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ANNUAL PROGRESS REPORT

1 July 1967 - 30 June 1968

Volume II

AD

RCS MEDDH-288 (R1)

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

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

Annual Progress Report
1 July 1967 - 30 June 1968

Volume II

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

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SUMMARY

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

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

RESEARCH AND TECHNOLOGY RESUME

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(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.

(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.

(U) PROGRESS - JUL 67 THRU JUN 68 THE SPECIFICITY OF THE IMMUNOELECTROADSORPTION TEST WAS STUDIED. THERE WAS A DIRECT RELATIONSHIP BETWEEN OPTICAL THICKNESS AND GLOBULIN CONCENTRATION, NO SPECIFIC ANTI-S. MANSONI OR DINITROPHENYL ANTIBODIES WERE DETECTABLE. A SOLUBLE ANTIGEN FLUORESCENT ANTIBODY TEST FOR AMEBIASIS WAS DEVELOPED. THE CLINICAL EVOLUTION PIPE STEM FIBROSIS (LIVER) IN CHIMPANZEES WITH SCHISTOSOMIASIS WAS SIMILAR TO THAT IN MAN. A SOLUBLE ANTIGEN FLUORESCENT ANTIBODY TEST FOR TRICHINOSIS IN MAN AND ANIMALS WAS DEVELOPED. IN VITRO ANTIGEN-INDUCED HISTAMINE RELEASE FROM BLOOD PLATELETS OF RABBITS INFECTED WITH S. MANSONI HAS OBTAINED BOTH IN PRESENCE AND ABSENCE OF PASSIVE CUTANEOUS ANTIBODIES. MICE AND RABBITS INFECTED WITH TRICHINELLA SPIRALIS PRODUCED HOMOCYTOTROPIC ANTIGEN-LIKE ANTIBODIES DETECTABLE BY PASSIVE CUTANEOUS ANAPHYLACTIC REACTIONS AFTER A 4-HOUR LATENT PERIOD. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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Project 3A014501B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 165, Parasitic diseases

Investigators.
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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. Lack of specificity of the immunoelectroadsorption test using Schistosoma mansoni antigens and a conjugated hapten.

In 1942 Rothen and Landsteiner demonstrated that defined antigens layered on chrome-plated slides would combine with their respective antibodies. This specific interaction could be measured by a difference in film thickness obtained with homologous or heterologous antiserum. Rothen later described an improved method of measuring film thickness on a barium stearate coated slide with the use of an ellipsometer. Mathot et al. attempted to use the above technics with crude antigen preparations, but they found only a small difference in film thickness when comparing specific or nonspecific antisera. The results were difficult to reproduce and the technic was not sufficiently sensitive for detecting antibody. Negative electric current applied to the slide during the adsorption of antigen and positive current for adsorption of antisera greatly increased the thickness of the layers and permitted the use of crude antigen preparations for specific antibody adsorption. This technic was termed "immunoelectroadsorption."

The immunoelectroadsorption (IEA) test has been applied in the diagnosis of arthropod-borne virus diseases, the mouse leukemia virus (Friend), and in the study of helminthic and protozoan infections. The authors indicated that the technic was sensitive and easily adaptable to various crude antigen preparations.

The following studies were initiated to evaluate the immunoelectroadsorption technic in Schistosoma mansoni infections in man and animals, and to investigate the specificity of this technic both with a complex parasite antigen and with a relatively simple hapten.

Animals. Young adult ICR mice and WRCF rats derived from the Wistar strain were used for the preparation of antisera. All animals were fed a standard diet. The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

Buffers. Triethanolamine buffered saline (TBS), pH 7.2, was prepared by adding 7.5 g NaCl, 1.04 g $MgCl_2 \cdot 6H_2O$, 0.167 g $CaCl_2$, 2.8 ml triethanolamine and 17.7 ml 1N HCl q.s. to one liter with distilled water. Veronal buffer pH 7.6 was prepared by adding 10.3 g of sodium barbital and 17.5 ml 1N HCl q.s. to one liter with distilled water.

Antigens. S. mansoni antigen was prepared from lyophilized adult worms by the technic described by Chaffee. The antigen was lyophilized and stored at -20°C and aliquots of the same preparation were used throughout the studies. Antigen was diluted in triethanolamine buffered saline (TBS), pH 7.2, and filtered through 0.45 and 0.22 micron Millipore filters before adsorption onto metalized slides.

Dinitrophenyl bovine serum albumin (DNP-BSA) and dinitrophenyl rabbit gamma globulin (DNP-RGG) were prepared as described by Eisen; DNP-BSA contained 30 haptenic groups per mole of protein and DNP-RGG contained 26 groups.

Dinitrophenyl-e-L-lysine hydrochloride (DNP-lysine) and 2,4-dinitrophenol (DNP) were prepared in a 0.05% and 0.1% solution (w/v) of TBS respectively; these solutions were then used for antigen adsorption onto slides.

Antisera. All antisera were diluted in 0.05 M veronal buffer at pH 7.6 and filtered through 0.45 and 0.22 micron Millipore filters before adsorption.

For the preparation of S. mansoni antisera, 50 mice were each exposed percutaneously to 200 cercariae. Eight weeks after exposure the mice were bled and the pooled serum was used as mouse S. mansoni antiserum. Normal mice were bled and the pooled serum served as control serum.

Rat anti-S. mansoni serum was obtained from rats 35 days after exposure to 400 cercariae each.

Mouse anti-Plasmodium berghei serum was obtained from animals after repeated inoculations of irradiated parasitized mouse red blood cells as described by Wellde and Sadun.

Rat anti-P. berghei serum was obtained from animals repeatedly injected with 2×10^7 parasitized rat red blood cells. The rats were bled 10 days after the last challenge.

Adult rats were injected in the footpads with 0.5 mg of DNP-BSA in complete Freund's adjuvant and booster doses were given intradermally with 100 μg of DNP-BSA in saline on days 10, 20 and 35. All rats demonstrated Arthus reactions and they were bled 10 days after the last challenge. The serum was pooled and used as anti-DNP-BSA serum.

Anti-DNP antibodies were isolated from rat anti-DNP-BSA serum as described by Eisen.

Rat gamma globulin was obtained from normal rat serum by collecting the first protein peak from a diethylaminoethyl (DEAE) cellulose column equilibrated with 0.01 M, pH 7.5 phosphate buffer; rat globulin was obtained by precipitation of serum at 50% saturation with $(NH_4)_2SO_4$. Rabbit gamma globulin was obtained from Mann Research Laboratories.

Serum was collected from patients with the following well-documented infectious diseases: schistosomiasis, Chagas' disease, trichinosis, toxoplasmosis, amebiasis, hookworm, ascariasis, strongyloidiasis, malaria, opisthorchiasis, filariasis, visceral leishmaniasis, coccidioidomycosis, histoplasmosis, syphilis, and leprosy. Five sera from individuals with clinically established systemic lupus erythematosus were also included.

Normal human sera were collected from healthy donors who were undergoing physical examinations as candidates for appointment to a military academy.

Immunoelectroadsorption (IEA) technic

Testing of samples was performed with strict adherence to the described IEA technic with the exception of the minor modifications stated herein. Metalized slides (0.6 cm x 12 cm) coated with Inconel were used in all the studies. Adsorption of the antigen was performed as follows: one ml of diluted antigen was placed in a 0.8cm x 6cm pyrex test tube. A clean metalized glass slide connected to the negative pole of a D.C. supply source was inserted into the test tube along with a platinum wire connected to the positive pole of the D.C. source. The current was maintained at 300 micro amps for 60 seconds in all the experiments. After 60 seconds slides were removed from the antigen solution, rinsed with distilled water and dried with forced air at room temperature. DNP antigen-coated slides were prepared by immersion in 0.05% DNP-lysine or 0.1% DNP in TBS for 30 minutes at room temperature. The thickness of the antigen layer was measured with an ellipsometer. For antibody adsorption the slides were then immersed in a test tube containing 0.5 ml of diluted serum to be tested, and the polarity was reversed (i.e.,the slides for antiserum adsorption were connected to the positive pole and the platinum wire to the negative pole of the D.C. power source). The current was maintained at 300 micro amps for 30 seconds. The slides were then rinsed and dried and the thickness of the antiserum layer was measured with ellipsometer. The difference in thickness between the antigen layer and the antiserum layer was used to determine the relative amount of antibody in the particular sample.

To approximate the amount of antibody in an individual sample, the optical thickness of identical dilutions of the sample and a normal serum were determined. The "limiting dilution" was defined by Mathot et al. as the dilution at which the optical thickness of the sample approaches the optical thickness of the normal serum and the ratio of sample/normal serum optical thickness approaches unity. A 1.4 to 1 ratio was selected as the point at which an antiserum was considered reactive with the adsorbed antigen.

The thickness of the layer is expressed in numerical units representing degrees of rotation of the ellipsometer analyzer; these units can be converted to angstroms by using a barium stearate calibration curve. Under the experimental conditions used, one unit on the ellipsometer is equivalent to 2.4 angstroms. All results are expressed as units of optical thickness and these units can be used as an index of the relative antibody concentration of different antisera at a given dilution.

Serum proteins. Total serum protein values were determined on individual samples using the ultramicro adaption of the Biuret technic as described by Williams et al. The protein concentration of rat globulin, gamma globulin and anti-DNP antibody was estimated by spectrophotometric adsorption at 2800 angstroms using 0.0091 as the optical density per μg N per ml. Serum electrophoresis was used to estimate the albumin to globulin ratio in whole serum.

Passive hemagglutination. Tannic acid-treated sheep erythrocytes were prepared and the cells were sensitized with DNP-RGG or DNP-BSA according to previously described methods. The patterns were read and scored by the method of Stavitsky. Titers are expressed as the reciprocal of the greatest dilution giving a 2 plus reaction.

I. IMMUNOELECTROADSORPTION IN SCHISTOSOMIASIS

A. Effect of antigen and antiserum dilution: Preliminary studies indicated that the IEA technic using adsorbed S. mansoni antigen could differentiate between infected and normal mouse sera. To determine the optimal concentration of antigen for antisera adsorption, varying dilutions of antigen were adsorbed onto the slides and reacted with constant dilutions of normal and S. mansoni antisera (Table 1). The optical thickness of adsorbed antigen varied inversely with the dilution used. At any of the dilutions studied a significant difference was noted between the optical thickness of adsorbed normal mouse serum and antiserum. The optimal antiserum/normal serum ratio of 1.65 was obtained with an antigen dilution of 1:20 which was used throughout these studies.

The sensitivity of the technic was determined by testing increasing dilutions of mouse anti-S. mansoni sera obtained 8 weeks after exposure to cercariae (Table 2). At a dilution of 1:320 a significant difference in optical thickness was still noted between normal sera and sera from infected animals.

Table 1

Results obtained in the IEA test using different dilutions of S. mansoni antigen for adsorbing normal and anti-S. mansoni mouse sera diluted 1:20

Antigen dilution	Optical thickness (units)			Antiserum/ normal serum
	Antigen	Normal serum	S. mansoni antiserum	Ratio
1:10	5.7	10.9	17.9	1.64
1:20	4.0	9.7	16.0	1.65
1:40	3.0	6.8	10.8	1.59
1:80	2.6	6.2	9.2	1.49

Table 2

Results obtained in the IEA test using S. mansoni antigen diluted 1:20 for adsorbing different dilutions of normal and anti-S. mansoni sera

Serum dilutions	Optical thickness (units)		Antiserum/ normal serum
	Normal serum	S. mansoni antiserum	Ratio
1:10	10.4	19.1	1.84
1:20	8.5	16.0	1.88
1:40	7.8	12.9	1.65
1:80	6.1	9.8	1.60
1:160	5.3	8.5	1.60
1:320	4.6	7.1	1.54

B. Specificity of the S. mansoni antigen with animal sera: To study the specificity of IEA with S. mansoni antigen, sera from anti-P. berghei, anti-S. mansoni and normal rat and mouse pools were compared at a constant dilution of 1:20 (Table 3). Positive results were obtained with both anti-S. mansoni and anti-P. berghei mouse sera. The relatively high 1.8 ratio obtained with the combination of anti-P. berghei sera and S. mansoni antigen suggested that antigenic cross-reactivity existed between these parasitic infections or that proteins were adsorbed non-specifically in sera from plasmodial infected rats.

With rats, anti-P. berghei sera were more reactive than normal sera, but they were less reactive than anti-S. mansoni sera. Antigenic cross-reactivity appeared to be present to a lesser degree in rat infections than in mouse infections.

Table 3

Comparison of results in the IEA test using S. mansoni antigen (1:20) for adsorbing S. mansoni and P. berghei antisera

	Mouse sera diluted 1:20			Rat sera diluted 1:20		
	Normal	Anti-S. mansoni	Anti-P. berghei	Normal	Anti-S. mansoni	Anti-P. berghei
Optical thickness	8.9	13.0	16.1	8.1	11.5	10.0
Ratio	-	1.46	1.81	-	1.42	1.24

C. Specificity of S. mansoni antigen with human sera: Serum samples from patients with proven schistosomiasis, from patients with a variety of infections other than schistosomiasis, and from patients with systemic lupus erythematosus were studied. All sera were diluted 1:20 and tested for the thickness of adsorption with slides coated with S. mansoni antigen and with slides containing no antigen (Table 4). Positive reactions were obtained with all sera tested. The highest ratios were noted with sera from patients with lupus erythematosus, opisthorchiasis and filariasis. The presence or absence of S. mansoni antigen adsorbed on the slide did not greatly alter antiserum adsorption even with sera from patients with schistosomiasis.

The values of optical thickness obtained from the individual human sera were divided into groups on the basis of a numerical range. The mean gamma globulin concentration of the sera in each group was then determined. As expressed graphically in Fig. 1, there was a direct

relationship between the thickness of the adsorbed layer determined by IEA and the gamma globulin content of the serum samples. The gamma globulin concentration produced a higher optical thickness on antigen-free than on antigen-coated slides (Table 4, Fig. 1). This difference in optical thickness was noted even when sera from patients with schistosomiasis were adsorbed onto both S. mansoni antigen-coated slides and antigen-free slides.

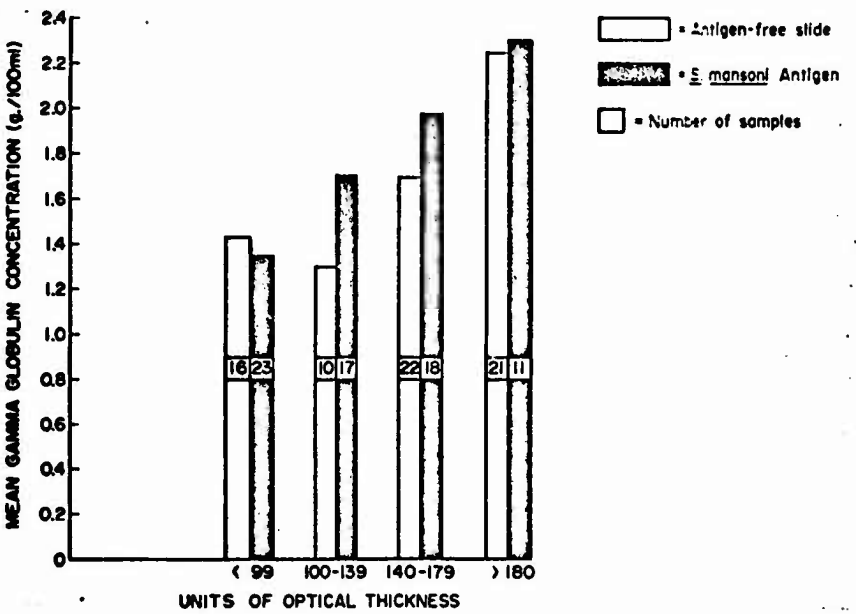


Fig. 1. Mean gamma globulin concentration in samples of human sera grouped according to units of optical thickness obtained with the IEA technic.

II. EFFECT OF GAMMA GLOBULIN AND ALBUMIN ON IEA

The effect of nonspecific gamma globulin and albumin on the adsorption thickness obtained from samples was studied. Aliquots of normal serum were prepared; increasing amounts of rabbit gamma globulin or bovine serum albumin were added, and the samples were then adsorbed onto slides coated with S. mansoni antigen. As shown in Fig. 2a the presence of increasing amounts of albumin had little or no effect on optical thickness. Conversely, increasing concentrations of gamma globulin (Fig. 2b) progressively increased the optical thickness. Similar results were obtained when the same samples were adsorbed onto antigen-free slides.

Table 4

Comparison of results obtained in the IEA test using slides with and without S. mansoni antigen for adsorption of sera from 65 patients with various types of infections or disorders

Diagnostic status	Number tested	Optical thickness (units)					
		S. mansoni antigen slides			Antigen-free slides		
		Mean	Range	Ratio*	Mean	Range	Ratio
Normal	16	5.6	4.0-7.8	-	7.2	5.8-8.1	-
Schistosomiasis	5	13.5	11.5-17.4	2.40	16.9	14.9-19.8	2.34
Chagas' disease	3	9.8	8.5-11.8	1.74	14.9	11.2-18.7	2.06
Coccidioidomycosis	5	16.0	14.1-19.6	2.84	17.7	13.9-22.7	2.45
Histoplasmosis	5	17.9	12.9-27.8	3.19	20.1	14.4-28.0	2.78
Syphilis	5	13.5	11.7-14.9	2.40	16.8	12.6-19.3	2.32
Leprosy	5	17.4	13.4-20.7	3.10	18.7	15.5-24.9	2.57
Malaria	3	12.7	10.8-13.8	2.26	17.2	14.8-21.2	2.38
Filariasis	3	18.7	8.5-38.5	3.33	25.9	11.9-52.5	4.61
Opisthorciasis	1	42.4	42.4	7.55	51.8	51.8	7.15
Hookworm	1	9.5	9.5	1.69	13.1	13.1	1.81
Ascariasis	1	10.2	10.2	1.81	11.3	11.3	1.56
Trichinosis	1	14.3	14.3	2.54	15.0	15.0	2.07
Strongyloidiasis	1	9.0	9.0	1.60	12.2	12.2	1.69
Visceral leishmaniasis	1	12.7	12.7	2.26	23.3	23.3	3.22
Toxoplasmosis	2	14.3	13.1-15.4	2.54	14.9	14.3-15.4	2.05
Amebiasis	2	12.9	10.2-15.5	2.29	13.0	10.9-15.1	1.80
Lupus erythematosus	5	28.7	22.2-36.7	5.09	32.2	23.0-47.3	4.46

*Ratio = sample/normal serum

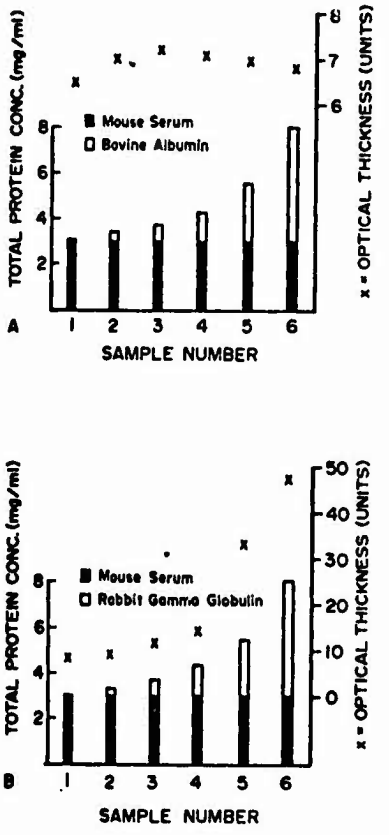


Fig. 2. The effect of albumin (A) and gamma globulin (B) on the optical thickness obtained from samples of normal mouse sera.

III. ANTIBODY DETECTION WITH IEA AND PASSIVE HEMAGGLUTINATION

A pool of rat anti-DNP-BSA sera was studied for the presence of antibody directed against the hapten DNP by using both IEA and the passive hemagglutination technics. DNP-lysine, DNP or DNP-BSA in a solution of TBS was adsorbed onto slides for use in IEA studies. Tanned sheep red blood cells were sensitized with DNP-RGG or DNP-BSA for hemagglutination tests. Initial studies with increasing dilutions of anti-DNP-BSA sera gave decreasing optical thicknesses and hemagglutinating titers. To determine the effect of rat globulin on these results anti-DNP-BSA sera were serially diluted in rat globulin. Four samples containing decreasing amounts of antisera and increasing amounts of normal globulin were then tested. As shown in Fig. 3 a continual increase in adsorption thickness was obtained from samples 1 through 4. This occurred

with the addition of increasing amounts of globulin to samples 2 through 4 as the antiserum concentration decreased in samples 1 through 3. The hemagglutinating titer decreased with decreasing amounts of antisera; however, a positive titer of 64 was obtained with normal rat globulin. When the immunizing antigen, DNP-BSA, was adsorbed onto the slide, the results were the same as reported above.

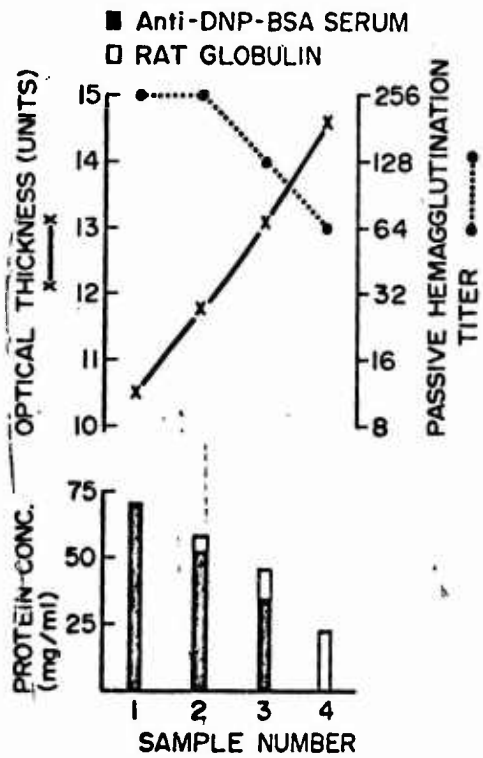


Fig. 3. Immunoelectroadsorption and passive hemagglutination reactions on samples of anti-DNP-BSA serum, normal rat globulin, and combinations of the two. Cells were sensitized with DNP-RGG, and the slides were coated with DNP-lysine.

Purified anti-DNP antibody was prepared and the anti-hapten antibody was tested for hemagglutinating and adsorbing capacities. Samples of antibody were mixed with increasing amounts of rat gamma globulin. The results of antisera adsorption and passive hemagglutination are shown in Fig. 4. Although the ellipsometer readings were low due to a decreased amount of protein, the hemagglutinating titers indicated that a significant

amount of specific antibody was present. The ellipsometer readings varied according to the nonspecific gamma globulin concentrations, whereas hemagglutinating titers decreased with decreasing concentrations of antibody. The normal rat gamma globulin containing no known anti-DNP antibodies gave a hemagglutination titer of 250.

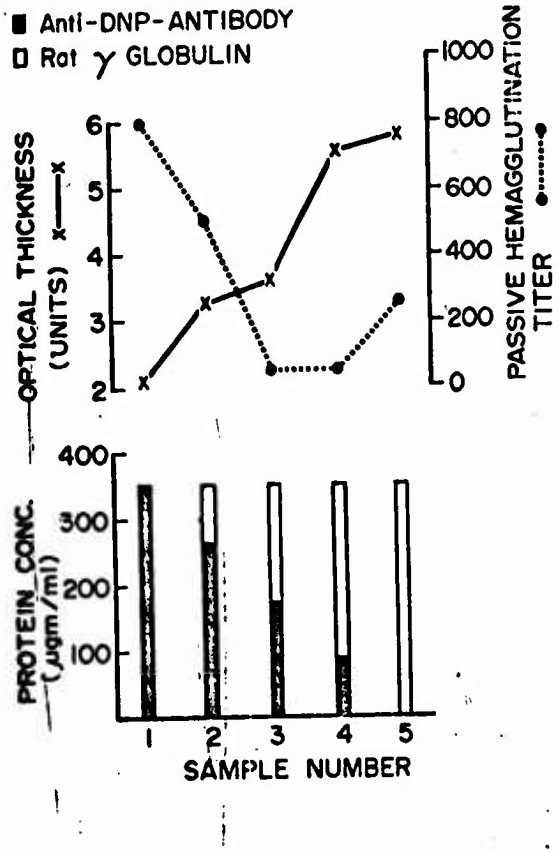


Fig. 4. Immunoelectroadsorption and passive hemagglutination reactions on samples of anti-DNP antibody, rat gamma globulin and combinations of the two. Cells were sensitized with DNP-RGG and the slides were coated with DNP.

Immunoelectroadsorption and passive hemagglutination studies were compared in anti-DNP-BSA sera after the addition of increasing amounts of DNP-BSA (Table 5). The hemagglutinating titer was completely inhibited with 400 μg of DNP-BSA. In contrast, the addition of the immunizing antigen to the antisera had no consistent effect on the IEA test.

An aliquot of anti-DNP-BSA serum was studied by IEA and passive hemagglutination technics before and after adsorbing out anti-hapten

antibody. Sheep red blood cells sensitized with DNP-RGG were used for adsorbing the antibody. The anti-hapten antibody titer detected by passive hemagglutination was reduced from 1920 to 30. The thickness of the adsorbed antiserum layer on a DNP-lysine coated slide increased in antisera repeatedly adsorbed with DNP-RGG coated sheep cells. This is the reverse of the results obtained with passive hemagglutination and could not be explained on the basis of a change in protein concentration determined by adsorption at 2800 angstroms.

Table 5

Comparison of passive hemagglutination and immunoelectroadsorption tests with antisera after addition of DNP-bSA

DNP-BSA added (μg/ml)	Passive hemagglutination titer*	Immunoelectroadsorption Optical thickness (units)**
0	6400	13.6
200	200	11.6
400	0	12.2
900	0	13.6

*Cells sensitized with DNP-BSA
**Slide adsorbed with DNP-BSA

The results of these experiments indicate a lack of specificity of the immunoelectroadsorption technic with a complex parasite antigen (S. mansoni) and with a relatively simple conjugated hapten antigen-antibody system. In animal schistosomiasis, a significant difference on the basis of adsorption thickness between serum specimens obtained from infected animals and those obtained from uninfected controls was observed. This is in agreement with results obtained in previous studies with other infectious diseases. However, when slides were adsorbed with S. mansoni antigen and then reacted with mouse anti-P. berghei sera, a greater optical thickness was obtained than with the homologous system. This led to a series of investigations designed to determine whether the results could be attributed to immunological cross-reactivity between P. berghei and S. mansoni antigens or to nonspecific adsorbing substance(s) present in the serum of infected animals. Investigations with human sera collected from individuals with a variety of infectious or degenerative diseases indicated that the "false positive" results were due to nonspecific substances present in the serum specimens and that a direct relationship between the optical thickness and the globulin concentration in a given serum was evident. The elevated gamma globulin concentrations produced a higher optical thickness even in the absence of antigen on the slides.

When the protein concentration of a given sample was increased by the addition of albumin, there was no significant change in the adsorbing characteristics of the sample as determined by optical thickness on a S. mansoni antigen-coated slide (Fig. 2A). In contrast, if the total protein concentration of the samples were duplicated by adding nonspecific gamma globulin instead of albumin, a progressive increase in optical thickness was noted when the various samples were adsorbed onto an antigen-coated slide (Fig. 2B). The reason for the difference in nonspecific adsorbing ability between albumin and globulin was not determined.

The specificity of IEA and passive hemagglutinating technics for antibody detection was compared under conditions of increased globulin concentrations. The hemagglutinating titers were directly related to the concentration of antisera or specific antibody present in a given sample. However, positive reactions at low titers were obtained by adding cells sensitized with DNP-RGG to nonspecific globulin and gamma globulin. This indicates that a certain population of gamma globulin molecules from normal animals can react with DNP or the red cell membrane altered by sensitization. "Natural antibodies" are known to exist and have been described for many substances. As noted in Fig. 4, the optical thickness increased when the total protein concentration of the samples remained constant. This is of interest and suggests that the antigen-coated slide is more reactive with nonspecific gamma globulin than with the purified antibody.

When antibody activity in a given sample was studied both with IEA and hemagglutinating technics, it was apparent that optical thickness was a function of globulin concentration and was not dependent on specific antibody content. This was confirmed by antigen inhibition and antibody adsorbing studies, both of which failed to effect the IEA results. Added evidence of nonspecificity of the IEA technic was obtained with antigen-coated and antigen-free slides. The presence of a known antigen on the slide did not enhance the adsorption of the antiserum layer or the ability to differentiate between homologous or heterologous antisera. The possibility that specific antibodies may be adsorbed onto an antigen-coated slide cannot be excluded. However, it was obvious that under the conditions described for the use of this technic the adsorption of nonspecific globulin was of such magnitude that it may have masked any specific antigen-antibody reaction.

Although the full assessment of the possible utilization of the IEA technic in the serology of infectious diseases must await further experimentation, the results of these studies leave little doubt as to the lack of specificity of this test both with S. mansoni antigens and a conjugated hapten. Furthermore, they emphasize the necessity of conducting rigid specificity studies before assuming that the results obtained with a new serologic test are directly attributable to antigen-antibody reactions.

2. Soluble antigen fluorescent antibody test (SAFA) for amebiasis.

The value of an indirect fluorescent antibody test for the serodiagnosis of parasitic infections has been established in recent years.

The use of soluble antigens in this test by placing them on a matrix of cellulose acetate filter paper was suggested by Parchetto and applied to parasitic infections by several investigators. This technic obviates the need for maintaining the parasite in the laboratory where the tests are performed, permits the investigator to select and purify the antigen to be employed, and provides means of mechanically reading the test.

Serologic tests for the immunodiagnosis of amebiasis using Entameba histolytica extracts as antigen had a long and rather discouraging history before the development of hemagglutination, complement fixation, and precipitin tests using extracts of axenic or monobacterial amebic cultures. Recently, Goldman described an indirect fluorescent antibody test for amebiasis in which intact formalin-fixed amebae from cultures with mixed bacterial flora were used as a source of antigen. With this test he reported positive results in 73 percent of cases of confirmed intestinal amebiasis and 51 percent of cases with extra-intestinal amebiasis. However, nearly one-third of individuals without amebic infections were also found positive at low titers.

Although it is evident that antibody activity against E. histolytica can be demonstrated by various serologic technics, the performance of routine testing procedures has been handicapped heretofore by various technical difficulties. Prominent among these are the unavailability of standardized bacteria-free antigens and the lack of relatively simple and inexpensive instruments which permit an objective reading of the test. Now, however, these two handicaps can be obviated by the commercial preparation of an antigen derived from axenically cultured E. histolytica for use in a soluble antigen fluorescent antibody (SAFA) test.

This report describes a SAFA test developed for the laboratory diagnosis of amebiasis. The sensitivity and specificity of this test were evaluated with sera from humans with proven amebiasis and from individuals with other communicable or degenerative diseases as well as with sera from healthy controls.

Sera. Human sera from 303 well documented cases were tested by the soluble antigen fluorescent antibody test. Of these, 52 were obtained from proven amebiasis patients. Diagnosis was established on clinical grounds, serologic tests and, in most cases, by the recovery of E. histolytica organisms in stools, aspirates or tissue sections. The examinations were performed by experienced workers. A total of 99 control sera from individuals with proven viral, bacterial or parasitic infection other than amebiasis was used in determining the specificity of the test. Normal sera were obtained from 52 individuals who were undergoing physical examination as candidates for appointment to a military academy. All of the specimens were stored at -20°C until the time of testing. Some specimens were lyophilized; others had been preserved with merthiolate, since preliminary tests indicated that lyophilization or merthiolate in the amounts used for serum preservation did not interfere measurably with the reactions. All sera were diluted 1:8 with 0.85 NaCl containing

2 percent Tween 80 in 0.05 M tris-2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) buffered (pH 8.0) and tested for reactivity. Twofold dilutions of sera reacting at 1:8 were then tested to determine their titer.

Antigen. The antigen was obtained from axenically cultured E. histolytica (NIH-200 and HK-0 strains) in TPS-1 media in a concentration of 10^7 amebae/ml. The optimal dilution of this antigen in the complement fixation test was 1:200 and in the hemagglutination test was 1:80. The optimal antigen dilution for the SAFA test was determined by box titrations against two serum specimens with hemagglutination titers of 1:36 and 1:81,000 respectively. A 1:20 dilution of the stock antigen was used in these experiments.

Test procedure. These tests were conducted essentially as described by Toussaint and by Sadun and Gore. A negative control serum was used to set the fluorometer dial at zero. The optimal dilution of conjugate was determined by box titrations. In most instances a 1:20 dilution of labelled antihuman globulin was used. On the basis of previous tests, arbitrary values were established for the interpretation of fluorometer dial readings. A reading of 6 or less was recorded as non-reactive. A reading of 7 through 9 was interpreted as a weak (doubtful) reaction and a reading of 10 or more was recorded as a positive reaction.

Results obtained with the SAFA test are summarized in Table 6. The findings with sera from amebiasis patients illustrate the relative sensitivity of this procedure. The findings with sera from patients with other conditions and from healthy individuals provide an index of the specificity of this test. Frequent cross reactions were observed with sera from visceral leishmaniasis patients. Only two serum specimens (4%) from individuals with proven amebiasis failed to react in this test, whereas 96% of specimens from 152 healthy controls gave negative reactions. As indicated in Figure 5, the reactive specimens from healthy controls were positive at low titers (up to 1:32). Conversely, most of the specimens from individuals with proven amebiasis reacted at much higher titers.

Clinical information was available on 35 of the 52 amebiasis patients. Of these, 15 had extra intestinal amebiasis, 12 were classified as patients with amebic dysentery and 15 were asymptomatic. As indicated in Table 7, positive reactions at high titers were obtained in all of the extra intestinal amebiasis patients, lower titers were observed for patients with amebic dysentery and the lowest titers were observed in the asmymptomatic individuals. The only two persons with proven amebic infection who did not react in this test at a titer of 1:8 were in the undefined category.

In order to obtain information on the reproducibility of results with this test, serum specimens from 6 infected individuals and 6 uninfected controls were each divided into aliquots and tested at 6 different times with the same lot of antigen and antiglobulin. None of

Table 6

Results of the Soluble Antigen Fluorescent Antibody Test for Amebiasis

Diagnostic Status	No. Tested	Reaction No.	Reaction %	Weak Reaction No.	Weak Reaction %	No Reaction No.	No Reaction %
Amebiasis	52	42	80	8	16	2	4
Healthy Controls	152	6	3	2	1	146	96
Infections other than Amebiasis	99	12	12	9	10	78	78
Parasitic Infections	62	8	13	7	11	47	76
Paragonimiasis	3	0	0	0	0	3	100
Toxoplasmosis	10	0	0	1	10	9	90
Malaria	4	0	0	1	25	3	75
Visceral leishmaniasis	16	7	44	3	18	6	38
Filariasis	12	0	0	1	8	11	91
Chagas' disease	6	1	17	1	17	4	66
Echinococcosis	1	0	0	0	0	1	100
Schistosomiasis mansoni	4	0	0	0	0	4	100
Schistosomiasis hematobia	6	0	0	0	0	6	100
Other Infections	37	4	18	2	9	31	73
Gastroenteritis	10	0	0	0	0	10	100
Bacillary dysentery	7	1	14	0	0	6	86
Plague	8	1	13	1	13	6	74
Leprosy	5	0	0	0	0	5	100
Syphilis	7	2	28	1	14	4	58

Fig. 5

DISTRIBUTION OF TITERS IN THE SOLUBLE ANTIGEN FLUORESCENT ANTIBODY TEST FOR AMEBIASIS

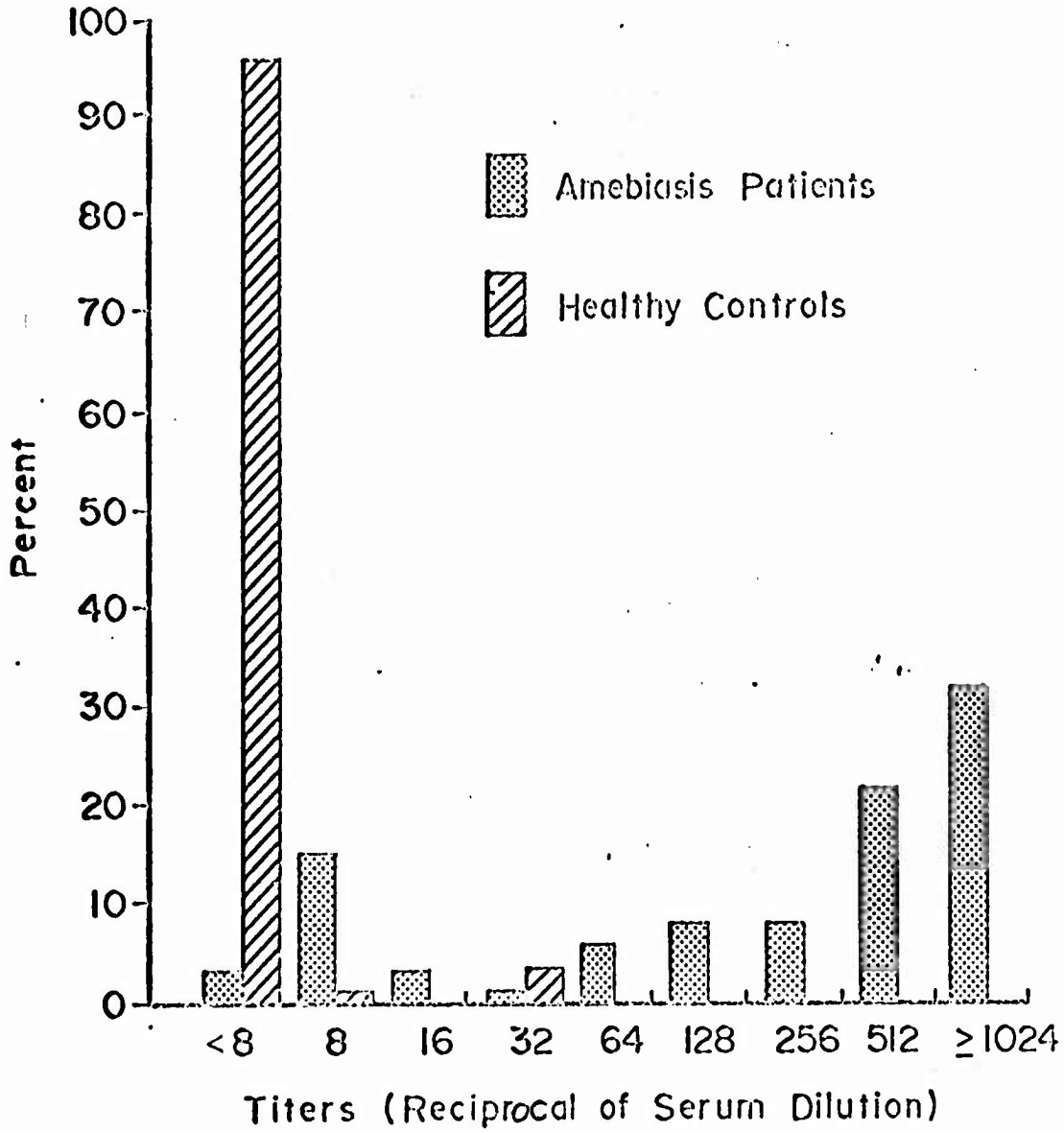


Table 7

Serum Titers Obtained from Amebiasis Patients

Clinical Category	<1:8	1:8	1:16	1:32	1:64	1:128	1:256	1:512	>1:1024	Total
Extra-intestinal amebiasis							1	5	9	15
Amebic dysentery					2	1	2	4	3	12
Asymptomatic			2	1	1	3	1			8
Undefined	2	7						3	5	17
Total	2	7	2	1	3	4	4	12	17	52

the 36 specimens from uninfected controls gave positive reactions (Table 8). Conversely, only 2 of the 36 specimens from proven infections gave a negative reaction. Both of these came from a specimen which, even when positive, reacted at the lowest titer (1:8). In all but one instance the highest and lowest titer obtained with a given specimen was within a fourfold dilution.

It is evident from these results that the use of the SAFA test employing as antigen a commercially available preparation from axenically cultured Entameba histolytica is a feasible procedure. A relatively high degree of sensitivity, specificity, and reproducibility of results was observed. However, the occurrence of cross-reactions with a number of sera from patients with visceral leishmaniasis and other infectious diseases should be investigated further before the reliability of this test can be fully assessed. In general, antibody titers could be correlated with the degree and location of organ involvement.

The results suggest that the SAFA test may provide a relatively simple and reliable procedure for the diagnosis of amebiasis of particular value to small laboratories if reagents can be made available from commercial sources. Moreover, the actual reading of the test is rapid and objective since it can be obtained by means of a fluorometer. A greater experience with this test and the use of more purified antigenic extracts should reduce the number of doubtful reactions and offer possibilities of greater standardization.

3. Experimental production of bilharzial pipe stem fibrosis in the chimpanzee.

The morbidity of endemic, human schistosomiasis mansoni and japonica is primarily due to bilharzial pipe stem fibrosis of the liver ("Symmers' clay pipe stem fibrosis"). The diagnostic criteria of this lesion have never been formally defined, but the following features are considered characteristic: (1) Grossly evident, diffuse, stellate fibrosis and enlargement of the large and medium sized portal field; (2) Variable portal inflammatory infiltration and crowding of the portal areas with schistosome eggs and granulomas (active stage) or with scar tissue containing egg shells and pigment (late stage); (3) Conservation of lobular architecture except for focal porto-central fibrous banding or post-necrotic scarring usually confined to the subcapsular parenchyma; (4) Relatively normal hepatocytic structure and function (except terminally and in the presence of complications); and (5) Lesions of the intrahepatic portal radicles, such as phlebosclerosis, narrowing, collateral formation or organizing thrombi with a distinctive circulatory anomaly on injection study, frequently with presinusoidal portal hypertension. Together these features result in a disease pattern distinct from other forms of human liver cirrhosis and virtually pathognomonic on gross examination (Fig. 6).

Granulomatous inflammation with mild fibrotic enlargement of the small portal fields is commonly found in uncomplicated S. mansoni and S. japonicum infection. This condition occurs both in natural and experimental hosts,

Table 8

Results of Repeating the Soluble Antigen Fluorescent Antibody Test Six Times Using the Same *E. histolytica* Antigen and the Same Antiglobulin

Serum Specimen	Diagnosis	Number of times given titer was obtained								
		<8	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
RM	Normal Control	6								
TE	"	6								
BA	"	6								
KI	"	6								
DH	"	6								
AP	"	6								
7072	Amebiasis							1	2	3
1195	"							1	4	1
7B	"						6			
7151	"				5			1		
5957	"		1	1	4					
5956	"	2	4							

including man, and frequently it can be reversed by effective treatment. Conversely, pipe stem fibrosis has thus far been reported only in man and in goats infected with a non-human schistosome, S. spindale.



Fig. 6. Gross appearance of bilharzial pipe stem fibrosis in a 42 year old heavily infected male patient at post-mortem examination, Bahia, Brazil, 1951.

Among laboratory animals, portal fibrosis, irregularity of the liver surface and portal hypertension have been produced in mice, amyloidosis of the liver in aging hamsters, and irregular, broad-banded liver scarring in albino rabbits spontaneously recovering from experimental S. mansoni infections. Most primate species develop more bilharzial pathology of the gut than of the liver, and only a few heavily infected green monkeys have shown liver scarring.

Although laboratory animals develop lesions which fulfill some of the criteria of pipe stem fibrosis, they do not reproduce to serve as useful models of the human prototype. To our knowledge, this is the first description of experimental pipe stem fibrosis produced in a non-human host by a schistosome species pathogenic to man. A more detailed analysis of the pathophysiologic sequence and pathogenesis of experimental pipe stem fibrosis will follow this preliminary report.

Twelve chimpanzees of West African origin including seven which had been previously splenectomized for purposes of an unrelated experiment were infected with the Puerto Rican strain of S. mansoni. Prior to exposure, these animals were free of schistosomes or other naturally acquired parasites of significance and were in good health. They were supplied with an adequate diet at all times. In a first experiment,

six chimpanzees were exposed to a single dose of 250 to 2000 cercariae. Following exposures, detailed clinical, parasitologic, immunologic and radiologic studies were conducted for seven months, at which time the animals were autopsied and their worm loads determined. In a subsequent series, six additional chimpanzees were divided into three matched pairs, each comprising one intact and one previously splenectomized subject. The first pair of chimpanzees was exposed to a single dose of 1000 cercariae; the second pair was exposed monthly to 100 cercariae, and the third pair was exposed monthly to 250 cercariae. Exposures were made according to published methods. The animals in this series were studied for nearly two years after exposure to infection (Table 9). The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

The present report summarizes some observations made on three heavily infected animals (1000-2000 cercariae) of the initial group which were sacrificed seven months after exposure and all six of the group studied for two years after exposure including one animal which was autopsied after two years.

The following parasitologic, clinical, serologic, pathologic and radiologic observations were made:

Procedure	Technique	Interval	Repetitions
Physical exam	Veterinary clinical obsvn.	a. weekly (for 15 weeks then monthly)	18
		b. monthly	24
Weight	Standard	a. weekly (15 weeks then monthly	18
		b. monthly	24
Temperature	Rectal, thermocouple	a. weekly (15 weeks then monthly	18
		b. monthly	24
Stool egg counts	AMS III	a. bi-weekly after 7 weeks	40
		b. weekly	94
Serology, schistosomiasis	Passive cutaneous anaphylaxis and slide flocculation (cercariae, adult ES, egg antigens	a. monthly	7
		b. monthly	24

Procedure	Technique	Interval	Repetitions
Hematology	Hematocrit, WBC and RBC counts, differential WBC	a. weekly b. weekly, 1 yr, then monthly	28 54
Biochemistry	Bromsulfalein clearance, SGO-T, SGP-T, total protein and electrophoresis	a. not done b. monthly	-- 24
Liver biopsy	Menghini needle	a. monthly b. monthly	7 24
Liver biopsy	Surgical	a. not done b. semi-annually	-- 2
Esophagoscopy	Standard	a. not done b. 2 years	-- 1
Barium swallow	Standard	a. not done b. 2 years	-- 1
Splenoportogram	Standard	a. semi-annually b. annually	1 2
Portal pressure	Standard	a. not done b. 2 years	-- 1

a. 7 month experiment
b. long-term experiment

Surgical biopsies were done under generalized sodium pentobarbital anesthesia, with the pathologist in attendance. Significant gross findings were recorded, and splenoportograms and vein pressure measurements were selectively performed at these times. All liver specimens were sectioned serially and stained with hematoxylin-eosin, Masson's trichrome, Wilder's reticulum, and Verhoeff-van Gieson elastica stains. Chimpanzee No. 15 had a stormy post-operative course after each biopsy and died after the second surgical liver biopsy. Complete post-mortem studies included worm counts by the Perf-O-Suction techniques and portal angiograms as described earlier, except that in animal No. 15 a latex medium was used instead of a modified Schlesinger mass. Pathologic descriptions were based mainly on the findings at autopsy and at surgical biopsy, since the alternating needle biopsies yielded only limited histopathologic information (other than confirmation of active infection and, in some instances, of portal fibrosis).

Table 9. Maximum and mean number of eggs per gram of feces (NEPGF) for given cercarial exposures

Animal no.	No. cercariae per exposure	No. exposures	Total no. cercariae	Duration of infection (weeks)	Maximum NEPGF	Mean NEPGF	Peak NEPGF (week)
5	1000	1	1000	30	104	35	19
8	2000	1	2000	30	85	34	7
10	2000	1	2000	30	49	19	14
2	1000	1	1000	100	539	180	51
38	1000	1	1000	100	107	20	47
15	100	21	2100	100	314	76	40
42	100	24	2400	100	469	121	100
22	250	24	6000	100	207	44	51
41	250	24	6000	100	1335	368	100

The results of parasitologic, clinical, serologic and radiologic observations will be reported in detail upon completion of the experiments at a later date. Therefore, only those findings which might have a bearing on the pathologic observations will be summarized here.

1. Parasitologic, clinical and serologic observations (Table 9).

The onset of patency and fecal egg excretion varied individually and were related to the size of exposure. Each chimpanzee maintained reasonably steady average egg counts over the period of observation. There was an imperfect correlation between cercarial doses and levels of egg-excretion, with discrepancies between the members of each pair. However, in general, higher exposures resulted in higher egg-outputs.

In the animals exposed to a single cercarial dose, the most dramatic clinical changes were seen during the third month after exposure. A temperature rise up to 102.5°F was noted in those chimpanzees (Nos. 8, 5, and 2) which were exposed once to large numbers of cercariae. Some animals showed diarrhea, anorexia, and decreased activity during this period. Eosinophilia occurred in eight of nine chimpanzees about the eighth or ninth week. Transitory hepatomegaly and ascites were noted about three weeks later in two of the chimpanzees which were exposed once to large numbers of cercariae (Nos. 8 and 5). Subject No. 41 began to develop ascites and abdominal distention about 16 months after exposure, and his blood count and hematocrit dropped to 2,670,000 and 25% respectively. In one chimpanzee (No. 41) a definite submucosal esophageal varix was observed endoscopically nearly two years after the first exposure. In another animal (No. 22) increased vascularity was noted. No superficial abdominal portocaval collaterals were seen. A few months later, Subject No. 42 began to show some increase in abdominal distention with possible ascites, and a drop in hematocrit. No intestinal bleeding was evident in either of these animals.

The pattern of serologic reactions was similar to that in previous observations. Positive reactions in the flocculation tests were observed by the sixth week. The titers increased rapidly and a plateau was usually reached 8-12 weeks after infection. Passive cutaneous anaphylaxis tests became positive in some animals at 12 weeks and remained positive up to 7 months after infection.

The results of serum globulin determinations indicated a marked degree of hyperglobulinemia, ranging from 3.9 to 7.2 gms percent, a magnitude rarely observed in man, and this was sustained over a long period of time. Serum albumin levels remained essentially normal.

2. Gross pathologic and radiologic observations

In general, the liver did not become permanently enlarged after infection. However, the consistency of liver tissue was increased in most cases, especially after two years. Pigmentation and fine superficial granularity of the liver appeared by the seventh month and were

found in all but one subject (No. 38) after two years. At that time, Chimpanzee No. 41 showed distinctive, coarsely linear scarring and pitting of the liver surface, forming a widely spaced, geographic pattern. On cross-section, prominence of the portal fields appearing as a delicate white linear pattern radiating from the hilus toward the liver capsule was observed as early as seven months after exposure in one subject (No. 5). After one year, two additional chimpanzees (Nos. 41, 42) showed a more prominent development of this lesion, while three of them (Nos. 2, 15, 22) showed only subtle prominence of small portal fields (Fig. 7). Gross alterations were absent in one animal (No. 38). After two years, Case No. 41 had developed the classical macroscopic features of pipe stem fibrosis, described above (Fig. 7). In this subject, the spleen was indurated and enlarged to the level of the iliac crest. Lesser splenic induration was noted in one additional animal (No. 42) one year after infection.

The extrahepatic portal tributaries appeared distended in four post-mortem angiograms, done at seven months and two years, but this was not evident in the few satisfactory splenoportograms obtained during life. Variable anastomoses of the portal tributaries with the azygos, hemi-azygos, and intraarachideal plexus, and occasional filling of intercostals and diaphragmatic branches were seen by the seventh month on post-mortem angiogram and on direct inspection of injected specimens. Two years after infection, esophageal anastomoses were demonstrated by clinical angiogram. Anatomically, these anastomoses consisted of small thin-walled subserosal and mediastinal veins running straight alongside and across the lower esophagus anteriorly and laterally. The only submucosal varix of the series was observed endoscopically at the end of two years. At that time, intrapperative portal pressure ranged from a minimum of 130 to a maximum of 234 with a mean of 187 mmHg. These values were higher than those of three uninfected controls which showed a minimum of 131, a maximum of 184 and a mean of 151 mmHg. However, due to wide individual variation, differences were not statistically significant. No consistent differences in angiographic observations or portal pressure findings occurred between intact and splenectomized chimpanzees.

3. Histopathologic observations

Disregarding lesser individual variations of liver pathology, four degrees of portal lesions were distinguished in this series: Stage 1. Mild portal inflammation without fibrosis (No. 38 up to 200 weeks and most of the early needle biopsies); Stage 2. Moderate fibrosis of the small and some medium sized portal fields (Nos. 2, 10, 15, 22); Stage 3. Fibrosis of the large portal fields (Nos. 5, 8, at 30 weeks, 41 and 52 weeks, 42 at 50 and 100 weeks); Stage 4. Established pipe stem fibrosis (No. 41, after 100 weeks). Each degree represents the addition of increasingly severe lesions to those found at the preceding level, thus suggesting a progression both in severity and in time. However, in actual experience, serial transition has thus far been observed only from Stage 1 to Stage 2 and from Stage 3 to Stage 4.

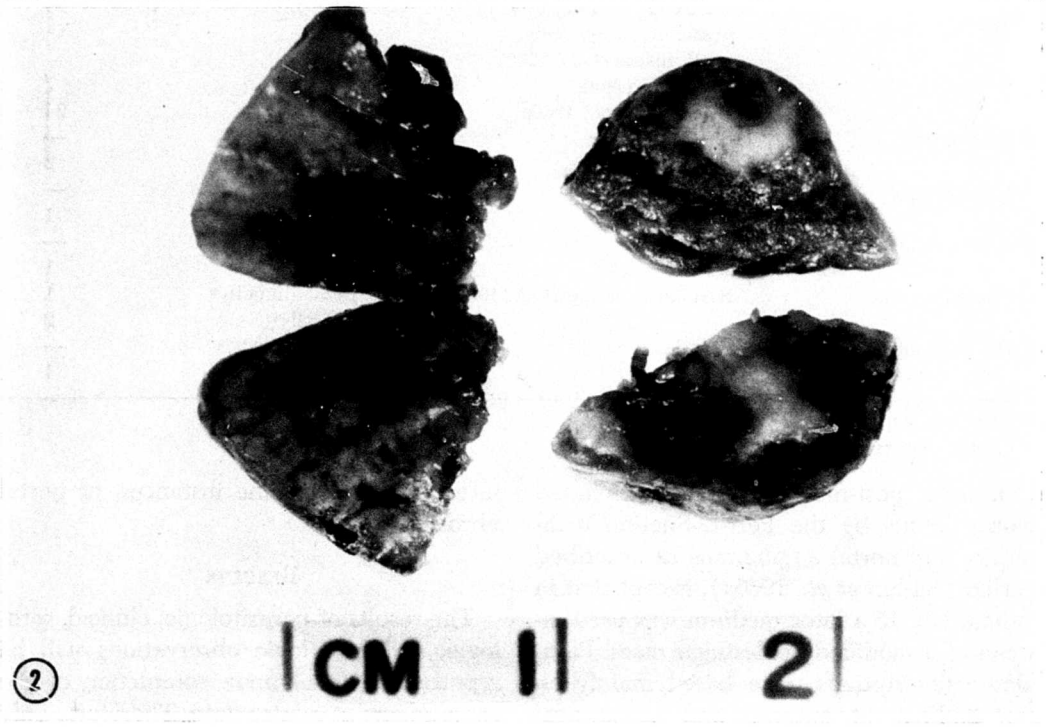


Fig. 7. Appearance of surgical liver biopsy specimens (100 weeks after exposure) in cross-section magnified approximately x4, chimpanzees No. 15 (left) and No. 41 (right); No. 15 shows subtle prominence of small portal fields (Stage II); No. 41 shows stellate fibrosis and marked expansion of large portal fields, a pattern typical of "pipe stem fibrosis" (Stage IV).

The portal lesions of Stage 1 did not differ significantly from those described in many other primates or in early human infection.

Similarly, the lesions of small portal fields in Stage 2 were not unlike those of uncomplicated chronic bilharzial infection of the human liver. Pseudotubercles and eggs were concentrated in the small portal triads, most of which were actively and diffusely inflamed and enlarged. Sheet-like lymphoid cell aggregates surrounding proliferating venules (portal collaterals) in an arrangement resembling granulation tissue were seen frequently. Substitution of small portal radicles by granulomas was common and, occasionally, there was endophlebitis of larger radicles (Fig. 8). Portal fibrosis developed both concentrically around involuting pseudotubercles, and in diffusely inflamed areas adjacent to granulomas (Fig. 9). Older, less cellular fibrotic foci often retained a rounded shape or contained residual egg shells. These dense fibrotic areas, forming patches and fibrous spurs at the margins of portal fields, became progressively more numerous in successive specimens seen over the two year period. Some of the middle-sized portal fields were also enlarged. These fields were usually surrounded by smaller, satellite triads bearing pseudotubercles, but were themselves free of inflammation. The reason for their fibrotic condition was not histologically evident (Fig. 10). No significant hepatocellular changes were found in this group except for mild centrolobular fatty change in one case (No. 38). Bile duct proliferation was focal and slight, and the lobular liver architecture was normal.

In the cases belonging to Stage 3, lesions of small portal fields were intense. In the early specimens of this group, pseudotubercles were numerous; later, stellate fibrosis was marked, with formation of fibrous bands between adjacent small portal fields or connecting with central veins. In addition, granulomatous inflammation extended from the smaller into the medium-sized triads, usually occupying their periphery while the central portion of the triads remained uninflamed and relatively "clean" looking; in this central portion, particularly in the adventitia of otherwise normal portal radicles, dense fibrosis was often found, together with dilated lymph vessels, and with areas of active fibroblastic proliferation around bile ductules and arterioles. These lesions, best visualized with the help of trichrome staining, were seen in even purer form in the largest portal fields (Fig. 11), which were only sporadically and marginally affected by granulomatous inflammation at this stage. The mildest and earliest degrees of central fibrogenesis and lymphatic dilatation were found at 30 weeks as the histological counterpart of the delicate portal prominence observed grossly at autopsy.

Development of Stage 4, i.e., of full-fledged pipe stem fibrosis, was marked by deposition of numerous eggs and pseudotubercles in the larger portal fields, together with diffuse inflammatory infiltration rich in eosinophils and plasma cells. Thus, the proximal, large portal triads, which earlier appeared "clean-looking" were not as densely populated by eggs as their distal branches and showed similar inflammation and structural anarchy. Simultaneously, there were increased fibrosis

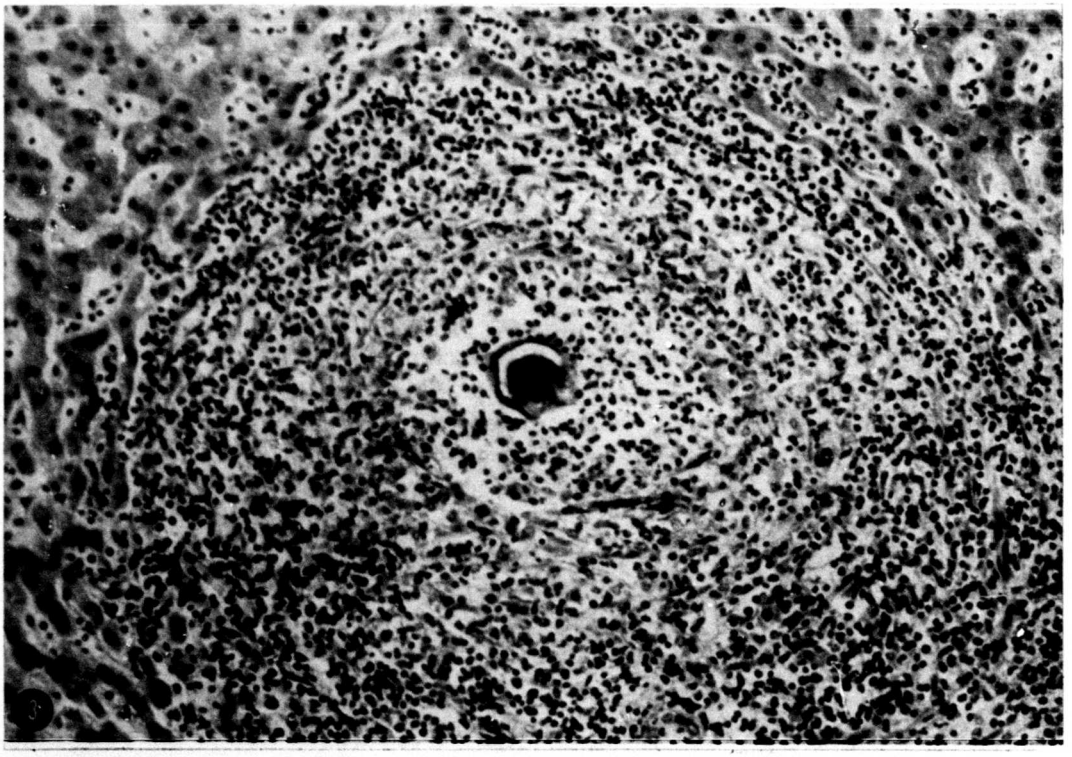


Fig. 8. Chimpanzee No. 15, hematoxylin-eosin, x160. Bilharzial endophlebitis: The lumen of an intrahepatic portal radicle is almost obliterated by numerous leukocytes surrounding a free schistosome egg; the inflamed intima is outlined poorly and the entire vessel is being transformed into a pseudotubercle. Many of the dark staining leukocytes are eosinophils.

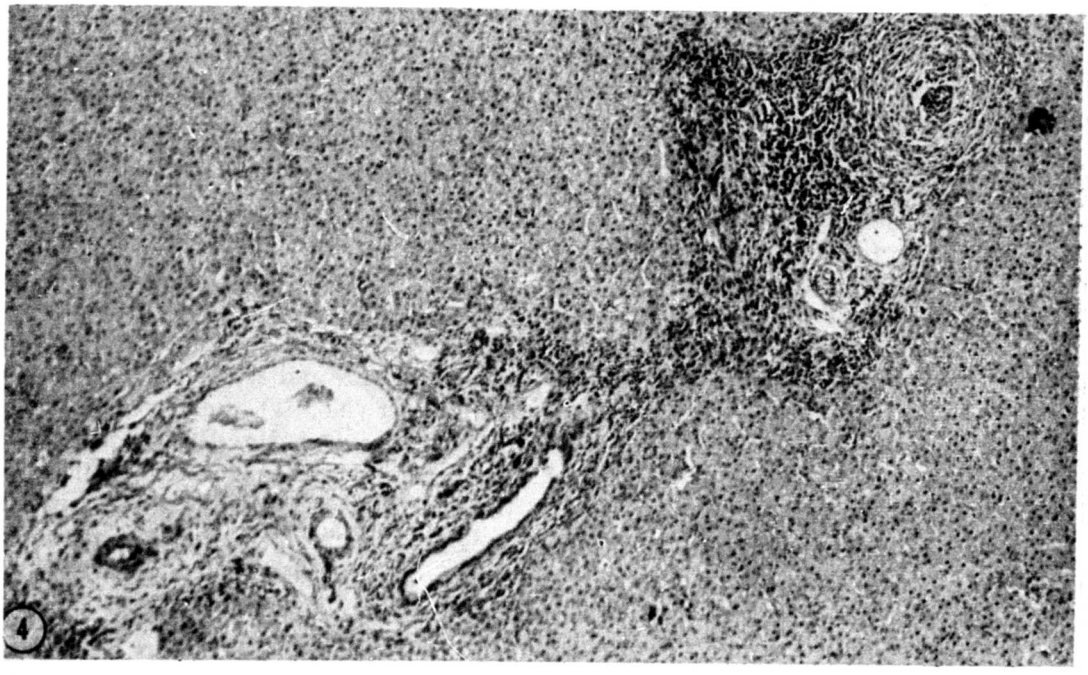


Fig. 9. Chimpanzee No. 41, 50 weeks after infection. Hematoxylin-eosin, x45. Two smaller portal fields have coalesced. The right one shows concentric fibrosis around a granuloma, with adjacent, sheet-like round cell infiltration; the left one shows diffuse fibroblastic proliferation particularly around the arteriole and bile duct.

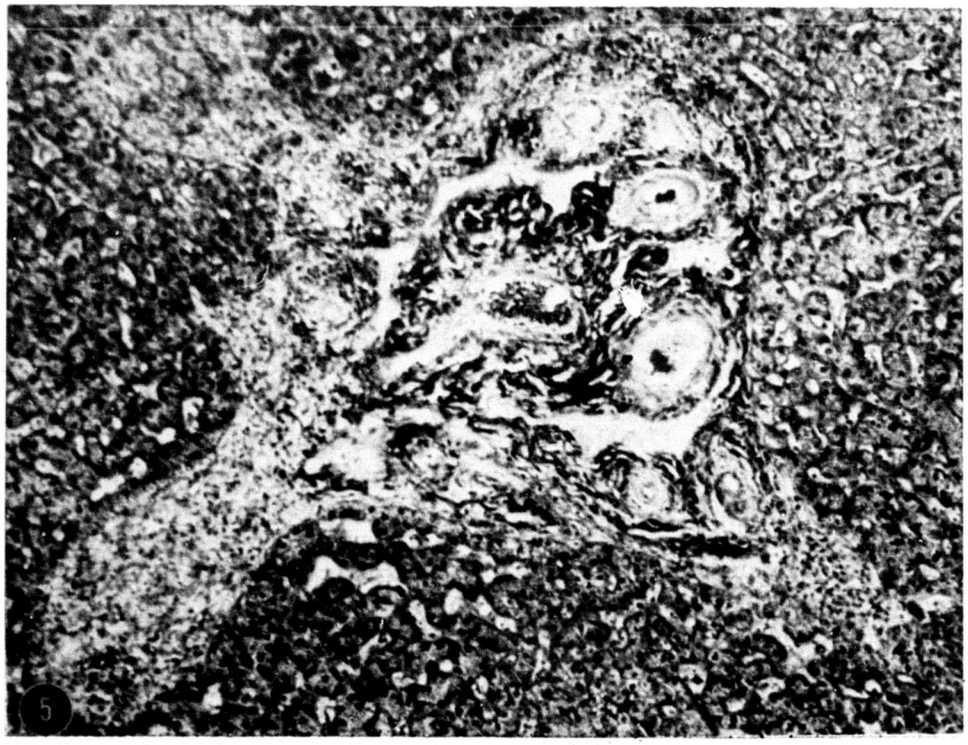


Fig. 10. Chimpanzee No. 15, Masson's Trichrome x72, 100 weeks after exposure. This medium sized portal field shows three inflamed fibrous spurs connecting with smaller satellite triads. The periphery shows inflammatory infiltration which is absent in its center; the portal radicle shows marked adventitial fibrosis and is surrounded by dilated, empty-appearing lymphatic vessels.

