efficiency of data acquisition, and enables rapid duplication as well. Hence, the slower, more expensive practice of photographing recorder presentations of spectral absorbancies will be replaced by rapid, relatively inexpensive punch cards containing spectra information for distribution to the U.S. Army Area laboratories where ultraviolet spectrophotometers are utilized for analytical purposes.

To date, a program for IBM 1401 Data Processing System has been devised to identify ultraviolet spectra. The spectra, used for drug identification usually by visual comparison with standardized ultraviolet spectra, can be scanned in the spectrophotometer, and the resultant scan quickly compared by the computer for rapid identification. The advantage of the computer comparison is that a far greater number of standard spectra can be used to give greater reliability and more precise identification in a fraction of the time required for visual comparison. Other computer programs, in preparation, will include computations to quantitate, from spectral absorbancies, the amount of drug or toxic substance present, as well as programs to process gas chromatographic data.

An attempt is being made to integrate present techniques of gas chromatography and spectroscopy into a coordinated system of rapid specimen screening for drugs and toxic substances. One approach has been to study simple devices or equipment modifications which enable simultaneous monitoring of effluent gas chromatography vapors. The vapors are either trapped and read directly in a spectrophotometer or condensed and dissolved in a solvent for subsequent scanning in the spectrophotometer. For the most part the system is operational whenever ultraviolet spectroscopy is applicable, but no simple method has been devised as yet to circumvent the limitations of insensitivity associated with infrared spectrophotometry.

Pending the completion of projected computer programs for processing ultraviolet data, the investigation of toxic plant substances such as polypeptides, toxalbumins, and glycosides has been temporarily curtailed. Since the analyses of these compounds depend largely on ultraviolet spectral determinations, considerably more data can then be processed expeditiously without overexpanding present capabilities.

Summary and Conclusions:

Final version of a highly portable biochemistry laboratory utilizing microliter techniques is being assembled. Twenty-two ultramicro clinical chemistry procedures have been checked for inclusion in the assembly.

Highly satisfactory methods for separation of protein components and isozymes from various tissues have been developed and are in operation. These include adaptations of microzone, starch gel, and acrylamide electrophoresis and the separation of the LDH isozymes.

Variations within and between tissues have been demonstrated and the methods have been satisfactorily applied to differential diagnosis of disease.

Both coulometric and atomic absorption methods have been used for determination of chromium in tissues. Atomic absorption appears to offer the necessary sensitivity for determination of nanogram amounts of chromium.

A rapid and sensitive method for determination of selenium in soils and in biological tissues has been developed.

Various applications of coulometry included generation of molybdenum (V) as a highly useful reducing agent. This has been applied to rapid and accurate calibration of micropipettes.

A fluormetric determination of phenylalanine was standardized for assistance in early diagnosis of phenylketonuria.

Thin layer chromatography has been applied to the study of serum lipoproteins and the lipids contained therein. The use of two consecutive solvent systems resolves the serum lipids into four classes. Cobalt and calcium heparin complexes are being analyzed with the thin layer chromatography technique. A method for the extraction of loosely bound serum lipids is being developed as a measure of lipoprotein stability.

A rapid data retrieval method for comparative analytical toxicology has been initiated. Data will be available to all Army Medical Laboratories where gas chromatography, infrared, and ultraviolet instrumentation is utilized.

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Publications:

Schur, P. H., and Christian, G. D., The Role of Disulfide Bonds in the Complement Fixing and Precipitating Properties of 7S Rabbit and Sheep Antibodies, J. Exp. Med., 20, 531 (1965).

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Christian, G. D., Knoblock, E. C., and Purdy, W. C., Use of Highly Acid Supporting Electrolytes in Polarography; Observed Changes in Polarographic Waves of Selenium (IV) Upon Standing, Anal. Chem., 37, 425 (1965).

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PROJECT 3A025601A821
COMBAT SURGERY

Task 01
Combat Surgery

RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A0464	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT / WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
01 07 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a CURRENT NUMBER CODE	10b PRIOR NUMBER CODE
62156011 3A025601A821 01 120	None

11 TITLE

(U) Acute renal injury and failure (21)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
003500 Clinical medicine; 012900 Physiology; 016200 Stress physiology	09 54	NA	OTHER / DA

16 PROCURE. METHOD	17 CONTRACT GRANT a DATE / b NUMBER / c TYPE / d AMOUNT	18 RESOURCES EST	a PROFESSIONAL MAN YEARS	b FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	4	61
		CURRENT FY 66	4	65

19 GOV'T LAB INSTALLATION ACTIVITY

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20 PERFORMING ORGANIZATION

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21 TECHNOLOGY UTILIZATION	22 COORDINATION
NA	NA

23. KEYWORDS Kidney; body fluids; hemodialysis; urine; anuria; nephritis; uremia; shock; trauma

24. (U) Tech Objective - (1) To elucidate the response of the kidney to host injury including the pathogenesis of acute kidney (renal) failure and of uremia, in order to develop effective, practical and safe means of prevention and of improved treatment; (2) to develop improved techniques of renal transplantation and substitution for application in military surgery.

25. (U) Approach - (1) Documented experimental models of renal failure, hemorrhagic shock and transfusion reaction with measurement of blood flow and metabolic activity are modified by timed treatment with a variety of metabolic, toxic, diuretic, volume-expanding and vasoactive chemical and physical agents; quantitative behavioral measures trace behavioral decrements in experimental uremia; metabolic activity of brain enzymes is measured in the presence and absence of uremic materials; (2) function tests constitute indices of renal transplant survival and functional efficiency.

26. (U) Progress (Jul 64 - Jun 65)(1) Reduced renal tubular fluid flow appears to be the pathogenetic fulcrum in acute renal failure: Effectiveness of preventive agents and maneuvers is strongly time-dependent. Adrenergic blockade by Phenoxybenzamine improves renal perfusion and function during hemorrhage; rates of cerebral glycolysis in the presence of uremic materials were influenced by contaminating anticoagulants and bacteria, indicating further sampling controls; behavioral decrements were quantitated in three uremic states and were reproduced by some but not all fractionated materials derived from human dialysates; (2) to date reduced kidney blood flow remains a more effective measure of graft rejection than e.g. functional responses to volume loads.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY a. COMSEC OR COMSEC RELATED X / b. NOT RELATED	28	29 OSD CODE	30 BUDGET CODE
		AR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
1439a	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) CFY+1	36

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A025601A821, COMBAT SURGERY

Task 01, Combat Surgery

Work Unit 120, Acute renal injury and failure

Investigators.
Principal: Lt Col Paul E. Teschan, MC
Associate: Capt Albert W. Dibbins, MC; Nancy B. Cummings, M.D.; John A. Gagnon, M.S.; Natalie L. Lawson, M.S.

Description.

The studies reported here concern (1) modifications in the renal hemodynamic response to hypotension by systemic use of adrenergic blocking agents, (2) evaluation of means to produce and prevent acute renal failure (ARF), (3) attempts to determine the nature of uremia in terms of a behavioral bioassay and of cerebral biochemical enzymatic changes, (4) physiological changes in renal auto- and homotransplants, and (5) lethality of alpha toxin of Clostridium perfringens Type A.

Progress.

(1) The effects of hemorrhage and adrenergic blockade on the renal response to changes in perfusion pressure.

Autoregulation, the characteristic feature of the renal circulation which allows the kidney to maintain relatively constant perfusion despite changes in perfusion pressure, has been shown to be abolished under conditions of unmodified hemorrhagic hypotension. Since the kidney undergoes vasoconstriction as a result of the high levels of circulating catecholamines which are released during hemorrhage or trauma, the effect of alpha adrenergic blockade on the renal response to changes in perfusion pressure was studied. The glomerular filtration rate and renal plasma flow were measured in the presence and absence of a phenoxybenzamine blockade during graded hemorrhagic hypotension and retransfusion.

When normotensive dogs were blocked with 1 mg./kg. of phenoxybenzamine the mean blood pressure fell 25 mm. Hg. while the glomerular filtration rate and the renal plasma flow increased 26 per cent and 36 per cent, respectively. When this was followed by graded hemorrhage to 80 mm. Hg., these values were maintained near control and at 60 mm. Hg. the glomerular filtration rate and renal plasma flow were still 68 and 89 per cent of control. In a second group of animals hemorrhaged in the same manner but not blockaded, the arterial pressure, glomerular filtration rate, and renal plasma flow declined in a linear fashion. Under these

circumstances glomerular filtration rate and renal plasma flow were 26 per cent and 36 per cent of control at 60 mm. Hg. Induction of sympathetic blockade with phenoxybenzamine at this pressure resulted in a significant increase in both glomerular filtration rate and renal plasma flow although the pressure was maintained at 60 mm. Hg.

It is concluded that the increase in sympathetic activity during hemorrhage which appears to protect other vital organs, impairs renoprotective autoregulation when perfusion pressure is decreased. This impairment may be resolved in the dog by the induction of an alpha adrenergic blockade which maintains renal hemodynamic integrity even at shock levels.

(2) Production and prevention of acute renal failure.

(a) ARF and protection in rats: An experimental model of spontaneously-reversible azotemic acute renal failure in rats was utilized to assess the renoprotective efficiency of a number of substances. Hypertonic sodium chloride, mannitol, mannose, sucrose, sodium sulfate, urea and THAM, given intravenously, were shown to prevent ARF with varying efficiency if administered within one hour of attempted induction. Isotonic saline, hypertonic dextrose, and albumin solutions failed to prevent the lesion; and dextran in two molecular weight ranges appeared to accentuate the lesion rather than afford protection.

In collateral studies the response of urinary flow rate to these materials was measured at known arterial pressures. To date, in contrast to agents which fail to prevent ARF, each of the protective compounds induces a significant diuresis. This correlation is under further study.

In conjunction with the Department of Surgical Metabolism and Pathology of this Division, the renal histochemical alterations which occur in this experimental model are being studied.

(b) Effects of mismatched blood transfusions on primate (Rhesus) kidney function: In an effort to produce an experimental primate model for the study of acute renal failure (ARF), 16 Rhesus monkeys, found to possess a human type B antigen with anti-human A material in their serum were administered small quantities of human A blood over a 4-month period to increase their anti-A titer. They were then hemorrhaged (20 ml. per kg.) for 2 hours and retransfused with an equal quantity of human A blood. The acute response to the transfusion ranged from a fulminant incompatible reaction to little or no observable clinical abnormality.

Those experiencing the most severe reactions usually died within 24 hours while the majority survived with little or no renal dysfunction

despite markedly elevated plasma hemoglobin levels. Subsequent analysis revealed that A_1 and A_2 specific antibodies had increased in only one-third of the animals. However, there was a substantial increase in the anti-O and anti-B titers as a result of the development of nonspecific hetero-agglutinins to human red cells.

This project is presently being continued to determine the feasibility of raising titers by repeated injections of homologous blood between monkeys possessing different factors. Since initiation of the study, a means has been made available to us whereby specificities of red cells of Rhesus monkeys, tentatively identified as A, B, C, D, and E, have been identified by means of rabbit-anti-Rhesus red cell sera.

If this is accomplished, study of the mismatched transfusion reactions and their effects on the kidney can be made in the absence of an elevated hetero-agglutinin titer due to species differences.

(3) Studies on uremia.

(a) Primate behavior: These studies have utilized primates conditioned to a simple behavioral schedule, testing modes of performance which appeared to be deficient in uremic man. Various models of uremia have been utilized including bilateral nephrectomy, bilateral ureteral ligation, continuous urine reinfusion, and infusion of 6 per cent urea.

Monkeys were maintained in a chair-type restraining apparatus with automatic recording, timing, and programming circuits attached. After a period of 2½ to 5 weeks' habituation to the chair, avoidance behavior was established. Uremia was then induced by means of one or more methods.

Experiments have demonstrated that the methods used to produce experimental uremic states are important variables in determining the degree of measured behavioral impairment. The impairment as measured in these conditioned animals does not appear to be directly related to a fixed plasma urea nitrogen concentration, alterations in plasma electrolytes, or blood pH. More significant performance changes were experienced at a lower plasma urea nitrogen level in the animals undergoing urine reinfusion than in other groups.

Preliminary experiments also indicate that uremic behavioral decrements may be induced by some but not all materials derived from dialysis of uremic humans.

(b) Biochemical alterations: (1) Modifications in the aerobic glycolysis cycle have been previously described in systems utilizing uremic plasma and brain minces. These studies are being continued to find a

possible uremic toxin(s). Oxidation of C^{14} labeled glucose and pyruvate to $C^{14}O_2$ by rat brain mince incubated in uremic and in control serum and in their sub-fractions are being compared. (2) Ten to twenty liter aliquots of hemodialysis baths from uremic patients are being concentrated, extracted with ethyl acetate and any crystalline material recrystallized from ethyl acetate to isolate tricarboxylic acid cycle compounds. Methyl derivatives of these compounds are being prepared for analysis by gas chromatography in an effort to determine the pattern of tricarboxylic acids in the dialysate from uremic patients. Earlier pilot studies had shown large amounts of tricarboxylic acids in the hemodialysate fluid. It is hoped to isolate a fraction either from the serum or dialysate which is related to production of the metabolic alterations in uremia or production of the uremic state in the behaviorally conditioned primates.

(4) Physiological changes in renal autotransplants and homotransplants.

Renal auto- and homotransplants in the dog have been utilized to study the physiological effects of transplantation and also as a model for study of a kidney undergoing a period of complete anoxia. The animals are unilaterally nephrectomized and after allowing the remaining kidney to undergo compensatory hypertrophy for two to three months the kidneys are either autotransplanted, or homotransplanted into a nephrectomized recipient animal. The American vascular stapler is utilized to perform the arterial anastomoses.

Prior to transplantation the glomerular filtration rate, renal plasma flow, renal tubular sodium reabsorption, and tubular water absorption are measured on successive days. Following transplantation these same parameters are measured daily until rejection in the homotransplants; for the first 5 days and then at selected intervals in the autotransplants.

During the first five days following transplantation both groups of animals show a gradual reduction in glomerular filtration rate and renal plasma flow with the degree of reduction being more severe in the homotransplants. After this period of time there is no further significant change in the autotransplants. After 5 days the homotransplants show an abrupt reduction in both glomerular filtration rate and renal plasma flow with cessation of urinary function within 48 to 72 hours.

In the autotransplanted group there is adequate maintenance of both tubular sodium and water reabsorption. In the homotransplanted group there is a gradual decrease in the ability to reabsorb tubular sodium. No distinct pattern of water reabsorptive abnormalities is seen.

Histologic studies of the rejected homotransplants have shown marked cellular infiltration with obliteration of normal architecture and loss of both tubular structures and glomeruli.

On the basis of these studies it appears that a period of complete anoxia results in loss of a variable amount of functioning renal tissue as manifested by the change in glomerular filtration rate and renal plasma flow in the autotransplants. However, the tubules which remain appear to handle sodium and water in a normal manner. More severe changes in homotransplants are probably due to further loss of functioning tissue by the rejection process.

(5) Clostridial toxin.

Alpha toxin from Clostridium perfringens Type A filtrate was prepared in the Department of Surgical Bacteriology, Division of Communicable Disease and Immunology, WRAIR.

(a) An attempt was made to tag the purified alpha toxin with I^{131} Using the method of toxin incubation with I^{131} and removal of free iodine with a quaternary ammonium resin column, it was found that the toxin was inactivated. Tagged toxin could be produced by incubating the material with I^{131} and dialyzing the combination against ion free water. This produced a compound with a high level of radioactivity and a somewhat diminished lethality. Further use of this compound awaits a demonstration that I^{131} is bound in stable combination to the toxin fraction.

(b) Purified alpha toxin was determined to have an equi-lethal LD_{50} among three species, the mouse, the rat, and the dog, in concentrations of 5 micrograms of toxin per mg. of test animal. Using an LD_{100} level of 10 milligrams per kilogram, the effect of the toxin on dogs was studied.

Intramuscular hind-limb injection of the toxin in the unanesthetized animal produced prostration within one hour and a fulminant clinical course characterized by hypotension and anuria; central nervous system irritability with nausea, vomiting, and convulsions; gross hemolysis. In anesthetized animals in which the glomerular filtration rate, renal plasma flow and blood pressure were measured, the picture was that of a hypovolemic hypotension due to the accumulation of large amounts of hemorrhagic edema in the injected extremity and pelvic area. In an effort to prevent the hypovolemia 6% dextran was infused at a rate considered to be equal to the rate of fluid loss into the soft tissue. Despite the prevention of hypotension and maintenance of both glomerular filtration rate and renal plasma flow at pre-injection levels death ensued in 8 to 10 hours. Plasma from these animals injected intraperitoneally into mice was non-lethal.

It is concluded that the alterations in renal function seen in the preparation are a result of fluid loss and may be combated by the

administration of suitable volume expanders. Changes in renal function, however, are not responsible for lethality of the toxin.

Summary and Conclusions.

(1) The effects of hemorrhage and alpha adrenergic blockade on the renal response to changes in perfusion pressure have been studied. At 60 mm. Hg., the glomerular filtration rate and renal plasma flow in a blockaded dog are significantly higher than in an untreated animal.

It is concluded that increase in sympathetic activity during hemorrhage designed to protect other vital organs impairs the mechanism designed to protect the kidney at decreased perfusion pressures. This effect may be ameliorated in the dog by induction of an alpha adrenergic blockade which maintains renal hemodynamic integrity.

(2) (a) An experimental model producing acute renal failure (ARF) in the rat has been used to study the type of protection produced by different treatment schedules. Effective protective agents studied to date include a variety of osmotic diuretics. The rate of diuresis produced by each compound appears to be directly correlated with degree of protection from ARF afforded.

It is concluded that rate of renal tubular fluid flow at the time of attempted induction may be the most important factor in the initial mechanisms of pathogenesis and protection.

(b) Heterologous blood transfusion combined with shock has been used in an attempt to produce a primate model of ARF in Rhesus monkeys. Acute responses have varied from fulminant reactions with death in 24 hours to little or no clinical abnormalities. The majority have survived without renal dysfunction.

The study is being continued to explore the possibility of producing the ARF lesion by homologous transfusion between primates with different blood factors.

(3) (a) Behaviorally conditioned primates have been utilized to study modes of performance which appear to be deficient in uremic man. Uremia in these animals has been produced by various procedures.

Behavioral impairment has been regularly observed uncorrelated with plasma urea nitrogen, pH, or electrolyte changes. Intravenous urine reinfusion has consistently produced more significant performance changes at lower plasma urea nitrogen levels than other methods. Such changes have also been induced by certain materials derived from uremic humans.

(b) Modifications of the aerobic glycolysis cycle of brain minces incubated in uremic sera have been studied; and compounds precipitated from ethyl acetate extracts of hemodialysate fluids have been analyzed and shown to contain large amounts of tricarboxylic acids.

It is hoped to isolate a fraction related to the production of metabolic and psychologic aberrations seen in uremia.

(4) Canine renal auto- and homotransplants have been studied to compare acute changes in renal hemodynamics and tubular function. Both groups have shown a gradual decrease in renal hemodynamics with the depression being more severe in the homotransplants, rapidly progressing to anuria. Autotransplants show no basic change in tubular function while homotransplants show sodium reabsorptive abnormalities.

Changes in autotransplants are probably due to nephron loss during a period of complete anuria; changes in homotransplants may be attributed to combined effects of anoxia and rejection.

(5) (a) Alpha toxin of Clostridium perfringens Type A has been tagged with I^{131} in an effort to produce a tracer compound. The stability of the combination and its biochemical characteristics have not been assessed.

(b) Intramuscular injection of the alpha toxin has been shown to produce a hypovolemic shock picture. Fluid replacement maintains renal hemodynamic integrity but does not prevent death. Hence the latter may not be attributed to the former.

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1. Murphy, G. P., Sharp, J. C., Lawson, N. L., Greer, R. B., and Johnston, G. S.: The Chronic Functional and Morphologic Alterations caused by Prolonged Experimental Nephrotoxic States in the Rat. Invest. Urol., 1(6): 529-551, May 1964.

2. Murphy, G. P. and Gagnon, J. A.: The Hemodynamic Alterations induced by Low Molecular Weight Dextran in Normotension and Hypotension. Invest. Urol., 1(6): 574-581, May 1964.

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6. Murphy, G. P. and Gagnon, J. A.: The alterations in glomerular filtration Rate, Osmolar, and Free Water Clearance during Mannitol Osmotic Diuresis. J. Urol., 92(1): 17-24 (July) 1964.

7. Murphy, G. P., Sharp, J. C., Johnston, G. S., and Helms, J. B.: Cross-species Measurement of Regional Circulatory Alterations during Osmotic Diuresis and Other States. I. Observations of Primate, Ovine, Canine, Fowl and Reptile. Invest. Urol., 2: 82-91 (July) 1964.

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13. Huser, H. J. and Murphy, G. P.: Renal Transplantation in the Rhesus Monkey. III. Hematological and Immunological Studies. Invest. Urol. 2: 273-285 (Nov) 1964.

14. Murphy, G. P., Mundy, R L., and Ewald, R. A.: The Physiologic Properties of Glyco-Alginate, a new Japanese Plasma-Volume Expander. Surgery, 56(6): 1099-1108, (Dec) 1964.

15. Murphy, G. P. and Sharp, J. C.: Timed Behavior in Primates during various Experimental Uremic States. J. Surg. Res., 4: 550-553 (Dec) 1964.

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16. Sharp, J. C. and Murphy, G. P.: Conditioned Avoidance Behavior in Primates during Various Experimental Uremic States. Nephron, 1: 172-179, 1964.

17. Murphy, G. P. and Gagnon, J. A.: Renal Alterations in Dogs during Renal Arterial Constriction, Hemorrhagic Hypotension and Osmotic Diuresis. J. Surg. Res., 5(1): 11-22 (Jan) 1965.

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RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6465	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 62156011 3A025601A821 01 121

10b. PRIOR NUMBER/CODE: NONE

11. TITLE: (U) Metabolic and nutritional problems associated with injury (21)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002300 Biochemistry; 016200 Stress Physiology; 010100 Microbiol.	09 54	NA	OTHER / DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	9	214
		CURRENT FY 66	9	235

19. GOV'T LAB/INSTALLATION/ACTIVITY
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TEL 202-576-5162 TYPE: DA

21. TECHNOLOGY UTILIZATION: Injury and Infection

22. COORDINATION: NA

23. KEYWORDS: Injury; infection; adhesive; metabolism; hyperbaric oxygenation; bilirubin; microorganisms; thyroxin

24. (U) Tech Objective - Major areas of investigation: (1) Factors responsible for the morbidity and mortality associated with peritonitis and other surgical infections, (2) the mechanisms of action of hyperbaric oxygen in respect to its effects on bacterial infection and metabolic pathways, and (3) the potential use of plastic adhesives for restoring tissue continuity following injury.

25. (U) Approach - (1) Infections established by either endogenous and exogenous microorganisms are studied as to causal bacteria, toxins, and adjuvant factors and by pathophysiological changes produced, enzyme systems and metabolic pathways effects, and the immunologic response of the host. (2) Hyperbaric oxygen treatment of infected animals provides information as to toxicity and effects on pathways of oxygen metabolism. (3) The adhesive polymers are studied by histological, biochemical, and radioisotope methods to determine biological receptivity and the toxicity of breakdown products.

26. (U) Progress (Jul 64 - Jun 65) - (1) Living bacteria have been shown to be an integral factor in the lethality of peritoneal infections, however, other factor(s) are needed. The adjuvant effect of hemoglobin appears to result from interference with the defense of the host. Histochemical procedures on tissue reveals abnormal enzyme distribution; metabolic alterations are manifested by excess lactate levels. (2) Exposure of animals with peritoneal infections to hyperbaric oxygen tends to increase lethality, whereas metabolic acidosis is minimized. (3) Cyanoacrylate polymers, while moderately histotoxic, appear useful for tissue repair.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Reports, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		AR	1

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1439a	NA

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Project 3A025601A821, COMBAT SURGERY

Task 01, Combat Surgery

Work Unit 121, Metabolic and nutritional problems associated with injury

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Description.

The Department of Surgical Metabolism and Pathology has conducted a multi-disciplinary approach to metabolic and nutritional problems associated with injury. Studies were initiated or continued in the areas of

I. The factors associated with lethality in peritonitis and other surgical infections attributable to pharmacologic, physiologic, and metabolic effects of microorganisms or their toxins and metabolites. Research included the use and study of endogenous and exogenous bacterial systems for

(A) characterization of the role of the microflora,

(B) identification of the role of endotoxin using the egg embryo assay,

(C) the clarification of the adjuvant role of hemoglobin in bacterial lethality in both intraperitoneal and subcutaneous infections, and

(D) elaboration of the metabolic pathways affected by the infection.

II. The mechanism of action of hyperbaric oxygen in respect to its effect on bacterial infections and metabolic pathways associated with oxygen utilization.

III. Wound healing

(A) the potential use of adhesive polymers for restoring tissue continuity following injury,

(B) biological receptivity of polymers, and the possible toxicity of degradation products utilizing histological, biochemical,

and radioisotope methods,

(C) the development and utilization of spray gun procedures for applying adhesive polymers, and

(D) evaluation of the American Vascular Stapler for use in common bile duct anastomoses.

IV. The metabolism of bilirubin in primates with complete common duct obstruction, including several studies on the metabolism and excretion of bilirubin in children with biliary atresia.

V. The antithyroid properties of the sulfonamides were re-examined with special emphasis on the ability of the thyroid to return to normal function following prolonged drug treatment.

VI. The microbiology of patient isolators during prolonged use. Studies include:

(A) the behavior of patient's microflora, and

(B) contamination levels within the occupied isolator.

Progress.

I Studies on the factors responsible for morbidity and mortality in peritonitis and other infections.

The host response associated with morbidity and mortality in infection has been attributed to various pharmacologic, physiologic and biochemical parameters. The factors most cited, however, have been the bacteria and/or their toxins and metabolites. Related studies have attempted (1) to characterize the role of microflora, (2) to identify the role of endotoxin and quantitate its concentration in blood and peritoneal exudates, and to explore the significance of a newly-demonstrated toxin in sera and peritoneal exudates of stressed animals, (3) to investigate the adjuvant role of hemoglobin in lethal bacterial infections, (4) to identify consequent histopathologic changes, and (5) to determine by histochemical and biochemical procedures the metabolic pathways affected.

Two experimental models have been developed and utilized: (1) an endogenous bacterial system based on the in vivo ligation of the appendix; (2) an exogenous bacterial system employing intraperitoneal inoculation of a single organism strain (E. coli 0111:B4) with added hemoglobin. The latter, E. coli-hemoglobin system has been expanded to include studies on the role of hemoglobin on the rate of growth and migration of the organisms and its possible effect on the defense mechanisms of the host.

I(A) Studies on the agents responsible for lethality in an experimentally produced endogenous peritonitis.

The agents which potentiate lethality in peritonitis have been designated as bacteria, bacterial toxins or bacteria plus adjuvant materials. The perpetrator of the observed complex physiological response has resisted isolation and identification. This study was undertaken in an attempt to differentiate among the bacteria, bacterial toxin or other factors as the cause of death in experimental peritonitis.

Peritonitis was produced in the dog by dividing the appendiceal blood supply and ligating its base. In preliminary experiments the exudate was permitted to collect in the peritoneal cavity; the results were disseminated peritonitis and rapid death. In order to collect and study the lethal exudate, the experimental procedure was modified to include a non-porous plastic bag sutured to the cecum about the base of the appendix. The plastic bag acted as an exudate reservoir and prevented death. The exudate (25-50 cc) was removed after four days and studied bacteriologically and biochemically. Portions of each exudate from 20 different experiments were fractionated and the lethality of the fractions studied by intraperitoneal injections into rats.

The exudates contained a large number of microorganisms which were quantitatively and qualitatively similar. The groups and numbers of organisms found in the exudates were Clostridia 1×10^4, Coliforms 1×10^9, Lactobacilli 1×10^{10}, and Staphylococci 1×10^6. Hemoglobin concentrations in the exudates varied from 1.2 gms per cent to 9.5 gm per cent, with over half the fluids having concentrations greater than 4.0 gm per cent. The most lethal exudates had hemoglobin concentrations of 4.0 gm per cent or above.

The exudate was fractionated into fluid and solid components by ultracentrifugation at 20,000 rpm for 20 minutes. The supernatent fluid was filtered through a Seitz filter to remove any remaining bacteria. A portion of this ultrafiltrate was heated to 60° C. for 15 minutes to destroy exotoxins. The heated or unheated ultrafiltrate was then recombined with the sediment or used alone in the subsequent rat injection studies.

The sediment, on the other hand, was resuspended in various liquid media for subsequent injection. The sediment was combined with ultrafiltrate saline, dog serum and 4 gm% hemoglobin in saline. A portion of the sediment was heated to 80° C. for 2 hours, which killed the organisms but did not destroy their endotoxin. Another portion was treated by ultrasound to achieve the same effect. These treated sediments were then resuspended in ultrafiltrate.

These various fractions and combinations were injected into Walter Reed white rats and mortality noted at 24 hours. Rats weighed 200 to 250 grams and received 5 cc/Kg of a given mixture intraperitoneally. Seventy per cent of the rats died when challenged with unaltered peritoneal exudate. However, when only the ultrafiltrate was injected (either heated or unheated) only 2 of 68 animals (3%) died. This demonstrates that the soluble toxins present in the original exudate were not present in sufficient quantity to kill. When the sediment was unsuspended in saline or serum only 37% of 103 animals succumbed. This demonstrates that the sediment is necessary to kill but the mortality was only half that of the unaltered fluid. The conclusions were that the ultrafiltrate must be responsible for enhancing the effectiveness of the sediment. This was demonstrated also when recombination of the sediment with the ultrafiltrate resulted in a mortality of 77% in 68 animals. Suspension of the sediment in 4 gm% hemoglobin, rather than saline or serum, caused death in 70% of the animals. This indicated that the factor in the ultrafiltrate which increases the effectiveness of the sediments was probably the hemoglobin. Heat killing the bacteria or sterilization with ultrasound eliminates the lethal properties of the sediment. Only 3% died of 45 animals after injection of heated sediment with ultrafiltrate; and only 13% of 30 animals were killed when the exudate was treated with ultrasound. The results indicate that living bacteria are essential for the lethality of this model. Endotoxin is not destroyed in either of these latter preparations.

I(B) Studies on the role of toxins in the lethality of bacterial peritonitis.

Demonstration and quantitation of toxins in plasma, exudates and tissue are essential for the understanding of bacterial shock, since the pathologic, physiologic, and biochemical changes associated with shock cannot be fully interpreted until the responsible agents are elucidated. Therefore, work was continued on the development and utilization of the sensitive chick embryo assay for endotoxin determinations. Experimentation was oriented toward: (1) the detection and quantitation of bacterial endotoxins in exudates and sera from animals with bacterial peritoneal infections, and (2) the demonstration of a hemorrhagic vascular toxin in sera from stressed animals.

(1) Detection and quantitation of bacterial endotoxins. The methodology employed and results from preliminary investigations of the chick embryo assay were previously reported (Progress reports, 1963-64). These investigations have been extended to include, (a) standardization of the assay procedure, and (b) the application of the assay to the detection of endotoxin or endotoxin-like activity in pathologic menstrua. Intermediate candling of inoculated eggs was performed at 3, 6, and 12 hours; all eggs were examined at 24 hours.

The results of the inoculations were recorded in terms of /1/ time of death of the embryo, /2/ gross pathology of the embryo at death or 24 hours postinoculation sacrifice, and /3/ appearance of the embryonic brain and other selected organs at time of death or sacrifice. Inocula contained either standard graded doses 0.001 mcg to 10.0 mcg per 0.5 ml, of commercial endotoxin from E. coli; 0111:B4 or pathologic menstrua obtained from animal models with bacterial peritoneal infections. Dilutions were made in sterile pyrogen-free saline or normal rat sera. Sera and peritoneal exudates were obtained from the two basic experimental models; one employing appendiceal ligation in the dog, the other using exogenous infections of E. coli 0111:B4, and hemoglobin in rats.

The reproducibility of the assay, as standardized for the 11-day embryos, was determined in a series of separate titrations to investigate possible seasonal variations. The results obtained indicated that while a variation in susceptibility to endotoxin was manifested in individual titrations, no marked variations reflective of seasonal changes were detectable. The separate LD_{50} values obtained from these titrations extended from 0.0068 mcg to 0.0316 mcg per 0.05 ml inocula volume. Data obtained from these experiments and value from standard graded endotoxin controls of other experiments yielded a mean LD_{50} of 0.0184 mcg and a standard deviation of ± 0.0075. These values indicated a reproducible assay sensitivity in the range of 0.01 mcg or less.