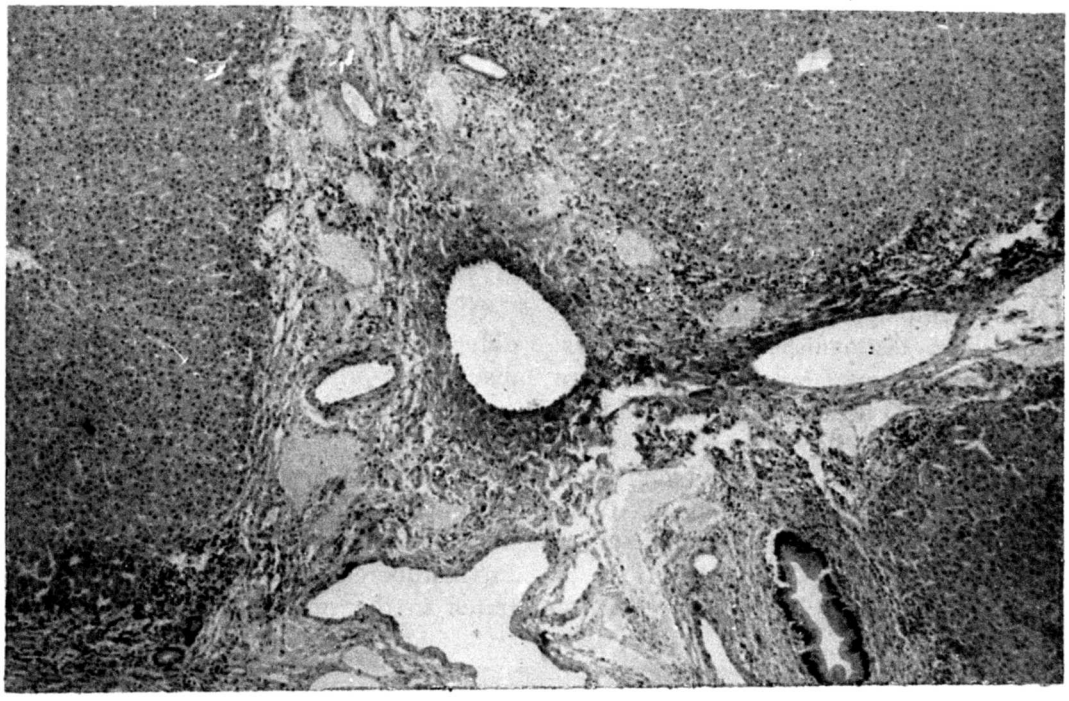


Fig. 11. Chimpanzee No. 42, hematoxylin-eosin, x45, 100 weeks after exposure. Similar changes as in Fig. 5 in a larger portal field; note the dense, hyaline appearance of the collagen around the portal radicle. The total size of the triad is enlarged; the adjacent parenchyma shows normal lobulation and intact central veins.

and coalescence of all the portal structures; the smaller fields were now either broadly connected to the larger ones or had entirely merged with their periphery. As a result, these extensive fibrous areas, crowded with granulomas, formed irregularly scalloped bands and patches sharply contrasting on cross-section with the orderly and intact liver parenchyma between them (Fig. 12). Where these bands abutted on the liver capsule, retraction was occasionally seen.

At this stage, lymphatic dilatation had largely disappeared in the portal fields, while active fibroblastic proliferation with focal interstitital edema and collagen deposition was widespread. At the interphase between the pseudotubercles and the liver parenchyma, bordering the portal fields, there was encroachment of fibrous tissue on the trabeculae with atrophy of individual liver cells, proliferation of ductules, and occasional sheet-like aggregates of plasma cells reproducing the picture of "piece-meal necrosis" described in chronic hepatitis (Fig. 13). Small and medium-sized portal radicles were difficult to locate, even with trichrome staining. Several had undergone obliteration and recanalization of lumina, and showed irregular, cleft-like venous channels between the disrupted muscle fibers of the media (Fig. 14). There was occasional arteriolar thickening, but the central veins remained normal, and bile duct proliferation was moderate. Biopsy and autopsy specimens of ten human cases of pipe stem fibrosis from the northern Brazilian endemic focus showed similar lesions (Fig. 15), varying only in detail and stage development.

Findings in organs other than the liver in chimpanzees killed 30 weeks after exposure have been reported elsewhere. In one additional post-mortem study conducted 100 weeks after exposure (No. 15) the findings were comparable, except for presence of an acute, superficially ulcerative colitis due to Balantidium coli infection, and of widespread, non-specific lymphadenitis of undetermined etiology. Death was due to barbiturate overdose and was accompanied by terminal mucous plugging of bronchioles and by pulmonary congestion and edema.

One of nine heavily infected chimpanzees developed the classical lesions of bilharzial pipe stem fibrosis; at least one additional animal showed a precursor stage of this lesion, and several others had variable, lesser degrees of fibrosis, predominantly of smaller portal fields. Portal fibrosis was induced in chimpanzees by Schistosoma mansoni infection alone, in the absence of other indentifiable contributory factors. The earlier precursor changes of pipe stem fibrosis were seen at seven months and the full-fledged picture developed within two years after exposure. Observations are being continued to determine the ultimate evolution of these lesions. Although pipe stem fibrosis developed after multiple exposures, lesser portal fibrosis was also seen after a heavy single exposure. In all cases, portal fibrosis was associated with sustained, high egg output in the stools. Severe fibrosis occurred at a time when the number of eggs in the stools had not declined significantly.

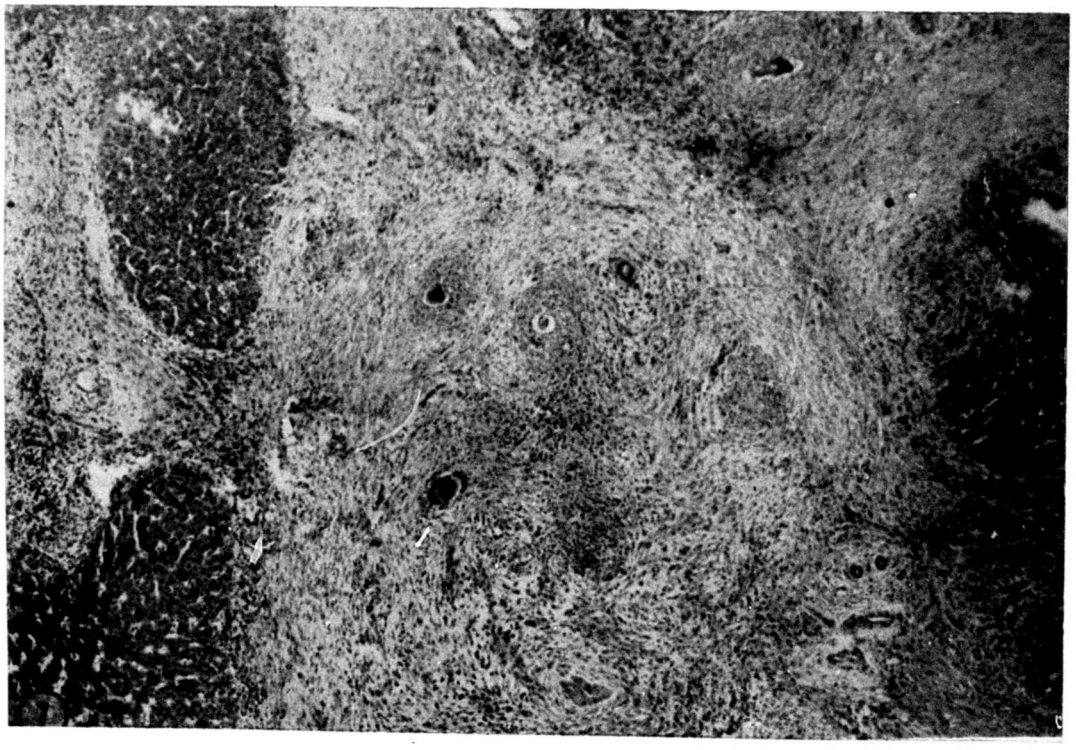


Fig. 12. Chimpanzee No. 41, hematoxylin-eosin, x45, 100 weeks after exposure. Full-fledged pipe stem fibrosis. This large portal field shows obliteration of landmarks by multiple granulomata, diffuse inflammatory infiltration and fibroblastic proliferation. Adjacent portal fields are connected to it by broad, fibrous stalks. The liver parenchyma, sharply demarcated by the fibrous stands, shows an orderly trabecular structure.



Fig. 13. Chimpanzee No. 41, hematoxylin-eosin, x450, 100 weeks after exposure. The upper margin of the field includes the peripheral fibrosis surrounding an involuting pseudotubercle. The lower 1/3 shows liver cell trabeculae bordering the triad. A bandlike aggregate of inflammatory cells, including neutrophils and plasma cells, is encroaching on the trabeculae. Necrosis of isolated cells and ductular proliferation are seen. A dilated ductule containing leukocytes is seen to the left and below center.

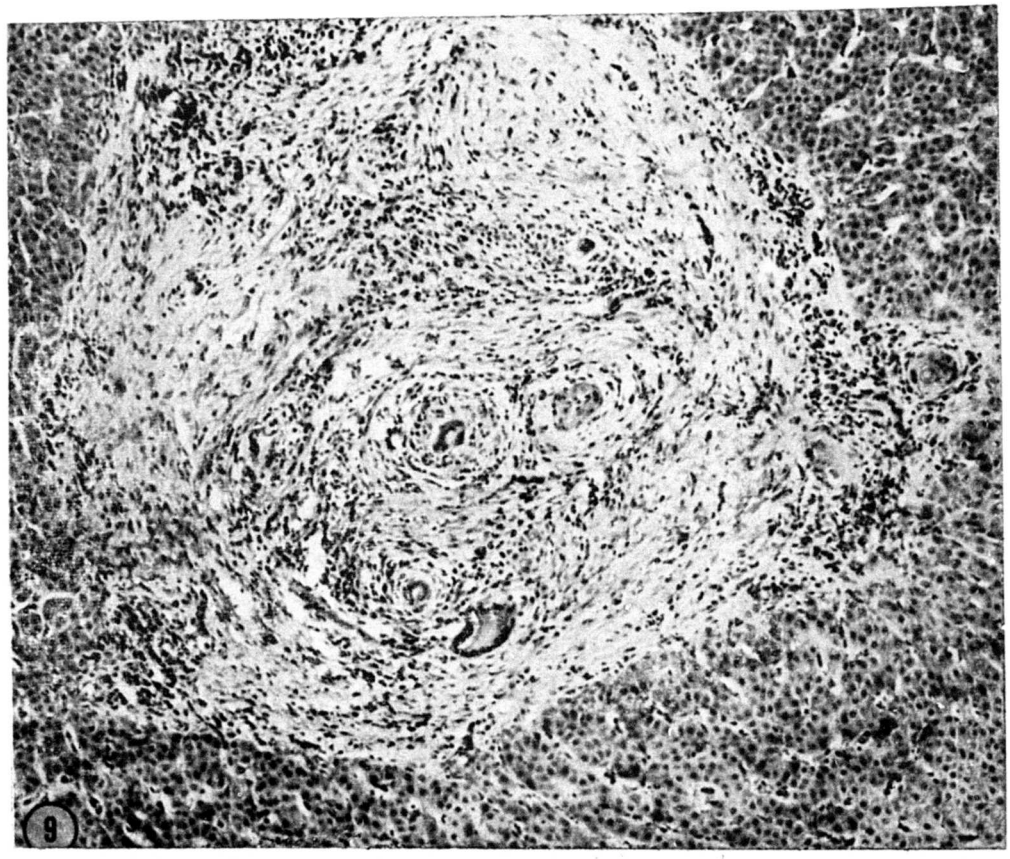


Fig. 14. Chimpanzee No. 41, hematoxylin-eosin, x72, 100 weeks after exposure. A middle sized portal field shows structural anarchy similar to that of Fig. No. 12. The former location of the main portal radicle is marked by the ellipsoid fibrous nodule in the upper right hand corner of the field. Numerous partly collapsed, slit-like venules are seen throughout the inflamed fibrous tissue. Pigmented Kupffer cells stand out in the sinusoids of the adjacent, orderly liver parenchyma.

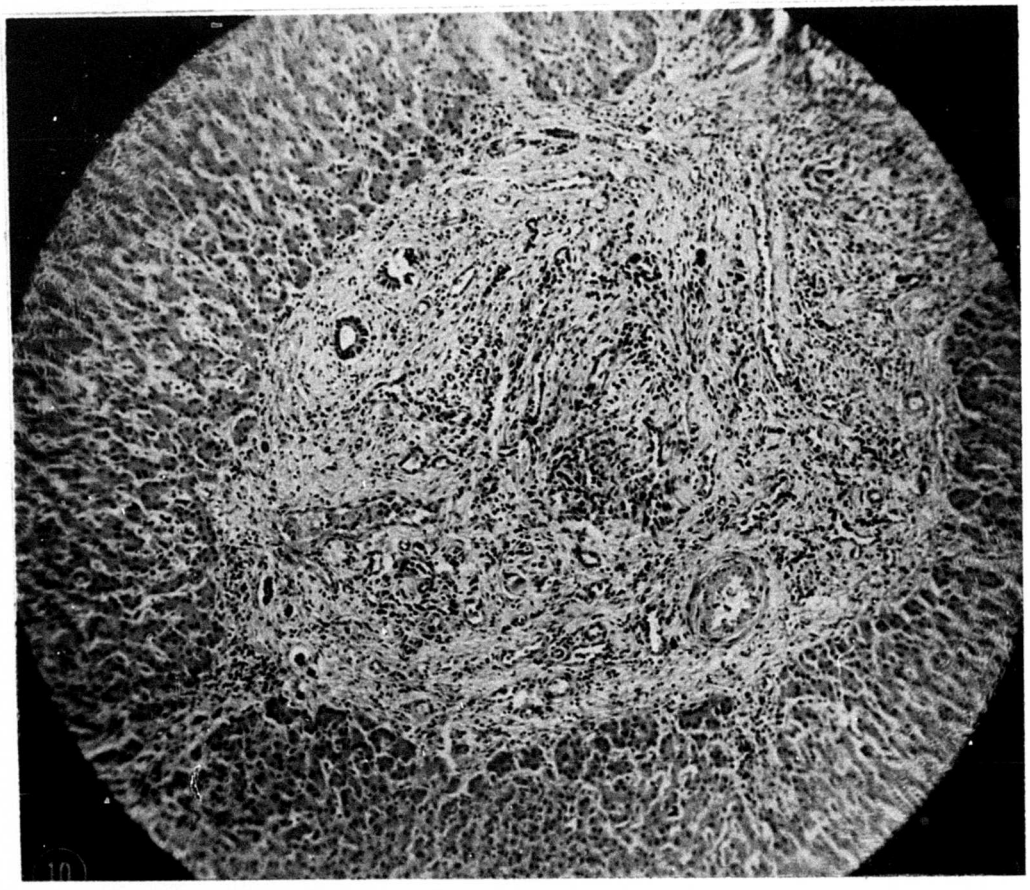


Fig. 15. Human autopsy case of bilharzial pipe stem fibrosis, Bahia, Brazil, 1951. Hematoxylin-eosin approximately x60. The same features illustrated in Fig. No. 14 are evident, but fibrosis and arterial thickening are somewhat more advanced.

Portal fibrosis was correlated with heavy egg deposition in the portal triads and intrahepatic portal radicles, accompanied by granulomatous as well as diffuse inflammation. On serial histologic observations, these lesions were seen to involve progressively the small, the medium sized, and finally the large portal fields from the periphery toward the hilus. In addition, fibrogenesis associated with phlebosclerosis and lymphatic dilatation was seen to begin in the central portion of the larger portal areas, at a time when granulomatous inflammation was still confined to more distal portal branches. The pathogenesis of this precursor change, unassociated with in situ inflammation, remains obscure. Evidently, besides the coalescence of granuloma scars observed in mice there are other histological precursor events in the chimpanzee (and probably in man). The full sequence and pathogenesis of these events require further study and it is still uncertain which factors determine the variable speed and completeness of individual progression of pipe stem fibrosis. Morphologically and angiographically the portal circulation of man and of the chimpanzee are very similar, and both hosts show concentric fibrosis of healing pseudotubercles. Another analogy is the finding of marked hypergammaglobulinemia, plasma cell infiltrates, and occasional piece-meal necrosis in liver tissue. However, these and other similarities between bilharzial infection of man and chimpanzee remain to be fully explored.

Although there was histologic evidence suggesting presinusoidal portal obstruction in the liver, portal pressures in the chimpanzee when compared with man were only moderately elevated. This could be due to the rich anastomotic network demonstrated angiographically at a relatively early stage of bilharzial infection. As in other primates, esophageal anastomoses were largely adventitial. Since submucosal varices have previously been reproduced only by vascular liver surgery, the finding of a true varix in one animal is the first such observation in experimental liver disease, including schistosomiasis. The clinical evolution of pipe stem fibrosis in the chimpanzee was similar to that in man. After an initial, acute stage, there was a prolonged asymptomatic interval, which developed gradually into a compensated stage of pipe stem fibrosis. As in man, this stage was characterized by extensive liver pathology, splenomegaly, and portal collateral formation, with relatively minor hepatocellular change. There are indications that the chimpanzee with the most advanced pipe stem lesion is now beginning to decompensate.

These studies on chimpanzees have permitted observations analogous to those of severely infected human population. As we follow the evolution of this infection, we hope to obtain a further insight into the natural history of bilharzial liver disease and to determine possible ways in which it can be arrested or prevented.

4. In vitro histamine release from blood cellular elements of rabbits infected with Schistosoma mansoni.

Recently, Zvaifler et al. described a homocytotropic antibody in rabbits infected with Schistosoma mansoni which produced a 72-hour

passive cutaneous anaphylaxis (PCA) in normal rabbits challenged with cercarial antigen. This anaphylactic antibody was inactivated by heating at 56°C and by dimercaptoethanol, migrated as fast gamma globulin electrophoretically and had a sedimentation coefficient close to 7S. Human reaginic antibody is capable of sensitizing leucocytes so that by the addition of antigen, histamine is released. Barbare and Zvaifler, using dinitrophenyl-bovine serum albumin (DNP-BSA) in Freund complete adjuvant, suggested that rabbit PCA antibody is capable of sensitizing rabbit platelets in vivo and demonstrated antigen-induced histamine release in vitro in the absence of free plasma factors and free antibody.

The present studies were designed to determine if S. mansoni infections in rabbits "sensitize" platelets and/or leucocytes for in vitro histamine release following the addition of S. mansoni cercarial antigen in the absence of added plasma. The results of these studies were compared with those obtained by PCA and slide flocculation (SF) tests. Furthermore, attempts were made to determine whether this in vitro reaction system could be utilized for a quantitative study of immediate hypersensitivity in rabbits infected with S. mansoni.

Animals. Albino rabbits weighing 2,500 to 3,000 grams were used in these experiments. All animals were fed a standard diet. The principles of animal care as promulgated by the National Society for Medical Research were observed. Rabbits were exposed percutaneously to either 5,000 or 25,000 cercariae each and were bled for histamine and serologic studies every other week beginning the day of exposure. At the end of each experiment, the rabbits were killed and the visceral organs were examined for the presence of gross pathologic changes resulting from infection with S. mansoni. Each animal was perfused by the Perf-O-Suction technic and examined carefully for completeness of blood clearance and possible retention of adult worms. Immediately after perfusion, the worms were separated by sex, counted, and observed microscopically.

Antigen. S. mansoni cercariae were recovered from the laboratory colony of Australorbia glabratus (Biomphilaria glabrata) snails, each infected with 5 to 10 miracida. A lipid free somatic antigen was prepared according to the method of Chaffee et al. After lyophilization, the antigen was reconstituted with distilled water to a concentration of 0.43 mg of nitrogen protein per ml, determined by the micro-Kjeldahl method. From this standard preparation, 0.5 ml of a 1:100 dilution in Tyrode's solution was found to be minimal for 100 percent histamine release.

Rabbit antiserum. The globulins from appropriate serum pools were precipitated with 50% saturated ammonium sulphate. The precipitate was reconstituted to one-half the original serum volume with phosphate buffered saline (PBS) pH 7.2, and dyalized against the same buffer for 48 hours at 4°C. The globulins were then fractionated by column chromotography utilizing Sephadex G-200 and diethylaminoethyl (DEAE) cellulose; the samples were eluted from the G-200 column with PBS and the DEAE columns were eluted sequentially using selected phosphate buffers with

slight modifications of the method described by Askonas et al., as reported previously.

At the same time, another aliquot of blood was collected separately and allowed to clot for serum separation. After the blood had clotted, the serum was separated by centrifugation and stored at -70°C. It was tested for PCA and SF activity with adherence to the methods published previously.

Histamine determinations. Appropriate amounts of blood were collected from the medial artery of the ear in a siliconized syringe and transferred to cold polypropylene tubes containing sufficient amounts of sodium heparin to yield a final concentration of 7 µg of heparin per ml of whole blood. The blood was then centrifuged at 1600 G's for 15 minutes at 3°C. The supernatant plasma was discarded and the packed cells were resuspended and washed 3 times with Tyrode's solution before reconstituting to the original blood volume. These suspensions contained red and white cells as the platelets. Aliquots of 0.5 ml of these washed cells were used in a total assay volume of 1.5 ml and tested in duplicate for total, antigen-induced and spontaneous histamine release. A pure suspension of platelets was obtained from blood collected as described, and centrifuged for 50 minutes at 30 G's at 3°C. The platelet rich plasma supernate was then washed with Tyrode's solution and divided into aliquots.

Histamine was extracted according to the technic described by Shore et al., and modified by Barbaro and Zvaifler. Fluorescence was read on a Turner Model III Fluorometer activated at 360 mµ and emitted at 450 mµ.

The first experiment was to determine whether histamine release could be induced in rabbits infected with S. mansoni and to compare the results with the production of PCA antibody. Eight rabbits were exposed to 25,000 cercariae each and bled every other week for 38 weeks. The results (Table 10) indicate that 5 of the 8 infected rabbits produced detectable PCA antibody.

Seven of the 8 rabbits showed significant levels of antigen-induced histamine release. This occurred as early as 3 weeks after exposure to infection in 1 animal and 4-7 weeks after exposure in most of the others. Histamine release was detected both in rabbits in which PCA antibody had been demonstrated and in those which showed no detectable levels of PCA antibody. With the single exception of rabbit No. 7, antigen-induced histamine release occurred throughout the experiment. A considerable variability in worm burdens was observed in the animals at necropsy (Table 10). No obvious correlation between the presence of anaphylactic antibodies and worm burden was observed. All of the animals exposed to infection gave positive results with the slide flocculation test. As indicated in Figure 16, antigen-induced histamine release was detectable in the absence of demonstrable circulating PCA antibody but at levels lower than in PCA positive rabbits (Figure 17). However, it was not possible to obtain PCA antibody production without accompanying histamine

Table 10. Cellular, Serologic, and Parasitologic Findings in Rabbits Infected with *S. mansoni*.*

Rabbit:	No. 1			No. 2			No. 3			No. 4			No. 5			No. 6			No. 7			No. 8		
Week	HR	PCA	SF	HR	PCA	SF	HR	PCA	SF	HR	PCA	SF	HR	PCA	SF	HR	PCA	SF	HR	PCA	SF	HR	PCA	SF
													Exposed to 25,000 Cer.											
0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—
1	—	—	—	—	—	—	—	—	—	—	—	—												
2													—	—	—	—	—	—	—	—	—	—		—
3	—	—	—	—	—	—		—	—	+	—	—												
4													+	—	—	+	—	—	—	—	—	—		—
5	—	—	+	+	—	+	+	+	+	+	+	+												
6													+	—	—	+	—	—	—	—	—	+		—
7	+	—	+	+	+	+	+	+	+	+	+	+												
8													+	+	—	+	—	—	—	—	+	+	+	+
9	+	—	+	+	+	+	+	+	+	+	+	+												
10													+	+	+	+	—	+	+	—	+	+	—	+
14	+	—	+	+	—	+	+	+	+	+	+	+												
15													+	+	+	+	—	+	—	—	+	+	—	+
16	+	—	+	+	+	+	+	+	+	+	+	+												
17													+	+	+	+	—	+	—	—	+	+	—	+
18	+	—	+	+	—	+	+	+	+	+	+	+												
21													+	+	+	+	—	+	—	—	+	+	—	+
24	+	—	+	+	—	+	+	+	+	+	+	+												
25													+	+	+	+	—	+	—	—	+	+	—	+
27	+	—	+	+	—	+	+	+	+	+	+	+												
													Exposed to 20,000 Cer.											
31													+	+	+	+	—	+		Died		+	—	+
32	+	—	+	+	+	+	+	+	+	+	+	+												
33													+	+	+	+	—	+				+		+
34	+	—	+	+	+	+	+	+	+	+	+	+												
35													+	+	+	+	—	+				+	—	+
36	+	—	+	+	+	+	+	+	+	+	+	+												
37													+	+	+	+	—	+				+	—	+
38	+	—	+	+	+	+	+	+	+	+	+	+												
Worm recovery at necropsy		1500			952			11			1150			718			1176			ND			863	

* Abbr.: HR, histamine release; PCA, passive cutaneous anaphylaxis; and SF, slide flocculation.

Figure 16

Typical Histamine Release Pattern in Rabbit With S. Mansoni (PCA-)

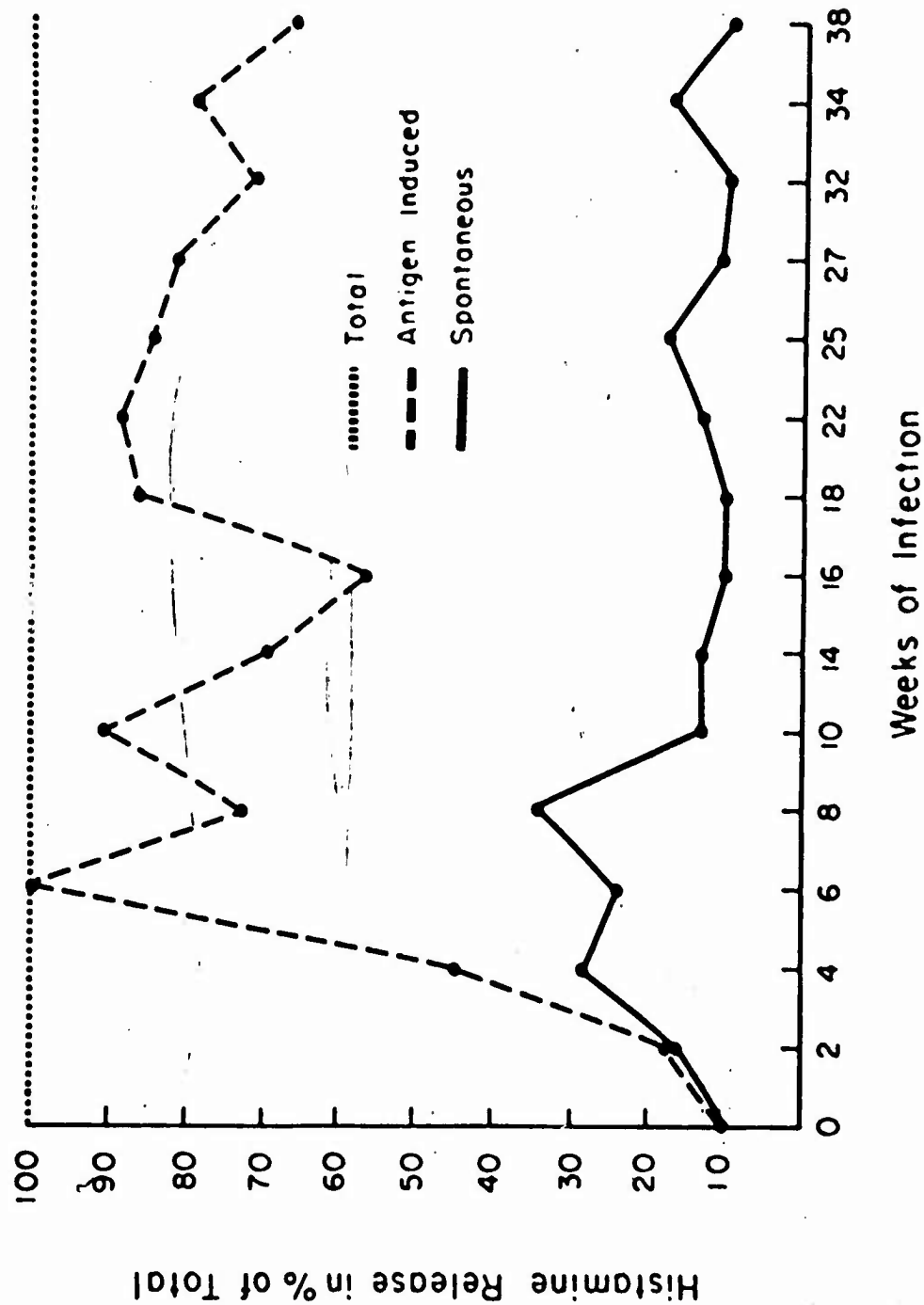
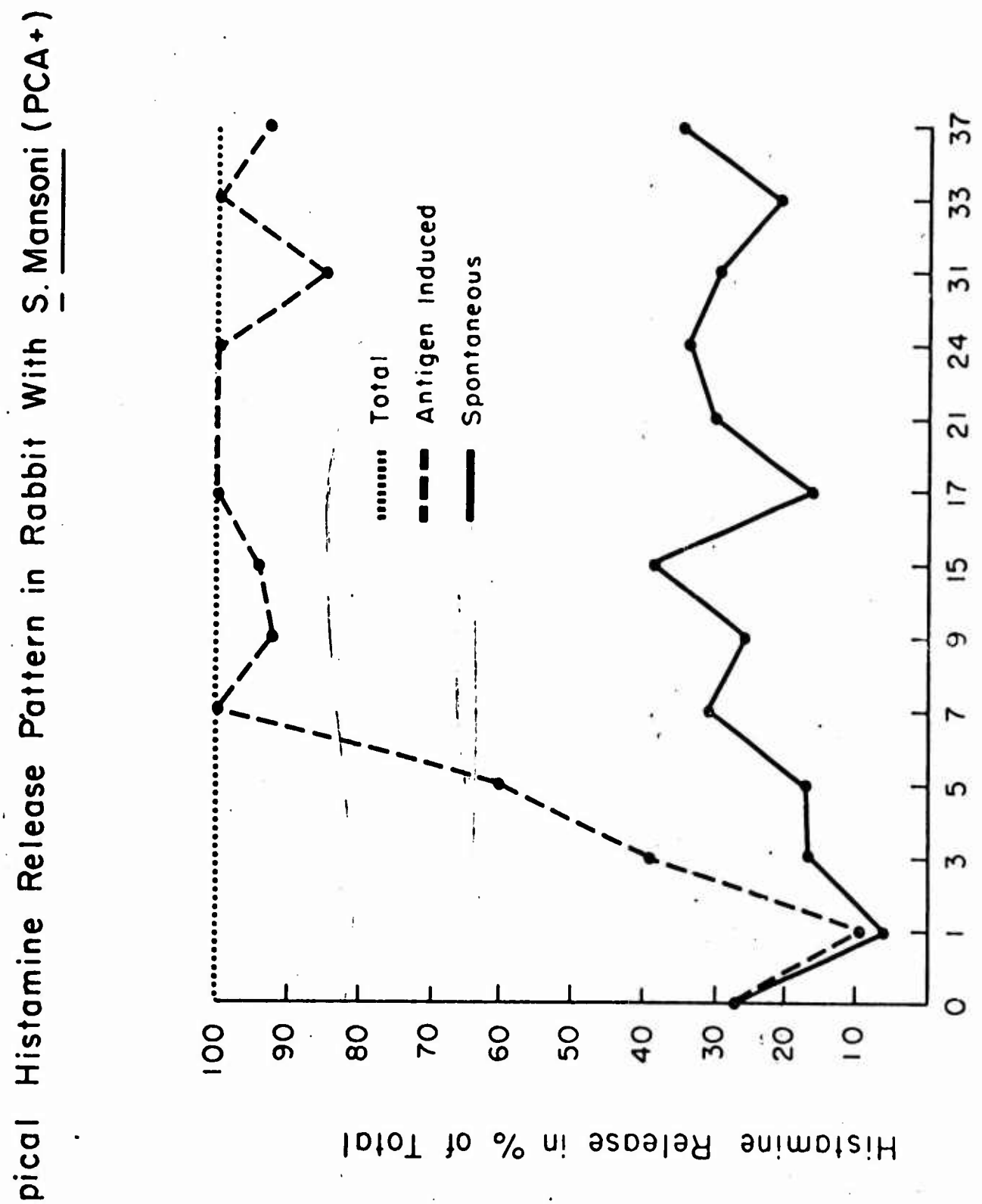


Figure 17


Typical Histamine Release Pattern in Rabbit With S. Mansoni (PCA+)
Histamine Release in % of Total
100
90
80
70
60
50
40
30
20
10
0
1
3
5
7
9
15
17
21
24
31
33
37
Weeks of Infection
Total
Antigen Induced
Spontaneous

release. The time course development of PCA antibody activity and of *in vitro* antigen-induced histamine release indicated that, in general, *in vitro* histamine release was present in more animals, was detected earlier and persisted longer than PCA antibody activity in the serum of infected animals. This incomplete correlation between the time course of the two activities suggests that more than one antibody may be responsible for the sensitization of platelets and for the observed histamine release, or may indicate that *in vitro* histamine release is a more sensitive method of measuring immediate hypersensitivity.

Additional experiments were designed to determine whether platelets are directly sensitized following *S. mansoni* infections or whether substances released from sensitized leucocytes following an antigenic stimulus trigger the release of histamine from platelets. Platelets in supernatant plasma from the uninfected rabbits were washed and divided into aliquots. Washed cells from an infected rabbit still containing platelets but in lesser quantity were added to the aliquot of platelets from an uninfected animal. As indicated in Table 11, the amount of histamine in this mixture was significantly greater than that obtained when antigen was added to the sensitized cells only. Conversely, there was no discernible antigen-induced histamine release from the washed platelets, obtained from infected or non-infected animals, in the absence of leucocytes. Similarly, platelets from normal rabbits failed to release histamine when the supernate of sensitized cells were added after having been exposed to the antigen for 30 minutes at 37°C.

Table 11

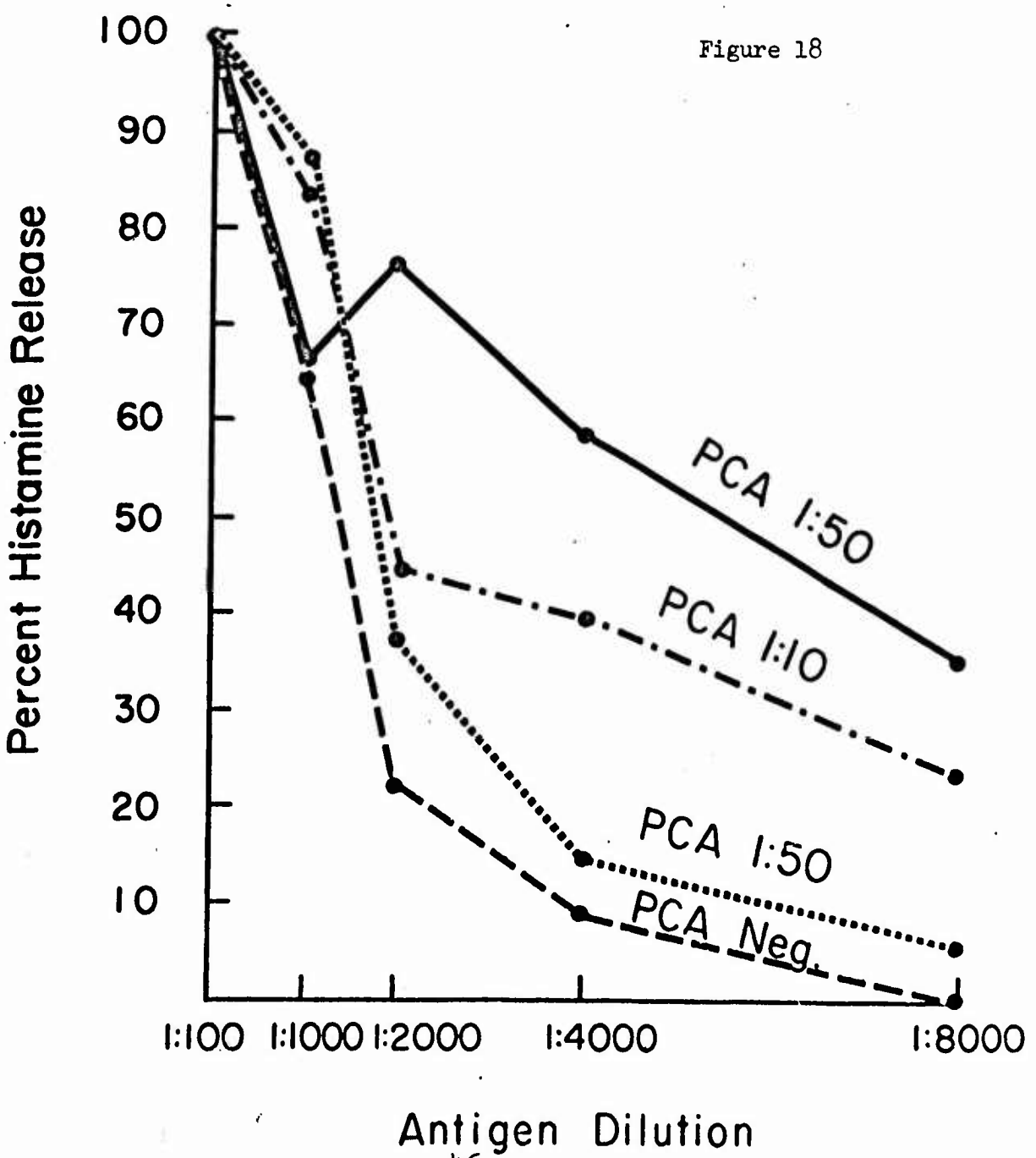
Histamine Release (μg)

Cellular Contents	Spontaneous	Antigen-induced	Total
Normal platelets	.245	.243	1.170
Sensitized platelets and cells	.122	1.350	1.528
Sensitized platelets and cells Normal platelets	.622	2.603	2.700

In an attempt to determine whether a quantitative *in vitro* system could be established, the relationship between antigen concentration and the percent of histamine release was studied. As shown in Figure 18, a 50% histamine release was observed at different antigenic dilutions in different animals. Although no obvious correlation between the amount of antigen required to permit a 50 percent histamine release and PCA titers was detected, the animal in which no demonstrable PCA reaction was present required a higher antigenic concentration for a 50 percent histamine release.

Relationship Between Antigen Concentration And Percent Histamine Release

Figure 18



The antigenic concentration required for a 50% histamine release at various intervals following exposure to infection was studied in a series of 5 rabbits, 2 of which were exposed to 25,000 S. mansoni cercaria and 3 to 5,000 cercariae each. As shown in Table 12, increasingly lower concentrations of antigen were required in both groups of animals from the third to the ninth week following exposure to infection. During this time, it seemed that the cells from animals exposed to 25,000 cercariae demonstrated 50% histamine release with less antigen than those exposed to only 5,000 cercariae.

Table 12

Antigen Dilution at which 50% Histamine Release Occurs

No. of cercariae	Rabbit No.	Weeks after exposure				
		0	3	5	7	9
25,000	93	0	1:800	1:3000	1:4000	1:4000
25,000	94	0	1:400	ND	1:4000	1:4000
5,000	1	0	1:1000	1:1000	1:2000	1:4000
5,000	2	0	<1:100	1:1000	1:2000	1:0000
5,000	3	0	<1:100	1:1000	1:2000	1:4000

Molecular sieving and ion exchange chromatography were employed to separate some of the antibodies present in the rabbit antiserum. The globulin in the combined sera from 4 rabbits exposed to 25,000 cercariae, all of which had high titers of PCA antibodies, was precipitated with 50% saturated ammonium sulphate. Passage of the reconstituted precipitate through Sephadex G-200 chromatography column yielded 4 fractions as shown in Figure 19. Ion exchange chromatography was then employed to separate some of the antibodies present in the fraction which contained most of the PCA activity. Aliquots of each of the 4 fractions obtained were analyzed for their ability to produce PCA reactions and for their flocculating activity. As reported with fluorescent antibody tests on sera from rabbits infected with Dirofilaria uniformis, the fractions containing most of the IgG immunoglobulin had all of the detectable flocculating activity whereas electrophoretically faster immunoglobulins contained PCA activity but no detectable flocculating activity.

The finding that only some of the infected rabbits produced a demonstrable amount of PCA antibody is in agreement with previous results obtained with infections or artificial immunization. In all the rabbits with circulating homologous PCA antibody, it was possible to obtain antigen-induced in vitro histamine release. Conversely, in vitro histamine release was observed in a number of rabbits with a detectable PCA

Fractionation of PCA Serum from Rabbits with S. mansoni

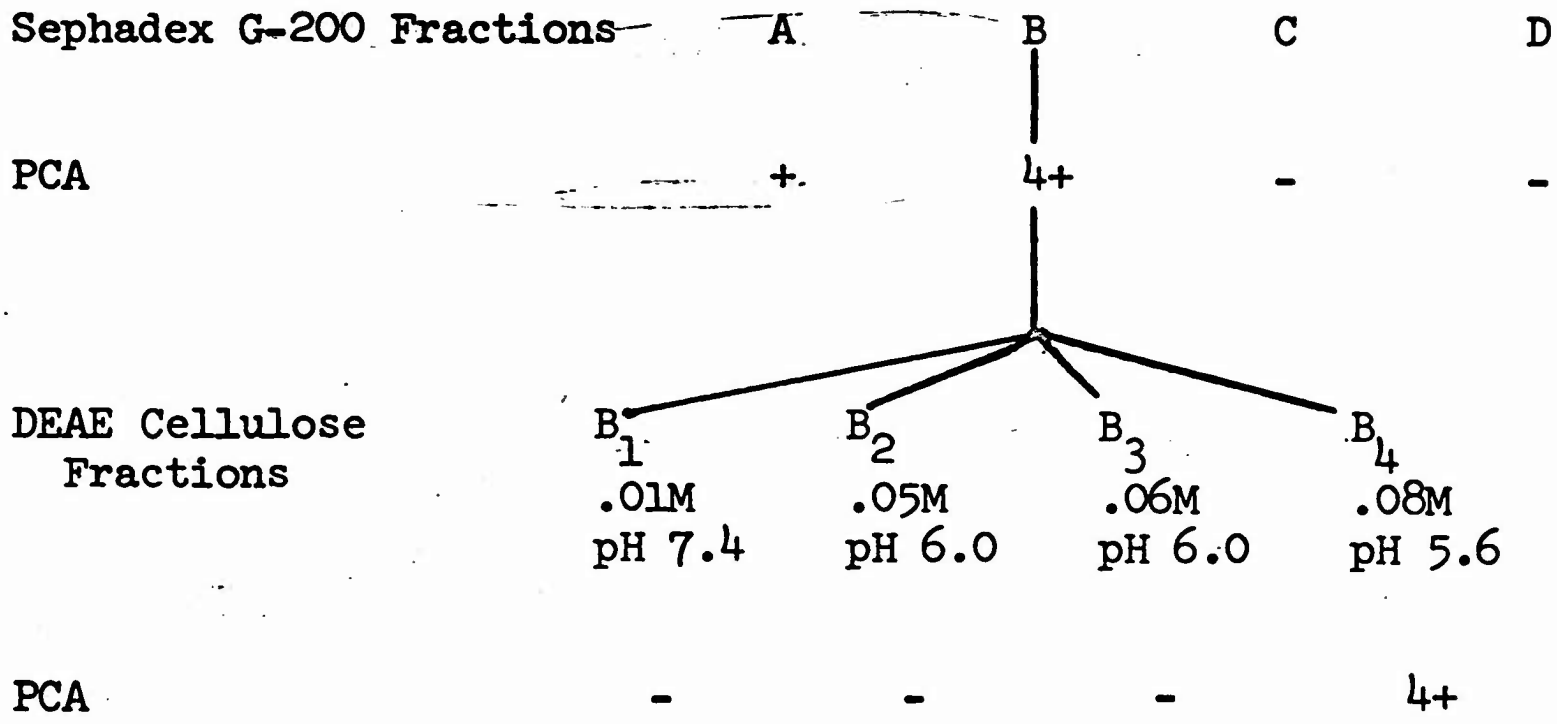


Figure 19

antibody activity. This consideration, added to the fact that antigen-induced histamine release appeared earlier and persisted longer than PCA activity, suggests that the release of *in vitro* histamine from the platelets is a more sensitive reaction and/or that more than one antibody is responsible for the former phenomenon.

Washed platelets from uninfected rabbits are capable of histamine release only when mixed with washed sensitized cells challenged with antigen. This suggests that some mechanism exists for histamine release from platelets obtained from unsensitized rabbits. Determination of a 50% histamine release by serial dilution of antigens indicated that this reaction is very sensitive and that titers are directly related to the length of infection. As shown in previous experiments conducted with rabbits infected with filarial parasites, the flocculating and the PCA activities are found in different immunoglobulin classes.

Studies are in progress to determine the possible relationship between histamine release, PCA antibodies, immunity and intensity of infection of rabbits to *S. mansoni*.

5. A soluble antigen fluorescent antibody (SAFA) test for the immunodiagnosis of trichinosis in man and experimental animals.

Jackson exposed larvae and adults of *T. spiralis* to fluorescein-labeled antisera from infected rabbits and found that the cuticle of living parasites did not fluoresce. Conversely, Sadun et al. observed that the cuticle of formalin-fixed larvae stained brilliantly after exposure to immune sera by an indirect fluorescent antibody technic. Since fluorescence was not observed when normal sera were tested under the same conditions, a sensitive and specific test for the serodiagnosis of trichinosis was developed. Evaluation of this procedure with sera from healthy individuals and from patients with trichinosis, other parasitic infections and non-parasitic diseases indicated that this technic had considerable promise as a diagnostic aid for trichinosis. Adapting this test to blood obtained by finger puncture and dried on absorbent paper also gave excellent results, which suggests that this relatively simple method of collecting, mailing and testing small amounts of blood from endemic areas could be used to great advantage in epidemiologic investigations for trichinosis.

The fluorescent antibody test (FAT) for trichinosis has been evaluated and slightly modified by a number of authors. Sulzer suggested as a source of antigen the use of larval cuticles which had been digested with pepsin so that internal structures could be removed. He indicated, however, that by using only the cuticle as antigen one may fail to detect antibodies directed against metabolic products of the larvae.

The FAT for trichinosis is relatively easy to perform, is highly sensitive, adequately specific and dry blood specimens may be used. However, this test still possesses the main disadvantages of technics employing whole organisms as antigen. As pointed out by Anderson, lack

of flexibility is a problem frequently encountered in the use of whole organisms since one cannot purify or fractionate the antigen as can be done for soluble antigens. The requirement for whole organisms limits the use of this test to laboratories that maintain the helminthic life cycle for other purposes, and the highly subjective interpretation of positivity in weakly reactive antisera places some serious limitations on the widespread use of such technics.

The use of a soluble antigen fluorescent antibody (SAFA) test by fixing a drop of antigen onto a matrix of cellulose acetate filter paper was suggested by Paronetto. This technic, which permits the investigator to select and purify the antigen to be employed, to obtain an objective reading of the test with a fluorometer, and to compensate for any non-specific fluorescence, has been applied to the diagnosis of parasitic infections by several investigators.

The current report summarizes results of studies in which the SAFA technic was used in an attempt to develop a reliable, objective and simple test for the laboratory diagnosis of trichinosis. This procedure was evaluated with sera from humans and experimentally infected animals. Also, attempts were made to determine whether blood smears dried on absorbent paper could be used.

Sera. Human sera were obtained from 28 cases of documented trichinosis. The diagnosis in the patients was established by clinical evidence and by previous positive findings in other serologic tests. To determine the specificity of the SAFA test, 104 control sera from individuals with other parasitic infections were used. In addition, 90 normal sera were obtained from healthy donors who were undergoing physical examinations as candidates for appointment to a military academy. All untreated sera were stored at -20°C until employed in this test. Some sera were treated with merthiolate and others were lyophilized.

Mice, rats and rabbits were used as experimental animals. The rats used were young adults (Wistar strain) weighing 130-150 gm each. They were given 3,000 to 4,000 larvae each by mouth to provide fresh larvae for preparing the antigen and for infecting the other experimental animals. At least 30 days after the rats were infected the larvae were separated from the muscles by artificial digestion in a pepsin-hydrochloric acid mixture. The mice used in this study were young adults (Walter Reed, ICR strain) weighing 18-22 gm. The rabbits were New Zealand albino, young adults weighing 2,500-3,000 gm at the beginning of each experiment. All animals were fed a standard diet. The mice and rats were bled by cardiac puncture and the rabbits were bled from the central ear vein before infection and at regular intervals throughout each experiment. After the blood had clotted, serum was separated by centrifugation and stored at -20°C until ready for use. Small amounts of rabbit blood were dried on filter paper, as previously described. Subsequently, the blood was eluted at pre-determined weekly intervals and tested. The principles of animal care as promulgated by the National Society for Medical Research were observed.

Soluble Antigen Fluorescent Antibody (SAFA) Test

A lipid-free somatic extract of Trichinella larvae was prepared at 4°C in anhydrous ether. Aliquots of the finished product in the form of powder were sealed in vials and stored at 4°C. Solutions were made when needed by dissolving 19 mg of powdered extract in 1.0 ml of distilled water.

The SAFA test was conducted essentially as described for schistosomiasis and amebiasis. A normal control serum was used to set the fluorometer dial at zero. Fourfold serum dilutions from 1:16 through 1:1024 were made. In determining test results sera which gave fluorometer dial readings of 20 or more were considered to be reactive and titers were expressed as a reciprocal of the highest dilution giving a positive reading.

Serologic response in infected rabbits, mice and rats

Sera from 12 rabbits inoculated with 25,000 Trichinella larvae were tested weekly for 38 weeks. The time course development of detectable antibodies is summarized and presented graphically in Fig. 20. The first positive reactions were obtained 2 weeks after infection. The titer in both groups increased rapidly up to 6 - 7 weeks following infection after which it remained elevated throughout the experiment. An anamnestic response seemed to occur following reinfection. The results obtained in 200 mice (Fig. 21) and 6 rats (Fig. 22) followed the same pattern as that observed in the rabbits.

Sensitivity and specificity of the SAFA test with human sera

The results obtained with human sera are summarized in Table 13. The findings with specimens from trichinosis patients illustrate the relative sensitivity of this procedure. The findings with sera from patients with other conditions and from healthy individuals indicate the degree of specificity of the test. Only 2 serum specimens from individuals with proven trichinosis failed to react in this test, whereas 98% of the specimens from 90 healthy controls gave negative reactions. As indicated in Fig. 23, the reactive specimens from healthy controls were positive at low titers (1:16). Conversely, most of the specimens from individuals with proven trichinosis reacted at much higher titers. Most cross-reactions were observed with sera from schistosomiasis patients. It is of interest to point out that of the 7 positive reactions obtained with sera from individuals infected with S. mansoni, 5 were from lyophilized sera and 2 from 20 frozen specimens.

The use of eluted blood in the SAFA test

An attempt was made to determine whether the technic described for the collection, preservation, shipment and testing of small amounts of dried blood for the diagnosis of trichinosis in the fluorescent antibody test would be applicable to the SAFA test. Dried blood smears and serum

samples obtained at the same time from an infected rabbit were tested at weekly intervals. A comparison of the results (Table 14) indicated identical qualitative and closely correlated quantitative determinations of antibodies. In general, somewhat higher titers were obtained when fresh serum was used.

The results indicate that the soluble antigen fluorescent antibody technic using T. spiralis larvae as antigen can be used successfully in the laboratory diagnosis of trichinosis. Preliminary findings contained in this report suggest that this test possesses adequate sensitivity and specificity. Except for schistosomiasis, cross-reactions with sera from individuals infected with other parasitic agents rarely occurred. The observed reaction between trichinosis and schistosomiasis is in agreement with previous reports and must be attributed either to cross-reactivity with a related antigen present in the 2 helminths or to an anamnestic response resulting from nonspecific stimulation of an already existing antibody.

Dried blood specimens obtained on absorbent paper could be tested satisfactorily by the SAFA technic. This is in agreement with previous observations conducted in the use of fluorescent antibody tests for schistosomiasis, for filariasis, trypanosomiasis, leishmaniasis and trichinosis. If these results are confirmed by tests of samples from infected humans in various parts of the world, they will establish for this technic many advantages over conventional tests based on serum obtained by venipuncture. By collecting dried blood smears on absorbent paper adequate sampling could be obtained in endemic areas, even from infants; collection could be made by relatively untrained personnel without regard to sterility of the specimens, and the samples could be mailed to a central laboratory where the test could be performed by adequately trained laboratory personnel.

The results of single and multiple doses of Trichinella larvae in rabbits, mice and rats indicate that infection with this parasite produced an antibody response of sufficient magnitude to be detectable by the SAFA test. In general, satisfactory and consistent results were obtained throughout the various experiments. All of the infected animals showed a detectable serologic response and no false positives were observed in any of the sera obtained from the animals before infection. The time course development of antibodies produced curves which followed almost parallel lines. Antibodies were detected 2 weeks after inoculation and reached a peak approximately 6 weeks after infection. The antibody level was sustained throughout the experiments. These results are in close agreement with those obtained using metabolic and somatic antigens in the bentonite flocculation test in rabbits exposed to graded infections with this parasite.

Table 13

Results of the SAFA Test for Trichinosis with Human Sera

Diagnostic status	No. tested	Positive reaction	
		Number	%
Trichinosis	28	26	93
Healthy controls	90	2	2
Other parasitic infections	104	17	16
Malaria	8	2	
Amebiasis	5	1	
Chagas' disease	11	1	
Filariasis	18	2	
Hookworm	11	1	
Strongyloidiasis	5	0	
Trichuriasis	9	1	
Ascariasis	8	2	
Echinococcosis	2	0	
Schistosomiasis	27	7	

Table 14

Comparison of Results of SAFA Test for Trichinosis Performed with Eluted Blood and Serum

Specimen No.	Week from collection	Titers in SAFA test	
		Blood	Serum
1	0	1024	1024
2	1	1024	1024
3	2	1024	1024
4	3	256	256
5	4	256	1024
6	5	256	1024
7	6	1024	1024
8	7	256	1024

Fig. 20. Time course development of SAFA in rabbits following single or multiple infections with T. spiralis larvae

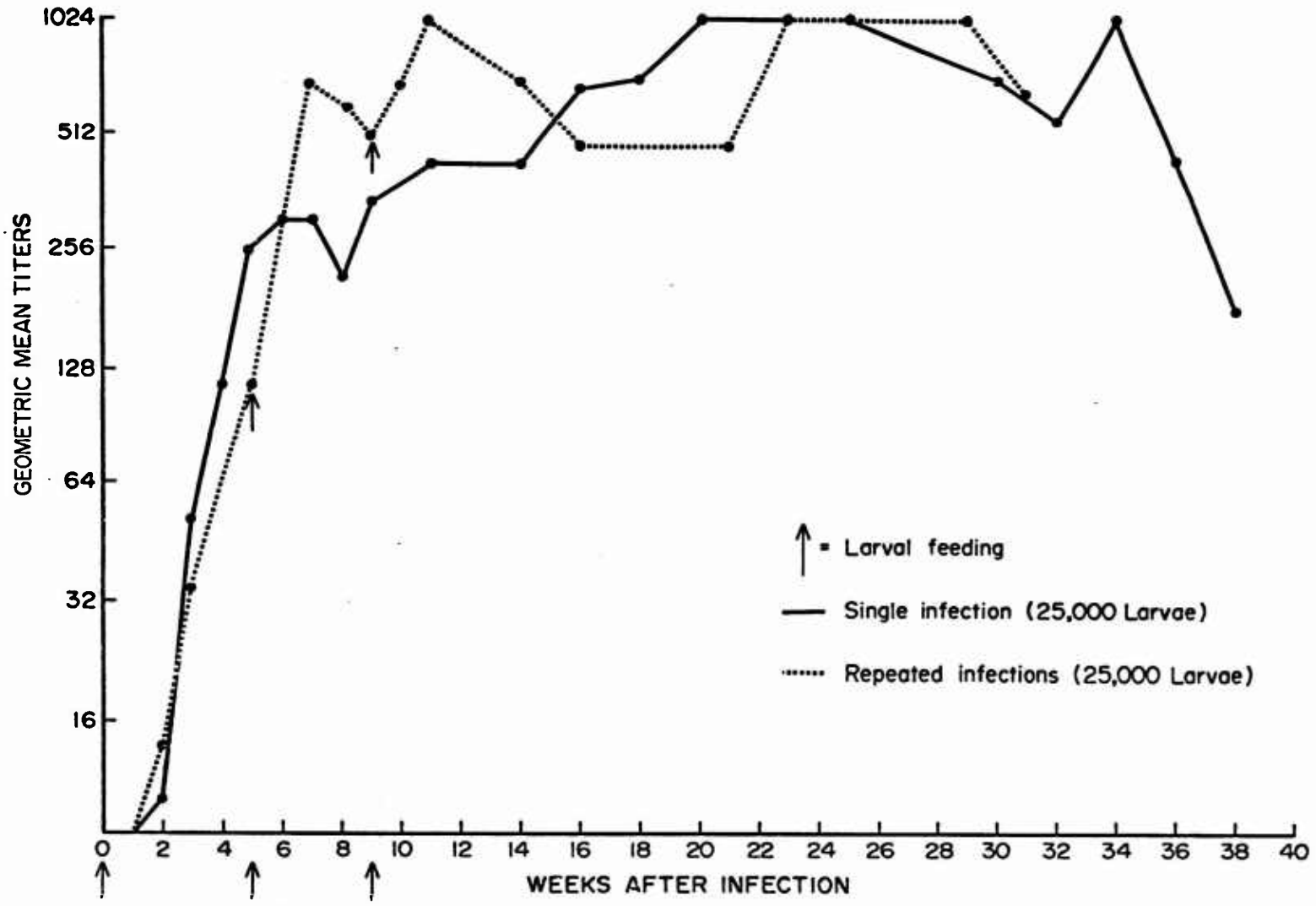


Fig. 21. Time course development of SAFA in mice following a single infection with T. spiralis larvae

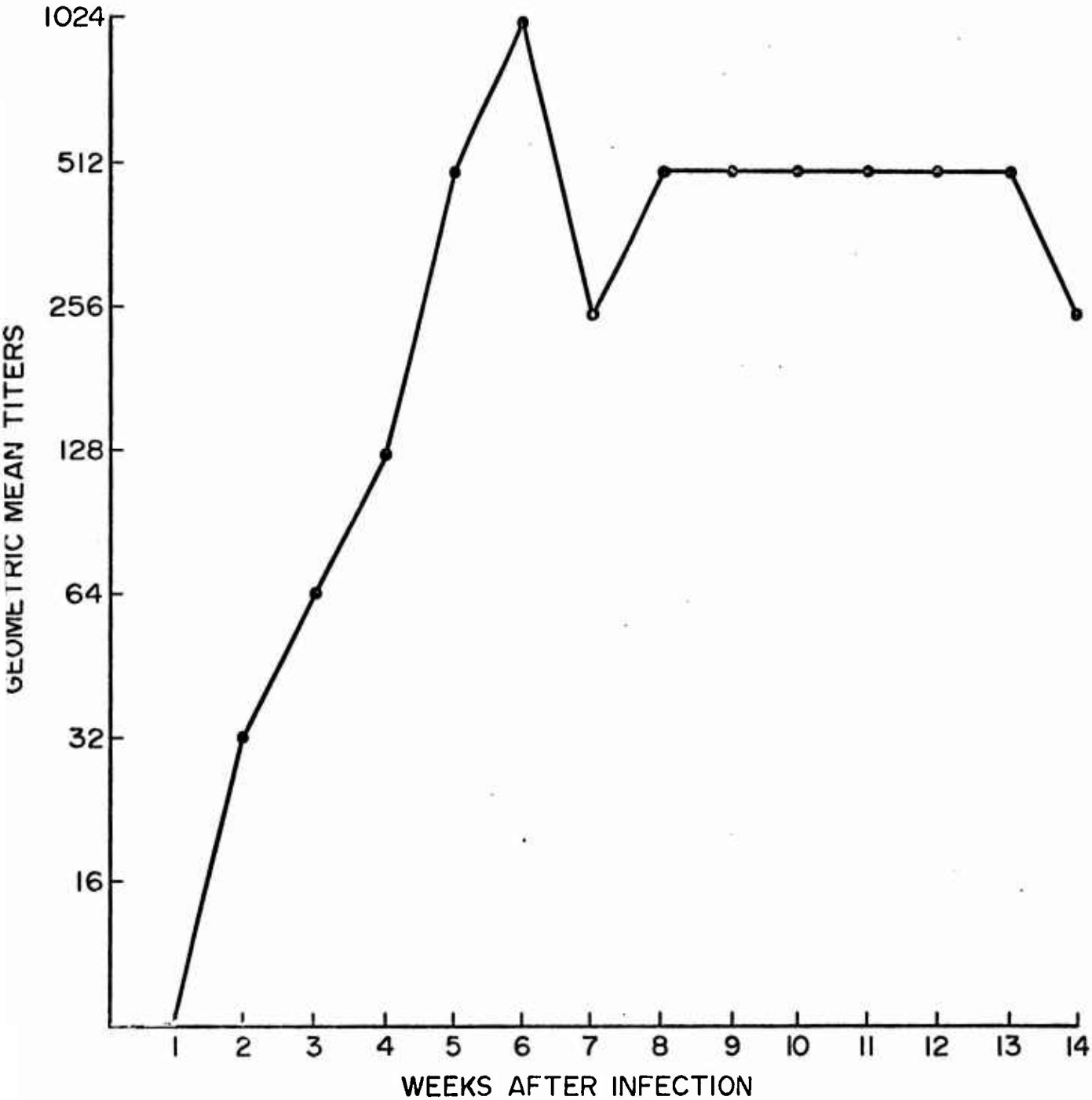


Fig. 22. Time course development of SAFA in rats following a single infection with T. spiralis larvae

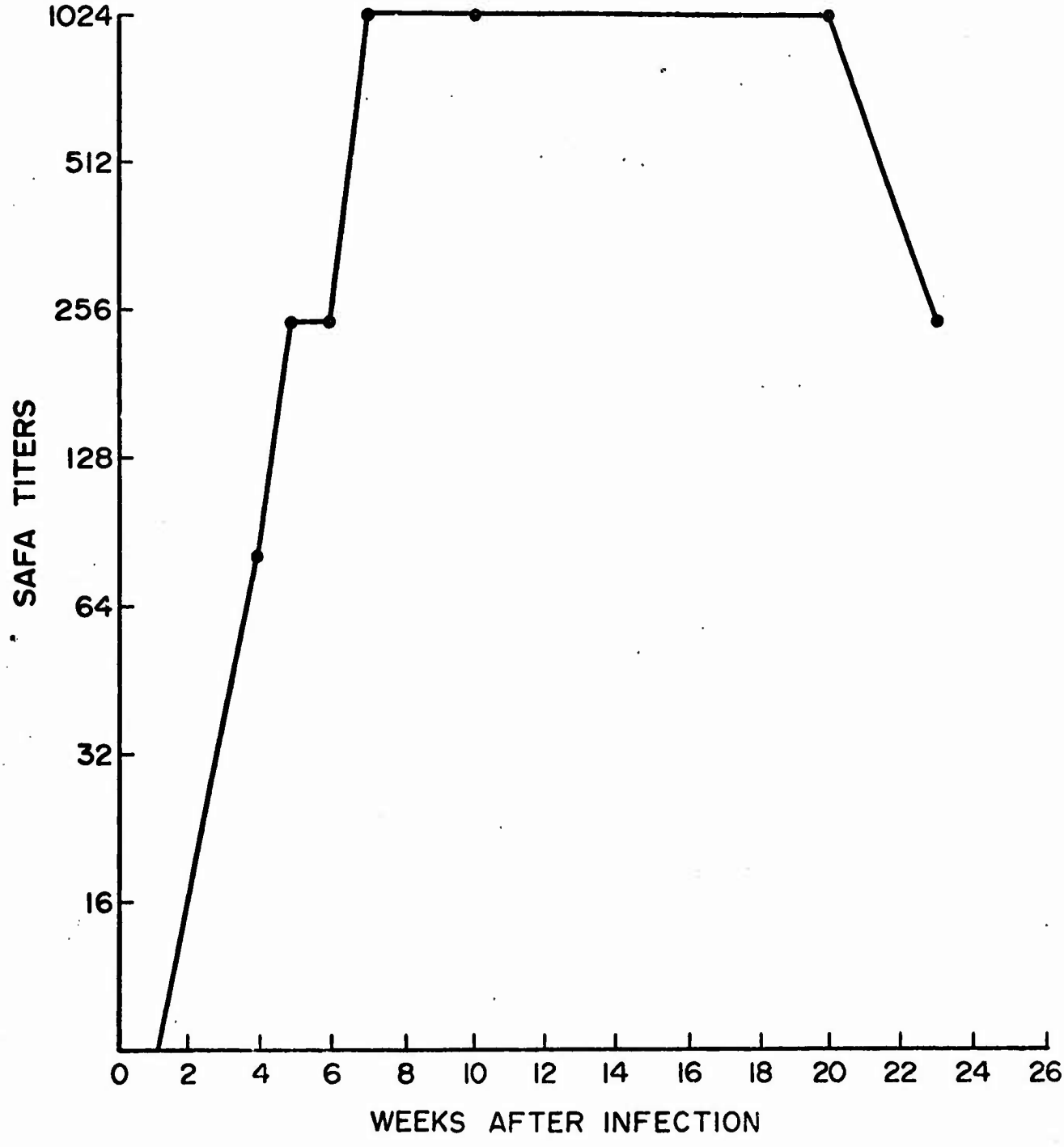
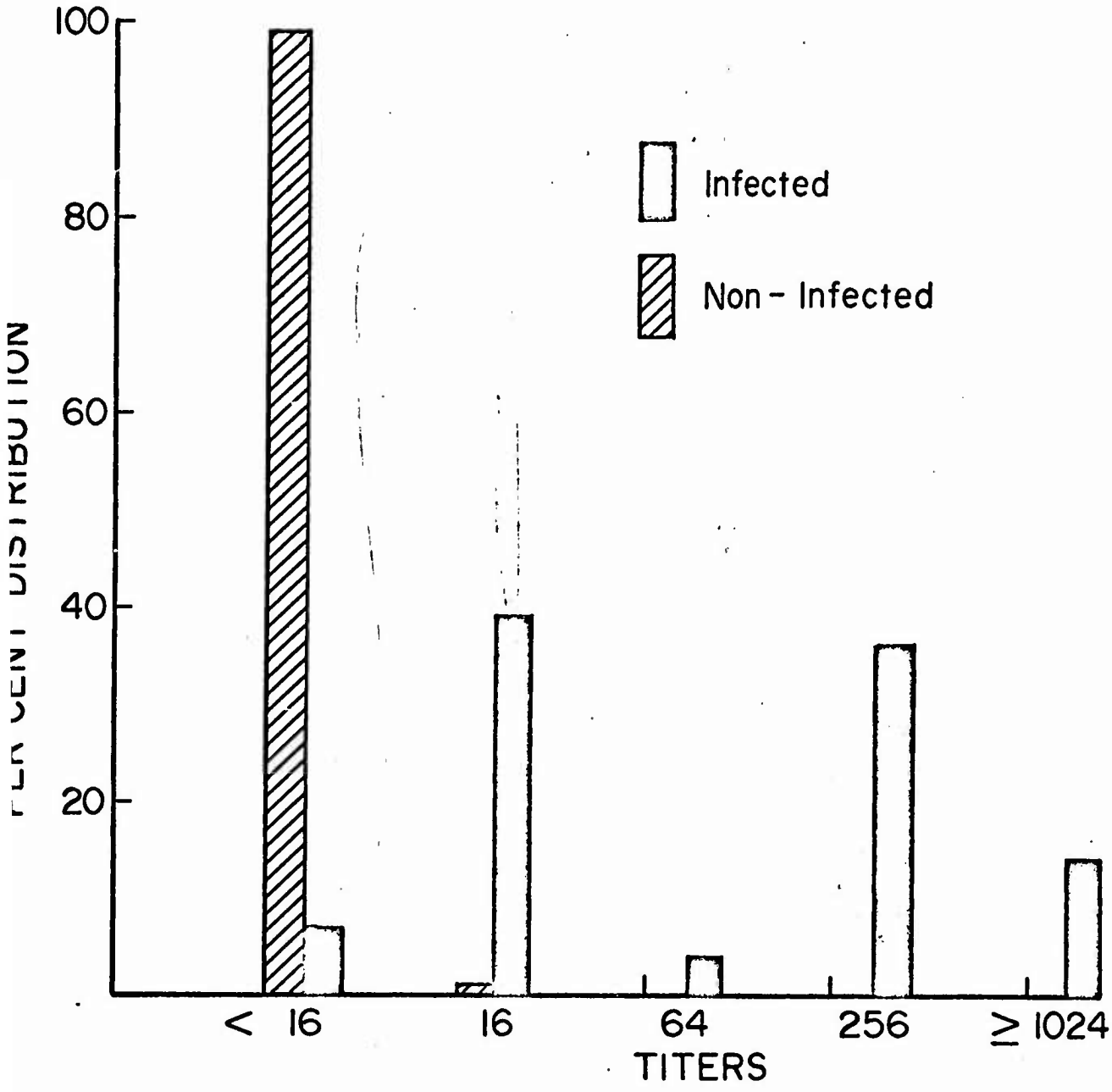


Fig. 23. Distribution of SAFA titers among sera from T. spiralis infected and non-infected humans



6. Demonstration of homocytotropic reagin-like antibodies in mice and rabbits infected with Trichinella spiralis.

Reagin-like antibodies are known to occur under natural conditions in man and lower animals but their artificial induction has been obtained only recently in laboratory animals such as rats, rabbits and mice. These antibodies have been referred to as reagin-like, mast cell sensitizing, anaphylactic, passive cutaneous anaphylactic, and homocytotropic antibodies. In general, they migrate in electrophoresis faster than IgG antibodies and are inactivated by heating at 56°C or when treated with 2-mercaptoethanol. When introduced into the skin they persist at the injection site for many days or weeks, in contrast to IgG antibodies that disappear within 1 or 2 days. It is this property of skin persistence which provides the most useful measure of homocytotropic reagin-like antibody.