The sensitivity of endotoxin contained in serum or plasma was compared with that found in saline. A series of titrations was conducted using serum or plasma preparations containing graded amounts of standard endotoxin. Parallel controls, which contain endotoxin in saline, were run simultaneously. The concentrations of endotoxin employed were 0.5, 0.1, and 0.05 mcg per ml. Portions of the endotoxin-plasma or endotoxin serum mixtures were heated at 56° C. for 30 minutes prior to inoculation. Results of these experiments showed that heating of the endotoxin serum mixtures at 56° C. for 30 minutes resulted in an apparent two-fold reduction in toxicity, while the 80° C. treatment resulted in complete inactivation.

Endotoxin assays were performed also on sera and exudates obtained from various experimental animal models employed in the study of bacterial peritonitis. Two assays were performed on individual exudates and a third on pooled exudates from rats succumbing to infection following intraperitoneal injections of a suspension of viable cells of E. coli; 0111:B4 in 4 per cent hemoglobin. Endotoxin or endotoxin-like activity was detected in all these assays in levels exceeding the equivalent of 10 mcg or more of standard E. coli 0111:B4 endotoxin per ml of the original sample. The criteria for endotoxin activity was the retention of toxic activity following heat inactivation, and the products of characteristic

hemorrhagic lesions in the brains, livers and dermal tissues of the 11 day-old chick embryo. The results are tabulated below.

TABLE I

Demonstration of endotoxin or endotoxin-like activity in rat peritoneal exudate

Preparation	Dilution	Per cent of Assay Embryos Showing Endoxotin Reactions		Per cent Normal
		Lethal	Nonlethal	
Exudate, rat 64-18-6, heated at 65° C. for 30 min.	1:1	100	0	0
	1:10	100	0	0
Endotoxin conc. (eq. 0111:B4 ET) = > 10.0 mcg/ml				
Exudate, rat 64-18-9 heated at 65° C. for 30 min.	1:1	100	0	0
	1:10	100	0	0
Endotoxin conc. (eq. 0111:B4 ET) = > 10.0 mcg/ml				
Endotoxin standard (0111:B4 ET) LD_{50} = 0.0147 mcg				
Pooled exudates, rats 64-18-2, 7, 10; heated at 65° C. for 30 min.	1:1	100	0	0
	1:10	100	0	0
	1:100	14	14	72
Endotoxin conc. (eq. 0111:B4 ET) = ± 100 mcg/ml				
Endotoxin standard (0111:B4 ET) LD_{50} = 0.0273 mcg				

In addition, four endotoxin assays were performed on sera and exudates obtained from dogs in which endogenous bacterial peritonitis was induced by appendiceal ligation (Section I(A)). In dog No. 218, dispersion of exudate into the peritoneum was prevented by enclosing the ligated appendix in a nonporous plastic bag. Exudate obtained from the dog at sacrifice was found to contain endotoxin activity equivalent to 0.5 mcg of standard endotoxin per ml. Assay of serum from the same dog suggested the possible presence of endotoxin activity equivalent to less than 0.05 mcg per ml. In dog No. 626,

when the lethal exudate was permitted to diffuse into the peritoneal cavity, specimens were collected post mortem. Assay of serum for endotoxin was negative, while peritoneal exudates indicated a level of endotoxin activity equivalent to 0.1 to 0.2 mcg per ml as compared with standard endotoxin preparations.

These results appear to warrant the following conclusions: (1) the chick embryo assay for the detection of endotoxin is feasible, (2) the assay procedure is characterized by a remarkable degree of reproducibility with a mean level of sensitivity of 0.0184 mcg ± 0.0075, and (3) the assay procedure can be successfully applied to the detection of endotoxin in sera and various exudates in the range of 0.1 to 20 mcg per ml when compared with a standard endotoxin preparation.

(2) Demonstration of a hemorrhagenic vascular toxin in sera from stressed animals. This material is concerned with the initial report on the characterization of a unique factor present in the sera of rats following trauma or periods of acute stress. The effects associated with this factor are described as they were observed in the 10- to 13-day chick embryo.

Sera were obtained from standard normal young adult white rats (Walter Reed strain) under one or more of the following conditions. Unanesthetized rats were stressed by immobilization in restraining cages for periods extending from 30 minutes to 6 hours, or rotational trauma for 15 to 30 minutes. Blood was obtained from the experimental (stressed) and control (nonstressed) rats from the exposed abdominal aorta under Na pentobarbital anesthesia (40 mg/Kg intraperitoneally). Blood was collected in sterile tubes and the serum removed. Several experiments employed normal dog sera which were obtained by venipuncture of the jugular vein without prior anesthesia.

The chick embryo assay was performed as previously described in Section I(B)(1). In collateral experiments chick embryos were treated with one or more of several pharmacological preparations diluted in either serum or pyrogen-free 0.9 per cent saline. These included epinephrine, norepinephrine and serotonin.

The hemorrhagic effect of normal rat serum for the 12-day chick embryo was first observed in embryos which had received, as controls for endotoxin assays, 0.05 ml of sterile pooled rat serum. The sera which comprised the pool had been obtained from normal rats bled under sodium pentobarbital anesthesia. When examined 24 hours following inoculation, 3 of 6 embryos receiving this serum exhibited a diffuse pattern of pinpoint hemorrhages and dilated vessels throughout the postlateral aspects of the optic lobes of the embryonic mesencephalon. All other regions of the brain as well as all other tissues and organs were normal. Other embryos receiving material from the same serum pool,

which had been heated at 56° C. for 30 minutes prior to inoculation, and all embryos receiving normal saline were normal.

Occurrence of the lesions described above was best correlated with the manner by which the rats had been handled prior to anesthesia and bleeding. A series of further experiments was undertaken in order to extend this association and to provide a description of the factor and its hemorrhagenic effects. The acronyms SCHF and SCHE, Serum Cerebrovascular Hemorrhagenic Factor and Serum Cerebrovascular Hemorrhagic Effects, were devised for ease of reference.

The association between appearance of the SCHE lesions and the mode of handling of rats prior to anesthesia and bleeding was confirmed by the following experiments. Sera were obtained from a normal rat and from one subjected to 15 minutes' traumatic abuse prior to anesthesia and bleeding. Dilutions were prepared in sterile pyrogen-free saline; portions of diluted and undiluted sera were heated at 56° C. for 30 minutes. The various preparations were inoculated into selected chorioallantoic veins of 11-day embryonated eggs. The inoculated eggs were candled at intervals and individual eggs selected for examination at intervals extending up to 48 hours. The embryos were scored in terms of the following reactions: experimental artifact deaths (EAD); superficial cephalic hematomas (CH); SCHE lesions in the optic lobes; nonspecific reactions or deaths (NSR or NSD); and totally normal appearance (Norm). The results show that the SCHF is present in normal rat sera but in relatively small amounts; physical trauma greatly increases the effective SCHF levels; the lesions associated with SCHF are not produced in embryos receiving heat inactivated sera. The differences between sera from traumatized and normal rats were statistically significant ($p < .05$).

The production of SCHE lesions by sera from normal immobilized or traumatized rats was assessed. Bloods were obtained as described; portions of the different sera were heated at 56° C. for 30 minutes. Inoculations were made in 11-day embryonated eggs; randomly selected eggs from each experimental group were opened and the embryos examined at 1, 2, 4, 6, and 8 days postinoculation. The results permitted two conclusions. /1/ increased levels of SCHF in rat sera are found following both types of stress, and /2/ the lesions associated with the SCHE are confined to the optic lobes and are not lethal to the developing embryo. Additional experiments also indicated that while SCHE lesions did not interfere with subsequent embryonic development, embryos in which severe lesions had been induced were incapable of normal hatching. Examination of such embryos at the 22 day of development indicated that while embryonic development had been normal in all other respects, the mesencephalon and part of the diencephalon had undergone marked necrosis and were not morphologically recognizable.

A series of experiments were undertaken to determine the relationship between SCHF and various normal catecholamines and to heat lability. A portion of the data obtained in these experiments is tabulated:

TABLE II
Demonstration of Synergism Between SCHF and Norepinephrine and the Independent Heat Sensitivity of SCHF

Preparation	General effects produced in 11-day chick embryos EAD	CH	NSR	SCHE	Norm
Epinephrine in SCHF reactive serum; (mcg per 0.05 ml)					
5.0	2/6	2/4	1/4	4/4, 4+*	0/4
5×10^{-2}		0/4	1/4	4/4, 4+	0/4
5×10^{-4}		0/4	1/4	3/4, 3+	1/4
5×10^{-6}		0/4	0/4	4/4, 2+	0/4
5×10^{-8}		0/4	0/4	4/4, 1+	0/4
Serum alone		0/4	1/4	2/4, 1+	1/4
Epinephrine in pre-heated** SCHF reactive serum; (mcg per 0.05 ml)					
5.0	1/5	1/4	1/4	0/4	3/4
5×10^{-2}		2/4	0/4	0/4	2/4
5×10^{-4}		0/4	1/4	0/4	3/4
5×10^{-6}		0/4	0/4	0/4	0/4
5×10^{-8}		0/4	0/4	0/4	0/4
Serum alone		0/4	0/4	0/4	4/4
Epinephrine in saline; (mcg per 0.05 ml)					
5×10^{-2}		2/3	0/3	0/3	1/3
5×10^{-4}		1/3	0/3	0/3	2/3
5×10^{-6}		0/3	0/3	0/3	3/3
5×10^{-8}		0/3	0/3	0/3	3/3
Saline alone		0/3	0/3	0/3	3/3
Epinephrine in SCHF reactive serum diluted 1:1 in non-reactive serum***; (mcg per 0.05 ml)					
5×10^{-2}		0/4	0/4	3/4, 2+	1/4
5×10^{-4}		0/4	0/4	2/4, 1+	2/4
5×10^{-6}		0/4	0/4	2/4, 2+	2/4
Serum mixture alone		0/4	0/4	2/4, 1+	2/4
Nonreactive serum alone		0/4	1/4	0/4	3/4
Epinephrine in pre-heated SCHF reactive serum diluted 1:1 in nonreactive serum; (mcg per 0.05 ml)					
5×10^{-2}		0/4	0/4	0/4	4/4
5×10^{-4}		0/4	0/4	0/4	3/4
5×10^{-6}		0/4	0/4	0/4	4/4
Serum mixture alone		0/4	1/4	0/4	3/4

*SCHE lesions were scored on a basis of 1+ to 4+ depending on the extent of the reaction.
**Heated at 56° C. for 30 minutes prior to addition of epinephrine or other materials.
***Normal rat serum found to be devoid of SCHF activity both without and with epinephrine.

/1/ Catecholamines alone in physiological doses are without marked effect in the developing chick embryo.

/2/ The addition of epinephrine to stressed sera and to occasional normal sera will result in synergistic enhancement of the SCHE reactions. This synergism is not obtained with norepinephrine or serotonin.

/3/ SCHF in rat serum is heat labile as demonstrated in experiments in which stressed serum was heated prior to addition of epinephrine. The SCHF activity cannot be restored by addition of normal rat serum (complement) to inactivated SCHF-containing serum.

Experiments using several sera obtained from normal dogs suggested the presence of a factor similar to SCHF as described in normal rat sera. However, it was also indicated that the factor as present in dog sera was either more active or present in greater amounts and could produce a lethal effect in the embryonated egg.

Serum was collected from approximately 50 ml of blood obtained via jugular venipuncture. Aliquots were removed and some sterilized by filtration through membranes. All specimens were then either refrigerated or heated at 56° C. for 30 minutes. These procedures gave eight different final preparations according to the presence or absence of platelets in the original samples, and whether or not the samples had been filtered or had been heated. These preparations were inoculated into 12 day embryonated eggs. The results indicated that dog serum contains an SCHF similar to that observed in rat sera. This factor may be lethal for the chick embryo; but it is not associated with formed elements such as platelets and is heat labile.

The SCHE lesions were sharply localized. Grossly, embryos exhibiting SCHE lesions were normal in all respects except for the localized hemorrhagic areas within the mesencephalon. These were distributed in the superior, lateral and superior portions of the optic lobes and were usually bilateral. They varied from small well defined foci to complete hemorrhagic involvement of the entire lobe. Extracranial hemorrhage was rare. Microscopically, the lesions varied from small, linear, punctate, perivascular hemorrhages, unassociated with necrosis, to extensive perivascular hemorrhage with vascular necrosis and massive liquefactive necrosis of the surrounding brain substance. Older lesions consisted

either of resolution with vascular engorgement and small perivascular hemorrhages, or progressive necrosis of the entire lobe.

The findings presented above may be summarized by the following: A cerebrovascular hemorrhagenic factor, SCHF, has been demonstrated in sera from normal and stressed rats. A similar if not identical substance has also been demonstrated in dog sera. The serum levels of SCHF, as indicated by the extent of reactions produced in the optic lobes of developing chick embryos, are greatly increased by traumatization or stressing of donor animals prior to bleeding. The factor is heat labile, being inactivated by heating at 56° C. for 30 minutes; activity is not restored by the addition of unheated serum (complement). The factor cannot be associated with hemoglobin, mast cells or platelets. The lesions associated with the factor are restricted entirely to the optic lobes of the developing chick embryo. They were not lethal in the case of rat SCHF, but are lethal when induced by undiluted canine SCHF. The reaction produced by SCHF is synergistically augmented by the addition of epinephrine but not by other catecholamines.

I(C). Studies on the adjuvant effects of red cells on the lethality of Escherichia coli:

The present series of study were undertaken to establish the role of hemoglobin in lethal, E. coli peritonitis and subcutaneous infections. Emphasis was placed primarily on early changes produced by hemoglobin on (1) the growth of the bacteria, (2) the migration of bacteria from the peritoneal cavity, and (3) the defense mechanisms of the host. In addition, (4) histopathologic studies were conducted, and (5) subcutaneous infections (E. coli-hemoglobin) were compared with those of the peritoneum.

(1) The growth of bacteria. Experiments were designed initially to study the effects of hemoglobin on the migration of E. coli from the peritoneal cavity of the dog. Fourteen adult mongrel dogs weighing 8 - 12 kg. were studied in seven different experiments. Conditions were as follows: Group I contained ten animals which were anesthestized with nembutal, connected to a respirator, and the following structures cannulated for sampling: external jugular vein, carotid artery, portal vein, thoracic duct, and urinary bladder. Blood pressure and pulse were monitored continuously. Two animals were used in each experiment, and were given intraperitoneal injection (5 ml/Kg body wt) of either E. coli suspended in nutrient broth or an E. coli-hemoglobin mixture in nutrient broth. In two of the five experiments, the E. coli were tagged with I^{131}.

In Group II (four additional animals) the experiment technique was changed to eliminate the factors of anesthesia, surgical procedures, artificial respiration and position which could possibly affect results.

The animals were allowed to awaken following intubation of only the external jugular vein and carotid artery. The following day peritonitis was induced as in the preceding experiments using E. coli: tagged with I^{131}.

In all experiments, hourly blood specimens were taken for bacterial counts, hematocrit, white cell counts, and for the detection of I^{131} labelled E. coli. Lymph specimens were collected at the same time and bacterial counts were performed. At death or sacrifice peritoneal exudate and portions of the lung, liver, and spleen were removed for bacterial counts and, where applicable, checked for radioactivity. Radioactive counting was performed in a liquid scintillation counter and a gamma spectrometer. Simultaneously, groups of rats were injected intraperitoneally with E. coli and E. coli-hemoglobin to test lethality of the inocula.

Rats injected with E. coli and hemoglobin experienced a 75% mortality; demonstrating the lethality of the inoculum, no deaths were recorded when E. coli alone were injected.

The number of viable bacteria in both arterial and venous blood in all epxeriments remained below 1×10^3 per ml in all samples but one, and approximately 60% of the specimens were sterile. However, positive blood cultures were more frequent in the dogs receiving the E. coli and hemoglobin mixture. In the experiments where labelled organisms were used, radioactivity was found in all blood specimens, but results could not be correlated with viable bacteria counts. The radioactivity in the blood was about equally divided between the cells and serum. Trichloracetic acid precipitates of the serum showed that over half of the serum iodine was unbound. This would indicate that the bacteria were being cleared and destroyed at about the same rate whether or not hemoglobin had been added to the inocula. In two experiments in which lymph flow was negligible, the blood level of viable organisms was similarly low. This indicated that the loss of organisms due to lymph flow was not a major contribution factor to low blood levels found.

The blood white cell response to the intraperitoneal injections of E. coli and hemoglobin and of E. coli alone were strikingly different. An immediate leukopenia was noted in the animals which received E. coli alone. In contrast, the animals receiving the E. coli and hemoglobin mixture showed a delayed, less, or some cases no drop in white blood cell counts. This effect could be attributed to interference by hemoglobin with white cell or bacterial migration, with phagocytosis by the leukocytes or to a combination of these factors.

The diffusion or migration of bacteria from the peritoneal cavity was also studied in five experiments by cannulating the right thoracic

duct and analyzing the lymph for viable organisms, and in two instances, for radioactivity. In four of the five experiments the output of viable organisms into the lymph of the animals (E. coli alone) exceeded that of the animals receiving E. coli and hemoglobin, i.e. larger numbers were found in more of the lymph specimens.

At sacrifice or death, the peritoneal exudate and organs were removed and studied for viable E. coli and radioactivity. The volume of peritoneal exudate was greater, where E. coli and hemoglobin mixtures were injected and the viable bacteria count per ml was larger, usually by factor of 100. The radioactivity per ml, however, was similar, which indicated increased growth of bacteria within the peritoneum in the presence of hemoglobin.

The liver, lung, and spleen of all animals contained large numbers of viable E. coli and similar levels of radioactivity suggesting that RES clearance was not affected by the differences in inocula. Apparent differences in radioactivity and in colony counts seemed correlated with the presence or absence of shock at sacrifice, but further experiments are needed to establish this observation.

(2) The migration of bacteria. Among the many postulates concerning the role of hemoglobin in converting a nonlethal E. coli peritonitis to a lethal one is the possibility that hemoglobin retards the clearance of bacteria from the peritoneal cavity. Walter Reed white rats (200-250 gm) were given an intraperitoneal injection (5 cc/Kg body wt) of a standard lethal E. coli-hemoglobin mixture; a second group received E. coli alone. Bacterial counts of the material were obtained prior to injection, and groups of the challanged animals were sacrificed each hour for five hours to determine the number of bacteria remaining in the peritoneal cavity. Complete recovery of the residual organisms was assured by injecting 20 cc of saline into the peritoneal cavity at the time of sacrifice, and after proper mixing, 1 cc was withdrawn for quantitative bacterial counts. Results indicate that when 1 cc of a mixture containing 1.5×10^{11} E. coli alone was injected intraperitoneally only 5×10^{6} bacteria were present one hour later. There was little change in counts over the next four hours. When 5 mg% hemoglobin was present in the media, 5×10^{9} organisms were found at one hour and at all times thereafter. Previous studies have shown that when an animal dies as a result of E.coli-hemoglobin injection, i.e. at 24 hours peritoneal bacterial counts are always greater than 1×10^{9}. If an animal survived, or was challenged with E. coli without hemoglobin, counts were always less than 1×10^{9} and averaged about 1×10^{4} after 24 hours. The increased bacterial growth in the peritoneum, associated with the E. coli-hemoglobin system and implying increased endotoxin production, may account for the physiological responses observed in this model.

Blood culture data in these experiments indicate that more organisms are present in the blood stream in the animals challenged with E. coli

and hemoglobin than in the controls. Thus, a defect in host defense mechanism could also be assumed since a decreased peritoneal clearance of organisms would be expected to produce fewer organisms in the blood stream. Therefore, an indirect measure of bacterial absorption from the peritoneum was also studied.

Proteins are absorbed from the peritoneum via the same route as bacteria, i.e. by diaphragmatic lymphatics. A measure of protein absorption should therefore provide information on lymphatic clearance of all substances similarly cleared. Plasma albumin was tagged by the addition of 1 cc of 0.4 per cent Evans blue dye to 4 cc of plasma. Tagged albumin (5 cc/Kg body wt) was injected intraperitoneally into rats 2 hours following a standard intraperitoneal injection of E. coli (Group 1), E. coli and hemoglobin (Group 2) or nutrient broth (Group 3). Blood samples were obtained from all animals 1½ hours after injection of the tagged plasma and analyzed spectrophotometrically for Evans blue dye. Results show that the mean concentration of dye in the plasma of Group 1 was 15.7 mg/L, Group 2, 1.27 mg/L and Group 3 25.2 mg/L. Even after accounting for changes in plasma volume, which may occur in peritonitis, absorption of protein from the peritoneum is markedly decreased by the hemoglobin-E. coli injection. Values are approximately 1/10 of those found with injections of E. coli without hemoglobin.

A similar study was carried out in the dog so that hourly changes in dye concentration in the plasma could be followed. From these data a curve was obtained representing the rate of albumin absorption. The tagged plasma was injected intraperitoneally 3 hours following an injection of one of the following: nutrient broth, hemoglobin (4 gm%) in broth, Escherichia coli in broth, E. coli plus hemoglobin (4 gm%) in broth. Similar doses of dye and tagged plasma were used as previously described and blood samples were drawn each hour for 5 hours and then at 24 hours.

The mean absorption at 5 hours based on regression analysis was 18.0 mg/L with nutrient broth, 17.1 mg/L with hemoglobin, 11.4 mg/L with E. coli and 5.4 gm/L with E. coli plus hemoglobin. Again hemoglobin-E. coli mixtures markedly retarded peritoneal absorption of plasma as compared to E. coli suspensions alone. Hemoglobin in broth without organisms did not affect absorption.

The results from these direct and indirect studies indicated that an important effect of hemoglobin when in combination with an E. coli suspension was to prevent the rapid clearance of bacteria from the peritoneum. Therefore, their growth was allowed to continue perhaps also relatively unhindered by the systemic defense mechanisms of the host.

Colloidal iron was investigated as a lymphattic marker for peritoneal absorption studies. Although the material can be seen grossly when the Prussian blue reaction is done, and tends to be picked up by lymphatics

less readily when hemoglobin and E. coli are injected, this technique did not offer the advantages of the quantitative Evans blue-albumin procedure.

(3) Defense mechanisms of the host. Previous studies in the dog (Section I(C)(1)) on the effects of hemoglobin on the migration of E. coli from the peritoneal cavity indicated an interference with the white cell proliferation, migration, or phagocytosis. This observation provided the basis for the study of white cell levels in the blood and peritoneal exudate of the rat. Experiments were conducted therefore in an attempt to evaluate the role of hemoglobin on this phase of host's defense.

Group of rats were injected intraperitoneally with either E. coli in nutrient broth or E. coli and hemoglobin in nutrient broth and sacrificed at intervals of 1/2, 1, 2, 3, 4, 6, 8, and 24 hours. Animal receiving E. coli alone revealed a white cell response similar to that found in intravenous injections. There was a rapid and immediate decrease in white blood cells. Subsequent sample, collected at hourly intervals, showed a continuous low blood level for 2 to 4 hours after which time the white blood cell count returned slowly to control values. The white blood cell response in the E. coli-hemoglobin animals differed from that found in the E. coli group in three respects: (a) There was a marked delay, 1/2 to 1 hour, in decrease of white blood cell counts, (b) the decrease in white blood cell counts was more gradual, and (c) the total decrease in white blood cell counts was always less. The white cell count in the peritoneal exudate was considerably lower in the E. coli-hemoglobin animals, and remained lower during the first four hours of the experiment.

The results indicate that hemoglobin in the presence of E coli either partially inhibits the migration of white cells from the circulation to the site of infection, or inhibits the initial migration of the bacteria, into the general circulation and hence lessening the leukopoietic stimulus. The delay in systemic leukopenia could also result from an inhibition of clearance of the bacteria from the peritoneal cavity. Histological examination of the peritoneal exudate showed that hemoglobin crystals were present for at least 4 hours. The presence of the hemoglobin crystals also apparently interfered to some extent with phagocytosis of the bacteria by the white cells. Further studies are indicated.

(4). Histopathologic studies on animals with E. coli-hemoglobin induced peritonitis. Histopathologic studies were carried out at serial sacrifice and at autopsy on animals which have received a lethal intraperitoneal inoculation of E. coli and hemoglobin.

Rats. The pathologic alterations include vascular changes, lymphoid tissue changes, and inflammatory reactions. The vascular reactions are stasis and congestion, with frequent venous thromboses. Frequently hyaline

thrombi are seen as well as red cell thrombi. These changes are seen in lungs, intestines, liver, kidneys and brain. In the liver centrilobular necrosis is frequently seen associated with thrombi in the central veins. Necrosis of the gut is also seen, associated with marked congestion of the submucosal vessels which frequently contain red cell conglutinations. Necrosis of tubular epithelial cells is frequently seen.

In the spleen, lymph nodes, and Peyer's patches, phagocytosed cellular debris is found in the follicles. Necrosis of lymphocytes is seen in these tissues and also in the thymus. The peritoneal inflammatory reaction varies from mild to pronounced, with many colonies of bacteria being present in some animals. The exudate consists of polymorphonuclear leukocytes and fibrin, and is seen over the surface of the spleen, liver and intestines and within the omentum. The peritoneal surface is coated by fibrin, leukocytes, bacteria and hemoglobin, and the diaphragmatic lymphatics are engorged with bacteria, which often are within leukocytes. Hemoglobin particles are frequently seen within the lymphatics of the diaphragm and in the peritoneal inflammatory reaction.

Dogs. An inflammatory exudate is present over the peritoneal surfaces and within the omentum. The diaphragmatic lymphatics contain neutrophils, bacteria and hemoglobin. The retrosternal lymph nodes contain phagocytosed bacteria. The changes with the lung, kidney, spleen and liver of the dog are less severe than in the rat. Only a few dogs showed lung changes similar to those in the rat.

(5) Subcutaneous infections. Investigations of the adjuvant effects of hemoglobin on E. coli lethality were expanded to include other sites of infection. The subcutaneous tissue of the rat was chosen as a convenient area in which to study both the locale and systemic response. Studies were initiated to observe the tissue responses, the bacterial migration and the biochemical changes associated with subcutaneous injections of E. coli-hemoglobin suspensions.

Groups of rats were injected with several inocula, nutrient broth, hemoglobin-nutrient broth, hemoglobin alone, E. coli alone, and E. coli-hemoglobin. The animals were sacrificed at intervals of ½, 2, 6, 12 and 24 hours. In two experiments animals were injected with E. coli and hemoglobin, at separate sites, both subcutaneously and intraperitoneally and sacrificed at 4, 8, 12, 24, 28 and 72 hours. In all experiments, bacteriologic, blood chemistry and histologic studies were performed.

Histological studies reveal that a well developed inflammatory reaction was established in the dermis by 6 hours. A mild, sterile inflammation was produced by the broth, probably due to its peptone content. Hemoglobin, where injected, was often present in the tissues in the form of crystals of various sizes and shapes and usually a marked inflammatory response was seen, consisting of polymorphonuclear leukocytes. Macrophages were seen where small particles of hemoglobin 1-2 micra

in size, were present. E. coli alone and E. coli-hemoglobin both elicited extensive vascular congestion and necrosis and a polymorphonuclear response. With E. coli-hemoglobin, however, numerous colonies of bacteria persisted in the center of the lesions among the hemoglobin particles, apparently isolated from the hosts' defense mechanisms. With E. coli alone, the bacteria were greatly diminished by 12 hours, most being seen within polymorphonuclear leukocytes by this time.

A slight bacteremia (10^2, 10^3) appeared during the first 6 hours and at 72 hours in those animals receiving E. coli intraperitoneally and hemoglobin subcutaneously. Sporadic animals yielded positive blood and organ cultures throughout this experiment. A repeated experiment, felt to be better controlled, revealed a positive blood culture at six hours only in those animals receiving E. coli intraperitoneally and hemoglobin subcutaneously. The blood in all other groups was clear and remained clear. After 6 hours the liver was cleared of bacteria and remained clear until 72 hours, when some animals in all groups yielded a few colonies of bacteria. In the subcutaneous injection sites, where E. coli was injected alone, the counts fell from an initial 10^7 to 10^1, or 0 by 72 hours, whereas when E. coli and hemoglobin were injected together the bacterial counts were still 10^6 at 72 hours. Contrary to the experience of Krizek and Davis (J. Trauma, 5:85-94, Jan. 1965), no rats receiving subcutaneous injections or injections of E. coli and hemoglobin at different sites died. Also, bacteria did not migrate from the original inoculation site into a sterile hematoma. The bacterial counts in injection sites of our animals were also lower than in Krizek and Davis' study.

(D) Histochemical studies of the activity of selected enzymes following the intraperitoneal injection of E. coli and hemoglobin. Metabolic alterations occurring in bacterial shock have been attributed frequently to a tissue anoxia which results in a breakdown of energy-producing systems of the cells. Therefore, histochemical study of the enzymes directly responsible for oxygen utilization and energy production were investigated in order to gain further insight into metabolic abnormalities associated with infection and shock. The enzymes studied were succinic dehydrogenase, cytochrome oxidase, ATPase, acid phosphatase, TPN diaphorase, and DPN diaphorase. Other histochemical techniques, i.e. Schiff stain for glycogen, were also utilized.

A lethal infection was induced in Walter Reed strain white rats with an intraperitoneal injection of E. coli and hemoglobin. At various time intervals up to 24 hours the rats were killed by CO_2 asphyxiation and the tissues removed. Tissues were then frozen, and sections cut on a cryostat for cytochrome oxidase, succinic dehydrogenase, and TPN and DPN diaphorase studies. The other tissue was fixed in buffered formalin for paraffin embedding and studied for ATPase, and acid and alkaline phosphatase activity.

Preliminary results have indicated that the liver is one of the organs chiefly affected in the E.coli- hemoglobin model for producing lethal peritonitis. Alterations in the liver may be observed in hematoxylin and eosin sections by 1 hour. The changes are well advanced by 6 hours and reach a maximum in 12-24 hours. Initially, a severe congestion occurs, with red cell thrombi and platelet thrombi plugging the central veins. Early, scattered hepatic cells are seen with dark nuclei and cytoplasm, and later, frank necrosis occurs in the centrilobular areas, which is manifest as an eosinophilic homogenization of the cytoplasm and disintegration of nuclei. Glycogen is absent in such areas, as determined by the Schiff stain. With the Nitro-blue tetrazolium technique for succinic dehydrogenase, the hepatic cells in the necrotic areas are stained intensely. These necrotic areas are surrounded by a light zone of fine blue-black granules, possibly representing lipid, since the granularity disappears after acetone treatment. The dark staining areas are not affected by acetone washes nor do the areas stain without succinate substrate. This indicates that the staining in these areas is not due to a lipid nor is it nonspecific staining. A possible explanation for increased staining in the necrotic tissue is that mitochondrial membranes are damaged, allowing substrate to come into contact with enzyme. In the necrotic areas alkaline phosphatase activity is increased in the sinusoids but absent in the pericanalicular region. Acid phosphatase is decreased in the necrotic areas. Pericanalicular ATPase activity is reduced in the necrotic areas, and platelet thrombi within central veins reveals intense ATPase activity. Other enzymes systems are currently being investigated.

II. Influence of hyperbaric oxygen on experimental peritonitis.

In the past several years there has been increased interest in the effects of hyperbaric oxygen on bacterial infections. Interest was first aroused by reports of the beneficial effects of hyperbaric oxygen on aerobic infections. Subsequent studies with aerobic organisms demonstrated a bacteriostatic effect of oxygen at high partial pressures. Since a facility for the use of oxygen at high pressures was available studies were initiated on the effects of oxygen on peritoneal infections.

Cumulative experience in the last two decades has emphasized the complexity of peritonitis, particularly from a microbiological point of view. The organisms, which are most predominant in clinical infections, are probably the most significant factor. These are represented by both the anaerobic forms and the coliform organisms. In these initial studies an uncomplicated model of peritonitis was selected, namely the intraperitoneal injection of E. coli-hemoglobin suspensions. This model was chosen for these studies because the degree of toxicity after such an injection is both predictable and easily reproducible: with this model the untreated animal dies in 20 to 24 hours and the physiological findings are consistent with those reported for peritonitis and endotoxin shock.

The rationale for the use of hyperbaric oxygen is based not only on its possible bacteriostatic effects, but also on the possible beneficial effects it might have in combating the anoxia associated with endotoxin shock. Several recent studies have suggested that it is useful in this regard.