Zvaifler and Becker have shown that rabbits immunized with natural or conjugated proteins produce in the early course of immunization an antibody able to induce passive cutaneous anaphylaxis (PCA) in this same species after a latent period of more than 1 day. The PCA antibody activity persists at skin sites for many days, is heat labile, as inactivated by treatment with mercaptoethanol, is non-precipitating and does not fix complement. More recently, Mota and Peixoto were able to detect an early homocytotropic reaginic antibody in mice immunized with ovalbumin or bovine serum albumin plus adjuvant. In contrast with mouse IgG, mouse reaginic antibody remains in the passively sensitized skin for many days.

It is known that animals infected with parasites produce large quantities of reagin-like antibodies whose role in host resistance is still uncertain. The presence of relatively high levels of reagin-like antibodies have been detected in serum of rats infected with Nippostrongylus brasiliensis, of humans, chimpanzees and rhesus monkeys infected with Schistosoma mansoni, of rabbits infected with S. mansoni and of rabbits infected with D. uniformis.

No information is available as to the immunoglobulin classes in which antibodies detected by the passive cutaneous anaphylaxis (PCA) and soluble antigen fluorescent antibody (SAFA) tests are located. This is important since antibodies belonging to different immunoglobulin classes have different biological properties.

The present experiments were designed to study the production and properties of homocytotropic reaginic-like antibodies in mice and rabbits infected with Trichinella spiralis and to compare their relationship to antibodies detected by the fluorescent technic. An attempt was also made to determine in which immunoglobulin fraction antibodies detected by the two tests are located.

Animals

Mice (ICR and Bagg strain) weighing 18-25g and albino rabbits weighing 2500-3000g at the beginning of each experiment were used for PCA reactions and for the T. spiralis infections. All animals were fed a standard diet. The mice were bled either by puncture of the ophthalmic venous plexus or by cutting the brachial plexus under ether anesthesia and collecting the extravasated blood with a Pasteur pipette. The rabbits were bled from the central ear vein before infection and at regular intervals throughout each experiment. After the blood had clotted, serum was separated by centrifugation and stored at -20°C until ready for use. The principles of animal care as promulgated by the National Society for Medical Research were observed.

Experimental infections

The mice were fed 100 washed T. spiralis larvae each and some were reinfected with an equal number 9 weeks after the primary infection. The rabbits were fed 25,000 washed larvae each and some were reinfected twice with the same number of larvae, 5 and 9 weeks after the primary infection. T. spiralis larvae for infecting animals and for preparing antigens were obtained from young adult rats 6 to 8 weeks after they were fed 3,000 to 4,000 washed T. spiralis larvae. The rats were killed and the larvae were separated from the muscles by artificial digestion in a pepsin-hydrochloric acid mixture.

Passive cutaneous anaphylaxis (PCA)

PCA reactions in mice were performed as described by Mota and in rabbits as described by Zvaifler and Becker. In both species PCA reactions were induced 72 hours after sensitization, and the reactions were recorded 30 minutes after antigen injection. They were defined as positive when the area of cutaneous bluing was greater than 5 mm in diameter.

Treatment with heat and reduction and alkylation

Samples of antiserum (0.5 ml) were dialyzed against 250 ml of 0.1M mercaptoethanol for 3 hours at room temperature and then dialyzed in 500 ml 0.02 M iodoacetamide for 4 hours. Before testing, samples were dialyzed against several changes of phosphate-buffered saline for 18-24 hours at 4°C. Control samples were either (1) dialyzed against phosphate-buffered saline in place of mercaptoethanol and then treated with iodoacetamide as described above or (2) dialyzed only against phosphate-buffered saline. Samples of antiserum were heated at 56°C in a water bath for 4 hours. Unheated samples served as controls.

Fractionation of rabbit antiserum

Chromatography on diethylaminoethyl (DEAE) cellulose columns was performed by sequential elution with slight modifications of the method

described by Peterson and Sober. Four and one-half ml of antiserum were applied to a column 2.4 cm in diameter and 47 cm in length; sequential elution was performed with the following phosphate buffers: 0.01 M, pH 7.5; 0.03 M, pH 7.5; 0.05 M, pH 7.5; 0.08 M, pH 7.5 and 0.1 M NaCl and 0.1 M HPO_4, pH 7.5. Ten ml samples were collected at a rate of 1 ml per minute. The protein concentration of the eluates was determined by spectrophotometry at 280 mμ and protein peaks were combined and concentrated to approximately 3 ml by ultrafiltration at 4°C. The protein concentration of each fraction (A through D) was determined by ultraviolet absorption at 280 mμ using 0.0104 as the optical density per μgm N per ml for rabbit antibodies. The concentrated samples were dialyzed against 0.01 M, pH 7.5 phosphate buffer for 8 to 10 hours at 4°C before testing them for antibody activity. The immunoglobulin content was evaluated by immunoelectrophoresis using goat antisera (Hyland Laboratories, Los Angeles, Calif.) directed against rabbit serum or γ globulin.

Antigens

A lipid-free somatic extract of T. spiralis larvae was prepared at 4°C in anhydrous ether and lyophilized. Aliquots of the finished product, in the form of a powder, were sealed in vials and stored at 4°C. Solutions were made when needed by dissolving 10 mg of powdered extract in 1.0 ml of physiological saline.

In the cross-reactivity studies, lipid-free somatic Schistosoma mansoni and Dirofilaria uniformis antigens were prepared according to the method of Chaffee et al.

Soluble antigen fluorescent antibody (SAFA) test

This test was conducted essentially as described for trichinosis and for schistosomiasis. A normal control serum was used to set the fluorometer dial at zero. Four-fold serum dilutions from 1:16 through 1:1024 were made. In determining test results, sera which gave fluorometer dial readings of 20 or more were considered to be reactive, and titers were expressed as reciprocal of the highest dilution giving a positive reading.

Mice

A series of experiments was designed to determine whether mice infected with T. spiralis develop reagin-like antibodies. In each experiment all the mice were given 100 T. spiralis larvae and some of them, taken at random, were killed at weekly intervals. The serum samples obtained each time were pooled and the pools were tested for the presence of homocytotropic reagin-like antibodies by PCA reactions. Nine weeks after the primary infection, one-half of the remaining mice were reinfected with 100 larvae each while the others were kept as primary infection controls. The results of quantitative PCA determinations are summarized in Fig. 24. Nine weeks after infection, challenge produced a sharp increase in the serum antibody level. The mouse anti-T. spiralis

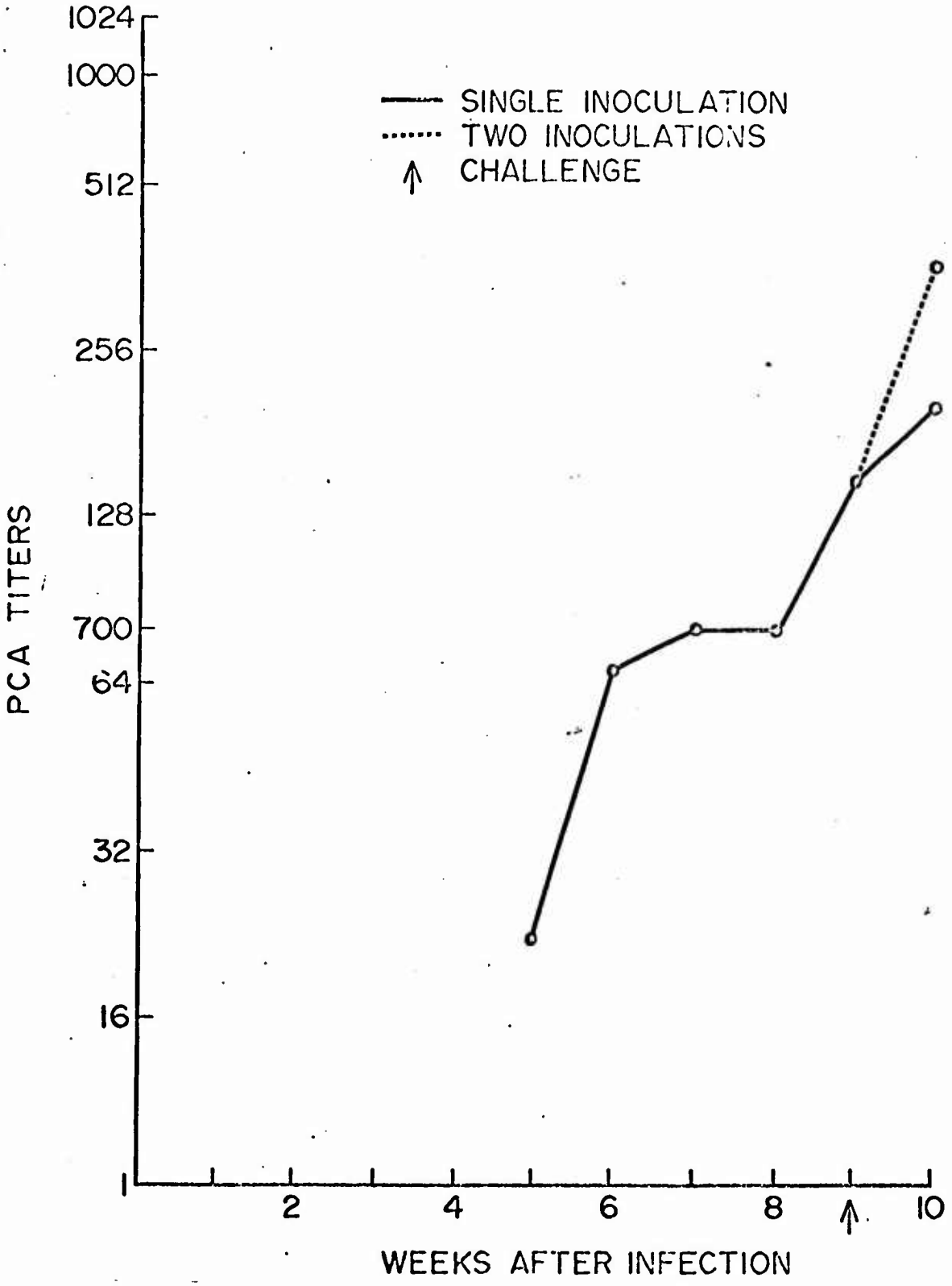


Fig. 24. Reagin-like antibody titers (PCA) in sera from mice inoculated with one or two doses of 100 Trichinella spiralis larvae.

reagin-like antibodies had a marked lability to heat and reduction and alkylation (Table 15). Reduction alone had no effect.

Table 15

Effect of Heat and Reduction and Alkylation on PCA Activity of Mouse Anti-T. spiralis Sera

Treatment	Titer of Serum Pool #1	Titer of Serum Pool #2	Titer of Serum Pool #3
Unheated	50	ND*	200
Heated	0	ND	0
2-mercaptoethanol	50	40	ND
Iodoacetamide	50	40	ND
Dilaysis only	50	40	ND
2-mercaptoethanol and Iodoacetamide	0	0	ND

*ND

The time-course development of reagin-like and fluoresc t antibodies in mice infected with T. spiralis was compared in a series of experiments. The results (Fig. 25) indicated that antibodies detected by PCA and SAFA tests followed independent courses. In 2 groups of mice, the reaginic antibodies reached a peak titer 9 weeks after infection and persisted at a low level throughout the experiment (16 weeks), but the SAFA titers reached a peak 5-6 weeks after infection and remained at a high level afterwards. In another group of animals infected in the same way, the reagin-like antibody decreased rapidly after the tenth week and could no longer be detected 11 weeks after infection, although the antibody level as determined by the SAFA test remained high. Thus, the dissociation between antibodies detectable by SAFA and by PCA was evident in all groups.

Rabbits

A first experiment was designed to determine the effect of a single inoculation of T. spiralis on the development of homocytotropic and fluorescent antibodies. Six rabbits were each given 25,000 T. spiralis larvae and were bled at weekly intervals for 38 weeks. The time-course development of PCA and fluorescent antibody activity in these 6 rabbits is summarized in Fig. 26. SAFA and PCA antibodies were detected as early as 2 weeks after infection and persisted for the duration of the experiment. The titers in both tests rose rapidly and persisted at high levels

Fig. 25. Time-course development of SAFA and PCA titers in sera from mice infected with a single dose of 100 Trichinella spiralis larvae.

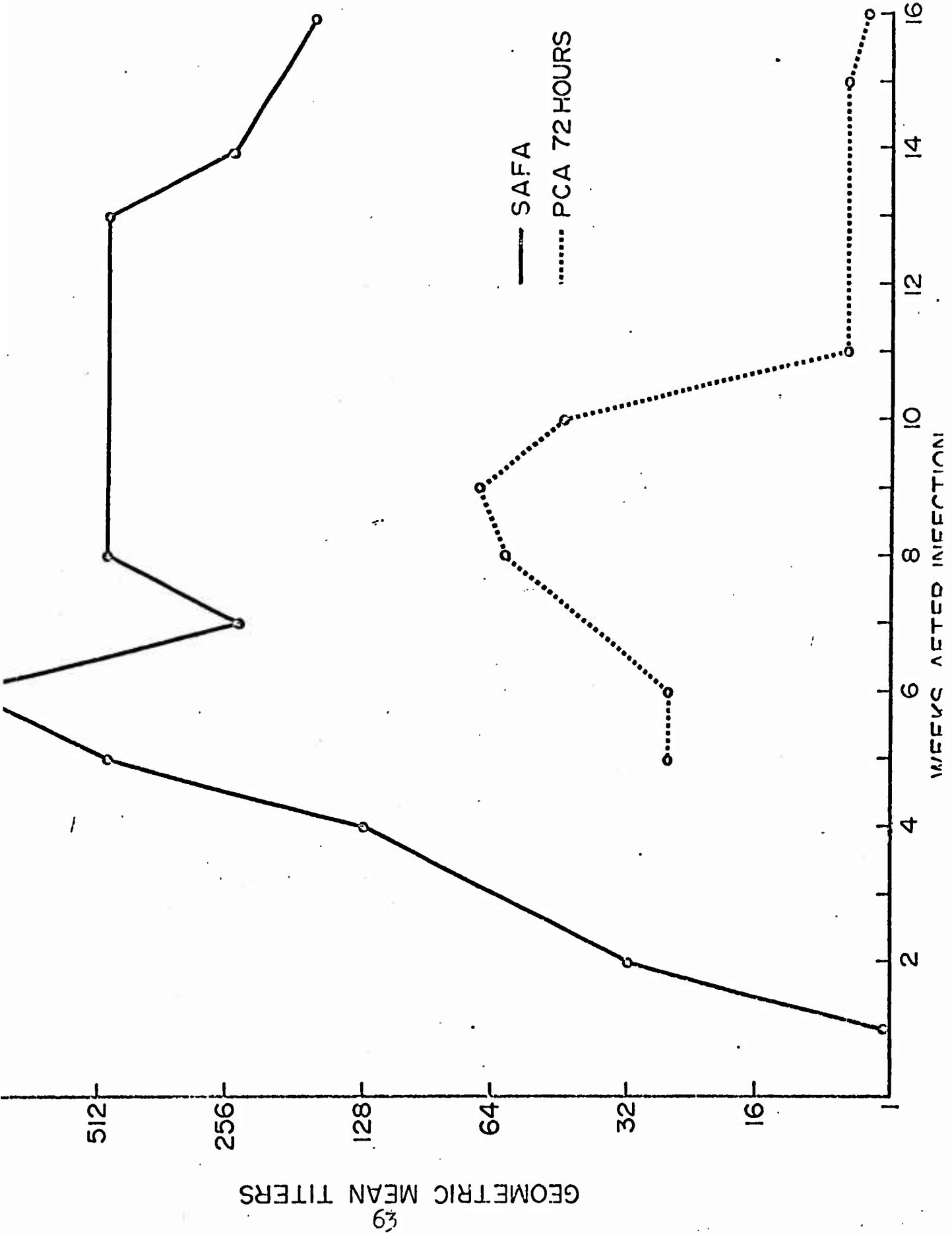
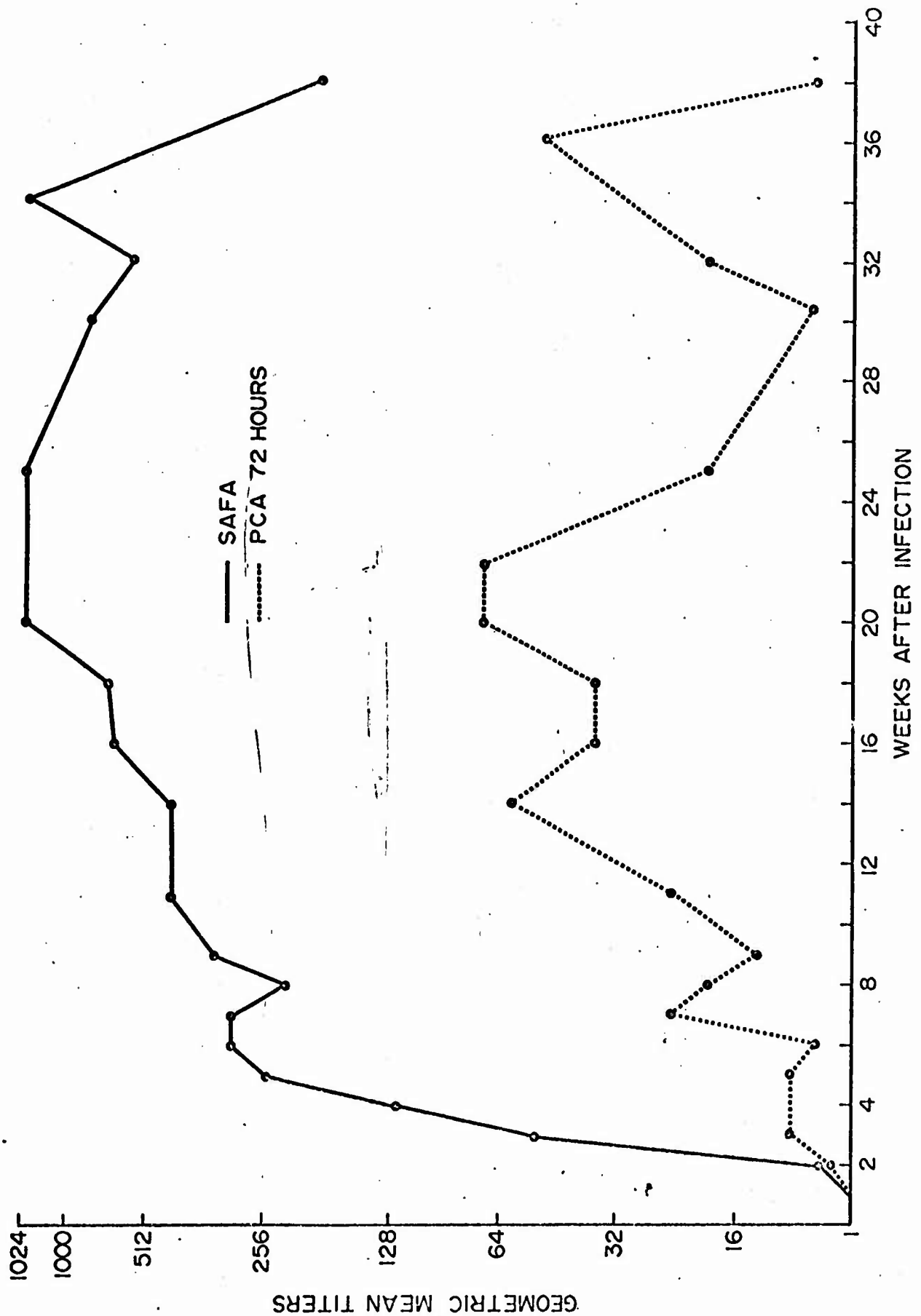


Fig. 26. Time-course development of SAFA and PCA titers in sera from rabbits infected with a single dose of 25,000 Trichinella spiralis larvae.



in most of the animals. Although all 6 of the infected rabbits developed fluorescent antibodies, only 3 of the 6 developed PCA antibodies. There was no apparent correlation between PCA and fluorescent antibody activity in the 6 rabbits.

A second experiment was set up to compare the SAFA and PCA activity in 6 rabbits following multiple infections with T. spiralis. Each animal was infected with 25,000 larvae and reinfected 5 and 9 weeks after the primary infection. As indicated in Fig. 27, although all 6 rabbits developed fluorescent antibodies after the first infection, PCA activity was detected in only 1 rabbit (No. 300) after the primary infection and in 3 others (Nos. 303, 305 and 307) after reinfection. No PCA activity was detected at any time in 2 animals (Nos. 302 and 303). SAFA titers rose quickly and reached a high level 6 weeks after infection.

Separation of antibodies responsible for SAFA and PCA tests

Serum specimens collected from rabbits 3 and 22 weeks after infection with T. spiralis were tested by PCA and SAFA tests before and after heating at 56°C for 4 hours or after exposure to mercaptoethanol, iodoacetamide and dialysis (Table 16).

Table 16

Effect of Heat and Reduction and Alkylation on PCA and SAFA Activity of Rabbit Antisera Obtained 3 and 22 Weeks after Infection with T. spiralis

Treatment	Titer of Pool 4 3 wks		Titer of Serum #302 22 wks		Titer of Serum #300 22 wks	
	PCA	SAFA	PCA	SAFA	PCA	SAFA
Unheated	0	64	0	1024	500	1024
Heated	0	64	0	1024	10	1024
2-mercaptoethanol and iodoacetamide	0	<16	0	1024	0	1024
Iodoacetamide	0	64	0	1024	50	1024
Dialysis only	0	64	0	1024	50	1024

As indicated in Table 16, the PCA antibodies were heat labile and were inactivated by reduction and alkylation. Conversely, no reduction in SAFA titers was observed in 2 of 3 antisera after treatment with mercaptoethanol. It must be pointed out that diminution in SAFA titer after reduction and alkylation occurred only with an antiserum collected soon after infection.

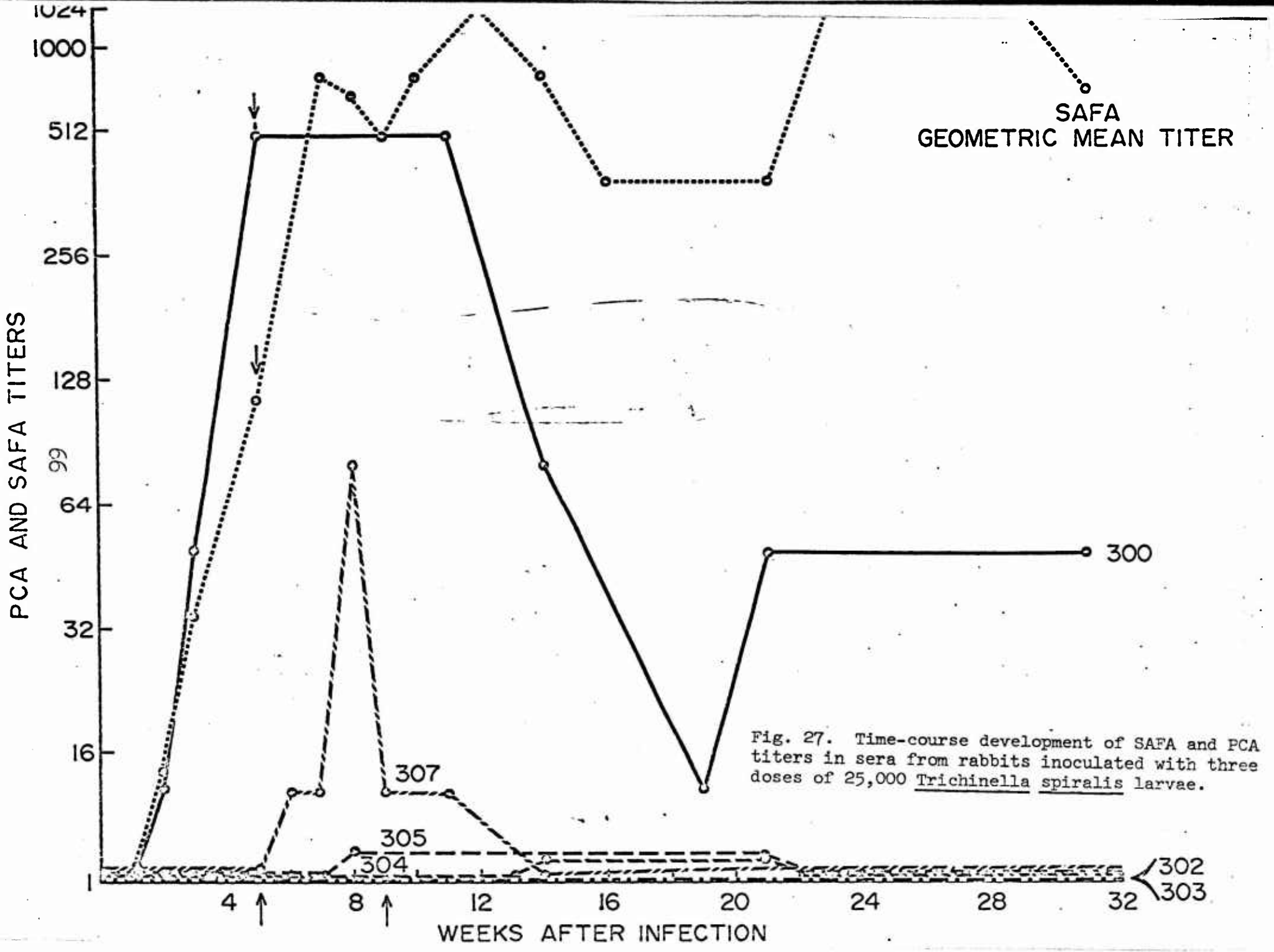


Fig. 27. Time-course development of SAFA and PCA titers in sera from rabbits inoculated with three doses of 25,000 Trichinella spiralis larvae.

Ion exchange chromatography was employed to determine elution patterns of some of the antibodies present in a 22 week rabbit antiserum pool which gave a SAFA titer of 1024 and a PCA titer of 100. Aliquots of each of the 4 fractions obtained (Fig. 28) were analyzed for their ability to produce anaphylactic reactions in the skin of rabbits and for their SAFA activity. Fractions A and B representing electrophoretically slow gamma globulin contained the major amounts of immunoglobulin and most of the detectable fluorescent antibody activity. Neither of these fractions reacted in the PCA test. Fraction C contained most of the PCA activity and only some fluorescent antibody activity at a low titer. Electrophoretically faster immunoglobulins (Fraction D) contained passive cutaneous anaphylactic activity but no detectable fluorescent antibody titer.

Cross-reactivity among antigens from T. spiralis, S. mansoni and D. uniformis

Cross-reactivity studies in the SAFA and PCA tests were made in rabbits infected with different helminths as a possible means of detecting other differences between fluorescent and reagin-like T. spiralis antibodies. Serum specimens from rabbits infected with either T. spiralis, S. mansoni or D. uniformis were tested by the SAFA technic. Cross-reactivity was observed between T. spiralis and S. mansoni (Table 17). Furthermore, S. mansoni antiserum also cross-reacted in the SAFA test with D. uniformis antigen. However, no cross-reactivity between T. spiralis and D. uniformis was observed in this test. Additional cross-reactivity experiments were set up by the use of the PCA test. Serum from rabbits infected with either T. spiralis, S. mansoni or D. uniformis was inoculated into the skin of recipient rabbits. Some of these animals were challenged with T. spiralis antigen, others with S. mansoni antigen and others with D. uniformis antigen. As shown in Table 17, no cross-reactivity was detected in this test between T. spiralis and S. mansoni. Conversely, cross-reactivity was observed between T. spiralis and D. uniformis.

Table 17

Cross-reactivity Among Helminth Antigens as Detected by PCA and SAFA Tests

	Antiserum					
	T. spiralis		S. mansoni		D. uniformis	
Antigen:	PCA	SAFA	PCA	SAFA	PCA	SAFA
T. spiralis	+	+	-	+	+	-
S. mansoni	-	+	+	+	-	-
D. uniformis	-	-	-	+	+	+

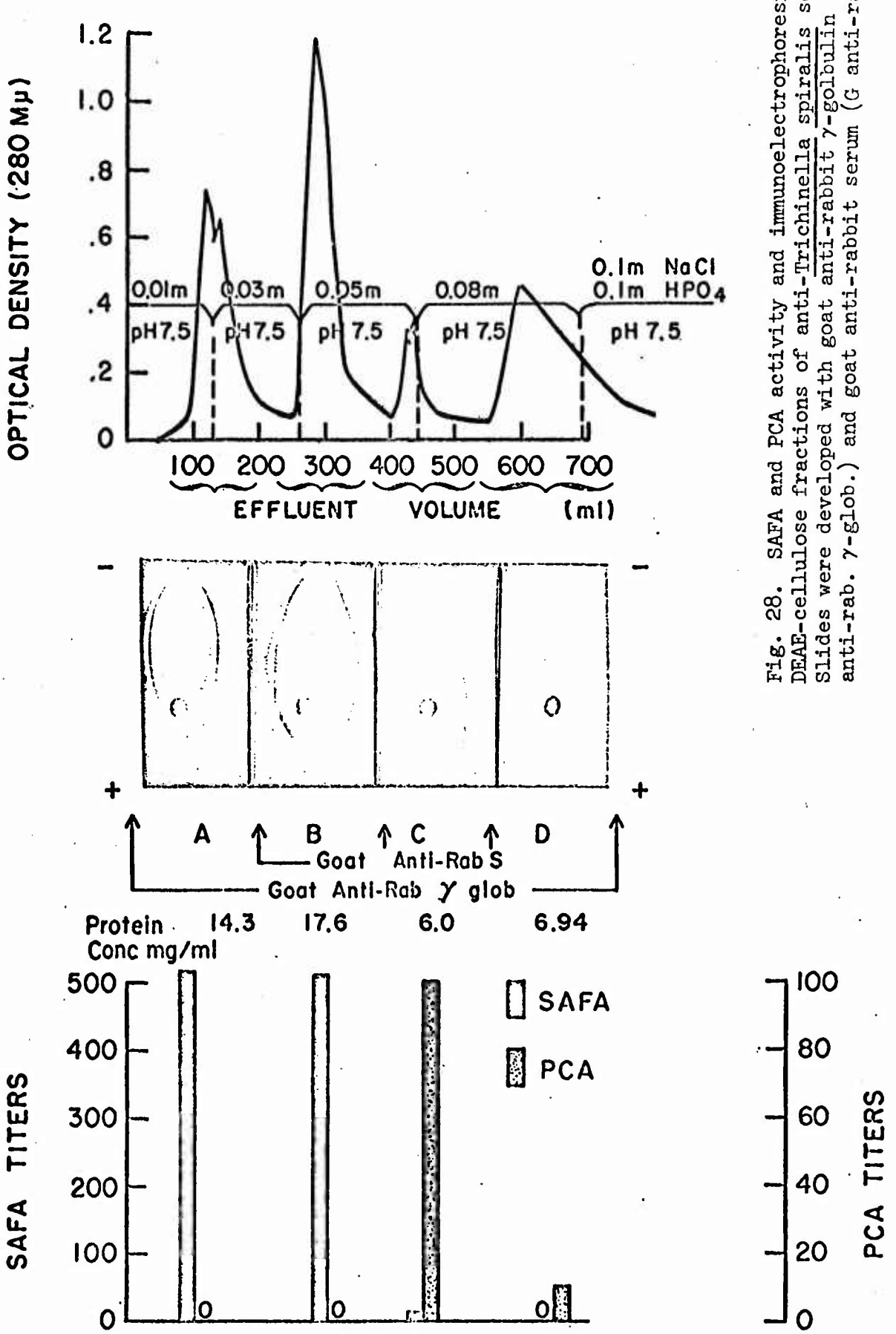


Fig. 28. SAFA and PCA activity and immunoelectrophoresis of DEAE-cellulose fractions of anti-Trichinella spiralis serum. Slides were developed with goat anti-rabbit γ-golbulin (G anti-rab. γ-glob.) and goat anti-rabbit serum (G anti-rab. S).

The results of these investigations show that mice and rabbits infected with Trichinella spiralis are capable of producing different types of antibodies one of which is similar to human reagin. The results obtained with rabbits are in agreement with and extend those reported previously for Schistosoma mansoni and Dirofilaria uniformis. The level of PCA antibodies in these 2 helminthic infections rose abruptly several weeks after infection, reached a peak titer early after infection and fell rapidly. However, in rabbits infected with T. spiralis, PCA activity was detectable as early as 2 weeks following infection, did not reach its peak until 18 weeks later and was still present 38 weeks after infection. As observed in experiments using artificial immunization or helminthic infections in rabbits, the amount of reagin-like antibodies produced varied greatly from animal to animal and in some rabbits could not be detected at any time during infection. Even among rabbits which were infected with a total of 75,000 larvae each in 3 doses, one-third of the animals failed to show a detectable PCA activity.

As observed in earlier studies with other helminths, although SAFA activity was detected in all the infected animals, PCA activity was demonstrated only in some of them. Both in mice and rabbits the time-course development of reagin-like antibodies followed a pattern quite different from that of fluorescent antibodies. This difference was particularly striking in the rabbits given more than one infection at various intervals. The disappearance of homocytotropic antibodies in one group of mice and in some rabbits inoculated only once with T. spiralis has also been observed in various animal species with other parasitic infections.

The instability of mouse and rabbit PCA antibody upon reduction and alkylation contrasted with the failure of such treatment to alter the activity of antibodies detectable by the SAFA technic in rabbit serum obtained later (22 weeks) in the infection. This is in agreement with previous observations and supports the view that reagins lose their in vivo biological activity following alkylation and reduction under conditions in which IgG antibodies retain their in vitro activity.

Cross-reactivity studies of PCA antibodies in rabbits infected with T. spiralis, S. mansoni and D. uniformis revealed another important difference between homocytotropic and fluorescent antibodies. Although in the SAFA test cross reactions occurred between T. spiralis antigen and S. mansoni antiserum, no such cross-reactivity was detected in the PCA reactions. However, cross-reactivity in the PCA test was observed when T. spiralis antigen was used to challenge rabbits which had received D. uniformis antiserum. Cross-reactivity between S. mansoni and T. spiralis had been observed with most of the conventional tests.

The results of experiments with column chromatography and immunoelectrophoresis indicate that the rabbit homocytotropic antibody activity detected by PCA reactions does not belong to the IgG class of rabbit immunoglobulin. This is in agreement with earlier results showing that rabbit anaphylactic antibodies had a faster electrophoretic ability than

IgG globulin. SAFA and PCA activities of fractions of rabbit antiserum obtained by DEAE chromatography were located in different fractions. Whereas most of the detectable fluorescent antibody activity and none of the PCA activity was found in the electrophoretically slow gamma globulin (presumably IgG), electrophoretically faster immunoglobulins contained PCA activity but no detectable fluorescent antibodies. These results agree with those reported for filariasis.

Attempts to characterize the fluorescent antibody activity indicated that it was contained in the electrophoretically slower immunoglobulins. Sera obtained from rabbits as early as 3 weeks and as late as 22 weeks after infection were tested to determine the effect of heat and reduction and alkylation on the SAFA test. Fluorescent antibody sensitive to reduction and alkylation, presumably IgM,was present in sera obtained from rabbits 3 weeks after infection. Conversely, antibody resistant to heat and to alkylation and reduction, probably IgG, was observed 22 weeks after infection. Our data are not sufficiently extensive to indicate the length of time IgM antibody persisted after infection, but on the basis of the present results it appears that most, or all, of it had disappeared in rabbits by the twenty-second week of infection.

The possible role of reagin-like antibodies in the physiopathology of T. spiralis infection is now under investigation.

7. The immunological response of mice infected with Trichinella spiralis. Biological and physicochemical distinction of two homocytotropic antibodies.

The antibody formed even to a single antigen may consist of a population of molecules with varying physicochemical properties and varying biological activity. Recent studies on the biological behavior of these different antibody molecules led to the finding that some of the antibodies produced by a given species are not able to mediate anaphylaxis in the same species although they are able to do so in other species. On the other hand, other immunoglobulins were able to sensitize only the same species that produced them or a closely related species. These latter antibodies were named homocytotropic while the former were named heterocytotropic. Some homocytotropic antibodies are characterized by their sensitivity to heat and to alkylation and reduction as well as by their remarkable property of remaining in the sensitized skin for many days. It is this property of skin persistence which provides the most specific means of characterization of this type of homocytotropic antibody which will be named throughout this report "reagin-like antibody." This type of antibody is usually present at a high level in the serum of animals infected with helminthic parasites.

The recent observation that after a single antigenic stimulation mice can produce 2 distinct homocytotropic antibodies, one able to induce passive cutaneous anaphylactic (PCA) reactions only within a few hours after sensitization and another sensitizing for PCA even after a

72 hour latent period, prompted us to investigate the production of similar antibodies by mice infected with Trichinella spiralis.

ICR, Bagg and hairless mice bred at Walter Reed Army Institute of Research, weighing 18 to 25g, were used throughout. The principles of animal care as promulgated by the National Society for Medical Research were observed.

Each mouse was fed 100 washed T. spiralis larvae both in the primary and later infections. The larvae were obtained from young adult rats approximately 6 weeks after they were fed 3000 - 4000 washed T. spiralis larvae. The rats were killed and the larvae were separated from the muscles by artificial digestion in a pepsin-hydrochloric acid mixture.

A lipid-free somatic extract of T. spiralis larvae was prepared at 4°C in anhydrous ether. Aliquots of the finished product, in the form of a dry powder obtained by lyophilization, were sealed in vials and stored at 4°C. Solutions were made when needed by dissolving the dry extract in saline (NaCl 0.85 percent).

Larvae were collected from the ground meat of infected rats after digestion in pepsin-HCl mixture for 90 to 120 minutes. They were washed at least 6 times by sedimentation in distilled water, by the dilution method and placed in a 1:10,000 merthiolate-physiological saline solution for 10 minutes. After two more washings in sterile physiological saline solution, 500,000 larvae were transferred into each of several 100 ml Erlenmeyer flask(s) containing 15 ml of medium 199 with 20% fetal bovine serum, 15,000 units of penicillin and 15 mg of streptomycin. A drop of the culture in each flask was examined under the microscope, and if dead larvae were found, the batch was discarded. If no dead larvae were present the mixture was shaken gently and incubated at 37°C for 72 hours. At the end of this time the cultures were observed under the microscope. Practically all of the larvae were motile. The few which were immobile showed no visible signs of internal degeneration. The larvae were separated from the preparation by high speed centrifugation. The supernatant fluid was stored at -20°C until used as antigen.

Larvae were collected from infected rats as above. They were washed by sedimentation in 5 changes of distilled water. Without further delay, they were placed in a Ten Broeck tissue grinder in an ice bath and homogenized for 20 minutes and used immediately as antigen.

Blood was obtained either at the end of the experiment by cutting the brachial plexus under ether anesthesia and collecting the extravasated blood with a Pasteur pipette, or during the course of immunization by puncture of the ophthalmic venous plexus. The blood was allowed to clot in an ice-bath and the serum was separated by centrifugation in a refrigerated centrifuge. Sera were kept at -20°C until used.

Ovary's method for PCA was used for detecting and estimating antibody with the exception that a latent period of 72 hours was also used.

Two or 3 intradermal injections of 0.05 ml antiserum or antiserum dilution were made on each side of the mouse's dorsal skin with a sharp hypodermic needle, and PCA reactions were elicited after a latent period of either 4 or 72 hours. The animals were injected intravenously with 0.5 ml of a 0.25 percent solution of pontamine sky blue in saline containing 0.5 mg antigen. Twenty to 30 minutes after antigen injection, the animals were killed with ether, the skin was inverted and the lesion diameter was measured on the inner surface of the skin with a transparent ruler. Antibody was estimated by determining the highest dilution of antiserum which induced PCA reaction. PCA titers were stated as the reciprocal of the highest serum dilution giving a skin reaction. A minimum of 6 mice was used for each determination. Hairless mice were most frequently used for PCA reactions because they did not need to be shaved and also they provided more consistent PCA reactions than either Bagg or ICR strains of mice. However, due to difficulty in obtaining hairless mice in sufficient numbers, Bagg mice were sometimes used.

Samples of serum (0.5 ml) were dialyzed against 250 ml of 0.1 M 2-mercaptoethanol for 3 hours at room temperature and then dialyzed against 500 ml 0.02 M iodoacetamide for 4 hours. Before testing, samples were dialyzed against several changes of phosphate-buffered saline for 18-24 hours at 4°C. Control samples were either (1) dialyzed against phosphate-buffered saline in place of mercaptoethanol and then treated with iodoacetamide as described above, or (2) dialyzed only against phosphate-buffered saline.

To determine the effect of heating on the ability of anti-T. spiralis mouse serum to induce PCA, the antiserum was heated for 60 minutes in a water bath at 56°C.

Chromatography on DEAE-cellulose was performed by applying 3 ml antiserum to columns measuring 2.5 cm in diameter and 30 cm in length packed under 5 pounds of pressure. Gradient elution was performed by gradually combining 3 phosphate buffer solutions having the same pH of 8.0 but differing in their molarity by addition of NaCl: 0.01 M, 0.1 M and 0.15 M. The mixture of these solutions was made by using the variable gradient device for chromatography as described by Peterson and Sober. Three ml samples were collected at the rate of about 0.7 ml per minute. The protein concentration of the eluates was determined by spectrophotometry at 2800 angstroms; protein peaks were combined and their volume was reduced to the original serum volume by positive pressure filtration. The concentrated samples were then tested for their ability to induce PCA either 4 or 72 hours after sensitization.

Induction of antibody production by infection with T. spiralis

The appearance and persistence of antibodies after a single infection with T. spiralis were studied in 2 groups of animals infected in the same way about 2 weeks apart. The animals of each group were bled weekly from 4 to 16 weeks after infection. The sera obtained at

each time interval were pooled and tested for their ability to induce PCA reactions after a latent period of 4 to 72 hours. In both groups, 4 and 72 hour PCA antibody activity appeared in the circulation 5 weeks after infection and each attained its maximum titer about the ninth week. From then on there was a striking difference between the two groups. In one (Fig. 29) the antibody responsible for the 72 hour PCA reaction disappeared from serum after the 10th week whereas the 4 hour PCA antibody remained in the other group (not shown here) both antibodies remained in circulation until the 16th week after infection when the animals were killed. At that time the reagin-like antibody content was very low (PCA titer of 10 after 72 hours).

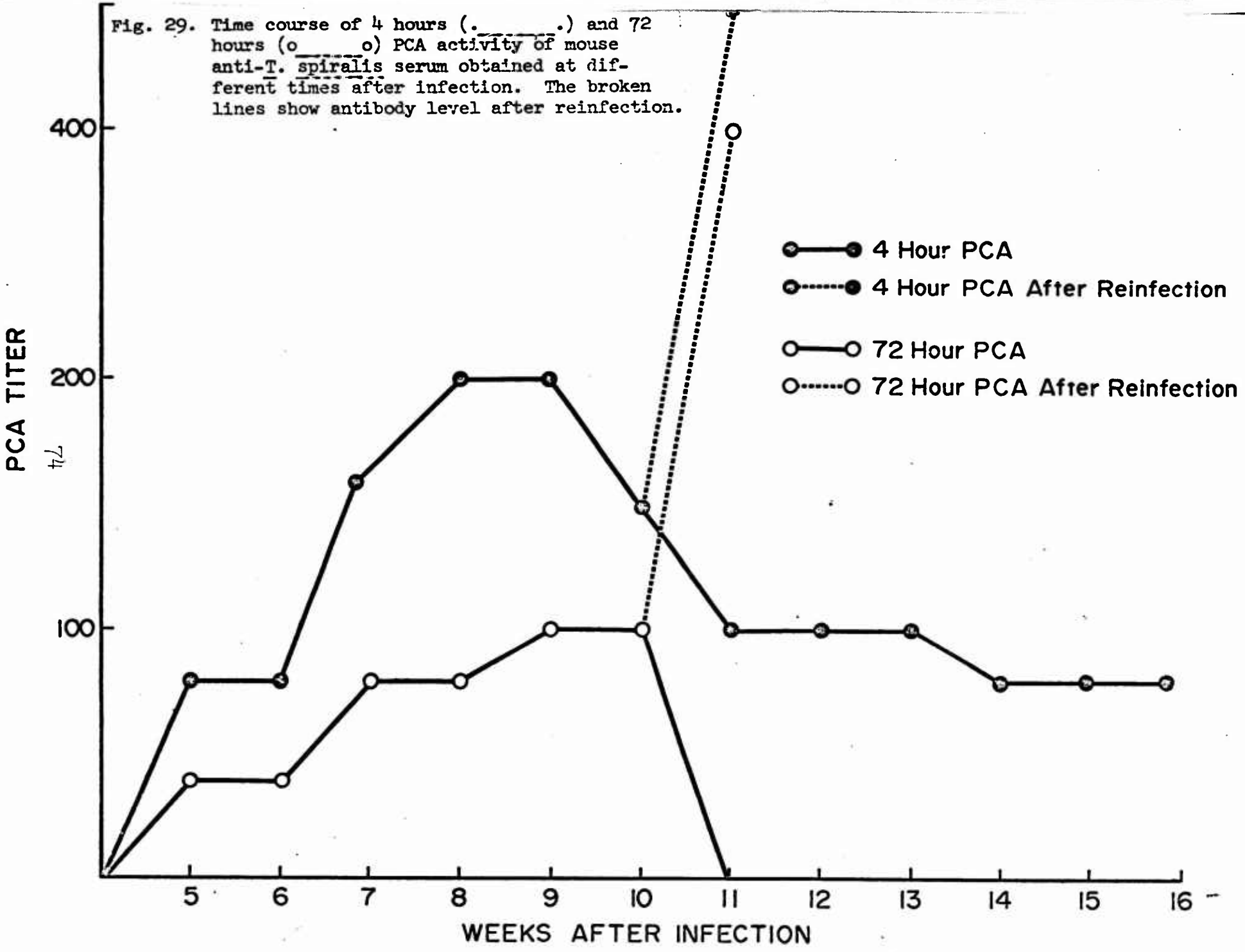
Effect of a second infection. Ten weeks after primary infection of the first group of mice they were divided into two groups, one of which received a second infection. The other group was not reinfected but was kept as a control to test the antibody level in the absence of reinfection. A week later both groups were bled and the pooled sera of each group were tested for PCA activity after 4 and 72 hours. Both of these activities rose sharply a week after the secondary infection (Fig. 29, broken lines).

Effect of repeated infections. In order to observe the effect of multiple infections, a group of mice was infected with T. spiralis and bled from the ophthalmic plexus for antibody determination 9 weeks later. The animals were then reinfected 6 times, at weekly intervals. Antiserum was collected 6 days after the 3rd infection and 10 days after the 6th infection. Antisera obtained after the primary infection and after the 3rd reinfection were pooled while antisera obtained after the last reinfection were tested individually. The results of this experiment, summarized in Fig. 30, show that although the level of the reagin-like antibody increased after 3 reinfections, it was very much reduced after the 6th reinfection and had disappeared from the sera of some of the animals. On the other hand, the 4 hour PCA antibody was still detectable at high titers even after the 6th reinfection.

Induction of antibody production by "dead" T. spiralis antigen

Although it was realized that it is very difficult to duplicate by artificial immunization the same type of antigenic stimulation provided by a living infection, experiments were performed to find out whether immunization of mice with "dead" T. spiralis antigens would induce an immunological response similar to that induced by a living parasite infection. A group of mice received a single subcutaneous dose of 100 μg T. spiralis larval antigen adsorbed onto aluminum hydroxide. Ten days later the animals were bled from the ophthalmic plexus and their sera were pooled and tested for PCA 4 and 72 hours after sensitization. The results of this experiment showed that immunization with an extract of T. spiralis larvae was able to induce the production of antibody activities with the same PCA characteristics as those present in the serum of mice infected with T. spiralis (Table 18).

Fig. 29. Time course of 4 hours (._____.) and 72 hours (o_____o) PCA activity of mouse anti-T. spiralis serum obtained at different times after infection. The broken lines show antibody level after reinfection.



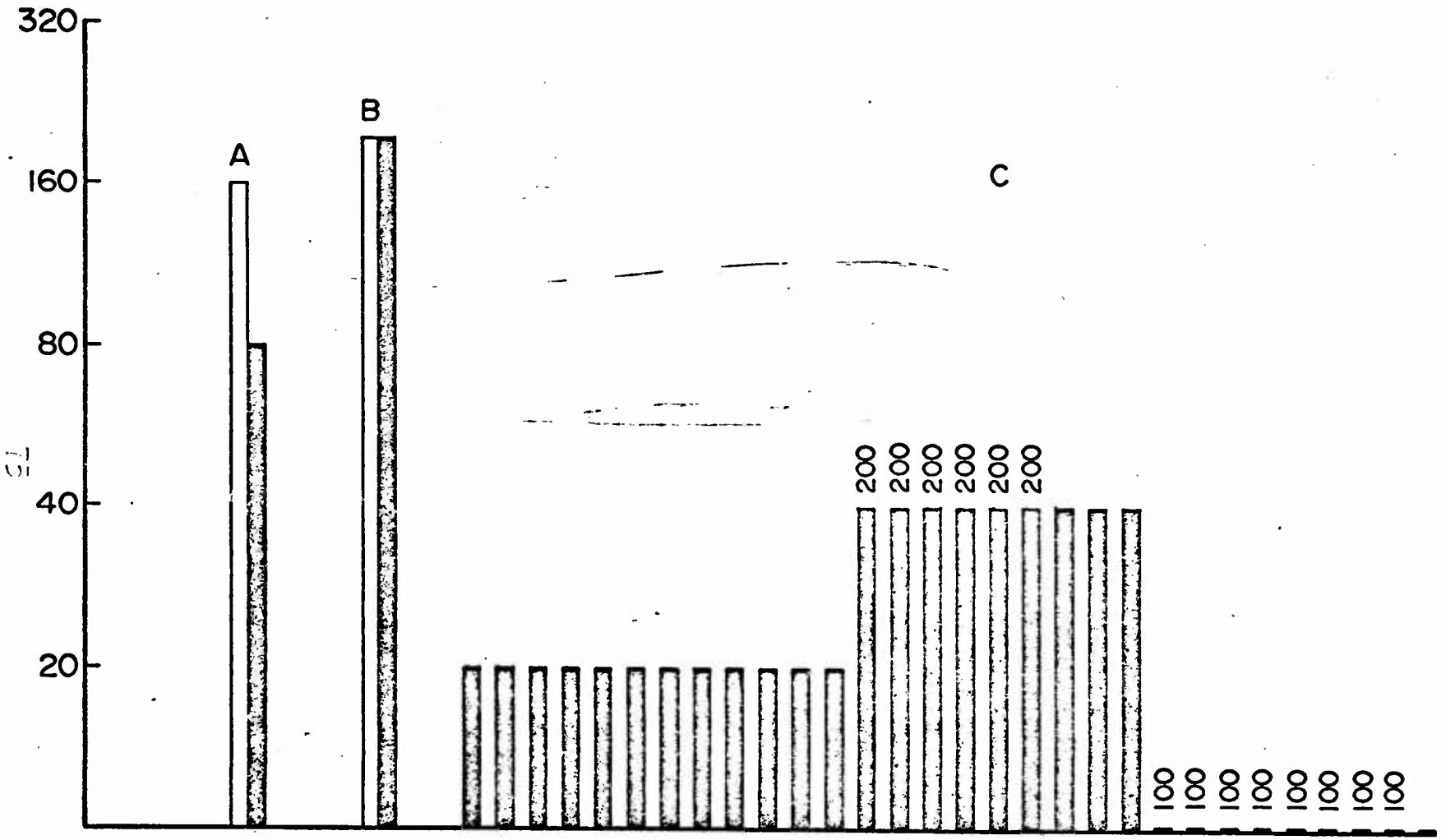


Fig. 30. Antibody level of sera of T. spiralis infected mice; A) Four (▯) and 72 (▮) hours PCA titer of pooled sera obtained 9 weeks after a primary infection; B) Four and 72 hour PCA titer of pooled sera obtained one week after the last of 3 additional infections; C) 72 hour PCA titer of individual sera obtained 10 days after the 6th and last additional infection. Each vertical column represents one serum. Figures above the columns show 4 hour PCA titer for the same serum when it was possible to obtain it.

Effect of repeated doses of antigen. The same animals used in the previous experiments subsequently received weekly intradermal injections of 0.1 ml saline containing 50 μg T. spiralis antigen for 6 weeks. Seven days after the 3rd injection and 10 days after the last injection, they were bled and their sera pooled. The pools were then tested for their ability to induce PCA 4 or 72 hours after sensitization. The content of the 72 hour PCA antibody increased after 2 additional injections of antigen and then disappeared after 3 subsequent injections (Table 18). However, the same treatment resulted in a steady increase in the 4 hour PCA antibody.

Table 18

Production of antibodies to T. spiralis antigen by mice after a single or multiple injection of "dead" antigen. Antibody content of antisera determined by PCA reactions induced 4 or 72 hours after sensitization.

Type of antigen	Number of injections	Amount of antigen injected	PCA titer 4 hr	PCA titer 72 hr
Larval extract	1	100 μg	50	20
	3	50 μg	100	100
	6	50 μg	200	0
Fresh washed larvae	1	10^3 larvae	20	5
Metabolic antigens	1	1.5×10^6 larvae	20	10

Immunization with fresh larvae suspension and metabolic antigens. Since drying and lyophilization of larvae can produce denaturation of larval antigens that may be important for the induction of reagin-like antibodies, a group of mice was injected with 0.5 ml of a freshly-prepared T. spiralis larvae suspension (1000 larvae/ml) plus aluminum hydroxide.

Metabolic antigens which are products of excretion and secretion of the worms are present in relatively low concentration in an extract of larvae. Since these antigens are constantly produced in the infected organism they may represent an important antigenic stimulus missing in the larval extract. Accordingly, a group of mice was also injected with 0.25 ml of the metabolic antigen preparation plus aluminum hydroxide. The results of these experiments (Table 18) showed that neither the fresh larvae suspension nor the metabolic antigens preparation were more effective in inducing the production of reagin-like antibodies than the dried and lyophilized extract of larvae.

Effect of treatment with 2-mercaptoethanol. As shown in Table 19, treatment of mouse anti-T. spiralis sera with mercaptoethanol followed by alkylation with iodoacetamide resulted in a considerable decrease in the ability of antisera to induce PCA after 4 hours and in the total loss of their ability to induce PCA after 72 hours.

Table 19

The Effect of Reduction and Alkylation on PCA Reactivity of Sera from Mice Infected with T. spiralis

Treatment	Average PCA Titers*			
	Untreated		Treated	
	4 hrs	72 hrs	4 hrs	72 hrs
2-mercaptoethanol	80	50	80	50
Iodoacetamide	80	50	80	50
Dialysis only	80	50	80	50
Mercaptoethanol and Iodoacetamide	80	50	10	0

*Reciprocal of highest antiserum dilution giving a skin reaction.

Effect of heating. The effect of heating at 56°C for 60 minutes on the ability of different mouse anti-T. spiralis sera is shown in Table 20. Although the ability of the antisera to induce PCA after 4 hours was not changed, their ability to induce the same reaction after 72 hours was completely lost.

Table 20

The Effect of Heat on PCA Reactivity of Sera from Mice Infected with T. spiralis

Antiserum	PCA Titers*			
	Unheated		Heated (56°C 1 hr)	
	4 hrs	72 hrs	4 hrs	72 hrs
TW	200	100	200	0
SP	400	50	400	0
TI	80	40	80	0
TB	400	400	400	0

*Reciprocal of highest antiserum dilution giving a skin reaction.

Chromatographic separation of the 4 and 72 hour PCA activity. Chromatography of mouse anti-T. spiralis serum on DEAE-cellulose column resulted in eluates able to induce PCA only after 4 hours and eluates able to induce PCA after 4 hours and 72 hours. Fig. 31 shows the distribution of PCA activity in the several eluates obtained using mouse anti-T. spiralis pool T collected 6 weeks after a single infection (PCA titer after 4 hours: 80; after 72 hours: 40). As it can be seen, three eluates, B, C and D, were able to induce PCA after 4 hours but not after 72 hours. Actually, they were not able to induce PCA even when challenge was done only 24 hours after sensitization. Thus, the antibody eluted with the first fractions is not able to remain in the skin for more than a few hours. On the other hand, eluates E, F and G were able to induce PCA after 4 hours and after 72 hours. These eluates may contain only one antibody able to induce PCA after 4 hours as well as after 72 hours or they may contain 2 antibodies, one responsible for the 4 hour PCA and another responsible for the 72 hour PCA.

Reagin-like antibodies are known to occur under natural conditions in man and lower animals and their artificial production has been obtained recently in laboratory animals such as rats, rabbits and mice. These antibodies have been designated by a variety of terms: reagin-like, mast cell sensitizing, anaphylactic, passive cutaneous anaphylactic and homocytotropic antibodies. For reasons still undetermined, animals infected with helminthic parasites produce large quantities of these antibodies whose role in host resistance is not yet precisely known. The presence of relatively high levels of a reagin-like antibody has been detected in serum of rats infected with Nippostrongylus brasiliensis; of humans, chimpanzees and rhesus monkeys infected with S. mansoni; of rabbits infected with S. mansoni and of rabbits infected with D. uniformis. Previous indirect evidence for the existence of a "mast cell sensitizing" antibody in mice infected with T. spiralis was provided by the work of Briggs who showed that mast cells of mice infected with T. spiralis were disrupted when contacting specific antigen. Our results show that mice infected with T. spiralis produce at least 2 homocytotropic antibodies, one able to induce PCA reactions only within a few hours after sensitization and another sensitizing for PCA even after a 72 hour latent period. Like the early antibody induced by injecting mice with ovalbumin the 72 hour PCA antibody obtained from T. spiralis infected mice is completely destroyed by heating and by treatment with mercaptoethanol. On the other hand, PCA reactions induced 4 hours after sensitization were not changed by heat and were only partially reduced by treatment with mercaptoethanol. As can be seen in Fig. 31, the antibodies responsible for these activities not only differ in their biological properties but also have different physicochemical properties which allow their separation by chromatography on DEAE-cellulose. It is possible that these different eluates still represent a heterogeneous antibody population. In spite of this, however, we think that the large majority of antibody molecules present in the initial and last eluates are different as indicated by their different properties. The 4 hour PCA activity may be due to 7S γ1 antibody which, so far, is the only mouse immunoglobulin known to have homocytotropic activity. The 72 hour PCA activity, which attains very high levels in

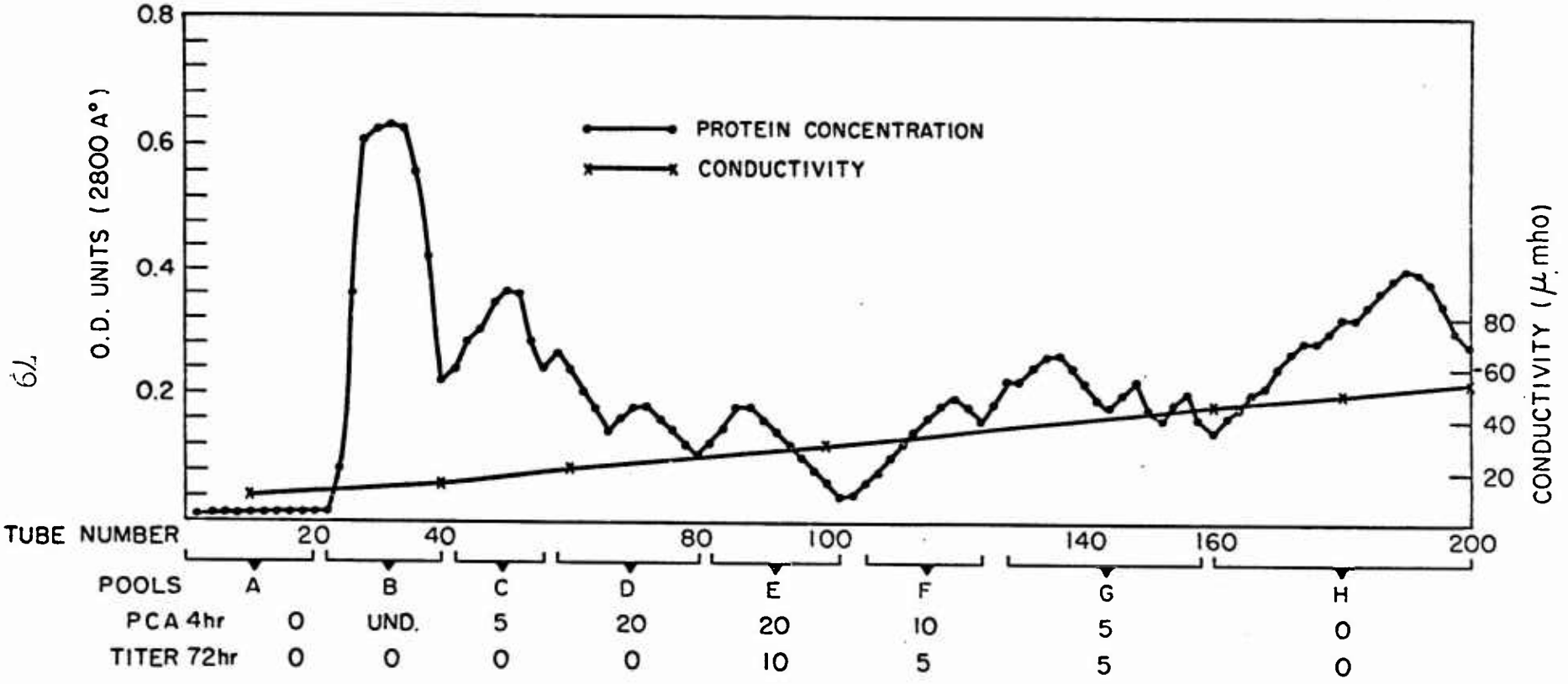


Fig. 31. Activity of pooled eluates obtained by DEAE-cellulose chromatography of mouse anti-T. spiralis serum. Each pool was tested for its ability to induce PCA 4 and 72 hours after sensitization.

sera of T. spiralis infected mice, may be the same observed to occur early in sera of mice receiving a single dose of antigen. The immunoglobulin class of this antibody is yet unknown; it may be a new type of immunoglobulin similar to the human IgE described by Ishizaka and Ishizaka or a special part of the 7S γ1 population as suggested by McCamish.