(A) Influence of hyperbaric oxygen on mortality from peritonitis. Initial studies were performed with rats. Animals were injected intraperitoneally with either E. coli-hemoglobin mixtures, E. coli alone, hemoglobin alone or nutrient broth. The animals were then treated with hyperbaric oxygen. Since deaths occurred only in the group receiving E. coli and hemoglobin mixtures, the hemoglobin and nutrient broth controls were eliminated in subsequent experiments.

After animals were challenged with an intraperitoneal injection of a standard E. coli-hemoglobin suspension, they were divided into four groups for treatment. Group 1 received no further treatment, Group 2 was treated with oxygen at 1 atmosphere, Group 3 was treated with air at 3 atmospheres of pressure and Group 4 received oxygen at 3 atmospheres. No other therapeutic measures were used.

Available data on oxygen toxicity indicate that rats can tolerate safely 3 atmospheres of oxygen for 2 hours. Experience with the treatment of anaerobic infections has shown the efficacy of multiple periods of treatment. Therefore, a 2-hour period of treatment was arbitrarily chosen. After a 4-hour interval, this was followed by a second 2-hour treatment.

Animals treated at 3 atmospheres of pressure (either air or oxygen) were brought to the hyperbaric chamber facility at the Naval Medical Research Institute at Bethesda. Animals which received air at 3 atmospheres were placed in an open cage and the cage placed directly into the large compression chambers. Animals which received 3 atmospheres of oxygen were placed in a sealed off infant isolette which was then placed in the main compression chamber. Oxygen was allowed to flow into the isolette at rates known to prevent CO_2 accumulation. The excess oxygen leaked into the main chamber through small defects in the sealed off isolette. Gas samples from the isolette and the main chamber were monitored by gas chromatography to be certain of a 100 per cent oxygen atmosphere in the isolette and 20 per cent oxygen in the main chamber. In addition, CO_2 analysis revealed no significant build-up of this gas in either environment. Temperature and humidity were controlled by ventilation of the main chamber.

Animals receiving oxygen at 1 atmosphere were placed in airtight containers modified to permit a flow of oxygen adequate for maintenance of a 100 per cent oxygen environment. Soda lime was added to the containers to prevent possible CO_2 increase.

Three different experimental conditions were studied: (1) therapy immediately after the intraperitoneal injection of a lethal E. coli-hemoglobin mixture; (2) therapy two hours after a lethal injection of a E. coli-hemoglobin mixture, and (3) therapy two hours after a non-lethal injection of a E. coli-hemoglobin mixture.

The mortality statistics in these three experiments can be found in TABLES III, IV and V. All data were submitted to Chi square statistical analysis and p values are shown.

TABLE III

Lethal E. coli-hemoglobin peritonitis: Therapy at onset and 6 hours later

	Treatment			
	None	O_2 1 Atmosphere	Air 3 Atmospheres	O_2 3 Atmospheres
No. rats	35	20	20	20
Deaths	22	10	13	16
% mortality	63	50*	65	80*
Av. time to death (hours)	21.1	21.3	22.0	11.2+

TABLE IV

Lethal E. coli-hemoglobin peritonitis: Therapy at 2 and 8 hours after onset

	Treatment			
	None	O^2 1 Atmosphere	Air 3 Atmospheres	O_2 3 Atmospheres
No. rats	35	35	30	30
Deaths	22	21	19	23
% mortality	63	60	63	76*
Av. time to death (hours)	21.1	21.3	22.1	13.1+

* χ^2 test $p > .05$
+ χ^2 test $p < .001$

TABLE V

Nonlethal E. coli-hemoglobin peritonitis: Therapy 2 and 8 hours after onset

	Treatment			
	None	O_2 1 Atmosphere	Air 3 Atmospheres	O_2 3 Atmospheres
No. rats	20	40	40	40
Deaths	0	0	8	5
% mortality	0	0	20*	13*

χ^2 = test $p < .01$

In studies 1 and 2 hyperbaric oxygen had no beneficial effect. The difference in per cent mortality in each of the treatment groups was not statistically significant.. However, a significant decrease in survival time was demonstrated for the animals placed in 3 atmospheres of oxygen. This showed a marked detrimental effect of oxygen, when used under the conditions of this experiment. Moreover, study 3 shows that treatment with either oxygen or air of 3 atmospheres of pressure caused a significant number of deaths in a situation where no treatment resulted in 100 per cent survival.

Pathologic examination of those animals dying showed no evidence of oxygen toxicity. The pathologic changes noted at death were identical in all groups. (Section I, (C)(4). Since the groups treated in 3 atmospheres of oxygen died at 12 hours after onset of peritonitis, as compared to untreated animals which died at 21 hours, the entire pathologic picture was accelerated in the former group. Untreated animals sacrificed and studied 12 hours after the onset of peritonitis had much less severe histologic lesions when compared with the animals who died from peritonitis at 12 hours.

The total number of bacteria present in the peritoneal cavity at the time of death was determined by standard bacterial counting methods. Greater than 1×10^9 bacteria were present in the peritoneum of all animals who died regardless of the treatment group. Therefore, oxygen apparently had no bacteriostatic effect in this model.

(B) Influence of hyperbaric oxygen on blood gases. In these experiments arterial pO_2, pCO_2 and pH were measured in the rat to evaluate the effects of the 3 atmospheres of oxygen on these parameters. Normal rats as well as those subjected to E. coli-hemoglobin peritonitis were studied.

A model was devised, whereby sufficient quantities of arterial blood could be obtained, while the animal was awake and in the compression chamber. Twenty-four to 48 hours prior to the experiment rats weighing 250-275 grams were anesthetized with nembutal. The common iliac artery was approached extraperitoneally in the groin and cannulated with polyethylene tubing. The tube was passed into the lower aorta below the renal vessels. The other end of the tube was then tunneled through the subcutaneous tissues to the back of the neck, filled with heparinized saline and capped. To obtain an arterial blood sample when fully conscious, the animal was placed into a snug lucite cylinder. A hole in a portion of the wall of the cylinder exposed the intra-arterial cannula and permitted sampling. Since 3 cc of blood was needed for each determination a rat was bled only once and then sacrificed.

Three atmospheres of oxygen was administered in an infant isolette placed within the main pressure chamber. The ports of the isolette were

fitted with rubber gauntlets so that the investigator could place his arms within the isolette and obtain blood samples. Gas samples from the isolettes were analyzed and showed the efficacy of the method. A 95 to 100 per cent oxygen environment was maintained within the isolette even during sampling.

Blood samples were analyzed within the main pressure chamber using an Instrumentation Laboratories gas analyzer designed to function under hyperbaric conditions.

The animals which had been previously cannulated were divided into 2 groups. One group received a standard E. coli-hemoglobin intraperitoneal injection and the other received an equal volume of saline. Two hours following intraperitoneal injection the animals were placed in an environment of 3 atmospheres of oxygen. One to three animals from each group were sampled and sacrificed at each of the following times: prior to the intraperitoneal injection, just prior to compression to 3 atmospheres, 30 minutes, 60 minutes and 120 minutes after compression, and finally 30 minutes following return to 1 atmosphere of air.

Results of pO_2 analyses showed no differences between the experimental and control groups. Arterial pCO_2 rose from 90 to 92 mm Hg at 1 atmosphere to 1000 to 1550 mm Hg at 3 atmospheres of oxygen. Values after returning to room air at the completion of the experiment were identical to those prior to compression. Arterial pO_2 values were somewhat decreased from the start and remained stable during treatment in both groups. They ranged from 27 to 23 mm Hg in the group with peritonitis and in the control group from 36 to 28 mm Hg. The pH values paralleled the pCO_2 findings and ranged from 7.50 to 7.59 in the group with peritonitis, and 7.44 to 7.54 in the controls. This respiratory alkalosis might be due to hyperventilation secondary to handling.

These findings suggest a possible cause for the harmful effects of oxygen which were noted in the mortality studies described above. In vitro bacterial studies have demonstrated inhibition of bacterial growth by oxygen when partial pressures of oxygen have exceeded 1½ atmospheres or approximately 1100 mm Hg. At values below 1100 mg Hg, growth is increased with increasing oxygen tensions. The highest partial pressures of oxygen measured was 1550 mm Hg, however, this was in the arterial system. Although direct tissue oxygen tensions, and hence intraperitoneal oxygen tensions, cannot be accurately measured, other studies have shown that tissue oxygen tensions are lower than those present in arterial blood. The values probably fall somewhere between those found in arterial and venous blood, and are probably closer to the latter. Therefore, it is quite possible that the increased partial pressure of oxygen in the peritoneum actually enhances the growth of the organisms and accounts for the acceleration of pathophysiologic processes.

Further studies are now underway using dogs which may help to clarify some of these findings. In addition, measurements of blood lactate and pyruvate levels will be performed as indicators of metabolic changes.

Results from other investigators, which suggest a possible beneficial effect of hyperbaric oxygen in endotoxic shock, are of interest. These studies were performed utilizing purified endotoxin injections, and the effects of oxygen are directed only to the shock state. Models which utilize living organisms would seem to be more closely related to actual clinical situations. Bacterial models take into account not only the possible effects of oxygen on living organisms but also its effect on shock. The possibility exists that endotoxin shock is not the primary factor in lethal peritonitis.

III. Studies on the mechanics of wound healing.

The department has continued investigation into the use of plastic and mechanical adjuvants to speed and improve wound healing. Previous studies have included the properties and histotoxicity of alkyl-cyanoacrylate tissue adhesives, the use of methyl-2-cyanoacrylate for the closure of skin incisions and sealing pancreatic wounds, and a preliminary evaluation of the American Vascular Stapler for anastomosing small blood vessles. Current studies extend this research and include (A) the biological receptivity of various adhesive polymers and their metabolic degradation, (B) the development of a spray gun for dispersing polymers and (C) the continued evaluation of the American Vascular Stapler.

(A) The degradation and excretion of methyl-2-cyanoacrylate tissue adhesives. The potential use of methyl-2-cyanoacrylate for tissue repair has been reported previously (Progress Report 1963-1964). Although these adhesive polymers are somewhat histotoxic, they possess several properties which favor their continued study: /1/ they adhere to a variety of wet tissues, /2/ healing can occur at the bonding site, and /3/ they are slowly removed from the bonding site. Hence it might be possible to close a wound with subsequent replacement of the closure material by contiguous tissue. So long as the rate of degradation is sufficiently slow, and the products of degradation biologically metabolized, biodegradability is a salutary attribute of a tissue adhesive. Studies were initiated therefore to study the metabolic degradation of C^{14} labelled polymers.

Radioactive methyl-2-cyanoacrylate monomer was prepared by allowing C^{14} formaldehyde to react with cyanoacetic acid. The synthesis was performed by the U.S. Army Medical Biomechanical Research Laboratory. The radioactive adhesive was then implanted subcutaneously into a total of 29 rats. In 25 of the rats, Ivalon sponges placed subcutaneously were used as a matrix for adhesive implantation; in the other 4 rats, the adhesive was delivered directly into the subcutaneous space of the

abdominal wall. The rats were placed in individual metabolic cages and sacrificed periodically up to 154 days. The urine, feces, tissues and implant sites were analyzed for radioactivity in a liquid scintillation spectrometer. Also, a specimen of liver, spleen and kidney from each animal was examined histologically.

The intense and persistent inflammatory response which developed about the cyanoacrylate - containing sponge implant was indistinguishable from that previously described for non-radioactive methyl-2-cyanoacrylate. Microscopically, the polymer within the sponge appeared as refractile plates, as noted earlier. Sections of liver, kidney and spleen were normal, and revealed no abnormalities attributable to the presence of chemical degradation products of cyanoacrylate. Lymphopoiesis in the spleen, a radiosensitive organ, was indistinguishable from that seen in control animals.

Ivalon sponges were used as a matrix for the implanted radioactive adhesive in most animals so that at sacrifice the amount of radioactivit remaining at the site of implantation could be determined. There was no significant difference between the radioactivity excretion patterns of animals in which the Ivalon was used as a matrix, and animals in which the adhesive was merely implanted subcutaneously. This is in accord with the previous findings that the tissue response elicited by methyl-2-cyanoacrylate is not appreciably modified by the sponge matrix. Total radioactivity excreted via the urine after 154 days for both the Ivalon and subcutaneous groups varied from 32.9 per cent to 60.6 per cent and averaged 46.1 per cent of the total implanted dose. Urinary excretion of radioactivity was rapid for the first several weeks and then gradually levelled off. Total radioactivity excreted via the feces after 154 days varied from 2.9 per cent to 13.1 per cent and averaged 5.5 per cent.

From the two animals sacrificed one hour after Ivalon sponge implantation, 96.6 per cent of the total implanted radioactivity was recovered from sponges. Progressively less radioactivity was recovered from sponges during the succeeding sacrifice periods. At seven days, 30 days, 90 days, and 154 days after implantation, 85.4 per cent, 47.0 per cent, 24.2 per cent and 6.6 per cent, respectively, of the total implanted radioactivity remained and was recovered from the Ivalon sponges.

No radioactivity was ever recovered from the brain, liver, spleen, or kidneys of the animals at the various sacrifice periods. Also, there was no radioactivity recovered from the fat and muscle specimens removed from the animals sacrificed at 154 days.

The radioactivity recovered in the urine was not volatile upon distillation and was found entirely in the residue. The radioactive

entity was, however dialyzed through the cellophane membrane which indicates that the molecular weight was quite low.

The results showed that methyl-2-cyanoacrylate is completely degraded and excreted over a 5 month period. No adhesive or breakdown product was deposited in any body tissue. Over 50 per cent of the radioactivity was excreted via the urine and feces. The remaining radioactivity was unaccounted for in this study and presumably was slowly expired as carbon dioxide. The radioactive moiety being excreted in the urine is currently under study. The study described does not preclude non-radioactive compound being retained in the tissue, and further experiments are planned with methyl-2-cyanoacrylate tagged in the number two carbon. The basic mechanism for the degradation of the polymer awaits further experimentation.

Current investigations are underway to study the degradation of some of the less necrotoxic higher homologues of alkyl-2-cyanoacrylate. Also, the potential uses and metabolic degradation of polylactate polymers are being studied in conjunction with the U.S. Army Medical Biomechanical Research Laboratory.

(B) Development of a tissue adhesive spray gun for the application of adhesive polymers. Methyl-2-cyanoacrylate has proven to be an effective adhesive and sealing agent experimentally. It is difficult to handle, however, because it polymerizes almost instantaneously on contact with moisture. To make handling easier a spray gun was developed in conjunction with the U.S. Army Medical Biomechanical Research Laboratory.

The gun consists of a Paasche type FH artist's air brush driven by nitrogen or an inert gas. A polyethylene bottle containing the adhesive has a teflon nozzle which is placed in the stream of air emanating from the gun. As air is forced through the gun and over the nozzle, adhesive is sucked up into the stream and dispersed in an even, small pattern.

The gun has been used experimentally on liver wounds and traumatic wounds of the extremities for hemostasis and has proven to be effective.

(C) Evaluation of common bile duct anastomoses with the American Vascular Stapler. Evaluation of an American-made vascular stapler was continued in selected experiments. This instrument, modified from the Russian instrument, uses staples from a preloaded sterile cartridge. The American Vascular Stapler was used to perform end-to-end common bile duct anastomoses in the Rhesus and two African green monkeys. Bilirubin and alkaline phosphatase determinations were performed post-operatively at frequent intervals. Two monkeys were sacrificed at 6 weeks, 2 at 20 weeks, and 3 at 36 weeks. The anastomotic site was examined grossly and microscopically.

Several of the monkeys became jaundiced in the immediate post-operative period, and nonjaundiced monkeys showed a temporary rise in alkaline phosphatase levels. These findings were attributed to edema at the anastomotic site; they subsequently disappeared. The animals remained healthy until sacrifice. At sacrifice the anastomotic site could be located grossly but only with difficulty. There was no evidence of bile leakage and the common duct looked completely normal. On microscopic examination minimal scarring was seen, but the lumen was not narrowed in any of the animals.

From these findings it can be summarized that a perfect common duct anastomosis can be attained consistently with the American Vascular Stapler, and that in certain selected clinical instances of duct trauma, stricture, or organ transplantation its use should be considered.

IV. Bilirubin metabolism under conditions of common duct obstruction.

The metabolism of bilirubin in the primate, under conditions of complete common duct obstruction, has been reported closely to resemble that of man, and to differ significantly from other species of animals. The current investigations continue studies on the metabolism and excretion of bilirubin-C^{14} in experimental obstruction in primates, and includes data on the metabolism and excretion of bilirubin-C^{14} in children with biliary atresia.

(A) Metabolism and excretion of bilirubin-C^{14} in primates with experimental obstructive jaundice. Rhesus monkeys were prepared for experimentation by ligating the common bile duct. Both pre- and post-operatively the animals were maintained in upright primate chairs to facilitate blood sampling and urinary and fecal collection. Each chair was equipped with a metabolic urine and feces separator. After an average of 5 weeks a "steady state" was reached wherein the serum bilirubin level plateaued and thereafter remained relatively stable. Because serum bilirubin levels remain stable in the face of continual hemoglobin breakdown and bilirubin formation, it is assumed that bilirubin excretion and/or metabolism equals bilirubin production. Bile pigments were recovered in the urine and stool of the monkeys in the "steady state" of obstructive jaundice, but not in quantities sufficient to account for daily bilirubin production. Therefore, bilirubin-C^{14} obtained from the Department of Medicine, The Johns Hopkins University and Hospital, was injected intravenously into the monkeys, and serum, urine, and feces monitored for 14 days.

The isotope, after injection, distributed itself between the extravascular and intravascular spaces in the ratio of 4.8 to 1. In the past, bilirubin was believed to distribute in the same ratio as albumin, 1.4 to 1. This study shows that bilirubin dissociates from albumin and binds, probably to tissue lipids and protein, mostly extravascularly.

The serum half life of bilirubin was found to be 3.6 days, a 20-fold increase over normal animals. Bilirubin excretion was almost entirely by the kidneys. Of the radioactivity recovered in the urine the majority was in the form of bilirubin. The remaining urinary radioactivity unaccounted for is probably in the form of soluble bilirubin breakdown products.

Daily turnover of bilirubin was increased 2 to 3 fold over normal. This was probably from decreased erythrocyte survival, known to occur frequently in jaundiced states.

Thus, from this study it was seen that in obstructive jaundice more bilirubin than normal is produced and excreted. In addition the kidney is the primary excretory route for bilirubin.

(B) Metabolism and excretion of bilirubin-C^{14} in children with biliary atresia. Three children with biliary atresia at Washington, D.C. Children's Hospital were studied with bilirubin-C^{14} obtained from the Department of Medicine, The Johns Hopkins University and Hospital. After intravenous injection of the radioisotope, serum, urine, and feces were monitored for radioactivity for periods up to 14 days.

Serum half life of the bilirubin-C^{14} appeared to be a good index of clinical well being in the children. One child with an isotope half life of 3.5 days, indicating that bilirubin was being excreted rapidly, is alive and well at 2½ years. A second child with a half life of 8.8 days, indicating a slower clearing of bilirubin, died of liver failure at 6 months. The third child with a serum half life of 7.0 days is just starting to decompensate at 8 months.

The radioactive bilirubin distributed itself principally extravascularly in contradistinction to what has been previously thought. Until now it was assumed that bilirubin is distributed, like albumin, in the ratio of 1.4 to 1 between the extravascular and intravascular spaces. In these children, however, the ratio was 7.2 to 1 indicating that bilirubin was being dissociated from albumin and binding extravascularly.

Of the radioactivity recovered, the majority appeared in the urine. Only tracer amounts were present in the feces.

Daily turnover of bilirubin was found to be increased several fold. Cr^{51} red cell survival studies indicated that the children were destroying red cells at several times the normal rate, thus accounting for the increased bilirubin production.

This study, therefore, showed (1) in children with biliary atresia the bilirubin is distributed chiefly extravascularly (2) bilirubin

production is increased, due to increased red cell destruction and (3) bilirubin is excreted almost entirely by the kidneys.

V. The effects of sulfonamides on thyroid metabolism.

The thyroid gland is particularly sensitive to sulfonamide administration. The nature of sulfonamide inhibition has been the subject of continuing investigation, but the mechanisms involved remain obscure. The wide therapeutic use of the sulfonamides prompted further studies of the nature and reversibility of their pathophysiological and biochemical effects.

Sulfaguanidine (SG), 2 per cent of diet by weight, rapidly produced observable histologic and biochemical changes in the thyroid (WRAIR Progress Reports, 1963-1964). The inhibition of thyroxine synthesis was evident in 3 days and complete inhibition of iodoamino acid production was found at 7 days. Previous studies in a limited number of animals have indicated that the thyroid will return to normal thyroxine synthesis in 7 days after cessation of sulfonamide administration. More extended studies reveal a reversal of the observed effects within 14 days following 7, 14, and 21 days of sulfonamide ingestion. The methodology has been reported previously (WRAIR, Progress Reports 1963-1964).

Body weights of the control and SG rats, recorded daily throughout the experiment, paralleled each other for about 14 days. After 14 days the body weight of the control rats increased in a normal pattern, while those of the SG rats remained constant. Based on just this observation, it could be assumed that adequate reserves of thyroid hormone were available for normal growth maintenance for about 14 days. At 7 days, however, gland weight had doubled, and the production of iodoamino acids as determined by chromatography had ceased. Table VI compares the thyroid weights (mg thyroid per 100 gm body wt) in controls, in SG treated and in SG-treated animals after 14 days on a controlled diet.

TABLE VI
Average Thyroid Gland Weight

	mg thyroid per 100 gm body weight		
Days	Controls	SC	SG Reversion
7	5.3	10.2	7.3
14	6.5	14.3	6.9
21	5.9	15.8	6.1
28	4.8	16.0	

Histological changes were identical with those previously reported (WRAIR Progress Reports 1963-1964). The glands from the reversion studies

showed essentially normal histology. It was noted that the weights of the thyroid return to approximately normal values in the 14 day reversion studies. Also, chromatography of gland extracts from 7-day SG and 14-day reversion studies revealed the presence of all the iodoamino acids normally found in the thyroid. This would indicate that the gland was able to return to its normal size and function following 7 days' sulfonamide treatment.

The tyrosine content of these thyroid glands was studied also to determine if sulfaguanidine inhibited its uptake or incorporation into thyroglobulin. Decreased level of tyrosine or thyroglobulin in the presence of SG would indicate a lack of matrix for thyroxine production. Saline homogenates of pooled thyroids from control and experimental animals were analyzed for bound and free tyrosine. Thyroglobulin was removed from the gland homogenates by isoelectric precipitation at pH 6.5; the remainder of the protein was precipitated by the addition of trichloroacetic acid to 5 per cent weight per volume. Tyrosine determinations were performed on the supernatant solution and acid hydrolysate of the precipitates. The tyrosine content of the thyroglobulin fraction (mg per 100 gm thyroid weight) was considerably higher in the SG fed animals than in the controls, while the amount of tyrosine in the soluble structural protein and supernatant was less than half of control animals. This would indicate that thyroglobulin was being continually synthesized in the SG treated animals. The largest amounts of tyrosine in thyroglobulin fraction occurred at 7 and 14 days, but was still slightly higher than that found in control animals at even 28 days. The lower level of tyrosine in the soluble structural proteins and the supernatant solution would indicate that available tryosine was being incorporated mostly into thyroglobulin. This could reflect the gland's attempt to produce thyroxine even in the absence of the iodoamino acids. The 14-day SG reversion studies indicated that the thyroid was approaching normal function. Lower levels of tyrosine were found in the thyroglobulin fraction when compared with the control animals and near normal levels were found in the other fractions.

VI. Studies on the microbiological aspects of patient isolation.

Continued consultative service and support have been provided to the Department of Nursing, WRAIR, in connection with an evaluation of patient isolators (WRAIR Progress Report 1963-1964), with particular reference to sterilization, sanitization, and maintenance of the microbiological integrity of one system for patient isolation. The material presented in this report is derived from data obtained during two clinical applications of the isolator system and is concerned with the behavior of the microbial population within the isolator during prolonged periods of occupancy.

(A) Observations on patient's microflora during prolonged confinement in an isolator. The effects on the external microflora of prolonged patient confinement and concurrent procedures for patient hygiene were examined. Detailed results were obtained from one adult volunteer confined within the isolator for twelve days. No antimicrobial chemotherapy had been prescribed. Additional data were obtained from a child with the diagnosis of aplastic anemia for whom isolator confinement was prescribed as a life-saving measure; the period of confinement in this latter situation extended for 8 days with one interruption when the patient was removed in order to receive a series of marrow transfusions. This case involved extensive treatment with staphcillin (8 grams/day intraperitoneally), Keflin (500 mg, q.i.d. postoperatively) and tetracycline (250 mg. q.i.d. postoperatively). Prior to isolation several episodes of Staphylococcus aureus or Klebsiella pneumoniae, bacteremias had occurred.

Both patients, prior to introduction into the isolator, were bathed and shampooed with a commercial skin cleansing lotion containing 3 per cent hexachlorophene. Partial and complete patient baths were administered to both patients during the periods of isolator confinement. In the case of the adult volunteer, a commercial "antibacterial" bar soap containing 2 per cent hexachlorophene was employed at the onset and the hexachlorophene lotion during the latter period of isolation. The child received hygienic care with 3 per cent hexachlorophene preparations throughout his period of isolation confinement. More detailed information regarding the various nursing care and related procedures is given in the report from the Department of Nursing, WRAIR.

Bacteriological samples were collected daily, using moistened sterile swabs, from the skin of forearm, abdomen, thigh, back, anal and perineal areas, and from the oro- and nasopharynx.

The individual swabs were eluted in sterile broth and used to inoculate various standard bacteriological media. The procedures employed provided semi-quantitative estimates of the microbial populations. Definitive bacteriological identifications were made according to accepted taxonomic and diagnostic criteria and procedures. Normal values for the different microflora of the adult volunteer-patient were obtained prior to confinement by means of identical procedures.

(1) Data obtained from the study of the patient-volunteer's microflora during his 12 days of confinement permitted four conclusions: (a) No migration of microorganisms from attendants or hospital environment into the isolator could be detected. (b) No microorganisms were recovered from the patient which were not also recovered either at the onset of the isolation period or under conditions of normal exposure. (c) Skin surfaces, which had been

bacteriologically denuded through application of intensive skin hygiene procedures, are repopulated by bacteria of fecal origin. (d) Comparative information of value on the selection and application of agents intended for use in personal hygienic and bathing procedures was also obtained.

The microflora of the pharyngeal and nasopharyngeal orifices of the adult patient-volunteer remained within the normal range. The organisms most frequently recovered from the various skin surfaces included Staphylococcus aureus, Escherichia coli and several strains of S. epidermidis. Both nontypable strains of Klebsiella-aerobacter and strains of Klebsiella pneumoniae, Type III, were also regularly recovered.

Data obtained during the first half of the study with the adult patient-volunteer indicated that procedures employed for patient hygiene were having only a limited effect upon the recoverable skin microflora. Following substitution of the 3 per cent hexachlorophene skin lotion for the 2 per cent bar soap product used during the initial period, a marked reduction was observed in the total number of microorganisms recoverable from all of the monitored skin surfaces.

The microfloral recovery rates from the different skin regions of the adult patient seemed to vary directly with the extent of exposure of the individual surfaces to fecal contamination: Rates were highest on the abdomen and lower back. Evidence of fecal contamination was obtained through isolation of S. aureus and of a distinctive strain of S. epidermidis, for both of which the patient was an intestinal carrier.

(2) A total of 33 skin surface specimens were taken from the child during his 9 days of confinement within the isolator; in addition, 21 specimens were collected from the various body orifices. The predominant organisms recovered from the positive cutaneous cultures were Candida albicans (42%) and S. epidermidis (21%); also recovered were a nontypable Klebsiella pneumoniae and a species of Pitrospora (2 recoveries each). Negative cultures were reported for 9 (27%) of the specimens. The isolates of K. pneumoniae were biochemically identical to isolates recovered from the blood during several episodes of bacteremia; antibiotic sensitivities included kanamycin, streptomycin and neomycin.

All of the specimens obtained from the various body orifices yielded positive cultures. Bacteriological analyses of these cultures were markedly uniform and failed to indicate colonization of the patient by exogenous microorganisms. Klebsiella pneumoniae and C. albicans were recovered from all cultures taken from the patient's throat; group D and alpha hemolytic streptococci were recovered from all but one specimen. Cultures of rectal specimens yielded only a limited number of different isolates. These included K. pneumoniae (all specimens), C. albicans (all but two specimens), enterococci

(all but 3 specimens) and various gram-negative forms including Escherichia coli and Proteus vulgaris. All cultures taken from the child were negative for S. aureus.

Cultures taken at autopsy yielded results which were in agreement with those described above. Local abscesses which yielded mixed cultures of K. pneumoniae and C. albicans were found in the following tissues: lung, kidney, bone marrow and liver.

It is significant in terms of the purpose of the patient isolator that the microflora of the child's various cutaneous surfaces and orifices did not fluctuate to any extent during this period of isolation prior to death and were in essential agreement with the results of post mortem cultures. While isolator care did not permit recovery, it did prevent the onset of additional secondary infections in an otherwise extremely susceptible host.

(B) Contamination levels within the occupied isolator. Detailed examinations of the levels of microbial contamination within the occupied patient isolator were conducted during the two studies described above. The data so derived provided information regarding the sources, extent, and patterns of contamination within the isolator and permitted formulation of procedures for maintenance of maximum levels of sanitization.

Samples for bacteriological examination were collected daily, using sterile moistened swabs, from selected interior surfaces of the isolator, and from various fomites within the isolator. In addition, samples were also collected from exterior surfaces and fomites in the immediate vicinity of the isolator. The swabs used for specimen collection were eluted in broth; inoculations were made onto standard bacteriological media using a semi-quantitative plating procedure. Identifications were made according to accepted diagnostic and taxonomic procedures.

Airborne microbial contamination within the isolator and in the area of the isolator was monitored through samplings taken twice daily. This was accomplished with the use of 2 commercial Fort Detrick-type rotary, impaction slit samplers coupled to a vacuum pump; flow rate averaged 10 liters per minute. Bacteriological examination of the collection plates (blood agar) was limited to determination of morphology (colonial and microscopic), gram reaction, and production of hemolysis.

(1) A total of 165 specimens were collected from the various internal sampling sites during the period the isolator was occupied by the adult patient-volunteer. Of these, 118 or 71.5 per cent, were bacteriologically negative on culture. No pathogens were recovered

other than two known to be carried by the patient. The specimens from which positive cultures were obtained yielded a total of 55 different isolates which were subsequently identified. The composition of these isolates was as follows:

TABLE VIII
Positive Bacteriological Recoveries from Interior of Occupied Patient Isolator (Patient Volunteer)

Total number of specimens	165
Number of specimens yielding positive cultures	47
Number of separate bacteriological isolates	55

Composition of recovered isolates	Recovery rate*
Staphylococcus epidermidis	32 (58%)
Staphylococcus aureus	12 (22%)
Klebsiella-aerobacter**	8 (15%)
Streptococcus faecalis	3 (5%)
Bacillus sp.	2 (3%)
Escherichia coli	1
Clostridium sp.	1
Alpha hemolytic streptococci	1

* Per cent of total number of isolates
** Including both typable and non-typable strains

All of the above isolates could be directly equated with organisms of patient origin except for the unidentified clostridia and bacilli. The predominant organism was found to be S. epidermidis; several strains of this organism were encountered and that most frequently isolated within the isolator was found to be identical to a strain being carried intestinally by the patient. The isolates of S. aureus and K. pneumoniae were also identical to strains which were routinely recovered from stool specimens and rectal swabs obtained from the patient. The above results were employed in formulating schedules for concurrent sanitization of the isolator during the current and subsequent occasions of isolator occupancy.

(2) During the child's second period of occupancy, because of the critical nature of the situation, bacteriological monitoring of the isolator was limited to that deemed absolutely essential. However, 54 specimens were collected during the 9 days' confinement. Of these, only 8 (15 per cent) were bacteriologically positive on culture. Only two organisms were recovered from the above positive specimens; these were: Candida albicans and Klebsiella pneumoniae.

As noted earlier, these two organisms also predominated in specimens collected from the child's skin surfaces and body orifices. Neither organism was recovered from any of the monitored surfaces in particularly large numbers and all of the isolations were made from surfaces or fomites with which the patient had direct contact.