It is of considerable interest to know why a living parasite infection is such an efficient stimulus in inducing the production of reagin-like antibody. This has been attributed to different causes such as the 1) nature of parasite antigens, 2) continuous presence of antigen and 3) location of the antigen in the intestinal tract. The nature of the parasite antigen does not fully explain this since injection of parasite antigens, although able to induce the production of reagin-like antibody, induced only a low level of the antibody as shown in Table 18. It is possible that the continuous stimulation by antigen is an important factor in inducing the production of reagin antibody although repeated stimulation with T. spiralis antigen resulted in the disappearance of the reagin-like antibody from serum. However, if the constant presence of antigen is an important factor for stimulating the production of antibody it would probably be so only in the beginning of the infection since the antibody could not be detected in some T. spiralis infected mice in which contact with antigen was continuously taking place. Therefore, the constant presence of antigen by itself is apparently not sufficient to keep the production of antibody at a high level. This is in agreement with previous observations showing that repeated contact with antigen not only failed to increase the antibody level but also accelerated the disappearance of the antibody from serum. In apparent contrast with this, a second infection of mice with T. spiralis produced an anamnestic response with a sharp increase in the antibody level as shown in this paper and previously observed by Ogilvie in studying infections of rats with N. brasiliensis,and by Zvaifler et al. in rabbits infected with S. mansoni. However, during artificial induction of reagin-like antibody with "dead" antigen, the reagin-like antibody of rats immunized with a single dose of antigen did not always disappear from serum after one additional dose of antigen but, on the contrary, it was increased in some animals and only decreased and disappeared after additional doses. Besides, as shown here, mice receiving a second injection of T. spiralis "dead" antigen also responded with an increase in the reagin-like antibody. A further explanation for the high level of reagin antibody induced by helminth infection is that the location of the parasites in the host may influence the type of antibody produced. However, this does not seem to be a decisive factor since reagins are produced in the host by a variety of elminths whose location is as different as that of S. mansoni, T. spiralis, Nematospiroides dubius and Dirofilaria uniformis. Since infection with living parasites is such a potent inducer of reagin-like antibody, we thought it was possible that many repeated infections would be required to bring down the antibody production by infected animals. As can be seen in Fig. 30, six additional infections were necessary to cause the disappearance of the reagin-like antibody from the serum of infected animals. In fact,even at this time,the antibody was still present in some of the animals at a low level. Thus, there may be only

a quantitative difference in the production of reagin-like antibody by "dead" antigen or by a living parasitic infection. The latter induced a much higher and sustained production of the antibody. In both situations the production of reagin-like antibody seems to be stimulated after the initial constant or repeated contact with antigen and then gradually subsides. This may occur either in animals under continuous and long-lasting antigenic stimulus, after a single infection with T. spiralis, or in infected animals receiving additional antigenic stimulation on being reinfected. This is in agreement with the previous suggestion that antibody-forming cells are able to produce reagin-like antibody only after the initial contacts with antigen, losing this ability after prolonged contact with antigen. However, the problem of why a living parasitic infection is so effective in inducing the production of reagin-like antibody is still unresolved.

Summary and Conclusions.

1. The specificity of the immunoelectroadsorption test was studied. There was a direct relationship between optical thickness and globulin concentration; no specific anti-S. mansoni or dinitrophenyl antibodies were detectable.

2. A soluble antigen fluorescent antibody test for amebiasis was developed.

3. The clinical evolution of liver pipe stem fibrosis in chimpanzees with schistosomiasis was similar to that in man.

4. In vitro antigen-induced histamine release from blood platelets of rabbits infected with S. mansoni was obtained both in the presence and absence of passive cutaneous antibodies.

5. A soluble antigen fluorescent antibody test for trichinosis in man and animals was developed.

6. Mice and rabbits infected with T. spiralis produced homocytotropic reagin-like antibodies detectable by passive cutaneous anaphylactic reactions after a 72 hour latent period.

7. T. spiralis infected mice developed a second homocytotropic reagin-like antibody which produced passive cutaneous anaphylaxis only after a 4 hour latent period and which could be separated from 72 hour antibody on DEAE-cellulose.

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RESEARCH AND TECHNOLOGY RESUME

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			CURRENT FY 69	13	330

19. GOVT. LAB/INSTALLATION/ACTIVITY

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21. TECHNOLOGY UTILIZATION: NA

22. COORDINATION: NA

ENTEROVIRUS DISEASES, VIRUSES, IMMUNOLOGY, BEHAVIOR, ECOLOGY, RESPIRATORY TRACT INFECTIONS, ARBOVIRUS INFECTIONS.

23.

(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.

(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.

(U) PROGRESS - JUL 67 THRU JUN 68 THE EXTENT OF DISABILITY RESULTING FROM ACUTE RESPIRATORY DISEASE IN BASIC COMBAT TRAINEES HAS BEEN FOUND TO VARY FROM ONE TRAINING POST TO ANOTHER DURING A GIVEN YEAR, BETWEEN YEARS FOR A GIVEN POST. FACTORS INFLUENCING THIS VARIATION ARE BEING SOUGHT AND IDENTIFIED. THE SPECIFIC ETIOLOGIES OF ARD WERE FOUND TO VARY BETWEEN POSTS, AND CONTINUE TO BE INFLUENCED BY BOTH NATURALLY OCCURRING AND VACCINE INDUCED IMMUNITY. APPLICATION OF ORALLY ADMINISTERED LIVE ADENOVIRUS TYPE 4 VACCINE CONTINUES TO FOSTER EMERGENCE OF OTHER RESPIRATORY PATHOGENS AS EPIDEMIC PRODUCING STRAINS. PRELIMINARY EXPERIMENTS SHOW THAT THE INTRODUCTION AND DISSEMINATION OF OTHER ADVENTITIOUS ADENOVIRUSES CAN BE BLOCKED OR INTERFERED WITH BY INFECTING THE NASOPHARYNX OF PREVIOUSLY IMMUNIZED INDIVIDUALS WITH LARGER DOSES OF VACCINE ADENOVIRUS TYPE 4. BOTH KILLED AND LIVING ADENOVIRUS TYPE 7 VACCINES HAVE BEEN PREPARED FOR EVALUATION IN MAN. THE NATURAL HISTORIES OF EEE AND WEE VIRUSES CONTINUE TO BE STUDIED IN TIDEWATER MARYLAND, DATA SUGGEST THAT THE DISTRIBUTION OF THE NATURAL SYLVAN VECTOR IN CERTAIN STUDY AREAS IS NOT RANDOM AND IS INFLUENCED BY NATURAL ENVIRONMENTAL PROTECTION OFFERED BY MARSHES TO IMMATURE FORMS OF THE VECTOR. SYSTEMS HAVE BEEN DEVISED FOR THE PURIFICATION, CHARACTERIZATION AND DIRECT OBSERVATION OF DENGUE II VIRUSES, WHICH WHEN PROPAGATED IN CELL CULTURE SYSTEMS OCCUR IN AT LEAST 3 DIFFERENT DENSITIES AND 2 DIFFERENT FORMS, PURIFIED VIRUS IS BEING SOUGHT FOR IMMUNIZATION PURPOSES. MOST NEWLY FOUND ANTIBODY TO TYPHOID VACCINE IS A MACROGLOBULIN MOIETY WHICH IS PRIMARILY ANTIFLAGELLAR IN SPECIFICITY. SURFACE ANTIBODY IN THE RESPIRATORY TRACT APPEARS TO BE CRUCIAL IN PROTECTION AGAINST RESPIRATORY VIRUS INFECTIONS, STUDIES CONTINUE OF ITS SPECIFICITY, MODE OF ACTION AND OF METHODS FOR ITS INDUCTION. IMPROVED METHODS FOR RAPID DIAGNOSIS OF VIRUS INFECTIONS AND FOR ACCURATE QUANTITATIVE ANTIBODY ASSAYS CONTINUE TO BE SOUGHT. FOR TECHNICAL REPORTS, SEE WRAIR ANNUAL PROGRESS REPORT, 1 JULY 67 - 30 JUNE 68.

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TEXT NOT REPRODUCIBLE

Project 3A014501B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 166, Viral Infections of Man

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Description.

To define the etiology and ecology of human virus infections, particularly those of military medical significance; to devise and evaluate means for precise diagnosis, control and/or prevention of disease. Studies have applied virological, immunological, epidemiological and physiological approaches to understanding disease caused by respiratory, arthropod-borne and other viruses, the factors influencing transmission among men, other vertebrates and invertebrates, and their survival in nature.

Progress.

I. Evaluation of Living Attenuated Adenovirus Type 4 Vaccine for Suppression of Acute Respiratory Disease (ARD) in Recruits. Induced Respiratory Tract Infection with Vaccine Virus.

An earlier experiment (Annual Report, this project, 1966-1967) showed that the vaccine strain, adenovirus type 4 (AV-4) could be safely administered by intranasal instillation to volunteer subjects who had received the same virus by mouth in enteric coated tablets three weeks previously. Exposure to approximately 100,000 TCD_{50} of virus resulted in the successful colonization of the upper respiratory tract of 8 of 12 volunteers who shed virus for 2-15 (mean 8.8) days, asymptomatically. No evidence for transmission from infected virus-shedding volunteers to either susceptible or immunized unchallenged persons was obtained. Extended to include naturally immune persons (rather than induced immunity the result of natural infection) at Fort Dix, N.J., the procedure was shown to be

a safe and effective way of establishing active adenovirus infections of the upper respiratory tract.

To determine whether such nasal infection, if induced in a large proportion of a BCT Company would interfere with the introduction and subsequent transmission of other adenoviruses, particularly AV-7, two field experiments were conducted at Fort Dix, N.J.

A. Evaluation of Induced Nasal Infection with AV-4, Vaccine Strain to Interfere with Transmission of Natural AV-7, Fort Dix, N.J., Oct-Dec 1967.

The first study was made in three companies of BCT at a time when the incidence of hospitalized ARD was rising; disease was shown to have been associated previously with AV-4 (60%) and with AV-7 (30%).

1. Design of Study. Three companies in a single cohort of BCT's (E-3-3, B-6-3 and B-2-3) were studied. Each recruit in the companies were given living enteric AV-4 vaccine (Wyeth Lot 16C1-00201) at the outset of BCT. The original plan was to instill fluid AV-4 vaccine (Wyeth Lot 16C1-00201) to each man in two companies (E-3-3 and B-6-3) at the end of the third week of BCT, 21 days after oral immunization. Placebo nose drops were planned for the 3rd Co. (B-2-3). Each company would be followed for ARD associated with adenoviruses in the conventional manner. One platoon from each company would be monitored for transmission of adenoviruses by twice weekly study of each man in the platoon for silent pharyngeal infection. However because an unexpected increase in incidence of ARD occurred at the beginning of the study, and because early evidence for variation in incidence of disease between companies was found, the basic design was changed. An outbreak of ARD occurred in B-2-3 during the 2nd BCT week and in E-3-3 during the 3rd BCT week, while few ARD admissions occurred in B-6-3 by the end of the 3rd BCT week, the time of intranasal AV-4 instillation. The changes were as follows:

a) Two platoons of each of the two companies E-3-3 and B-6-3 were immunized intranasally at the end of the 3rd BCT week.

b) The remaining two platoons of these companies received a placebo intranasally.

c) Transmission of ADV type 7 was monitored in all members of one of the two platoons in each company who received nasal virus (during the entire study) and in all members of one platoon of each company who received placebo (from the time of intranasal AV-4 instillation to the end of the study).

2. Methods:

a) Enteric immunization: Living enteric oral ADV type 4

vaccine, Wyeth (16C1-00201) was given to all members of the three platoons at commencement of BCT.

b) Intranasal Vaccine AV-4 Instillation (end of 3rd BCT week): Platoons 3&4 of B-6-3 and platoons 1&2 of E-3-3 received 0.5 ml of a dilution of Adenovirus Type 4, Wyeth Lot No. OPL 24 (NS) 04 intranasally. Platoons 1&2 of B-6-3 and 3&4 of E-3-3 received 0.5 ml of HBSS intranasally. (Table 1)

Table 1 Distribution of Test and Control Subjects
Study #1 Fort Dix Oct-Dec 1967

Company	Platoons	Material instilled intranasally
B-6-3	1&2	0.5 ml HBSS
	3&4	90,000 TCD_{50} AV-4 in 0.5 ml HBSS
E-3-3	1&2	30,000 TCD_{50} AV-4 in 0.5 ml HBSS
	3&4	0.5 ml HBSS

c) The schedule for sample collection for the study platoons is shown in Table 2. In addition, an admission throat wash and acute and convalescent blood samples were obtained on each member of the study companies hospitalized with ARD.

Throat washings were obtained by having trainees gargle 15 ml of HBSS containing 0.4% BPA. 0.5 ml of each TW was inoculated into each of 3 tubes of Human Embryonic Kidney tissue culture tubes. The tubes were read daily for 18-21 days. Adenovirus isolates were harvested and typed in neutralization tests with Hyperimmune Adenovirus antisera. Enterovirus isolates were typed in neutralization tests with Poliovirus I, II and III pool antiserum. Herpes virus isolates were tentatively identified by cytopathic effect and chloroform sensitivity.

Table 2 Schedule for Collection of Clinical Samples
Study Platoons Fort Dix Oct-Dec 1967

Co.	Platoon	Status	Blood	Nasal Wash	Throat Wash
B-6	1st	Placebo	ND*	ND*	Biweekly 4-7BCT week
	4th	Nasal virus	0,3,7BCT week	0,3,7BCT week	Biweekly 0-7BCT week
E-3-3	1st	Nasal virus	0,3,7BCT week	0,3,7BCT week	Biweekly 0-7BCT week
	4th	Placebo	ND*	ND*	Biweekly 4-7BCT week

* Not Done.

d) Neutralization tests were performed on 0,3, and 8 week sera of all BCT's of the nasal virus platoon and on acute and convalescent serum on all hospitalized BCT's with 32-320 TCD_{50} of ADV type 4 and with ADV type 7 in tube cultures of human embryonic kidney cells.

3. Results:

a) Virus Excretion in Study Companies:

AV-4 excretion: As shown in Figures 1 and 2 and Table 3, excretion of AV-4 was not detected in either the B-6-3 or E-3-3 placebo platoons. Two of 36 BCT's in the E-3-3 nasal virus platoon and 9 of 36 BCT's in the B-6-3 nasal virus platoon excreted ADV-4 from the nasopharynx for a duration of ½ to 1½ week.

AV-7 excretion: Fifteen of the 36 BCT's in the nasal virus platoon studied in company E-3-3 were found to excrete AV-7 prior to intranasal AV-4 instillation. (Due to the change in design of the study, no information on Av-7 excretion prior to the time of intranasal AV-4 instillation in the placebo platoons of E-3-3 and B-6-3 are available). After immunization 7 of the 36 BCT's given nasal virus and 9 of the 36 BCT's given placebo excreted AV-7. In B-6-3, AV-7 associated ARD occurred in the training cycle with only 1 of 35 BCT's in the nasal virus platoon excreting AV-7 prior to nasal AV-4 instillation. After instillation 10 of 35 BCT's in the nasal virus platoon and 20 of the 36 in the placebo platoon excreted Av-7.

None of the 11 BCT's in E-3-3 and B-6-3 who excreted vaccine AV-4 post nasal instillation later excreted AV-7; 6 of the 11, however, were judged not susceptible to AV-7 infection (0 week serum AV-7 Neut titer of $\leq$1:4). Conversely none of the 16 BCT's of vaccine virus platoons in

Table 3 Virus Excretion
Study Platoons, Nasal Interference Study, Fort Dix 67

	Nasal Virus (1st platoon)			E-3-3 Placebo (3rd platoon)		
		Virus Excretion			Virus Excretion	
		AV-4	AV-7		AV-4	AV-7
	No.	No.	No.	No.	No.	No.
A) Pre-nasalvirus	36	0	15	36	Not studied	
B) Post-nasalvirus	36	2	7	36	0	9

	Nasal virus (4th platoon) B-6-3			Placebo (1st platoon)		
		Virus Excretion			Virus Excretion	
		AV-4	AV-7		AV-4	AV-7
	No.	No.	No.	No.	No.	No.
A) Pre-nasalvirus	35	0	1	36	Not studied	
B) Post-nasalvirus	35	9	10	36	0	20

both companies who excreted AV-7 in the nasopharynx were found to excrete vaccine AV-4 in the nasopharynx after nasal vaccine AV-4 instillation.

b) Effect of Intranasal AV-4 on ARD Hospitalizations. Most all hospitalized disease in both the nasal virus and the two placebo platoons of E-3-3 occurred prior to intranasal AV-4 instillation (Table 4); only 3 AV-7 associated admissions occurred after nasal virus instillation, two of them in the placebo group. On the other hand, only four AV-7 associated hospitalizations occurred prior to administration of nasal virus in B-6-3. After intransal instillation, six AV-7 associated hospitalizations occurred in the two nasal virus platoons in this company is opposed to ten AV-7 associated admissions in the two placebo platoons.

c) AV-4 Associated ARD Hospitalizations. One of the 142 BCT's who received AV-4 vaccine virus intranasally was hospitalized with AV-4 associated respiratory illness.

Discussion.

Definitive data concerning the effect of interference by vaccine AV-4 instilled intranasally into BCT's upon the transmission of AV-7 in BCT companies were not obtained in this study due to first the necessity to change the design of the study after its commencement and second, the small numbers of BCT's that could be studied at this stage in the development of the AV-4 interference project. Nevertheless certain qualitative interferences seemed possible from this study:

First, vaccine AV-4 can be safely administered intranasally three weeks post enteric AV-4 immunization in BCT's.

Second, the intranasal instillation of vaccine AV-4 virus into a company (E-3-3) in which respiratory disease due to AV-7 was well established did not seem to influence the transmission of AV-7 in the company.

Table 4 ARD Hospitalizations
Nasal Interference Study Fort Dix Oct-Dec 1967

E-3-3

NASAL VIRUS (1st and 2nd platoon)

	Strength	No. ARD Hosps	No. AV-4 Hosps (isolation)	No. AV-7 Hosps (isolation or 4 fold Ab)
Pre-nasal virus	72	16	0	12
Post-nasal virus		1	0	1

PLACEBO (3rd and 4th platoon)

	Strength	No. ARD Hosps	No. AV-4 Hosps (isolation)	No. AV-7 Hosps (isolation or 4 fold Ab)
Pre-nasal virus	73	9	0	5
Post-nasal virus		3	0	2

B-6-3

NASAL VIRUS (3rd and 4th platoon)

	Strength	No. ARD Hosps	No. AV-4 Hosps	No. AV-7 Hosps
Pre-nasal virus	70	2	0	1
Post-nasal virus		11	1	6

PLACEBO (1st and 2nd platoon)

	Strength	No. ARD Hosps	No. AV-4 Hosps	No. AV-7 Hosps
Pre-nasal virus	73	4	0	3
Post-nasal virus		11	0	10

Third, the intransal instillation of AV-4 vaccine virus into a company in which AV-7 associated ARD was beginning to emerge might possibly influence AV-7 transmission in the company.

B. Further Evaluation of Induced Nasal Infection with AV-4 Vaccine, Fort Dix, N.J., Feb-Apr 1968. A second study involving seven BCT companies was done to determine whether the suggested reduction in transmission of AV-7 in one of two companies following nasal instillation of AV-4 vaccine virus in the earlier study could be confirmed.

1. Design of Study. Serum BCT companies were selected from cohorts of incoming recruits which had received living attenuated, orally administered adenovirus vaccine upon arrival at Fort Dix. These companies were formed in February 1968. Each company was monitored for etiology of hospitalized ARD during the active eight week basic training cycle. Two platoons in each company were followed twice weekly for upper respiratory tract viral flora; these were selected in advance of nasal instillation of virus. One of these, together with yet another platoon not followed in detail for virological flora was given an estimated 100,000 TCD_{50} of vaccine AV-4 intranasally as soon as possible after the end of the 3rd week of BCT. The other studied platoon and one other received a placebo instillation of Hanks BSS at the same time. Baseline serum specimens were obtained from each man in the intensively studied platoons in each company at the outset of basic training, at the time of nasal instillation of virus, and again at the completion of the study. Laboratory studies of the subjects were made as described under IA above.

2. Effect upon Hospitalization for ARD. The numbers of men, identified by company, participating in the study, the hospitalizations for ARD associated with AV-4 and AV-7 before and after nasal instillation of virus, and the rates for hospitalization are summarized in Table 6. The data show no significant reduction in hospitalization for ARD was affected by the procedure (rate for test group post nasal instillation, 0.13; for controls, 0.18). AV-7 appeared to be the primary pathogen associated with disease in these patients; no disease attributable to AV-4 was obtained in the test group after the 3rd week of BCT, and five patients had AV-4 associated disease in the control group; this again establishes the effectiveness of oral immunization for type specific disease.

3. Effect upon Transmission and Infection by AV-7 in Intensively Studied Platoons. Patterns of type specific virus shedding among men in intensively studied platoons were determined. The numbers of persons yielding various adenoviruses before and after nasal

Table 6 AV Specfic hospitalizations for ARD Induced Nasal Infection, Fort Dix, Feb-Apr 1968

Unit	Strength	Number Men Hospitalized; Test Group[1/]							
		Pre-challenge				Post-challenge			
		Total	AV4	AV7	AV21	Total	AV4	AV7	AV21
A-3	95	7	0	2	0	18	0	12	0
A-6	84	1	0	0	0	7	0	6	0
B-1	88	5	0	2	0	9	0	5	0
B-6	105	7	1	0	0	13	0	9	0
D-2	97	5	1	1	0	7	0	3	0
D-5	100	2	0	0	0	16	0	5	0
E-4	85	4	0	1	0	14	0	6	0
Total	654	31(.05)	2(.004)	6(.01)	0	84(.13)	0	46(.07)	0
						Control Group[1/]			
A-3	105	17	1	5	0	15	1	11	0
A-6	89	8	2	1	0	14	1	9	0
B-1	101	10	1	3	0	16	0	4	0
B-6	107	12	0	5	0	17	1	10	0
D-2	104	11	0	4	0	10	0	5	0
D-5	112	11	0	4	0	34	1	14	1
E-4	111	8	1	1	0	25	1	13	0
Total	729	77	5(.006)	23(.03)	0	131(.18)	5(.006)	66(.09)	1

[1/] Receiving 13,000-73,000 TCD_{50} of AV-4, vaccine virus intranasally 22-26 days after onset BCT. (Test Group) or Hanks BSS (Control Group).

instillation of AV-4, or placebo, and the relationship of these patterns to the presence or absence of N antibody to AV-7 at the outset of BCT are summarized in Tables 7 and 8. It will be noted that no significant reduction in infection rates for AV-7 virus were effected by intranasal instillation of vaccine AV-4 virus (Test Group) as compared to the controls either among these were considered susceptible to AV-7 (ie. those possessing no detectible N antibody to AV-7 at serum dilution of 1:2) or those with serological evidence for natural infection prior to BCT.

4. Immunological Confirmation of Vaccine AV-4 and Natural AV Infections. Serological tests to assess N antibody responses to induced and natural AV infections are in progress and will be reported at a later date.

Table 7 Patterns of AV Infection of the Respiratory Tract Intensive Platoons, Fort Dix, Feb-Apr 1968

Unit	Interval susceptibility AV7 [1]	Number Men in Indicated Category							
		Test Group				Control Group			
		Total	AV-4	AV-7	Other [2]	Total	AV-4	AV-7	Other
A-3	Pre/yes	39	2	8	--	36	1	14	--
	Post/yes		5	19	--		1	14	--
	Pre/no	10	2	1	--	17	3	0	--
	Post/no		2	1	--		1	3	--
A-6	Pre/yes	29	0	1	1(I)	27	2	3	--
	Post/yes		5	21	--		2	18	--
	Pre/no	13	1	0	--	17	0	1	--
	Post/no		6	2	--		2	4	--
B-1	Pre/yes	22	3	2	--	26	3	5	--
	Post/yes		1	15	--		1	15	--
	Pre/no	18	1	1	--	20	--	--	--
	Post/no		7	2	1(V)		2	4	--
B-6	Pre/yes	36	0	13	--	35	4	13	--
	Post/yes		1	20	--		0	13	1(V)
	Pre/no	18	0	1	--	18	5	1	--
	Post/no		3	0	--		1	3	--
D-2	Pre/yes	30	1	7	--	35	3	11	--
	Post/yes		5	13	--		0	10	--
	Pre/no	20	3	0	--	15	1	0	--
	Post/no		3	0	--		4	1	--

Table 7 (Continued)

Unit	Interval susceptibility AV7[1/]	Number Men in Indicated Category							
		Test Group				Control Group			
		Total	AV-4	AV-7	Other[2/]	Total	AV-4	AV-7	Other
D-5	Pre/yes	30	2	0	--	34	4	9	--
	Post/yes		3	17	--		0	20	--
	Pre/no	24	0	1	--	26	1	2	--
	Post/no		3	1	--		3	3	--
E-4	Pre/yes	26	1	2	--	32	4	6	--
	Post/yes		4	17	--		1	23	--
	Pre/no	16	1	0	--	12	0	0	--
	Post/no		3	2	--		4	2	--

1/ Pre or post nasal instillation of AV-4/N antibody to AV-7 at 1:2 or >.

2/ Other adenoviruses received (type).

Table 8 Summary: AV Infection of the Respiratory Tract
Intensive Platoons, Fort Dix, Feb-Apr 1968

Interval[1/] susceptibility AV7	Number Men (Rate) In Indicated Category					
	Test Group			Control Group		
	Total	AV-4	AV-7	Total	AV-4	AV-7
Pre/yes	212	9(.04)	33(.16)	225	21(.09)	61(.27)
Post/yes		23(.11)	122(.58)		5(.02)	113(.50)
Total		32(.15)	155(.73)		26(.12)	174(.77)
Pre/no	119	8(.07)	4(.03)	125	10(.08)	4(.03)
Post/no		27(.23)	8(.07)		18(.14)	18(.14)
Total		35(.29)	12(.10)		28(.22)	22(.18)

1/ As in Table 7.

5. Several explanations for the apparent failure of induced AV-4 vaccine virus infection of the upper respiratory tract might obtain. First, in this second study, natural transmission of AV-7 virus was underway in each of the seven companies before nasal instillation of AV-4 vaccine. The subsequent increased transmission of AV-7, attested to by rising virus recovery rates from the intensively studied platoons in the test group, confirmed observations made in Co. E-3-3 of the pilot study (see I A above). Coupled with the reduced recovery of AV-4 from the test subjects after nasal instillation as compared with earlier studies in human volunteers (see Annual Report this project 1966-1967), the influence that natural AV-7 infection interferred with the colonization of the respiratory tract with vaccine virus, is further strengthened. It remains remotely possible that the amount of vaccine virus introduced nasally in the present experiments was less than optimal. This, however, does not seem likely since it was but 4-6 fold less than that which successfully colonized volunteer subjects (Annual report this project 1966-1967). It seems clear, however, that the approach does not offer the degree of effectiveness in reduction of hospitalized ARD that would warrant further experimentation.

II. Changes in Serum Immunoglobulin Levels Following Multiple Immunization of Army Recruits in Basic Combat Training.

During winter months, recruits in basic combat training are plagued by respiratory illnesses. Of paramount importance is ARD, or the acute respiratory disease due to types 3, 4 or 7 adenoviruses. When one of these agents becomes prevalent on a base, new recruits, with few exceptions, become infected within two weeks of arrival. About half can be expected to ultimately have overt ARD, and about half of these men will require 3 to 5 days of hospitalization. The time lost from training and the cost of the massive hospital load are significant problems to the military.

During the winter of 1965 factors that could relate to ARD susceptibility were studied in a single platoon of basic trainees at Fort Dix, New Jersey. One parameter - the immunoglobulin levels of these men - is the subject of this report.

A. Study Population. Forty-eight men, comprising a single platoon, were studied from the time they arrived at the Fort Dix reception station until the end of their eight weeks of basic training. Each day individuals were questioned about respiratory symptoms and their oral temperatures recorded. Weekly bleedings and biweekly nasopharyngeal washes were cultured for viruses on Hep-2 or HEK and RMK.

These men became victims of a widespread ARD epidemic due to type 4 adenovirus during this training period, and there were 24 hospitalizations in the group. When an individual was hospitalized, cultures were repeated and acute and convalescent sera obtained for adenovirus complement-fixing titrations (See Annual Report 1965-1966).

The serum IgG, IgA, and IgM immunoglobulin levels of 37 of these 48 men were measured on days 0, 17, 23, 31 and 58 of training. Of the eleven not included, five were dropped because they had no evidence of adenovirus infection (four of these had neutralizing antibody for type 4 adenoviruses at the outset of training), five had incomplete sera or insufficient clinical data to rate the severity of their ARD, and one was infected with type 3 adenovirus instead of type 4 which was prevalent at the time of the study.

B. Immunoglobulin Measurement. Total serum IgG, IgA, and in some instances IgM were measured by radial diffusion using commercially produced* agar plates and standards. The mean variation and 95% confidence limits between duplicate measurements of the same serum were 87 $\pm$ 12mg/100ml for IgG, 15 $\pm$ 2 mg/100ml for IgA, and 7 $\pm$ 2.8mg/100ml for IgM. When it became apparent that IgM increased more than the others, a micro-quantitative precipitation method was developed to confirm this change in IgM and to measure it more accurately. For this procedure human IgM globulin and monospecific rabbit anti-human IgM were prepared as described by Fahey and McLaughlin. Euglobulins, obtained by dialysis of plasma against distilled water, were subjected to Pevicon-Geon-block electrophoresis. IgM fractions, free of γ_2 macroglobulins were applied to a Sephadex G-200 column. The leading portion of the first protein peak delivered was pure IgM as assayed by immunoelectrophoresis (Fig. 1A).

Analytical ultracentrifugation performed by Dr. Joseph Bellanti at Georgetown University identified a single homogenous 18S component in this preparation. IgM protein concentrations were measured with a Beckman Du-2 spectrophotometer assuming an E 1%/1cm of 11.85 at 280 mu.

Rabbit anti-IgM was prepared by injecting two animals with three monthly intradermal doses of 1 to 5 mg IgM each. The first injection contained IgM emulsified in Freund's complete adjuvant and the subsequent two in incomplete adjuvant. Serum obtained three weeks after the last injection precipitated IgM, IgA, and IgG but no other serum proteins as judged by immunoelectrophoresis. The latter specificities were absorbed with a fraction of normal human serum eluted from a diethylaminoethyl cellulose column with .01 M. pH 7.5 phosphate buffer in .1 M. NaCl

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* (Hyland Labs, Los Angeles, California).

containing IgA and IgG but no IgM. The absorbed antiserum usually produced a single broad immunoelectrophoretic precipitation arc occupying the usual position of IgM. Occasionally this broad line appeared to be split into two or more parallel arcs of the same shape and mobility. Dr. H. Metzger of the National Institutes of Health provided four purified Waldenstrom's macroglobulins to test the possibility that different allotypes of k chains were recognized by the antiserum. Similar splitting of the IgM arcs was observed and the phenomenon was considered an artifact.

The micro-technique of quantitative precipitation utilized 12 X 75 mm tubes, and disposable micro-pipettes or calibrated serological pipettes. Most commonly, a mixture containing .01 ml of human serum and .05 ml of anti-IgM diluted to .25 ml with PBS was incubated for one hour at 37° and three days at 4°C. After the precipitates were washed twice with 1.5cc of cold PBS, their protein contents were measured by the Lowry modification of the Folin-Ciocalteu method.

That the absorbed rabbit anti-IgM precipitated only IgM was confirmed by comparing quantitative precipitation curves of it versus purified IgM and whole serum. Both antigens formed equal maximum amounts of precipitate at equivalence and IgM levels in the whole serum were the same when calculated from several points on the purified IgM curve. This whole serum with standardized IgM content was used to prepare reference curves for each quantitative precipitation assay. The mean variation and 95% confidence limits of this assay for 250 duplicate determinations was 2.7 $\pm$ .3mg IgM/100ml serum. Determinations were made in duplicate. When they differed by more than 6 mg/100ml or 6% of the total they were repeated. IgM levels were approximately the same measured by radial diffusion or quantitative precipitation.

C. Immunoglobulins during BCT. Under normal circumstances the serum concentrations of the major human immunoglobulins remain constant in normal adults. Although IgA and IgG levels of recruits were in the most part consistent with this observation, IgM concentrations were not.

IgG, IgA and IgM concentrations of 37 men in basic combat training were measured on days 0, 17, 23, 31 and for most on day 58 also. Since normal levels varied widely, changes of immunoglobulins were calculated for each individual, the mean changes from baseline are given in Table 9. The IgG mean was never above 0 change at 95% confidence limits. If any change occurred, it was relatively small contrasted to the total serum IgG level and the error of radial diffusion assay. The highest mean change would only represent a 5% increase of the 1000mg/100ml average baseline concentration. IgA was above baseline at 95% confidence limits on days 31 and 58 of training. The changes were still small, however.

The largest mean increase was about 12% of the baseline 180mg/100ml average level. In contrast, mean IgM changes were above baseline at 95% confidence limits on all days studied. The changes were also larger. Thirty-three of the 37 men had more than a 20% increase at some time between days 17 and 31, and the mean increase on day 23 was

Table 9 Immunoglobulin Changes During Basic Combat Training

	Change from Baseline			
	Day 17	Day 23	Day 31	Day 58
IgM	39.5* (29.6 to 49.4)	50.2 (37.7 to 62.7)	40.2 (30.8 to 49.6)	13.2 (6.2 to 20.2)
IgA	-.6 (-6.9 to 5.7)	2.6 (-5.7 to 10.9)	22.4 (11.7 to 33.1)	19.0 (9 to 29)
IgG	-25.4 (-66.8 to 16.0)	33.8 (-13.6 to 81.2)	50.9 (-10.3 to 112.1)	55.5 (-3.0 to 114.0)

* Mean of changes from baseline and its 95% confidence limits in mg/100ml serum.

more than 50% above initial average IgM levels. A relation between starting concentration of IgM and the amount of increase was not apparent on any day of the response. This increase was transient. By day 58 levels were approaching their beginning concentrations. Serum taken at 111 days was available from 9 men remaining at Fort Dix after completion of BCT. The IgM concentrations at this time are compared with peak changes and baseline values in Table 10. Although peak changes averaged 88% above baseline, by day 111 IgM averaged 3% below baseline and ranged from 26% above to 23% below initial values. Therefore, IgM tended to return toward starting concentrations in spite of the high increases.

This IgM increase was unexpected in view of prior reports that such levels remain fairly constant in normal adults under normal circumstances. Recruits, however, are in a unique situation that cannot be considered normal. They have considerably more physical and mental stress than civilians, their incidence of respiratory infections, especially ARD, is high, and they receive a battery of protective immunizations during basic combat training. Any or all of these factors could be responsible for the IgM change.

D. Immunoglobulins in Physicians at MFSS following Immunization. When physicians enter the army they have a form of army training at the Medical Field Service School (MFSS) in San Antonio, Texas. It differs from basic combat training in practically every respect except basic immunizations. Physicians live off base in commercial housing and eat, for the most part, in restaurants. Their working hours are regular and most are spent in classrooms. There is almost no physical stress, probably much less mental stress, and ARD is not found in this group. The immunoglobulin levels of 20 physicians in a single MFSS class during fall of 1967 were measured.

Table 10 Tendency for IgM to Return to its Starting Concentration

Recruit	Day 0 M	Highest M level	% Change from Day 0	Day 111 M level	% Change from Day 0
3	66	96	45	73	11
5	154	181	18	132	-14
6	34	69	103	43	26
10	68	208	206	67	-1
11	69	160	132	61	-12
28	70	158	126	81	16
36	86	120	40	75	-13
45	142	151	6	121	-15
46	73	161	120	56	-23
Ave			88		-3

Table 11 compares the immunization schedule of these men with that of the Dix 65 recruits. The minor differences are due to the shorter training period of physicians - 5 weeks versus 8 for recruits - and to changes in the army's needs between 1965 and 1967.

IgG, IgA and IgM concentrations were assayed by radial diffusion on days 0, 7, 14, 21, 27 and 35. Mean changes in immunoglobulin lines are presented in Table 12. Again, if any IgA or IgG changes occurred, they were small while IgM increased to an even greater extent than it had during BCT. Since MFSS students and BCT recruits have little in common other than the multiple immunizations, circumstantial evidence implicates immunizations as the cause of the IgM increases of both groups.

E. Immunoglobulins Following Typhoid Vaccine. Direct evidence that immunization can increase total serum IgM was obtained by immunizing eight children with one to two .5ml subcutaneous injections of a standard commercially produced S. typhosa vaccine*. Table 13 summarizes their mean IgG, IgA and IgM changes calculated in the same manner as for physicians and recruits. Again IgG and IgA changed little, if at all, while IgM was distinctly higher (in all eight children) during the second post-immunization week. Further, the sera with peak IgM concentrations were absorbed with S. typhosa antigens. Since this treatment removed most of the increase from baseline, the majority of new IgM appeared to be specific anti-typhoid antibody. This data directly implicates typhoid immunization as the cause of the children's total serum IgM increase.

F. ARD and Immunoglobulin Changes. Although these data indicate commercial vaccines can increase IgM and perhaps IgA and IgG to

Table 11 Immunization Schedules

	Recruit's training week					Physician's training week	
	1	2	4	6	8	1	5
Typhoid		X		X		X	X
Tetanus	X			X		X	X
Influenza	X					X	
Typhus		X					X
Plague						X	
Cholera						X	X
Polio (Sabin)	X		X		X	X	
Smallpox		X				X	
Yellow fever	X					X	

Table 12 Immunoglobulin Changes During Medical Field Service School Training of Physicians

	Change from Baseline				
	Day 7	Day 14	Day 21	Day 27	Day 35
IgM	14.5** (3.0 to 26.0)	52.5 (22.2 to 82.8)	57.1 (31.3 to 82.9)	63.0 (...0 to 84.0)	42.0 (26.6 to 57.4)

* E. Lilly and Co., Indianapolis, Indiana.

** Mean of changes from baseline and its 95% confidence limits in mg/100ml serum.

Table 12 (Continued)

	Change from Baseline				
	Day 7	Day 14	Day 21	Day 27	Day 35
IgA	3.4 (-1.9 to 8.7)	.6 (-6.2 to 7.4)	9.2 (-2.2 to 20.6)	2.5 (-5.3 to 10.3)	5.7 (-2.2 to 13.6)
IgG	23 (-59 to 105)	76 (-7 to 159)	77 (-32 to 186)	85 (9 to 161)	25 (-80 to 130)

** Mean of changes from baseline and its 95% confidence limits in mg/100ml serum.

Table 13 Immunoglobulin Levels Following Typhoid Immunization of Children

	Change from Baseline			
	Day 7	Day 10	Day 14	Day 28
IgM	30* (16 to 44)	45 (25 to 65)	35 (17 to 53)	20 (1 to 39)
IgA	5 (-9 to 20)	21 (-3 to 45)	17 (-3 to 36)	17 (1 to 35)
IgG	54 (12 to 96)	47 (-8 to 102)	67 (-40 to 174)	115 (48 to 188)

* Mean change and 95% confidence limits in mg/100ml serum.

a smaller extent, one cannot assume that all of the recruits' immunoglobulin changes are due to such vaccines. These men receive another form of antigenic stimulation from the adenovirus infections of ARD. Since recruits make neutralizing, hemagglutination-inhibiting, and complement-fixing antibody following ARD, part of the changes may be due to this natural form of immunization. The relationship between the severity of ARD and the immunoglobulin changes of individual recruits was examined to test this possibility. This required a system to rate the severity of ARD for each Fort Dix trainee.

The timing of adenovirus infection was reasonably clear from isolation and serological data. It was also evident that some recruits handled these infections better than others, but defining the severity of ARD in individuals was more difficult. The presence or absence of hospitalization,

of various respiratory symptoms and signs, and daily temperature measurements were available to rate severity. In deference to simplicity, objectivity, and loading (biasing) the data, fever was the only parameter used. The number of days that oral temperature was above 37.5°C during ARD was counted for each individual. Many recruits had intermittent fever throughout training, so in order to be included in the individual's series the days of elevated temperature had to be separated by no more than one day of normal temperature.

There appeared to be some relation between degree of IgA and IgM change and the severity of ARD when rated in this manner. Individuals with fever for longer periods tended to have higher increases of both immunoglobulin classes. The contrast between immunoglobulin changes was most distinct when recruits with 0 or 1 day of fever were compared with those who had more than one day of fever (Tables 14, 15).

Table 14 IgA Change and Severity of ARD During Basic Combat Training

	Days of ARD Fever	
	Less than 2	2 or more
Day 17	-9.9* (-18.9 to -.9)	4.5 (-3.3 to 12.3)
Day 23	-6.9 (14.1 to .3)	11.0 (0 to 22.0)
Day 31	-4.2 (-11.1 to 2.7)	36.9 (24.4 to 49.4)
Day 58	2.5 (-7.5 to 12.5)	27.9 (14.9 to 40.9)

* Mean of changes from baseline and 95% confidence limits in mg/100ml serum.

Table 15 IgM Change and Severity of ARD
During Basic Combat Training

	Days of ARD Fever	
	Less than 2	2 or more
Day 17	20.8* (8.1 to 33.5)	49.6 (37.3 to 61.9)
Day 23	28.1 (12.1 to 44.1)	62.2 (46.3 to 78.1)
Day 31	20.8 (7.5 to 34.1)	50.6 (39.6 to 61.6)
Day 58	3.6 (-5.0 to 12.2)	20.8 (13.1 to 28.5)

* Mean of changes from baseline and 95% confidence limits in mg/100ml serum.

The 95% confidence limits for IgM did not overlap on any day in these two groups. The 95% confidence limits of IgA overlap on day 17 and to a slight degree on day 28 but not day 31 or 58. A similar relationship between IgG change and days of ARD fever was not apparent.

Another ARD study was made at Fort Dix during the winter of 1968. The purposes differed from those of the 1965 study. Consequently, a much larger number of recruits were included, but data concerning disease severity and the number of bleedings obtained were much more limited. The only parameter available to rate ARD severity was the presence or absence of hospitalization with respiratory disease during the training period. Three platoons with high rates of hospitalization were selected for measurements of IgM in day 0 and 17 or 23 sera. As shown in Table 16, there was little if any difference in the average degree of IgM change between recruits requiring hospitalization and those not requiring it. However, when day 0 to 17 and 23 IgM changes observed for the 1965 Dix recruits were compared in the same manner differences were also small.

Table 16 IgM Changes of Recruits Hospitalized with Respiratory Illness vs. Those Not Hospitalized

Platoon - Dix 1968	Hospitalized	Not Hospitalized
D-5		
No. Recruits	16	40
Ave % Change IgM		
Day 0 to 17	41%	36%
A-6		
No. Recruits	10	36
Ave % Change IgM		
Day 0 to 21	51%	65%
A-3		
No. Recruits	19	31
Ave % Change IgM		
Day 0 to 21	53%	38%
Dix 1965		
No. recruits	15	22
Ave % Change		
Day 0 to 17	61%	54%
Day 0 to 23	74%	62%

G. Significance of Observation. Serum levels of the major human immunoglobulins including IgM remain remarkably constant in individuals over time. Allansmith et al., for example, found it unusual for IgM to vary more than 20% in a 6-month study of 15 normal adults.

There is considerable evidence, however, that under proper circumstances antigenic stimulation can increase serum IgM. Intensive immunization of animals appears to accomplish this, and certain infections of man - notably mononucleosis, trypanosomiasis and malaria - are associated with high serum IgM. The normal infant is born with only about

10% of the adult IgM level, but within days of first contact with the microbial flora and other antigens of extrauterine life serum IgM begins to rise. If infectious agents traverse the placental barrier, IgM production can increase during prenatal life, and in such cases levels as high as those of adults have been found at birth.

The studies reported here provide further evidence that IgM levels are directly influenced by antigenic stimulation. Recruits had distinct increases in IgM during eight weeks of basic training. Physicians in MFSS had similar changes, and since these groups have little in common besides the battery of protective immunizations that begin near the time of induction the immunizations appear to be responsible for the increase. Direct evidence of this was derived from the response of children to commercial S. typhosa vaccine. Total serum IgM levels increased in every case, and most of the newly produced IgM was specific anti-typhoid antibody.

As would be expected, the high IgM levels following immunization were transient. Concentrations were falling by the latest bleeding in all three study groups, the recruits, physicians and children. Further, the baseline day 0 IgM levels, the peak level and the level 111 days after beginning BCT were compared in nine recruits. In spite of high peak increases averaging 88% above baseline, the IgM concentration had a marked propensity to return to starting concentrations. By 111 days the mean change from baseline was -3%, and the extremes were barely outside the ±20% variation found in normal adults by Allansmith et al.

It was also interesting that no correlation could be found between the degree of IgM change in mg/100ml and the starting baseline concentrations. Persons with high baseline levels were not more likely to have higher IgM responses than men with low baseline levels. Apparently, when examined in this manner, the resting concentration of IgM did not reflect upon the capacity of an individual to respond to new antigenic stimuli.

The most interesting observation was the association of high IgA and IgM responses with more severe ARD as rated by duration of fever. Although the association seemed clear, the number of cases examined was small and the experiment should be repeated as a prospective type study. Speculation as to the reason for this association includes at least two possible explanations. The most direct explanation is that the antigenic stimulation of the adenovirus infection was more intense in the more severe ARD cases. This could add to the vaccine stimulation, the summation of effects inducing larger IgM and IgA changes.

An alternate possibility exists, however. If all of the immunoglobulin change is due to vaccine and if there is a limit to the rate

of immunoglobulin production at a given time, the recruits with most intense vaccine responses could be at a relative disadvantage in dealing with antigens of adenoviruses. These individuals might then have more severe and prolonged ARD. This would explain the coincidence of peak IgM (day 23) and IgA (day 31) levels and the highest rate of ARD during BCT (3rd and 4th week), and perhaps be related in part to the unique occurrence of ARD among basic trainees. Civilian populations do not have a similar disease, and, in fact, the responsible agents are uncommon among civilians. Further support can be derived from Figure 5. At 17 days recruits with more than one day of ARD fever already had higher mean IgM changes at 95% confidence limits than those with 0 or 1 day of fever. The mean IgA levels followed this same pattern on day 17, although the 95% confidence limits of the two groups still overlapped. Yet only one recruit had ARD fever before day 17, and the largest number of men with fever were found on day 26. The differences between immunoglobulin changes of the mild and severe groups thus seemed to precede the onset of ARD. The navy has previously asked a question similar to this without knowledge of these immunoglobulin changes. Postulating that vaccines, by antigenic interference, could affect the severity of subsequent ARD, they tested the effect on ARD of withholding recruit immunizations. Hospitalization rates were approximately 20% lower in recruits who received the majority of immunizations in the second half of training compared to those given the routine schedule.

The army repeated a similar experiment at Fort Dix in 1962, but were unable to confirm this association The hospitalization rate was similar in delayed and routine immunization groups. The failure of recruits requiring hospitalization for ARD to have higher IgM responses than those who did not during the 1968 study also tends to refute this hypothesis. The conditions of the 1965 and 1968 studies were considerably different, however. In the latter case a large number of men were included, but the only data concerning occurrence of ARD was need for hospitalization. Only two bloods - day 0 and either 17 or 23 - were available from these three platoons, and, finally, the men were immunized with type 4 adenovirus vaccine at the outset of training.

III. Studies of Rubella.

A. Variables Influencing the HI Test. The rubella HI antibody test is a sensitive and useful method for estimating rubella antibodies. The test is accurate, reproducible and the various parameters of the test are well accepted. Several additional variables applicable to this test have been investigated.

1. Effect of Antigen Dose on Serum Antibody Titer. Dilutions of kaolin treated, chick red cell absorbed acute and convalescent sera collected from a single individual were reacted with varying

amounts of rubella hemagglutinating antigen for ½ hour at 4 degrees C and for 90 minutes at 4 degrees following addition of 1 day old chick red blood cells. The HI antibody titers are shown in Table 17.

Table 17 Effect of Antigen Dose on Rubella HI Antibody Titers

	Units Hemagglutinin					
	64	32	16	8	4	2
ACUTE SERUM TITER	8[1/]	8	16	8	16	16
CONV. SERUM TITER	128	256	256	64	512	256
DEMONSTRATED FOLD INCREASE	16	32	16	8	32	16

1/ Reciprocal of antibody titer.

It is apparent that the amount of hemagglutinin does not significantly influence the serum titers of either the early or the late sera. Both high and low doses of antigen gave similar antibody titers and significant increases between the paired sera.

2. Effect of Divalent Cations in the Diluent. 0.02 M phosphate buffered saline without calcium or magnesium ions showed a 4-8 fold decrease in rubella hemagglutinin titer compared with phosphate buffered saline containing 0.5 micromolar magnesium and 0.9 micromolar calcium ions. This phenomenon was observed over a pH range of 6.7-7.9.

In 0.01 M phosphate buffered saline with calcium and magnesium rubella hemagglutinin activity was maximal, from pH 6.8-7.48.

Thus, it appears that divalent cations are important in enhancing rubella HI activity, and pH control is not critical in demonstrating maximal hemagglutination.

3. Removal of Inhibitor. The nonspecific inhibitor of rubella hemagglutination is heat stable and readily removed by most lots of kaolin. The inhibitor is also easily removed by acetone extraction, but a significant number of antibody negative sera exhibit slippage at the lower serum dilutions and may lead to erroneous readings.

B. Rubella HI Antibody Responses in BCT. In cooperation with Dr. Paul Parkman of the Division of Biologics Standards, sera from a company of men experiencing an outbreak of rubella while undergoing BCT

at Fort Dix, N.J. in 1962 were retrieved from storage and tested for rubella HI antibody. Sera Stored at -20°C were inactivated at 56°C for 30 minutes, kaolin and chick red blood cell absorbed, and diluted in DGV buffer containing 0.2% bovine plasma albumin. Serial two-fold dilutions from 1:8-1:2048 were made in plastic disposable tray (Linbro) with an automatic diluting machine (Automatic Serial Diluter, American Instrument Co.) at the Division of Biologics Standards. Final volumes in the test were 0.2 ml serum dilution, 0.2 ml antigen, and 0.2 ml of 0.16% dayold chick red blood cells. Plates were incubated at 4°C for approximately 90 minutes and read.

Previous extensive laboratory work had identified in the 246 men comprising the company a total of 26 subclinical infections, 23 of which had associated virus isolations. Neutralizing antibody responses, using between 5-10 interfering doses of virus, identified 30 men without detectible antibody at a 1:2 dilution of serum. All 30 susceptibles experienced rubella infection during the 8 weeks of BCT.

Table 18 compares the number of susceptibles at the onset of BCT identified by the HI test with that by neutralization. All 30 susceptibles by neutralization had HI antibody titers $<$1:8. Thus the rubella HI test correlates extremely well with the absence of neutralizing antibody in identifying those individuals without demonstrable antibody.

Table 18 Rubella Virus Infections in BCT

Co. B, 2nd TR, Ft Dix, N. J. 1962

Platoon	# Men	# SUSCEPTIBLE BY Neut.	HI	# with INFECTION OVERT	SUBCLINICAL	TOTAL
1	62	9	9	3	6	9
2	62	5	5	1	4	5
3	61	7	7	0	7	7
4	61	9	9	0	9	9
TOTAL	246	30	30	4	26	30

Sera were available from 29 of the 30 men with rubella infection, and all 29 had significant HI antibody responses. Table 19 compares the response between 0 and 8 week sera for the 29 men with rubella infection. The greater sensitivity of the HI test compared to the neutralization test is readily seen. Thus each of 29 individuals responded with an 8 fold or greater HI antibody response while only 24/30 men responded with a similar neutralizing antibody response. Of these 29 infected individuals the magnitude of the HI response was similar for both subclinical and overtly infected men.

Table 19 Demonstrated Fold Increase in Rubella Antibody Response in BCT

INCREASE	BY NEUT.	BY HI
2x	4	0
4x	2	0
8x	4	3
16x	10	8
32x	10	11
64x	0	6
128x	0	0
256x	0	1
NOT DONE	0	1
TOTAL	30	30

Initial and eight week sera were studied in 216 of 246 men; 211 of these individuals had titers that were either stable or varied only two fold. Four individuals with pre-existing antibody titers (1:16, 1:32, 1:128, 1:256 respectively) demonstrated 4 fold rises in HI antibody. One additional man with a 0 week titer of 1:256 had a 4 fold fall. These results suggest that in individuals with pre-existing HI antibody 4 fold increases in HI antibody occur infrequently in situations of intense prolonged exposure. Whether these are mild, true anemnestic antibody responses or simply variations in the test is impossible to evaluate from the data.

C. Rubella HI Antibody in Pregnant Women. Two prenatal clinic populations have been surveyed for rubella HI antibody as part of a combined laboratory-clinical study. The objectives included 1) testing the feasibility of a laboratory's offering routine large scale screening for rubella antibody, and 2) acquisition of epidemiologic information concerning historical recall of German Measles by patients and also, the various relationships to age and race in regard to the prevalence of rubella HI antibody. Originally, we had hoped to acquire information about endemic and epidemic infection in relation to congenital rubella infection; however, this proved to be impossible, since most patients presented themselves after the first trimester of pregnancy.

Since August 1967, at Walter Reed General Hospital and from November 1967, at Dewitt Army Hospital, Ft. Belvoir, all patients entering the obstetrical prenatal clinics have had serum collected for rubella HI antibody assay at WRAIR. Each patient was queried for a history of past rubella infection.

Rubella HI antibody titers were determined on unheated, kaolin treated, chick red blood cell absorbed serum using day old chick red cells, the microtiter system, and phosphate buffered saline containing calcium and magnesium and 0.2% bovine plasma albumin as diluent. Rubella HI antigens were either obtained commercially or made in BHK21 cell culture. For the latter antigen supernatant fluid was centrifuged for 3 hours at 30,000 rpm in a #30 rotor in the Model L ultracentrifuge followed by tween-ether treatment of the pellet. Both antigens gave similar serum antibody titers.

Table 20 shows the composition of the populations by clinic and race. A total of 2194 sera from both installations have been surveyed through May 31, 1968. The two clinics differ significantly in their racial composition; at WRGH the ratio of white to black is roughly 2:1, while at DAH nearly 90% of the prenatal population is white.

Table 20 Composition of Prenatal Clinic Populations

	WRGH	DEWITT
WHITE	679 (59)[1/]	934 (89)
BLACK	361 (31)	49 (5)
OTHER	12 (1)	10 (1)
NOT GIVEN	99 (9)	50 (5)
TOTAL	1151	1043

1/ () % of total.

Tables 21 and 22 compare the historical responses with the presence or absence of demonstrable rubella HI antibody. The two installations show remarkable similar results. In effect, it did not matter whether a patient answered "yes", "no", or "I don't know" to a past history of german measles. With either response she had a similar opportunity, 1 in 10, of being seronegative (i.e., titer < 1:8), and conversely 9 out of 10 chances of being seropositive (i.e., titer ≥ 1:8). The overall incidence of seronegative individuals in the combined populations was 218/2194 or 9.9%.

Table 21 Correlation of History of Rubella with Rubella HI Antibody Titer, WRGH

HISTORY	TOTAL	# HI POSITIVE	# HI NEGATIVE	% SERONEGATIVE
Yes	365	334	31	8.5
No	618	557	61	9.9
?	95	82	13	13.6
Not Given	73	66	7	9.6
TOTALS	1151	1039	112	9.7

Table 22 Correlation of History of Rubella with Rubella HI Antibody Titer, Dewitt Army Hospital

HISTORY	TOTAL	# HI POSITIVE[a/]	# HI NEGATIVE[b/]	% SERONEGATIVE
Yes	498	456	42	8.4
No	305	265	40	13.1
?	186	165	21	11.3
Not Given	54	51	3	5.5
TOTALS	1043	937	106	10.1

a/ HI titer ≥ 8.
b/ HI titer < 8.

Table 23 compares the distribution of rubella HI seronegatives by age and race for the two clinic populations. The overall incidence of rubella HI seronegatives is 10.1% and 9.8% for Dewitt and WRGH respectively. By 5 year age intervals for all groups, the rates for seronegatives tend to hover around 10% through the second decade and to decline thereafter. This same general trend is seen in the two white populations, with the exception of the WRGH 35-39 year age group where there is possibly some bias in the rate because of the small number of individuals.

Table 23 Distribution of Rubella HI Seronegatives by Race and Age

DEWITT

AGE	WHITE #/# test	BLACK #/# test	OTHER & NOT GIVEN #/# test	ALL GROUPS #/# test
<19	16/148 (10.8)[a]	1/13 (7.7)	1/1	18/162 (11.1)
20-24	52/446 (11.6)	1/16 (6.2)	0/3	53/465 (11.0)
25-29	22/193 (11.4)	0/12 (0)	1/5	23/210 (10.9)
30-34	5/75 (6.7)	2/5 (40)	0/3	7/83 (8.4)
35-39	3/62 (4.8)	0/3	0/2	3/67 (4.5)
>40	0/10	0/0	0/0	0/10 (0)
AGE NOT GIVEN				2/46
TOTALS	98/934 (10.5)	4/49 (8.2)	2/14 (14.3)	106/1043 (10.1)

WRGH

AGE	WHITE #/# test	BLACK #/# test	OTHER & NOT GIVEN #/# test	ALL GROUPS #/# test
<19	15/121 (12.2)	3/112 (2.7)	3/16	21/249 (8.5)
20-24	37/295 (12.5)	9/163 (5.5)	3/26	49/484 (10.1)

Table 23 (Continued)

AGE	WHITE #/# test	BLACK #/# test	OTHER & NOT GIVEN #/# test	ALL GROUPS #/# test
25-29	19/160 (11.9)	6/51 (11.8)	1/19	26/230 (11.3)
30-34	4/66 (6.1)	3/19 (15.8)	2/8	9/93 (9.7)
35-39	3/26 (11.5)	0/15 (0)	0/2	3/43 (7.0)
>40	0/11 (0)	0/1 (0)	0/2	0/14 (0)
AGE NOT GIVEN				4/38 (10.5)
TOTALS	78/679 (11.5)	21/361 (5.8)	9/73	112/1151 (9.8)

a/ (%)

In contrast, the black population at WRGH showed the reverse trend. In the under 25 year age groups, the percentage of seronegatives is distinctly lower than for whites. Thus, for under 20 years the black rates were five times lower than for the comparable white population, and in the 20-24 age group, approximately two times lower than for the white population. By age 25-29, the rates for both races are similar, and above this age the numbers of individuals are too small for meaningful comparison. The negro population at DAH is too small for significant analysis.

Table 24 gives the distribution of titers for the two groups. The range of titers and the geometric mean titers are similar for both groups. The majority of titers tend to cluster about the geometric mean titer. Approximately 5% of titers are in the 1:8-1:16 or low range while approximately 7% are in the 1:512-1:1024 or high range.

Table 24 Distribution of Rubella HI Antibody Titers

TITER[1]	DEWITT	WRGH	BOTH
8	106[2]	112	218 (9.9)[4]
8	2	1	3 (0.1)
16	48	57	105 (4.8)

Table 24 (Continued)

TITER	DEWITT	WRGH	BOTH
32	145	146	291 (13.3)
64	236	271	507 (23.1)
128	293	305	598 (27.2)
256	150	164	314 (14.4)
512	55	85	140 (6.4)
1024	8	10	18 (0.8)
2048	0	0	0
TOTALS	1043	1151	2194
GMT[3/]	1:101	1:105	1:103

1/ Reciprocal of antibody titer.
2/ # with indicated titer.
3/ Geometric Mean Titer for all sera with 1:8 titer.
4/ ( ) % of total.

Assay of neutralizing antibody by the standard AGMK Echo-11 interference method has been performed on 118 of the 218 HI negative sera. All of these 118 have had neutralizing antibody titers 1:2 with the great majority of these titers being clearly :2. Fifty seven sera with positive HI antibody titers have also had neutralizing antibody assays. Of these 57, 48 have shown clearly demonstrable neutralizing antibody; however, nine sera from individuals with repeatedly low HI antibody titers after either kaolin absorption or 2x acetone extraction have no clearly neutralizing antibody at a 1:2 dilution. The lack of reproducibility of the interference method (between 1 in 2 and 1 in 3 tests are unsatisfactory) has precluded resolution of this problem. A sensitive plaque assay for neutralizing antibody may help to resolve these discrepancies.

The rubella HI antibody assay has provided a useful tool for large scale screening in two prenatal clinics. Cooperation from clinic personnel has been excellent, and laboratory feedback has provided the

obstetrician with useful information in this day of increasing patient and physician awareness of rubella in pregnancy. Numerous instances of reported exposure following collection of the "baseline" routine serum have demonstrated the value of the early sera previously collected. When the followup sera is tested simultaneously with the earlier sample the great majority of followup titers have remained stable. Following reported exposure, three serologically documented infections in initially seronegative individuals, all in their second or third trimester of pregnancy, have been found.

It is apparent from these data that a history of german measles is of no value to the clinician. Assuming that the presence of rubella HI antibody indicates past experience with rubella, two well known facts probably account for the majority of inappropriate historical responses. First, rubella in the absence of an epidemic has no distinguishing clinical features; and secondly, the presence of serum antibody in individuals with no history of german measles is evidence of the frequent occurrence of inapparent infection. The finding of a lower incidence of susceptibility in the younger negro population is of interest epidemiologically, particularly because other workers have reported the reverse. It should be emphasized that this distribution of antibody should in no way be relied upon by the clinician. Large scale screening has proven to be both practical to the clinician and feasible for the laboratory.

IV. Studies of Interferons.

The source of interferon *in vivo* during the course of viral infections remains unknown. There is evidence, however, that cells of the reticulo-endothelial system play a special role in interferon synthesis and defense against viruses. Peritoneal macrophages are of interest as an easily accessable source of cells which are components of this system to serve as models of interferon synthesis.

Earlier work demonstrated that these cells (rabbit peritoneal macrophages) serve as a rich source of interferon following virus induction. It was observed further that these cells respond with interferon production following stimulation with endotoxin. While endotoxin is known to stimulate interferon production in intact animals, an *in vitro* model of endotoxin-induced interferon production was lacking. Following the demonstration that virus-induced macrophage interferon and endotoxin-induced macrophage interferon possess different physico-chemical properties, it seemed of interest to investigate further the synthesis of endotoxin-induced interferon, and to compare its action with that of virus-induced interferon. The purpose of these studies is to attempt to clarify the question of whether interferons of different physico-chemical properties are synthesized by the same cells by different mechanisms, and to analyze the conditions under which cells are protected by these interferons, in an effort to determine whether there is a common pathway resulting in cellular resistance to viral infection. That is, to determine whether different interferons of the same species can be differentiated on the basis of the manner in which they induce the synthesis of viral-inhibiting protein in a cell population.

A. Methods. Rabbit peritoneal macrophages are obtained following the stimulation of a sterile peritonitis with a suspension of shellfish glycogen in saline. Medium 199 or Earles-LAH with 10% FBS served as culture media - Newcastle Disease Virus (NDV) was used to induce virus-induced interferon (VIF) - E. coli lipopolysaccharide B was used to stimulate endotoxin-induced interferon (EIF). Interferons were assayed in rabbit kidney cells, using vescicular stomatitis virus as challenge virus. The interferon titer is taken as the highest dilution capable of reducing the number of VSV plaques by 50%.

B. Observations on Induction. In an effort to determine whether VIF and EIF are synthesized by different mechanisms, the effects on the production of each was measured under several circumstances. First, the effect of the temperature of incubation of the macrophages was determined. When cultures of these cells were stimulated with NDV and placed at either 22°, 30°, 33° or 37° for 24 hours, the yield of interferon at that time was the same regardless of the temperature of incubation. In contrast, repeated testing has shown that the 24 hour yields of EIF are greater from cultures incubated at 22° than at 37°. When samples of fluids from these cultures are taken at intervals during this period, it is apparent that EIF synthesis proceeds at a more rapid rate at 37° than at 22°, but the total yield is less in 24 hours. The fact that the 24 hour yield of EIF from macrophages is greater at 22° than at 37° suggests that possibly EIF is performed in these cells and is simply released during the 24 hour period in vitro. It had been shown previously, however, that large doses of Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, inhibit EIF production by macrophages. More recently, additional studies have confirmed this observation and established that a dose of Actinomycin D as low as 2.5 /ml is sufficient to inhibit EIF synthesis. Thus, de novo synthesis of EIF apparently proceeds more efficiently at 22° than at 37°. Experiments are in progress to determine whether the minimal effective dose of Actinomycin D is the same for EIF and VIF synthetic mechanisms.

Several investigators have reported that the administration of gluco-corticoids to animals results in impairment of interferon synthesis and increased susceptibility to virus infection. A recent study by Postic and Comorus (P.S.S.B.M. 1963), however, demonstrated that cortisone excited a markedly inhibitory effect on EIF in intact rabbits and only a minimal effect on VIF synthesis.

The effect of cortisol on EIF synthesis has been tested in the rabbit macrophage culture system to determine whether the same inhibitory effect can be demonstrated in vitro. Macrophage cultures treated with cortisone (hydrocortisone sodium succinate) 100 /ml were compared with control cultures for their EIF synthetic capacity during a 24 hour period

of incubation at 22°. After 4, 8, 12 and 24 hours of incubation, culture fluids were removed and tested for interferon content. Cortisone exerts a marked inhibitory effect on the synthesis of EIF. Thus, preliminary work indicates that this cultural system can serve as an in vitro model for the interaction between cortisol and interferon synthesis. Work in progress includes studies to determine a minimal effective dose of cortisone as well as studies to determine the effect of cortisone on VIF synthesis by macrophages. One recent experiment showed that cortisol (100 /ml) has no effect on the synthesis of VIF by rabbit kidney cells induced by NDV.

Interferons exert their actional effect by inducing cells to synthesize a new protein; it has been reported in the literature that this synthesis can be blocked by inhibitors of macromolecular synthesis, indicating that interferon itself is devoid of antiviral activity. The studies described below were carried out to determine whether rabbit VIF and EIF can be distinguished on the bases of their action in rabbit kidney cells. The VIF used in these studies was synthesized in RK cells, while the EIF was produced in macrophages.

The activity of the interferons (VIF and EIF) were measured by treating cultures of RK cells with appropriately diluted samples and then challenging the cells with a large dose of VSV. Eight hours later, supernatant fluids were taken and the eight hour yield of VSV determined. The amount of restriction in virus yield in interferon treated cultures compared with controls was taken as a measure of interferon effect. Table 25 depicts the results of an experiment conducted to compare the efficiency of VIF and EIF action at varying temperatures. At 37°, treatment of RK cells for four hours with either VIF or EIF resulted in an

Table 25 The Effect of Temperature on the Action of Interferon

Temp. of Incubation	VSV Yield in 8 Hours		
	30°	33°	37°
Control	1.8×10^4 (1.0)	2.4×10^5 (1.0)	3.4×10^7 (1.0)
VIF Treated	2.0×10^3 (0.11)	2.6×10^3 (0.011)	6.3×10^2 (0.0018)
EIF Treated	2.3×10^3 (0.13)	2.4×10^3 (0.01)	7.2×10^2 (0.0023)

eight hour yield of VSV of approximately 0.2% of untreated controls. Interferon effect at 33° was less efficient with both preparations

reducing the virus yield to 1% of controls. At 30°, interferon treated cells produced a virus yield of approximately 12% of controls. This experiment demonstrated that while interferon effect is temperature dependent, with decreasing efficiency as the temperature decreases, no difference could be detected between VIF and EIF.

Next an experiment was carried out to measure the time required for VIF and EIF to protect cells against VSV. Since messenger RNA and protein synthesis must proceed before cells are protected, Actinomycin D can be used to block RNA synthesis at various times after interferon is added to cells. The degree of cellular protection then reflects the amount of messenger RNA synthesis which went on before the Actinomycin D was introduced. Table 26 lists the results of an experiment in which Actinomycin was added to cells 0, 1, 2, 3 and 4 hours after treatment of cells with VIF and EIF. Eight hour VSV yields from the RK cells were measured. In

Table 26 The Effect of Actinomycin D on the Action of VIF and EIF

Duration of Treatment		8 Hr. Yield of VSV	Fraction of Control
Media (Control)	Actinomycin D		
4	0	5.9×10^5	
4	0	8.3×10^5	
4	1	8.5×10^5	
4	2	8.2×10^5	
4	3	6.6×10^5	
4	4	5.1×10^5	
	MEAN	7.1×10^5	1.0
EIF	Actinomycin D		
4	0	8.7×10^3	0.012
4	1	3.1×10^4	0.044
4	2	5.2×10^4	0.073
4	3	1.5×10^5	0.21
4	4	3.1×10^5	0.44
VIF	Actinomycin D		
4	0	4.9×10^3	0.007
4	1	2.9×10^4	0.041
4	2	8.2×10^4	0.12
4	3	2.9×10^5	0.41
4	4	6.2×10^5	0.87

the case of both interferons, the longer the interval between the addition of interferon and the addition of Actinomycin D, the greater the protecting effect, reflecting the amount of messenger RNA synthesized in the interval. Again, VIF and EIF cannot be distinguished in this manner. Both induce cells to synthesize messenger RNA at approximately the same rate.

V. Laboratory Studies of Dengue Viruses.

A. Properties of Hemagglutinin. Studies concerned with the separation and analysis of the components of dengue virus populations have been extended during the past year. Preliminary results of sedimentation of dengue virus in sucrose gradients were reported previously (see WRAIR Annual Report, 1967).

Dengue virus (type II) derived from suckling mouse brain and from tissue culture (continuous rhesus kidney LLC-MK_2 and continuous green monkey kidney BSC-1) have been examined. Technicues for the preparation of virus grown in tissue culture were described in last year's report.

1. Methods. Mouse brain virus is prepared as follows: 20-24 litters of 2-3 day old mice are inoculated IC with .01 ml of a 10^2 dilution of high mouse passage dengue virus (type II). When most mice are moribund (usually day 5) they are sacrificed and stored intact at -60°C until ready for use. At that time, the mice are thawed and the brains aspirated. A 20% suspension in TRIS (.02 M, pH 8.2) or PBS (.01 M, pH 7.4) is homogenized by two one-minute cycles (separated by a one-minute pause) at full power in a Servall omnimixer, and then clarified by centrifugation at 2800 RPM for 20 minutes at 4°. The supernatant is treated with protamine sulfate, 5 mgm/ml, with intermittent shaking for 30-60 minutes at 4°C, and then centrifuged at 10,000 RPM for 30-45 minutes. This supernatant is then centrifuged for 2.5 hours at 40,000 RPM in a Spinco model L ultracentrifuge (#40 rotor). Supernatant fluids are discorded, and the pelleted virus resuspended in 2.5-3ml of .02 M TRIS - .2% BPA or .01 M PBS - .2% BPA. After ultrasonic vibration (2 one-minute cycles, full power, 10 KC Raytheon Sonic Oscillator) to disrupt aggregates, the concentrated virus preparation is ready to layer on a sucrose gradient. Sucrose stock solutions 5% and 25% in .01 M PBS or .02 M TRIS are sterilized by autoclaving at 10 pounds pressure for ten minutes. 27ml 5% to 25% sucrose gradients are performed in 1" x 3" cellulose nitrate tubes; 1.0 - 2.5ml samples are layered on top by means of pipette.

Sucrose gradients are centrifuged for three hours at 25,000 RPM in the model L ultracentrifuge (SW25.1 rotor), after which 1 ml fractions are collected, dropwise, through a needle inserted in the bottom of the tube. Hemagglutinin content of the fractions is measured by the microtiter technique (.25% goose cells, pH 6.2). Complement-fixing antigen

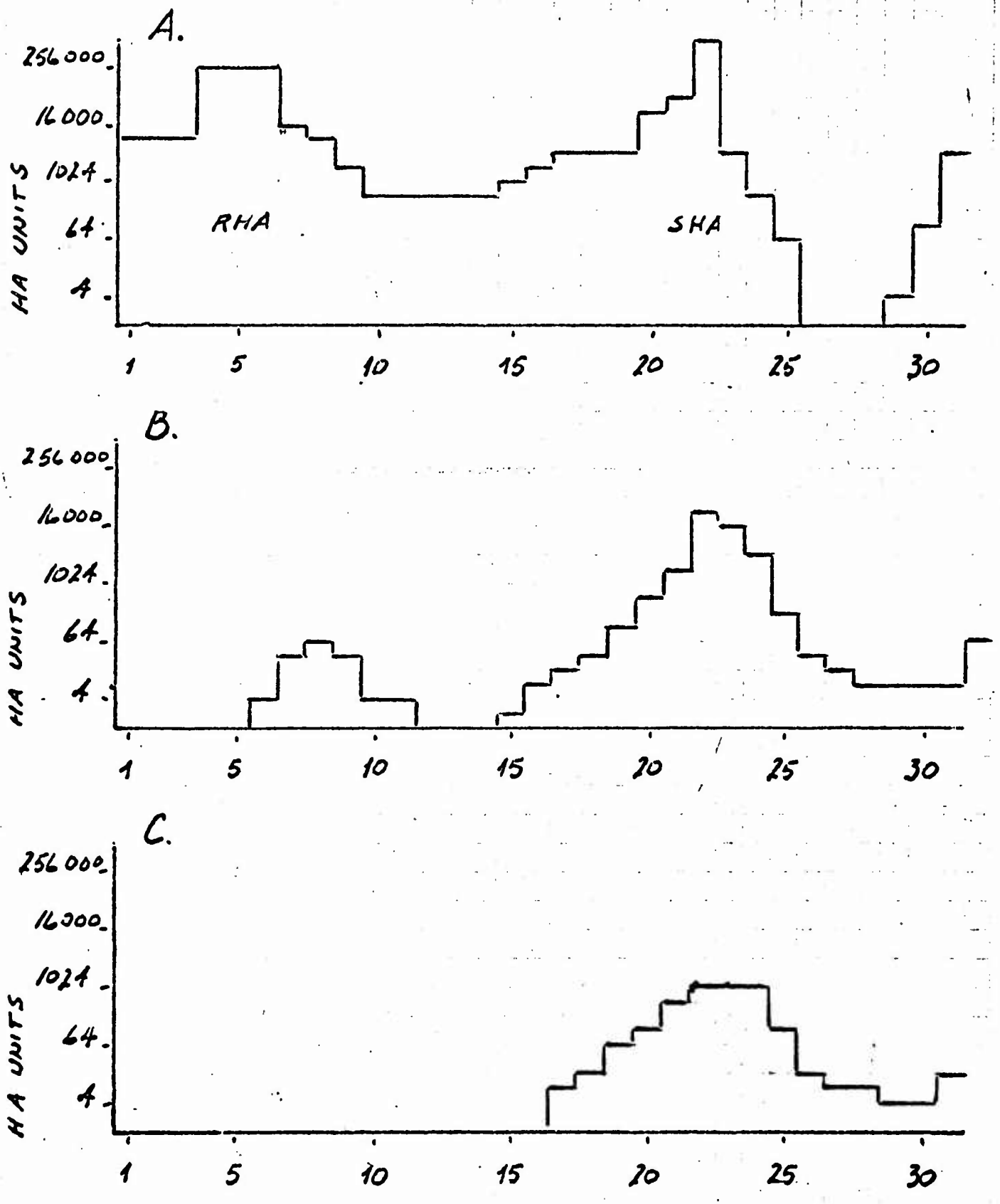
also is detected by the microciter technique; fractions are detected serially in two-fold steps in normal saline and mixed with an equal volume (0.025 ml) of mouse hyperimmune ascitic fluid (anti-dengue type II) diluted 1:10. This ascitic fluid has a CF antibody titer of 1:512 when tested against eight units of dengue type II CF antigen. Two exact units of complement (in normal saline containing 10 mg % M_g50_4) in .05ml are then added; after incubation overnight at 4°C, .05ml of 1.5% sensitized sheep cells are added and the test is read after one hour at 37°C.

2. Observations on Sedimentation of HA. Preliminary results with cell culture derived virus, reported last year, revealed that two components of hemagglutinin were separated by sedimentation in a sucrose gradient. Identical results have been obtained with mouse brain virus; that is, two distinct and widely separated peaks of HA activity are observed. Gradient A of figure #1 illustrates a representative gradient; the more rapidly sedimenting HA component (RHA) peaks at fraction #5, while the more slowly sedimenting HA particle (SHA) peaks at fraction #22. A third peak of HA activity is observed at the top of the gradient (fraction #31) and is thought to represent virus which has failed to sediment due to association with lipid. Further separation and purification of the HA components can be accomplished by repeated centrifugation in gradients, as evidence by Gradients B and C in figure #1. In this experiment, fractions comprising the SHA component of Gradient A were pooled and the sucrose removed by dialysis overnight against 100 volumes of PBS. This sample was then sedimented in a second sucrose gradient (Gradient B); while total HA activity is somewhat reduced, most consists of SHA. Low levels of activity are present further down the gradient, indicating that the sample contained some RHA. Gradient C of figure 1 depicts the results of a second purification step; a pool of fractions containing SHA from Gradient B was again dialyzed and sedimented in sucrose. Hemagglutinin assay of the fractions revealed a relatively homogeneous SHA component.

Electron microscopic studies, described in detail elsewhere, have shown that the RHA peak is comprised of intact virions, which are spherical particles measuring approximately 450Å in diameter; capsid subunits measuring 70Å in diameter can be identified. The SHA particle is spherical and measures 140Å in diameter; that is, SHA apparently does not consist of isolated capsid subunits derived from intact virions, since these particles are twice as large as identifable subunits.

Repeated experiments with sedimentation of mouse brain dengue virus have confirmed the observation that two HA peaks are regularly present, and that these HA components have the same sedimentation characteristics as those which make up tissue culture-derived virus. In addition, a third peak of HA activity is apparent in some gradients. This peak is

Figure 1 Sedimentation Characteristics of Dengue II HA in Sucrose Gradient (5-25%) Virus from BSC-1 Cells



ill-defined and usually contains considerably less HA activity than either of the two major components. When present, this component is observed to sediment in a position approximately mid-way between the other two components. One of the more prominent examples of this peak is illustrated in figure #2. Again, this minor component is observed in virus preparations derived from tissue culture as well as mouse brain.

When examined through the electron microscope, HA sedimenting in this zone appears to be fragments of disrupted virions.

RHA does not withstand the stress of repeated centrifugations in sucrose as well as does SHA. This is illustrated in figure #3, which depicts the sedimentation in a 5-25% sucrose gradient of virus grown in LLC-MK_2 cells. Two peaks of hemagglutinin were obtained, and pools of each were prepared and again subjected to sucrose gradient centrifugation after overnight dialysis against borate saline. Gradients I & II of figure #3 represent the results of sedimentation of these pools. Gradient I received RHA, which was represented by a sharply defined peak of high titer in the original gradient; in contrast, repeat centrifugation resulted in a considerable loss of total activity as well as a diffuse spreading of the hemagglutinin throughout the gradient. SHA (Gradient II), however, again sedimented such that the HA activity was almost quantitatively recovered in a relatively symmetrical peak in the same position in the gradient as that from which the pool was derived. These results indicate not only that RHA breaks up readily, but also that the fragments possessing HA activity are fairly diffusely distributed. There is no evidence to suggest that SHA is derived from, or is made up of fragments of RHA. Certainly no increase in SHA is evident as RHA breaks up with repeated centrifugations. It is possible, however, that the HA minor component found in the mid-zone of the gradients does represent fragments of RHA, since it has been observed that HA activity in that zone increases as RHA breaks up. When fractions are selected from the mid-zone of a gradient and sedimented in sucrose again, the HA activity again appears in the mid-zone, indicating that while some HA activity is lost in the process, those particles or fragments which retain HA activity remain intact.

The relative instability of RHA is variable from one experiment to another; that is, repeat centrifugations have been carried out with RHA resulting in recovery of a symmetrical peak of HA activity in the same position in the gradient. Factors responsible for this variablity remain to be defined. While dialysis of RHA overnight against PBS, TRIS or borate saline results in some loss in activity, this is equally true for SHA.

Additional evidence which indicates that SHA does not represent fragments of RHA is derived from the observation that both HA components

Figure 2 Sedimentation Characteristics of Dengue II HA in Sucrose (5-25%); Virus from Mouse Brain

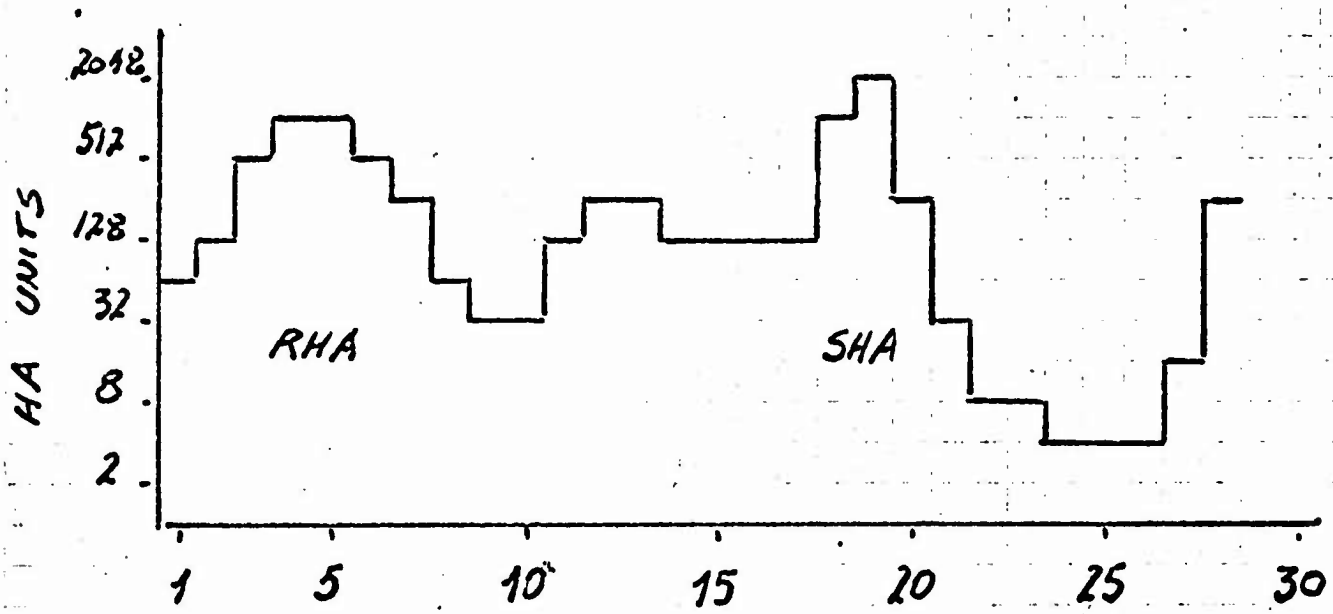
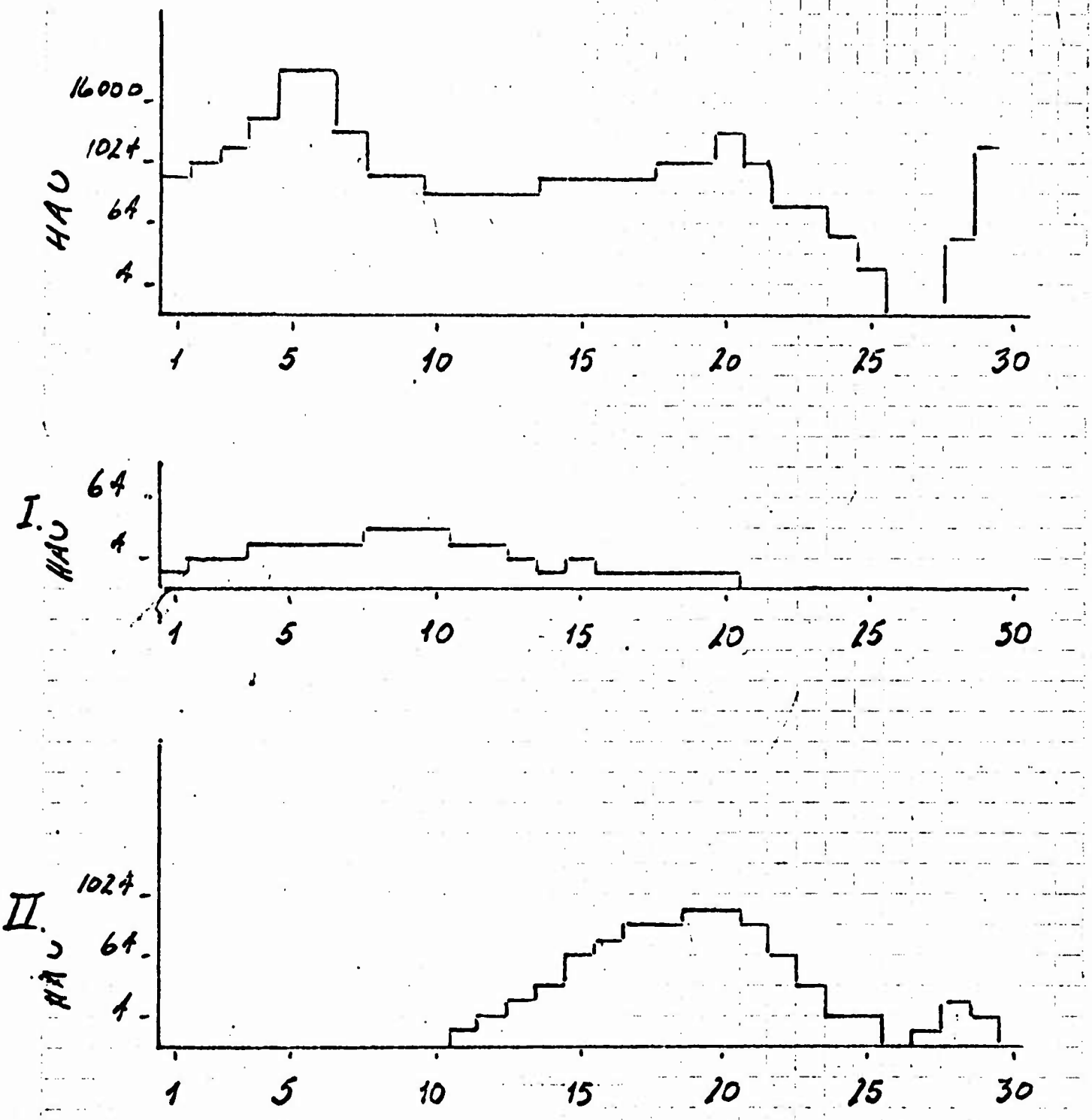


Figure 3 Sedimentation Characteristics of Dengue II HA; Virus from LLC-MK_2 Cells



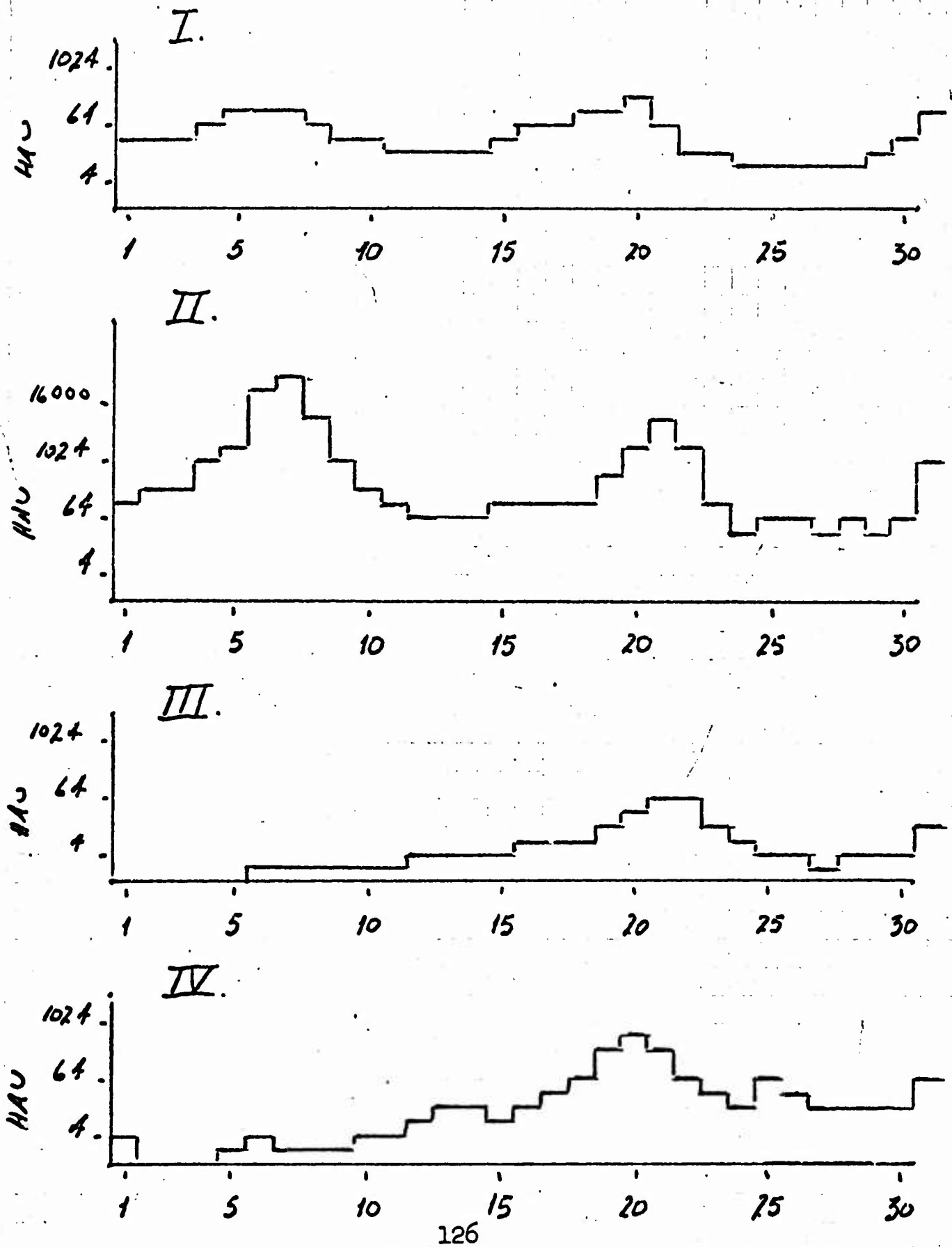
are present in infected mouse brain in approximately equal proportions prior to pelleting of the virus by centrifugation. Gradient I of figure #4 is a representative example of the distribution of HA in a sucrose gradient of unpelleted virus from a 20% mouse brain suspension. Gradient II of figure #4 depicts the results of sedimentation in sucrose of mouse brain virus which had been concentrated by pelleting; clearly, effective concentration of both RHA and SHA has been achieved, with a relative increase in RHA over SHA. The fact that pelleting of dengue virus from a mouse brain suspension results in a relative increase of RHA could be expected from the sedimentation behavior of the two particles. Gradient III of figure #4 demonstrates that the supernatant fluid contains a considerable amount of SHA, and only barely detectible amounts of RHA, explaining the relative increase of RHA in pelleted virus. SHA, then, does not make its appearance during the virus concentration procedure; rather it is present in infected mouse brain, and in all probability is not derived from fragmentation of RHA.

Dengue virus can be precipitated from a suspension of infected mouse brain, or from TRIS buffer, by the addition of alcohol. Pre-chilled absolute alcohol is added to the virus-containing fluids to a final concentration of 25%. After one hour at 4° (with occasional agitation) the precipitate can be separated by centrifugation and re-suspended in a small volume to effect virus concentration. Preliminary experiments with ethanol precipitation indicate the following: a) this technique is considerably less efficient than pelleting dengue virus by centrifugation from a mouse brain suspension; b) RHA and SHA can be identified after alcohol precipitation and gradient centrifugation; however, it appears that there is a considerable loss of RHA in the process. That is, when alcohol-precipitated virus is analyzed by sucrose gradient centrifugation, most remaining HA consists of SHA. In some instances alcohol precipitation leads to an increase of HA activity which sediments in the gradient mid-zone as well as a loss of RHA, suggesting that this HA activity is derived from fragments of RHA. Gradient IV of figure #4 is an example of the sedimentation of alcohol-precipitated virus in which almost all precipitated HA consists of SHA. In this instance, RHA was apparently destroyed since no HA could be detected in the supernatant fluid.

In a separate experiment, an aliquot of mouse brain virus was pelleted by centrifugation and resuspended in TRIS buffer; precipitation of virus by alcohol from one aliquot yielded the same proportions of RHA and SHA as was contained in the original suspension, as evidenced by sucrose gradient centrifugation of treated and untreated samples.

Efforts to disrupt RHA in such a way as to produce fragments which possess HA activity have been unsuccessful. Methods employed have included treatments of RHA with ether, chloroform, desoxycholate, heat,

Figure 4 Sedimentation Characteristics Dengue II HA Infected Mouse Brain



sonic vibration and mechanical agitation. Each of these treatments resulted in a loss of total HA activity, the extent of the loss being dependent on the duration of the treatment.

While RHA appears to be somewhat more fragile than SHA when subjected to repeated centrifugations in sucrose, SHA appears to be the more labile of the two components when subjected to heat. The loss of HA activity of these two particles when heated to 46° is depicted in figure #5A. In this experiment the two HA peaks obtained from a sucrose gradient and adjusted to contain comparable amounts of HA and sucrose. Four replicate aliquots of each were then placed in a 46° water bath, and two samples were taken from each at ten-minute intervals to determine residual HA activity. As shown in figure #5A, there is an approximate 10-fold reduction in SHA in the first ten minutes. Subsequent heating caused a further loss of activity at a considerably slower rate. There appeared to be a linear loss of RHA for the duration of the heating, with considerably less inactivation occurring during the first 30 minutes than that observed with SHA. Since the sample of SHA undoubtedly contained some RHA (as is always the case after one centrifugation), the more gradual inactivation slope seen after 30 minutes may actually represent inactivation of contaminating RHA. Several heat-inactivation experiments with RHA and SHA have been performed, and some have yielded conflicting results. It appears that several factors may alter such a test and some of these factors remain ill-defined.

The complement-fixing activity has been measured of fractions collected following sucrose gradient centrifugation of both mouse brain and tissue culture-derived dengue virus. A representative example of such a determination is shown in figure #5B. The HA activity of fractions of mouse brain virus is shown in the upper portion (A) with clearly defined peaks of RHA and SHA. The CF activity of the same fraction is shown in the center of figure #5 (B), three zones of CF antigen are delineated. Two CF peaks coincide exactly with RHA and SHA peaks substantiating the fact that both particles contain CF antigen. Of even greater interest is the observation that a third peak of CF antigen sediments near the top of the 5-25% sucrose gradient in a region where there is no discernable corresponding peak of HA activity. The peak of this CF antigen was found in fractions 26 and 27; a pool of these two fractions was made, dialyzed against .02 M TRIS and layered on another 5-25% sucrose gradient. Fractions collected from this gradient were then examined for CF and HA antigen; fraction 21 had an HA titer of 1:2, while all other fractions were negative. The CF activity of the fractions is shown at the bottom of figure 5 (B); it appears that quantitative recovery of the CF activity was achieved and the sedimentation characteristics were unchanged. That is, the peak of the CF activity again appears in fraction 27. It is clear from figure 5 (B) that the ability to fix complement is a relatively insensitive measure of those particles designated RHA and SHA compared to a

Figure 5 (A) Thermal Inactivation of Dengue II RHA and SHA

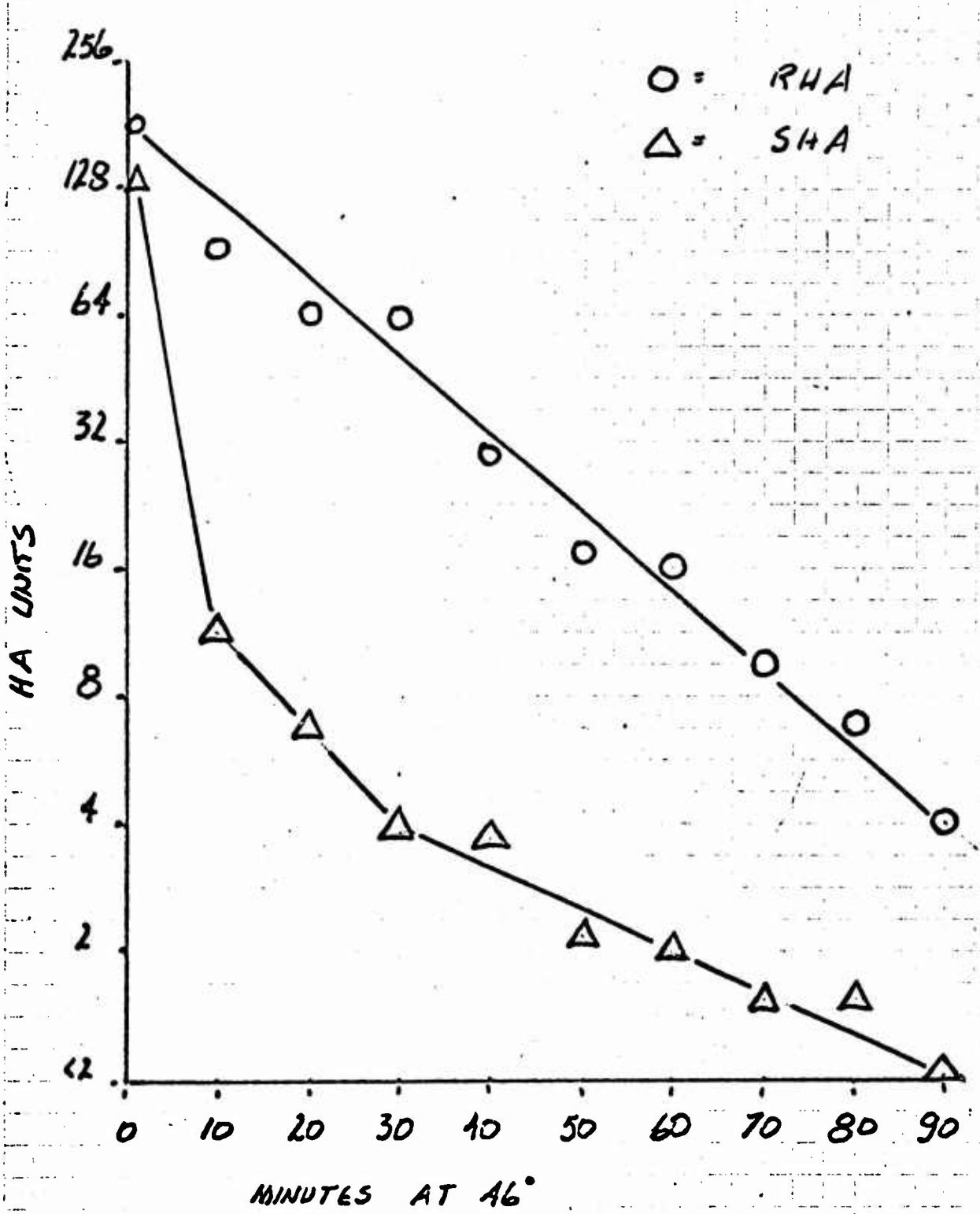
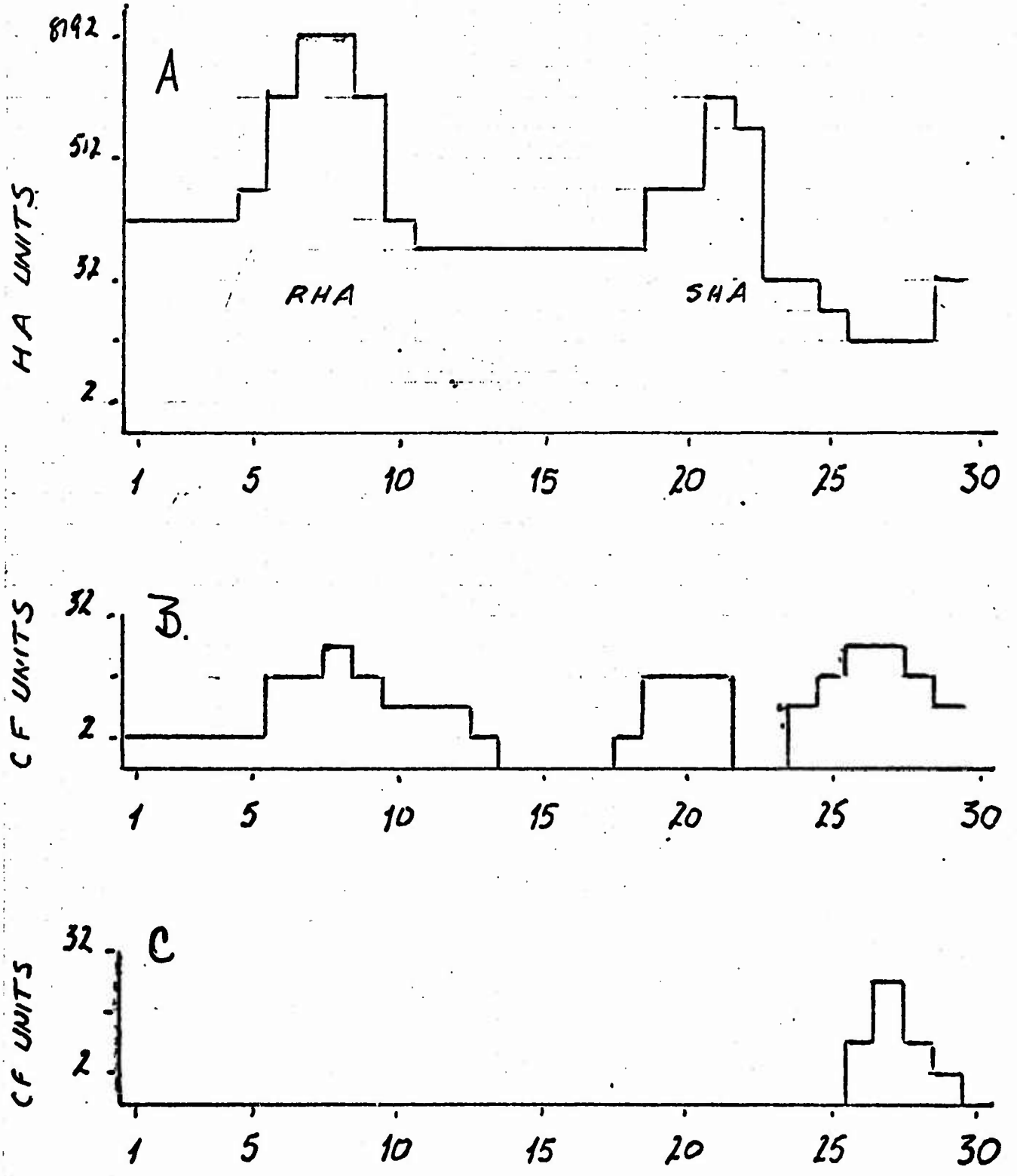


Figure 5 (B): Relationship of CF Antigen to RHA and SHA of Dengue II Virus



measure of hemagglutination. In contrast, the third or very slowly sedimenting CF component is accompanied by no peak of hemagglutinin. To exclude the possibility that HA activity in that area is masked by inhibitor which may sediment at that zone of the gradient, fractions 19-30 of the gradient depicted in the lower portion of figure 5 (D) were tested for hemagglutinin-inhibitory (HI) activity. Antigen for the HI test consisted of SHA; eight units were used and no evidence of inhibition was detected in any fraction. Thus it appears that this very slowly sedimenting particle found near the top of a 5-25% sucrose gradient possesses CF activity but no HA activity. Further, the addition of this antigen to goose red cells does not alter the capacity of the cells to agglutinate when HA antigen is added. Whether the CF antigen attaches to these cells at all is yet to be determined.

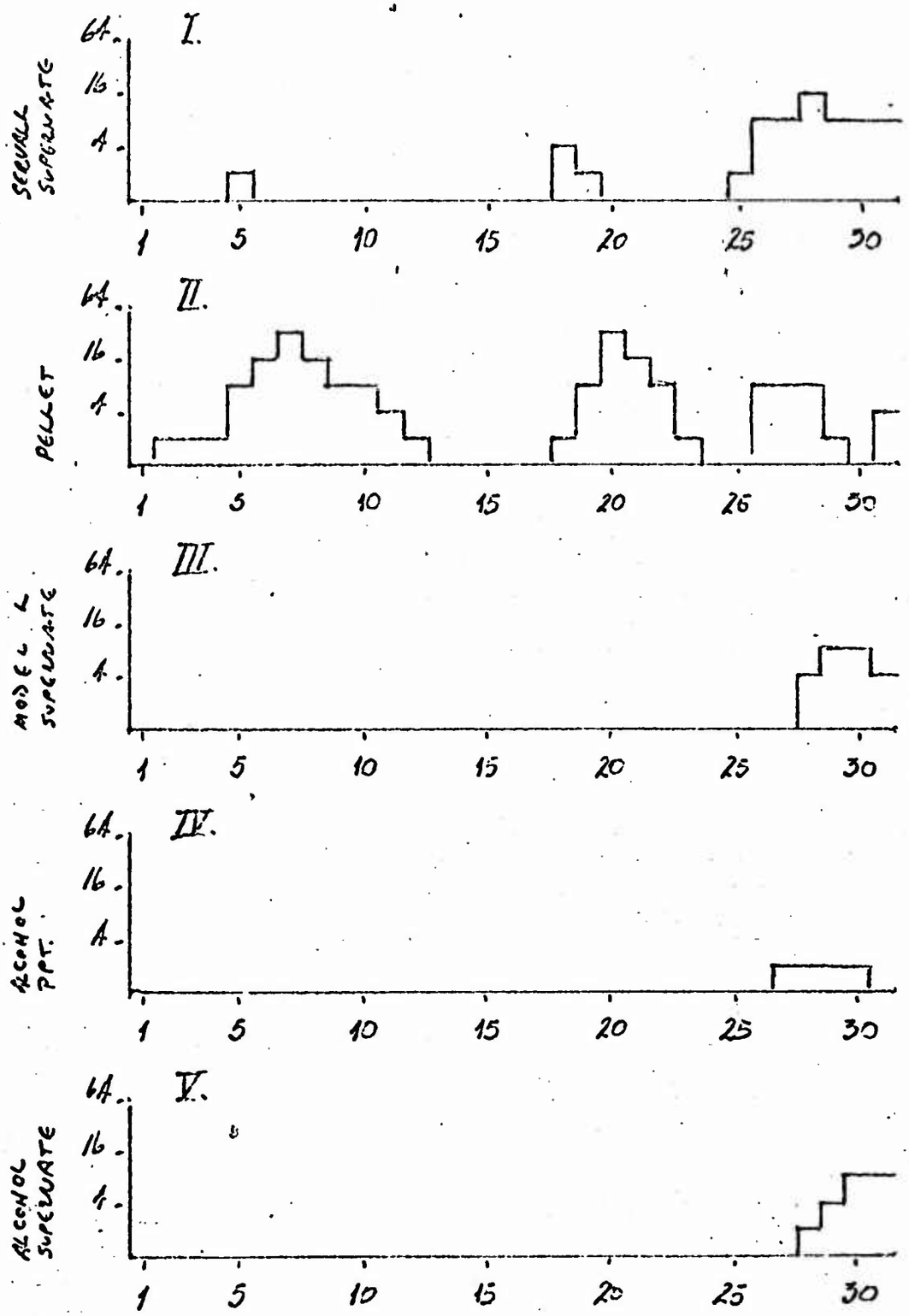
The same three peaks of CF activity are found with virus derived from LLC-MK_2 cells as well as from mouse brain.

The CF antigen which sediments very slowly in sucrose is found in relatively large amounts in a 20% suspension of infected mouse brain. Gradient I of figure #6 depicts the results of sedimentation of a sample of protamine treated mouse brain. By far the largest amount of CF activity is located near the top of the gradient. Gradient II illustrates that while CF activity corresponding to RHA and SHA increase as a result of pelleting these components, the CF antigen near the top of the gradient is relatively decreased. This indicates that centrifugation at 40,000 RPM for 2.5 hours provides for a virus concentrate consisting mainly of the larger RHA and SHA particles. A substantial portion of the slow CF antigen remains in the supernatant fluid after centrifugation at 40,000 RPM, as illustrated in Gradient III. Gradients IV and V of figure #6 show the distribution of CF antigen in a sucrose gradient after alcohol precipitation of mouse brain virus. As was the case with HA antigen, this technique is a very inefficient method for recovering CF antigen. The small amount of CF antigen present in the precipitate consits of the slowly sedimenting antigen while even more of the same antigen remains in the supernatant fluid (Gradient V). The absence of any CF activity corresponding to RHA and SHA reflects the adverse effect alcohol exerts on these particles, particularly RHA.

Electron microscopic examination of a sample taken from the zone corresponding to the slowly sedimenting CF antigen revealed spherical particles measuring 70Å in diameter, with an appearance identical to the 70Å subunits comprising surface subunits of intact virions.

Work is in progress to characterize this antigen further and to determine whether this 70Å particle is also the CF antigen responsible for the CF activity of the intact virus. Emphasis is being placed on

Figure 6 Effect of Protamine Sulfate upon CF Activity of Sucrose Density Gradient Separated Dengue II Virus; Infected Mouse Brain



finding methods to concentrate and purify the antigen. Animal inoculation will be used to obtain antibody directed against this antigen, which will then be tested for potency and specificity.

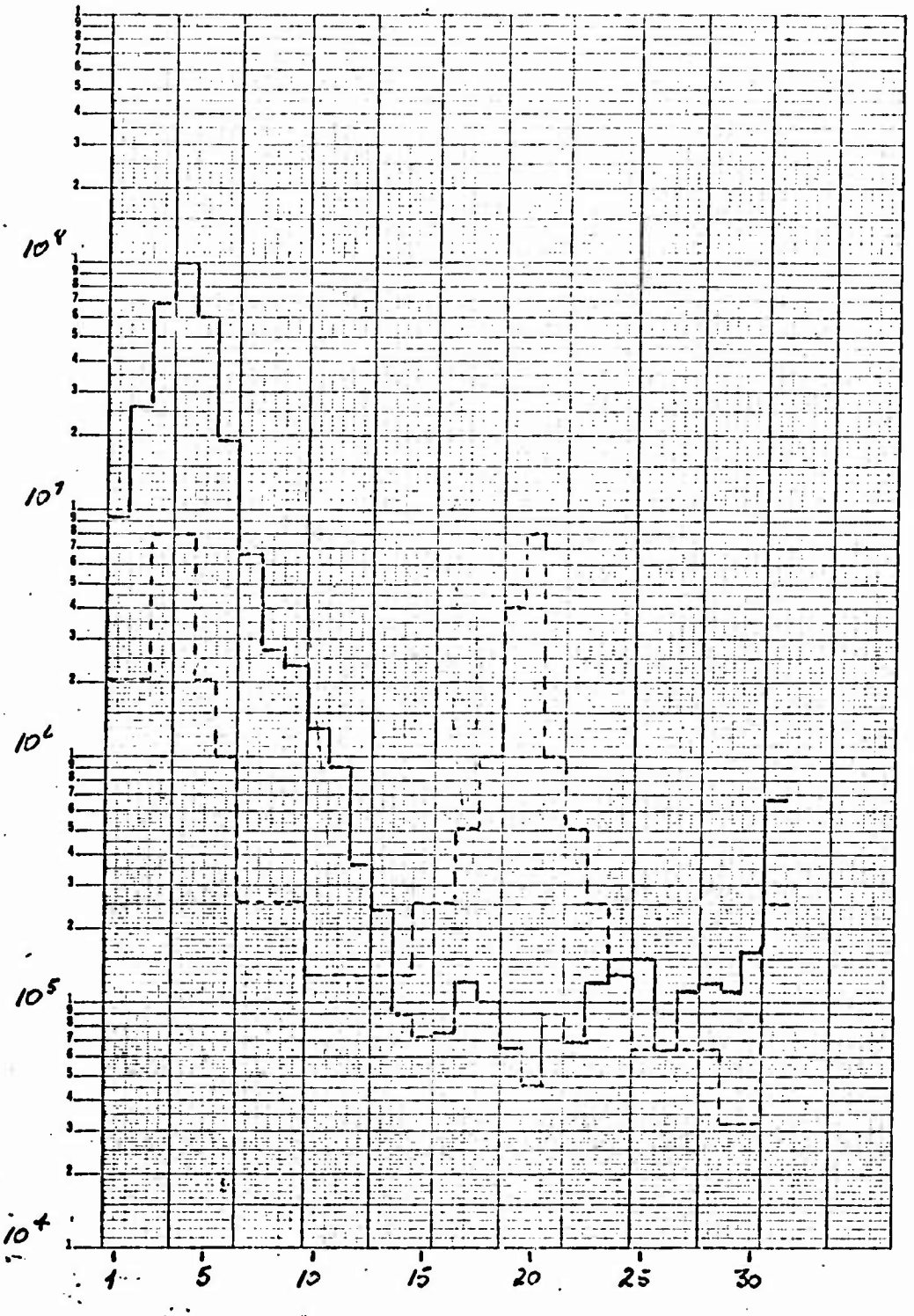
Infectivity determinations have been made of sucrose gradient fractions after sedimentation of both tissue culture grown and mouse brain virus. Early efforts using the BSC interference technique were reported last year. Since that time the more accurate and sensitive plaque assay has been employed. Details of this method are described below. It appears that infectious virus sediments in sucrose as a simple homogeneous peak corresponding to the RHA particle. (As mentioned above, electron microscopic examination has documented the appearance of RHA to be intact virions). Figure #7 illustrates this observation. Infectivity of fractions after sedimentation of mouse brain virus was determined by plaque formation in pig kidney (PS) cell cultures and is expressed as plaque forming units (PFU). Maximal titer (10^8) was achieved in gradient fraction 4, which corresponds to the peak HA activity of the intact virus or RHA. In this gradient, equivalent amounts of hemagglutinin were found in the RHA and SHA components, as represented by the dotted line in figure #7. There is, however, no corresponding peak of infectivity associated with the sharply delineated HA peak of SHA. Thus, infectivity is a property of RHA alone. SHA, by virtue of its inability to replicate (or at least to produce plaques) and its small size, is an incomplete virus. Whether it contains RNA or consists simply of a protein shell remains to be determined.

It is apparent from figure 7 that infectious virus is present in every fraction. This is due not only to the fact that the virus is not sedimented to equilibrium, but also to the fraction collection technique of needle puncture through the bottom of the tube. An increased amount of infective virus is noted at the top of the gradient. As noted earlier, in the HA activity there is thought to represent intact virus which fails to sediment due to some association with lipid.

Identical results have been obtained with virus grown in tissue culture. Likewise, plaque assays performed in BSC cells rather than PS cells of gradient fractions have confirmed the observation that only RHA is capable of plaque formation.

B. Properties of HA Inhibition. It has been observed that in sucrose gradients of tissue culture-derived virus a zone of hemagglutination-inhibition exists near the top of the gradient, usually beginning one or two fractions beneath the top and extending into the gradient for 3-6 fractions. Most typically, this inhibitory activity manifests itself as a prozone effect with HA appearing in affected fractions on dilution. This same effect is sometimes observed in gradients of mouse brain virus, but usually is less evident. Efforts have been made to determine the

Figure 7 Relationship of Infectivity to HA in Sucrose Gradient Fraction Dengue 11 Virus; Virus Grown in BSC-1 Cells



PFU/0.2 ml.

HAU/0.05 ml.

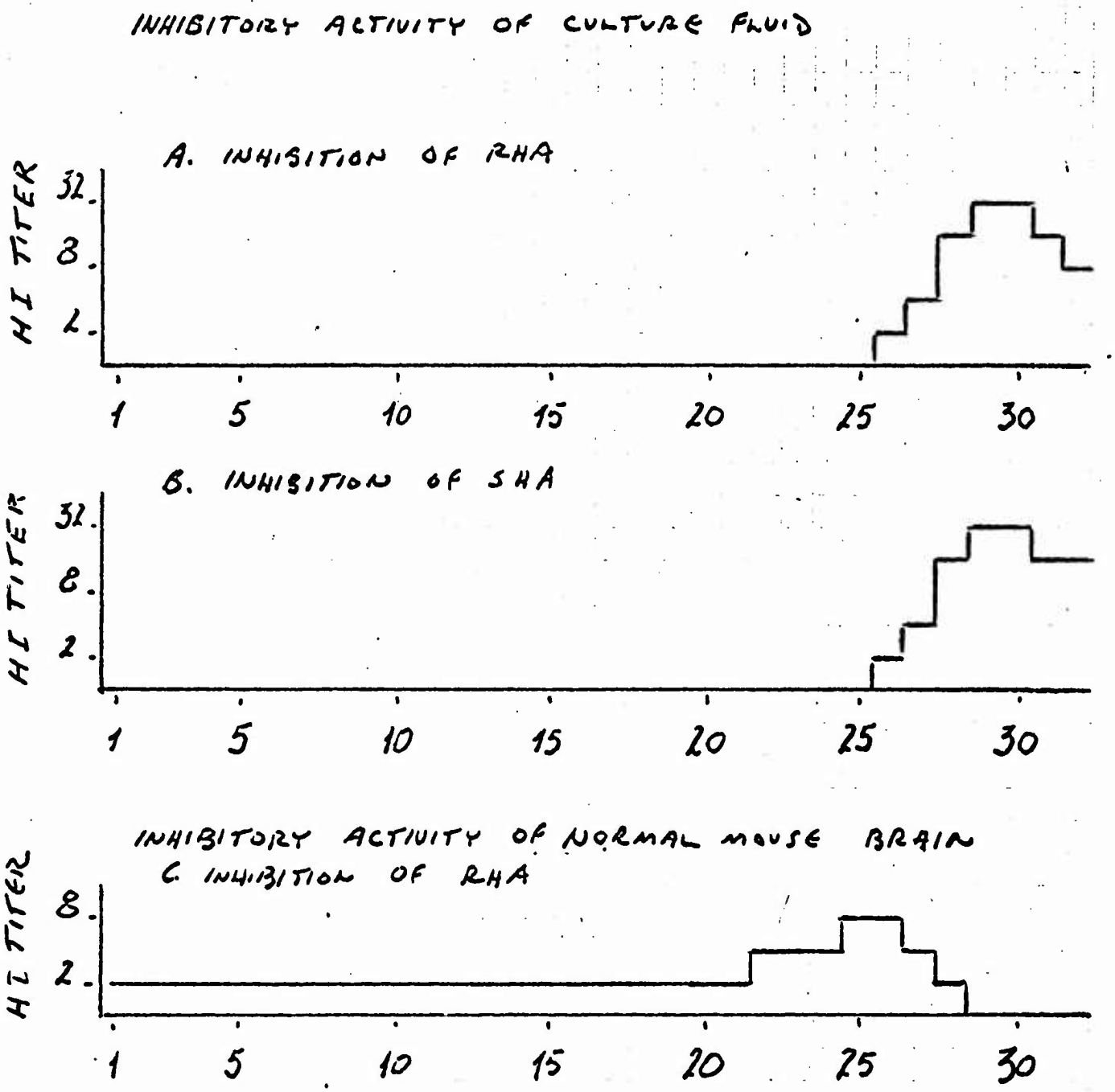
GRAPHIC NOT REPRODUCIBLE.

source of this inhibitory activity, to compare the inhibitor in mouse brain with that in cell culture as preliminary steps toward characterization of these materials. To determine the sedimentation behavior in sucrose of the inhibitor in tissue culture fluid, uninfected LLC-MK_2 cells were disrupted by repeated freeze-thaw cycles followed by sonic vibration and suspended in tissue culture fluid containing 10% fetal bovine serum (FBS). After the debris was removed by centrifugation, a sample of this fluid was sedimented in a 5-25% sucrose gradient. The fractions were then tested for inhibitory activity in an HI test using both RHA and SHA (16 units of each). Results are depicted in figure #8. A peak of HI activity titering 1:32 in fractions 29 and 30 is present and extends from the top of the gradient down to fraction 26. The pattern of inhibition is identical in panels A and B of figure 8, indicating that the inhibitor is equally effective against RHA and SHA. The source of the inhibitor in fluids from tissue cultures is serum. When LLC-MK_2 cells are washed free of serum and then disrupted in media containing no serum, the clarified fluid contains no inhibitor. In contrast, 100% FBS (inactivated at 56° for 30 minutes) has an HI titer of 1:128 when tested against 16 HA units of RHA; 20% FBS has a titer of 1:64 and 2.5% FBS titers 1:16. Protamine treatment of FBS (5 mg protamine sulfate per ml of either 100% FBS or 10% FBS in Hanks Salt Solution for 30 minutes at 4°) has no effect on serum inhibitor.

Normal mouse brain likewise contains inhibitor of dengue HA; however, there appear to be several differences between this inhibitor and the one found in FBS. A 20% suspension of normal mouse brain in TRIS was prepared by homogenization and clarification (10,000 RPM 40 minutes) and found to have an HI titer of 1:64-128 against 16 units of RHA. In contrast to FBS inhibitor, mouse brain inhibitor is removed completely by protamine treatment. The distribution of mouse brain inhibitor in a 5-25% sucrose gradient is illustrated in panel C of figure 8. While a small peak of HI activity is evident at fraction 25-26, inhibitory activity is found throughout the gradient with the exception of the top four fractions. This HI activity was measured against 16 units of RHA.

Neither the serum inhibitor nor mouse brain inhibitor affect CF activity; since RHA and SHA both demonstrate CF activity, this antigen can serve as a marker to locate these particles in sucrose gradients after HA activity has been masked by the inhibitors. This experiment was performed to determine whether serum or tissue inhibitor binds to these particles and whether the sedimentation behavior in sucrose is altered as a result of contact with inhibitor. SHA and RHA were mixed with either 20% FBS or 20% normal mouse brain and then sedimented in sucrose. RHA and SHA diluted in TRIS buffer were also sedimented and served as controls. In the case of serum inhibitor, it was observed that while the original samples placed on the gradients possessed no HA activity due to the presence of

Figure 8 Inhibition of Dengue II-Virus HA in Cell Culture Fluids



serum, sedimentation resulted in the appearance of RHA and SHA at their appropriate positions in the gradient. Mouse brain inhibitor, on the other hand, obscured SHA and RHA, and neither could be identified after sedimentation. This difference can be explained by the fact that mouse brain inhibitor distributes itself throughout the gradient; hence, centrifugation fails to separate HA from inhibitor. It is clear that serum inhibitor merely masks HA activity of RHA and SHA; not only does HA reappear when separated from inhibitor in the gradient but also it sediments in characteristic fashion. Tests for detection of CF antigen revealed that while RHA and SHA were obscured in the gradient by the presence of mouse brain inhibitor, the corresponding CF antigens were located in their usual positions indicating that the inhibitor had no effect on the sedimentation of either particle.

Studies were next carried out to determine whether either inhibitor could alter the surface of goose erythrocytes in such a way as to impair or prevent subsequent hemagglutination by RHA. Red blood cells were added to either 20% FBS or 20% normal mouse brain for 30 minutes at room temperature (protamine-treated mouse brain and acetone-extracted serum served as controls). Cells were then pelleted and one aliquot was washed. The washed and unwashed cells were then tested for their capacity to agglutinate in the presence of RHA, and the titers of inhibitors were measured before and after absorption with red blood cells. Results are shown in Table 27. The HI titer of serum inhibitor was unaffected by incubation with red blood cells, and the ability of red blood cells to agglutinate in the presence of RHA was unaffected by contact with serum.

Table 27 HA Inhibitory Activities in Culture Media and Normal Mouse Brain

	TITER OF INHIBITOR			
	20% FBS		20% Mouse Brain	
	Acetone Extracted	Normal	Protamine Treated	Normal
Before RBC Absorption	<10	160	<10	320
After RBC Absorption	<10	160	<10	80

Table 27 (Continued)

TITER OF ANTIGEN (RHA)

	RBC's Absorbed with:			
	20% FBS		20% Mouse Brain	
	Acetone Extracted	Normal	Protamine Treated	Normal
RBC's Washed After Absorption	400	400	200	200
RBC's Not Washed After Absorption	400	200	200	25

A four-fold reduction in HI titer of mouse brain inhibitor was effected by incubation with red blood cells and these same red blood cells demonstrated an impaired capacity to agglutinate when added to RHA. This impairment was reversible, since cells agglutinated normally after washing. It appears, then, that mouse brain inhibitor, but not serum inhibitor, binds reversibly to goose red blood cells. Whether this observation indicates a different mechanism of inhibition for the two inhibitors or simply reflects the more particulate nature of mouse brain inhibitor remains to be determined. Studies are in progress to determine whether red blood cell absorption could serve as a method of concentration and purification of the tissue inhibitor.

C. Densities of Dengue Virus Components. Estimates of the density of dengue virus have been made employing the technique of isopycnic density-gradient centrifugation in a solution of cesium chloride. The procedure consists in adding 3.9 ml of the virus sample to be tested to 1.4 ml of a saturated solution of cesium chloride in 0.01 M TRIS buffer, and sedimenting the virus to equilibrium by centrifugation at 39,000 RPM for 40 hours. Fractions are collected dropwise, HA activity measured, and the refractive index read with a B&L refractometer. Refractive indices of the fractions are then converted to density values according to the formula -- Density=10.8601 x refractive index - 13.4974 (Ifft et.al). The linearity of the gradients is confirmed by regression analysis of the refractive indices.

When dengue virus is pelleted from a protamine-treated suspension of infected mouse brain and then sedimented to equilibrium in CsCl, three separate and distinct zones of HA activity are observed. Figure 9 illustrates the appearance of these peaks of HA activity and the slope of the density gradient as measured by refractive indices. The major hemagglutinin has a density of 1.22, while the other two peaks correspond to densities of 1.31 and 1.19.

Essentially similar results have been obtained in repeated tests. The mean value for the middle HA peak of nine separate samples of mouse brain virus is 1.22. The mean for the lighter peak is 1.18. The small dense HA peak was observed in only three of these eight trials; the mean of these three values is 1.31.