On one occasion during the second study more detailed bacteriological data was obtained on surface contamination within the isolator. These came from a series of cultures taken before and after a routine isolator sanitization procedure in which the surfaces of the tent surrounding the patient's bed were cleaned with a 1:750 solution of benzalkonium chloride. The bacteriological sampling in this instance differed from before in that plates of blood agar were used as the sampling vehicle. These were entered into the tent directly and the exposed agar surfaces pressed firmly against the plastic material; the cont act area was 55 cm. A total of 10 sites were so monitored. The plastic surfaces of the various sites yielded an average of 6 organisms per 55 cm^2 prior to sanitization; the average recovery rate following treatment with the benzalkonium chloride solution was 0.22 per 55 cm^2. Excluded from these calculations were the results obtained from one test site which had been in direct contact with the patient; prior to sanitization, this site yielded in excess of 100 organisms per 55 cm^2, and following sanitization, the recovery rate was reduced to 10 organisms per 55 cm^2. Comparison of the various rates indicated that a reduction in excess of 90 per cent of the recoverable surface contamination was effected by the sanitization procedure.

(3) Data obtained from the monitoring of airborne contamination levels within the occupied isolator provided both a measure of the degree of isolation achieved by the isolator system and information essential for proper utilization of the system. The results of the airborne microbial contamination studies from the first period of occupancy revealed certain inadequacies in either design or application of combined air filtration and supply systems. This information was subsequently used as justification for a modification in the isolator's air supply system and for several equipment changes.

A definite deficiency in the rate of ventilation within the isolator was revealed during the first period of patient occupancy. During the first four days that the isolator was occupied by the adult patient volunteer, the levels of airborne microbial contamination within the isolator remained well below those of the external room air; the average counts for both air volumes during this period were 19.6 and 45.6 organisms per 1,000 liters of air, respectively. In contrast, during the last 7 days of occupancy, the interior and exterior bacterial counts per 1,000 liters of air were, on an average 80.6 and 18.9, respectively. The highest counts obtained from the isolator interior air volume during this period extended from 130 to 187

organisms per 1,000 liters; the highest external air volume bacterial counts, in contrast, did not exceed 50 organisms per 1,000 liters.

It was determined that the excessively high levels of airborne microbial contamination which prevailed within the isolator resulted from the use of ultra-high efficiency filters for filtration of both the intake and exhaust air streams of the isolators air supply. Accordingly, it was recommended that subsequent applications of the isolator for reverse isolation employ only filtration of the intake air stream. The effects of this change are seen in the data below compiled during the second period of occupancy. It should be noted that recoveries of airborne contaminants from the room air volume adjacent to the isolator during this second period included numerous isolations of C. albicans. As cited above, this was found to be the predominant contaminant within the isolator during the second study.

TABLE IX
Daily Concentrations of Airborne Microorganisms-Second Study

Day of Isolation	Total Count Interior Air Volume*	Total Count Exterior Air Volume
Day 2 AM	3	140
Day 3 AM	7	93
PM	0	80
Day 4 AM	7	89
Day 5	10	203
Day 6 AM	13	50
Day 7 AM	0	43
Day 8 PM	3	133

*Counts corrected to values per 1,000 liters of air.

The findings presented above regarding the levels of surface and airborne contamination within the occupied patient isolator demonstrate the following: (a) confinement within the isolator does not insure absolute protection against secondary bacterial invaders. In both studies, the involved patient harbored and introduced into the isolator one or more potentially pathogenic strains of bacteria including S. aureus and K. pneumoniae. (b) Isolation as provided by the isolator system under study does provide a highly effective barrier between the patient and the indigenous microflora of the surrounding hospital environment: no penetration of the isolation barrier was demonstrated.

Summary and Conclusions.

The Department of Surgical Metabolism and Pathology has continued a diversified, multi-discipline research program on the metabolic and

nutritional problems associated with injury. Major areas of study have included: (I) the factors responsible for morbidity and mortality in peritonitis and other surgical infections, (II) the mechanisms of action of hyperbaric oxygen and its effect on infection, (III) the mechanics of wound healing, (IV) the metabolism of bilirubin in experimental biliary obstruction and in patients with biliary atresia, (V) the effects of sulfonamides on thyroid gland function, and (VI) the microbiological aspects of patient isolators.

Studies on the factors responsible for morbidity and mortality in peritonitis and other infections have been extended to include: (A) the characterization of the role of the microflora, (B) the identification of the role of endotoxin and its quantitation by the chick embryo assay, (C) the investigation of the adjuvant effects of hemoglobin in lethal infections, (D) the study of histopathologic changes, and (E) the histochemical identification of affected metabolic pathways. Two experimental models have been developed and utilized, an endogenous system based on appendiceal ligation, and an exogenous bacterial system using inocula of E. coli and hemoglobin.

Peritonitis, produced by appendiceal ligation, provided a lethal exudate. Fractionation of the exudate into bacterial and sterile portions showed that a bacterial population was necessary for lethality, but that an adjuvant factor was present in the sterile filtrate. Analyses for hemoglobin and endotoxin revealed hemoglobin level which usually exceeded 4 per cent and low levels of endotoxin. The role of each of these components as lethal adjuvant is currently under investigation. Using the chick embryo assay for endotoxin a neurotoxic factor was demonstrated, which may play a role in bacterial shock. Preliminary attempts were made to characterize this material.

Hemoglobin, as an adjuvant in E. coli lethality, was examined in both intraperitoneal and subcutaneous infections. Emphasis was placed primarily on early effects of the presence of hemoglobin on the growth of the bacteria, the migration of the bacteria from infective sites, and the defense mechanisms of the host. The bacterial counts of the infected site were always higher when E. coli were injected with hemoglobin as compared with E. coli alone. Bacteremia was absent or of small magnitude in either situation. Hemoglobin did not enhance in vitro bacterial growth but it interfered with protein migration from the peritoneal cavity and modified the host leukocyte response; therefore it is assumed either to have inhibited bacterial migration or interfered with the host defense mechanisms, or both. Subcutaneous infections in collateral studies produced analagous findings, but were nonlethal in contrast to published results from other laboratories.

Histopathologic study of E. coli-hemoglobin infections in the rat showed vascular and lymphoid inflammatory reactions. The vascular

reactions included stasis and congestion with frequent, hyaline and red cell, venous thromboses. Centrilobular necrosis was noted in the liver and was frequently associated with thrombus formation. Changes were noted within one hour after infection and were well advanced by six hours. Liver necrosis was associated with an alteration in the pattern of enzymatic activity.

The action of hyperbaric oxygen in infection was studied, with emphasis on treatment of experimental peritonitis and effects on blood gas levels. The use of hyperbaric oxygen in experimental peritonitis proved to be detrimental in shortening the survival time in animals treated by 3 atmospheres of pressure. These findings may possibly be related to increased bacterial growth under the experimental conditions, but blood gas and pH analyses revealed no significant differences between control and experimental animals.

The Department has continued investigations into the mechanics of wound healing. Studies were extended (1) to establish the biological receptivity of the adhesive polymer, methyl-2-cyanoacrylate, and its metabolic degradation products, (2) to evaluate a spray gun for dispensing polymers, and (3) to evaluate the American Vascular Stapler in common bile duct anastomoses.

Methyl-2-cyanoacrylate C^{14} monomer, implanted subcutaneously in rats, was completely degraded and excreted over a 5-month period. Neither the adhesive or its radioactive metabolic products were deposited in any body tissue. The radioactivity was excreted predominately via the urine and feces; radioactivity unaccounted for in this experiment was presumably expired as carbon dioxide. The radioactive moiety excreted in the urine was not volatile but was sufficiently small to be dialyzed through cellophane membrane. The alkyl-cyanoacrylates are effective tissue adhesives, but are difficult to apply in some situations. A spray gun was developed, therefore, to dispense under pressure a fine spray of adhesive. The spray gun was successfully used for hemostasis in liver wounds. The American Vascular Stapler was found to be useful in performing common duct anastomoses in selected clinical studies on duct trauma, stricture, or organ transplantations.

The metabolism of bilirubin during common duct obstruction was investigated utilizing radioactive bilirubin C^{14}. Studies were conducted on primates with experimental common bile duct obstruction and children with biliary atresia.. The serum half-life of bilirubin in primates with complete duct obstruction was 3.6 days. This was a 20 fold increase over the value in normal animals. Bilirubin excretion was almost entirely via the kidney, and the labelled compound was recovered from the urine mostly as bilirubin. The increased bilirubin turnover probably resulted from decreased erythrocyte survival. The

half-life of radioactive bilirubin in children with biliary atresia appears to be a good index of clinical well being. The shorter the half-life of the labelled bilirubin, the better the prognosis. The radioactive bilirubin was found to be distributed principally in extravascular sites, and as in laboratory primates was excreted primarily via the kidney.

Studies on the inhibition of thyroid metabolism by the sulfonamides were continued. The long-term therapeutic use of the sulfonamides prompted further investigation of their pathophysiological and biochemical effects with special emphasis on the reversibility of their action. Rats fed sulfaguanidine, 2 per cent of diet by weight, showed normal increases in body weight for only about 14 days. This fact would indicate a reserve of thyroid hormone sufficient to maintain growth for short periods of time. Thyroid gland weights were twice control after 7 days on sulfaguanidine, and were 4 times larger after 28 days. The gland size returned essentially to normal size after 14 days on a drug-free diet. Iodoamino acids, production of which is inhibited by the sulfonamides, were present in the glands after 7 days on the drug and 14 days on a control diet. Thyroid histology was also essentially normal. This indicates that the gland had returned to normal functional activity. Tyrosine analyses on fractions of thyroid gland homogenates suggested that thyroglobulin synthesis was not inhibited by the sulfonamides.

The microbiological aspects of patient isolation were extended in conjunction with the Department of Nursing, WRAIR. Studies included two clinical applications concerned with (1) the behavior of patients microflora during prolonged isolator confinement, and (2) levels of contamination within the occupied isolator. The recovery of culturable microflora from the skin varied directly with the extent of exposure to fecal contamination, e.g. the abdomen and lower back. Evidence of fecal contamination was obtained through recovery of a bacterial strain which was carried intestinally by the patient. Microbiological monitoring of the isolator showed that most bacterial isolates could be directly equated with organisms of patient origin. Schedules for sanitization of the isolator during patient occupancy were formulated from these studies. Sanitization measures were effective in reducing recoverable surface contamination by 90 per cent. Studies on airborne contamination resulted in a modification of the isolator ventilation system, which reduced internal contamination. Results showed that patient isolation provides a highly effective barrier between the patient and indigenous microflora from the surrounding environment.

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RESEARCH AND TECHNOLOGY RESUME

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19 GOV'T LAB INSTALLATION ACTIVITY
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21 TECHNOLOGY UTILIZATION: Clinical surgery

22 COORDINATION: NA

23 KEYWORDS: Wound healing; adhesive; fracture fixation; fractures; neurosurgery; tendon injury

24. (U) Tech Objective - To investigate the problems of wound healing at the clinical procedure level, with emphasis on the development of procedures to assure or accelerate healing of wounds of types sustained in warfare.

25 (U) Approach - The majority of the investigations involve the direct testing in animals of surgical methods designed to accomplish the above objectives. The use of the cyanoacrylate adhesives as tissue adhesives is being investigated in bone, peripheral nerve, cardiac and vascular surgery. Healing of fractures and of large skin grafts is being evaluated under conditions of augmented vascular flow. Studies of tendon healing have been extended into investigation of healing potential in areas of extensive scarring.

26 (U) Progress (Jul 64 - Jun 65) - The overall tissue potential of the cyanoacrylate adhesives indicates at present that bone is probably inhibited in its healing, but further comparative testing of individual agents and techniques is continuing. Similar continued testing of local toxicity in neurorrhaphy is under way. Methods have been developed for skin grafting on vascular pedicles, and for implanting arteries into bone to accelerate healing under conditions of diminished vascularity. These methods will provide the model situations for further investigation.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project No. 3A025601A821, COMBAT SURGERY

Task 01, Combat Surgery

Work Unit 122, Wound Healing

Investigators:

Principal: Col. William F. Macdonald, MC

Associate: Maj. Bruce Butler, MC, Maj. William Burkhalter,MC, Capt. Robert B. Rutherford, MC, Capt. Maurice J. Elovitz, MC, Capt. Martin L. Dalton, MC, Capt. Ralph A Lehman, MC, Capt. Robert L. West, MC, Capt. John Cranston, MC, Lt. Paul Jennings, VC

Description:

Tendon Healing: The previously reported studies of tendon healing using freeze-dried tendon homografts in the digital flexors of dogs are continuing. Long-term survivors are being sacrificed to determine the absence of any delayed rejection phenomena. An additional project has been undertaken to identify the influence of the nature of the graft bed on the results of tendon grafting.

Investigation of Cyanoacrylate Adhesives as "Bone Glues:" The investigation of the potential use of the cyanoacrylate adhesives as bone glues was begun during the previous year. Although the substances appear to be unsuitable because of interference with fracture healing, studies have continued to determine the least toxic members of the group, and to find the most efficient gluing techniques.

Experience with the American Vascular Staplers: Following the pioneer work of Androsov in Russia in 1953 and the subsequent work of theJapanese under Nakayama, an "American Vascular Stapler" has been produced, which is claimed to be a more precise and delicate instrument than its predecessors, with the additional advantage that a range of staples (1.5 to 3.5 mm LD) come pre-sterilized in mounted bushings, facilitating the loading of the stapler and thereby decreasing the occlusion time necessary. It was suggested that these features might allow surgeons without significant vascular experience to handle vascular trauma in a mass casualty situation. It therefore became of interest to the military to test the performance of this instrument. During the previous year, a series of dogs was begun, anastomosing the divided femoral artery with standard suture technique on one side and with the Stapler on the other.

Peripheral Nerve Injury: Efforts to improve the technique of peripheral nerve repair have concentrated largely upon the use of wrappers at the site of repair. Previous nerve studies at this institution have involved the use of collagen wrappers and have shown some advantage to the utilization of this material. Because of this it was felt that a wrapper which did not provoke very much reaction in the surrounding tissue or the epineurium might be advisable. With this in mind a study with silicon tubing was instituted. Various methods of repair with suture material and/or cyanoacrylate adhesives have been employed in chimpanzees.

Neural Toxicity of Isobutyl Cyanoacrylate: The development of this family of tissue adhesives has provoked much interest in their possible usage in various surgical repairs. Because it has been found that the methyl monomer was quite toxic to various tissues, it was thought worthwhile to investigate the properties of other adhesives in this chemical family. Isobutyl monomer was selected because of its physical properties and this has been placed upon the cortex after exposing the pia and also about the peripheral nerves of dogs. It has also been placed upon the optic chiasm of monkeys.

Control of Increased Pressure in Hydrocephalus with Acetazolamide: Ventricular perfusion studies showing that this compound can lower cerebrospinal fluid production have suggested that this compound might be helpful in lowering the pressure of hydrocephalus. Dogs were made hydrocephalic and the compound introduced into the ventricular system directly.

Small Vessel Anastomosis: The interest in small vessel repair and its possible use in cerebrovascular surgery has provoked the following study in small vessel anastomosis. A method is being used to approximate vessels less than 2 millimeters in outside diameter by means of a suture technique employing a needle as a stint within the lumen of the vessel. Studies are aimed at finding out what particular factors such as various materials and mode of suturing are important.

Preformed Collagen Tubes as Common Bile Duct Replacements: The multiplicity of injuries to the common duct in modern life and in combat casualties make very necessary the development of a fitting prosthesis for this structure, in injuries in which its length is so comprised that direct anastomosis cannot be made. Much work has been done in recent years at other institutions, which has suggested that various materials, among them preformed tubes of fibro-collagen of animal production might be used both as autografts and homografts. We have produced these preformed collagen tubes and have provided a safe and stable method of storage.

Use of Acrylate-amide Prosthesis in Replacement of Section of Common Bile Duct and use of Eastman 910 Adhesive and other Adhesives in the Anastomosis: Future prostheses for the common bile duct were studied. A defect in the common duct was replaced with acrylamide prostheses. An anastomotic prosthesis was made using Eastman 910 and octyl-2 cyanoacrylate adhesive.

Dacron Diaphragmatic Grafts. Various prostheses have been utilized to replace the diaphragm experimentally and clinically. However, none of these has proven to be entirely satisfactory, with the chief difficulties being shrinkage, fragmentation, and failure of muscle and fibrous connective tissue healing and ingrowth.

Direct Arterial Diversion to Supplement Bone Circulation: The shunting of new arterial circulation to bone is being studied for two purposes:

1) Would the bone circulatory system accept a new source of blood

from a main artery? If so, would this new "nutrient artery" produce a new vasculature within the bone?

2) Provided that the normal bone accepts a new blood supply, would a fractured bone, where delayed union or non-union has been induced, heal with this new vessel as its only source of circulation?

Progress:

Tendon Healing: The long-term survivor dogs who received the freeze-dried flexor profundus homografts in late 1963 are at present reaching the time for sacrifice. Later reports will discuss the microscopic findings in this series. A study has been begun in dogs, using the flexor profundus tendons in the forepaw, to assess the influence of the nature of the graft bed on the success of tendon grafting. Tendon lacerations are performed and surgically repaired in traumatic fashion. One month later, the area is explored and the scar tissue excised. In half of the animals, an immediate tendon graft is performed. In the other half the wound is closed and grafting is performed one or two months later. The animals are being sacrificed at appropriate intervals to determine the effect of grafting into the area of freshly excised scar tissue, as compared with grafting into the area after healing has matured.

Investigation of Cyanoacrylate Adhesives as "Bone Glues:" Using the same operative method previously described, fractures of the left femur are produced in anesthetized dogs. Open reduction is then performed. In the initial series, the cyanoacrylate was introduced into the fracture line, reduction was obtained, and a plate and screws were applied. Animals were sacrificed at 15, 30, 45, and 60 days. Octyl and isobutyl cyanoacrylates were tested. No fractures followed a normal course of healing. Essentially it appeared that a normal bridging callus was formed except that at the fracture line itself, the callus dwindled away into fibrous tissue. As this group was completed, reports were received from those working with the cyanoacrylates as soft tissue adhesives that butyl cyanoacrylate appeared to have a very low toxicity. A new group of animals was begun, using methyl as a control against butyl. These animals are being sacrificed at fifty days. The technique has been changed in that the glue is no longer introduced into the fracture line. The reduction is obtained and the glue is applied to the outer cortex of the bone. X-ray studies on this group indicate an inhibition of formation of any external callus. Tissue studies on this second group are not yet completed.

Experience with the American Vascular Stapler: During this year the femoral artery series was completed and a similar series, anastomosing the brachial artery, was carried out to test the possibility that the stapler may prove to be more advantageous in smaller vessels. Although neither of these two series have had the final (one-year) follow-up with arteriograms and gross and microscopic studies of the anastomoses, the initial impressions are as follows:

1) The stapler can do only end-to-end anastomoses.

2) It is suitable only in vessels of equal diameter whereas the suture technique is more accommodating to disparities between luminal sizes.

3) The stapler's range of bushings is too limited so that presently only vessels of two to four millimeters in diameter can be handled by it.

4) A stapler slightly smaller than the measured diameter of the vessel must be used.

5) One loses more vessel length than expected during the cuffing procedure, which is mechanically still crude in this instrument.

6) Collateral branches which need to be preserved may limit the applicability of the stapler, e.g., in the popliteal artery where the geniculate collaterals should be preserved.

7) There is more suture line bleeding using the stapler than with standard suture techniques and usually this must be controlled by additional placement of sutures.

8) The cuffing slightly compresses the anastomosis.

9) The present instrument is unsuited for deep or narrow operative fields.

10) The staples can slip out of the bushings unnoticed and this may be discovered only after the clamp is removed and severe bleeding ensues.

11) It does not greatly speed up the anastomosis of vessels.

Peripheral Nerve Injury: Silicon tubing has been placed around one nerve of each pair with a standard method of repair being done contralaterally. These repairs have been done in a series of a dozen chimpanzees utilizing the radial, ulnar, and peroneal nerves. The actual suture technique has involved the use of a limited number of sutures. In some cases sutures were omitted and an adhesive, isobutyl cyanoacrylate, was used to approximate the nerve ends. In addition a few studies have been done in which an anastomotic site has been wrapped with a thin sheet of silastic or with mircropore tape. As yet only preliminary results have been obtained because the animals are still to be followed for a long duration of time. It is hoped to obtain various functional tests of regeneration including strength-duration curves of the muscles and skin resistance measurements. The few animals in which histological results are currently available are promising, though it is too early to draw any firm conclusions.

Neural Toxicity of Isobutyl Cyanoacrylate: The studies are so far incomplete. However, results so far show that this material has a moderate degree of neural toxicity. This is not so great as the methyl compound, but it may be sufficient to preclude its clinical use. Furthermore, the compound does not seem to provoke the degree of fibrous reaction which might be desired if it were to be used in aneurysm surgery. Finally, it is noteworthy that unlike the methyl monomer, the isobutyl remains present in the tissue for very long periods of time, exceeding six months in many cases.

Control of Increased Pressure in Hydrocephalus with Acetazolamide: Studies of five dogs were done. A good degree of hydrocephalus was induced in each one. This was evident both by measurement of ventricular pressure (which usually exceeded 200-250 millimeters of water) and by autopsy (demonstration of dilated ventricles). Acetazolamide was injected in quantities sufficient to give levels of approximately 100-200 milligram percent in the cerebral spinal fluid (introduction of 0.1 or 0.2 cc of 40 mg/cc strength solution of acetazolamide). Acute measurements of spinal fluid pressure were made 0, 12, 24, 36, and 60 hours after the injection and showed no consistent variation from pretreatment levels. Pressure measurements were made with a needle of 20 gauge through a guide implanted in the skull. The needle was introduced through the guide percutaneously and lowered to the point at which cerebral spinal fluid first began to appear. The needle was then connected to the Sanborn Recorder by means of tubing and the pressure measured. Prior to autopsy, methylene blue was injected through the needle in the same fashion in order to be sure that no fluid escaped around the needle tract. All animals were anesthetized with a combination of thorazine and pentobarbital, in order to avoid the effects of pentobarbital alone. When used alone this latter compound will increase the cerebral spinal fluid pressure, whereas when used with thorazine only 1/3 the dose is necessary and the pressure does not rise.

Small Vessel Anastomosis: The studies thus far performed are still inconclusive. Tevdek and Mersilene sutures have been used and as yet the results of this comparison are not available. In addition, the comparison of anastomosis when procaine is used to irrigate the vessel externally, and when it is not used is being evaluated in order to determine whether the vasodilating action of this compound is of any help. The direction in which the needle is placed within the vessel is also under evaluation.

Preformed Collagen Tubes as Common Bile Duct Replacements: Sheets of fine mesh stainless steel grids were formed into tubes a centimeter to a quarter centimeter in diameter and about 7 centimeters in length. These tubes were implanted in the backs of dogs and caused a formation of a fibro-collagenous layer (in the shape of a tube) which was removed. These tubes were harvested and freeze-dried in liquid nitrogen under high vacuum. They were stored in sealed glass containers. Progress was made to the point that we are ready to implant these in suitable animals.

Use of Acrylate-amide Prosthesis in Replacement of Section of Common Bile Duct and use of Eastman 910 Adhesive and other Adhesives in the Anastomosis: A series of long-term survivor dogs have been completed in the study and were sacrificed at varying intervals over the past year. Thirty-seven dogs were studied, the majority having anastomoses made using the octyl-2-cyanoacrylate monomer. No other form of suturing was attempted.

Dacron Diaphragmatic Grafts: Ten adult mongrel dogs were subjected to left thoracotomy and excision of the central portion of the left hemidiaphragm, with replacement with a porous dacron graft which we felt would be more likely to incorporate tissue healing and ingrowth of connective tissue. These animals were sacrificed from 3-13 months after surgery. Autopsy findings revealed complete healing without hernia in any of the animals. The graft was well incorporated. Beyond six months there

were early signs of immune reaction, not unlike the early reaction seen in transplantation. At present we are studying in five small puppies weighing under 4,000 grams, the effect of complete excision of the entire left hemidiaphragm and replacement with a dacron graft. These animals will be sacrificed at periods greater than 12 months.

Direct Arterial Diversion to Supplement Bone Circulation: The initial experiments to date have been concerned with the technique of arterial insertion. The tibia has been selected as the bone for arterial insertion, and the anterior tibial artery as the new nutrient artery. Evaluating the procedure has been by means of direct inspection, arteriography, and histological sectioning of the experimented bone. Should this technique show promise, then it will be applied to fractured bones in the near future.

Summary and Conclusions:

Tendon Healing: Studies of tendon healing are continuing. Late results of the use of freeze-dried homograft tendons are being assessed. An investigation of the influence of the graft bed on the results of tendon grafting is under way.

Investigation of Cyanoacrylate Adhesives as "Bone Glues:" The use of the cyanoacrylate adhesives as bone glues does not appear to be feasible at this time. The glue joint itself is not sufficiently strong to serve as the sole fixation method. The agents appear to exert a specific inhibitory effect on bone union.

Peripheral Nerve Injury: The methods currently employed suggest that the use of silicon tubing about the area of nerve repair may be at least as favorable to nerve regeneration as the standard methods of repair used at the present time. Furthermore, this method has some advantages in being simpler to perform.

Neural Toxicity of Isobutyl Cyanoacrylate: Studies thus far have shown this material is probably not advisable in various neurosurgical applications.

Control of Increased Pressure in Hydrocephalus with Acetazolamide: This compound seemed to have no effect in reducing the cerebrospinal fluid pressure in hydrocephalus. There is no reduction in pressure in hydrocephalus. Certainly there is no reduction in pressure evident under the conditions in which this was measured (dogs with obstructing hydrocephalus induced by cisternal injection of Kaolin).

Small Vessel Anastomosis: The use of a needle as a stint within vessels of very small diameter greacly facilitates the suture technique and may possibly be useful.

Preformed Collagen Tubes as Common Bile Duct Replacements: Preformed fibro-collagenous tubes may be successfully grown in the period of one to two months in the subcutaneous tissues of the back of the dog. These tissues may be stored by means of the freeze-drying technique to periods of one year. Further studies are now awaited in which these

materials will be implanted as prosthetic devices. It is also hoped to assess their antigenicity which is altered by the freeze-drying process.

Use of Acrylate-amide Prosthesis in Replacement of Section of Common Bile Duct and use of Eastman 910 Adhesive and other Adhesives in the Anastomosis: Thirty-seven prosthestic replacements have been made, with long-term survivors ranging up to one year. All dogs appear to have done well in weight, with the exception of one. They were sacrificed at varying intervals with the majority being carried to the one-year termination date. Preliminary results indicate the merit of this technique of common bile duct replacement. Studies are now being summarized prior to publication.

Dacron Diaphragmatic Grafts: A new type dacron prosthesis is being tested in dogs. Although it heals well and is incorporated into the connective tissue, at one year real signs of rejection are apparent. Therefore, more chronic studies are in progress utilizing puppies to be sacrificed in the future. This work is in progress and has not been completed.

Direct Arterial Diversion to Supplement Bone Circulation: Animal studies are being performed to determine the feasibility of increasing blood flow to the tibia by implantation of the anterior tibial artery. At present, it appears that this artery is too small in the dog to allow successful cuffing and insertion.

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23. KEYWORDS: Shock; shock, hemorrhagic; shock, surgical; shock, toxic; shock, traumatic; irreversible shock; blood coagulation; wound infection; debridement; vasodilator agents

24. (U) Tech Objective - To improve the methods of treatment of the severely injured to minimize morbidity and mortality associated with combat trauma.

(U) Approach - On the assumption that the common denominator in all types of shock is a diminished capillary perfusion, investigation of the role of disseminated intravascular coagulation in this phenomenon is under way. Particular emphasis is placed
25. on the phenomenon of irreversibility in shock. The influence of vasoconstrictors and vasodilators is being evaluated, as well as that of substances which may prevent red cell and platelet aggregation. Abnormalities of plasma clotting factors in the shock state are being evaluated.

Investigation in the treatment of clostridial infections in rabbits is directed at the development of methods of protection against gas gangrene either as a supplement to wound debridement or as an expedient method in emergency situations.

26. (U) Progress (Jul 64 - Jun 65) - The shock studies have documented the consumption of all the plasma clotting factors in the shock state, in endotoxin shock as well as in hemorrhagic shock. This emphasizes the importance of disseminated intravascular coagulation in the mechanism of shock. The studies of clostridial infections have confirmed the efficacy of Sulfamylon as a protective agent.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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Project No. 3A025601A821, COMBAT SURGERY

Task 01, Combat Surgery

Work Unit 123, Responses to Trauma

Investigators:

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Description:

Disseminated Intravascular Coagulation: The role of disseminated intravascular coagulation in the shock state is the subject of continuing investigation. Detailed studies of the clotting factors are necessary for the elucidation of this process.

During the past year techniques for assay of all clotting factors were worked out. It is now possible to assay all these factors and this has been done in both endotoxin and hemorrhagic shock in dogs. It has been found that irreversible hemorrhagic or endotoxin shock results in a coagulation defect characterized by a deficiency of all clotting factors, many of which go down to less than 10%. These clotting defects can be prevented by volume administration and vasodilators.

Coagulation Changes in Hemorrhagic Shock: Previous work in this laboratory with an irreversible hemorrhagic shock preparation has shown drops in fibrinogen, platelets, and prothrombin time suggesting that disseminated intravascular coagulation takes place. These observations have been correlated with other pathologic findings and have been supported further by the experimental success of pretreatment with heparin and fibrinolysin in this same preparation.

Significance of the Rate of Fibrinogen Decline Following Hepatectomy in Dogs: The marked drop in fibrinogen, which has been observed in the hemorrhagic shock preparation in this laboratory, has been offered as further evidence for the occurrence of DIC. Theoretically, there are four possible explanations for this fibrinogen drop. Two of them, i.e., a dilutional effect and the effect of fibrinolysin, had been eliminated by previous study. While other evidence suggests that this fibrinogen drop is indeed due to consumption by intravascular coagulation, it still remained to eliminate the fourth possibility, that absent or decreased fibrinogen production by a damaged liver could cause the degree of fibrinogen drop seen in these shock studies. Hepatectomy affords opportunity for this study.

Histopathologic Studies on Porto-Systemic Encephalopathy (PSE): Porto-systemic encephalopathy occurs in approximately 30% of cases with portacaval shunts. While its etiology remains unsettled, it seems to be related to blood ammonia levels which in turn are influenced by three factors: 1) the amount of portal blood shunted around the liver; 2) decrease in liver function; 3) the protein intake. The interrelationship of these three factors was previously pointed out by work in the last two years

in this laboratory. A high degree of porto-systemic encephalopathy could be demonstrated histologically in dogs with total portacaval shunts, in which all three of the above factors were operative. If, however, the shunt was made below the last portal collateral, liver function was better maintained and the histologic changes of porto-systemic encephalopathy were not seen unless the dogs were put on a high protein diet. The degree of histologic change seen correlated well with ammonia tolerance curves in the different experimental groups. It remains, however, to show that it is ammonia and not some other substance being shunted around the liver which is the cause of these histologic changes.

Significance of Histamine Levels after Porto-Systemic Shunting: The incidence of peptic ulceration associated with portacaval shunts in cirrhotic patients ranges from 10 to 30%. It has been theorized that this results from histamine rich portal blood reaching the systemic circulation without passing through the liver. It has been previously shown in dogs that shunting only the blood returning from the gastrointestinal tract below the ligament of Treitz will cause an increase in gastric secretion, while shunting of the portal blood from above this point will not. It would seem important therefore in confirming the histamine theory of gastric hypersecretion following portacaval shunt to show that the histamine in the portal blood also comes mainly from the small intestinal venous return.

Study of Experimental Clostridial Infections in Massive Open Wounds: Previously this department, in co-operation with the department of bacteriology, has studied histotoxic clostridial infection using a standard rabbit wound preparation. The wound is made by excising a one-inch square of skin on the posterolateral thigh of the rabbit, deeply incising the exposed muscle and thoroughly crushing it. Into this wound is mixed an amount of previously analyzed dirt. The previous year's work dealt with the effect of debridement delay on mortality and it showed that there was no discrete "golden period" but rather a progressive increase in mortality with delay until, at 18 hours and beyond, the mortality rate exceeded the controls. There was also found an unexpected protection against gas gangrene by both tetanus anti-toxin and tetanus toxoid.

Fate of Dearterialized Spleen: Continued clinical usage of the splenic artery for revascularizing various intra-abdominal organs has led to the speculation by many investigators as to the ultimate fate of the dearterialized spleen. A study of this problem has been performed in dogs.

Pleural Responses to Trauma: Knowledge of the anatomical response to pleura is of moderate surgical value and yet few reports have been entered into the literature. This experiment was unique in that it is a study of pleural response to trauma on the usually encountered modalities of trauma; that is thoracotomy, pleural abrasion and hemothorax, using a three dimensional approach afforded by the Häutchen *en face* preparation. Using this preparation in addition to routine stains, we have effectively studied the pathological effects of trauma on the pleura.