In order to correlate the results of density-gradient centrifugation in CsCl with those obtained using sucrose gradients, samples of RHA and SHA were obtained by sedimentation of mouse brain virus in sucrose and then subjected to density gradient centrifugation in CsCl. Figure 10 is an example of the results of density gradient centrifugation in CsCl of RHA. Two peaks of hemagglutinin are obtained, one with a density of 1.22 and the other corresponding to 1.18. Thus the two HA peaks in a CsCl gradient derived from RHA correspond to the two major HA peaks found in the original mouse brain virus population. Figure 11 illustrates the results of density gradient centrifugation of SHA. This sample of slowly sedimenting HA, originally derived from infected mouse brain, was purified by two sedimentations in sucrose gradients prior to centrifugation in CsCl. This purified SHA sedimented in CsCl as a simple, sharply defined peak of hemagglutinin in a zone corresponding to a density of 1.23. Analysis of four separate samples of SHA derived from mouse brain has yielded a mean density value of 1.23. In no instance has a second HA peak been observed following sedimentation of SHA which corresponds to the second HA peak seen with sedimentation of RHA. These results are interpreted to indicate that RHA and SHA are of the same or approximately the same density. Since RHA is known to fragment easily with the appearance of the minor "mid-zone" peak of HA in sucrose gradients, it is thought that the HA peak in CsCl with a density of 1.18 represents these fragments. This would explain why this peak appears in CsCl gradients of original mouse brain virus and RHA but not SHA. There is electron microscopic confirmation of this interpretation. Particles seen in the HA peak with a density of 1.18 appear to be fragmented and partially fragmented virions. Particles present in the single HA peak at 1.23 in gradients of SHA have the typical appearance of these SHA particles. Thus, fragmentation of RHA in CsCl, as in sucrose, does not result in the appearance of increased amount of SHA, but rather of fragments of lighter density than either of the two particles. Additional confirmation stems from the fact that when "mid-zone" HA is selected from a sucrose gradient for density gradient centrifugation in CsCl, the peak

Figure 9 Densities of Dengue II Virus; HA

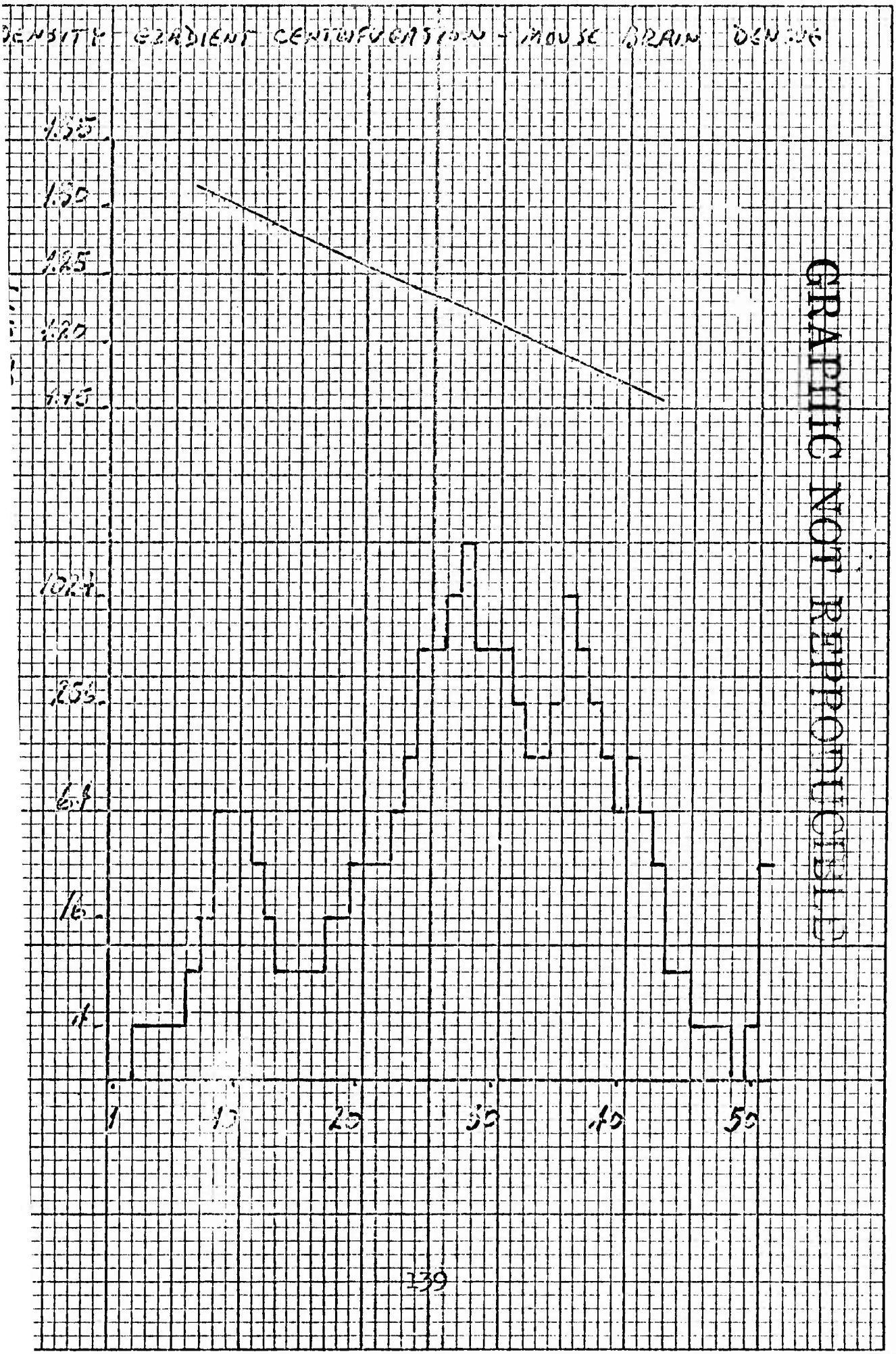


Figure 10 Density of Dengue II RHA Determined in CsCl

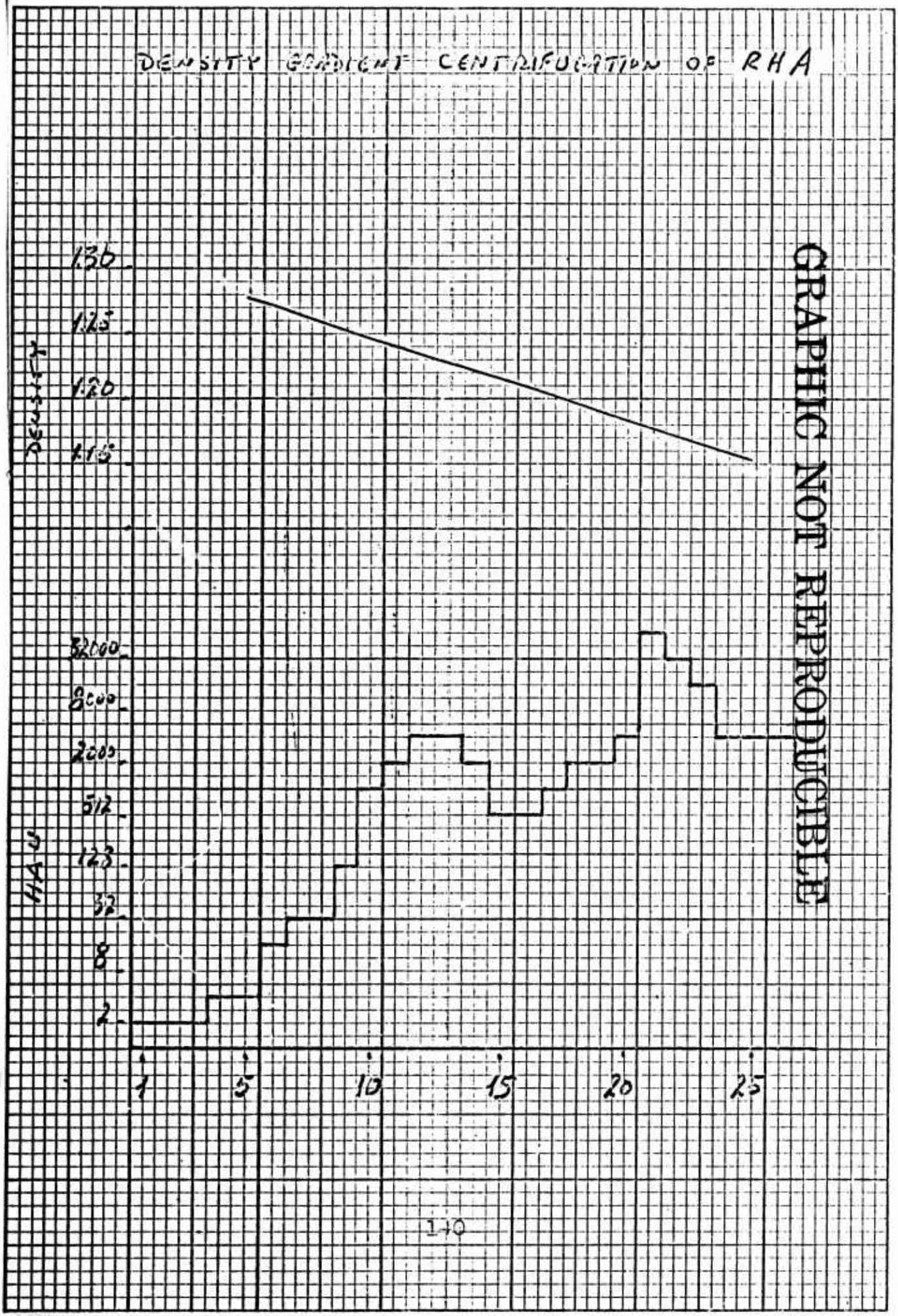
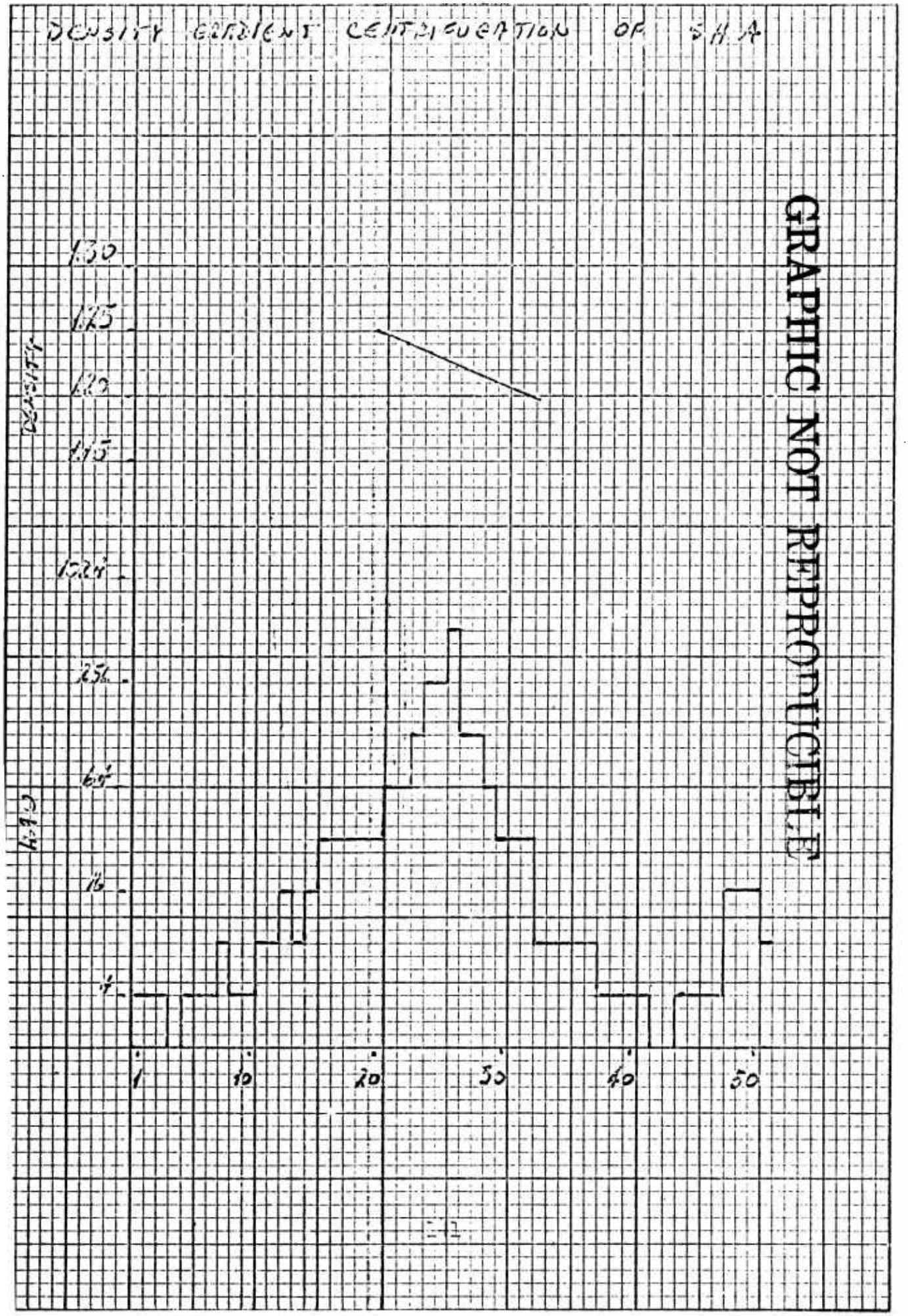


Figure 11 Density of Dengue II SHA in CsCl



of HA activity corresponds to a density of 1.18. Due to variability from test to test, it is impossible to say at this time whether RHA and SHA are of the same or slightly different densities.

Dengue virus grown in LLC-MK_2 cells yields similar results in CsCl gradients. Two peaks of hemagglutinin are seen. In two tests, the more dense particle sedimented in zones corresponding to 1.24 and 1.23. The lighter HA peak had a density of 1.18. Again, two HA peaks resulted from sedimentation of RHA derived from tissue culture, with mean values for three tests of 1.24 and 1.19. SHA sedimented in a single peak at 1.23. Repeated tests will be required to determine whether the slightly greater density values for RHA from LLC-MK_2 cells compared to RHA of mouse brain origin is significant. It is interesting to speculate that dengue virus grown in mouse brain may contain more lipid that virus grown in cultured cells, explaining the apparent lower density of the former.

Occasionally, CsCl gradient centrifugation of both mouse brain and virus derived from LLC-MK_2 cells has yielded a small peak of HA activity at a density corresponding to 1.27. The significance of this peak, as well as the one at 1.31, remains to be determined.

D. Interference by Dengue Virus Components. Cells in culture-infected with dengue virus are resistant to infection with a variety of other viruses. This property, which is related to interferon produced during the course of virus growth, has served as the basis of an assay system for dengue virus for several years.

This capacity to produce interference has been examined further during the past year. It has been observed that infected cells manifest resistance to superinfection as early as 24 hours after initiation of infection, and the degree of resistance is dependent on the amount of input virus. This phenomenon has been observed in several types of cells, including L cells (mouse fibroblasts) and primary rabbit kidney (RK) cells. The method used to detect interference consists of a challenge of monolayer cultures with 100 PFW of vesicular stomatitis virus (VSV) 24 hours after the inoculation of dengue virus in serial dilutions. The interference end-point is taken as the highest dilution of dengue virus which protects a culture to the extent that VSV plaques are 50% reduced in number. Both tissue culture grown and mouse brain virus induce interference, in direct proportion to the virus titer of the preparation. Low levels of interferon are present in the fluids of cells demonstrating resistance, suggesting that the interference is interferon mediated.

In an effort to determine whether intact virus (RHA), incomplete virus (SHA) or both are capable of inducing interference, sucrose gradient

fractions have been tested for their capacity to protect cells against VSV. In the experiment depicted in figure 12, sedimentation in sucrose of mouse brain virus was used to separate the two HA peaks which in this instance were of approximately equal activity. Fractions from this gradient were tested on RK cells for interference capacity; fractions 1-7 diluted 1:40, interfered with VSV plaque formation with a peak of activity at fraction 3-4. Fractions 0-31 were tested at a 1:20 dilution and no significant interference was demonstrated. It is clear from these data that fractions possessing interference activity correspond to the RHA zone and that no peak of interference activity is observed which corresponds to the SHA zone. Hence, it appears that an intact dengue particle (RHA) is required to protect cells from superinfection with VSV.

Next, efforts were made to determine whether an infectious particle is required to mediate interference; that is, a study was done to determine whether a complete dengue particle which has been rendered noninfectious by UV irradiation can protect cells against VSV. A sample of RHA was exposed to a source of UV irradiation (15W. G.E. Germicidal low pressure mercury vapor lamp, emitting light 90% of which is at a wave length of 2537$\mathring{A}$) at a dose rate of 2000 ergs/cu^2/sec. Samples were removed every 15 seconds for two minutes and then every 30 seconds for an additional minute. All were then assayed for infectivity in PS cells and for interference capacity in RK cells. The results are shown in figure 13. There is an exponential loss of infectivity with UV irradiation, with less than 0.1% of the original infectivity persisting after two minutes. Of interest is the observation that the capacity to produce interference, depicted in the figure by the dotted line and expressed as plaque depressing dose 50, falls off at the same rate as infectivity. No initial shoulder is observed in the initial phases of the slope, indicating that the functional loss is the result of one lesion. The face that the two slopes are parallel suggests that a single "hit" in any portion of the viral RNA eliminates the interference capacity as well as the infectivity. Repeated experiments will be required to determine whether loss of capacity to interfere is truly related to loss of infectivity, or whether the former is lost at approximately the same rate but independently of the latter.

This is of particular interest because the cells used to demonstrate dengue interference (L cells or RK cells) fail to support a complete cycle of dengue virus replication, at least as this is measured by the production of progeny capable of killing mice. If interference capacity is related to infectivity, this property may result from an abortive infection in these cells. Work is in progress to determine whether there is any other evidence for such an infection in protected cells, as well as to determine whether the interferon present in the fluids of protected cultures is responsible for the interference.

Figure 12 Interference by Dengue II Virus Components

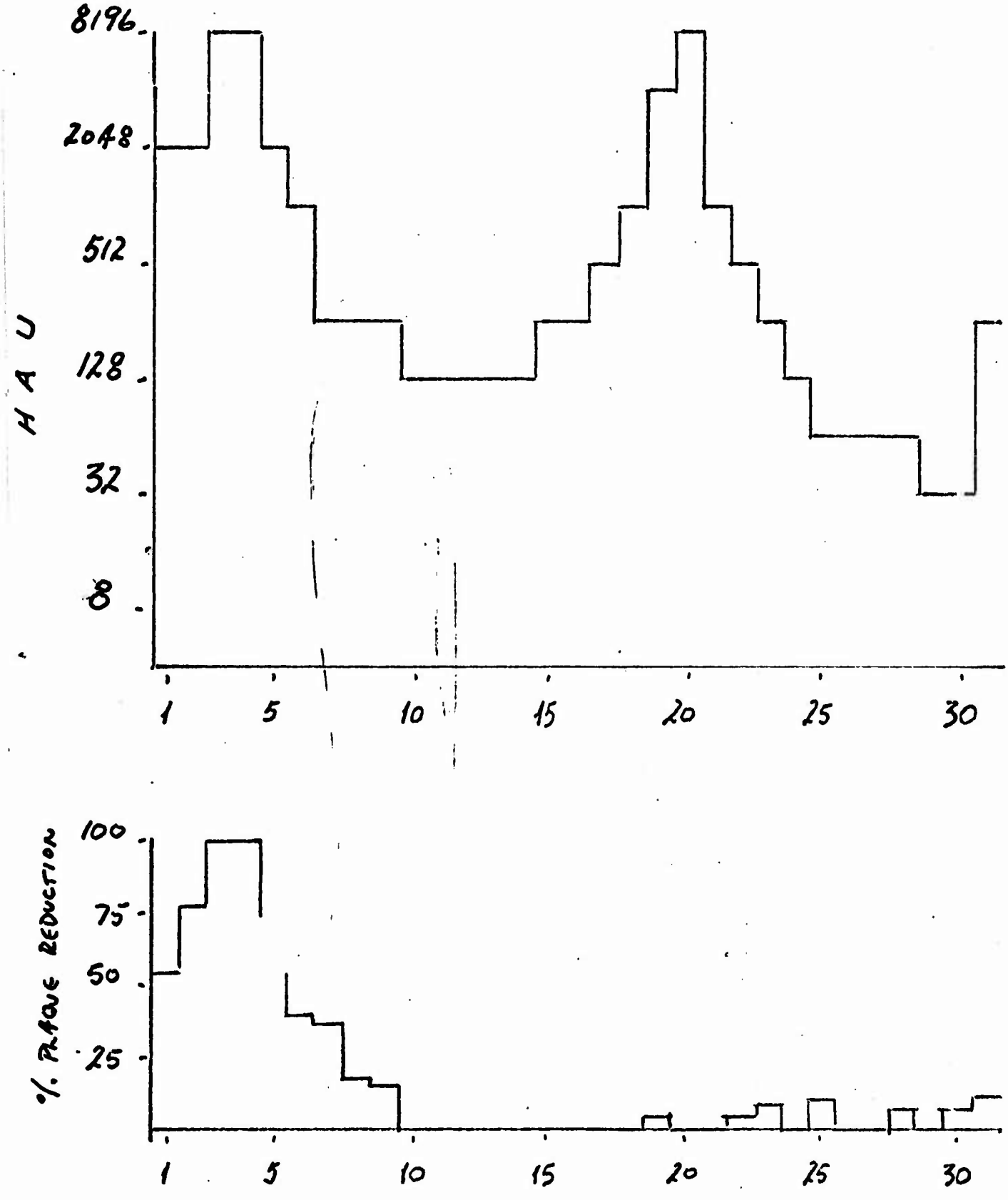
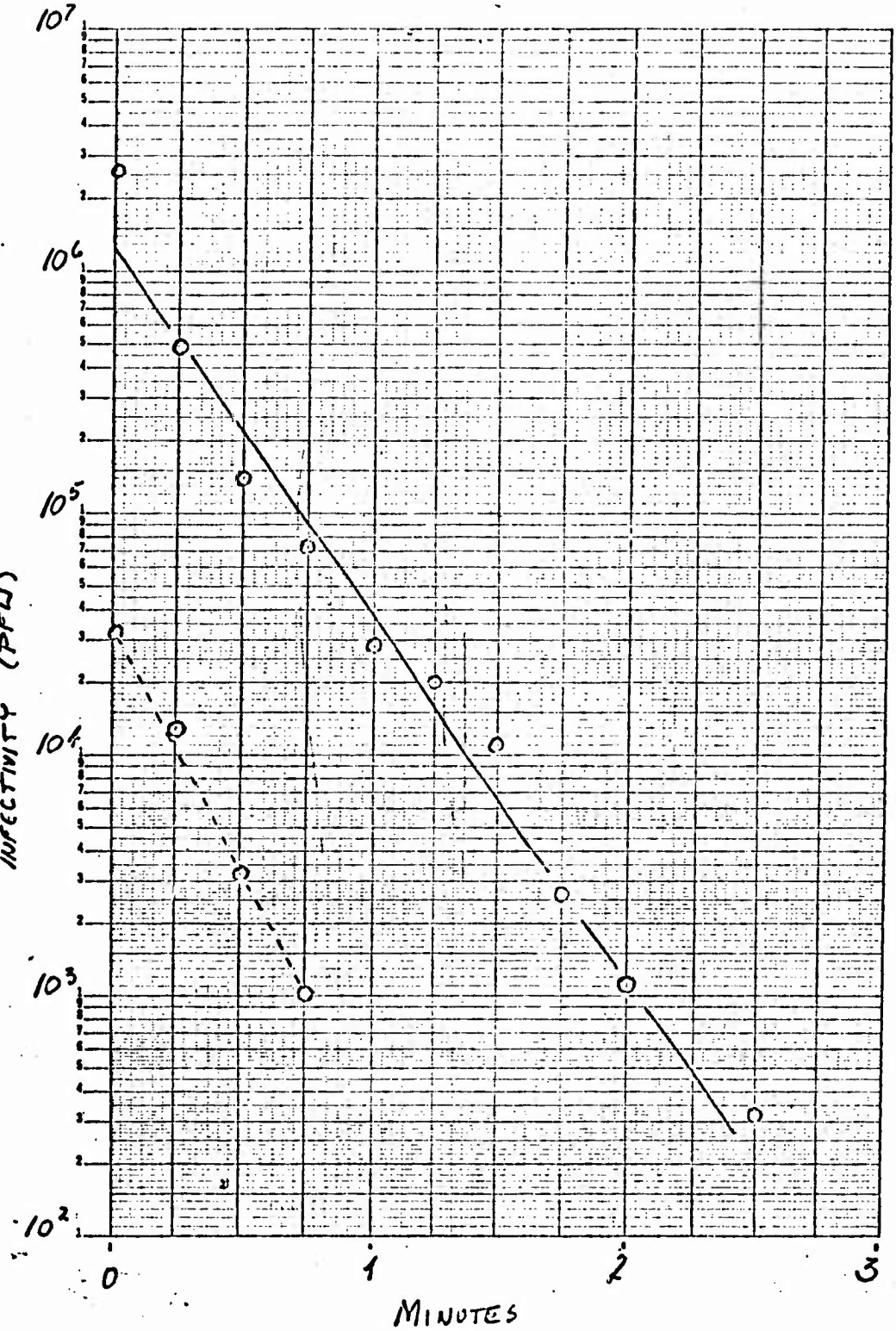


Figure 13 Interference by Ultraviolet Mediated Dengue II Virus



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E. Assay of Dengue Virus Infectivity. A dengue virus plaque assay in continuous green monkey kidney cells (BSC-1) was described in last year's report. This system has been used extensively during the past year and has served as a sensitive and reproducible assay method. Because of recent difficulties encountered in maintaining this cell line however, another cell line is currently under investigation as an assay system. The PS strain of stable porcine kidney cells was kindly suggested by Dr. Sonya Buckly of the Yale Arbovirus Research Unit. These cells are grown as stationary monolayer cultures with Hanks-BME as growth media, dispersed with trypsin and seeded into plaque flasks. When confluent, flask cultures are inoculated with the dengue virus sample and overlayered with agar-containing media (as described for BSC-1 cells in last year's report). Cells are stained with neutral red after seven days and plaques are scored the following day. Thus far, tests with these cells have been limited to Dengue Type II. These plaques are clear, sharply demarcated areas of necrotic cells measuring 1 mm in diameter. Studies are in progress to determine the sensitivity of these cells compared to BSC-1 cells and to observe the efficiency of plaquing in these cells of strains of other dengue virus serotypes.

In the course of performing plaque assays of a high mouse passage Type II Dengue virus, it became apparent that both large and small plaques developed in BSC-1 cells under agar overlay. In an effort to determine the significance of this observation, large and small plaques were selected and purified by three cloning steps. Each was then passed in BSC-1 cultures under fluid media at weekly intervals for ten weeks. In the course of these passages it was observed that the large plaque progeny produced both large and small plaques, while the small plaque variant remained small. Repeated testing, however, demonstrated that there is considerable variation in the plaque size of a given virus stock in BSC-1 cells from one test to another. The reasons for such variability are not clear, but probably include such factors as age, state of nutrition and cell density of the BSC cultures.

At each of the ten passage levels, the small plaque population grew to a maximum yield approximately one day less than that achieved by the large plaque strain. Since this suggested a possible difference in growth rate, each was tested in mice in an effort to detect differences in virulence. No such differences were observed. The relationship of the mouse LD_{50} to plaque titer was the same for each strain.

Mouse immune ascitic fluids were prepared by injection of both purified strains. Cross neutralization tests failed to detect any antigenic differences between the two. When the large and small plaque strains were assayed for plaquing efficiency in another cell line (BHK-21), they could not be distinguished on the basis of size. Since the same

agar overlay was used as for BSC-1 cells, it would seem that the difference in plaque size observed in BSC-1 cells is not due to differences in sensitivity to polysaccharide inhibitors in agar.

The variability of plaque size mentioned above, and the reappearance of small plaques in the large plaque strain, in the absence of any demonstrable differences in antigenicity or virulence, have precluded any further studies with these strains.

More recently, variations in plaque size of another dengue virus (D-V SM17BSC) has been observed in PS cells. Two different plaques can be distinguished with no difficulty. The majority of the population consists of 0.5-1mm clear, sharply defined plaques, while a smaller number of plaques consisting of large clear areas (2-3mm) contained a core of stained cells. Both are neutralized by Type II hyperimmune ascitic fluid with equal efficiency. The distinct differences in morphology of these two plaques are such that this virus seed and assay system would be a promising model for future studies concerned with the significance of variations in Dengue virus plaque morphology.

Studies with dengue plaque reduction neutralization tests in BSC-1 cells were begun last year, and preliminary results were reported in last year's report. More recently, efforts have been directed at the development of a system for a kinetic analysis of neutralization, in the expectation that this method will detect minor antigenic differences between dengue virus strains, as well as serve as a model to investigate the dynamics of dengue virus neutralization and the factors affecting this reaction.

Preliminary studies showed that dengue virus neutralization proceeds at a rapid rate at room temperature in the presence of antibody of high titer. Kinetic studies, therefore are conducted at room temperature to minimize the effects of thermal inactivation. The technique consists in incubating virus and antibody (in the form of hyperimmune mouse ascitic fluid) in equal parts. Samples are taken at intervals and immediately diluted 1:100 cold media to stop the neutralization reaction. Virus and non-immune ascitic fluid serves as the control mixture. At the end of the test, samples are diluted further and inoculated into BSC-1 or PS cell cultures. Standard plaque assays used and residual infectivity in each sample is plotted as percent of control (0 time sample). Neutralization proceeds so rapidly in this test that a true 0 time sample cannot be obtained after virus is added to immune ascitic fluid. Therefore, the virus-normal ascitic fluid mixture must be taken as the control. It has been observed in some tests that 90% or more of the infectivity is neutralized by potent antibody almost instantaneously.

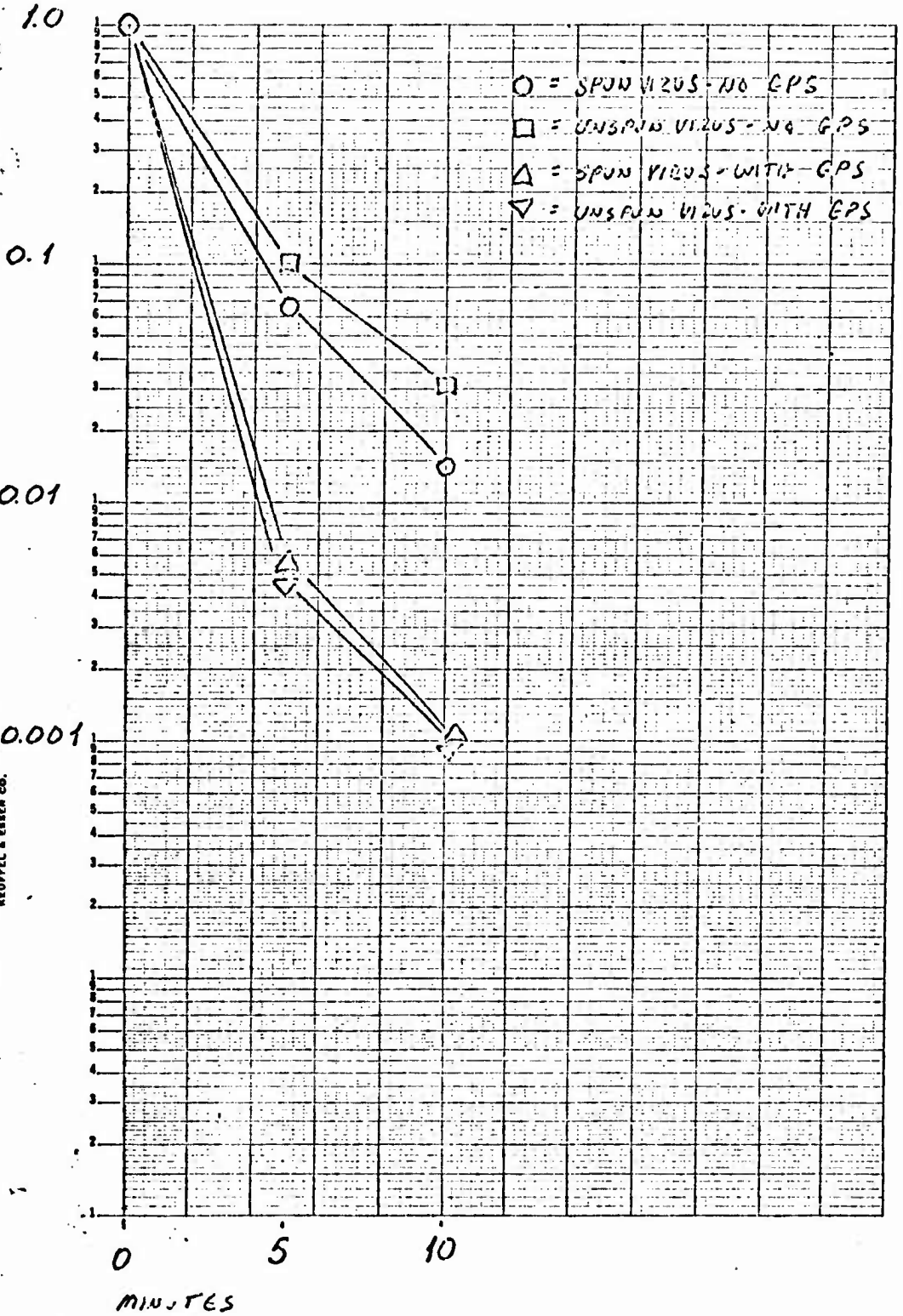
The amount and specificity of antibody present in the test and the temperature of incubation govern the rate of virus neutralization. Other factors may alter the reaction however, and affect the amount of infectivity persisting at the end of the reaction. Two of these have been examined. It has been reported recently that virus aggregation is responsible for the non-neutralization fraction of many viruses persisting after incubation with antibody (Walles & Melnick J. of Virol 1:479, 1967). To determine whether virus aggregation was affecting dengue virus neutralization, a sample of Dengue virus Type II (SM31) was diluted and centrifuged at 12,000 RPM for two hours at 4° and then compared in a neutralization test to an aliquot of the same sample not subjected to centrifugation. It was found that while centrifugation reduced the virus titer by 50%, presumably by removal of aggregated particles, antibody neutralized spun and unspun samples at the same rate. Further, the non-neutralizable fraction was not reduced by prior centrifugation.

The addition of fresh unheated guinea pig serum to a reaction mixture is known to enhance the neutralization of certain viruses. In an effort to increase the rate of dengue virus neutralization and reduce the non-neutralizable fraction, virus-antibody mixtures have been incubated in the presence of 10% guinea pig serum. It was found that the result was a potentiation of antibody activity, with both an increased rate of neutralization and a reduction in the magnitude of the non-neutralized fraction. Figure 14 depicts this affect and illustrates that, in contrast, prior centrifugation of virus has no significant effect on its subsequent neutralization by antibody.

In the experiment depicted in figure 15, the potentiating effect of fresh guinea pig serum was measured when two concentrations of antibody were used in the test. It was observed that the reaction proceeded at a more rapid rate at the higher concentration of antibody regardless of whether guinea pig serum was present or not. The most striking effect of guinea pig serum is the reduction in the residual activity at ten minutes, particularly with the lower concentration of antibody. In the presence of guinea pig serum, ten-fold less antibody accomplished the same reduction in infectivity during this period.

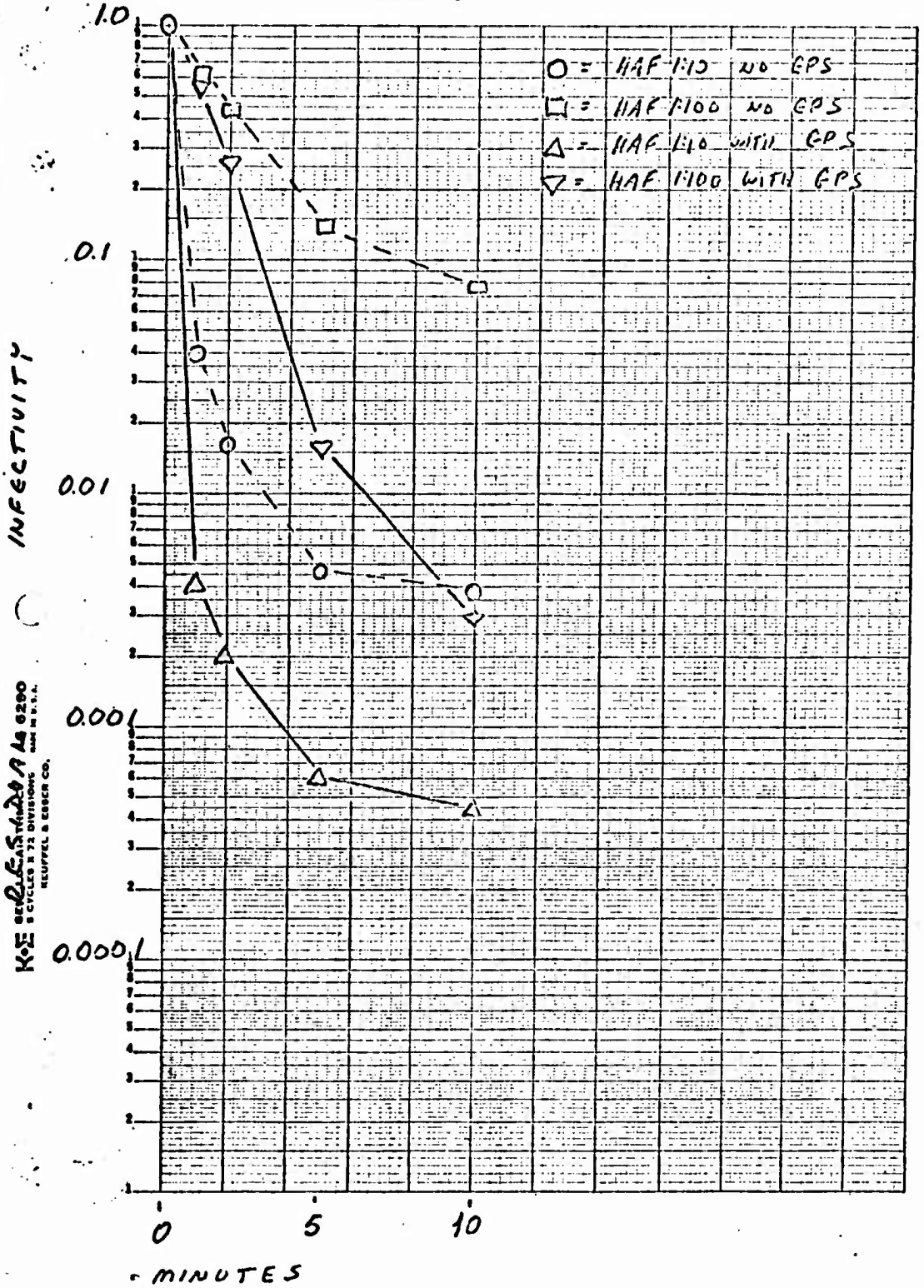
The kinetic analysis of neutralization is currently being tested as a system for the detection of intra-typic strain differences among Dengue viruses and for the detection of possible minor differences in antigenic composition of the various components of a given Dengue virus population. To approach the first goal, dengue strains from different geographic areas of the world, isolated by various investigators over a number of years, have been collected and stocks of each prepared in LLC-MK_2 or BSC-1 cells. It is planned to systematically test certain of these viruses in this system, to compare the kinetics of neutralization by a given antibody preparation in an effort to detect minor antigenic differences. An analysis of the antigenic composition of the defined

Figure 14 Exhaltation of Dengue Virus Neutralization by Normal Guinea Pig Serum



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Figure 15 Quantitative neutralization by Antisera in Presence of Normal Guinea Pig Serum



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members of a dengue virus population (RHA, SHA, "slow" CF antigen) will be attempted by the preparation of antibody directed against each component, and a measurement of the specificity of such antibody when compared with antibody directed against unfractionated virus. Thus far, ascitic fluids have been obtained from mice injected with RHA and SHA. Preliminary studies show that these antibody preparations cannot be differentiated when tested against RHA or unfractionated type II Dengue virus. It is recognized that most of the SHA preparations used for immunization contained some RHA, possibly obscuring any antigenic differences existing between these two particles. Future efforts will be directed toward immunization of animals with purified SHA. As mentioned earlier, studies are underway to obtain concentrates of purified slowly-sedimenting CF antigen. When available, this preparation will be used for animal immunization. An analysis of the specificity of this antibody will be of great interest and will be conducted with this kinetic neutralization system.

F. Electron Microscopy of Dengue Virus-Infected Cells in Culture and of Purified Dengue Virus. Electron microscopic studies have been carried out on Dengue II-infected cells from MK cell culture to define the intracellular location of virus replication and to document the pathological changes occurring in the infected host cells.

Standard methods were utilized for preparation of thin sections of infected cell cultures. These procedures include glutaraldehyde (2% in 0.1 M sodium cacodylate, pH 7.4) fixation, washing in buffer, en bloc staining in aqueous uranyl acetate (1%), dehydration in graded alcohols and embedding in Epon. Cultures of BSC and MK-2 cells were examined at varying intervals following inoculation with Dengue virus.

Dengue particles were first seen in cells on the third to fourth days following inoculation of the culture (figures 16-1; 16-5). Particles were present both outside and inside the cells. The virus, as visualized in thin sections is 50-55 mu in diameter and consists of a 25-30 mu electron-opaque core surrounded by a thin ($20\mathring{A}$) electron dense rim. Some particles exhibit a morphologic pattern which suggests that a $70\mathring{A}$ thick, unit-membrane forms the outer covering of the virus (Figure 16-4).

Intracellular Dengue virus particles are primarily located in dilated cisternae of both rough and smooth endoplasmic reticulum. An occasional particle is seen lying in the cytoplasm outside these cisternae. The endoplasmic reticulum of infected cells contains other structures (Figures 16-2; 16-5) whose relationship to the virus particles is not clear. These are membrain-bounded vesicular or cigar-shaped structures which vary from 90 to 110 mu in diameter. They are usually devoid of contents but may contain either invaginated portions of their wall

(Figure 16-5) or delicate strands of fibrillar material (Figure 16-4) or both. They have not been found to contain morphologically intact virus. These intracisternal membranous structures may occupy major portions of the cytoplasm of infected cells. Along with focal regions of cytoplasmic degeneration (Figure 16-3) the occurrence of the intracisternal membranous bodies constitute the main pathological changes in Dengue-infected cells prior to alterations which signal cell death and generalized degeneration.

Electron microscopy has also been utilized for identification of partially purified virus and related particles which have been obtained by density-gradient centrifugation. These studies were carried out to correlate the structure of subviral particles with their biological activities and to gain insight into the organization of intact, infectious Dengue virus particles.

The usual methods for negative staining give poor results when applied to preparations of Dengue virus. Thus, phosphotungstate gives less resolution of the virus surface than does uranyl acetate which was used in this study as a1% aqueous solution. Uranyl formate (2% aqueous) also provides adequate contrast for visualization of Dengue virus. Prefixation with glutaraldehyde (1% in 0.1 M cacodylate, pH 7.4 followed by water washing) also gives more satisfactory results than negative staining of either the unfixed particles or osmium-fixed Dengue viruses.

Centrifugation of supernatant (after cell and debris removing preliminary centrifugation) material derived from infected newborn mouse brains on 5-25% sucrose gradients yields two peaks of hemagglutinating activity. The faster sedimenting peak corresponds to 20% sucrose, approximately, and has been called rapid (sedimenting) hemagglutinating material (RHA). This peak contains the bulk of infectivity found in the various fractions of the gradient. Particles present in the RHA fractions are shown in Figures 16-6; 16-7. Spherical particles measuring 45 to 50 mu are the dominent constituent in the fraction. The surfaces of the particles display substructure which depends on the staining conditions. Particles surrounded by large amounts of the negative staind (Figure 16-1) exhibit fairly distinct surface protrusions from which the stain is excluded. These are approximately 70_A across. A definite geometric arrangement has not been eludicated for the protrusions which resemble the capsomeric or morphologic units present on most animal and plant viruses. Many of the particles have "tails" or fragments of material adherent to or protruding from their spherical exteriors (Figure 6). The diameter of the majority of particles correlates with the cross section dimensions of viruses seen in previously described Dengue-infected cell cultures. It is assumed, therefore, that these spherical 45 to 50 mu particles represent the intact virus.

The slowly sedimenting peak of hemagglutinating activity (SHA) corresponds, approximately, to 10% sucrose and is virtually devoid of infectivity after three successive purifications by centrifugation and dialyses. The SHA material, when purified, is comprised almost entirely of "doughnut" forms which are 135 to 145$\mathring{A}$ in diameter (Figure 16-8). These particles or doughnuts have a central region in which negative stain accumulates. This central zone may also contain bits of material which exclude the stain and which appear to be partial or complete septations of the particles.

If the RHA is allowed to stand for several days in the refrigerator (approximately 4°C) the infectivity of the preparation significantly decreases. The morphology of particles of the RHA also changes over this period of time. Figures 9 and 10 show particles from RHA fractions which were allowed to stand for five days following their separation in a sucrose gradient. The outer portion of the viruses appear to be falling apart and seems to be the origin for smaller particles which surround the fragmented virus. The majority of these smaller particles or subunits are 70$\mathring{A}$ in diameter and have a central region in which negative stain accumulates. Some instances are noted in which the previously described 135 to 145$\mathring{A}$ doughnut forms are adherent to partially fragmented viruses (Figure 16-9). In addition, an occasional micrograph shows several 70$\mathring{A}$ particles clustered about and apparently adherent to a central, 130 to 140$\mathring{A}$ structure (Figure 16-10). These 135 to 145$\mathring{A}$ particles (hereafter referred to as 140$\mathring{A}$ particle or "doughnuts") are similar to the doughnuts of the same dimension that constitute the SHA except that the centers of the former accumulate less negative stain than the doughnuts. It is not clear whether both the 140$\mathring{A}$ particles of the SHA and the smaller 70$\mathring{A}$ particles derived from fragmented viruses form the outer layers of the intact particle. The possibility exists, and finds support in some micrographs, that the 140$\mathring{A}$ "doughnuts" are aggregate forms of the 70$\mathring{A}$ particles arranged in tetrahedral or cubic forms.

Recent studies utilizing gradients of 2-10% sucrose have indicated non-sedimenting particles which have complement fixing properties and another fraction, corresponding to 4½% sucrose, which promotes hemagglutination but does not fix complement. The electron microscopic appearance after negative staining of complement fixing component is shown in Figure 11. There appears to be a small particle which measures 70$\mathring{A}$ across, has a central, stain accumulating focus, and is similar in morphology to the 70$\mathring{A}$ seen on fragmenting particles. The 4½% sucrose fraction has two components. One of these is the 140$\mathring{A}$ "doughnuts" and the other consists of threadlike material, 35-40$\mathring{A}$ in diameter, which is positively stained with uranyl acetate (Figure 16-12). The size and configuration of the filamentous material is suggestive of nucleoprotein.

A limited number of fractions from cesium chloride gradients have been successfully visualized by electron microscopy. This paucity of study is due to technical difficulties which arise when material from cesium chloride solutions is negatively stained. RHA, when placed on a cesium chloride gradient, yields two peaks that can be discerned by the optical system of the ultracentrifuge. The denser component (d=1.24) has been shown to consist of spherical virus particles (Figure 13) whose morphology is identical to that of particles from the original RHA fractions. The second component (d=1.18) appears to be partially fragmented virus particles (Figure 16-14). Centrifugation of the SHA in cesium chloride results in one peak with density = 1.25, corresponding closely to that of the intact, spherical virus particles derived from the RHA. This peak from SHA material is composed of 140$\mathring{A}$ "doughnuts" (Figure 15). The high density of this SHA-derived material is confusing. The possibility exists that RNA contaminates the doughnut-shaped particles (as shown in 2-10% sucrose gradients and Figure 16-7) and accounts for the phenomenon.

At the present time, it appears that the 70$\mathring{A}$ particle is the subunit which comprises the capsid of Dengue virus. However, this is only speculation until there is definition of arrangement of 70$\mathring{A}$ particles in some geometric pattern. The relationship of the 130-140$\mathring{A}$ "doughnuts" to the structure of the intact virus is not clear. One possible relationship, that of a stable aggregate of 70$\mathring{A}$ particles, is based on tenuous evidence. RNA content determinations of this fraction, both before and after RNA as treatment, could clarify the situation. This might also shed light as to whether the doughnut represents a defective, RNA-containing particle.

Figure 16

Fig 16-1: Dengue virus particles are present in a specimen of BSC-1 cells 23 days after infection. The individual particles are 50 mu in diameter, are spherical and contain an electron-dense core 25-30 mu in diameter. The accumulation of virus is present in a dilated cistern of the endoplasmic reticulum. X40,000

Fig 16-2: Both intact viruses and membranous vesicular and cigar-shaped structures are present in the endoplasmic reticulum of this BSC-1 cell three days following inoculation with dengue-2. X40,000

Fig 16-3: These portions of two dengue-infected BSC-1 cells displays the main pathologic alterations present in these cultures. The cytoplasm in the cell of the micrograph on the left contains a demarcated focus in which nearly all normal architectural detail has been lost and most elements display markedly dark staining. In an adjacent portion of the same cell and in the neighboring cell, membranous bag-like structures fill many cisternae of the endoplasmic reticulum. X20,000

Fig 16-4: In a six-day post-infection culture of BSC-1 cells, many viruses and intracisternal membranous structures are present. Some of the virus particles (encircled area) exhibit a trilaminar 75 $\mathring{A}$ thick coating which is similar in profile and dimension to those of adjacent membranes of the endoplasmic reticulum. Note the fine filaments in the membranous structure shown by the arrow. X55,000

Fig 16-5: An occasional virus (circle) and many membrane-bounded structures fill the endoplasmic reticulum of this dengue-infected cell. Invaginations of the wall and electron-dense filaments are present in the interiors of some of the membrane-bounded intracisternal sacs. X55,000

Fig 16-6: Spherical virus particles populate this rapidly sedimenting hemagglutination fraction from a 5-25% sucrose gradient. The particles vary in diameter (43-50 mu) depending on the amount of negative stain in which they are embedded. Surface protrusions (about 70-80 $\mathring{A}$ in diameter) are present on the surfaces of the particles but a pattern of symmetry is not apparent. X70,000

Fig 16-7: Higher magnification of a preparation similar to that in Figure 6 offers little enhancement of surface detail. X140,000

Fig 16-8: These particles were originally obtained in the slowly sedimenting HA fraction of a 5-25% sucrose gradient. Following two additional centrifugation and dialysis steps, these "doughnuts" are the only particles present in the specimen. The "doughnuts" are 140$^{\circ}_{A}$ in diameter and sometimes are seen to have septae extending into their hollow centers (circle). X140,000. Figure 8b shows the "doughnut" at higher magnification. X280,000

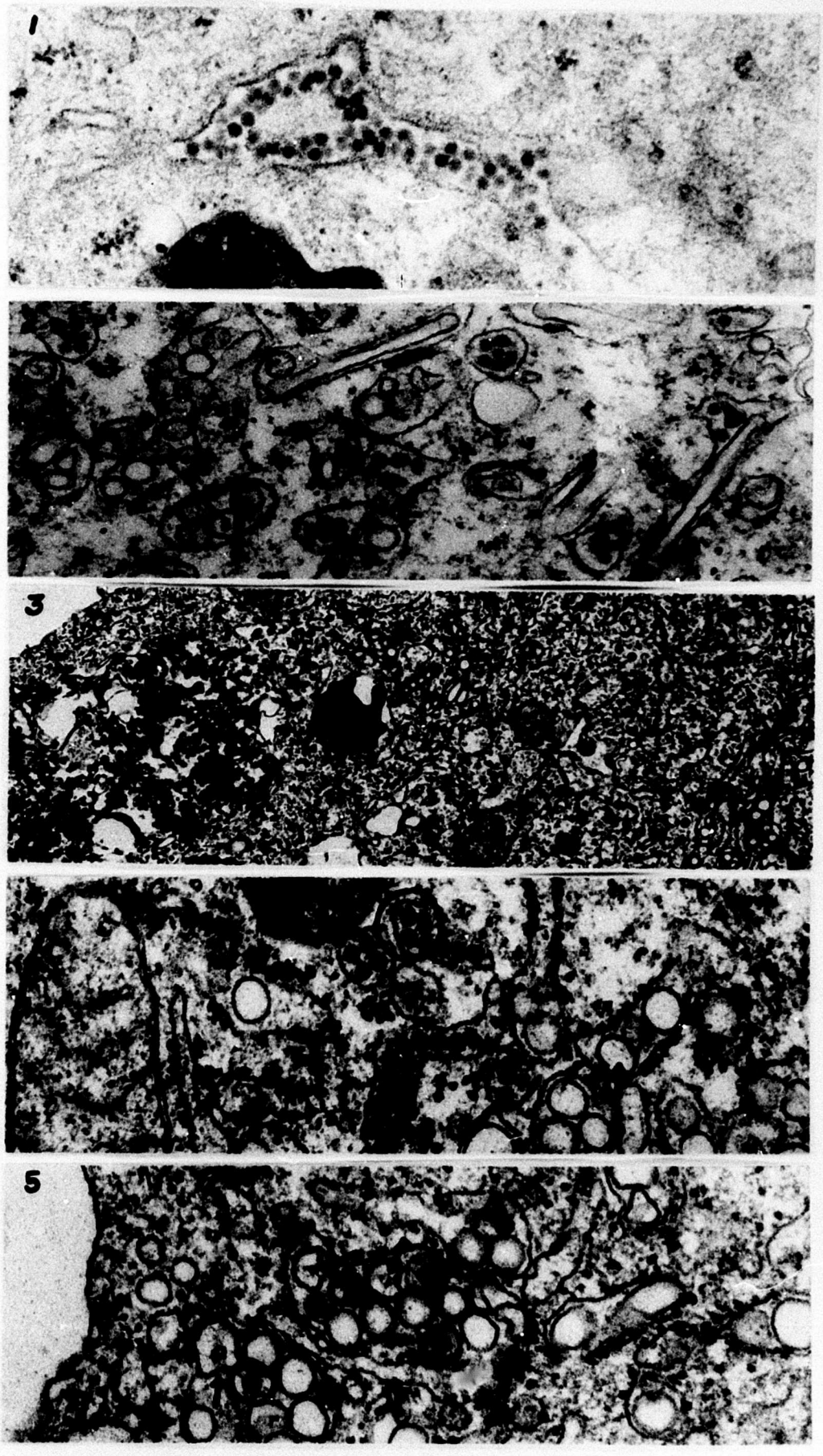
Fig 16-9: These virus particles were obtained from the RSA fraction from a 5-25% sucrose gradient and were stored at 4°C for five days prior to examination. The majority of viruses seem by negative staining to be fragmented. One partially disrupted particle has on its outer surface a 140$^{\circ}_{A}$ doughnut (circle) which is identical in size and morphology to another "doughnut" lying unattached in the specimen (arrow). Most of the viruses have 70$^{\circ}_{A}$ subunits projecting from their surfaces. X70,000

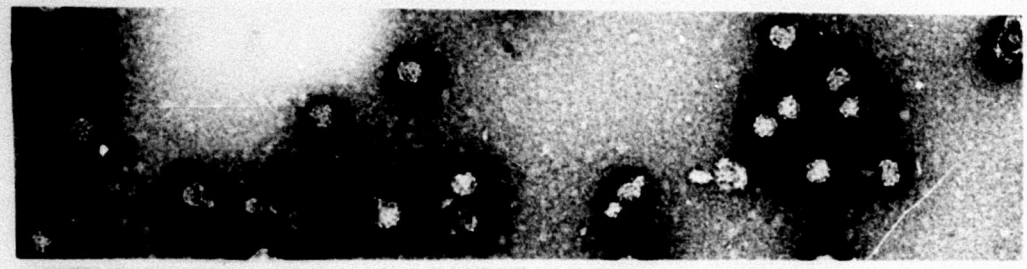
Fig 16-10: A preparation similar to the one depicted in Figure 9 contains many 70$^{\circ}_{A}$ particles or subunits which appear to derive from the surfaces of partially disrupted viruses. The 70$^{\circ}_{A}$ particles are clustered about and seem attached to a central component which is 140$^{\circ}_{A}$ in diameter in the encircled structure. This 140$^{\circ}_{A}$ center resembles the "doughnuts" previously described. X140,000

Fig 16-11: This complement fixing fraction from a 2-10% sucrose gradient is composed of abundant 70$^{\circ}_{A}$ particles (circle) which appear to have hollow or stain accumulating centers. X140,000

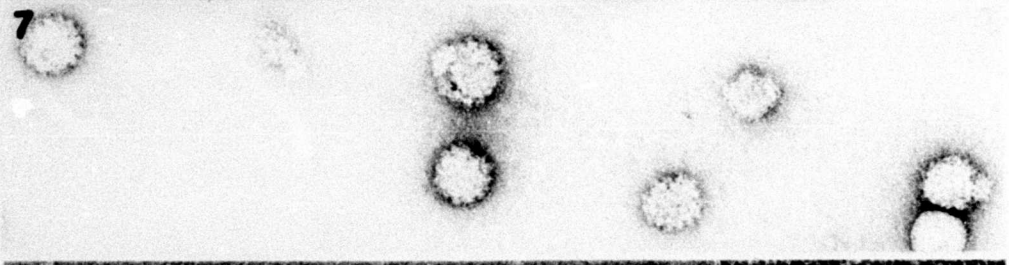
Fig 16-12: Staining of this preparation (rapidly sedimenting peak in 2-10% sucrose) with 1% uranyl acetate for 15 minutes produced both positive and negative staining. Two 140$^{\circ}_{A}$ "doughnuts" have scanty stain around their peripheries and in their centers (circle). Positive staining has occurred on the 35-40$^{\circ}_{A}$ wide filamentous material shown by arrows. The latter may represent RNA derived from previously complete viruses. X140,000

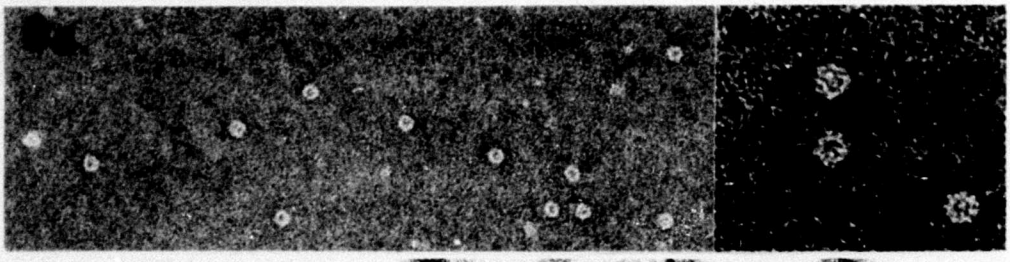
Figs 13, 14, 15: These particles were first purified on 5-25% sucrose gradients (Fig 13 & 14 = RHA; Fig 15 = SHA) and were subsequently separated in cesium chloride density gradients. The intact virus (Fig 13) and the viral fragments (Fig 14) have different densities in the cesium gradient. In a separate cesium gradient, the 140$^{\circ}_{A}$ "doughnuts" (Fig 15) have a density similar to that of intact virus. Note the 70$^{\circ}_{A}$ particle or subunits (arrow) which project from a partially disrupted virus in Fig 14. X140,000


1
3
5

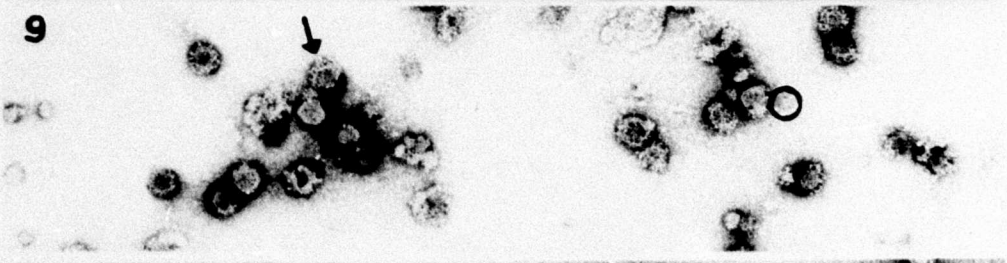


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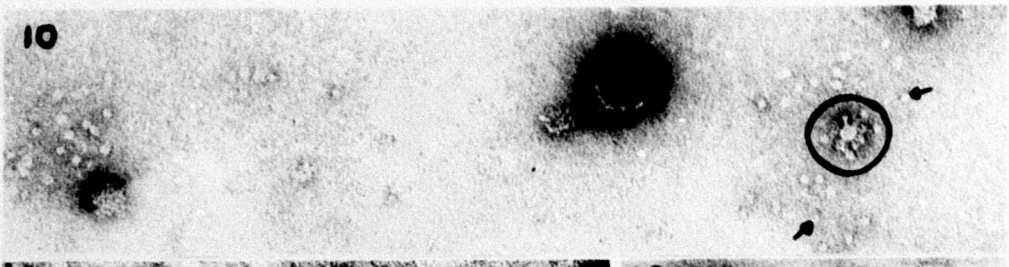




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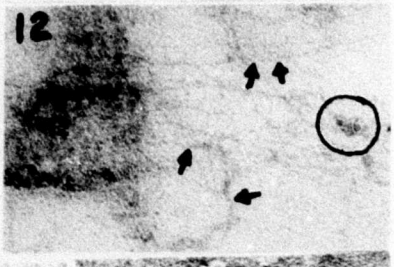
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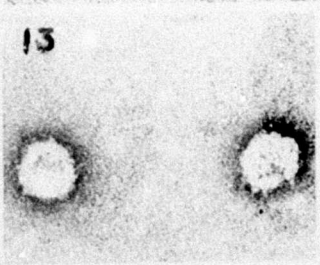
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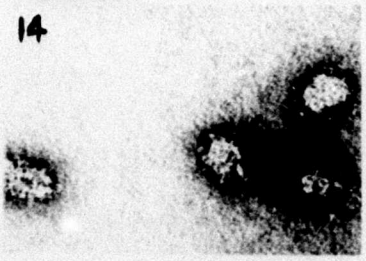
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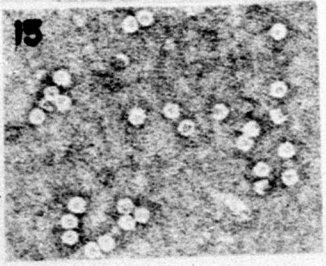
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14



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VI. Studies in Arbovirus Ecology.

The continued success in isolation of EEE and WEE viruses from Culiseta melanura, the relatively high percentages of birds which were shown to have neutralizing antibody against WEE, and the isolation of WEE virus from two birds during 1966 led us to continue studies of the ecology of mosquitoes and birds during 1967. Unlike 1965, the virus most frequently isolated from mosquitoes in 1966 was WEE (in 1965 it was EEE). Birds also showed a much higher percentage of antibody reactors against WEE than against EEE. During the same year it was shown by blood meal identification techniques that C. melanura was almost exclusively an avian feeder. Thus, it appeared possible that the endemic phase of the virus life-cycle is primarily maintained in birds and C. melanura mosquitoes. Mammals and reptiles tested for WEE and EEE antibodies had comparatively small percentages (<1%) of antibody reactors with the exception of the larger, fairly long-lived mammals, such as raccoons and skunks, which because of their longevity have an increased probability of becoming infected; but, even these had lower percentages of positives than those observed in much shorter-lived birds. Further, when birds were considered in regard to their temporal relationship to the swamp, it was found that the numbers that had antibodies were also disparate: the birds spending the greatest amount of time in the swamp while the mosquitoes were active, either because they were permanent residents or summer breeders, had far larger numbers of antibody reactors (20%-53%, respectively) than birds that were transient or only over wintered in the swamp (1.5%-2.5%). This means that infections were incurred primarily during the time mosquitoes were active and abundant.

All of this information began to form a fairly satisfactory picture of the relationship between the birds, the mosquitoes, and the viruses. During 1967 the mosquito and bird ecology studies were continued with some additional approaches, including use of sentinel quail, passerines, turtles, and snakes. The additional information, although confirming some of the previous findings, has opened a series of new questions and has left us with the impression that the ecology of these viruses and their associated vertebrates and invertebrates is much more complicated than what appeared to be the case during earlier phases of the study.

A. Methods of Approach.

1. Study Area. A general description of the Pocomoke River Swamp study area appeared in last year's Annual Report. Briefly, the study area, located about five miles southwest of Pocomoke City, Worchester Co., Maryland is demarcated by a series of east-west trails at one chain (66') intervals numbered from one on the south and to 40 on the north end of the grid. The grid is further divided by a series of north-south lines (at one chain intervals) beginning on the west edge with "J" and continuing through "X" on the east side. The total area enclosed by the grid is a little less than 60 acres. The northern edge of the grid roughly parallels the Pocomoke River, the eastern and

southern edges border the Pocomoke River Swamp which is about 3000 acres in size, with vegetation approximately equivalent to that in the study grid. The western edge borders an upland area which has sandy and clay soils with oaks and loblolly pines; the understory is much more sparse than in the swamp. This upland area extends south and includes Groton's farm (about 1½ miles SW of the study grid), but to the west it adjoins tidal salt marshes along the Pocomoke River which takes a turn south at this point.

In the study grid (and the Pocomoke River Swamp in general) the substrate consists of an interwoven root mat with a matrix of peaty soil and fragments of dead and dying vegetation. During summer there is little open water, but water can be reached nearly anywhere by digging one foot or less into the vegetation root mat.

From the old logging railroad (RR), which is actually south of the grid, north to approximately #10 line, Loblolly Pines are the dominant trees, mixed with Red Maples, increasingly to the north. The canopy in this section of the grid is nearly complete and up to 80' high. In the central part of the grid there are scattered Cedars and Red Maples standing 20'-60' above a thick understory of shrubs, ferns, and saplings interwoven with Green-briar. Tree growth becomes denser toward the north end, and tall Red Maples and Cedars form a nearly closed canopy. Ground cover is more sparse and the soil more silty and mucky than in the middle of the grid, probably because of the periodic inundation by the Pocomoke River. Pools of open water can be seen in this area, especially after the river has been high.

The western edge of the grid, bordering the upland area is also mucky probably because of a small creek running roughly parallel to line "J". In this area there are tall Red Maples, Sour Blackgums, Gums, and Oaks invading from the adjoining upland area.

2. Mosquitoes. Larvae - During late April of 1967 a series of larval traps were placed in various small pools in the 60 acre study grid where C. melanura larvae had been captured during the previous summer. Most of the traps (12) were made of clear plastic and some were made of opaque white plastic. Both types consisted of an inverted funnel, the neck of which was inserted into a larger cylindrical container that held a reservoir of water; a small amount of air in the reservoir kept the trap afloat. These traps were usually checked once a week. Captured larvae were identified, counted, and in some cases an attempt was made to estimate the developmental stage of the larvae of C. melanura.

Five artificial oviposition sites were constructed in various parts of the swamp, consisting of opaque plastic containers buried in the substrate.

Because of the success in obtaining larvae in newly dug holes in the floor of the swamp, in 1966 and early 1967, which led us to suspect

that larvae might be living in capillary water held in the matrix of roots, stems, and dead and dying vegetation, we installed five energence funnels (7 June), with bases 30 inches in diameter, in various places on the study grid (15-16Q, 17O, 17P, 30Q, 40Q). The base of each funnel was inserted into the "dry" floor of the swamp; a shallow, circular ditch, 30 inches in diameter, was dug around the base of the funnel and the edge of the base was packed with soil to prevent escape or ingress of adult mosquitoes. Care was taken to ensure that no open water was present inside the funnels. The funnels were checked twice a week.

On several occasions samples of the substratum in the swamp were brought back to the laboratory and were analyzed for larvae and eggs.

3. Adult Mosquitoes. Adult mosquitoes were captured with CDC miniature light traps (CLT) operated at six sites in the swamp at heights of 5 feet and 15 feet from the ground (RR = just south of the grid, 6K, 17Q, 20Q, 30Q, 40Q). CLTs were also operated in nearby farmyards (Groton's - 1½ miles SW of the study grid, and Bishop's - about 5 miles south of the study grid but still bordering the Pocomoke River Swamp). The traps at the five foot height in the swamp and in the nearby farms were operated once each week and during a second night each week the high and low traps in the swamp only were compared.

Adult mosquitoes were also captured with a vacuum device at various sites in the swamp (2-RR, 6-7K, 17#O-S, 18-22J, 38-40Q), and at one site in the upland area west of the swamp. The vacuum sweeps were made over a prescribed course of 264 feet. The sweeps were taken in a standardized manner at a height of six to twelve inches from the ground; irregularities in the terrain such as hollows at bases of trees, were consistently swept. The order in which the various sites were sampled was maintained constant so that weekly comparisons could be made without regard to time of day. This, of course, makes comparisons between sites more difficult since the time of day during which sweeps were made might have influenced the numbers obtained. Nevertheless, all of the sites were sampled during the morning, usually within a two hour period.

On 9 May, 10 wooden resting boxes were placed near the south edge of the study grid; these had various size openings: two each with ¼", 1", 2", 4", and 7" holes. Mosquitoes were collected from these at weekly intervals. Mosquitoes were also aspirated from Groton's farm buildings near the study area at about 2000 hours on 26 July 1967.

At various times mosquitoes and larvae were also collected on Assateague Island, where intensive studies had previously been conducted (see Annual Reports, 1961, 1962, 1963). Captured mosquitoes were brought to the field laboratory, killed in a freezer at -20° to -65°C, and were usually sorted during the same day. Sorting was done on paper which covered a sheet of wet ice in an enamel pan, with the aid of a binocular dissecting microscope. Mosquitoes were segregated into pools according to species, sex, and presence of eggs in the abdomen. Mosquitoes containing a blood meal were cut in half; the blood-filled

abdomens were placed in gelatin capsules, sealed in Wasserman tubes for later shipment to the School of Public Health at the University of California, Berkeley for blood meal identification at the laboratories of Dr. D. H. Tempelis (Tempelis and Lofy, 1963). The head and thorax were saved for virus isolation attempts. The pools of mosquitoes, up to 100 mosquitoes per pool but usually no more than 25, were refrozen at -65°C and stored until they were shipped in sealed plastic bags on dry ice to WRAIR for virus isolation work.

At WRAIR virus isolation attempts were made in a cubicle set aside for this purpose. Also, in the adjoining cubicle no work was done with known North American arboviruses. During the days the mosquito pools were processed; the involved personnel did not handle known North American arboviruses even in the other laboratory rooms.

Pools containing from one to five mosquitoes were transferred from tubes, which were kept on dry ice, into sterile 1.0cc syringes in which they were triturated and mixed with diluent (BME; 20% FBS-heat inactivated; antibiotics - 100 units of penicillin and 100 micrograms of streptomycin/ml; fungizone - 5 micrograms/ml). In an attempt to prevent "overdiluting" the small mosquito pools the following amounts of diluent were added to the pools: one mosquito, 0.10cc; two mosquitoes, 0.15cc; three, 0.20cc; four, 0.25cc; five, 0.30cc.

The syringe full of medium and ground mosquito material was emptied into Kahn tubes in which the material was stored in an ice bath until it could be inoculated onto a media free monolayer of hamster kidney cell culture. The inoculated cell cultures were incubated without medium at 37°C for one hour or longer after which the monolayers were rinsed twice with media (BME; 10% FBS-heat inactivated; antibiotics - 500 units penicillin and 500 micrograms streptomycin/ml; fungizone - 5 micrograms/ml) to remove the mosquito debris; 1.0cc of the latter media was then added to each cell culture tube. Cells were examined under a microscope each day for five days for cytopathogenic effect (CPE).

The syringe procedure was discontinued 25 July 1967. Thereafter, all pools were processed by tissue grinders. The larger pools were triturated in TenBroeck tissue grinders throughout 1967. Grinders were kept chilled in an ice bath. The amount of diluent in which the pools were triturated varied according to the sizes of the pools: 1-15 mosquitoes, 0.5cc; 16-25, 0.8cc; 26-50, 1.0cc; 51-100, 1.5cc. During trituration each grinder was enclosed in a plastic bag to aid in prevention of cross contamination of pools. The triturated mosquitoes were transferred into one dram sterile vials and centrifuged at 3000 rpm for 15 minutes at about 4°C. After centrifugation 0.1cc of the supernatant was added to each of three tubes containing monolayers of hamster kidney cell cultures (HKCC) from which the medium had been previously removed. The remainder of the supernatant was stored at -65°C for further reisolation attempts. Inoculations were incubated at 37°C for one hour or longer after which the monolayers were rinsed and the medium was replenished; rinsing was discontinued 18 July 1967. The

cells were examined each day for CPE for five days. In cases of bacterial fungal contamination or toxicity the isolation attempts were repeated using the remaining material.

When the inoculated pools were observed to have CPE with 25-50 per cent of the monolayer destroyed, the remaining cells were knocked off the walls of the culture tubes by vortexing and 0.5cc of FBS (heat inactivated) was added to each culture tube as a protein support. The tubes were stored at -65°C until the sample could be reinoculated in HKCC.

Inoculations producing CPE for the second time were tested for WEE and EEE viruses by the neutralization test.

4. Wild Birds. The survey of wild resident and migrant birds was continued; this report covers January 1967 through December 1967. Some birds were captured with live traps but mist nets were primarily used. In addition to the 29-30 nets operated in, and in the vicinity of, the Pocomoke River Swamp ten nets were placed on Assateague Island where very few birds with antibody against EEE or WEE had been captured during 1960-1964. In and in the vicinity of the Pocomoke River Swamp, nets were usually operated 3 days a week and on Assateague usually once a week. Nets were periodically moved when it appeared that the capture rate was decreasing, presumably because the birds learned the locations of the nets.

The bird populations were also followed by direct field observation and attempts were made to determine species composition territoriality, habitat associations, reproduction, etc.

Blood samples were obtained from captured birds and in some cases the same bird was bled on more than one occasion. Depending on the size of the bird 0.2 to 0.5 of blood (heparinized) was taken with a 27 gauge needle and a 1 ml syringe by jugular venipuncture. After they were bled, the birds were banded and released. The blood samples were transferred into Wasserman tubes and were stored on ice until they were taken to the field laboratory to be centrifuged at about 1500 rpm for 15 minutes. The plasma was drawn off after centrifugation and was stored in separate 2 dram vials, along with the cells remaining in the Wasserman tubes, at -65°C until they were shipped to WRAIR and could be tested.

In the laboratory at WRAIR the blood cell fractions were broken up and diluted 1/5 with BME (with 20% FBS - heat inactivated, antibiotics, and fungizone in the same proportions as used for mosquito pools). From each sample 0.1 cc of the suspended cells were inoculated into each of two tubes containing monolayers of primary hamster kidney cells. The tube media had been previously removed, and the inoculums were incubated (absorbed) at 37°C for two hours or longer on the media free monolayers. This was followed by a rinse and the addition of 1.0cc of standard medium (BME, 10% FBS, etc.). The inoculated cell cultures were maintained at 37°C and were read each day for 5 days. If CPE occurred, standard procedure as described for virus isolation attempts from mosquitoes was followed.

The plasma was tested for the presence of HI and neutralization antibodies. Blood samples with a volume of 0.1cc or less were diluted 1:5 in FBS (heat inactivated).

For HI tests, 0.05cc samples of the plasma were extracted with acetone (total volume 13cc); the chilled acetone was added to the sample while the conical was vortexed. The conical containing the plasma sample and acetone was centrifuged at 1500 rpm for 5 minutes, the acetone was poured off and the conical was inverted over a paper towel to drain for 2 to 3 minutes. The pellet was loosened and dispersed on the sides of the conical by vortexing and the material was dried by vacuum. The samples were incubated at 37°C for approximately 8 hours and stored in dessicators until they could be tested. The samples were rehydrated with 0.5cc of borate saline (pH 9.0), 0.05cc of packed goose cells (washed in saline) were added, and the mixture was placed in ice water for 20-25 minutes. The samples were centrifuged at 1600 rpm for 5 minutes and the supernatant was poured into separate Kahn tubes.

Microtiter plates were used with 0.025 of borate saline added to each cup, 0.05 for controls. Samples that were previously diluted were tested from 1:50 through 1:1,600 (two-fold dilutions) and the previously undiluted samples were tested 1:20 through 1:640.

Antigens used were: 1) WEE M3249/65, originally isolated from a mosquito pool from the Pocomoke River Swamp, and cultured in primary chick embryo fibroblast cell culture. 2) EEE M2448/64, used from February through 31 August 1967, also an isolate from mosquitoes from the Pocomoke River Swamp, propagated in mouse brain. 3) EEE C_3SM3A, Cambridge strain, used through 21 December 1967, originally isolated from a horse brain and propagated in mouse brain. 4) EEE M2449 was used after 21 February 1968, also an isolate from mosquitoes from the Pocomoke River Swamp, propagated in primary chick embryo cell culture.

Antigens were previously titrated and for the test adjusted to 4-5 units, 0.025 of which was added to each cup, except the serum controls. This was followed by an hour incubation. After which 0.05cc of 0.25% goose cell suspension was added to each cup. The plates were sealed and vortexed, and were read one hour later.

For neutralization tests all the samples of plasma were diluted to a final dilution of 1:10. The diluting medium was 199 with appropriate amounts of FBS (heat inactivated) to equilibrate the percentage of FBS in the small samples that were previously diluted 1:5 and those that had sufficient material so initial dilution was not necessary.

Samples of the diluted plasma were dispensed into tubes and stored at sub-zero temperatures until they could be tested. Prior to testing the samples were heat inactivated at 60°C for 30 minutes and then placed in an ice bath. Between 1.5 to 2.5 logs of antigen were added to each plasma sample (the volume of virus suspension was 0.2cc, previously

titrated at 2.0 logs; in the test the titrations of such a volume would vary between 1.5-2.5 logs). The plasma-virus mixtures were incubated at 37°C for 30 minutes and were inoculated into tubes containing hamster kidney cell culture. The cells were examined daily for 4 days for signs of CPE. If no CPE was observed the sample was considered to have neutralizing antibody against the virus with which it was tested, either EEE or WEE or both.

The strain of WEE virus used was isolated from a pool of 100 C. melanura mosquitoes collected 13 July 1965 in Pocomoke River Swamp and identified by cross neutralization tests with the Massachusetts strain. This WEE virus strain had undergone two passages in primary hamster kidney cell culture (HKCC) and one passage in continuous HKCC (BhK-21).

The strain of EEE virus used was isolated from a pool of 22 C. melanura mosquitoes collected 1 September 1964 in the Pocomoke River Swamp and identified by cross neutralization tests with the Cambridge strain. The EEE virus strain had two suckling mouse brain passages and one passage in BhK-21 cells.

The seed stocks were stored in basal medium eagles (BME Earles) with 20% heat inactivated fetal bovine serum (FbS), antibiotics (200 units of penicillin and 200 micrograms of streptomycin/ml) and fungizone (5 micrograms/ml).

5. Evaluation of Quail for use as Sentinel Birds. Since quail were among the top three species of wild birds showing WEE neutralizing antibody during 1966, this species was chosen for sentinel studies at the Pocomoke River Swamp in 1967. In order to properly evaluate the course of viremia and antibody production in this species in the wild the following pilot laboratory experiment was conducted.

Twenty-five commercially procured adult birds were tested to determine presence of WEE neutralizing antibody. The birds were bled with heparinized needle by jugular venipuncture; the plasma was tested undiluted and in four cases diluted 1/2 by the Metabolic Inhibition Test (3.0 logs of HeLa cell LC_{50}). All were negative.

Ten birds were inoculated subcutaneously over the pectoral muscle with 0.02cc of WEE virus (Pocomoke River Swamp strain), previously and in the test determined by titration to contain 30 $TCID_{50}$ (in HKTC). Half of the birds were bled every 12-16 hours for 3 days after inoculation. Cloacal swabs were also taken to determine whether virus was shed in the feces.

The remaining 15 birds were bled on the same schedule as the experimental birds.

The plasma samples collected at the various times were tested by HI tests. Virus isolation attempts from the blood and cloacal swabs

of the experimental birds were also made.

6. Sentinels. Sentinel flocks of quail were kept in various parts of the Pocomoke River Swamp and adjoining areas. Four flocks of ten birds each were taken to the swamp on 6 July 1967 and were maintained there through the period covered by this report (i.e., Demcember 1967). Each flock contained equal numbers of males and females, which were all kept together in a wire cage (2'x3'x1½') on wooden stilts (4' high). One end of the cage was enclosed in a wooden cover to protect the birds from bad weather conditions. Half of each flock was bled each week, thus individuals in the flock were bled during alternate weeks. Birds that died were replaced from a group of extra bobwhites maintained in the laboratory. Some birds were replaced when HI tests indicated that they had antibody against either WEE or EEE.

Flocks were kept at middle of the north end of the study grid in the Pocomoke River Swamp, 40Q, in the center of the grid, 20Q, and near the south end "RR". The fourth flock was kept in the "pasture" of Groton's farm, about a mile and a half south-west of the study grid. Near all of these locations mosquito traps (CLT) were also operated.

Beginning in October 1967 other sentinel birds, including red-wing blackbirds, grackles, catbirds, cardinals, white-throated sparrows, towhees, juncoes, wild caught qual, etc., were kept in large flight cages near the middle of the west edge of the study grid. These birds were all wild-caught and were bled at two week intervals.

Blood samples from the sentinel bobwhites and sentinel wild birds were treated in an identical manner with samples obtained during the wild bird survey.

In addition, several snakes and a group of box turtles and a few other turtles were kept in outdoor pens and were bled at various intervals.

B. Ecological Observations.

1. Mosquito Larvae. As was the case during last year, few C. melanura larvae were found in the large pools of water near the north end of the study grid, although Aedes canadensis larvae were abundant in such pools during the spring (3rd and 4th instar on 5 April). Additional typical natural sites were discovered that contained C. melanura larvae; these included root holes, stump holes, and holes ripped into the vegetative mat where trees were blown over. C. melanura larvae were found in April in three (15Q, 16Q, 17Q) of the 30 holes dug in January and February 1967, along the NS axis of the grid along the "Q" line. Four of the holes had larvae in them when they were sampled in February (6Q, 15Q, 17Q, 20Q) (see 1966 Annual Report). Altogether 74 C. melanura larvae were collected during a survey on 5 April in the

in the Pocomoke River Swamp. These represented nearly all stages of development, but most of them were 4th instar. The water temperature at this time was 8°C. No adult mosquitoes were found at this time.

The larval traps made of clear plastic began to yield results within 24 hours after they were placed in the swamp on 25 April. The traps made of opaque plastic were not successful and their use was discontinued. It appeared that C. melanura larvae are phototropic.

A summary of the results of the larval traps is given in Table 28. The great majority of the larvae caught in late April until 19 May were fourth instar which indicates that they were ready to pupate. Indeed, this was the time that the first C. melanura were caught in light traps. Probably C. melanura overwinters in the Pocomoke River Swamp as late instar larva.

The larval catch decreased after 19 May, perhaps because of many of them emerged as adults. This was reflected in the numbers caught by light traps. After 22 June through 13 July the numbers of trapped larvae increased slightly and most of the ones caught were in the first instar. These larvae apparently were the result of reproduction among the adults which emerged early in the season. Gravid C. melanura were observed as early as 6 June (Table 29) and were caught in high proportions in the middle of July in light traps. In vacuum sweeps, they were most numerous during early to middle June. Another increase in the numbers of larvae caught occurred late July; these larvae also included a large proportion of first instars (about 50%, 20 July; "most", 27 July) and probably represent reproduction occurring near this time (high percentage of gravid females was caught in the light traps and in the vacuum sweeps). The catch during early August probably represents an aging larval population, with mostly second instars observed. The largest numbers of larvae were caught on 23 August just prior to the time the largest numbers of adults were caught in CLTs (27 August) (Table 28).

During September about half of the larvae caught were 4th instar, the rest being either second or third instar, and very few first instars. This apparently represents the overwintering larval population, since not many adult C. melanura were captured in CLTs after 3 September. It appears, however, that adults continue to breed until cold weather sets in since a high percentage of gravid females were caught in late September and in vacuum sweeps as late as 15 October, which was the last catch of C. melanura for the season.

C. melanura could not be induced to breed in the artificial breeding sites constructed of white, opaque plastic. An egg raft of C. melanura was found in a natural breeding site on 29 June, but no eggs were recovered from soil samples taken from the swamp on 21 June; the samples did yield some larvae of Aedes canadensis.

Table 28. Larval trap results.

	Number of traps Operating	Number of trap days	Number of *C. melanura* Larvae	Number of larvae per trap day	
26 April	4	1	11	2.75	)
10 May	4	14	52	0.93	) mostly 4th instar
19 May	12	9	121	1.12	)
24 May	10	5	22	0.44	
31 May	12	7	33	0.39	
8 June	12	8	17	0.18	
14 June	12	6	11	0.15	
22 June	10	8	22	0.28	)
29 June	10	7	14	0.20	) mostly 1st instar
6 July	9	7	19	0.30	)
13 July	10	7	27	0.39	)
20 July	10	7	57	0.81	- about ½ 1st instar
27 July	10	7	84	1.20	- mostly 1st instar
4 Aug	9	8	121	1.68	- mostly 2nd instar
10 Aug	9	6	97	1.80	
17 Aug	10	7	110	1.57	
23 Aug	10	6	242	4.03	
1 Sept	9	7	76	1.21	1:1)
7 Sept	6	7	81	1.93	3:4)
14 Sept	9	7	34	0.54	1:1) 4th instar: 2-3 instar
21 Sept	9	7	41	0.55	2-3:1)
28 Sept	10	7	81	1.15	1:1)
			1373		

Table 29. Percentage of female C. melanura captured in the swamp that were gravid or engorged with blood.

	Light Traps		Vacuum Sweeps	
	% Gravid	% Engorged	% Gravid	% Engorged
7 May	0	0	0	0
14 May	0	0	0	0
21 May	0	0	0	0
28 May	0	0	0	0
4 Jun	4.4	0.5	25	10.0
11 Jun	3.2	0.2	18	0.4
18 Jun	3.7	1.1	44	23.6
25 Jun	2.0	0	11	9.5
2 Jul	1.3	0.6	11	0
9 Jul	2.9	1.5	14	0
16 Jul	5.7	0.3	15	10.5
23 Jul	20.2	2.9	10	3.2
30 Jul	3.9	0	30	5.5
6 Aug	3.0	0.8	29	4.7
13 Aug	4.6	0	14	1.4
20 Aug	0.2	0	14	6.9
27 Aug	0.8	0	19	4.8
3 Sep	1.2	0.2	5.8	1.1
10 Sep	0.01	0	5.5	0.5
17 Sep	12.0	3.5	20	10.0
24 Sep	1.7	0	27	3.3
1 Oct	9.3	0	9.5	24.0
8 Oct	5.6	0.4	6.5	0
15 Oct	5.4	0	22	0

On 6 July a male Culiseta melanura was found in one of the emergence funnels placed on the substratum at "16Q". Another male C. melanura appeared in another funnel (17P) a week later and a female Aedes canadensis was collected from the funnel at "40Q" on 20 July. These observations, together with the recovery of C. melanura larvae in pools dug into the vegetative mat in winter, give some evidence in support of the theory that C. melanura larvae can live in the saturated vegetative mat without the need for open water.

Although only two males emerged from the mat into the funnels, one must keep in mind that the area covered by an emergence trap is somewhat less than five square feet; five of them covered less than 25 square feet. At that rate, from one acre of swamp substrate 3484 C. melanura could emerge in the time period that the traps were operated, i.e. 7 June-6 July. The two C. melanura caught in these funnels may have emerged from larvae that overwintered in that particular site, in which case ones that emerged earlier than 7 June must have been missed. If larvae are capable of lateral movement through the vegetative mat, invasion from areas adjacent to the site could have occurred. Moreover, if C. melanura deposit eggs in the vegetative mat during the summer, then 3484 individuals emerging from one acre is a low estimate since the funnel would have prevented egg deposition in the enclosed vegetative mat and the subsequent productivity of adults in that spot.

Larvae were also collected on Assateague Island but no C. melanura were found. Numerous A. canadensis and A. cantator were observed in fresh water pools and A. sollicitans in brackish water. Larvae of other species included Aedes infirmatus, Psorophora ferox, P. howardii, P. ciliata, Culex territans, and what appeared to be Aedes grossbecki.

2. Adult Mosquitoes. In 1967, 28 species (45,225 individuals) of adult mosquitoes were collected in light traps by vacuum and other means (Table 30). About 70% of the total catch was Culiseta melanura (31,500) with Aedes canadensis being next most abundant (nearly 20% of the total catch). The total catch of C. melanura was about 2½ times larger than in 1966 (12,788) but less than ½ of the 1965 catch (68,786).

The first C. melanura was observed on 26 April north of the study grid across the Pocomoke River. It was found in a hollow of a tree which contained water and it appeared to have just emerged. Light traps operated in the swamp during the previous night caught only a few A. canadensis; the temperature was 41°F during the night. The first C. melanura were caught in light traps in the week of 23 April.

The light traps in the swamp were much more successful in catching C. melanura than those in peripheral areas (Table 31). This indicates that although C. melanura move out of the swamp into peripheral areas, the population remains denser in the swamp.

Although the CLTs in the swamp consistently caught more mosquitoes

Table 31. Mosquito species collected in 1967.

	Male	Female
Aedes atlanticud	1	48
aurifer	0	9
canadensis	5,556	3,173
cantator	68	591
cinereus	0	1
grossbecki		
infirmatus	0	43
sollicitans	3	93
taenionhynchus	1	49
thibaulti	0	2
triseriatus	7	23
vexans	41	300
species		1
Anopheles bradleyi	13	459
brad-crucians	1	170
punctipennis	1	15
quadrimaculatus	2	11
Culex pipiens pipiens	0	2
pipiens quinque-fasciatus	0	1
restuans	19	76
salinarius	93	2,696
territans	11	50
Culiseta inornata	1	2
melanura	6,143	25,357
Mansonia perturbans	0	2
Orthopedomyia signifera	0	2
Psorophora ferox	3	45
howardii	1	0
Uranotaenia sappharrina	0	3
	11,965	33,260
Total	45,225	

Table 31. Mean monthly number of female C. melanura captured per trap during the same nights of trapping.

	A	B	B/A
	In the Swamp	Peripheral Areas	
May	31.5	0.5	0.016
June	62.5	4.5	0.072
July	126.2	10.1	0.080
August	180.6	30.0	0.166
September	35.1	12.7	0.362
October	17.0	8.5	0.588

than those in the peripheral areas, the numbers of mosquitoes caught in the peripheral areas increased over the year. This may indicate that as the season progressed increasing numbers of C. melanura wandered out of the swamp into the peripheral areas.

There were some differences in the numbers of C. melanura caught in the various parts of the study grid (Table 32). The central part of the grid (17Q and 20Q), which has scattered trees and therefore is more open, had a greater abundance of females during April, May and June than the northern portion (30Q and 40Q), where very few C. melanura larvae were found or the southern portion (RRJ and 6K) where the canopy is complete and the emergence was apparently delayed. Later in summer (July, August, and September) the catch in the central and northern portions was about the same. Dispersal from the central portion may result in a more even distribution of adult C. melanura in the various parts of the swamp, including areas such as 30Q and 40Q.

The numbers of C. melanura caught by CLTs sometimes underwent large fluctuations over a short period of time, i.e. two days. Traps were operated at the five foot level on Monday nights and again on Wednesday nights in conjunction with traps at 15 foot heights at the same sites. The total catch of female C. melanura in the swamp when the low traps were operated alone (Monday nights) was 9080 while the catch in the low traps during the nights (Wednesday) the high traps were also operated was 12,495. In terms of the total numbers one might say that the high traps attracted more mosquitoes into the area because they were visible from a greater distance, even though they didn't catch as many as the low traps. When the catch record is examined (Tables 33 and 34) it is apparent that the disparity between the numbers caught by the low traps two days apart was often in favor of the nights in which the low traps were operated alone (Table 33). From this it appears that there are wide fluctuations occurring in the numbers of mosquitoes attracted by light traps over periods of only a few days. However, because the vacuum sweep samples do not fluctuate as widely, it is possible that much of the variation in the numbers caught by light traps represents variations in activity (e.g. dispersal, mating, feeding, etc.) as well as fluctuation of numbers. Also, there may have been increased activity following times of emergence which resulted in larger numbers caught in light traps. However, the vacuum sweeps were taken during the day, and the mosquitoes may already have had time to disperse over a wide area. Thus mosquitoes would not be caught in large numbers in the vacuum sweeps, which cover only a small area.

As was the case during last year, the CLT catch was greatly influenced by weather conditions, particularly cloud cover. Temperature also had an affect: not many C. melanura were caught until the mean temperature was over 50°F in the spring and none were caught after the mean fell below 50°F in the fall. All collections containing over 30 female C. melanura per light trap week were taken when the mean temperature was over 60°F.

Table 32. Mean numbers of C. melanura per trap-night.

	RRJ and 6K		17Q and 20Q		30Q and 40Q	
	Male	Female	Male	Female	Male	Female
April	3.7	6.5	4.25	5.5	0	0.33
May	4.0	7.7	2.69	31.4	.45	5.36
June	13.5	20.17	20.73	80.53	4.3	29.9
July	51.28	184.7	44.3	194.0	19.47	204.3
August	11.7	85.6	14.0	181.7	16.27	180.45+
September	10.15	36.38	11.75	44.37	25.0	37.5
October	26.25	15.8	2.1	14.1	3.63	7.75
Year average	17.2	51	14.3	78.8	9.7	65.1

Table 33. Numbers of C. melanura caught in light traps 5 feet above ground.

	Females			Males		
	Monday	Wednesday	Mon/Wed	Monday	Wednesday	Mon/Wed
May	679	56	12.1	126	10	12.6
Jun	1125	728	1.5	252	277	.91
Jul	3346	6432	.52	364	1880	.19
Aug	3024	4542	.66	87	376	.204
Sept	667	648	1.02	242	277	.87
Oct	239	89	2.67	51	11	4.6

Table 34. Largest differential in catch in any single week.

	Females			Males		
	Monday	Wednesday	Differential	Monday	Wednesday	Differential
May	306	11	-295	37	4	-33
June	393	203	-190	156	65	-91
July	435	4193	+3758	150	1632	+1593
Aug	2256	2917	+661	14	286	+272
Sept	7	115	+108	5	140	+135
Oct	212	37	-175	43	7	-35

Table 35 shows the relationship between cloud cover on the CLT catch.

Table 35. CLT catch (average number of C. melanura per trap night) under various weather conditions while the temperature was 55°F and above.

	Clear	Partly Cloudy (50% or less)	Partly Cloudy (50% or more)	Overcast	Rain or fog
	72			9.6	
	28.2	98.2	61.2	38.6	
	104.0	38.3	32.6	71.2	40.6
	6.1	60.0	36.8	698.8	35.5
		90.2	252.0	161.2	
		1.8	101.4	189.5	
			87.0		10.5
			59.0	26.2	73.6
			143.2	198.9	65.7
			82.6	23.0	564.0
			9.4	35.6	729.2
			18.0		31.0
			1.2		27.0
			7.0		
Average	52.6	57.7	68.56	145.25	185.7

The disparity in the sizes of the collections taken under various weather conditions is not as marked as during the previous year, but nevertheless the catch during completely overcast nights was from two to three times larger than the catch during clear or partly cloudy nights. Each of the different weather categories includes data from various times of year representing various catch sizes which should be evenly distributed between the categories, if there was no influence of cloud cover on the catch. Thus, the mean values for the collections should be about equal. Because they are not one is inclined to conclude that cloud cover, or some factor correlated with cloud cover, affects mosquito activity and influences the size of the catch.

Because the size of the CLT collections was so readily influenced by temperature, cloud cover, and various kinds of activity (swarming, etc.) and because the numbers of mosquitoes caught in light traps are subject to wide fluctuations in numbers of mosquitoes caught over a period of only a few days, conclusions about C. melanura population size and variation based on CDC miniature light trap measurements should be carefully qualified by considering these environmental variables.

The collections obtained by vacuum sweeps, although generally smaller, probably reflect better the sex ratio of the C. melanura population since the CLT collections were strongly biased toward females (Table 32). In the vacuum sweeps, about twice as many males were caught as females (Table 36); the same sex ratio was found in vacuum sweep samples during 1966.

The peaks in the numbers of C. melanura caught by CLTs were reflected in the vacuum sweep samples in late June and mid-July but not as much in late August, whereas the largest vacuum sweep collection for the season (in the week of 13 September) was not reflected in the relative numbers caught by light traps.

The vacuum sweeps and light traps also differed in the percentage of the female C. melanura catch that was gravid. In the CLT catch in the swamp only once during the first half of June, twice during July, once in early August, once mid-September, and during October (altogether 8 out of 20 samples) did the percent of gravid females among the total female C. melanura caught exceed 4.0% (Table 29); only on two occasions were gravid females more than 10% of the catch and only once as high as 20%. In the vacuum sweeps, on the other hand, from the first of June to mid-October, gravid females never comprised less than 5% of the catch; in 15 out of 20 collections that contained gravid females they exceeded 10% of the total collection and in seven the percentage of gravid females was 20% or higher, reaching as high as 44% in mid-June. This peak in gravid females, incidentally, was associated with the later appearance of mostly 1st instar larvae in the larval traps (late-June to mid-July).

Blood engorged mosquitoes were also more readily caught by the vacuum sweeps than by CLTs (Table 29). On eight occasions the percentage of engorged female C. melanura in the vacuum sweep samples exceeded 5%; on two occasions as high as 24%. The highest percentage of C. melanura containing a blood meal in the CLT collections was 3.5%; only on four occasions was the percentage higher than 1.0.

Thus, unlike vacuum sweeps, light traps are more prone to catch female C. melanura than males and their catch is more sensitive to weather conditions and other factors that alter the activity of the mosquitoes. Vacuum sweeps exceed the CLTs in the numbers of males caught and in the capture of mosquitoes that are less likely to fly (i.e. recently fed females and gravid females). Yet when the percentages of engorged and gravid female collections at 5' and 15' are compared, one finds very few differences (engorged: 5', 0.55%; 15', 0.43%; gravid: 5', 2.5%; 15', 3.6%). Apparently if gravid and engorged mosquitoes are disposed to fly, they are

Table 36. Numbers of C. melanura caught in vacuum sweeps in 1967.

	Males	Females
10 May	18	10
17 May	41	22
24 May	-	-
31 May	28	10
7 June	61	20
14 June	163	50
21 June	187	55
28 June	114	63
5 July	68	37
12 July	55	21
19 July	204	57
26 July	35	31
2 August	113	36
9 August	45	21
16 August	140	71
23 August	49	29
30 August	96	63
6 September	153	90
13 September	346	205
20 September	92	60
27 September	10	30
4 October	13	42
11 October	5	16
18 October	1	9
25 October	0	0
Total	2037	1054

as apt to fly to 15' as to 5' heights.

Surprisingly, nearly six times as many female Aedes canadensis were caught in vacuum sweeps as males. In CLT collections, however, males were nearly twice as numerous as females. A. canadensis was very numberous in the vacuum sweep samples in May through July, and especially in June (when female A. canadensis were five to nine times as abundant as C. melanura females). In August A. canadensis were uncommon, but their numbers increased again slightly in September.

The preceding information suggests that not only are A. canadensis and C. melanura different in terms of their relative abundance during the year, but that special distribution of the sexes and attraction to light in the two sexes also differ in these species.

The question of where C. melanura stays in the swamp and where it is active is of interest. When light traps were operated simultaneously at the same sites in the swamp at two heights, 5 and 15 feet from the ground, about half of C. melanura in the catch at the 15 foot height were males, whereas, less than a third were males at the five foot height (Table 37). Although the traps at 15 foot height consistently caught fewer mosquitoes during June through August the relative abundance of mosquitoes at the two heights was the same through time. During September and October the disparity between the catches at the two heights was greatly reduced; during the last week of the collections the collections from the high traps actually included more mosquitoes than those at the five foot level. This suggests seasonal variation in flight activity of C. melanura.

Only the resting boxes with the 7" and 4" openings were used by C. melanura; in the boxes with the 7" opening 19 male and 8 female C. melanura were caught; in the boxes with a 4" opening one male and 4 females were caught. Two Culex territans were caught in the 7" hole boxes and one C. pipiens was caught in a box with a 4" hole. The failure by C. melanura to use the boxes with 2", 1", $\frac{1}{4}$" openings may indicate that this species will not use small crevices and hollows as resting sites, but prefers cavities with larger openings.

Gravid and engorged females apparently stay close to the ground where they were readily caught in the vacuum sweeps.

To date 118 blood meals of C. melanura have been identified. With the exception of one that had a pig blood meal, C. melanura fed on birds. Among the blood engorged females that were caught in the light trap at 40Q, which was very near a sentinel quail pen, 83 out of 85 caught contained quail blood; 10 out of 13 engorged females taken by vacuum sweep in the same area contained quail blood.

In other areas that were not as close to sentinel quail pens passerine birds comprised 21 out of 28 blood meals identified; quail, 2 out of 28; other birds, 5 out of 28.

Table 37. C. melanura catch at 5' and 15'.

Date	Low Traps					High Traps						
	Males	Females	No. Traps	Male/ Trap	Female/ Trap	Males	Females	No. Traps	Male/ Trap	Female/ Trap	High Fem / Low Fem	High Male / Low Male
24 May	-	7	5	0	1.4	1	1	5	0.2	0.2	0.14	0
1 Jun	1	11	5	0.2	2.2	1	-	5	0.2	0	0	1
8 Jun	85	141	5	17	28.2	76	38	6	12.7	6.3	0.27	0.89
15 Jun	65	203	5	13	40.6	11	9	3	3.7	3	.015	0.17
22 Jun	107	163	5	21.4	32.6	6	8	2	3	4	0.05	0.05
29 Jun	20	231	6	3.3	38.5	1	9	1	1	9	0.05	0.04
6 Jul	47	507	5	9.4	101.4	20	56	2	10	28	0.11	0.44
13 Jul	1,643	4,193	6	273.8	698.8	890	509	4	222.5	127.2	0.12	0.43
20 Jul	22	806	5	4.4	161.2	21	100	4	5.2	25	0.12	0.95
27 Jul	137	716	5	27.4	143.2	177	135	5	35.4	27	0.19	1.3
3 Aug	31	210	2	15.5	105	66	66	3	22	22	0.22	1.42
10 Aug	286	368	5	57.2	73.6	26	9	4	6.5	2.2	0.02	0.11
17 Aug	41	263	4	10.2	65.8	105	81	5	21	16.2	0.24	1.0
24 Aug	25	2,917	4	6.2	729.2	3	883	3	1	294.3	0.43	0.12
31 Aug	25	944	5	5.0	188.8	17	430	4	4.2	107.5	0.57	0.84
7 Sep	80	417	4	20	104.2	27	156	4	6.7	39	0.37	0.33
14 Sep	4	54	3	1.3	18	12	61	3	4	20.3	0.06	3.0
21 Sep	53	62	2	26.5	31	22	24	2	11	12	0.38	0.41
26 Sep	140	115	5	28	23	80	84	4	20	21	0.73	0.57
5 Oct	3	36	2	1.5	18	13	52	3	4.3	17.3	1.44	4.3
12 Oct	10	31	4	1.2	7.7	7	37	6	1.2	6.1	1.0	0.79
19 Oct	4	41	2	2	20.5	1	16	2	.5	8	0.39	0.25
	2,829	12,436	94	30.1 ave.	132.3 ave.	1,583	2,754	80	19.8 ave.	34.9 ave.		

Of 11 blood meals of A. canadensis identified only two were bird and the rest were bovine. An A. cantator also contained a bovine blood meal and an Aedes aurifer was engorged with chicken blood.

All of the Aedes which had blood meals identified were caught in the swamp. This indicates that either deer blood was cross reacting with bovine antiserum in the tests or that the mosquitoes moved into the swamp after obtaining bovine blood from farm animals at least one and a half miles distant.

On 26 July 1967, beginning about 2000 hours, mosquitoes were collected in a cow shed on Groton's farm: 128 out of 139 Culex salinarius collected were engorged with blood. In the collection were also one each A. cantator, A. sollicitans, Aedes taeniorhyneus, two Anopheles bradleyi and four An. bradleyi-crucians, all of them engorged with blood. One gravid C. melanura was collected.

One EEE and six WEE virus isolations were made from mosquitoes in 1967. The single EEE virus isolation came from a pool of six female C. salinarius, caught on 6 July in a light trap (20Q). All six of the WEE virus isolations came from pools (10-25 individuals) of female C. melanura; three were from the middle of the study grid (17O, 17Q, 20Q) and two were from the northern part (30Q, 40Q). The most significant isolation (WEE) was that from a pool of 14 female C. melanura taken in a light trap in Groton's farm yard on 28 July. The pools of mosquitoes from which isolations of WEE virus were made were caught in the swamp on 30 and 31 (2) August and on 7 September (2).

As can be seen in the rates of virus isolation from mosquitoes during the last three years (Table 38), EEE virus has been isolated at a decreasing rate. Although quite a large proportion of female C. melanura were carrying WEE virus in 1966, the infection rate in 1967 was nearly eight times lower. However, C. melanura were about three times as abundant in 1967 as in 1966. The approximate numbers of potentially infective mosquitoes in 1966 were only about 2 2/3 times greater than in 1967.

Table 38. Isolation of viruses from female C. melanura.

	1965	1966	1967
EEE	1/734 females	1/8,232	1/25,357
WEE	1/1,521	1/515	1/4,225

3. Birds. Experimental Quail. No virus isolations were made from either the blood or cloacal swabs of the ten experimentally

infected quail. The first plasma samples that showed positive HI activity were drawn 14 days after the birds were inoculated with the virus; three had titers of 1/1280 and one 1/160. Of the six remaining birds three became HI positive 20 days after inoculation (one 1/320, two 1/160) and another 27 days post inoculation (1/640). Two birds did not become HI positive (last tested 2 months after inoculation).

Two of the HI positives were negative when tested 14 days after they first showed up as HI positive; one was negative one month after the first time it was shown to be positive. Three were still positive (1/20) a month and a half after they were first shown to be positive.

Sentinel Quail. Three virus isolations (all WEE) were made from the sentinel quail:

Date	Place
7 September 1967	20Q
19 September	RR (just south of study grid)
3 October	Pasture near Groton's farm

All three birds produced neutralizing antibody against WEE virus; one had antibody in the blood sample from which the virus was isolated, the other two had antibody in the blood sample drawn two weeks after the virus isolation (Table 39). Neutralizing antibodies persisted until the last sample tested (taken in mid-December), but HI activity was last in two after 27 November. All three showed cross reaction with EEE in HI tests at one time or another.

In the flock kept in the pasture of Groton's farm, all but one bird developed neutralizing antibodies against WEE. In most cases there was a simultaneous appearance of WEE HI activity but usually at a relatively low titer (1/40-4, 1/80-3, 1/160-1, 1/320-2) at the start and never more than 1/800. One bird had neutralizing antibody against EEE as well as against WEE; this was the only sentinel quail with EEE neutralizing antibody. In another bird HI activity preceded neutralization activity by one month. Six birds had short term EEE HI activity but none before mid-October after the C. melanura mosquitoes had ceased activity; this was most likely cross reaction activity. One bird had neutralizing antibody beginning 24 August, four beginning 30 August, two beginning 7 September, one 14 September, one 27 September, and on 16 October; quail brought out to the cage on 10 and 16 October showed sporadic WEE neutralizing activity but no HI.

The pattern of appearance of neutralizing antibody and HI activity in birds at 40Q was rather similar. Nine birds developed neutralizing antibody against WEE and were simultaneously HI positive (WEE). The first bird became positive on 30 August, four on 7 September, and four on 14 September.

Table 39. Sequence of HI and neutralization activity in three quail from which WEE was isolated.

	100 Male (replacement, brought to "Pasture" 19 Sept)		70 Male 20Q since 7 June		89 Male RR since 7 June	
	Neutralizing	HI	Neutralizing	HI	Neutralizing	HI
30 Aug			0	0	0	0
			WEE isolation			
9 Sep			0	0	0	0
14 Sep						
19 Sep	0	0	WEE+	WEE 1/160 EEE 1/20	0	0
					WEE isolation	
27 Sep	0	0				
3 Oct	WEE+	0	WEE+	WEE 1/20	WEE+	WEE 1/160 EEE 1/20
	WEE isolation					
10 Oct						
16 Oct			WEE+	WEE 1/40	WEE+	WEE 1/40
24 Oct	WEE+	WEE 1/40				
1 Nov			WEE+	WEE 1/160 EEE 1/40	WEE+	WEE 1/200 EEE 1/50
8 Nov		WEE 1/20 EEE 1/40				
14 Nov			WEE+	WEE 1/100 EEE 1/100	WEE+	WEE 1/80 EEE 1/80
27 Nov	WEE+	WEE 1/40	WEE+	WEE 1/200 EEE 1/50	WEE+	WEE 1/40
14 Dec	WEE+	WEE 1/100 EEE 1/25	WEE+	0	WEE+	0

In the middle of the study grid, 20Q, the first WEE neutralization positive blood was taken on 24 August; three more birds converted on 7 September, one on 14 September, one on 19 September, one on 27 September, one on 24 October, one on 1 November, and one on 8 November. All but one (24 October) had simultaneous HI activity (WEE); the 24 October convert began HI activity on 27 November (1/800).

Near the south edge of the study grid (RR) only four birds became positive for WEE neutralizing antibody; the first on 14 September, second on 27 September, third on 3 October, fourth on 16 October; the last one was not positive again until 14 December. All had simultaneous HI activity (WEE).

Nearly all the sentinel birds showed positive EEE HI activity at one time or another regardless of whether they had any WEE neutralizing antibody. Except in the single case where EEE neutralizing antibody was present, interpretation is difficult. The vast majority of the cases were probably just an artifact of the test. Most of the EEE activity occurred in November and was rather synchronized in the birds (up to 40% of the birds involved compared to 0-2.5% involvement until 24 October), this suggests that something in the blood resulting from physiological activity, perhaps hormonal, in response to the onset of the cold weather could have been responsible for mimicry of EEE HI antibody in the tests.

Many of the birds introduced into the flocks late in the season (mid-October) had one-time positive WEE neutralizing blood samples; no explanation for this can be offered at this time.

Because the sentinel birds at the various sites were kept together in a single cage, infection in each case was not necessarily initiated by a mosquito bite. Some birds could have become infected from fecal contamination or from wounds inflicted by infected birds. Transmission by ectoparasites can not be discounted.

The earliest appearance of WEE antibody was in the flock maintained in the pasture of Groton's farm; the latest at site RR, just south of the study grid. The RR site also had the lowest number of conversions (4/10) compared to the other sites (Pasture 10/11, 40Q, 9/10, 20Q, 11/11). It is perhaps not a coincidence that the largest numbers of wild birds occurred in the vicinity of the pasture and the fewest near the south end of the study grid.

4. Sentinel Wild Birds. The species kept in largest numbers were Red-winged Blackbirds. Among 34 birds 20, or 68%, had neutralization antibodies against WEE (WEE-NT) at least during one sampling period. Among 14 males, 13 were positive, whereas among 20 females only 7 were positive for WEE-NT at least once. No virus was isolated and none of the Red-winged Blackbirds had EEE-NT. Only 7 of the WEE-NT positives also had simultaneous positive WEE hemagglutinization inhibition activity (WEE-HI). Of the 11 birds that were positive WEE-NT and were bled at least on two occasions 5 had only one positive WEE-NT sample out of 4 samples taken, 4 had only 2 positive WEE-NT samples out of 4. Such

erratic patterns of antibody appearance suggest that in Passerine birds there might be wide fluctuations in the level of antibody over time. If so, results of neutralizing antibody surveys or of hemagglutinization-inhibition antibody (HI) surveys from wild birds which include only one blood sample per bird may not be reliable indicators of the true involvement of Passerine birds in the virus life-cycle in nature.

Nine Catbirds were placed in the sentinel cages on and about 10 October of which only one was WEE-NT positive during the initial bleeding and remained positive through the sampling period. Two others had a single positive WEE-NT blood sample, one on 17 October and one on 12 December; the rest of the blood samples were negative for WEE-NT, as well as EEE-NT. None of the three WEE-NT positives were WEE-HI positive at any time, but one of the WEE-NT negatives was WEE-HI positive once (28 November, 1/100). Six of the Catbirds had positive EEE-HI activity at least once, but this may have been an artifact of the EEE-HI tests (see discussion under sentinel quail).

Nine Swamp Sparrows were placed in one of the sentinel pens (two on 3 October, five on 10 October, one on 17 October, and one on 24 October). Only two were WEE-NT positive on initial bleeding and both of these died before a second bleeding could be obtained. Of the negatives only one lived long enough to obtain from it more than one blood sample - it lived through the sampling period but none of the samples were positive. The two WEE-NT positives were WEE-HI negative, but one was EEE-HI positive.

Of three House Sparrows that survived through the sampling period one was WEE-NT negative on initial bleeding (3 October) but was WEE-NT positive through the remainder of the sampling period; however, WEE-HI activity did not appear until 28 November. Another House Sparrow was WEE-NT positive through the sampling period, but WEE-HI positive only during the initial bleeding. The remaining House Sparrow was negative all through the sampling period.

Three Brown Thrashers were negative during initial bleeding and remained WEE-NT, WEE-HI, and EEE-NT negative through the sampling period; one had one-time EEE-HI activity (1/20).

Of three Robins that survived through the sampling period, one had one positive WEE-NT sample; the rest of the samples were all negative.

Four Cardinals were kept as sentinels: one had four positive WEE-NT samples out of eight; another had two. None of the samples were positive for WEE-HI, EEE-NT, or EEE-HI.

Other summer residents and permanent residents had a similar pattern of antibody appearance (Mockingbird, Grackle, Song Sparrow). Among winter residents, three Fox Sparrows (placed in sentinel pen on 28 November) and a slate-colored Junco (placed in sentinel pen on 17 October) had all negative blood samples. Among White-throated Sparrows, on the other hand, which appeared in the swamp somewhat earlier while some

C. melanura were still being caught, eight out of 11 that survived were WEE-NT positive at least once (two were positive twice), one of the WEE-NT positives was EEE-NT positive at a later time, and one of the WEE-NT negatives was EEE-NT positive on two occasions. None of the White-throated Sparrows were WEE-HI positive at any time.

Taken collectively, the wild bird sentinels had 73 positive WEE-NT blood samples, but only 18 positive WEE-HI samples. Although only three samples were positive EEE-NT, there were 33 that were positive EEE-HI.

The sentinel wild bird data indicate that WEE-NT and especially WEE-HI surveys of Passerine bird populations underestimate the involvement of Passerine birds in the life-cycle of WEE virus.

5. Wild Birds. During 1967 a total of 2062 wild birds were captured. These represented 83 species; an additional 32 species were observed, but not captured. Of the total captured, 1547 were bled, of which 1468 were tested for WEE neutralizing antibody (WEE-NT); 223 or 15.2% were positive compared with 17% last year. 1437 plasma samples were tested for WEE hemagglutinization inhibition activity (WEE-HI); 143, or 10%, were positive at >1/20; 44, or 3.1%, were positive at 1/40 or greater; 22, or 1.6%, were positive at 1/80 or greater - of these 22 only 10 were positive for WEE-NT. 223 were positive for WEE-NT but not WEE-HI.

Of the samples tested for EEE-NT 68 out of 1468, or 4.6%, were positive. Twenty-five percent were positive for EEE-HI at >1/20; 17% were positive at >1/40. As was mentioned in the discussion of sentinel quail, the EEE-HI test seems to be unreliable. Of the 365 samples that were EEE-HI positive (>1/20) only 13 were also positive for EEE-NT; 19 others were ± (one out of two HKCC tubes with CPE). The highest percentage of birds were positive for EEE-HI between 17 March and 7 April (50-64.6%); only 4 - 7% were EE-NT positive during that time.

The highest proportion of birds were positive for WEE-NT and WEE-HI in January and February (Table 40); the lowest in August, when the largest proportion of sentinel quail became positive WEE-NT. This might seem incongruous at first sight, but during the weeks of 7 and 14 January, all of the positive WEE-NT samples were from permanent residents (4 quail, 1 brown thrasher); during the weeks of 3 and 10 February six of the seven positives were quail and one was a White-throated Sparrow (which could have been infected while farther north during the fall). In August, among the 77 birds in which age could be determined 47 were immatures. These young birds would have had a short exposure time to potential infection and thus would contribute disproportionately to the numbers of negatives.

Forty-three species of birds had WEE-NT among which only the Fox Sparrow, the White-throated Sparrow, the Myrtle Warbler, and the Slate-colored Junco were not either permanent residents or summer residents (Table 14). Thus, the permanent residents and summer residents accounted for 93.5% of all positive WEE-NT samples. About half of the positives

Table 40. Biweekly summary of antibody occurrence.

	Total No. Caught	Total No. Bled	Total No. Tested Tube-Neut	% Pos. WEE-SN	% Pos.	No. Pos. EEE-SN	% Pos.	Total No. Tested	HI WEE-HI >1/40	% Pos.
7 Jan & 14 Jan	14	13	13	5	38	-	-	13	-	-
21 Jan & 28 Jan	-	-	-	-	-	-	-	-	-	-
3 Feb & 10 Feb	16	13	13	7	54	3	23	13	1	7.7
17 Feb & 24 Feb	56	51	51	7	14	2	4	43	1	2.3
3 Mar & 10 Mar	76	55	55	15	27	3	5	54	3	5.6
17 Mar & 24 Mar	56	30	30	2	7	2	7	29	1	3.4
31 Mar & 7 Apr	126	84	82	9	11	3	4	82	3	3.7
14 Apr & 21 Apr	65	36	36	6	17	-	-	36	2	5.5
28 Apr & 5 May	147	121	104	21	21	4	4	102	1	<1
12 May & 19 May	73	61	58	12	21	5	9	56	1	1.8
26 May & 2 Jun	77	61	60	14	23	4	7	58	1	1.7
9 Jun & 16 Jun	140	120	110	32	29	11	10	109	3	2.7
23 Jun & 30 Jun	129	113	110	15	14	7	8	110	3	2.7
7 Jul & 14 Jul	155	135	123	13	11	3	2.5	117	4	3.4
21 Jul & 28 Jul	124	117	111	16	14	1	<1	107	3	2.8
4 Aug & 11 Aug	82	74	62	3	5	1	2	67	1	1.5
18 Aug & 25 Aug	15	14	14	1	7	-	-	13	-	-
1 Sep & 8 Sep	123	122	118	11	9	1	<1	111	1	<1
15 Sep & 22 Sep	23	22	21	2	10	-	-	21	1	5
29 Sep & 6 Oct	39	34	34	6	18	-	-	34	2	6
13 Oct & 20 Oct	220	146	140	19	14	7	5	146	7	5
27 Oct & 3 Nov	283	104	103	4	4	9	9	101	5	5
10 Nov & 17 Nov	-	-	-	-	-	-	-	-	-	-
24 Nov & 1 Dec	7	7	7	1	14	-	-	7	-	-
8 Dec & 15 Dec	16	14	13	2	15	2	15	12	-	-
Totals	2062	1547	1468	223	15.2	68	4.6	1440	44	3.1

were summer residents. Among the winter residents only the Slate-colored Junco and the Fox Sparrow were caught only after C. melanura ceased to be caught. Moreover, these birds may have become infected earlier in the season while in areas farther north.

Only two virus isolations were made from wild birds during 1967. Both were from Myrtle Warblers, one caught on the 10th and the other on the 17th of October, just when the last C. melanura were captured. These birds were either infected by the last of the local mosquitoes or they may have brought the virus south from areas farther north. Neither bird had WEE-NT.

The relative importance of permanent residents, summer residents, winter residents, and transient species was discussed in last years Annual Report. In light of that discussion, the two virus isolations coming from a transient species that appears to be relatively little involved in the WEE virus life-cycle in areas from which it migrates in the fall (only one WEE-NT positive in 97 samples tested) (Table 41) is of great interest. This observation indicates that Myrtle Warblers, and perhaps other similar birds, represent a large susceptible population moving through the Pocomoke River Swamp area at a time when C. melanura are still active, and depending on the longevity of viremia and the longevity and motility of the infected birds, Myrtle Warblers may serve as a vehicle for transportation of virus from areas where mosquito activity is about to cease to areas farther south where mosquitoes are active later in the year and ultimately to areas where mosquitoes are active through the winter.

Among birds that were caught in significant numbers during last year (1966), the three species with the highest proportion of individuals with WEE-NT were Quail (63%), Tufted Titmouse (74%), and Pine Warbler (68%). In 1967 (Table 41) Quail (71%) were still among the top three species, but Tufted Titmice were only 33% positive WEE-NT and Pine Warblers only 25%. Red-eyed Vireos and Wood Thrushes, on the other hand, had a higher percentage of positive WEE-NT (69% and 38% respectively) than last year.

On Assateague Island 15 our of 154 birds bled, or 9.7%, were WEE-NT positive; seven, or 4.5%, were EEE-NT positive. Most of the WEE-NT positive samples were from Catbirds, two each from Yellow-breasted Chats, Brown Thrashers, and Pine Warblers; one each from Towhee, Red-winged Blackbird, and Gray-cheeked Thrush. Six of the EEE-NT positives were from Catbirds and one from a Brown Thrasher. It is not possible to determine from the existing data whether the birds on Assateague acquired their infections on the island or from the nearby mainland.

In the Pocomoke Swamp area the incidence of positive WEE-NT reactors among birds varied somewhat in the different types of habitats. The total catch from the areas netted was divided into five categories according to the type of habitat in which the birds were caught (Table 42).

Table 41. Bird species positive for WEE-NT.

Species	No. Bled	No. Pos. WEE-NT	% Pos.
Quail	41	29	71
Hairy Woodpecker	5	1	20
Downy Woodpecker	13	1	7.7
Red-bellied Woodpecker	8	3	38
Yellow-shafted Flicker	13	1	7.7
Eastern Kingbird	3	1	33
Crested Flycatcher	18	1	5.6
Acadian Flycatcher	19	4	21
Blue Jay	2	1	50
Red-winged Blackbird	43	2	4.7
Grackle	40	1	2.5
White-throated Sparrow*	89	7	7.9
Chipping Sparrow	2	1	50
Slate-colored Junco*	65	4	6.2
Swamp Sparrow	33	2	6.1
Fox Sparrow*	39	1	2.6
Rufus-Sided Towhee	54	10	19
Cardinal	82	14	17
Indigo Bunting	5	2	40
Summer Tanager	2	2	100
Red-eyed Vireo	16	11	69
White-eyed Vireo	71	16	23
Prothonotary Warbler	66	2	3.0
Worm-eating Warbler	46	3	6.5
Parula Warbler	8	1	13
Myrtle Warbler	97	1	1.2
Black-poll Warbler	3	1	33
Pine Warbler	12	3	25
Prairie Warbler	18	2	11
Ovenbird	41	2	4.9
Kentucky Warbler	5	1	20
Yellowthroat	80	11	14
Yellow-breasted Chat	10	2	20
Hooded Warbler	16	3	19
Mockingbird	1	1	100
Catbird	69	21	30
Brown Thrasher	25	6	24
Carolina Wren	36	3	8.3
Tufted Titmouse	39	13	33
Carolina Chickadee	55	12	22
Blue-gray Gnatcatcher	6	1	17
Wood Thrush	40	15	38
Robin	36	4	11

* Winter residents or transients.

Table 42. WEE-NT locality summary.

	Pasture			Swamp-Field			Swamp-Upland			Slab Road (Swamp)			Swamp			Totals		
	No. Bled	No. Pos. WEE-NT	% Pos.	No. Bled	No. Pos. WEE-NT	% Pos.	No. Bled	No. Pos. WEE-NT	% Pos.	No. Bled	No. Pos. WEE-NT	% Pos.	No. Bled	No. Pos. WEE-NT	% Pos.	No. Bled	No. Pos. WEE-NT	% Pos.
7 Jan	-	-	-													13	5	38
3 Feb	-	-	-													13	7	54
17 Feb	-	-	-													51	7	14
3 Mar	-	-	-	23	6	26	-	-	-	11	6	55	21	3	14	55	15	27
17 Mar	-	-	-	4	1	25	-	-	-	20	0	0	14	1	7	30	2	6.7
31 Mar	-	-	-	22	5	23	1	-	-	30	2	7	29	2	7	82	9	11
14 Apr	-	-	-	9	2	22	2	1	50	12	2	17	13	1	8	36	6	17
28 Apr	13	2	15	27	5	19	9	2	22	24	4	17	52	8	15	120	21	18
12 May	7	2	29	14	3	21	18	5	28	6	1	17	16	1	6	61	12	20
26 May	17	1	6	24	6	25	3	2	67	9	2	22	9	3	33	62	14	23
9 Jun	29	7	24	30	8	27	35	9	26	10	1	10	15	7	47	120	32	27
23 Jun	26	1	4	7	1	14	27	4	15	24	6	25	29	3	10	113	15	13
7 Jul	16	0	0	22	2	9	30	2	7	38	5	13	23	4	17	132	13	10
21 Jul	23	2	9	29	6	21	18	3	17	25	1	4	22	4	18	118	16	9
4 Aug	25	0	0	18	0	0	14	0	0	13	2	15	6	1	17	74	3	4.0
18 Aug	4	0	0	4	0	0	1	0	0	2	0	0	3	1	33	14	1	7.2
1 Sep	40	3	8	32	1	3	6	1	17	23	1	4	18	5	28	121	11	9
15 Sep	6	1	17	1	0	0	3	0	0	10	1	10	2	0	0	22	2	9
29 Sep	6	1	17	8	1	13	11	3	27	6	0	0	4	1	25	33	6	17
13 Oct	44	3	7	24	2	8	29	4	14	33	6	18	20	4	20	151	19	13
27 Nov	31	0	0	27	2	7	18	2	11	15	0	0	12	0	0	104	4	3.8
10 Nov	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24 Nov	-	-	-	-	-	-	5	1	20	2	0	0	-	-	-	7	1	14
8 Dec	-	-	-	7	1	14	3	1	33	3	0	0	-	-	-	13	2	15
Total	287	23	8	332	52	16	233	40	18	316	40	13	308	48	16	223		

Pasture: an upland area on Groton's Farm consisting mostly of tall Loblolly Pines, bordering a secondary forest of mostly Oaks to the north, brackish marshlands to the west, a swampy area to the south, and agricultural fields to the east. As a result of grazing by farm animals the understory and ground cover were sparse.

Swamp-Field: border areas between the swamp and agricultural fields, often parallel with roads.

Swamp-Upland: border areas between the swamp and upland forest.

Slab Road: swamp area similar to that in the middle of the study grid, but about one mile south of the grid.

Swamp: swamp area in the study grid; three nets in the middle of the grid, viz. 20Q, two near 17X, and one near 40Q, or the north end.

The lowest incidence of positive WEE-NT was in the "Pasture" area, i.e. 8%. If, however, the fall migrants are excluded from the catch in that area (i.e. birds caught after 12 October) 11% of the birds were WEE-NT positives. It should also be added that the catch from the "Pasture" area included more marshland birds, such as Red-winged Blackbirds, and birds from agricultural fields, such as Grackles, than that from any of the other areas. Presumably, such birds are less likely to come in contact with C. melanura mosquitoes than birds that live in the swamp or nearby forests and would, therefore, experience a lower risk of infection (Table 41, Red-winged Blackbird, 4.7% positive WEE-NT, Grackles, 2.5%). Fewer C. melanura mosquitoes were caught per light trap in this area than in the swamp (Table 31).

In the areas in and bordering the swamp the percentages of positive WEE-NT were about the same. This correlates well with the sentinel quail conversion rates. Unfortunately no netting was done near the south end of the grid (RR) where only four of the 10 sentinel quail became positive and conversions occurred comparatively late. However, direct observations indicated that fewer birds occurred in that (more homogeneous) part of the study area than in the others (ecotone habitats, edge effect).

Young birds occurred only slightly more often in the "Pasture" area than in the others (Table 43), thus their effect on the numbers of positives in each of the habitat categories is probably not very marked. However, when the incidence of young birds are considered temporally (Table 44), it appears that their appearance during the end of June was associated with approximately a 50% reduction in the rate of positives (WEE-NT) in the total population. As the numbers of young birds increased, the rate of positive WEE-NT decreased. Many of these young birds apparently had experienced a lower risk of infection because of a shorter exposure time and thus "diluted" the numbers of positives. Associated with the increased time of exposure of the young birds was an increase in the WEE-NT positive rate (late September, early October). However, the rate drops again in mid- and late October as large numbers of migrants

Table 43. Summary of occurrence of adult and immature birds in the different habitats.

Locality	Number Caught	Percent Adults	Percent Immatures	Percent Undetermined
Pasture	278	17	32	51
Swamp-Field	359	16.5	20	63.5
Swamp-Upland	260	17	17	66
Slab Road	338	12	25	63
Swamp	322	12.5	14.5	73

Table 44. Relationship between appearance of young birds and migrants and the incidence of positive WEE-NT.

Date	Adults	Immatures	Undetermined	% Immature among Determined (>70% Determinable)	% Migrants or Winter Residents	% Total Population Positive WEE-NT
14 Apr	-	-	65	-	-	17
28 Apr	-	-	147	-	-	18
12 May	-	-	73	-	10	20
26 May	-	1	76	-	7	23
9 Jun	-	3	137	-	0	27
23 Jun	-	26	102	-	<1	13
7 Jul	68	66	20	49	0	10
21 Jul	56	64	10	53	2	9
4 Aug	25	40	17	62	0	4
18 Aug	5	7	5	58	0	7
1 Sep	38	51	34	57	7	9
15 Sep	5	11	8	70	23	9
29 Sep	7	12	20	-	19	17
13 Oct	22	36	63	-	53	13
27 Oct	5	22	65	-	61	4

and winter residents were captured. These birds for the most part apparently came from areas where WEE virus was relatively less active, since the occurrence of antibody in the migrants was very low and some had none at all.

VII. Ultrastructural Alterations in Meningococci after treatment with Penicillin.

The morphological alterations in meningococci which have been exposed to penicillin have been studied by electron microscopy in an attempt to correlate changes in ultrastructure with the established chemical changes occurring in organisms exposed to this antibiotic.

Neisseria meningitidis was inoculated into side-arm flasks containing beef heart infusion broth (with 0.3% yeast extract) and incubated at 37°C with constant agitation. The optical density was measured at frequent intervals ans showed a straight line increase after an initial lag period. The time required for the optical density to double (roughtly equal to a generation time for the organism) is 50 min during this log phase of growth. While the culture showed log phase growth, as defined by OD determinations, penicillin was added to the culture in a final concentration of 100 u/ml. Incubation was continued, optical density determinations were carried out at frequent intervals, and aliquots were removed at desired times and were prepared for electron microscopic examination. The turbidity or optical density of the culture continued to increase for a period following penicillin addition. The interval between time of antibiotic addition and point at which the apogee of the OD curve was reached was roughly two-thirds the doubling or generation time.

The ultrastructure of untreated meningococci has been described in the section of this report dealing with reactions of the cells to the serum bactericidal system. The control cells which were fixed prior (Fig. 17-1) to addition of penicillin differ from that description only, mainly, that these cells have a prominent mesosomal structure. The mesosome consists of a series of membranous profiles which assume either coiled or straight configurations. These structures are often noted to be continuous with the cytoplasmic membrane of the cell.

Few changes in fine structure are present in meningococci which have been exposed to penicillin for 15 min (Fig. 17-2, 17-3, 17-4). A small number of the organisms have blebs or outpouchings of their wall. These blebs are located near the plane of cleavage between cells and appear to be composed solely of the outer membrane of the wall. Both the middle dense line and the cytoplasmic membrane are observed coursing across the neck or origin of the bleb and not protruding into the outpouching. The bleb usually has no electron dense contents. The second change noted after 15 min is a subtle decrease in the distinctness of the mesosome.

Figure 17

17-1: This cell from a fluid culture before addition of penicillin shows the usual arrangement of the cell wall laminae and a well-developed mesosome (M). x 70,000.

17-2: After 15 min exposure to penicillin, a small bleb (B) is present at the plane of division and has a continuous segment of the middle dense line (DL) coursing across its base. The mesosome (M) is somewhat less distinct than in control cells. x 70,000.

17-3: Another meningococcus after 15 min incubation with penicillin shows a small bleb (B) forming in the plane of division between the two daughter cells. x 70,000.

17-4: The bleb (B) on this cell, treated for 15 min, is clearly seen to consist only of the outer membrane of the wall and contains no electron-dense material. Note the middle dense line (DL) along the base of the bleb. The mesosome (M) is similar to that in Fig. 17-2 and exhibits poor definition of its membranous components. x 70,000.

17-5: In addition to blebs on their surfaces, these cells which have been exposed to penicillin for 30 min, have rarifactions in the peripheral portions of their cytoplasms. These areas are associated with segments of the middle dense line (DL) which are questionable intact. x 70,000.

17-6: Another cell from a culture 30 min after penicillin addition has a full-thickness defect in its wall (arrow) through which nucleoid material has been extruded. x 70,000.

17-7: These meningococci have enlarged nucleoid regions (N) after 30 min exposure to penicillin. x 35,000.

17-8: After 45 min in penicillin, these meningococci show several gradations in extensiveness of the alterations induced by the antibiotic. One cell (A) appears fairly normal except for an outer membrane bleb at its place of division. Another cell (B) has a full-thickness defect in its cell wall (encircled area) and appears to be losing nucleoid material through this gap. The cytoplasmic contents are only partially present in a cell (C) which shows more extensive changes. The wall of this meningococcus (C) contains recognizable middle dense line material in contrast to the absence of such material (arrow) in the adjacent cell (D) which is nearly completely lysed. x 35,000.

After 30 min exposure of meningococci to penicillin, the majority of organisms show blebs on their outer membranes (Fig. 17-5, 17-6, 17-7). Many of these outpouchings are nearly as large as the organism from which they arise. Other defects in the continuity of the bacterial wall are occasionally observed in the 30 min specimens. Full-thickness gaps in the wall are seen (Fig. 17-6) and may be associated with outpouring of nucleoid material. These strands of the nucleoid, which normally occupies a central position in the cytoplasm, are derived from the enlarged, somewhat distorted nucleoid, which often extends to a more peripheral position in the cytoplasm of cells after this duration of penicillin treatment (Fig. 17-7). Mesosomal structures are difficult to find in any of the organisms. In addition, some foci of the peripheral cytoplasm are rarified and appear poorly fixed. This change is unlikely due to a fixation artifact as the control cells do not show the same changes, and similar alterations have been seen in the penicillin treated organisms in several experiments utilizing modifications of fixation time, temperature, etc. These foci of rarified cytoplasm are often associated with a slight outward bulging of the overlying bacterial wall. This partially ballooned portion of the wall sometimes exhibits a markedly thinned, ragged middle dense line (Fig. 17-5).

By 45 min after penicillin addition (Fig. 17-8) many of the cells in the specimen have lost the bulk of their cytoplasmic contents and consist of membranous bags whose walls are comprised of two layers of unit-type membranes with little recognizable middle dense line material (Cell D, Fig. 17-4). These cells are obviously lysed forms. All of the cells in the specimen do not show the same extent in alterations of structure, and a considerable number have changes which were described above in cells treated with penicillin for shorter periods of time.

The fine-structural changes present in penicillin-treated meningococci consist, primarily, of disordered relationships among the cell wall laminae. Large blebs on the bacterial surface have been described in light microscopic studies on penicillin-treated E. coli by Hahn and Ciak. The present study correlates with these observations and demonstrates that the outpouchings consist only of outer membrane, the outermost lamina of the complex, Gram negative cell wall. However, the basis for formation of these wall defects is not entirely clear.

Penicillin has been shown to inhibit the final polymerization step in the synthesis of mucopeptide which comprises the rigid layer of the Gram negative bacterial wall. Mucopeptide has been reported by Murray, Steed, and Elson as being analogous to the thin layer, or middle dense line, of the cell wall. On the basis of these observations, one would expect to find gaps or other evidence of loss in structural integrity appearing first in the middle dense line of the wall in organisms which have been exposed to penicillin. The present study shows that the blebs which form on the cell wall are composed of outer membrane and overly an intact middle dense line. Two alternative explanations for these disparities seem plausible. First, the middle dense line may not

represent mucopeptide but may actually be a layer of globular protein, which is topically associated with mucopeptide, as suggested by Remsen and Lundgren. This concept finds support in the experiments of Work, et al. who finds that purified mucopeptide is not electron opaque after staining with the usual heavy metal stains used in electron microscopy. Against this explanation is the finding of disappearance of the middle dense line from organisms which have been treated with lysozyme. Studies in this and other laboratories have confirmed this ultrastructural consequence of lysozyme treatment. Because lysozyme or muramidase hydrolyzes mucopeptide, an associated globular protein would not be expected to disappear upon treatment with the enzyme and specific removal of mucopeptide from the cell wall.

The second possible explanation is that the final polymerization step in mucopeptide synthesis enhances or permits the attachment of the outer membrane, which is lipopolysaccharide in nature, to the mucopeptide polymer. Penicllin, in inhibiting the final polymerization step, would lead to synthesis of "defective" mucopeptide which would not bond to the outer membrane in the usual fashion. This would lead, then, to segments of outer membrane which are unstable because of defective or no attachment to the underlying mucopolymer which is known to be essential for maintenance of the structural integrity and shape of the cell.