Progress:

Disseminated Intravascular Coagulation: During the past year experiments have been continued on both endotoxin and hemorrhagic shock. A great amount of laboratory effort has been devoted to perfecting methods of assay of all clotting factors I thru XII. These methods have now been worked out along with a new method of determining fibrinogen. This involves salting out fibrinogen instead of precipitating it with thrombin. In addition, a method of determining the concentration of cryofibrinogen has been worked out. Cryofibrinogen is what is left of the fibrinogen molecule after peptides A and B have been split off and is the first step in coagulation. An elevation of this material is pathognomonic of beginning DIC.

It has also been found that factors V and XII are particularly decreased in DIC and account for the incoagulability of blood which has been found previously to accompany DIC. Factor V is relatively easy to assay on a continuing basis because Factor V deficient plasma may be easily made in the laboratory. In future work both in the laboratory and in the clinical shock study unit these new methods will be fully utilized to study DIC in both laboratory animals and patients. This clinical unit should begin activities in July 1965.

Specific decreases in various clotting factors found in hemorrhagic and endotoxin shock are listed.

Table 1
MEAN DECREASE IN CLOTTING FACTOR ACTIVITY*

Factor	Interval After Endotoxin 5 min. (%)	4 hr. (%)
Fibrinogen**	58.5	99.3
Prothrombin	25.0	37.0
Factor V	27.0	64.0
Factor VII	20.0	65.0
Factor VIII	47.0	31.0
Factor IX	45.0	41.0
Factor X	15.0	60.0
Factor XI	68.0	54.0
Factor XII	36.0	33.0

* Factors V-XII expressed as the percent of the total activity originally present on the animal's control plasma.
** Expressed in mg%

Table 2
MEAN DECREASE IN CLOTTING FACTOR ACTIVITY*

Factor	Four Hours After Hemorrhage (%)
Fibrinogen**	129
Prothrombin	75
Factor V	78
Factor VIII	41
Factor IX	84
Factor X	88
Factor XI	62

Factors VII & XII***

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* Factors V-XI expressed as the percent of the total activity originally present in the animals' control plasma.

** Expressed in mg%

*** Not quantitated in animals in this series

In addition it has been found that these coagulation changes are in part preventable by various methods including 1) regulation of pH by bicarbonate and TRIS buffer, 2) vasodilation with dibenzyline. This is true in both endotoxin and hemorrhagic shock. Other studies have been performed to study the mechanism of platelet agglutination, and its influence on DIC. Several substances which have been reported by other investigators to inhibit platelet agglutination have been examined. In the shock preparation, all have been found ineffective; platelet agglutination has occurred, as has disseminated intravascular coagulation.

Coagulation Changes in Hemorrhagic Shock: In an attempt to study the coagulation changes in more detail and to more closely correlate the success of certain treatment parameters with changes in the coagulation factors, studies with this standard shock preparation were extended to measure the following: Fibrinogen, Platelets, pH, Glass and Silicone Clotting Times, Hct, Fibrinolysis by Fibrinogen Incubation Methods, Prothrombin Times, Partial Thromboplastin Times, Prothrombin Utilization Rates, Total Serum Proteins and Assays for Coagulation Factors V, VIII, IX, X, and XI. Initial results show that most dogs, 1/2 hour after hemorrhage demonstrate a phase of accelerated coagulation as reflected by increase in prothrombin utilization rates and shortened partial prothrombin times. In addition, silicone clotting times shorten from an average normal of fifteen minutes to three. Subsequently, a state of relative "hypocoagulability" ensues. By the end of the four-hour period of shock, silicone clotting times usually rise to one to two hours. The prothrombin utilization rate slows and the partial prothrombin time increases. By this time fibrinogen, prothrombin times and platelets drop to approximately 50% of control levels and the clotting factors in animals thus far analyzed have shown a definite decline. Since it has previously been shown in vitro that acidosis enhances the coagulability of blood, even when heparinized, it was decided to buffer the acidotic dogs to see if this modified the clotting changes observed. To date the buffered dogs have consistently shown less evidence of intravascular coagulation in contrast to the concurrent controls.

Four dogs were also studied comparing the various clotting parameters by simultaneous samples taken from the portal vein and the inferior vena cava during the course of shock. No significant differences were detected.

Significance of the Rate of Fibrinogen Decline Following Hepatectomy in Dogs: Fibrinogen levels were monitored after hepatectomy in dogs. Using a technique that minimized portal occlusion and significant blood loss and the release of vasoactive substances into the blood, an initial period of gradual decline in fibrinogen could be demonstrated. During this early period the rate at which fibrinogen declined correlated very well with the natural decay rate estimated by radiosotope tagging

studies, indicating a fibrinogen half-life of 2½ days. Subsequently, however, the animals developed a rapid decline in fibrinogen the onset of which correlated with the onset of shock and acidosis. In two further study groups hepatectomized animals were given heparin or totally eviscerated. Only a gradual rate of decline was observed in these groups.

An additional number of hepatectomy dogs were studied with more frequent sampling for fibrinogen as well as other clotting parameters, arterial and venous pressures, pH's and blood gases. It could be seen from these that the late, accelerated phase of fibrinogen decline began with the onset of terminal shock and acidosis and correlates with the disappearance of other clotting factors.

Histopathologic Studies on Porto-Systemic Encephalopathy: During this year a series of normal rhesus monkeys (without portacaval shunts) were chronically infused with ammonia acetate, buffered to normal pH, so that their blood ammonia level was held near 200 mg% for two weeks. Of the first five monkeys fully studied thus far, three have shown definite histologic evidence of porto-systemic encephalopathy whereas the other two have shown only questionable changes. These changes were easily distinguished from the controls by a panel of pathologists. For this reason an additional seven monkeys have been similarly studied with the length of the infusion extended from two weeks up to six weeks. While this group of monkeys have been sacrificed the histologic studies are not yet completed.

Significance of Histamine Levels After Porto-Systemic Shunting: The venous return of the dogs' gastrointestinal tract was subdivided into three regions according to portal tributaries as follows:

1) The gastric blood returning through the gastrosplenic trunk (spleen removed)

2) The pancreatic and duodenal blood returning through the gastroduodenal vein

3) The small intestinal blood returning through common intestinal vein

By dividing appropriate collaterals and anastomosing one or the other of these main tributaries into the inferior vena cava, it was observed that shunting the small intestinal blood gave the highest peripheral histamine levels in the same range as a total shunt where no, or only a mild elevation was observed after a pancreato-duodenal venous shunt or gastric venous shunt was done. In addition, the elevation seen after a total portal shunt using portacaval transposition, could be returned to normal by shunting the small intestinal venous blood back through the portal bed.

Study of Experimental Clostridial Infections in Massive Open Wounds: Experimentation proceeded along the following lines:

1) Attempts to reduce the excessive mortality rate associated with late debridement by doing more radical excision, i.e., hip disarticulation, at 18 or 24 hours after wounding, was met with a further increase in mortality to 100%. Control animals with hip disarticulation alone all survived.

2) Attempts to elucidate the mechanism of protection of tetanus immunization against gas gangrene were made using sterilized dirt repopulated with only histotoxic clostridial strains. This met with considerable difficulty because of the extreme potency of these organisms. The dirt was cut serially from 1:5 up to 1:250 before a partial mortality (LD 80) was obtained. This amounted to only twenty organisms introduced into the wound. Using this particular dirt there was no protective effect from tetanus anti-toxin (control mortality 70%, TAT 80%).

3) The testing of topical chemotherapy was again begun. Preliminary studies show that sulfamylon, a homo-sulfonamide that is not inhibited by para-amino benzoic acid, gave the greatest protection, reducing the mortality to 10% against a control of 70%. Penicillin and polybacterin gave moderate protection, with mortality in the 40-50% range. Further testing of sulfamylon against a supralethal wound, using dirt restocked with five pathogenic histotoxic clostridial strains, showed a prolongation of survival from 27 hours, control average, to 65 hours using sulfamylon, with a 16-hour gap between the last death in the control series and the first death in the sulfamylon treated rabbits. When dirt was cut to give an LD 80, treatment with sulfamylon reduced the mortality rate to 20%. Control animals debrided 24 hours after wounding had a 100% mortality, which was reduced to 70% using sulfamylon. Hip disarticulation 24 hours after wounding offered no protection, with 100% of both the no-treatment controls and sulfamylon treated animals dying.

Fate of Dearterialized Spleen: Eight dogs were observed from one to four weeks following either complete dearterialization of the spleen or ligation of the main splenic artery; gross and microscopic changes have been studied. Results of this study indicate that the effects grossly and microscopically of splenic dearterialization are negligible.

Pleural Responses to Trauma: Using sterile technique, 42 rabbits under ether anesthesia were subjected to various combinations of trauma and sacrificed at one-to-three-week intervals. No adhesions were noted in the hemothorax group, but there were microscopic changes consistent with widespread trauma to the mesothelial cells even of hemothorax as well as of thoracotomy. After pleural abrasion for thirty seconds the mesothelial cells are completely absent, but regenerate within one week.

Summary and Conclusions:

Disseminated Intravascular Coagulation: During the past year techniques for assay of all clotting factors were worked out. It is now possible to assay all these factors and this has been done in both endotoxin and hemorrhagic shock in dogs. It has been found that irreversible hemorrhagic or endotoxin shock results in a coagulation defect characterized by a deficiency of all clotting factors, many of which go down to less than 10%. These clotting defects can be prevented by volume adminstration and vasodilators. Beginning approximately July 1965 a unit will be set up in WRGH for the clinical study of shock. Coagulation factors will be studied in human shock in the same way that it has been studied in experimental shock in animals.

Coagulation Changes in Hemorrhagic Shock: More detailed study into coagulation changes occurring in standard hemorrhagic shock preparation indicates that early in the period of shock there is a period of accelerated coagulation during which multiple clotting factors are consumed and subsequently, secondary to this consumption, a marked "hypocoagulability" results with all clotting factors being reduced to 50% or less of baseline. This correlates well with the oscillations in "coagulability" seen in human shock cases by Attar. The degree of these changes can be reduced experimentally in dogs by buffering the pH in a normal to high normal range. The portal blood does not appear to contribute disproportionately to these changes.

Significance of Rate of Fibrinogen Decline Following Hepatectomy in Dogs: The natural decay rate of fibrinogen in the dog barring further production is probably quite slow, in the range of one percent per hour, much as previously estimated by fibrinogen tagging studies. This gradual rate of decline can be observed initially following hepatectomy by careful technique, especially if frequent early sampling is done. Eventually as the dog goes into shock, this rate of disappearance becomes greatly accelerated by coagulation. This late phase of rapid consumption can be prevented by heparinizing the dogs or by removing the other abdominal viscera along with the liver. The findings further consolidate the concept that disseminated intravascular coagulation occurs during hemorrhagic shock in the dog, and explain the previous discrepancy between the two methods of estimating the natural decay rate of fibrinogen.

Histopathologic Studies on Porto-Systemic Encephalopathy: It appears that the histologic CNS changes seen with porto-systemic shunts can be reproduced by chronic ammonia infusions at a level seen in clinical cases. The appearance of these changes is not inevitable with infusion lasting only two weeks and the infusion period has thus been extended in further studies.

Significance of Histamine Levels After Porto-Systemic Shunting: The bulk of the histamine in the portal blood appears to arise from the small intestine. This is further circumstantial evidence that gastric hypersecretion after portacaval shunts results from histamine rich portal blood gaining direct access to the peripheral circulation.

Study of Experimental Clostridial Infection in Massive Open Wounds:

1) Further work strengthens the impression that beyond a certain point delayed debridement, if unsupported, carries a mortality in excess of the controls and this cannot be reduced by more radical debridement at this late stage.

2) A marked protective effect was obtained by using 20% sulfamylon instilled into the wound one hour after wounding.

Fate of the Dearterialized Spleen: The production of a dearterialized spleen by ligation of the splenic artery or by complete dearterialization results in little demonstrable change in that organ.

Pleural Responses to Trauma: The natural history of pleural changes associated with thoracotomy, hemothorax and pleural abrasion has been studied. Widespread mesothelial changes are seen in hemothorax as well as in thoracotomy. Very rapid mesothelial regeneration follows pleural abrasions.

Publications:

1) Hardaway, R. M., D. G. Johnson, D. N. Houchin, E. B. Jenkins, J. W. Burns, and D. R. Jackson: Influence of Stress on Fibrinogen. J. of Trauma 4: 673-676, 1964

2) Hardaway, R. M., D. G. Johnson, M. J. Elovitz, D. N. Houchin, E. B. Jenkins, J. W. Burns, and D. R. Jackson: Influence of Trauma and Hemolysis on Hemorrhagic Shock in Dogs. J. of Trauma, 4(5): 624-641, 1964

3) Hardaway, R. M., D. G. Johnson, D. N. Houchin, E. B. Jenkins, J. W. Burns, and D. R. Jackson: Studies on the Fibrinogen Replacement Rate in Dogs. Ann. of Surg., 160: 835-838, 1964

4) Hardaway, R. M.: Bleeding After Incompatible Blood Transfusion. Published in a book entitled "Surgical Hemorrhage" which is a Handbook for the management of bleeding in surgery and its specialties. Ed. Dr. Alex W. Ulin, Hahneman Med. Col. & Hosp., Phila., Pa. Pub. McGraw-Hill, 1964

5) Hardaway, R. M., M. J. Elovitz, W. R. Brewster, Jr., D. N. Houchin, N. L. Renzi, and D. R. Jackson: Influence of Vasoconstrictors and Vasodilators on Disseminated Intravascular Coagulation in Irreversible Hemorrhagic Shock. SG&O, 119: 1053-1061, 1964

6) Hardaway, R. M., M. J. Elovitz, W. R. Brewster, Jr. and D. N. Houchin: Clotting Time of Heparinized Blood. Influence of Acidosis. Arch. of Surg., 89: 701-05, 1964

7) Hardaway, R. M.: Pulmonary Microthrombi. Presented at Symposium on Pulmonary Embolic Disease pp. 65-72 Arthur A. Sasahara Editor, U. S. A. 1964

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6468	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 65	A. NEW	U RPT U WRK	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 62156011 3A025601A821 01 124

10b. PRIOR NUMBER/CODE: None

11. TITLE: (U) Experimental anesthesia (09)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002400 Bioengineering; 003500 Clinical medicine; 008800 Life support	05 58	NA	OTHER | DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	3	31
		CURRENT FY 66	3	35

19. GOV'T LAB/INSTALLATION/ACTIVITY
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ADDRESS: Washington, D. C. 20012
INVESTIGATORS PRINCIPAL ASSOCIATE: Barila, Lt. Col. T.G.; Meyer, Maj. J.A., Cohen, Capt. P.J.
TEL: 202-576-3669 TYPE: DA

21. TECHNOLOGY UTILIZATION: Clinical anesthesia and resuscitation

22. COORDINATION: NA

23. KEYWORDS: Anesthesia; heart, mechanical; respiration; respirators; heart massage; inhalation therapy; hyperventilation

24. (U) Tech Objective - To evaluate anesthetic agents and methods, and to develop and evaluate techniques for resuscitation and support of damaged circulatory and respiratory mechanisms.

(U) Approach - A combined effort with the Harry Diamond Laboratories has resulted in the development of a family of devices using the principles of fluid amplification as power and control sources. This group of devices includes the Army Artificial Heart Pump, a volume-cycled respirator, a pressure-cycled respirator and a mechanical heart massage assister. The hardware is in existence, at least in prototype models, and the present phase is one of testing and design refinement. Investigation is in progress on the effects of hyperventilation and intracranial pressure on cerebral blood flow.

(U) Progress (Jul 64 - Jun 65) - Improvements in the control capability have been incorporated into the heart pump design. The pump is being tested in left heart bypass procedures in animals, and an encouraging increase in duration of pumping without alteration of tissue pH is noted. The respirator designs are being modified currently and will be further tested. The heart massage assister is undergoing comparative testing with existing models. Hyperventilation has been shown to produce cerebral hypoxia, as does increased intracranial pressure at levels of impairment of consciousness.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		AR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
1439a	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project No. 3A025601A821, COMBAT SURGERY

Task 01, Combat Surgery

Work Unit 124, Experimental Anesthesia

Investigators:
Principal: Lt Col Timothy G. Barila, MC
Associate: Maj James A. Meyer, MC, Capt Martin L. Dalton, MC, Capt Peter J. Cohen, MC

Description:

Army Artificial Heart Pump: Continued evaluation of the Army Artificial Heart Pump is leading to further refinements in design. Animal studies are furnishing encouraging results on the effects of pulsatile flow, as compared with those of non-pulsatile flow.

Respirators: Design modifications are being introduced in the pressure-cycled and volume-cycled respirators which have been reported previously. A broad test program is underway in both animals and humans.

Mechanical Cardiac Massage Assisters: The devices available for performing mechanical external cardiac massage are undergoing comparative testing. Included in this group is a prototype unit developed by the WRAIR-Harry Diamond Laboratories group.

Control of Cerebral Circulation and Metabolism: This study is directed towards the further elucidation of the factors important in the regulation of cerebral circulation and metabolism. Studies of normal disease have been made. Among the factors examined have been cerebral oxygenation, arterial carbon dioxide tension, and the presence of increased intracranial pressure. In addition, the effects of drugs upon hemodynamic alterations produced by disease processes have been investigated.

Progress:

The investigators in this work unit have continued their joint efforts with the Harry Diamond Laboratories* in the development and testing of a family of fluid-powered and fluid-controlled devices for the long-term circulatory and respiratory support of the severely wounded soldier.

Army Artificial Heart Pump: Recent changes in the design of the amplifier have resulted in improved control characteristics. Eleven of these new amplifiers have been sent to pump evaluators together with a new operation and maintenance manual describing the pump with this latest amplifier installed. Only three yet require the new amplifiers. They will be sent as soon as fabrication is complete. Three studies were carried out during the

* K. E. Woodward, MEA, Research and Development Supervisor
H. Straub
J. W. Joyce
G. Mon
E. A. Wright

past year using the Heart Pump. The first was designed to measure renal blood flow during complete heart-lung bypass. Prior studies had shown renal lesions after prolonged bypass using non-pulsatile flow, and it was considered desirable to compare renal blood flow in pulsatile and non-pulsatile flow preparations. Using sterile technique, twenty dogs were subjected to either Army Artificial Heart Pump or depulsated Roller Pump complete heart-lung bypass for periods of three hours. Renal blood flow was measured and found to be essentially the same in each group. The discrete renal lesions were noted in all the roller pump dogs and in approximately 1/2 of the Army Heart Pump dogs.

A second study compared renal blood flow using the Army Artificial Heart Pump with that obtained by using depulsated roller pump perfusion on left heart bypass. In a series of eight dogs a method was used which allowed instantaneous change from one pump to the other. In this way each animal served as his own control. Once again no detectable difference in renal blood flow was observed. These two studies add further weight to the belief that renal lesions following prolonged perfusion are due to the nature of the renal blood flow, since the volume of flow shows no difference in either pulsatile or non-pulsatile modes.

In the third study, the Army Artificial Heart Pump was used for long-term assisted circulation. The Dennis-Senning Cannula was used to establish left ventricular bypass. Control animals survived perfusion for periods up to 48 hours. Dogs were then studied in endotoxin shock and in cardiogenic shock. Four endotoxin dogs showed a mean survival time of 13.5 hours, compared to 6 hours for the controls. Studies are being made in cardiogenic shock induced by Agress's spheres. A stainless steel version of the pump is about 90% complete. It will be less expensive to produce than the present plastic model and much more rugged. It offers a further advantage of being completely autoclavable, thus permitting the complete unit to be in the sterile operative field and under direct control of the surgeon.

Respirators: The volume cycled respirator has been redesigned to comply with suggestions made last fall. Tidal volumes have been increased from 1500 cc to 2400 cc, and cycling rates have been increased to satisfy the needs of panting patients. To accomplish the latter, the logic of control was altered to allow a second fluid amplifier to drive the bellows down to a reset position in sufficient time to allow panting patients to initiate the inspiration cycle. If an inspiration attempt is not made within a prescribed time, the respirator will automatically go into a controlled mode of operation.

The inspiration initiation control has been redesigned for both greater sensitivity and variation of sensitivity. Provision has been made for oxygen administration and for the attachment of a gas canister-type filter. The pressure-cycled respirator has been modified by the addition of a special breathing value, which reduced expiratory resistance, and added an expiratory pause. Tests of both respirators are continuing.

Mechanical Cardiac Massage Assisters: Mechanical external cardiac massage during electrically induced ventricular fibrillation was performed on sixteen dogs while adjunctive measures were employed to increase the efficacy of the effort. Cardiac output determined by using the dye dilution method and carotid artery blood flow measured by electromagnetic flowmeter, were used along with blood pressure measurements, to appraise the results of the effort.

Mechanical external massage was accomplished with the LSI-Butterworth Chest Compressor during the series of experiments. A single animal was used to compare results using the Army Cardiac Compressor, the LSI-Butterworth Compressor and the Westinghouse "Iron Heart." Sixteen dogs have been used in this study to date. After baseline measurements the results were taken following infusion of sodium bicarbonate, norepinephrine and a combination of the two. Small doses of norepinephrine (2 mgm/kg) increased total cardiac output and blood flow as produced by external cardiac massage in the fibrillating dog heart. Large amounts of norepinephrine (0.5 mg/min.) which produced a pressor response appeared to reduce cardiac output and carotid blood flow by an increase in peripheral vascular resistance while the propelling pressure stayed constant. Reduction of the metabolic acidosis by infusion of sodium bicarbonate resulted in a decrease in the aortic pressure and a rise in total cardiac output and carotid blood flow.

Control of Cerebral Circulation and Metabolism: Effect of hyperventilation on Cerebral Blood Flow. The ability of diminished arterial carbon dioxide tension to lower cerebral blood flow (CBF) is well-known. It is therefore of considerable interest to consider whether or not CBF can be diminished to such an extent that cerebral oxygenation is imperiled. Studies have been performed at the Naval Medical Research Institute to better define the effects of hyperventilation in awake man. Normal healthy young male volunteers have been studied during intentional hyperventilation of two gas mixtures: 1) 7% oxygen at an ambient pressure of three atmospheres absolute (inspired oxygen tension 160 mmHg); 2) 100% oxygen at an ambient pressure of three atmospheres absolute (inspired oxygen tension 2280 mmHg). During the period of hyperventilation of each of the gases a continuous eight channel electroencephalogram is recorded while end-tidal pCO_2 is constantly monitored by infrared analysis. The subject is unaware of the gas mixture he is inspiring at any one time, nor is he informed as to when the switch to another mixture is made. In many studies to date, profound alterations of the EEG are seen during hyperventilation with 7% oxygen. In all cases these alterations are completely and rapidly reversed when the hyperbaric oxygen is inhaled. During this period, there is no change in end-tidal CO_2 content. It thus appears that marked hyperventilation can, indeed, result in cerebral hypoxia. This work is to continue in both man and animals in a further attempt to define critical levels of oxygenation and carbon dioxide tension.

Other studies in progress involve the evaluation of patients with increased intracranial pressure (WRGH). When the level of consciousness is affected by the disease process, the measurement of both CBF and oxygen consumption yields the expected low values in both parameters. The institution of treatment with corticosteroids often results in a striking amelioration of symptoms. This is accompanied by an increase in both CBF and cerebral oxygen consumption. Work is to continue along these lines.

Summary and Conclusions:

Army Artificial Heart Pump: Continued refinement in design has resulted in improved control characteristics of the pump. Further studies of renal flow during perfusion confirm the beneficial effects of pulsatile flow on renal tissue, since there is no difference in rate of flow in comparing the Army Heart Pump with roller pumping. Preliminary results of a series of dogs in shock, with long-term assistance using the Army Heart Pump in left ventricular bypass, are encouraging. A stainless steel version of the pump has been produced.

Respirators: Both the pressure-cycled and the volume-cycled respirators are undergoing design modifications. Clinical testing is continuing.

Mechanical Cardiac Massage Assisters: A study to evaluate adjuncts to resuscitation during external cardiac massage was performed. A series of sixteen dogs was used to evaluate the effects of sodium bicarbonate, norepinephrine and a combination of the two infused during external cardiac massage. Small doses of norepinephrine increased cardiac output and carotid blood flow. Large doses reduced cardiac output and carotid blood flow. Reduction of metabolic acidosis with sodium bicarbonate increased cardiac output and carotid flow with a drop in aortic pressure.

Control of Cerebral Circulation and Metabolism: Studies are in progress on cerebral blood flow under various circumstances. Results of varying the oxygen content of the inspired air at three atmospheres have been identified. Studies are underway on rates of cerebral blood flow and oxygen consumption in increased intracranial pressure.

PROJECT 3A025601A822
MILITARY INTERNAL MEDICINE

Task 01
Military Internal Medicine

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
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10b. PRIOR NUMBER/CODE: NONE

11. TITLE: (U) Metabolic response to disease and injury (09)

12. SCIENTIFIC OR TECH. AREA: 003500 Clinical Medicine; 012900 Physiology; 002300 Biochemistry

13. START DATE: 08 63

14. CRIT. COMPL. DATE: NA

15. FUNDING AGENCY: OTHER | DA

16. PROCURE. METHOD: C. IN-HOUSE

17. CONTRACT/GRANT: a. DATE; b. NUMBER; c. TYPE: NA; d. AMOUNT

18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
PRIOR FY 65	20	185
CURRENT FY 66	20	200

19. GOV'T LAB/INSTALLATION/ACTIVITY
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ASSOCIATE: Shinaberger, Maj J.H.
TEL: 202-576-3529 TYPE: DA

21. TECHNOLOGY UTILIZATION: Medical Science

22. COORDINATION: NA

23. KEYWORDS: Anuria; hemodialysis; uremia; peritoneal diseases; body fluids; endocrine glands; kidney; shock; mannitol

24. (U) Tech Objective - Development of improved methods for prevention and treatment of renal failure.

25. (U) Approach - To continue efforts to improve the use of peritoneal dialysis including studies of the movement of fluid, solute and nutritive substances across the serosal membranes. Associated with this is an evaluation of fluid and electrolyte shifts within the body in response to acute stress.

26. (U) Progress (Jul 64 - Jun 65) - Peritoneal dialysis has been employed in a wide variety of cases. Various experimental models have been developed and employed to gain additional understanding of renal failure and uremia.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
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31. MISSION OBJECTIVE	32. PARTICIPATION
AR 705-5	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

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DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

ANNUAL PROGRESS REPORT

Project 3A025601A822, MILITARY INTERNAL MEDICINE

Task 01, Military Internal Medicine

Work Unit 120, Metabolic response to disease and injury

Investigators.
Principal: Lt. Colonel Kevin G. Barry, MC
Associate: Major James H. Shinaberger, MC; Capt Jack W. Coburn, MC; Capt Leroy Shear, MC; Capt John P. Malloy, MC; Capt Seymour Rosen, MC; Capt Murphy T. Scurry, MC; Capt Jessie Hano, MC; Gerald J. McCormick, Ph.D.; and Marjorie Knowlton, B.S.

Description

This work unit includes: (A) Development of improved methods for the prevention and treatment of renal failure. (B) Use of the peritoneal cavity for dialysis. (C) Study of the absorption from the peritoneal cavity of fluid, solute, and nutritive substances across the serosal membranes. (D) Fluid and solute shifts within the body in response to acute stress which are poorly defined and more poorly understood. (E) Adrenal steroid excretion is of importance in the stress reaction. The development of more discrete techniques for fractionation in the Steroid Laboratory represents a complemental study of the metabolic response to stress.

Progress

I. Prevention of Acute Renal Failure

1. Sustained hydration and judicious use of osmotic diuretics has markedly decreased the incidence of acute renal failure. However, fluid shifts in response to anesthesia and surgery are not well defined. Use of isotopic methods for simultaneous determination of blood, extracellular and intracellular volume shifts are under way.

2. The value of the osmotic diuretic mannitol is well established. However, it must be given intravenously. Isosorbide, structurally similar to mannitol, is absorbed from the gastrointestinal

tract, distributed in the total body water, and causes an osmotic diuresis. Animal and human studies have demonstrated both effectiveness and safety. Currently, the agent is being tested in patients. The least dividend will be in having a new agent similar in distribution and kinetics to urea, but of different structure. At most, isosorbide may prove to be an important oral osmotic diuretic and superior replacement for urea in clinical therapy.

II. Treatment of Renal Failure: Work in this area has centered primarily on improved techniques for peritoneal and hemodialysis. However, important strides have been made in study of nutrition, protein synthesis - degradation and infection which are important determinants of the final outcome.

1. Peritoneal and Hemodialysis: Modification of Equipment and Techniques. Modifications are needed to increase efficiency and decrease cost.

a. Recirculation Peritoneal Dialysis. A new technique of peritoneal-extracorporeal dialysis with continuous recirculation of dialysate through the Kolff Twin-coil Kidney has resulted in clearances of urea, creatinine, uric acid and phosphate much higher than previously reported. This technique has demonstrated the potential capability of the peritoneal membrane as a dialyzing system. This procedure has been performed eleven times without complications and is under further evaluation. It is more efficient and may become less expensive and safer than standard intermittent methods.

b. Use of Solutions in Plastic Containers. Physiologic solutions packaged in plastic are currently receiving clinical evaluation in collaboration with the Anesthesiology Service, WRGH. The goal is to develop plastic containers which will hold concentrated solutions or dry solute for reconstitution at the place of use. This should help with logistic problems relative to dialysis and other solutions.

c. Indwelling Quinton-Silastic Teflon Arteriovenous Shunt. In two military battle casualties with post-traumatic renal failure, repeated hemodialysis was performed using the indwelling Quinton-Silastic-Teflon arteriovenous shunt. One patient received five dialyses over a 14-day period and the other, ten dialyses over a 40-day period. Both patients diuresed despite severe wounds, and

the uremic syndrome was avoided in both. This experience would corroborate that of the U. S. Army Surgical Research Unit's successful use of this shunt for repeated hemodialysis in acute renal failure.

2. Peritoneal and Hemodialysis: Modification of Dialysis Solutions. As previously demonstrated in this laboratory, the physicochemical properties of toxins may be used to enhance their extraction from the body during dialysis. Incorporation of THAM in peritoneal dialysate enhanced barbiturate extraction. Currently, other modifications of dialysis solutions are being investigated.

a. Modification of Solutions for Peritoneal Dialysis: An Approach to Treatment of Intoxication with Highly Lipid Soluble Substances. Many intoxicants of clinical and chemical warfare significance are highly lipid soluble. This characteristic limits their effective removal by renal excretion, hemodialysis, and peritoneal dialysis. A common clinical example is intoxication with glutethimide. Studies comparing the effects of substituting emulsified cottonseed oil (Lipomul) for the usual aqueous dialysis solution on removal rates of glutethimide have been completed in 15 dogs. In aqueous dialysate, glutethimide peritoneal fluid to blood concentration ratio was 0.38 at 45 minutes (equilibrium). In lipid dialysate, concentration ratio reached 1.86 at 60 minutes and 3.3 at 90 minutes, representing a fivefold increase in removal rate of glutethimide when peritoneal dialysis was done with lipid-containing dialysate. These studies demonstrate a potentially effective means of removing lipid soluble compounds from the body.

b. Use of Lipid Dialysate for Increased Extraction of Glutethimide in Hemodialysis. Following the successful use of intraperitoneal lipid emulsion to increase removal of glutethimide, the principal was applied to hemodialysis to test the concept that lipid soluble substances might pass through the cellulose membrane into a lipid bath at an accelerated rate. Seven dogs were dialyzed with the Heaton Minicoil Kidney using Ringer Lactate bath as a control solution and cottonseed or olive oil as test baths. Animals withstood dialysis for six hours on the Minicoil well. Final bath: blood concentration ratio of glutethimide with oil dialysate (1.37) averaged 4.3 times that with aqueous dialysate (.032). In three

additional six-hour dialyses with cottonseed oil, bath: blood ratio was 2.5. No harmful effects of dialysis against oil dialysate were apparent.

This information was applied clinically in two patients with severe glutethimide intoxication. Lipomul, a 15 per cent cottonseed oil emulsion, was added to the Twin-coil dialysis bath for a total of 450 gm. lipid in the first and 1800 gm. lipid in the second dialysis. As in the animal studies, addition of lipid resulted in increased removal of glutethimide over control dialysis periods using the standard aqueous bath, but the calculated total amount of glutethimide removed in eight hours' dialysis was disappointingly low.

c. Studies of Other Exogenous and Endogenous Intoxications. A clinical study of the effects of substituting bicarbonate for lactate in peritoneal dialysis solution on removal rate of uric acid was done and showed no difference at comparable pH. The addition of albumin, however, resulted in a significant increased removal rate of uric acid and urea and provided in vivo evidence for protein binding of these substances.

d. Use of intraperitoneal THAM combined with recirculation through an extracorporeal lipid bath for treatment of seconal intoxication is under way. If successful, the method will be applied in patients with intoxication due to lipid soluble agents.

3. Enterodialysis. Enterodialysis has been reported as a promising procedure. To determine the feasibility of chronic enterodialysis using a prosthesis in proximal jejunum for inflow and a second prosthesis in distal ileum for collection of the fluid, two chronic studies were performed. Technical difficulties have been great. The majority of the dogs experienced vomiting and explosive diarrhea. Two working models have been studied in some detail. Using hypotonic lavage solutions, i.e., Na, 60 mEq/L and Cl, 54 mEq/L, electrolyte balance is maintained. Urea is cleared but at slow rates, less than 5 cc/minute. Creatinine is not removed. Both dogs studied have shown progressive weight loss and debility. The procedure has been abandoned.

4. Chronic Peritoneal Dialysis. A 36-year-old male with chronic glomerulonephritis, urine volume 60 ml/day, creatinine clearance less than 0.5 ml/minute, has been maintained with weekly

peritoneal dialysis for 15 months. There have been three episodes of bacterial peritonitis, all cured. Gradual weight loss of 100 pounds has occurred and marked calcification of blood vessels has resulted in peripheral gangrene. Edema and hypoalbuminemia required weekly infusions of serum albumin. Severe neuropathy of both legs and arms has resulted in almost complete incapacitation of the patient. Metastatic calcification of soft tissues is progressing despite attempts to prevent or reverse it. Arthralgia and acute arthritis have become a problem. Experience with this patient has demonstrated that although chronic peritoneal dialysis may be technically successful, a variety of metabolic abnormalities produced require further effort for solution.

5. Complications of Peritoneal Dialysis: Abdomino-thorico Communications. In two patients, on the institution of peritoneal dialysis, there was the abrupt appearance of a massive pleural effusion. This fluid contained glucose in concentrations above 1000 mgm/100 ml. while the simultaneous blood sugars were less than 200 mgm/100 ml. The pleural fluid likewise contained very low concentrations of protein and therefore resembled the dialysate in the peritoneal fluid. Such connections have been described in chronic ascitis, but because physicians doing peritoneal dialysis should be aware of this possible complication, a report of this is being prepared.

6. A Low Protein High Amino Acid Diet for Use in Renal Insufficiency. Very low protein diets as usually used in renal failure do not contain adequate amounts of essential amino acids and therefore can not maintain nitrogen balance. Furthermore, they are invariably unpalatable. Based on studies by Giovanetti in Italy showing that when minimal daily requirements of the essential amino acids are added to a basal 8-10 gram protein diet, uremic patients can reutilize endogenous urea for synthesis of nonessential amino acids and attain positive nitrogen balance, a similar diet has been developed in the Department of Metabolism. Nitrogen balances have been done on two patients with urea clearances below 2.0 ml. per minute. Both patients showed significant clinical and chemical improvement on the diet and one patient with virtually no renal function has been maintained on this diet and weekly peritoneal dialysis for seven months. Balance data confirmed Giovanetti's claim of inducing positive balance. The diet has been completely developed and a diet manual has been written and reproduced locally.

7. Uric Acid Metabolism. Changes in uric acid synthesis and excretion in response to stress are important. Three studies addressed to this problem are significant.

a. The Effects of Acute Total Caloric Starvation on Uric Acid Metabolism in Obese Human Subjects. Plasma concentration and urinary excretion of uric acid were measured daily in three moderately obese male subjects during a control period of low purine intake and during a short-term starvation. The miscible pool sizes and daily turnover rates of uric acid were determined with the use of uric acid-2-C^{14}. During starvation, urinary excretion decreased, plasma concentration rose, and miscible pool size increased. The uric acid retained was much less than the calculated increase in pool size, suggesting acceleration in de-novo synthesis of uric acid during starvation.

b. Studies of Uric Acid Kinetics in Advanced Renal Failure. Uric acid pool size and turnover rates in severe renal insufficiency have not been previously published. Working from Sorenson's 2-C^{14} uric acid method, a rapid and relatively simple method for studying these kinetics in severe renal insufficiency was developed. Useful data were obtained from the pilot study suggesting the technique offers a method not previously utilized for studying effects of various diets, drugs such as xanthine oxidase inhibitors, and dialysis on urate kinetics in renal failure.

c. Renal Failure (With Hyperuricacidemia) Following Exercise. Two (possibly a third mild case) cases were seen with the abrupt onset of renal insufficiency following strenuous exercise. Studies for myoglobin were negative (Initial samples on one patient are now being obtained). Striking in both cases was a striking increase in serum uric acid. Urine was free of urate crystals. A subsequent study of one of the patients did not reproduce the disease, but there was a rise in serum uric acid in excess of that expected on the reduction in renal excretion. A report of these cases is under way.

8. Protein Synthesis in Rats and Patients with Azotemia. Methodology has been developed and studies initiated to study rate and mechanisms of protein synthesis in patients with renal disease. Rate of incorporation of tagged Leucine by rat liver homogenates

into a TCA insoluble fraction and onto SRNA is being measured in rats made acutely uremic by penile ligation and in paired normal controls. Preliminary results indicate that incorporation is significantly greater in the uremic animals. Similar studies comparing uremic animals and normals subjected to starvation from one to three days indicate that incorporation decreases with time in the controls but not in the uremic group. These observations are being extended to determine the effect of cellular amino acid composition on incorporation and to determine the nature of the peptide into which leucine is being incorporated.

Parallel studies in humans have been initiated to determine rate of incorporation of S-35 labelled methionine into serum protein in patients with azotemia and in normal control subjects. Experiments in two control subjects (one of these patients was studied twice) indicate that incorporation rate measurements are repeatable. One uremic subject incorporated more S-35 methionine into serum protein than did any of the control subjects. These studies will be extended to include more subjects.

III. Pathogenesis of Acute Renal Failure: These studies include mainly prospective animal work utilizing primarily irradiation and exsanguination models and retrospective observations in patients.

1. Radiation Nephritis in Rats. It has been shown that a defect in concentrating ability and azotemia appear simultaneously in rats given 2500 r to both kidneys. Histologic study of an initial group of rats indicates that the damage is primarily to tubular cells and interstitial tissue with less damage to glomeruli and vessels. This differs from the generally accepted concept of pathology. In order to clarify this further, another group of rats, similarly radiated, was sacrificed at shorter intervals after radiation, just before and after the appearance of the standardly occurring physiologic abnormality. A technique has been devised by which a reproducible degree of renal damage may be created. It is possible that this technique may be used to (1) cause "uremia" in rats for other metabolic studies, or (2) to further study radiation nephritis; i.e., effect of other doses, effect of constant water load on the medullary pathology, etc.

2. Radiation Nephritis in Dogs. Great problems have beset this study. One group of dogs received 500 and 1000 r by

conventional X-ray and another received what was thought to be 250 r, 500 r and 750 r by the reactor (combination of gamma rays and neutrons). Unfortunately, after the study was well under way and many of the time-consuming physiologic measurements were made, it was found that there was gross error in the dosage from the reactor: the port delivering the dose had a very uneven dose field, and hence one kidney received much more radiation than the other with no way to quantitate the dosage received. A preliminary evaluation of the data from the radiation dogs indicates that there was a perceptible decrease in $T^{C}H_2O$ in dogs receiving 1000 r and in some a decrease in their GFR at a later time. By four or five months the $T^{C}H_2O$ appeared to return toward normal. When a defect in concentrating ability appeared, it was accompanied by the expected decrease in medullary solutes.

3. Metabolic Aspects of Response to an Acute Heat Stress. A study of the effect of potassium depletion revealed that in three of four subjects there was no change in their response to heat exposure. In a fourth, a more obese subject, there was a more rapid accumulation of body heat during potassium depletion, although there was no change in sweating performance or in the cardiovascular system. Additional findings included the observation that during mild to moderate hyperthermia and the concomitant respiratory alkalosis, there was no movement of potassium into cells as has been shown under normal conditions, but that an increase in serum potassium invariably was seen. During the potassium deprivation period, there was no rise in serum potassium during the heat exposure. From these preliminary observations, it may be concluded that there is not a universal decrease in tolerance to heat with decreased body potassium, although in isolated instances there may be. It is also possible that with more intense degrees of potassium depletion there may be a more profound effect not seen in the degree of potassium deficiency seen here. A preliminary report is in preparation.

4. Potassium Balance: A Comparison of Balance Technique and the Whole Body Counter as a Measure of Skin Potassium Loss. Data has been collected on four individuals during potassium deprivation with conventional balance technique and frequent counting with the whole body counter. It is hoped that these studies will clarify the accuracy of the Humco and, if accurate enough, may indicate a way of measuring total balance.

5. Decreased Body Potassium in Heat Stroke. A case of fatal heat stroke with acute renal failure was studied by the K_{42} exchangeable potassium method. A great deficit in body potassium was found - far in excess of that expected from measured losses. Because of the importance of these observations and a theoretic and possible role of potassium depletion in genesis of failure to sweat, this case is being reported and a case report has been prepared.

6. Gastrointestinal and Renal Potassium Loss in Sprue. A case of steatorrhea presenting as hypokalemia was studied. It was found that during a period of active steatorrhea, the patient not only lost inordinate potassium in his stool but that his renal potassium conservation mechanism was faulty. With regression of his steatorrhea on a glutin-free diet, he normally conserved potassium. The nature of the renal defect in potassium conservation is obscure; perhaps protein depletion plays a role. A case report is under way.

7. Renal Failure Following Aortography with Recovery. A case study is being prepared by Dr. Stark in which acute renal failure followed abruptly after aortography with Hypaque. The rarity of this and the course of recovery make this a worthwhile case to be reported.

8. Renal Failure Due to Renal Artery Occlusion. A case report is being prepared of a case with spontaneous atheromatous occlusion of the renal artery with anuria. Surgery was performed and the patient's renal failure recovered. Because of the possibilities of therapy, and the diagnostic and therapeutic features this case shares with a handful of similar cases, a case report is in preparation.

9. Sarcoid Nephritis. A case with generalized sarcoidosis with extensive involvement of the kidneys and renal insufficiency has been extensively studied. The effect of steroids has been evaluated. Although there was regression of the sarcoid lesions on steroids, this has been followed at a later time by the development of contraction of the kidney and again a dimuniton of renal function. A case report is being prepared.

IV. Studies of Peritoneal Absorption: Widespread use of peritoneal dialysis has demonstrated the relative safety of exposing the peritoneal

surface to fluid. The absorptive potential of the surface has not been adequately studied and exploited. The following explorations are intended to lead the way to clinical use of the absorptive potential of the peritoneal membrane.

1. Absorption of Antibiotics From the Peritoneal Cavity. Studies on the absorption of antibiotics from the peritoneal cavity and the rate of movement of antibiotics from the blood stream into the peritoneal cavity in humans have been completed and accepted for publication. When 1 gm. methicillin is given IP in 1000 ml. normal saline to patients with impaired renal function, blood levels in the therapeutic range are attained in one hour and persist at least six hours. Peritoneal fluid levels remain very high for six hours. When 1 gm. methicillin is given intramuscularly, IP levels are within the therapeutic range within one hour but are much lower than those attained with IP drug. Kinetics of tetracycline diffusion from blood to PF are similar to those for methicillin.

2. Absorption of Fluids and Electrolytes. Results of dog studies on the effect of dehydration have been completed and accepted for publication. Results demonstrate that absorption is increased by 40 hours of dehydration and that rate of absorption during the first two hours following IP saline infusion is directly related to transperitoneal osmotic pressure gradient. After two hours, blood and PF solute concentrations are equilibrated and absorption occurs more slowly with no relation to osmotic pressure. Studies concerning the effect of vasopressin are completed and a manuscript is in preparation. Results indicate that two units vasopressin given IP increase fluid and solute absorption, but only after two hours when the osmotic pressure gradient is zero. Physiologic doses of vasopressin given intravenously have no effect, suggesting that endogenous ADH does not regulate transperitoneal fluid and solute movement in vivo. Pharmacologic doses given intravenously also have no effect, suggesting that the IP vasopressin acts directly on the peritoneal membrane instead of through a systemic vascular effect.

3. Effect of Drugs and Physical Agents on Na^{22} Transport Across Isolated Peritoneal Membrane. These studies, utilizing rabbit peritoneal membrane in a leucite chamber have been completed and submitted for publication. Rate of Na transport was increased by increasing temperature, decreasing calcium concentration and adding vasopressin to the solution bathing the membrane. Dinitrophenol had

no effect on sodium transport. Results indicate that sodium transport across peritoneum can be modified. Indirect evidence suggest that peritoneal sodium transport is not an active, energy requiring process.

V. Endocrine Function Evaluation: The large number of patients with endocrine dysfunction offers a unique opportunity for study. In addition to clinical compilation, studies in depth involve primarily adrenal and gonadal steroids.

1. Adrenal Cortical Suppression and Stimulation. A standardized protocol utilizing ACTH stimulation, dexamethasone suppression, and SU-4885 is being used to study various groups of patients with pituitary and adrenal dysfunction. Included in this study are patients with acromegaly, Cushing's syndrome, adrenal insufficiency, adrenal androgen excess, and Klinefelter's syndrome.

2. Gas Chromatography Studies of Steroids. Gas-liquid chromatography is continuing to be utilized in the separation and quantitation of the urinary 17-ketosteroids-androsterone, etiocholanolone, and dehydroepiandrosterone. These steroids are being quantitated in patients with acromegaly, androgen excess, and various types of hypopituitarism in the basal state and after stimulation with chorionic gonadotropin. In addition, studies on the use of gas-liquid chromatography for the determination of urinary testosterone and cholesterol are being conducted.

3. Adrenal Androgen Biosynthesis. Studies conducted utilizing the *in vitro* incubation of dog, bovine, and monkey adrenal slices revealed that the *in vitro* system used was inadequate for studying adrenal androgen synthesis. At the present time, an *in vivo* system is being used utilizing the orchiectomized rhesus monkey. The effect of chronic intravenous infusion of various test substance on urinary excretion of androsterone, etiocholanolone, and dehydroepiandrosterone is being studied and preliminary results indicate this system may be excellent.

4. Renal Tubular Handling of Steroids. Preliminary studies have been conducted utilizing stop flow techniques on how the renal tubule reabsorbs steroids. It is planned to study the renal tubular handling of aldosterone, hydrocortisone, and testosterone.

Summary and Conclusions

The department has continued its basic direction in the study of metabolic response to disease and injury. Items of possible interest not deserving inclusion in the body of the report are included under Publications.

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PROJECT 3A025601A823
MILITARY PSYCHIATRY

Task 01
Military Psychiatry

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6470	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT	6. SECURITY WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
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10a. CURRENT NUMBER/CODE: 62156011 3A025601A823 01 030

10b PRIOR NUMBER CODE: None

11. TITLE:
(U) Social and preventive psychiatry (09:21:31:TH:VS)

12. SCIENTIFIC OR TECH. AREA	13 START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
007500 Human factors engineering; 013400 Psychology (indiv & group behav)	09 54	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT b. NUMBER	c. TYPE	d. DATE	d AMOUNT	18 RESOURCES EST.	a PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE		NA			PRIOR FY 65	11	360
					CURRENT FY 66	11	395

19. GOV'T LAB/INSTALLATION/ACTIVITY
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TEL 202-576-5261 TYPE DA

21. TECHNOLOGY UTILIZATION: Social and Community Psychiatry

22. COORDINATION: NA

23. KEYWORDS: Adaption; alcoholism; communication; psychiatry; group process; environment; social welfare; research anthropology

24. (U) Tech Objective - To investigate social and cultural factors influencing the development of adaptive and maladaptive behavior in military organizations, using results to maximize adaptive behavior while reducting maladaptive behavior, e.g. reduction of man days lost due to psychophysiological complaints, alcoholism, AWOL, basic training stresses, and improvement of the US soldier's ability to communicate with members of other cultures.

25. (U) Approach - To study the influence of individual, social and cultural factors in clinical and field circumstances in the US Army, SE Asia and certain portions of American civilian society on human behavior. The techniques employed are individual, psychiatric interviews, group process analysis, sociometric devices, questionnaires, social anthropologic investigation, semantic analysis, and analysis of the formal and informal structure of organizations. Since these studies are carried out using humans operating in varying environments, the major technical problems arise from unexpected environmental changes (e.g. intensification of the struggle in Vietnam).

26. (U) Progress (Jul 64 - Jun 65) - Data concerning alcoholism and AWOL soldiers has been partially reduced and reported. Some operating techniques have been used to clarify factors that effect the operation of research organizations. Information concerning factors which may influence the behavior of American advisors in Vietnam has been established. Research teams to study organizational, cultural and social matters in poverty areas, Thailand, and a military post have been organized. Studies of symbolic behavior continue. Data concerning the relationship between adaptation of basic trainees and the occurrence of adenovirus infection has been collected and is being analyzed. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		AR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
AR 705-5	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A025601A823, MILITARY PSYCHIATRY

Task 01, Military Psychiatry

Work Unit 030, Social and preventive psychiatry.

Investigators.
Principal: Lt Col Harold S. Kolmer, Jr., MC
Associate: Capt Peter G. Bourne, MC; Capt Horacio Fabrega, MC; Capt Herbert S. Gross, MC; Maj Harry C. Holloway, MC; Raymond L. Johnson, Ph.D; Capt Richard G. Morrill, MC; Julius Paul, Ph.D; William M. Smith, Ph.D; Edwin A. Weinstein, M.D.; Maj Bernard J. Wiest, MSC.

Description: 1. A series of studies into advisor-advisee relationships in the counterinsurgency setting and into communication across language and ethnic boundaries have been initiated during this year in Vietnam, Thailand, and Washington, D.C. The work in Vietnam initially involved the development of a series of group process conferences, under the direction of WRAIR team personnel, with military advisors in each of the four Corps areas and with two groups of Special Forces personnel. In addition, an experimental psychologist has been carrying out work in Vietnam involving the training of Vietnamese students in experimental techniques in order to introduce into this group concepts of the study of behavior in a systematic and controlled scientific fashion.

2. In Thailand a WRAIR anthropologist has been assigned to develop a program of studies into medical care practices among several ethnic minority groups, in partial collaboration with personnel of two anthropology projects supported by the Army Surgeon General's Office, also aiming at similar goals. Exploratory visits in Thailand were made by two psychiatrists of this division with the aim of initiating one or more additional psychiatric projects in Thailand during the next fiscal year, which will study advisor-counterpart relations in that country and will compare psychiatric data in the Royal Thai Army and in other segments of the Thai population with comparable data upon U.S. and other populations. In addition, a neurologist from this Division has also been assigned to Thailand to conduct epidemiological studies on several classes of neurological disorders.

3. A study of the Poverty Program in the Cardoza area of Washington, D.C. has focused on the use of "indigenous non-professionals." These are residents from the slum area who are paid and trained to act as "neighborhood workers," and who serve as links between the socially disadvantaged and the social agencies. This neighborhood worker group represents individuals from the subculture of poverty who are in the process of role, status, and value change and who show the effects of the cross-cultural communication barrier between themselves and the professional social workers supervising them. The model used and the data obtained in this cross-cultural setting in Washington may be compared to cross-cultural situations between Americans and host nationals in developing countries and between the city elite and the peasants within the host country.

4. A social psychiatric study of military organizations and small deviant groups is in progress. Presently under investigation is the determination of crucial organizational variables that may determine the social setting and behavior of individuals with such problems as alcoholism, indebtedness, psychophysiological-musculoskeletal reactions, and "incorrigible" behavior. Concurrently, explorations are being conducted to learn if meaningful information is to be gained about referential behavior of small deviant groups. The objective is to gain an understanding of the efficiency and efficacy of present-day health and welfare problem-solving, as well as the institutional myths and value differentiation of formal organizations and small groups that lead to varying solutions by command and the problem carriers.

5. A related study focuses on behavior of small human groups existing in various areas of the world. This has consisted of studying personal documents, diaries, log-books, and of making on-the-spot observations of groups in Antarctica and in other extreme environments.

6. Human clinical experimental studies of chronic alcoholic soldiers, 15 August 1961 to 30 June 1963, explored the feasibility of applying certain combat psychiatric principles, with various environmental and group-oriented therapeutic techniques, to a sample of one of the most disruptive forms of human behavior, in order severely to test the methods and derive reasonable inferences concerning certain aspects of "normal" behavior usually kept secret.

7. A study of the relation of social attitudes, the law and human behavior has used as a model the history of State eugenic sterilization laws in America. This has been explored in relation to the issues of due process of law; to the interpretation and shaping of law by medical administrators; and to the transformation of scientific or quasi-scientific ideas into law and other related factors. Preliminary work is underway on an historical-administrative study of Army directives and policies in the area of homosexuality beginning with the Uniform Code of Military Justice of 1950, intending to analyze intra-service differences in interpretation and enforcement of these policies, as well as the impact of Congressional pressures and outside criticisms on the military management of this problem.

8. The research program studying verbal behavior and its relation to neurologic, psychologic and social factors has continued. Emphasis has been placed on work with patients and the study of threats against the President. The classes of patients studied have been those with neurological damage, neurosis and those hospitalized prior to surgery. A program has been initiated to develop and apply computer methods for the content analysis of language and the corrollary development of measures of effectiveness of communication.

9. Another study attempts to delineate the linguistic and other behavioristic characteristics of hospitalized psychiatric patients with

altered and deviant sexual characteristics. Building on information gained from brain-damaged patients, the study attempts to clarify the pre-morbid symbolic indices of importance, especially as they relate around the patient's family. It is hypothesized that those persons whose behavior under stressful circumstances shows socio-behavioral and metaphorical characteristics which would be classified as sexual in content will demonstrate preoccupation with gender destructions in achieving identity by structuring reality.

Progress: 1. The WRAIR group conferences were held in Vietnam from July through November 1964. The team consisted of three psychiatrists and two anthropologists. The team held three day group conferences with groups of 12 advisors in each Corps area. The advisors were from Divisions, Corps and Provinces, plus advisors from other organizations. Monthly follow-up meetings were held in several locales. Additional meetings were held with two groups of Special Forces personnel.

Members of the MAC-V staff cooperated excellently and expressed the view that the study was critically important to the U.S. advisory effort. Several group sessions were recorded and transcribed at WRAIR for future reference and study.

With the support of the University of Saigon authorities and the collaboration of a Vietnamese psychologist, the University and the WRAIR medical research team inaugurated training of University students. Sixty students attended the didactic courses and a smaller group conducted animal studies, e.g. the Slow Loris and the Gibbon, in the laboratory. The program was discontinued when political upheaval led to the closing of the University of Saigon and administrative requirements restricted the laboratory's availability for student research. If the expected absence of the Vietnamese psychologist materializes, this will force the discontinuance of this part of the program at the end of this fiscal year.

2. The studies in Thailand are still in developmental or exploratory phases. Therefore, no substantial progress can be reported at this time, except that all WRAIR personnel now in Thailand have received effective support of the appropriate Thai government officials. A tentative offer of the assignment of a Thai psychiatrist counterpart has been made to the U.S. Army-SEATO Laboratory, Bangkok, in anticipation of the assignment of U.S. Army psychiatrist in Thailand.

3. The Cardoza study has taken place from February 1965 to June 1965 in Washington where the selection, training, utilization and performance of the neighborhood workers and supervisors have been observed. The methods employed have included observation of training and discussion groups, interviews with staff, individual neighborhood workers, and neighborhood residents, and experience with informal social groups. The purposes of this study have been to delineate the status, and changes in this interface group and to identify some of the core conflicts of such a group as they participate in social change.

4. The organization part of the study of military units began in February 1965 when access was gained to a bi-weekly on-post Outpatient Clinic Conference and to periodic small group meetings with the chaplains at Ft. Meade. Formal organizational study began with the assignment of two technicians to portray schematically the structure and personnel composition of all the units at Ft. Meade. A staff member has been present daily in the Outpatient Clinic to acquire socio-culture data of the Clinic. An administrative system exists for obtaining knowledge of specific problem individuals for future use in determining behaviors within specific small deviant groups. Concurrently, entry was made and feasibility studies continue in command-unit systems. An armored cavalry unit has permitted access to key figures and critical information in the organization. Work has proceeded in the acquisition of behavioral patterns of the NCO system relative to decisions and attitudes used by the NCO group to shape the social context and movement of problem carriers.

5. The Stefansson Polar Collection at the Dartmouth College Library was visited and photocopies of certain relevant manuscripts were obtained for study, primarily for the purpose of exploring relative areas of significance for future studies. Data concerning the development of informal group structure and certain measures of this development, in a small group in Antarctica, are being placed in manuscript form for publication.

6. A modified psychiatric treatment ward became operational on 15 August 1961 and remained so until 15 June 1963 to study the biosocial development and environmental circumstances of 34 chronic alcoholic soldiers. Individual and group psychotherapy was conducted and analyzed in connection with those aspects of group process which aid in formation of therapeutically beneficial groups. Field social anthropological studies were conducted within the military unit of the alcoholic soldier and, on a few occasions, with members of the parental family. Various attempts were made to adjust the administration of these studies so as to produce findings of increased informational value, after which the studies were readjusted and re-evaluated. Following termination of the data collecting phase of the study, techniques for obtaining a ten year follow-up on the results of treatment in the experimental ward have been developed and refined. The mass of data which evolved from the study has been analyzed and reduced and is currently being prepared for publication.

7. Compilation of a manuscript on the history and present status of state eugenic sterilization laws was aided by area library facilities, correspondence with each of the States presently or in the past participating in eugenic sterilization groups, field study in six selected states, and examination of all present and past sterilization laws and State and Federal court decisions relating to these laws, and a survey of pertinent medical, legal, and scientific literature pertaining to the subject of human sterilization. Academic library review has begun and feasibility and exploratory activity continues.

8. The study of threats against the President is in the writing-up process. Analogies have been made between such threats and the clinical phenomena of delusions. In both, similar personal problems gain public expression. Background biographical data on the writers of letters to the President has been compiled on a group large enough to allow for statistical generalizations. These data have been used to differentiate serious threats from those that are less serious.

The investigation of the relationship of aphasia due to disturbance of verbal behavior continues with emphasis on the effects of head trauma on the style of describing the environments. Patterns of verbal behavior classifiable as confabulation, delusion, amnesia and disorientation and the correlation with subsequent social behavior are being studied.

The studies of verbal behavior of psychotic patients are substance of two papers, entitled "Auditory Hallucination and Intellectual Deficit," and "Auditory Hallucination and Descriptive Language Skills." Two other studies are in the phase of data analysis. One is an investigation of the role of metaphor in communicative effectiveness and the other studies the difference between written and oral language of psychotic patients.

The development of computer methods for content analysis of verbal behavior has resulted in a package of computer programs and two preliminary papers. The former entitled "Classification Using Multidimensional Bi-Polar Scales." The other two papers are entitled "Content Analysis and Semantic Classifications," and "Toward the Content Analysis of a Psychiatric Interview."

An anti-disturbance project in collaboration with the Dept of Neuoendrocrinology, studies the adaptation to a painful surgical condition, prolapsed intervertebral disc in patients hospitalized on the Neurosurgical Wards, WRGH. Attention was directed to social role, assessment of psychological defenses, verbal behavior, and the pattern of hormone secretion.

9. During the Fall and Winter of 1965 careful scrutiny of recently admitted psychiatric patients enabled the selection of patients for study. These were subject to structured interviews involving items relating to their current problems and past life, particularly involving their parents. In addition, careful summary of hospital charts of these patients enabled the collection of information which served as background clinical data. The interviews obtained were taped and coded according to categories indicative of certain values and emphasis regarding parental relations. Metaphors chosen to structure current problems as well as characteristics of their sexual adjustment served as criteria for classifying the patients. Further analysis of data is currently being undertaken.

Summary and Conclusions: 1. The Advisor-advisee study in Vietnam is viewed as a successful pilot study and has demonstrated the feasibility of the group process technique in the field in studying the complex functions of the military advisor in the counterinsurgency situation. The changing situation in Vietnam has caused the termination of this phase of the study upon which two classified interim reports have been written and submitted to the CG, MAC-V. A proposal has been made for the training of military line personnel in group process techniques, but no action has been taken to date on this proposal. The experimental psychologist will be returned from Vietnam in early FY 1966 and will not be replaced, due chiefly to the unstable political-military situation in Saigon and the absence of a Vietnamese collaborator at the University of Saigon.

2. The Thai studies have been initiated or planned, and promise to represent a logical extension and development of the work in Vietnam and in Washington, D.C.

3. The data from the four month Cardoza study on the neighborhood worker in a cross-cultural interface has been collected and presented in a preliminary report. The possibility of identifying a similar interface group in the cross-cultural project in Thailand for purposes of comparison will be investigated.

4. The exploratory phase of the study of the organization of small deviant groups continues at Ft. Meade. Information input on referential behavior and problem-solving processes has begun. Health problem incidence is being successfully acquired and entry has been made into the command-unit and NCO social systems. An immediate next step is a survey of health and welfare problem incidence.

5. Studies of group processes are being made along three lines; personal documents from members of small groups in isolation, preparation for publication of data from studies already completed of small groups in Antarctica, and preparation for a field study of group processes in emerging elite groups in New Guinea.

6. A Milieu Therapy Ward, in addition to its value as a treatment method, serves as an excellent laboratory for the study of deviant behavior. Some problems of chronic alcoholism in the military have been studied by these established methods.

7. State sterilization laws were a useful vehicle for studying the internal dynamics of administrative law and procedure, especially their impact on individual rights, and the significant role that administrators play in shaping the law in various directions. The relation of scientific ideas to public policy was found to be a critical aspect of the entire relation between medicine, law, and society.

8. Several related studies have explored the symbolic use of language in several classes of individuals, including brain injured patients, psychotics, those with painful surgical conditions, and a group of people who have made threats against the President. These studies will continue in an attempt to establish relevant models of man's use of symbolic language under certain conditions of stress.

9. The study attempts to study the linguistic and behavioral characteristics of hospitalized psychiatric patients who showed altered and abnormal sexual characteristics. Use of language was the focus of study, and tape interviews subsequently typed were the means of collecting data. Categories reflecting sexual adjustment and important metaphorical trends served to code and organize the data. Preliminary nature of the evaluation procedure does not allow drawing conclusions at this time.

Publications.

Artiss, K.L., "Environmental-Transaction (Milieu Therapy) in Schizophrenia," Current Psychiatric Therapies, Jules H. Masserman, Ed., Grune & Stratton, Inc., New York, N.Y., April 1964.

Artiss, K.L., Kolmer, H.S., Kurke, L., "Modes of Communication Used by Military Patients Diagnosed as Schizophrenic (Acute), as Alcoholic (Chronic) and Military Offenders (AWOL)," published in Disorders of Communication, a publication of the Assoc. for Research in Nervous and Mental Disease, Vol. 42, Proceedings of the Association, 1964, pp. 367-372.

PROJECT RD 41-51 (DASA)
NUCLEAR WEAPONS EFFECTS RESEARCH

Task IIIA.1
Biomedical Aspects of Ionizing Radiation

RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6473	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT	6 SECURITY WRK	7. REGRADING	8. RELEASE LIMITATION	9 LEVEL OF RESUME
01 07 65	A. NEW	U	U	NA	QR	A. WORK UNIT

10a CURRENT NUMBER/CODE	10b PRIOR NUMBER CODE
NWER A4d DASA 03.0081	NONE

11. TITLE

(U) Durability of behavior following lethal radiation exposure (09)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
013400 Psychology; 017100 Weapons effects; 014100 Radiobiology	07 62	NA	MIPR DD

16 PROCURE. METHOD	17 CONTRACT GRANT a. NUMBER b. TYPE c. DATE d. AMOUNT	18 RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	2	75
		CURRENT FY 66	2	75

19 GOV'T LAB INSTALLATION ACTIVITY	20 PERFORMING ORGANIZATION
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21 TECHNOLOGY UTILIZATION	22 COORDINATION
X-ray effects	NA

23. KEYWORDS

Radiation; incapacitation; behavior; motor performance

24. (U) Tech Objective - Ultimate aim of this research is the specification of those behavior types which deteriorate rapidly and those which could be maintained at a functionally useful level. This represents a critical step in the analysis of radiation effects and the lack of such information is impeding to accurate military planning.

25. (U) Approach - Before considering the more "radio resilient" behaviors, it was necessary for this laboratory to determine the precise time course of general behavioral incapacitation through utilization of a highly motivated, shock avoidance task. Proceeding from this basic data, current work focuses on development of a more complex behavioral task calling for a series of closely-spaced, discrete, decisions on the part of a rhesus monkey following irradiation.

26. (U) Progre s (Jul 64 - Jun 65) - The last in a series of three exhaustive experiments establishing the precise time course to general behavioral incapacitation is presently drawing to a close. In the first two experiments, it was found that identically trained monkeys performed normally for a longer period of time if the same radiation dose was administered slowly (200 r/min) as opposed to a pulse. The last experiment is investigating to contributions of different radiation spectra to the temporal decoy of behavior.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965, and DASA-78, Nuclear Weapons Effects Research Semi-Annual Progress Summaries, 1 July 1965.

27 COMMUNICATIONS SECURITY a. COMSEC OR COMSEC RELATED X b. NOT RELATED	28	29 OSD CODE	30 BUDGET CODE
		AR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
CDOG Annex I para 1	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) CFY+1	36

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1221) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project RD 41-51, NUCLEAR WEAPONS EFFECTS RESEARCH

Task IIIA4 d, Biomedical Aspects of Ionizing Radiation

Work Unit 03.0081, Durability of behavior following lethal radiation exposure

Investigators.
Principal: Capt Joseph C. Sharp, MSC
Associate: 1st Lt Dennis D. Kelly, MSC; Barbara K. Keller, M.A.

Description.

Specification of those behavior types which deteriorate rapidly and those which could be maintained at a functionally useful level represents a critical step in the analysis of radiation effects; hence, the lack of this information is impeding to accurate military planning. Before focusing on the more "radioresilient" behaviors, it is the program of this laboratory to determine the precise time course of general behavioral incapacitation. At present, the last of a series of integrated experiments aimed at isolating the effects of radiation spectrum and dose rate in connection with this temporal decay of behavior is drawing to completion. Concurrently, a more complex behavioral technique is under development which calls for a series of closely-spaced, discrete, decisions on the part of a monkey following irradiation. Systematic analysis of this expanded situation should afford isolation of differential radiation effects upon several key behavioral systems.

Progress.

During the course of this investigation, facilities for the simultaneous housing and training of ten animals have been constructed at the Behavioral Radiology Laboratory, Forest Glen Section, Walter Reed Army Medical Center. Close working relations have been established with exposure facilities at the National Institutes of Health and the Armed Forces Radiobiology Research Institute, both in nearby Maryland. Equipment includes automatic timers, counters, programming devices, recorders, and other electronic apparatus for the presentation of environmental events, reinforcement contingencies, and the recording of behavioral data.

To date, the emphasis has been to establish the precise time course to general behavioral incapacitation following massive doses of ionizing radiations. In the first report of this laboratory (WRAIR Technical Report #3, 1964), animals exposed to 10, 20, and 40 Kr (gamma) at 2000 r/minute were monitored for behavioral degradation during and following exposure. It was found that 10K animals performed normally, on the average, for 58 hours, the 20K for 3 hours, and the 40K for 0.10 hours. In a more recent study this year, utilizing the

facilities at AFRRI, animals trained in an identical avoidance task were exposed to a pulsed, mixed source (60% gamma and 40% neutron). It was found that the 10K group performed normally for an average of 5.17 hours, the 20K for 2.49 hours, and the 40K group for 0.55 hours.

The differential effects obtained raised questions about the mechanism of radiation effects and suggested another experiment, currently drawing to completion, which should answer these questions. This is an exhaustive attempt to isolate the variables of radiation spectrum and dose rate by using a mixed source of radiation at 2000 r/min. Early data from this study indicate that radiation spectrum is a more critical determinant of survival time than the rate at which it is administered.

Summary and Conclusions.

Data derived as a result of accidental exposures of personnel and from animal experimentation is considered insufficient for forming military opinion of the degree of incapacitation to be expected at various times following exposures to lethal amounts of radiation. The major contribution of this program has been to establish the precise temporal course of general behavior decay with special reference to the radiation parameters (total dose, spectral distribution, and delivery rate) most critical in producing this debilitation.

Publications.

Sharp, J. C. and Murphy, G. P. Conditioned avoidance behavior in primates during various experimental uremic states. Nephron, 1, 172-179, 1964.

Sharp, J. C. The effects of prenatal X-irradiation on acquisition, retention, and extinction of a conditioned emotional response. Radiation Research, 24, 154-157, 1965.

RESEARCH AND TECHNOLOGY RESUME	1.	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
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11. TITLE

(U) Biological responses to total and partial body irradiation (09)

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C. IN-HOUSE	NA	PRIOR FY 65	3	75
		CURRENT FY 66	3	85

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21. TECHNOLOGY UTILIZATION	22. COORDINATION
Radiation Biology; Civil Defense; Nuclear Medicine	NA

23. KEYWORDS Radiation, biochemistry; radiation injury; radiation genetics; physiology; radiation; pathogenesis

24. (U) Tech Objective - The technical objective of this work unit is the quantitation of radiation injury, adaptation and recovery.

25. (U) Approach - The objective will be pursued through the interplay of biochemistry, physiology and pathology. Multiple parameters will be used to study the biological effects of radiation exposure.

26. (U) Progress (Jul 64 - Jun 65) - Biochemical studies included the measurement of serum levels of carbohydrate, lipid, and protein constituents as well as enzymes and principle cations and anions in a large irradiated dog population. Physiological studies were utilized to evaluate erythropoietic reserve in lethally irradiated rats treated with single donor and pooled donor marrow implants. Pathological examinations included vital organ responses to animals irradiated through grids.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965, and DASA-78, Nuclear Weapons Effects Research Semi-Annual Progress Summaries, 1 July 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
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31. MISSION OBJECTIVE	32. PARTICIPATION
CDOG Annex I para 1	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project RD 41-51, NUCLEAR WEAPONS EFFECTS RESEARCH

Task IIIA4 e, Biomedical Aspects of Ionizing Radiation

Work Unit 03.0741, Biological Responses to Total and Partial Body Irradiation

Investigators.

Principal: Lt. Col. Kent T. Woodward, MC

Associate: Major Charles R. Angel, MSC; Ann R. Berman, B.S.; Major Charles N. Conant, MC; John Davis, B.S.; Minnie H. Davis, B.S.; Captain Fletcher Hahn, VC; Lt. Col. Dan Hightower, VC; Major Merrill Johnson, MC; Adolph T. Krebs, Ph.D.; Mary M. McLaughlin, M.S.; Major LaWayne Stromberg,MC; George Huser, M.D., Dept of Hematology; Sol M. Michaelson,DVM, University of Rochester; Joe W. Howland, M.D., Ph.D., University of Rochester.

Description.

The overall objective of this work unit is the measurable definition of the extent of radiation injury to the intact organism and the consequent adaptation of the organism after maximum recovery has taken place. The experimental studies under this work unit are subdivided into biochemical, physiological and pathological effects.

1. Biochemical Effects.

The purpose of this subdivision is to measure and define biochemical changes in the mammal after exposure to ionizing radiation. A second purpose is to integrate biochemical changes with physiological function and pathological consequence.

2. Physiological Effects.

The purpose of this area is to functionally study the total organism as a consequence of exposure to ionizing radiation. A second purpose is the definition of stress tolerance.

3. Pathological Effects.

The intent of this subdivision is to integrate biochemical and physiological observations with the pathological consequence of exposure to ionizing radiation. Recovery and adaption of the radiated mammal are important contributions to the understanding of pathologic change.

Progress.

1. Biochemical Effects.

A series of 19 common biochemical parameters were measured in 195 dogs that have survived exposure to varying amounts of ionizing radiation. One hundred sixteen normal unirradiated dogs were also screened for normal biochemical values. The parameters are grouped on the basis of metabolic area as shown below.

Procedure	Carbohydrate	Lipid	Protein	Enzyme	Anions and Cations
Glucose	X				
Cholesterol		X			
Total Protein			X		
Albumin			X		
Urea N			X		
Creatinine			X		
Uric Acid			X		
Bilirubin			X		
Cholinesterase				X	
Alkaline Phosphatase				X	
Acid Phosphatase				X	
SGOT				X	
Sodium					X
Potassium					X
Carbon Dioxide					X
Phosphate					X
Chloride					X
Calcium					X
Magnesium					X

All of the procedures enumerated were performed by means of automated wet chemical analysis. Each one of the methods utilized was checked for consistency with appropriate control sera.

The experimental animals were grouped together on the basis of the degree of radiation exposure as shown on the following page.

Type of Radiation Exposure	Level (Rads)	Dog Strain
Fractionated whole body	150, 200-225, 250-300	Beagle
Single exposure, whole body	200-500, 500-700, greater than 700	Beagle and Mongrel
Single exposure, whole body		
1 rad/min.	400, 600	Beagle
11 rads/min.	450	
Upper body exposure	1100	Beagle
Lower body exposure	1500	Beagle
Treated with chemical protectants	700	Mongrels

Statistical evaluation of the data was accomplished by computation of group means, variances, standard deviations and standard error. Intercomparison of the group mean values was made using the student "t" test. The 1% level of significance was selected as representative of a true difference outside of chance alone.

The results of the evaluation of unirradiated dogs along with those changes that were statistically significant are presented in Tables 1 through 6.

TABLE 1

SERUM VALUES UNIRRADIATED CONTROLS

SERUM PARAMETER	BEAGLES		MONGRELS
	YOUNG	OLD	
Glucose	113.57 ± 13.52	105.71 ± 1.49	50.76 ± 3.17
Cholesterol	269.14 ± 20.04	211.84 ± 15.81	159.11 ± 5.76
Total Protein	6.69 ± 0.170	6.45 ± 0.106	7.26 ± 0.105
Albumin	2.88 ± 0.284	2.97 ± 0.106	2.80 ± 0.064

Values Represent Means ± Standard Error

TABLE 2

SERUM VALUES UNIRRADIATED CONTROLS

SERUM PARAMETER	BEAGLES		MONGRELS
	YOUNG	OLD	
Urea-N	20.25 ± 0.89	18.66 ± 1.25	18.18 ± 0.51
Creatinine	0.92 ± 0.016	0.88 ± 0.02	1.06 ± 0.020
Uric Acid	0.186 ± 0.015	0.399 ± 0.016	0.337 ± 0.03
Bilirubin	0.205 ± 0.015	0.128 ± 0.009	0.62 ± 0.058

Values Represent Means ± Standard Error

TABLE 3

SERUM VALUES UNIRRADIATED CONTROLS

SERUM PARAMETER	BEAGLES		MONGRELS
	YOUNG	OLD	
Cholinesterase	35.80 ± 1.94	40.46 ± 2.19	19.19 ± 2.07
Alkaline Phosphatase	3.94 ± 0.77	6.67 ± 1.29	6.16 ± 2.16
Acid Phosphatase	8.89 ± 3.54	4.39 ± 0.67	2.40 ± 2.08
Serum Transaminase (SGOT)	37.35 ± 3.99	20.59 ± 2.22	35.23 ± 3.31

Values Represent Means ± Standard Error

TABLE 4

SERUM VALUES UNIRRADIATED CONTROLS

SERUM PARAMETER	BEAGLES YOUNG	BEAGLES OLD	MONGRELS
Sodium	153.95 ± 0.360	153.69 ± 0.52	154.99 ± 0.396
Potassium	5.01 ± 0.070	5.16 ± 0.056	5.39 ± 0.050
Calcium	10.84 ± 0.088	12.04 ± 0.22	10.61 ± 0.077
Magnesium	1.59 ± 0.029	1.61 ± 0.024	1.71 ± 0.022
Carbon Dioxide	18.45 ± 0.32	22.89 ± 0.42	16.88 ± 0.27
Chloride	119.55 ± 0.58	114.41 ± 0.57	116.75 ± 0.40
Phosphate	5.00 ± 0.14	4.50 ± 0.133	5.05 ± 0.150

Values Represent Means ± Standard Error

TABLE 5

BEAGLES

SERUM VALUES, WHOLE BODY AND PARTIAL BODY EXPOSURE

SERUM PARAMETER	700 RADS WHOLE BODY	1000-1500 RADS UPPER BODY	900-950 RADS LOWER BODY
Cholesterol	189.18 ± 12.52	200.75 ± 14.24	306.26 ± 42.53
Serum Transaminase (SGOT)	30.86 ± 2.17	16.33 ± 3.36	26.94 ± 2.67
Alkaline Phosphatase	4.00 ± 0.43	5.36 ± 0.67	8.83 ± 1.62
Acid Phosphatase	2.40 ± 0.26	4.14 ± 0.51	5.49 ± 1.08
Calcium	11.32 ± 0.22	11.17 ± 0.24	11.54 ± 0.27

Values Represent Means ± Standard Error

TABLE 6

MONGRELS

SERUM VALUES, WHOLE BODY IRRADIATION

SERUM PARAMETER	UNPROTECTED	PROTECTED	
	500-700 RADS	MEA + CYSTEINE	PAPP + MEA + CYSTEINE
Phosphate	3.87 ± 0.21	4.17 ± 0.29	4.26 ± 0.15
Calcium	10.81 ± 0.15	9.88 ± 0.21	10.13 ± 0.14
Alkaline Phosphatase	8.55 ± 1.71	3.96 ± 0.82	4.21 ± 0.77
Cholinesterase	35.58 ± 3.46	40.66 ± 6.33	42.56 ± 5.96
Cholesterol	264.22 ± 18.85	261.75 ± 21.45	173.6 ± 8.89
Urea-N	29.78 ± 4.65	24.87 ± 1.33	23.25 ± 2.43

Values Represent Means ± Standard Error

2. Physiological Effects.

a. Biochemical Physiology of Survivor Dogs. Mammalian radiobiology is generally oriented towards description of radiation injury, interpretation of tissue and organ sensitivity on a morphologic basis, and quantitation of lethality. Little is being done to analyze the physiologic mechanisms by which radiation affects the animal's ability to maintain homeostasis. More quantitative information is needed to explain the interaction of physiological processes in the regulation of homeostasis after exposure to ionizing radiation.

Animals exposed to a level of external ionizing radiation high enough to result in overt injury but not sufficient to be incompatible with survival appear to recover. On closer examination, however, it becomes obvious that there is a residuum of nonrecoverable injury. There are few reliable indices of post-acute radiation functional injury and recovery or compensatory repair processes.

For these investigations healthy beagles one to three years of age, males or females were used. All animals were quarantined and processed for two to four weeks after receipt before being included in the experimental program.

Animals were bilaterally irradiated by exposure to 1000 kvp x-rays (HVL 3.0 mm lead) at a dose rate of 50-60 rad/min. for whole, upper or lower body irradiation. Some animals were whole body irradiated dorsally at a dose rate of 1 or 11 rad/min.

Table 7 indicates the effect of 450 and 600 rad (1 rad/min.) whole body on selected biochemical characteristics 2-2½ years after irradiation. The results indicate significant shifts in electrolyte: sodium, potassium and chloride as well as nonprotein nitrogenous constituents of blood serum.

Table 8 lists the same biochemical characteristics for 450 rads at 1 rad/min. and 400 rads at 11 rad/min. It should be noted that although there are significant shifts in electrolyte balance and nonprotein nitrogen constituents at a slower dose rate, these biochemical indices are in the normal range for dogs irradiated at 11 rad/min.

It is doubtful that 50 rads would be enough to cause the difference in response especially in view of the previous tabulation (Table 7) which showed no difference between 450 rads and 600 rads at 1 rad/min. It can be assumed, therefore, that the difference in dose rate is the critical factor.

Table 9 summarizes the residual radiation effects in dogs determined by physiologic testing and biochemical analysis of the serum.

TABLE 7

THE EFFECT OF X-RAY DOSE ON BIOCHEMICAL CHARACTERISTICS

BIOCHEMICAL CHARACTERISTIC	MEAN ± S.D.* SHAM (10)	RANGE (NUMBER OF DOGS) 450 rad (9)	600 rad (8)
Sodium (mEq./l)	148.53±2.02	153.7-159.2(8)	153.6-159.6(6)
Potassium (mEq./l)	5.13±0.28	4.42-4.54(2)	4.22-4.50(4)
Chloride (mEq./l)	109.58±2.27	114.4-120.3(6)	115.1-120.7(4)
Creatinine (mg./100 ml)	0.581±0.081	0.780-1.020(4)	0.740-1.900(5)
Urea-N (mg./100 ml)	22.92±3.95	9.7-14.4(5)	10.5-13.5(2)

* Standard Deviation

TABLE 8

THE EFFECT OF X-RAY DOSE RATE ON BIOCHEMICAL CHARACTERISTICS

BIOCHEMICAL CHARACTERISTIC	MEAN ± S.D.* SHAM (10)	RANGE (NUMBER OF DOGS) 450 rad-1R/min(9)	400 rad-11R/min(8)
Sodium (mEq./l)	148.53±2.02	153.7-159.2(8)	145.5-149.8(8)
Potassium (mEq./l)	5.13±0.28	4.42-4.54(2)	4.76-5.35(8)
Chloride (mEq./l)	109.58±2.27	114.4-120.3(6)	105.3-112.0(8)
Creatinine (mg./100 ml)	0.58±0.08	0.780-1.020(4)	0.470-0.650(8)
Urea-N (mg./100 ml)	22.92±3.95	9.7-14.4(5)	16.7-27.7(7) 14.0 (1)

* Standard Deviation

TABLE 9

RESIDUAL RADIATION INJURY IN X-IRRADIATED DOGS

PORTION OF BODY	RADIATION (1000 kvp X-RAYS) DOSE (R)	DOSE RATE (RAD/MIN)	TIME (YEARS)	ORGAN FUNCTION	
Upper	970-1820	55	4-5.5	Thyroid Hypofunction	(1)
				Pulmonary Alveolar-Capillary Block	(1)
				Adrenal Hyperfunction	(2)
				Hepatic Insufficiency	(2)
Lower	700-950	55	4-5.5	Nephritis	(1)
Whole	150-340	55	4-5.5	Nephritis	(2)
				Adrenal Insufficiency	(2)
	400	11	2-2.5	Nephritis	(2)
				Adrenal Insufficiency	(2)
	450-600	1	2-2.5	Adrenal Hyperfunction	(2)
				Hepatic Insufficiency	(2)

(1) Confirmed by Physiologic Testing and Pathology
(2) Presumptive-based Primarily on Biochemical Findings

Some very interesting conclusions and conjectures can be drawn from these data not only from the point of view of diminished physiologic reserve capacity as a manifestation of residual radiation injury but the influence of dose-time intensity factors in these findings. Suggestion of hepatic insufficiency is seen in certain whole and upper body irradiated animals. This evidence is based on decreased urea nitrogen, uric acid, cholinesterase, glucose and cholesterol levels in the presence of increased bilirubin. Presumptive evidence of adrenal hyperfunction is seen in whole body irradiated animals exposed to a moderate dose at a slow dose rate or in upper body irradiated dogs exposed to a high dose at a faster dose rate. This is in contrast to possible adrenal insufficiency indicated by decreased sodium and magnesium, increased potassium and calcium seen at a moderate dose, faster dose rate in whole body irradiated dogs. The adrenal hyperfunction could be a result of prolonged oversecretion of adrenal cortical hormone during pituitary

irradiation in whole and upper body irradiated animals resulting in a syndrome comparable to "diseases of adaptation."

It should be emphasized that the residual injury indicated by biochemical alterations is only presumptive at this point and requires additional testing before definitive conclusions can be made. This survey does, however, permit us to focus on specific organs such as the adrenal, kidney, and liver as well as the thyroid, cardiopulmonary and hematopoietic systems as critical areas displaying residual radiation injury.

The definitive findings reported to date of thyroid hypofunction, alveolar-capillary block, altered renal hemodynamic function (decrease in filtration rate and renal plasma flow), diminution in work capacity (exercise tolerance), defective thermal regulation as well as presumptive evidence of adrenal and hepatic dysfunction in dogs several months to years after x-irradiation are indicative of diminished physiologic reserve capacity in these animals.

b. Clinical Evaluation of Radiation Survivor Dogs.

(1) Studies have been continued on purebred beagle dogs which are long-term survivors of Co^{60} gamma irradiation.

(2) Seven of the dogs died during the period covered by the report.

Dog Number	Dose	M. Date of Irrad.	Cause of Death
1001	450 r	November 1961	Strangulated Intestinal Hernia
3555	819 r	August 1956	Hemorrhagic Cystitis
3809	795 r	July 1956	Septicemia (Unproven)
3877	450 r	October 1956	Cellulitis
3937	594 r	February 1957	Chronic Interstitial Nephritis
3982	450 r	June 1957	Fight Wounds
3782	594 r	December 1956	Acute Blast Cell Leukemia

Dog #3782 was the first dog in this group to develop leukemia. The bone marrow was most interesting in that in many smears there was an orderly transition from the predominant blast cells to mature plasma cells. On some slides a less definite progression was seen in

cells of the granulocytic series. The morphological characteristics of the blast cells themselves in the bone marrow and in the peripheral blood were not helpful in diagnosis.

An abnormal amount, and probably type, of gamma globulin was seen in the serum electrophoretic tracing.

Fe^{59} kinetics and Cr^{51} R.B.C. life span studies indicated an aplastic anemia. Peripheral blood smears and reticulocyte counts were consistent with the Fe^{59} and Cr^{51} studies.

Cytogenetic studies have been started on this animal and at the present time 6 of 9 chromosomal spreads from bone marrow material show a complement of 80, whereas the normal in dogs is 78.

(3) A number of dogs developed disease states requiring treatment.

Dog Number	Dose	Date Irradiated	Disease
1007	450 r	November 1961	Cystitis
3643	750 r	September 1957	Tumor of the Tongue (Excised - Grossly resembling a rhabdomyoma- Not yet confirmed)
3731	1157 r	April 1956	Multiple Skin Tumors
3826	450 r	October 1956	Pyometrium
3928	470 r	November 1956	Multiple Adenocarcinoma of Breasts
3648	640 r	February 1956	Hemangioma of Spleen
3719	1404 r	March 1956	Hemangioma of Spleen

These last two dogs were found to have large abdominal masses on physical examination. Both animals had exploratory laparotomies and massive splenic tumors were removed in each case. Both tumors had the microscopic appearance of benign hemangiomas although in one dog (3648) enlarged, hard abdominal lymph nodes were palpated at the time of surgery.

Hematologic investigations since the time of operation three months ago indicate progression of two different syndromes in these dogs. Fe^{59} kinetics and Cr^{51} studies in #3648, three weeks after splenectomy showed a plasma Fe^{59} disappearance curve with a pronounced hump between the sixth and twenty-second study day. The maximum R.B.C. Fe^{59} was 30% at

three days. The Cr^{51} T 1/2 for R.B.C.'s was 5.5 days. The bone marrow showed extreme erythroid hyperplasia; there was a 29% reticulocyte count in the peripheral blood which also showed target cells and Cabot rings. These findings are consistent with extra and intramedullary hemolysis and the post splenectomy state.

Bone marrow smears have recently (three months after splenectomy) shown abnormal cells which may be of metastatic tumor or endothelial origin. These cells have also been seen in the peripheral blood.

Cytogenetic studies have been started on this animal.

The only striking abnormality in the Fe^{59} and Cr^{51} studies on dog #3719 was a Fe^{59} R.B.C. uptake of 43.6% at five days. The bone marrow at the time of these studies showed moderate erythroid hyperplasia; there was a 1.7% reticulocyte count in the peripheral blood which also showed target cells and Cabot rings. This was thought consistent with the post splenectomy state and a moderate impairment of erythropoiesis.

A recent bone marrow (three months post splenectomy) shows cells consistent with a diagnosis of reticulum cell sarcoma or lymphosarcoma.

Cytogenetic studies are being done on this animal.

(4) Routine care and study of these dogs continue as before. Ophthalmologic examination continues and now six of the surviving beagles have posterior cataracts. One of these dogs is a non-irradiated control.

c. Hematologic Studies. Anemia is a common finding in the long-term survivor mammal. This may be due to a new homeostatic state as is true in the apparent anemia of the hypophysectomized animal; to impaired stem cell function or to decreased production of erythropoietin. Equipment has been assembled for the bioassay of erythropoietin in order to test the third alternative. Preliminary assays have been done this year but a dose response curve must still be constructed. Erythropoietin and anti-erythropoietic substances are of additional interest as to their effect on altering the sensitivity of the stem cell to radiation. The equipment used for erythropoietin assay can also be used in such a sensitivity study.

Erythrokinetic studies in mammals that have survived exposure to radiation are a means of studying the integrity of the hematopoietic system. Baseline studies of the animal as well as repeat studies after specific stress are necessary to measure the physiological reserve of

this system. Experiments have been performed in long-term radiation survivor dogs and sham irradiated dogs. Iron plasma clearances using Fe^{59} ferrous citrate and Fe^{59} red blood cell uptake were measured before and after removal of 20% of the blood volume. The results are summarized in Table 10.

Iron deficiency anemia in some animals and a varying blood volume in all animals not splenectomized accounts for some of disparate results. Finally, bleeding an estimated 20% of the blood volume may not be a very uniform stress. In the coming year,screening tests will be done to choose animals suitable for stressing of hematopoiesis. Exposure in a high altitude chamber will be explored as a more uniform stress compared to bleeding.

d. Late Radiation Effects in Primates. In collaboration with the Radiation Biology Department, USAF School of Aviation Medicine and the Yerkes Primate Center, Emory University, a survey of late radiation effects in primates has been initiated. Parameters of effect are (1) 14 biochemical and 5 serum enzyme measurements, (2) hematopoietic function, and (3) peripheral lymphocyte cultures for cytogenetic changes.

The Yerkes Primate Center maintains rhesus monkeys with radiation exposures 5 to 9 years previously to (1) single acute nuclear radiation from the Plumbbob 1957 weapon test series with graded doses from 280 to 810 rem, (2) fractionated low dose rate Co^{60} gamma rays with graded doses from 200 to 1000 rads, and (3) fractionated fast neutron exposures with graded doses from 14 to 600 rem.

TABLE 10

ERYTHROKINETICS IN DOGS BEFORE AND AFTER STRESS

DOG	RADIATION EXPOSURE TYPE, DOSE RATE AND TOTAL DOSE, RADS	ELAPSED TIME FROM EXPOSURE YEARS	IRON PLASMA CLEARANCE*		Fe^{59} UPTAKE**		SERUM IRON MICROGMS/100 ml	
			BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER
1	0	2 1/2	117	60	87(8)	85(3)	167	94
2	0	2 1/2	86	57	81(6)	87(3)	99	117
3	Gamma. 1 rad/min 750	2 1/2	106	36	117(10)	117(3)	167	51
4	Gamma, 1 rad/min 750	2 1/2	114	63	73(10)	88(3)	159	152

*Iron Plasma Clearance expressed in minutes

**Maximum uptake expressed in percent

( ) Day of maximum uptake

Results to date obtained from the group exposed to fractionated low dose rate Co^{60} gamma irradiation show little hematopoietic or cytogenetic change from normal aged control animals.

3. Pathological Effects.

In the past, it has been shown that grid-irradiated C-57 mice demonstrated a grid pattern by greying of the hair in the exposed areas. This pattern was evident on both the dorsal and ventral sides of the animal, indicating that the x-ray beam passed through the animal. ICR mice show a similar grid pattern by epilation of the hair in the exposed areas.

Based on this observation it was felt that there must be damage to the internal organs in the path of these beams. This damage must be a relatively late effect as it nas not been reported by previous investigators who held their animals the usual 30-60 days.

Therefore a pilot series of animals were irradiated at varying intensities (100 Kv, 200 Kv, and 300 Kv) and at varying dose levels ranging from 500 rads to 4,000 rads. These animals were then held for study of long term effects and sampled at varying time intervals.

From these animals, it has been found that the internal organs, primarily the kidneys, do exhibit a late injury, appearing between 6-9 months after irradiation. This injury is manifest by discrete cortical scars in the kidney which give the organ a "mulberry" appearance. These lesions have been shown histologically to follow an almost straight course through the entire thickness of the kidney. The renal lesions have been produced at all voltages and all doses above 1000 R that have been studied.

The lesions have been studied by routine histological sections stained with H and E, masson, elastic tissue, PAS - alcian blue and reticulin methods. Whole organs have been stained with the triphenyltetrazolium chloride method for the demonstration of the enzyme succinic dehydrogenase. These organs were then sectioned on the cryostat freezing microtome for further study.

More recent techniques utilized in the study of the renal lesions have included the following:(1) autoradiographs of kidney sections after injection of tritiated thymidine; (2) injection of the arterial vasculature with a solution of gelatin and micropaque from which microradiographs have been made; (3) injection of the vasculature with liquid silicone rubber for stereoscopic study of the microvasculature of the kidney and other organs.

A similar long-term study on mice irradiated through varying sized grids with 3 mev electrons has been started and is continuing.

It is hoped that these and further studies about to be started will elicit the pathogenesis of the renal lesions and provide better understanding of the mechanisms involved in radiation damage, particularly in the interphase area between irradiated and unirradiated tissue.

Summary and Conclusions.

Within the scope of the overall work unit biochemical, physiological and pathological effects as related to the total organism are being studied. The major emphasis has been in the delineation of the status of the radiation survivor.

1. Biochemical Effects.

Nineteen biochemical parameters have been evaluated in a survivor dog population. The results of this study raise the question of the integrity of the physiological reserve of the animal.

2. Physiological Effects.

Initial efforts have been made to integrate biochemical effects with the physiological reserve of the dog. Clinical evaluation of the survivor colony is being continued. Hematologic studies are being carried out.

3. Pathological Effects.

Mice are being studied for their pathological consequence after exposure to ionizing radiation.

Publications.

Angel, C. R., Woodward, K. T., Davis, J., Michaelson, S., and Howland, J.W.: A Survey of Biochemical Parameters in Irradiated Dog Survivors. *Radiation Research* 25: No. 1, p. 3, 1965 (Abstract).

Michaelson, S., Angel, C. R., Woodward, K. T., and Howland, J.W.: Biomedical Aspects of Radiation Injury and Recovery. *Radiation Research* 25: No. 1, p. 48, 1965 (Abstract).

RESEARCH AND TECHNOLOGY RESUME

1.	2 GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6475	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6 SECURITY RPT	WRK	7. REGRADING	8. RELEASE LIMITATION	9 LEVEL OF RESUME
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10a CURRENT NUMBER/CODE: NWER A4e DASA 03.0851

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11. TITLE: (U) Effectiveness of mixed radiation exposures (09)

12. SCIENTIFIC OR TECH. AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
014100 Rabiobiology; 017100 Weapons effects	07 63	NA	MIPR | DD

16. PROCURE. METHOD	17. CONTRACT GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18 RESOURCES EST	a PROFESSIONAL MAN-YEARS	b FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	5	125
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21. TECHNOLOGY UTILIZATION: Radiation protection in nuclear industries; nuclear medicine

22 COORDINATION: NA

23. KEYWORDS: Biological effectiveness; dose rate; particle; radiation injury; radiation mechanism; radiobiology; recovery

24. (U) Tech Objective - the objective of this work unit is to systematically investigate neutron radiation injury and recovery in biological systems. Specific technical objectives are (1) determination of the effectiveness of time intensity factor, (2) determination of the effectiveness of neutron exposures with random recovery intervals, and (3) determination of neutron energy spectral effects on physiologic systems.

25. (U) Approach - Various biological and points of radiation effects which are known to afford quantitative change to reflect extent of injury will be utilized. In mammals end points will be the effect of graded doses and intensities on lethality, survival time, hematopoietic depression, organ weight change, metabolic and trace element aberration, and response to split radiation dose and other stresses.

(U) Progress - (Jul 64 - Jun 65) - Data has been collected and analyzed for determination of the radiation environment at the south face of the Walter Reed Research Reactor. Dose rate studies (10, 50, 100, and 200 rad per minute) on mice have been
26. completed. The mean lethal dose for all rates was approximately the same. Analysis of this data for mean survival time is complete. Studies are in progress to determine strain differences.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965 and DASA-'78, Nuclear Weapons Effects Research Semi-Annual Progress Summaries, 1 July 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
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31. MISSION OBJECTIVE	32. PARTICIPATION
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DD FORM 1 AUG 64 1498 (Items 1 to 24 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project RD 41-51, NUCLEAR WEAPONS EFFECTS RESEARCH

Task IIIA4 e, Biomedical Aspects of Ionizing Radiation

Work Unit 03.0851, Effectiveness of Mixed Radiation Exposures

Investigators.
Principal: Lt Col Dan Hightower, VC
Associate: Lt Col K. T. Woodward, MC; Lt Col E. O. Jones, MSC; Capt F. F. Hahn, VC; Maj J. M. Brady, DC; Mr. R. E. Simpson, MS; Mrs. M. M. McLaughlin, MS

Description.

The objective of this investigation is to systematically investigate neutron radiation injury and recovery in biological systems. Specific objectives are: (1) determination of the effectiveness of time intensity factor (dose rate); (2) determination of the effectiveness of neutron exposures with random recovery intervals; (3) determination of neutron energy spectral effects.

Progress.

The major effort during this reporting period has been the investigation of dose rate on lethality and median survival time of ICR strain female mice exposed to mixed neutron-gamma radiation. Comparison of these effects has been made between ICR strain mice, C-57/BC, and Bagg-Swiss strain mice at comparable dose rates. The effect of age on the response of mice to mixed radiation exposure has been investigated. Median survival time has been studied in Bagg-Swiss mice as a function of total dose. In collaboration with the Los Alamos Scientific Laboratory, study of recovery from neutron exposures using random re-exposure intervals has been initiated (as described herein).

Dosimetry measurements are in progress to provide a second exposure area in the thermal column area of the Walter Reed Research Reactor for the study of thermal neutron effects on small experimental animals.

The radiation environment in the fission neutron exposure facility has been characterized. Graphite was removed from the horizontal thermal column of the Walter Reed Research Reactor providing a 12 x 12-inch cross sectional exposure position approximately 16" from the reactor core. An exposure rack 10 x 11 inches containing eight 1-1/2 inches diameter graphite tubes was used for positioning mice to the mixed radiation field. A bismuth gamma shield inherent to the reactor was located between the exposure rack and the reactor vessel.

The variation of gamma, total dose, and neutron flux across the exposure volume has been obtained. Use was made of lithium-fluoride gamma detectors for gamma dose, tissue equivalent Sievert-type ionization chambers for total dose, and gold and sulfur foils for neutron flux measurements. Ilford nuclear emulsions and threshold detector foils were used to determine the neutron energy spectrum. A neutron to gamma dose ratio of approximately 5-6:1 and a cadmium ratio of 4:1 were obtained. There was no significant variation in total dose over the animal exposure area. The composite radiation spectrum has been compared with those of other reactors used for animal exposures.

The transient dose (reactor period = 30 seconds) from startup to desired power (dose rate) was found to be approximately 0.05 rad/watt of peak power. Transient dose was significant only in exposures of less than 15 minutes' duration. The total dose rate at steady state power was approximately 0.10 rad/watt-min. providing a broad range of dose rates with little or no change in radiation spectrum.

Eight-week-old ICR female mice were exposed at dose rates of 10, 50, 100 and 200 rad/min. A minimum of 24 animals were exposed at each dosage level. The results are shown in Table I.

TABLE I

Dose Rate (rad/min)	$LD_{50/30}$ (95% C. I.)
10	408 (395-420)
50	402 (380-416)
100	413 (360-462)
200	407 (396-417)

Mortality of 8-week-old ICR female mice for various dose rates of mixed radiation.

Animals of the same strain and sex, but 12 weeks of age, were exposed at similar dose rates. Forty-eight animals were exposed per point with graded doses in increments of 50 rads. The results are shown in Table II.

TABLE II

Dose Rate (rad/min.)	$LD_{50/30}$ (95% C. I.)
10	460 (449-471)
100	435 (424-449)
200	466 (454-481)

Mortality of 12-week-old ICR female mice for various dose rates of mixed radiation.

These results are essentially in agreement with those of Vogel, Clark, and Jordan (1957), Spalding, Sayeg, and Johnson (1964), and Ainsworth, Leong, Kendall, and Alpen (1964) in that no dose rate effect from neutron irradiation was detected. They differ from the above investigators in that the median lethal doses obtained in ICR strain mice are approximately a factor of 2 higher. Data presented below indicate differences in strain sensitivity to radiation exposure, but such differences are not of sufficient magnitude to be accepted as a complete explanation.

In two of the cited references pulsed neutron irradiation results were compared to lower dose rates and no differences noted. These data are particularly important since the absence of neutron dose rate effect on mortality indicates significant irreparable injury.

The effect of age on radiation lethality was studied using animals at 16 weeks of age and a reactor dose rate of 100 rad/min. Data for 8, 12 and 16 weeks of age are shown in Table III.

TABLE III

Age (weeks)	$LD_{50/30}$ (95% C. I.)
8	413 (360-462)
12	435 (424-449)
16	433 (403-452)

Mortality of ICR female mice exposed to mixed radiation at a dose rate of 100 rad/min.

At all dosage levels where there were a significant number of deaths mean survival times were calculated. A trend toward shorter median survival times with increasing dose was obtained, whereas no significant difference was noted in median survival time at the approximate LD_{50} regardless of dose rate. Results are shown in Table IV.

TABLE IV

Age (weeks)	Dose Rate (rad/min)	Median Survival Time
8	10	10.8 (9.6-12.1)
8	50	11.4 (10.0-12.5)
8	100	11.4 (9.9-12.9)
8	200	12.2 (10.9-13.0)
12	10	12.1 (11.4-12.6)
12	100	10.9 (9.3-12.4)
12	200	11.5 (10.8-12.1)

Median survival time of ICR female mice at the approximate mid-lethal mixed radiation dose for various dose rates.

These data do not suggest a dose rate effect on the median survival time of mice. Vogel, et al, observed a vast majority of deaths between the fourth and ninth days, while those from gamma ray exposures occurred around the fourteenth day. They inferred different mechanisms of lethality. Ashikawa irradiated mice with 730 mev protons at rates of 100 and 1000 rads per minute. He observed no dose rate effect on median lethal dose, but obtained a pronounced effect of dose rate on median survival time. Seven hundred thirty mev protons have an LET similar to 100 kvp x-rays. By reducing dose rate the effectiveness of protons in producing gastrointestinal deaths was decreased.

The effect of total mixed radiation dose on the median survival time of 5-6-week-old female Bagg-Swiss mice has been extensively investigated for dosages from 300 rads (the approximate mid-lethal dose) through 15,000 rads. The median survival time declined over the range 300-700 rads and then remained relatively constant over the range 700-10,000 rads. Above 10,000 rads the median survival time begins to decrease. Survival time data are presented in Table V.

TABLE V

Total Dose (rad)	Median Survival Time
300	13
350	9
400	6
450	5
475	5
500	5
550	4.5
600	4.5
650	4.5
700	4.5
800	4.0
1,000	5.0
2,500	5.0
5,000	5.0
7,500	5.0
10,000	4.0
15,000	3.8

Survival time of 5-6 week-old Bagg-Swiss female mice as a function of total mixed radiation dose.

These data are in general agreement with that reported by Rothermel, et al (1956).

In collaboration with the Los Alamos Scientific Laboratory an experiment has been initiated to study the recovery of RF strain mice after fission neutron irradiation. The same exposure routine is being accomplished at LASL using a low dose rate Cobalt-60 gamma ray source. Three dosage levels selected were 100, 200, and 300 rem. Groups were initially irradiated to these levels of injury. For the neutron exposures, an RBE of 1.5 was selected. An irreparable injury fraction of 10% of each exposure and a repair half-time of seven days was used. After a recovery period randomly selected, ranging from 2 to 14 days, mice are re-irradiated to maintain equivalent residual injury (ERD). Blood counts following the first 3-4 exposures revealed a damage proportional to dose. This experiment is expected to continue for a minimum of 150 days.

A major dosimetry program has been initiated to define the radiation environment in a thermal neutron exposure position in the horizontal thermal column of the Walter Reed Research Reactor. The cadmium ratio is expected to be in excess of 500:1. Interest in this position arises from the mode of action attributed to thermal neutron capture gamma-rays in tissue.

Summary and Conclusions.

1. No dose rate effect from mixed radiation could be demonstrated on lethality of mice in the range of 10-200 rad/minute.

2. No dose rate effect from mixed radiation could be demonstrated on median survival time of mice in the range of 10-200 rad/minute.

3. Strain differences after mixed radiation exposures have been explored.

4. The survival time of mice following mixed radiation exposures is relatively constant from 700 to 10,000 rads.

5. Experiments are in progress to determine the effectiveness of fractionated fission neutron exposures and equivalent residual injury after random recovery intervals.

RESEARCH AND TECHNOLOGY RESUME

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(U) Biophysical aspects of radiation injury (09)

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21. TECHNOLOGY UTILIZATION	22. COORDINATION
NA	NA

23. KEYWORDS Bacteria; biophysics; DNA; Escherichia coli; ion transport; membrane permeability; protein synthesis; radiation injury; radiation-protective agents; RNA

24. (U) Tech Objective - To measure response to ionizing radiation injury at the cellular and molecular levels, and to determine the biochemical mechanisms involved in the induction and repair of specific cellular lesions. Information to be applied (1) in evaluating radiation damage to organs and organisms, and (2) in development of prophylactic and therapeutic procedures.

25. (U) Approach - Effects of radiation on DNA synthesis in bacteria; influence of alterations in DNA, RNA, and protein syntheses on radiation survival of bacteria; effects of radiation on isolated mammalian membranes; effects of ionizing radiation on ion transport across membranes.

26. (U) Progress - Inhibition of protein synthesis in logarithmic phase bacteria before irradiation enhances radiation survival apparently by forcing culture into premature stationary phase. Initial damage produced by a given radiation dose appears to be the same in logarithmic and stationary cultures, even though greatly different survivals are obtained. Data suggest that increased resistance of stationary cultures is due solely to their greater ability to repair and, thus, recover from the initial lesions. Studies of membrane permeability have been initiated using a constant temperature membrane transport apparatus.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965 and DASA-78, Nuclear Weapons Effects Research Semi-Annual Progress Summaries, 1 July 1965.

27. COMMUNICATIONS SECURITY a. COMSEC OR COMSEC RELATED X / b. NOT RELATED	28.	29. OSD CODE	30. BUDGET CODE
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31. MISSION OBJECTIVE	32. PARTICIPATION
CDUG Annex I para 1	NA

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DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C, WHICH ARE OBSOLETE.

Project RD 41-51, NUCLEAR WEAPONS EFFECTS RESEARCH

Task IIIA4 e,Biomedical Aspects of Ionizing Radiation

Work Unit 03.1071, Biophysical Aspects of Radiation Injury

Investigators.
Principal: Maj David M. Ginsberg, MSC
Associate: Dr. Robert T. Lofberg; Dr. John Jagger

Description.

The objectives of this work are to measure response to ionizing radiation injury at the cellular and molecular levels, and to determine the biochemical mechanisms involved in the induction and repair of specific cellular lesions. Two model biological systems are being used (1) isolated mammalian membranes and (2) bacteria. Radiation-induced biochemical changes in the model systems are being evaluated in terms of (1) their roles in the development of cellular morbidity, (2) their quantitative relationships to both the radiation dose and ultimate gross biological effects, and (3) the influence of alterations in chemical and physical environment before, during, and after irradiation.

Progress.

(1) Membrane Studies.

Damage to extracellular, cellular, and intracellular membranes probably plays a role in primary radiation injury to living organisms. Membrane water transport is significantly modified by radiation doses of less than 100 rads and the effects of radiation in membranes have been reported to be modified by many radioprotective drugs. In order to study radiation effects on isolated membranes, a constant temperature membrane transport apparatus was constructed and tested. Sodium with two isotopes available (permitting simultaneous directional measurement and general ease of analysis) was selected as a major parameter to be studied. In the initial experiments for this study, rat peritoneum was used as considerable information is available about water transport characteristics.

A peritoneal membrane was placed between two cells at 37^{o}. One cell contained isotonic sucrose solution; the other oxygenated Ringer's salt solution. Samples were taken periodically from the sucrose solution to determine the quantity of sodium which was transported across the membrane from the Ringer's solution. Sodium concentration in the sucrose cell was plotted versus time. When normal unirradiated membranes were used, smooth curves were obtained; however, the slope of the curve obtained was different for different portions of the peritoneal membrane (pure membrane versus muscular

membrane). Muscular tissue may exhibit some physical leakage which would increase transport. This observation necessitated modification of the experimental transport cell to eliminate possible cell leakage factors.

In one experiment a membrane was administered a radiation dose of 1000 rads using 250 kvp x-rays. The irradiated membrane exhibited an apparent discontinuity manifested as an increased transport of sodium. No conclusions are drawn from this single experiment.

These studies are continuing.

(2) Bacterial Studies.

a. Introduction: Since it appears likely that deoxyribonucleic acid (DNA) is a major site of initial lethal lesions, studies are being made of the effects of radiation on DNA synthesis in bacteria, and of the influence of alterations in DNA, ribonucleic acid (RNA), and protein syntheses on the radiation survival of bacteria. Previously reported studies at this laboratory showed that inhibition of protein synthesis in a logarithmic-phase culture of bacteria forces the cells into a premature stationary phase, where they become more resistant to radiation killing. The experimental evidence was consistent with the hypothesis that similar initial lethal damage is produced in logarithmic- and stationary-phase cultures, and that the greater radiation resistance of a stationary phase culture is due to an enhanced capacity to repair and, consequently, recover from the initial lethal damage.

Studies are currently underway to characterize the changes produced in DNA during inhibition of protein synthesis and to determine the relationship of these changes to modification of radiosensitivity. Results of these studies are still inconclusive.

In a related study the effects of the compound, β-mercaptoethylamine (MEA), on DNA and protein synthesis in logarithmic-phase cultures of *Escherichia coli* strain 15TAU-bar have been investigated, and the results are discussed below.

While the broad spectrum of MEA for protecting biological systems against effects of ionizing radiation has been demonstrated by many workers, the mechanism by which MEA affords protection is still unknown, and there is surprisingly little information about effects of MEA on bacteria. Nothing is known about biochemical effects of MEA on unirradiated bacteria, nor about the radioprotective effects, if any, of MEA in logarithmic-phase cultures.

b. Materials and Methods: *Escherichia coli* strain 15TAU-bar (requires thymine, arginine, uracil, methionine, proline and tryptophan) was grown aerobically at 37°C in Davis mineral salts

medium supplemented with thymine, 20 ug/ml; uracil, 50 ug/ml; and the four required amino acids, each, 140 ug/ml; plus glucose, one percent (w/v) as an energy source. To obtain early stationary-phase bacteria, cells from an overnight stationary phase culture were diluted 1:20 in fresh medium and grown 6-1/2 - 7-1/2 hours (yield: 3 x 10^9 viable cells/ml). Logarithmic cultures were obtained by diluting cells from the overnight cultures 1:200 in fresh medium and growing at 37°C for 3 hours (yield: 10^8 viable cells/ml).

Amount of protein synthesis was estimated by measuring the dry cell mass in 10-ml aliquots of culture. Amount of DNA synthesis was estimated by substituting ^{14}C-labeled thymine in the growth medium, and measuring radioactivity in either washed whole cells, or the boiling-water-insoluble fraction in 10-ml aliquots. Radioactivity was measured using a Packard Tri-Carb three-channel, liquid scintillation spectrometer. Samples to be counted in the Tri-Carb were collected on Millipore filters (Millipore Grade HA) and placed in scintillator fluid made of dioxane 1 liter; PPO, 6 g; POPOP 300 mg; and naphthalene, 100 g. Numbers of viable bacteria were estimated by colony counts on Difco nutrient agar plates incubated at 37°C for 24 hours. Total bacteria were estimated by hand-counting samples in a Petroff-Hauser chamber using oil immersion optics of a Spencer A/O phase microscope.

Bacteria were collected on membrane filters (Millipore grade HA), washed free of medium by several volumes of M/15 phosphate buffer (KH_2PO_4, 2.3 g and NA_2HPO_4, 5.9 g per liter distilled water), and suspended in M/15 phosphate buffer. The buffer suspension was exposed in air at room temperature to gamma-rays from a $^{60}Cobalt$ "Gamma-Cell" (Atomic Energy of Canada, Ltd). The dose rate was about 1300 rad/min.

c. Results and Discussion: Figures 1a and 1b show effects on DNA, and net mass syntheses when MEA is added (0.04 M) to a logarithmically growing (doubling time: 45 minutes) culture of *E. coli* TAU-bar at 37°C. Net mass synthesis was inhibited immediately, while DNA synthesis continued for about 90 minutes before stopping, total DNA increasing about 70%. Viable and total numbers also increase by about 70% during this time. A parallel culture, continued at 37°C without MEA, grew logarithmically for at least 3 hours before beginning to enter stationary phase. After 120 minutes in MEA the bacteria were washed with buffer on a membrane filter and resuspended in fresh, supplemented mineral salts medium without MEA. As shown in Fig. 1c, net mass synthesis resumes at the normal exponential rate within a few minutes after removal of MEA from the medium, while resumption of DNA synthesis lags for about 30 minutes. The same effects are observed when amino acids are removed from a logarithmic-phase culture for 120 minutes (see Fig. 2 for comparison). That washing on the membrane filter effectively removes MEA from the culture is shown by the fact that the inhibition of net mass synthesis is relieved immediately.

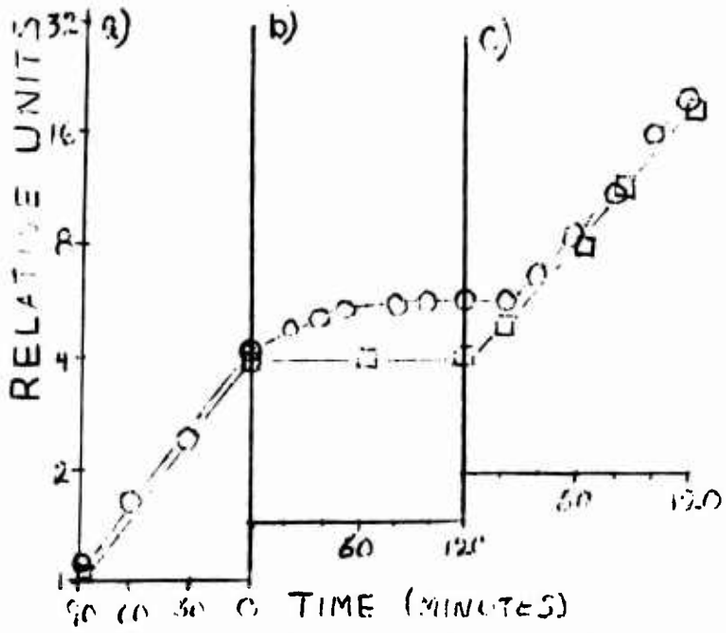

Figure 1. Growth of a logarithmic-phase culture in (a) supplemented minimal medium; (b) supplemented minimal medium with 0.04M MEA; (c) supplemented minimal medium after removal of MEA. ○ = radioactivity in DNA (1 unit = 160 counts per minute ^{14}C/ml culture); □ = washed dry mass (1 unit = 0.025 mg/ml culture).

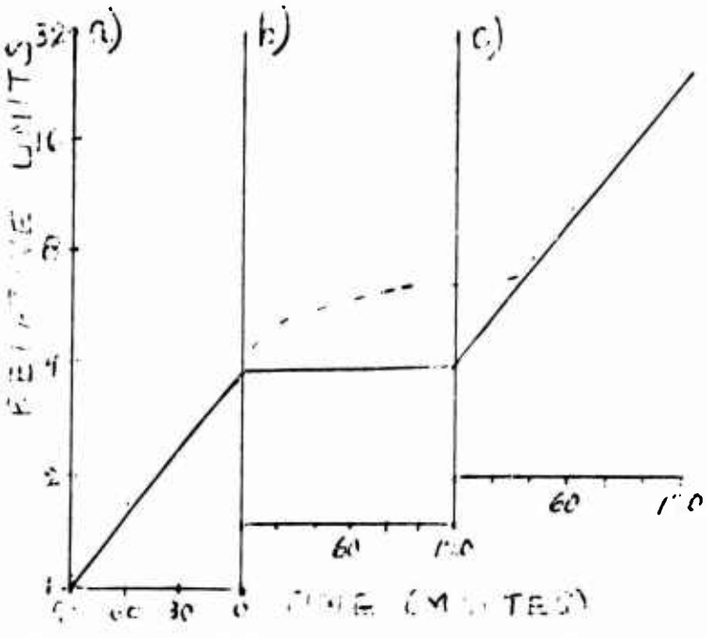

Figure 2. Growth of a logarithmic-phase culture interrupted for 120 min by removal of amino acids. (a) supplemented minimal medium; (b) minimal medium with thymine supplement only; (c) supplemented minimal medium (uracil and amino acids added after 120 min starvation). Dashed line = DNA synthesis; solid line = dry mass.

Figure 3 shows the gamma-radiation survival for PO_4 buffer suspensions of (a) a logarithmic phase culture, (b) an early stationary-phase culture and (c) a logarithmic culture after 120-minutes growth with MEA (the bacteria were washed free of extracellular medium and MEA prior to irradiation). The same visually-fitted curve can be drawn for the stationary and MEA-treated logarithmic cultures. A portion of the culture to which MEA was to be added, was removed and incubated without MEA for 120 minutes. Its radiation survival is that shown for a logarithmic culture. Also shown in Fig. 3 is the survival of an untreated logarithmic-phase culture irradiated in buffer with 0.04 M MEA. In order to determine whether protection by MEA present during irradiation is altered by the pre-irradiation growth with MEA, logarithmic- and early stationary-phase cultures were pre-incubated with MEA for 120 minutes. Samples from both cultures were washed, resuspended in phosphate buffer containing MEA (0.04 M), and irradiated. In addition a sample of the early-stationary-phase culture was washed free of MEA and irradiated without MEA. Figure 4 shows that the pre-irradiation treatment of an early stationary-phase culture by incubation in MEA does not change its radiation sensitivity (compare with Fig. 3). The data also show that MEA present during irradiation gives the same amount of protection to early stationary-phase and the 120-minutes pretreated logarithmic-phase culture.

The data presented indicate that MEA immediately inhibits protein synthesis but not DNA synthesis in a logarithmic phase culture (Fig. 1) and that after 120 minutes of incubation with MEA, the culture is like an early stationary phase culture, as determined by comparing radiation survival curves (Fig. 3). The parallel control culture (grown 120 minutes without MEA) was still in logarithmic phase. Thus, it is concluded that MEA forces a logarithmic culture into stationary phase, probably by immediately inhibiting protein but not DNA synthesis. That the presence of MEA during irradiation gives the same amount of protection to the logarithmic culture pre-incubated with MEA as to a normal early stationary-phase culture is consistent with the above conclusions that the MEA-treated logarithmic culture has been forced into early stationary phase. Furthermore, that a normal stationary-phase culture, pre-incubated with MEA for 120 minutes and irradiated without MEA present, is not protected by the pretreatment supports the interpretation that the pre-incubation treatment affects only the metabolic state of the culture. That is, since an early stationary phase culture has already stopped net mass increase and DNA synthesis, incubation with MEA should have no further effect on its metabolic state.

On the basis of these observations, it is suggested that in addition to the direct (physico-chemical) protection against ionizing radiation afforded by MEA present during irradiation, MEA

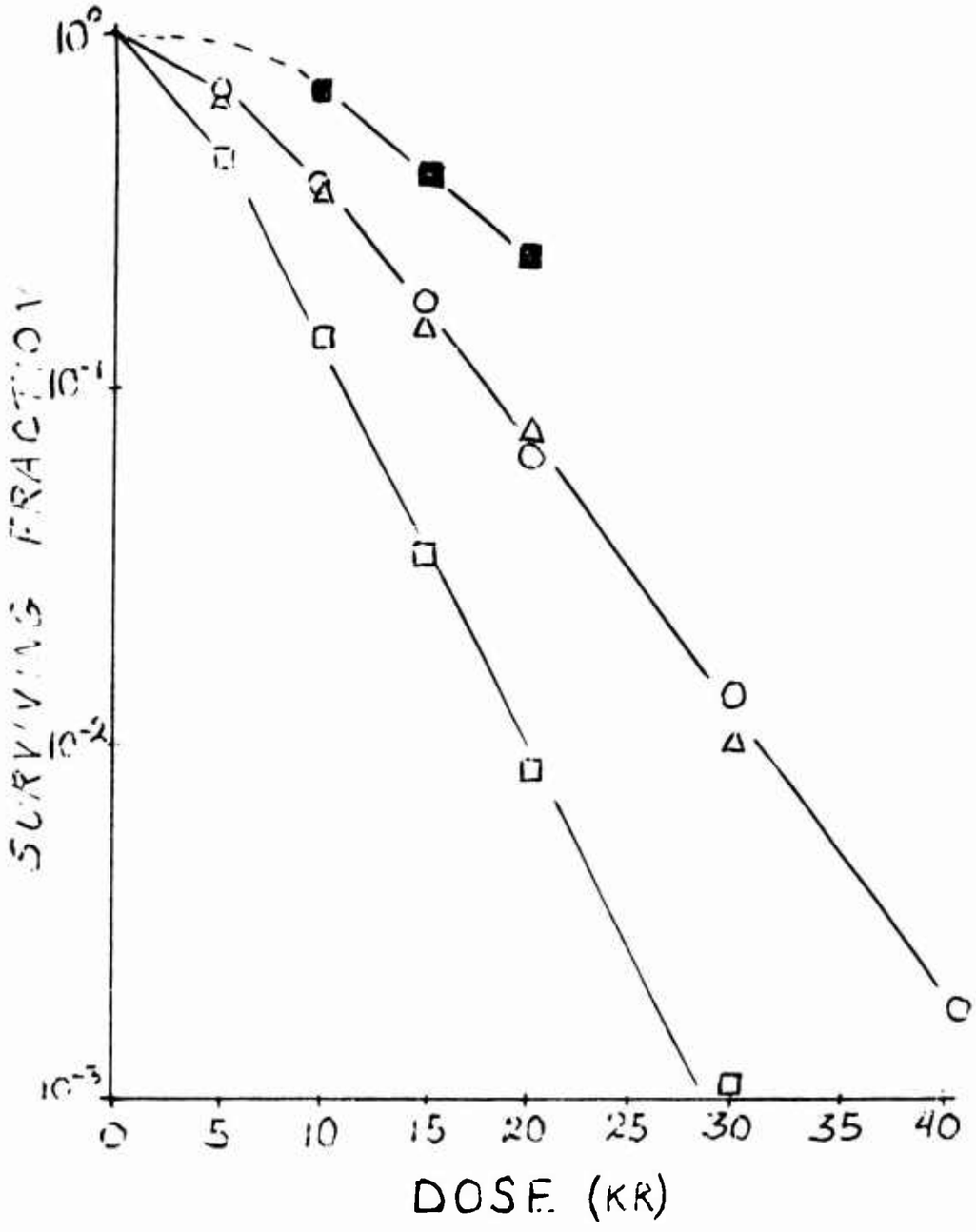

Figure 3. Radiation survival of a logarithmic-phase culture, □ irradiated in buffer; ■ irradiated in buffer with 0.04M MEA; ○ grown with 0.04M MEA for 120 min and irradiated in buffer; and, △ an early stationary-phase culture irradiated in buffer.

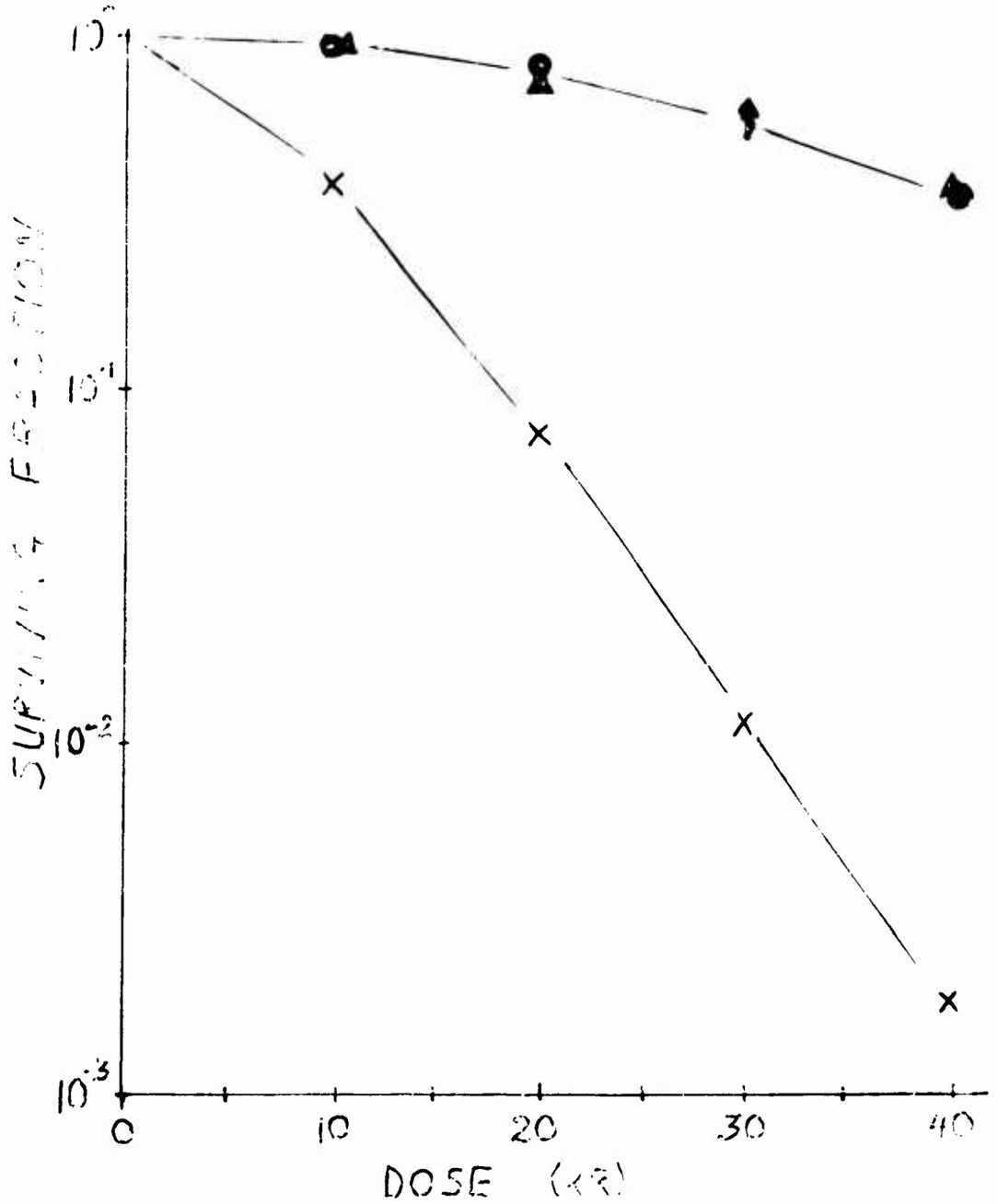

Figure 4. Radiation survival of an early stationary-phase culture, ▲ irradiated in buffer with 0.04M MEA; X grown with 0.04M MEA for 120 min and irradiated in buffer; ● a logarithmic-phase culture grown with 0.04M MEA for 120 min and irradiated in buffer with 0.04M MEA.

can also protect indirectly (biochemical) as a consequence of changes in metabolic state produced by growth of the culture with MEA before irradiation.

It also appears from these data (Figs. 3 and 4) that biochemical protection is effective in logarithmic cultures, but not in stationary phase cultures. It is also concluded that, in so far as direct protective effects of MEA are concerned, the MEA diffuses freely to the protected site(s) because (1) washing MEA-incubated cells before irradiation eliminates direct protection, and (2) maximum direct protection is obtained by adding MEA to the buffer suspension of bacteria immediately before irradiation. Furthermore, in so far as metabolic inhibition is concerned, any binding of MEA to critical constituents of the cell is probably very loose, because washing the cells into fresh growth medium without MEA immediately relieves the inhibition of net mass synthesis.

Summary and Conclusions.

The biophysical aspects of radiation injury are being studied using selected model systems. Techniques for the study of radiation effects on isolated membranes have been developed. Preliminary experiments using a sucrose-salt membrane transport cell showed the feasibility of the model system for radiation effects studies. The influence of changes in metabolic state on radiation sensitivity of bacteria is also being studied. The effects of β-mercaptoethylamine, a chemical modifier of radiation sensitivity, on growth and radiation survival of logarithmic-phase bacterial cultures were investigated. It was concluded that virtually all of the protection afforded to a logarithmic culture by pre-irradiation incubation with MEA is due to a change in growth state produced by inhibitory effects of MEA on protein syntheses, and that this protection is in addition to that afforded by the presence of MEA in the culture during irradiation.

Publications.

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Ginsberg, D. M. and J. Jagger. Radiation sensitivity of *Escherichia coli* strain 15TAU. II. Comparison between an arginine- and uracil-starved culture and a normal stationary-phase culture. *J. Gen. Microbiol.*, Vol. 39 (1965).

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