The effect of penicillin on the mesosome is of interest in that 1) the changes in mesosomal morphology are seen soon after addition of the antibiotic, and 2) the mesosomal region may function in synthesis of the cytoplasmic membrane or replication of the nucleoid or both. The alterations which are noted in this structure suggest that penicillin may act on a synthetic process concerned with plasma membrane production in addition to mucopeptide synthesis or that the mesosome is, in some way, intimately associated with mucopeptide synthesis.

The nucleoid of penicillin-treated cells appears to enlarge and become disproportionate in size in relation to the rest of the organism. This increase may be apparent because of a preferential accumulation of fluid in this nucleoid region as compared to the remainder of the organism. However, penicillin is well known to affect only organisms which are in a growth phase and that the cells continue to divide and remain viable for about one generation time following addition of the antibiotic. The increase in size of the nucleoid region may signal continuing synthesis of nucleoprotein which proceeds until the cell lyses because of its cell wall defects.

VIII. Ultrastructural Alterations in Meningococci after Treatment with Normal and Modified Rat Serum.

Electron microscopic studies have been carried out on Neisseria meningitidis exposed to normal rat serum in order to document the changes which accompany killing and lysis of the organism in this bactericidal system. Neisseria catarrhalis and Herellea sp. also have been studied

after treatment with rat serum to compare the changes in these serum-susceptible bacteria to those found in meningococci. The role of lysozyme in producing the final morphology of serum-killed meningococci was evaluated by exposing cells to lysozyme deficient serum with and without added egg white lysozyme. The "holes" which appear in the wall of meningococci after reaction of antibody and complement have been defined and have been localized as occurring in the outer membrane of the cell wall. Finally, "holes" in the walls of normal, untreated meningococci have been found and have been compared to the complement-produced "holes" in serum-treated organisms.

Five hr old cultures (Mueller-Hinton agar) of serum-sensitive meningococci (Type A, strain A_1), N. catarrhalis, and Herellea sp. (10^8/ml) were reacted at 37°C with 10% heated rat serum (56°C, 30 min) or 10% normal rat serum (Lewis rats) for 15 min. After this time aliquots were withdrawn for determining the number of surviving organisms. The remainder of the specimen was immediately processed by the method of Ryter and Kellenberger for electron microscopic examination. The same strain of meningococci was also examined by colony counts and electron microscopy at varying times after the addition of bacteria to the serum-containing reaction mixture to study the sequence of changes which occur in these cells. Lysozyme-deficient serum was prepared by absorption with bentonite which, in itself, is non-toxic to the cells. Additional lysozyme was added in the form of egg white lysozyme to the bentonite absorbed serum. Negative staining with 1% phosphotungstic acid was carried out on serum-treated, water-washed meningococci and on specimens which were directly withdrawn from a broth culture medium.

The ultrastructure of meningococci which have been exposed to heated rat serum (HRS) is identical to that of cells suspended in phosphate buffered saline (PBS-Dulbecco) and is shown in Figs. 18-1 and 18-2. The cytoplasm is composed of tightly packed ribosomes and a loose, net-like nucleoid region and is bounded by the cytoplasmic membrane. This membrane forms the innermost lamina of the organism's complex cell wall which is typical for Gram negative bacteria. The various parts of this cell wall and the interposed portions of the periplasmic space are shown in detail in Fig. 18-2. The outermost lamina, the outer membrane, is a rugose, trilaminar, unit-type membrane that is 75 Å thick. At irregular intervals, this outer membrane contacts the middle lamina of the wall, the middle dense line. Between these outer two layers of the wall is the outer periplasmic space. The inner periplasmic space is bounded by the middle dense line and the cytoplasmic membrane. The latter structure is, like the outer membrane, a typical unit-type membrane, 75 Å thick, with a trilaminar profile. HRS-treated meningococci show no electron-dense material on their external surfaces. This was a constant finding in numerous experiments and contrasts with the appearance of cells which have been grown in fluid medium and which may have darkly stained material on their surfaces.

Exposure of A_1-meningococci to normal rat serum (NRS) for 30 sec did

Figure 18

18-1: This cell exhibits the usual morphology of untreated or HRS-exposed N. meningitidis from a 5 hr culture. The cell wall consists of three layers (see Fig. 18-2). The rugose wall is devoid of any electron-dense material on its surface. The cytoplasmic contents are compact in appearance except for the loose, net-like nucleoid. x 70,000.

18-2: A portion of the wall of a HRS-treated meningococcus shows clear demarcation of the outer membrane (OM), middle dense line (DL), and cytoplasmic membrane (CM). These three laminae enclose the outer periplasmic space (OPS) and the inner periplasmic space (IPS). x 140,000.

18-3: A small amount of amorphous, electron-dense material (arrows) on the outer membrane is the only definite morphological alteration in this cell which has been exposed to NRS for 30 sec prior to fixation. x 70,000.

18-4: After 2 min exposure to NRS, several alterations in ultrastructure are present in this organism. Abundant material coats the exterior and has a fibrillar appearance. The inner periplasmic space (IPS) is widened and is relatively "empty" as compared to the outer periplasmic space. The electron-dense material in the outer periplasmic space appears to be continuous with the fibrillar material coating the cell and seems to penetrate the outer membrane. The overall density of the cytoplasm is only slightly decreased as compared to controls. x 70,000.

18-5 and 18-6: After a 5 min exposure to NRS these meningococci show severely deranged morphology. Large gaps in the cytoplasmic membrane (CM) are associated with a spillage of ribosomes (R) beyond the usual confines of the cytoplasm. Small foci of discontinuity (rectangular inset, Fig. 18-6) are present in the middle dense line. Fibrillar material appears to contact the remaining solid portions of the middle dense line and the material has a radial orientation. The particulate elements of the cytoplasm are widely separated to give the interior of the cell a "watery" appearance. Fig. 18-5 - x 70,000; Fig. 18-6 - 140,000.

18-7: N. catarrhalis shows essentially the same morphology as N. meningitidis after exposure of both to HRS. The middle dense line (DL) of N. catarrhalis is thicker than in meningococci.

18-8: Exposure of N. catarrhalis to NRS for 15 min results in morphological alterations similar to those in N. meningitidis after the same treatment. Large gaps of the cytoplasm are present and there is intracytoplasmic edema. The middle dense line (DL), however, is intact. x 70,000.

18-9: This cell of the genus Herellea has been exposed to HRS for 15 min. The cell has closely apposed wall laminae, extended fimbriae, and a compact cytoplasm. x 70,000.

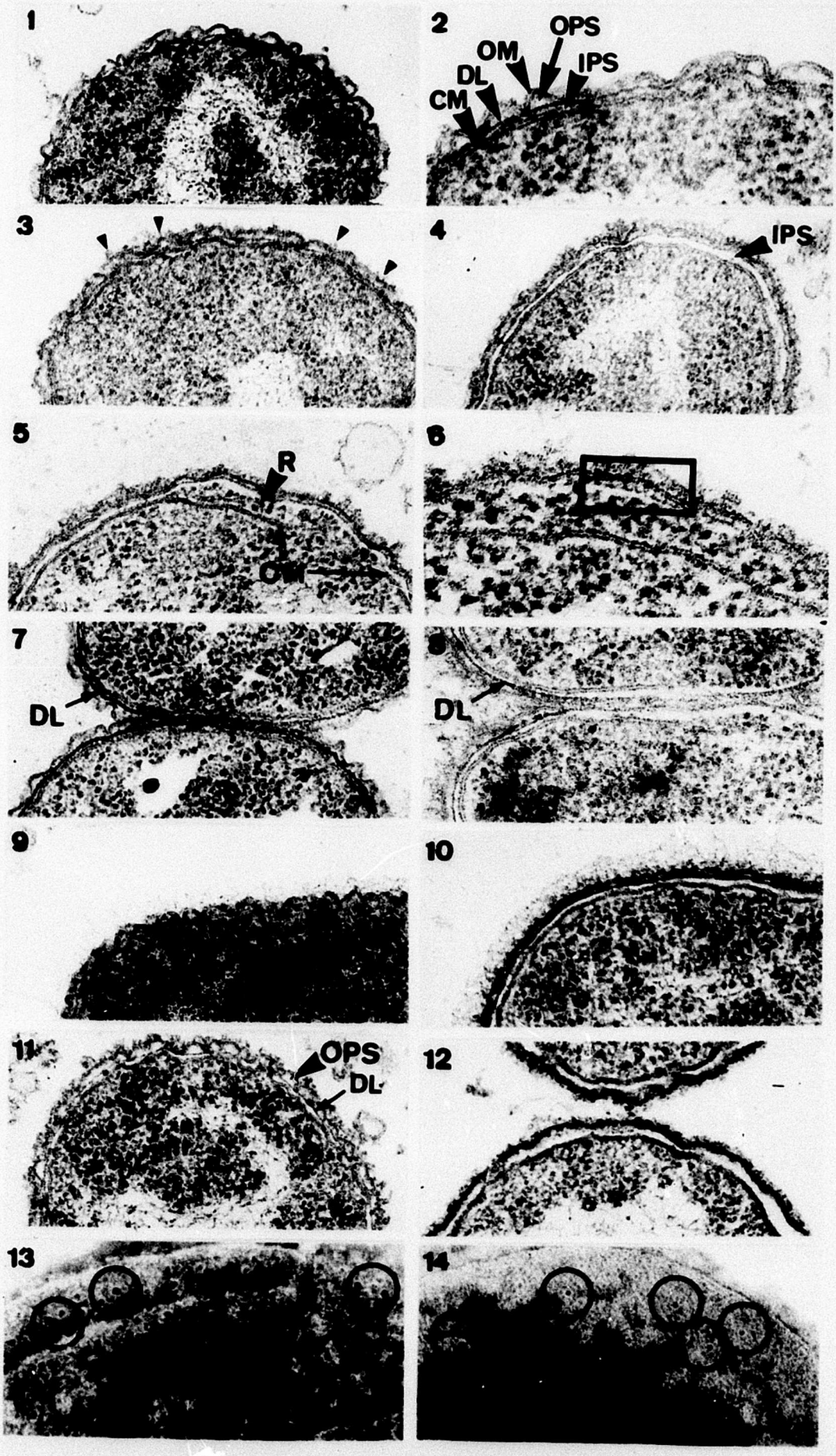
18-10: A 5 min exposure to NRS converts the morphology of this Herellea sp. cell to that resembling a meningococcus treated with NRS for 2 min. The fimbriae appear curved and admixed with electron-dense fibrillar material. Edema is present mainly in the inner periplasmic space. The cytoplasmic membrane and the middle dense line are intact. x 70,000.

18-11: A coating of electron-dense fibrillar material, enlargement of the periplasmic space, and slight intracytoplasmic edema are present in this cell which has been exposed to LDS for 5 min. The outer periplasmic space (OPS) is more prominent than the inner periplasmic space. The middle dense line (DL) is intact. x 70,000.

18-12: Addition of EWL to LDS is associated with disappearance of the middle dense line and enhancement of the intracytoplasmic edema. The cytoplasmic membrane is intact. x 70,000.

18-13: The entire surface of the A_1-meningococcus has discrete foci of accumulated phosphotungstate following exposure to normal rat serum, water washing, and negative staining. The foci, which resemble "holes", are 110 Å in mean diameter and are surrounded by light halos. x 100,000.

18-14: Direct negative staining of fluid from a 24 hr culture of meningococci (no intermediate washing step) results in small accumulations of negative stain (mean diameter = 82 Å). These foci or "holes" are also surrounded by a light halo and are of the same diameter as the foci seen in meningococci after water washing and heated serum treatment. x 100,000.


1
2
OPS
OM
IPS
DL
CM
3
4
IPS
5
R
CM
6
7
DL
8
DL
9
10
11
OPS
DL
12
13
14

not result in detectable killing of the organisms as compared to colony counts performed on HRS-or PBS-controls. The fine structure, however, is altered by this brief treatment. Small amounts of rather amorphous, electron-dense material are present on the exterior of the organisms (Fig. 18-3).

Two minutes after addition of normal rat serum to meningococci, approximately 50% of the organisms have been killed. The changes in morphology are shown in Fig. 18-4 and consist of an increased amount of electron-dense on the exterior of the organism and dilatation of the periplasmic space. The material which accumulates on the meningococcal surface has a fibrillar appearance and seems to protrude through the outer membrane to contact the middle dense line. Widening or dilatation of the periplasmic space primarily occurs in the inner compartment between the middle dense line and the cytoplasmic membrane. No discontinuity is seen in any of the three wall laminae.

After 5 min in normal rat serum, all the exposed organisms are dead. In addition to changes noted above, these meningococci exhibit breaks or gaps in their cytoplasmic membranes after 5 min in serum (Fig. 18-5). This is associated with "herniation" of ribosomes into the periplasmic space and with intracytoplasmic edema. Small disontinuous foci are present along the middle dense line. The fibrillar material seems to extend through the outer membrane and contact the remaining solid portions of the middle dense line (Fig. 18-6).

Additional exposure of meningococci to NRS results in accentuation of the changes present after 5 min. By 30 min, little internal architecture is recognizable in NRS-treated cells.

Both Neisseria catarrhalis and Herellea sp. are killed by brief exposure to NRS and exhibit marked alterations in fine structure. N. catarrhalis control specimens (either PBS or HRS treated) are similar in morphology to N. meningitidis except that the middle dense line is thicker (25 - 35 Å) in N. catarrhalis than in meningococci. After 15 min in NRS, N. catarrhalis shows accumulation of electron-dense fibrillar material, breaks in its cytoplasmic membrane, and marked edema. However, no discontinuity of the middle dense line is present (Fig. 18-8).

Herellea sp. differs from both N. meningitidis and N. catarrhalis in that treatment with NRS produces no breaks in the cytoplasmic membrane of Herellea sp. This organism, which bears extended fimbriae on its surface (Fig. 18-9), also does not show dissolution or discontinuities in its middle dense line (Fig. 18-10).

Removal of lysozyme from normal rat serum by bentonite absorption and exposure of A_1-meningococci to the lysozyme-deficient serum (LDS) produces little in the effectiveness of the bactericidal activity of the serum. Thus, LDS is as efficient as NRS in killing meningococci. The morphological changes associated with 15 min exposure of organisms to LDS is quite different from the picture of NRS-exposed meningococci.

LDS-treated cells (Fig. 18-11) bear fibrillar material on their surfaces and have periplasmic edema, but the widening of this space is primarily in the outer compartment. The middle dense line and the cytoplasmic membrane remain intact even after 15 min incubation of meningococci in LDS. Addition of egg white lysozyme (EWL) to LDS results in disappearance of the middle dense line and an increase in intracytoplasmic edema. However, the complete pattern of changes seen in NRS-treated cells is not reproduced by LDS + EWL.

Negative staining of meningococci treated with NRS revealed phosphotungstate stain accumulations which have the appearance of "holes" on the surface of the cells (Fig. 18-13). These "holes" have a mean diameter of 110 ± 3 Å. Penicillin treatment of meningococci induces the formation of blebs or outpouchings of the outer membrane of the wall. Penicillin-pretreated, NRS-exposed organisms had 110 Å "holes" on the blebs that were present. This finding localizes the defects ("holes") produced by serum to either the outer membrane of the mucopeptide layer (which may not be visualized due to lack of electron density). To further localize the lamina of the wall in which "holes" are produced, cells were negatively stained after exposure to ODS or LDS + EWL. The appearance of the "holes" on the meningococci was identical after these treatments. Because EWL removes mucopeptide, it was concluded that the outer membrane in the layer of the cell wall in which "holes" are produced by rat serum (antibody and complement).

Examination of serum-susceptible A_1-meningococci exposed to HRS or PBS, or of serum-resistant C_{11}-meningococci treated with NRS, demonstrated by negative staining that these organisms also have "holes" in their walls. These accumulations of negative stain are 82 ± 1.8 Å in diameter. These smaller "holes" were easily differentiated from the 110 Å lesions and also appeared less numerous than the larger variety. Because all the preparations previously described had been water-washed after serum treatment and before negative staining, the possibility existed that this preparative step introduced the 82 Å defects (Fig. 18-14). This was ruled out by placing a drop of a broth culture (24 hr) on a grid and mixing with negative step without intervening washing. "Holes" of the 82 Å diameter type were also seen in these specimens. Therefore, it was concluded that the 82 Å "holes" are present in normal organisms.

The sequence of changes which occur in meningococci upon exposure to normal rat serum suggest that the various laminae of the cell wall act, in concert, to protect the organism from the osmotic forces of the external environment. These protective mechanisms are overcome by the bactericidal substances present in rat serum. The components which act on the organisms were shown to be a recognition (antibody) mechanism and an effector (complement) mechanism.

It is assumed that the initial insult delivered to the cell wall by normal rat serum is the production of 110 Å "holes" by complement after preliminary attachment of antibody. Following this, edema of the periplasmic space occurs and the pattern of this fluid accumulation depends

on the integrity of the middle dense line (mucopeptide?). In LDS, in which no hydrolysis of mucopeptide would be anticipated and in which the middle dense line remains intact, the edema is mainly in the outer periplasmic space. Conversely, in NRS, which contains lysozyme, the edema is primarily in the inner periplasmic space. This suggests that the mucopeptide layer (or globular protein represented by the dense line) plays a role in the organism's osmotic regulation.

It was noted that addition of egg white lysozyme to bentonite-absorbed serum did not duplicate the morphological changes present in organisms exposed to normal rat serum. This suggests that bentonite removes some factor, in addition to lysozyme, that plays a role in the bactericidal reaction by its action on the cytoplasmic membrane. This is suggested because the cytoplasmic membrane remained intact in spite of marked periplasmic and intracytoplasmic edema in organisms treated with LDS + EWL. An alternative explanation for rupture of the cytoplasmic membrane after NRS treatment of meningococci is that this is an affect of an autolytic enzyme. Although N. meningitidis is autolytic, N. catarrhalis is not, and the finding of gaps in the cytoplasmic membrane of N. catarrhalis rules against this change being due to an autolytic mechanism.

The 110 Å "holes" which are produced in the wall of serum-susceptible meningococci by rat serum were localized as occurring in the outer membrane of the wall. This correlates with the supposed outer membrane-origin of Gram negative endotoxin and the production of "holes" by antibody and complement in partially purified endotoxin preparations.

The "holes" in normal meningococcal walls may have some relationship to the production of larger "holes" by complement or to some physiological function of the organism, or both. This study establishes that the small "holes" (82 Å) and the large "holes" (110 Å) form two distinct populations on the basis of size ($P < 0.01$ in test for difference of the two means). Elucidation of any further relationship awaits additional study.

Summary and Conclusions.

I. Two experiments were done in BCT troops at Fort Dix, N.J. to test the hypothesis that intranasal instillation of Av-4, vaccine virus, to persons previously immunized by the oral route would lead to successful colonization of the respiratory tract with vaccine cirus, and subsequent interference with the introduction and transmission of other naturally occurring adenoviruses. At the time of both experiments (Oct 1967; Feb 1968) the predominent virus on post responsible for ARD was AV-7; the tests there were done under appropriate circumstances. Nasal administration of vaccine virus three weeks post-immunization by the gastrointestinal route failed in both instances to interfere significantly with natural transmission of AV-7, and to reduce significantly hospitalization for disease caused by that virus.

II. Investigation of changes in classes of immunoglobulins and associated antibody activity in basic combat trainees showed that significant amounts of IgM were produced consistently by recruits, reaching maximal levels during the third week of basic training; IgG and IgA levels remained relatively constant. The magnitude of the IgM response apparently induced by endotoxin contained in typhoid vaccine, could not be correlated with either the occurrence of overt ARD or its severity.

III. The rubella HI tests made were reproducible by careful attention to control of several additional physico-chemical variables were found to be a useful adjutant to prenatal care of pregnant women. Epidemiological implications of data in over 2000 pregnant participants are discussed.

IV. Studies of the induction of interferon by various substances in rabbit peritoneal macrophages show that while two interferons from the same species differ in physico-chemical characteristics, and in conditions for optimal synthesis, they apparently exert their antiviral effect via a common pathway. Studies are in progress to extend these preliminary observations with particular emphasis on factors governing synthesis of EIF by macrophages and its activity under various environmental conditions.

V. Populations of dengue virus type II can be separated into components on the basis of their sedimentation behavior in sucrose gradients. Two major peaks of hemagglutinin (HA) activity exist; the more rapidly sedimenting HA (RHA) consists of the complete virion and is the only component which is infectious. The slowly sedimenting HA (SHA) is a spherical particle measuring 140 $\mathring{A}$ in diameter of unknown origin or function. The HA activity of this particle is inhibited by dengue-immune ascitic fluid as readily as in that of RHA. RHA and SHA exist in approximately equal proportions in infected mouse brain. There is no evidence to suggest that SHA results from fragmentation of RHA.

Both RHA and SHA contain surface complement-fixing antigens. In addition, a third zone of CF antigen activity is identified in sucrose gradients. This very slowly sedimenting CF antigen, which possesses no HA activity, consists of spherical particles 70$\mathring{A}$ in diameter. Their appearance is similar to that of capsid subunits visible on the surface of intact virions, and it is likely that this antigen consists of isolated subunits.

Estimates of particle density by equilibrium gradient centrifugation in cesium chloride indicate that RHA has a density of 1.22 and SHA has a density of 1.23. Mouse brain dengue virus sediments in CsCl such that particles of three densities are distinguished; 1.31, 1.22 and 1.18. The component with a density of 1.18 is observed when RHA is tested also, and most probably represents fragments of disrupted virions. This component is not found following sedimentation of SHA.

Inhibitors of dengue virus HA exist in serum (FBS) and normal mouse brain. Gradient studies have shown that while serum inhibitor remains at the tope of a 5-25% sucrose gradient, tissue inhibitor is distributed throughout the gradient. Neither inhibitor alters the sedimentation characteristics of RHA or SHA nor affects their CF activity. When RHA and SHA are separated from serum inhibitor by centrifugation, normal HA activity is restored, indicating that the reaction is reversible or simply that the inhibitor exerts a masking effect.

Monolayer cultures of BSC-1 and PS cells serve as good plaque assay systems for dengue viruses. A method for kinetic analysis of dengue neutralization is described in these cells. The incorporation of guinea pig serum in incubation mixtures in this test results in potentiation of neutralizing antibody. Studies are in progress to detect intra-typic strain differences among dengue viruses.

Other work in progress includes efforts to characterize further the virus components described. Particular emphasis will be placed on the concentration and purification of isolated CF antigen and subsequent measurement of its immunogenicity.

VI. Studies of the natural history of EEE and WCE virus in the Pokomoke Cypress swamp were continued. Bird and mosquito, but not mammalian infection was detected during the late summer and early fall of 1967 with both viruses using conventional virological methods. Climate, rainfall, height of tides and ambient temperature were again found to be variables apparently affecting vector concentrations and subsequent virus transmission. The significance of the findings and additional questions raised by them are discussed.

VII. Addition of normal and modified rat serum to suspensions of neisseria result in death of the organism accompanied by the production of defects in the outer periplasmic membrane. This reaction is similar to that obscured by treatment with specific antibody.

VIII. Treatment of neisseria with Penicillin results in a specific morphological sequence of counts which can be related to the bacteriocidal action of this antibiotic in the organism.

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24. (U) TECH OBJECTIVE - PRINCIPAL AIMS ARE- 1. SCRUB TYPHUS VACCINE DEVELOPMENT, 2. IMPROVED METHODS FOR IDENTIFICATION OF RICKETTSIAE, 3. IMMUNOLOGICAL STUDIES OF RICKETTSIAL DISEASES, 4. DETERMINATION OF THE ENZOOTICITY OF A NEW TYPHUS GROUP MEMBER IN THE U. S., 5. IMPROVED METHODS FOR LAB DIAGNOSIS OF SCRUB TYPHUS, 6. RELATIVE EFFECTIVENESS OF Q FEVER PHASE I AND II VACCINES, 7. OCCURRENCE OF RICKETTSIAL DISEASES IN S.E.ASIA, AND 8. IMPROVEMENT OF SPECIFIC THERAPEUTIC MEASURES FOR TREATMENT.

25. (U) APPROACH- 1. DETERMINE THE ANTIGENIC DIVERSITY AMONG STRAINS OF R. TSUTSUGAMUSHI. 2. EVALUATE FLUORESCENT ANTIBODY STAINING TECHNIQUES FOR RAPID, SPECIFIC RICKETTSIAL IDENTIFICATION. 3. UNDERTAKE IMMUNOCHEMICAL CHARACTERIZATION OF ANTIBODY RESPONSE FOLLOWING SCRUB TYPHUS INFECTION AND IMMUNIZATION. 4. SEARCH FOR A WILD ANIMAL RESERVOIR OF R. CANADA. 5. TRIAL OF SCRUB TYPHUS IS TEST IN OVERSEAS MILITARY LABS. 6. CHARACTERIZE PHASE OF Q FEVER VACCINES. 7. SEROLOGIC TESTING OF SERA FROM SUSPECT CASES IN THAILAND AND VIETNAM. 8. TEST FEWER ANTIBIOTICS FOR RICKETTSIOCIDAL ACTIVITY.

26. (U) PROGRESS - JUL 67 THRU JUN 68 1. AT LEAST 5 AND POSSIBLY 8 DISTINCTIVE SCRUB TYPHUS STRAINS HAVE BEEN FOUND. 2. ANTI-RICKETTSIAL FLUORESCEIN CONJUGATES ARE BEING TESTED. 3. THE INFLUENCE OF DOSE, ROUTE OF INOCULATION AND ANTIBIOTIC TREATMENT ON THE CF AND IF ANTIBODY RESPONSE OF MICE INFECTED WITH SCRUB TYPHUS ARE BEING CHARACTERIZED. 4. SEROLOGIC EVIDENCE OF TYPHUS GROUP INFECTION OF GLAUCOMYS VOLANS HAS BEEN FOUND. 5. REAGENTS FOR THE SCRUB TYPHUS IF TEST ARE BEING SUPPLIED TO 6 MILITARY LABS IN S.E. ASIA. 6. PHASE I VACCINE PILOT PRODUCTIONS ARE BEING STUDIED. 7. SCRUB TYPHUS ACCOUNTS FOR 10-30 PERCENT OF UNDIAGNOSED FEBRILE DISEASES IN U.S. PERSONNEL IN VIETNAM. 8. OF 16 ANTIBIOTICS TESTED THUS FAR ONLY TETRACYCLINES EXHIBIT RICKETTSIOCIDAL ACTIVITY. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 67 - 30 JUNE 68.

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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.

During the current reporting period, research efforts concerned with the scrub typhus vaccine development program included: (1) An antigenic analysis of 5 candidate prototype strains of R. tsutsugamushi recovered in Thailand which are distinctive from the standard Karp, Kato and Gilliam strains, and attempts to develop them for possible use in a polyvalent killed vaccine; and (2) characterization of the antibody response of mice following experimental infection. Investigations were initiated to evaluate the effectiveness of newly developed antibiotics for treatment of rickettsial diseases. Studies were carried out to identify an agent causing human disease in Thailand that had not been recognized previously. A field trial of the scrub typhus indirect immunofluorescent test was made by military laboratories in Southeast Asia. The immunologic and biologic properties of R. canada n. sp. were compared with the other members of the Typhus Group prior to investigating the presence of this newly discovered rickettsial species in the United States. In the course of development of a Q fever phase I vaccine, the phase composition of seed materials and pilot products was determined.

Progress.

1. Scrub Typhus Vaccine Development. Over the past several years the Department of Rickettsial Diseases has been evaluating the feasibility of developing a killed polyvalent vaccine which would protect man against the antigenically diverse strains of Rickettsia tsutsugamushi that exist in nature. Based upon reports from Japanese workers that all strains of scrub typhus rickettsiae recovered in Japan were related antigenically to either the Gilliam, Karp or Kato strains, the prospect of formulating a trivalent vaccine was promising. However, an antigenic analysis of 78 strains of R. tsutsugamushi isolated in Thailand revealed that in addition to the previously established prototype Gilliam, Karp and Kato strains, possibly 5 other antigenically distinctive strains existed. Classification of the scrub typhus strains was based upon the complement-fixing reactivity of immune sera from guinea pigs infected with the newly recovered agents. The antigens for the complement fixation tests were comprised of partially purified suspensions of the three prototype

strains and other distinctive Thai strains as they were recognized. The majority of the agents recovered in Thailand were found to be mixtures of 2 or more different antigenic types. In defining the composition of the mixture, only the strains related to those used for the preparation of antigens could be recognized. Other antigenically distinctive strains that might be present would not be detected. Years of additional time and effort would be required to define all of the different antigenic types that might be included in the Thai collection of strains without bringing the stated goal of vaccine evaluation any closer. At the present time, with the knowledge of the existence of at least 8 antigenically diverse strains, the practicality of developing an effective polyvalent killed vaccine may be seriously questioned. However, the prospects of success in finding other means of affording man immunity against this disease are even more remote. Therefore, during the past year one of the principal efforts has been concerned with antigenic characterization of the Thai strains which may represent new prototypes of R. tsutsugamushi and attempts to develop them for possible use in a polyvalent vaccine.

Mice successfully immunized with killed monovalent scrub typhus vaccines are afforded protection against only infection with the homologous strain. On the other hand, mice infected with any strain of R. tsutsugamushi survive lethal challenge with all other strains. Indeed, this immunity-challenge test provides the basis for definitive identification of scrub typhus rickettsiae. Studies were initiated during the recent year to investigate the nature and mechanism of the protection afforded mice by infection.

a. Characterization of Candidate Prototype Strains of Scrub Typhus Recovered in Thailand. Included among a collection of strains of scrub typhus recovered from patients, wild animals, and chiggers in Thailand were 5 strains which showed no relationship to the established prototypes, Gilliam, Karp and Kato. Serum from guinea pigs inoculated with suspensions of spleens from mice infected with TA 763, TA 716, TA 678, TA 686, and TH 1817 did not fix complement in the presence of the highly strain-specific prototype antigens. The adaptation of these unclassified candidate prototype agents to cultivation in embryonated hens' eggs was described last year. Since then a concerted effort has been made in an attempt to obtain the high concentrations of rickettsiae required for the production of satisfactory complement-fixing antigens. Serial passages of 2 or 3 lines of each of the strains were made in embryonated hens' eggs at approximately 10-day intervals. Except when bacterial contamination, or unidentified adventitious lethal agents from the eggs were encountered necessitating the use of stored frozen material, all the passages were carried out with freshly harvested yolk sac. A Giemsa-stained smear of each yolk sac was examined microscopically and the tissue containing the heaviest concentration of organisms was used to initiate the next passage or, if not needed for this purpose, it was stored for the preparation of complement-fixing antigens. The difficulties that have been encountered in obtaining sufficient numbers of heavily

infected yolk sacs can be exemplified with the experience with the "well-established" Kato prototype strain. Over 2 years ago only about one yolk sac of every 65 eggs inoculated with the 30th egg passage was satisfactory for antigen production. Currently, with the 86th egg passage, about 10% of yolk sacs of inoculated eggs are heavily infected. During the past year, yolk sacs heavily infected with the Thai candidate prototype strains have been found infrequently even though some of the agents are now beyond the 40th egg passage.

Complement-fixing antigens have been prepared from yolk sacs infected with the Gilliam, Karp and Kato strains which are highly strain-specific and devoid of anti-complementary and non-specific reactivity. These antigens do not react significantly with a 1:10 dilution of heterologous guinea pig sera which contained as much as 128 to 512 units of antibody. It has not yet been possible to prepare similar strain-specific antigens from yolk sacs infected with each of the 5 unclassified Thai agents. Table I summarizes the results of tests with the complement-fixing antigens that have been produced. The specific homologous reactivity of Gilliam, Karp and Kato antigens is evident. The homologous antibody titers with the TA 763 and TA 716 antigens are significantly higher than the heterologous reactivity with each other and with the Karp and Kato strains. Although these data show the strains are different, the possibility that each also contains the other cannot be unequivocally excluded. TA 678 antigen displayed only homologous activity. TH 1817 exhibited slight reactivity with sera from some TA 678 infected guinea pigs. It has not yet been possible to prepare any satisfactory antigens from TA 686 infected yolk sacs.

The data suggest that each of the 5 unclassified scrub typhus agents represent an antigenically distinctive strain. Efforts will continue to improve the cultivation of these candidate prototypes in embryonated eggs, and the quality of complement-fixing antigens prepared from infected yolk sac tissue, in order to confirm the purity and antigenic individuality of the strains.

b. Characterization of the Antibody Response of Mice Following Experimental Scrub Typhus Infection. Classification of scrub typhus organisms on the basis of the complement-fixing reactivity of the dominant antigenic component of selected prototype strains with immune sera from guinea pigs infected with wild strains did not simplify the problem of antigenic heterogeneity as expected. Thus far, 8 distinctive antigenic types have been found and it remains to be determined if wild strains with the same dominant antigenic component share the same minor antigens. Last year the preparation of killed vaccines from yolk sacs infected with the Karp and Kato strains which provided significant homologous protection was reported. Vaccines prepared with the prototype Gilliam strain, although comparable in rickettsial antigen content, were not satisfactory. Studies have continued with the Thai strain, TC 586, which has been identified as probably a Gilliam homotype, and is

being considered as a candidate to replace the prototype Gilliam in experimental vaccines. Cross vaccination-challenge experiments which are necessary to establish definitive identity have not been carried out because the yolk sac concentrations of TC 586 required for the production of a satisfactory vaccine have been attained only rarely. Similar problems have been encountered in the propagation of other vaccine-candidate strains in embryonated hens' eggs (see previous section). Studies undertaken in mice by previous workers have shown that killed scrub typhus vaccines provide protection principally against infection with the homologous strain. Furthermore, vaccines were effective against an intraperitoneal challenge only if administered by the same route. Subcutaneous inoculation of the same doses of vaccine was not effective. Trials undertaken last year using killed rat lung-spleen vaccines failed to show a relationship between the presence or absence or level of complement-fixing antibodies (CF) and those detected by indirect immunofluorescence (IF), with the ability of the mouse to survive challenge with the homologous strain.

In contrast with the homologous protection afforded by killed vaccine, mice convalescent from infection with one scrub typhus strain resist lethal challenge of all other strains. It was expected that an understanding of the basis of the immunity provided the mouse by infection might lead to a better means of protecting man against disease than could be achieved with a polyvalent killed vaccine. Studies were initiated to characterize the antibody response following infection with the prototype Gilliam, Karp and Kato strains and subsequent heterologous challenge. The effect of route of inoculation, size of infectious dose, and antibiotic therapy upon the antibody response following initial infection, as well as the effect of size of the challenge dose inoculated intraperitoneally were investigated. The time periods for treatment and the interval between infection and challenge were those routinely employed in immunity challenge experiments for the definitive identification of scrub typhus strains.

For convenience of presentation, only the results of the experiments with the Gilliam strain will be presented in their entirety. Groups of 64 white mice weighing 10-12 grams were inoculated either intraperitoneally or subcutaneously with 0.2 ml of a large dose of the Gilliam strain ($10^{7.1}$ ID_{50}) and comparable groups were inoculated by either route with a small dose ($10^{2.6}$ ID_{50}). Half of the mice in each group were treated with chloramphenicol by administering the antibiotic in the drinking water (2.5 mg/ml) continuously from the third day after inoculation through the 21st day. In order to minimize undesirable effects of frequent retro-orbital bleeding, the mice in one category were subdivided. Blood was first collected from one group 3 days after inoculation and the sera pooled; and the second bleeding was obtained from the other group 4 days later. Thereafter, animals in each group were bled at weekly intervals.

TABLE I

COMPLEMENT FIXATION TESTS WITH ESTABLISHED PROTOTYPE SCRUB TYPHUS STRAINS AND CANDIDATE PROTOTYPES RECOVERED IN THAILAND

IMMUNE GUINEA PIG SERA	COMPLEMENT-FIXING ANTIBODY TITER ANTIGENS (2-4 UNITS)						
	GILLIAM	KARP	KATO	TA763	TA716	TA678	TH1817
GILLIAM	≥1280	—[+]	—	—	—	—	—
KARP	—	1280	—	20	40	—	—
KATO	—	—	320	20	20	—	—
TA 763	—	—	—	80 to ≥1280[++]	<10-320[++]	—	—
TA 716	—	—	—	40 to 160[++]	320-≥1280[++]	—	—
TA 678	—	—	—	—	—	≥80	<10-10[++]
TA 1817	—	—	—	—	—	—	≥80
TA 686	—	—	—	—	—	—	—

[+] NEGATIVE AT INITIAL 1:10 SERUM DILUTION

[++] RANGE OF VALUES ARE TITERS OBTAINED WITH DIFFERENT GUINEA PIGS

During the period following the initial infection, except for losses attributable to scrub typhus infection in the untreated groups and in other groups to the trauma of bleeding, the serum pools contained equal aliquots from a maximum of 16 mice. Prior to heterologous challenge on day 36, all the mice in a single category were mixed and randomly sorted into 3 equal groups. One group was inoculated intraperitoneally with 0.2 ml of a large dose of the Karp strain ($10^{5.5}$ LD_{50}), another group received a small dose ($10^{1.5}$ LD_{50}) by the same route, and the third group served as an unchallenged control. For the purposes of bleeding, the redesignated categories were again subdivided and half the mice were bled on alternate weeks and the sera pooled. Following challenge, the maximum number of mice contributing to a serum pool was 5.

The antibody content of the serum pools was determined by indirect immunofluorescent tests with yolk sac smears of the homologous Gilliam strain as antigen. The antigens used to detect homologous and heterologous complement-fixing antibody were the same partially purified suspensions of the prototype Gilliam, Karp and Kato strains used for the antigenic analysis of the Thai strains of scrub typhus described elsewhere in this report.

Figures 1 and 2 depict the antibody response of mice following intraperitoneal inoculation of $10^{7.1}$ ID_{50} of the Gilliam strain during treatment with chloramphenicol and when antibiotic therapy was not given, as well as after subsequent challenge with the Karp strain. In both the treated and untreated animals homologous CF and IF antibody was first demonstrated 7 days after inoculation. In spite of the early appearance of antibody, most of the untreated animals succumbed to the infection and only 3 mice survived beyond the 14th day. By contrast, 20 of the treated mice were still living at that time. Although the homologous CF and IF titers appeared to be generally higher in the untreated group, the difference was probably due to variations in individual antibody response. The apparent fluctuation in the levels of antibody in successive bleedings in these illustrations, as well as in the succeeding figures, can be attributed to the same reason. Heterologous CF antibody was first demonstrated on day 31, and both treated and untreated groups had developed Karp and Kato antibodies prior to challenge. No significant changes in the titers of homologous CF and IF antibodies, or in the level of heterologous CF antibodies, which could be attributed to either the low dose ($10^{1.5}$ LD_{50}) or high dose ($10^{5.5}$ LD_{50}) of Karp administered as the challenge were observed. In all of the experiments to be described, all animals surviving until the 36th day resisted the subsequent high and low dose challenge irrespective of the route used to initiate infection, the infectious dose or treatment with chloramphenicol. Deaths in the post challenge period were extremely rare and were due to the trauma of retro-orbital bleeding.

Figures 3 and 4 portray the antibody response following the subcutaneous inoculation of $10^{7.1}$ ID_{50} of the Gilliam strain under the same

FIGURE 1

ANTIBODY RESPONSE OF MICE FOLLOWING INTRAPERITONEAL INOCULATION OF $10^{7.1}$ ID_{50} GILLIAM STRAIN DURING TREATMENT WITH CHLORAMPHENICOL AND AFTER SUBSEQUENT CHALLENGE WITH THE KARP STRAIN

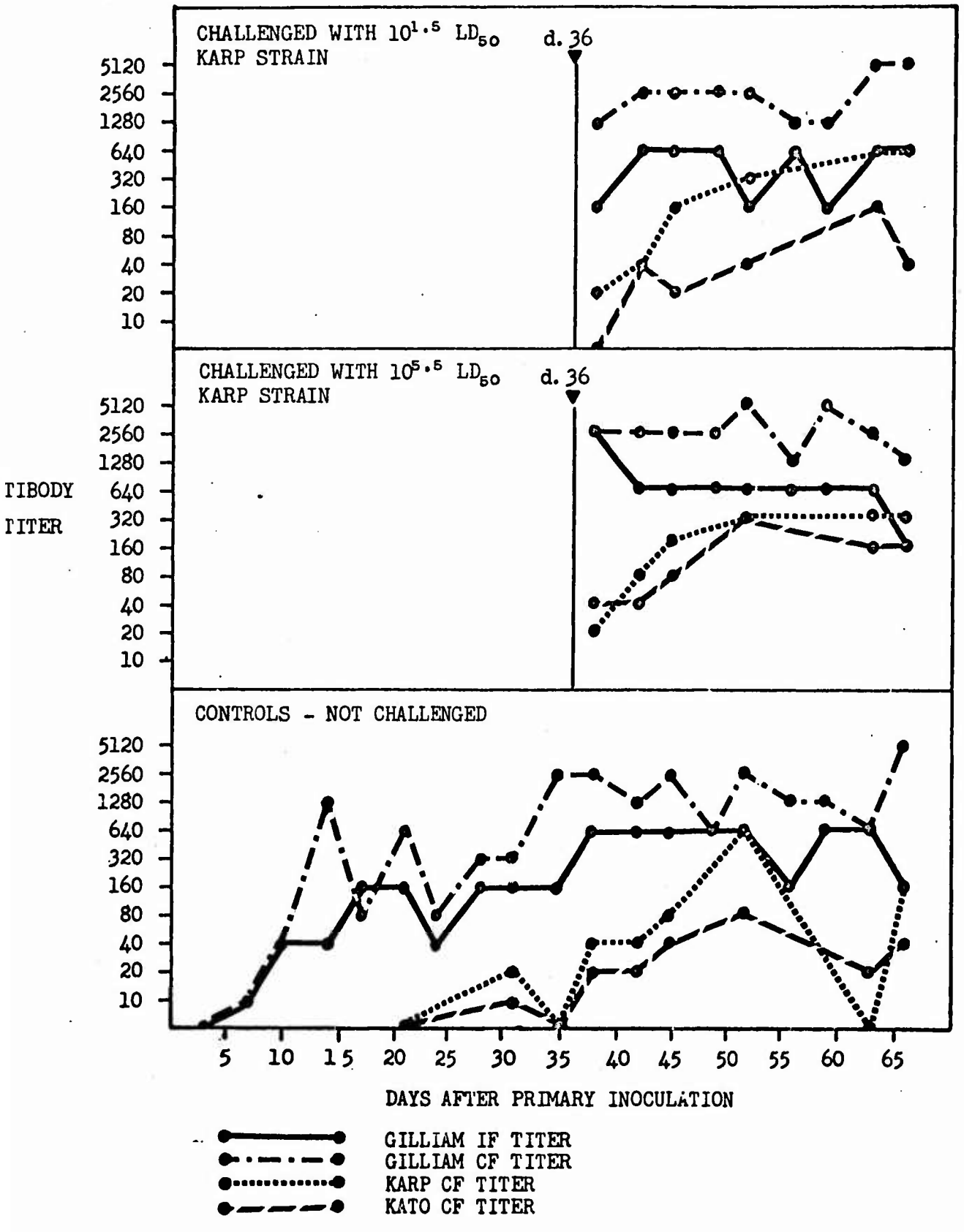


FIGURE 2

ANTIBODY RESPONSE OF MICE FOLLOWING INTRAPERITONEAL INOCULATION OF $10^{7.1}$ ID_{50} GILLIAM STRAIN AND AFTER SUBSEQUENT CHALLENGE WITH THE KARP STRAIN

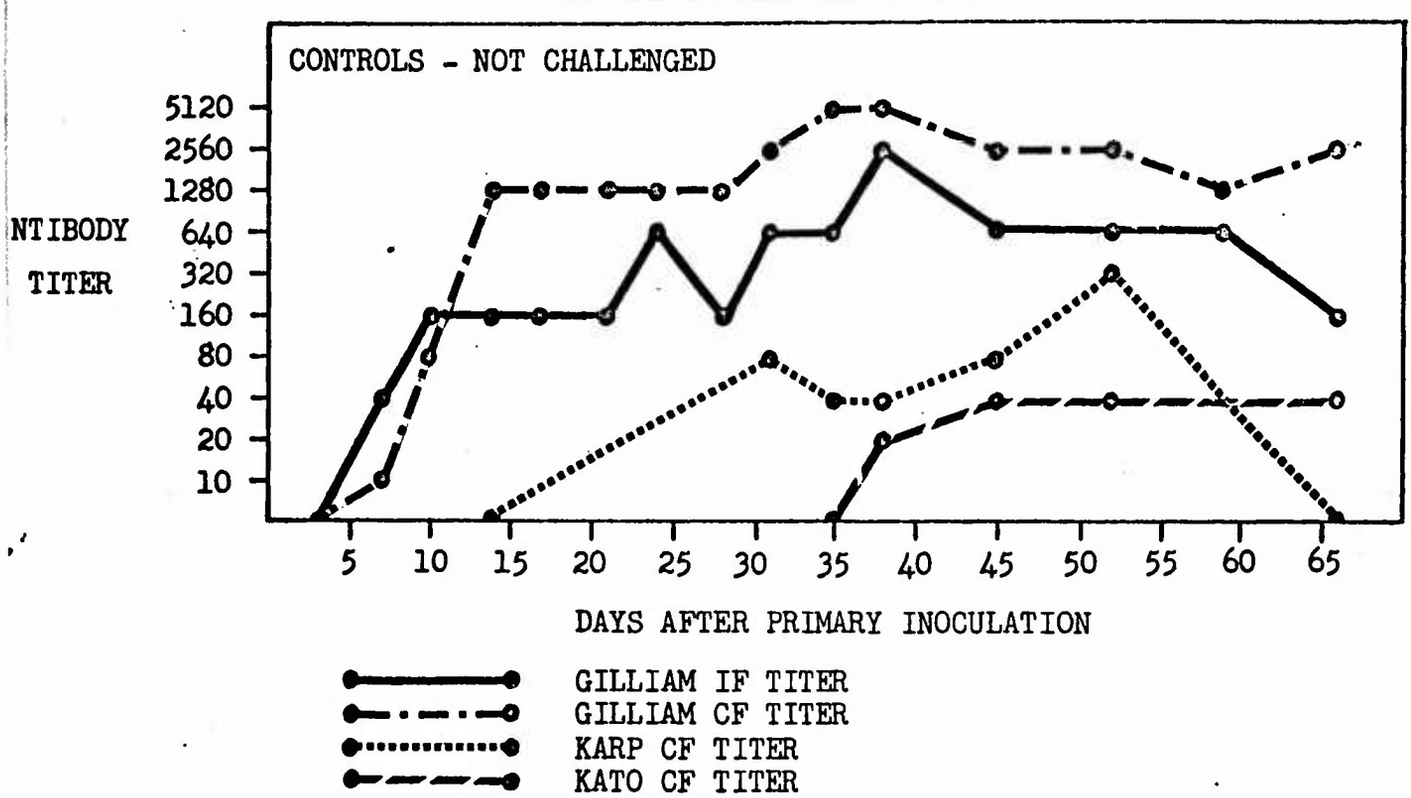


FIGURE 3

ANTIBODY RESPONSE OF MICE FOLLOWING SUBCUTANEOUS INOCULATION OF $10^{7.1}$ ID_{50} GILLIAM STRAIN DURING TREATMENT WITH CHLORAMPHENICOL AND AFTER SUBSEQUENT CHALLENGE WITH THE KARP STRAIN

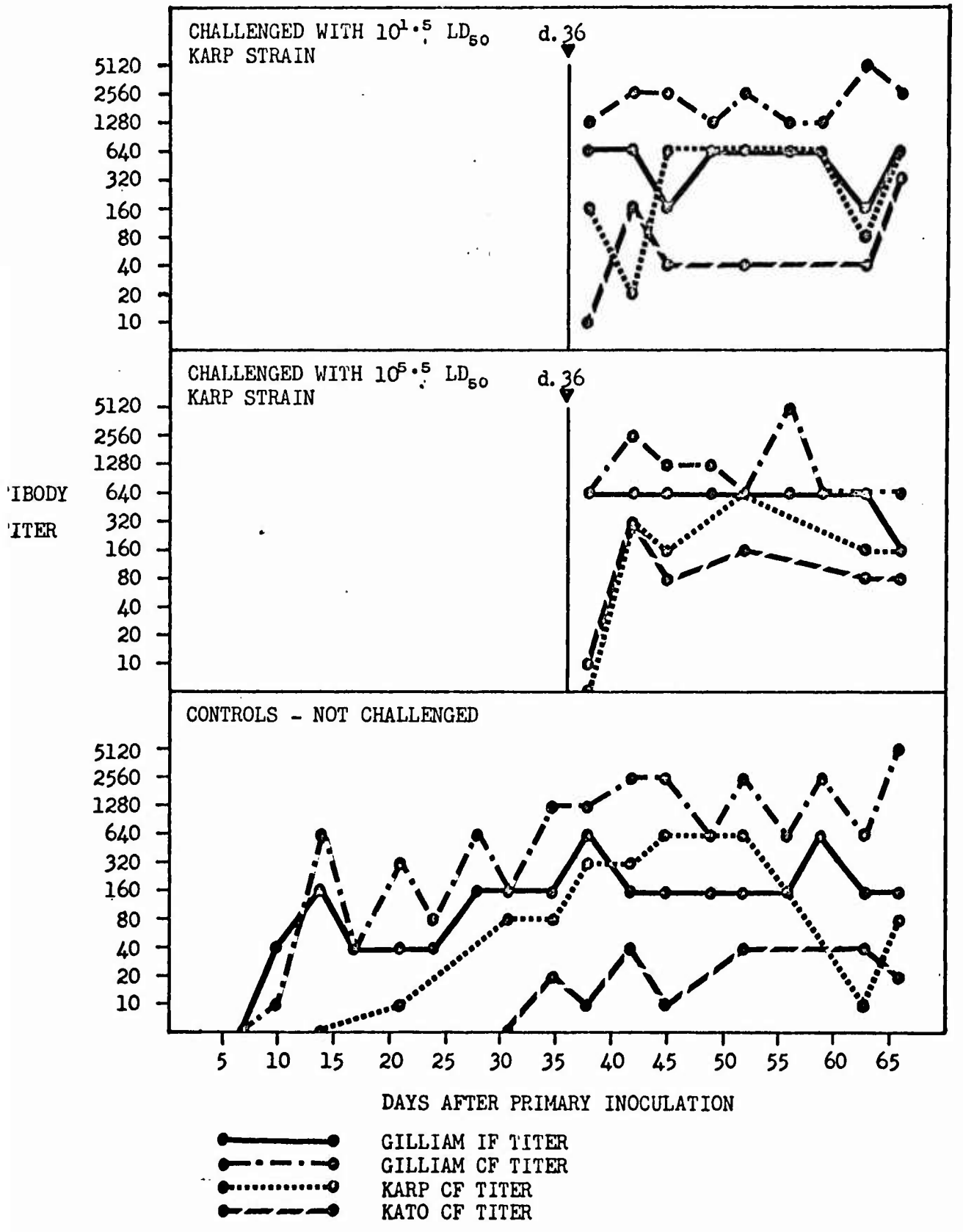
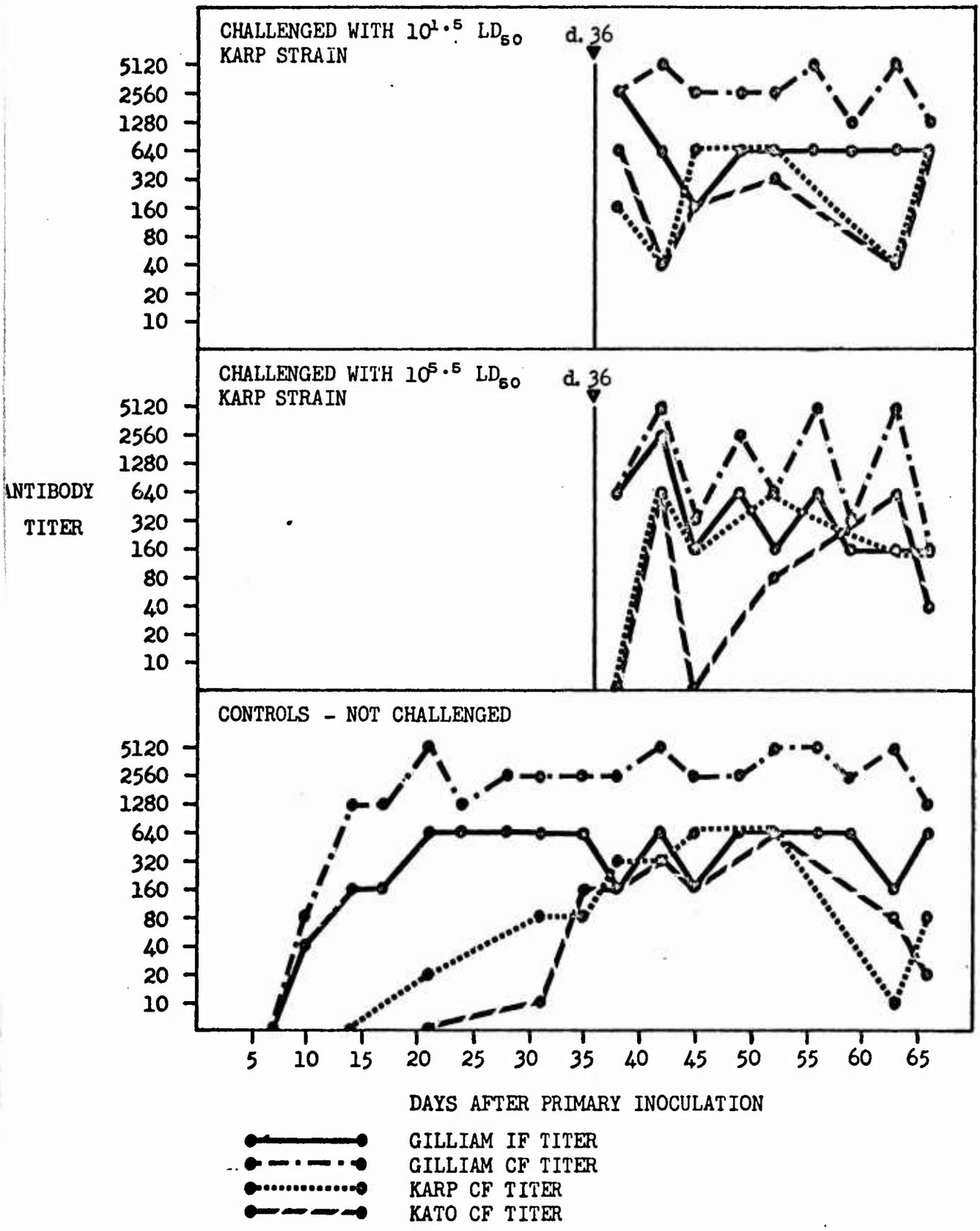


FIGURE 4

ANTIBODY RESPONSE OF MICE FOLLOWING SUBCUTANEOUS INOCULATION OF $10^{7.1}$ ID_{50} GILLIAM STRAIN AND AFTER SUBSEQUENT CHALLENGE WITH THE KARP STRAIN



experimental conditions described previously. The appearance of the homologous IF and CF antibodies in both treated and untreated was delayed until the 10th post-inoculation day. Thereafter, these antibodies rose more rapidly and attained higher titers sooner in the untreated animals than in the treated group. Karp CF antibody was present on day 20 and Kato CF antibody was not detected until 10-14 days later. Significant levels of both Karp and Kato CF antibodies were present on the day of challenge and the titers of both continued to rise irrespective of the dose of the Karp strain inoculated intraperitoneally.

When a small non-lethal dose of Gilliam ($10^{2.6}$ ID_{50}) was used to initiate infection by the intraperitoneal route, significant levels of homologous IF and CF antibodies were not found until 2 weeks later. (See Figures 5 and 6.) Comparison of subsequent response in the treated and untreated groups shows again the slight inhibitory effect of the antibiotic. The effect of variation of individual antibody responses on the level of antibodies demonstrated in the serum pools is more apparent in this experiment. Indeed, in one untreated group which was challenged with $10^{5.5}$ LD_{50} of the Karp strain, no Karp or Kato CF antibody was demonstrated prior to challenge although significant levels were present in the serum pools from the other groups. (See Figure 6.)

The antibody response following subcutaneous inoculation of the $10^{2.6}$ ID_{50} dose of Gilliam was not appreciably different from that observed after intraperitoneal injection. (See Figures 7 and 8.) Treatment of the animals with chloramphenicol was associated with a slower rate of antibody production but the ultimate homologous and heterologous titers attained before challenge were comparable.

Identical experiments were carried out with the Kato strain using $10^{5.6}$ LD_{50} as the high dose, and $10^{1.1}$ LD_{50} as the small dose, to initiate infection. Similarly, in another series $10^{5.7}$ LD_{50} and $10^{1.2}$ LD_{50} of the Karp strain were employed as infecting doses. Generally, the influence of route of inoculation, size of the infecting dose, and treatment with chloramphenicol upon the homologous antibody response was the same as found in the Gilliam tests. In the heterologous antibody response following Kato infection, Karp antibodies predominated over the Gilliam titers, whereas after Karp infection, the levels of Kato CF antibody were higher than the Gilliam titers. As in the Gilliam experiment, challenge of Kato-infected mice with the Karp strain, and challenge of Karp-infected mice with the Kato strain, did not significantly alter the levels of homologous and heterologous antibodies from those of unchallenged controls.

The results of these experiments to characterize the antibody response of mice following scrub typhus infection and subsequent lethal challenge with a heterologous strain were unexpected. It was postulated that following initial infection, the animal would develop homologous immunity evoked by the dominant antigenic component of the infecting strain. The common minor antigenic components would prime the animal so

FIGURE 5

ANTIBODY RESPONSE OF MICE FOLLOWING INTRAPERITONEAL INOCULATION OF $10^{2.6}$ ID_{50} GILLIAM STRAIN DURING TREATMENT WITH CHLORAMPHENICOL AND AFTER SUBSEQUENT CHALLENGE WITH THE KARP STRAIN

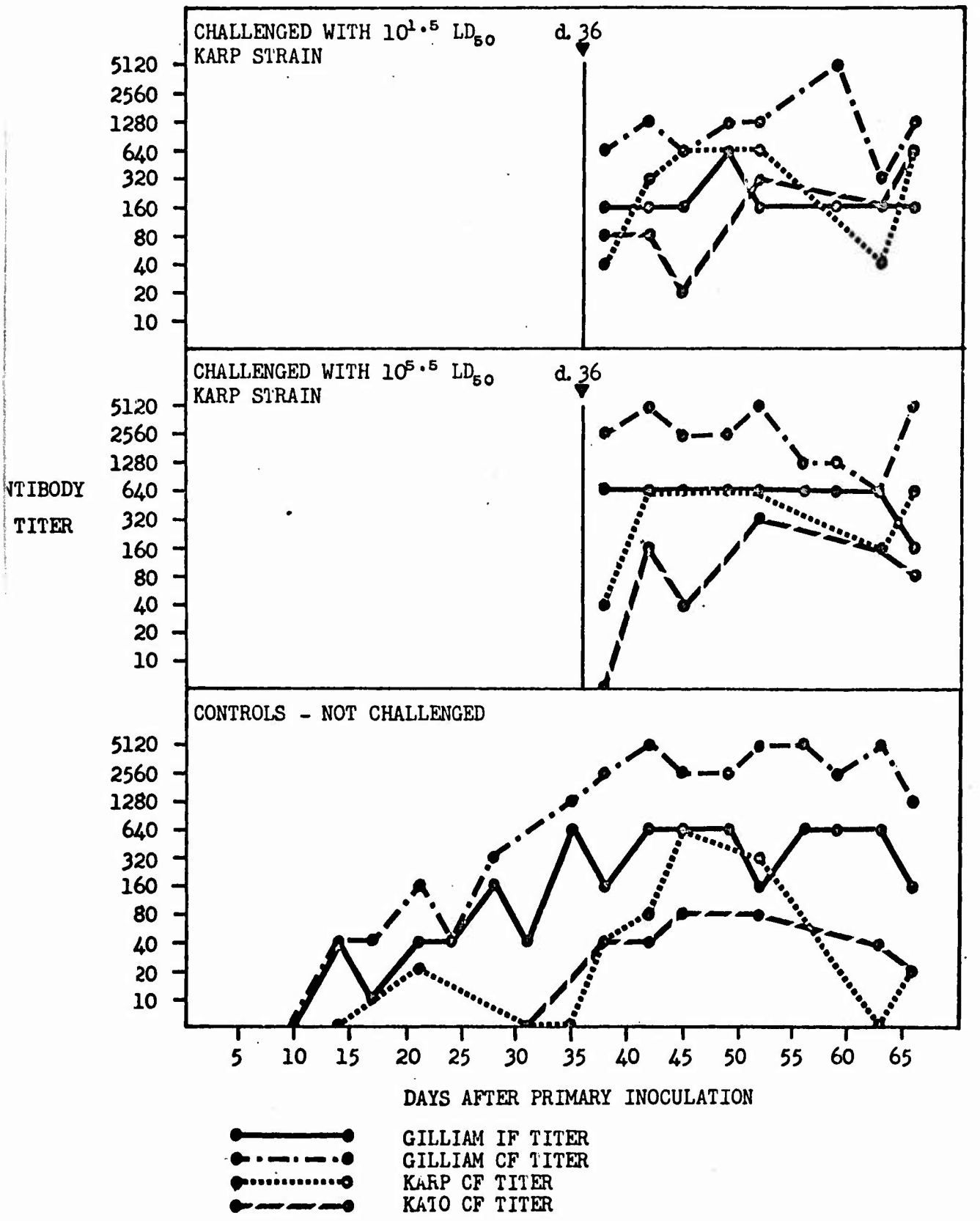


FIGURE 6

ANTIBODY RESPONSE OF MICE FOLLOWING INTRAPERITONEAL INOCULATION OF $10^{3.6}$ ID_{50} GILLIAM STRAIN AND AFTER SUBSEQUENT CHALLENGE WITH THE KARP STRAIN

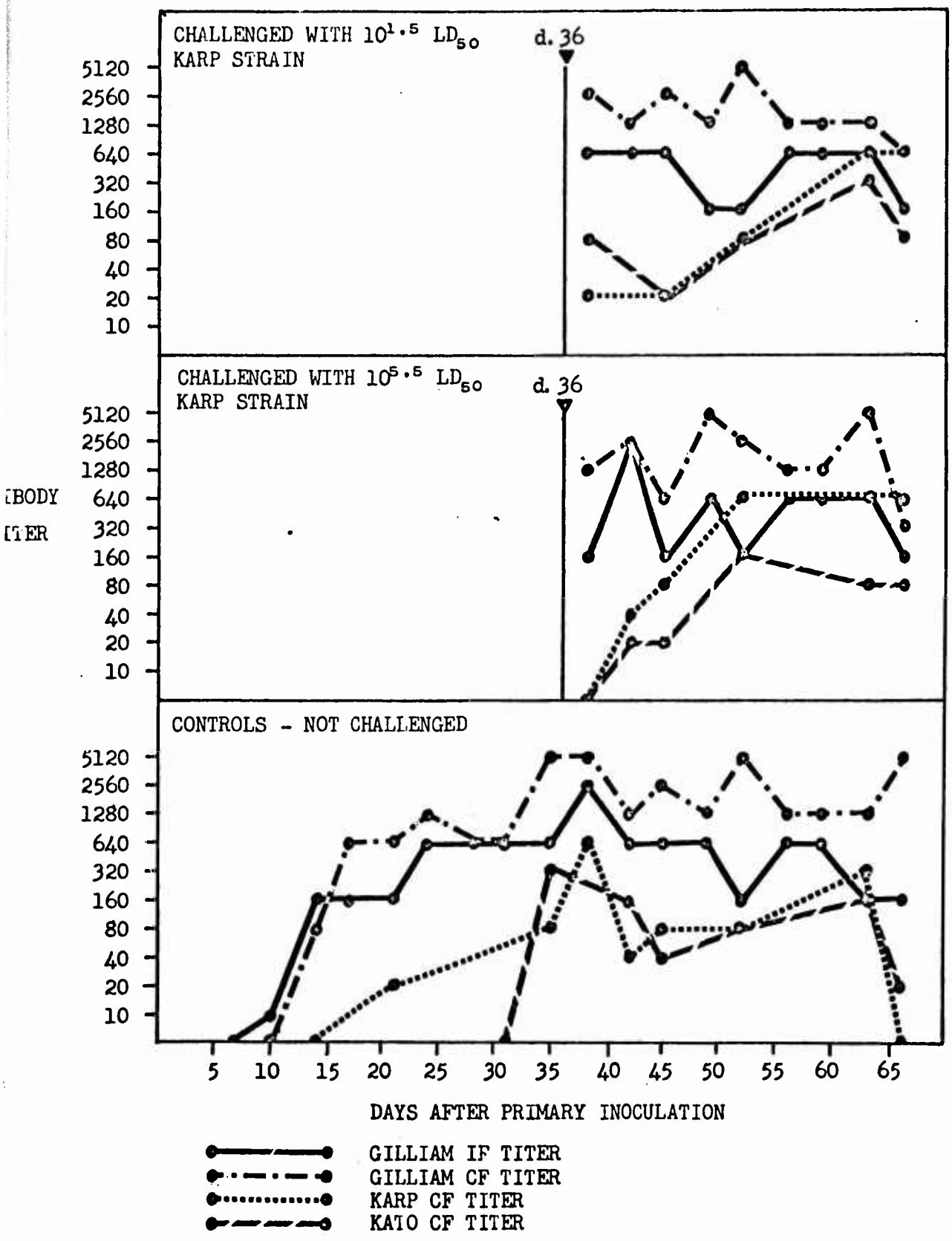


FIGURE 7

ANTIBODY RESPONSE OF MICE FOLLOWING SUBCUTANEOUS INOCULATION OF $10^{3.6}$ ID_{50} GILLIAM STRAIN AND AFTER SUBSEQUENT CHALLENGE WITH THE KARP STRAIN

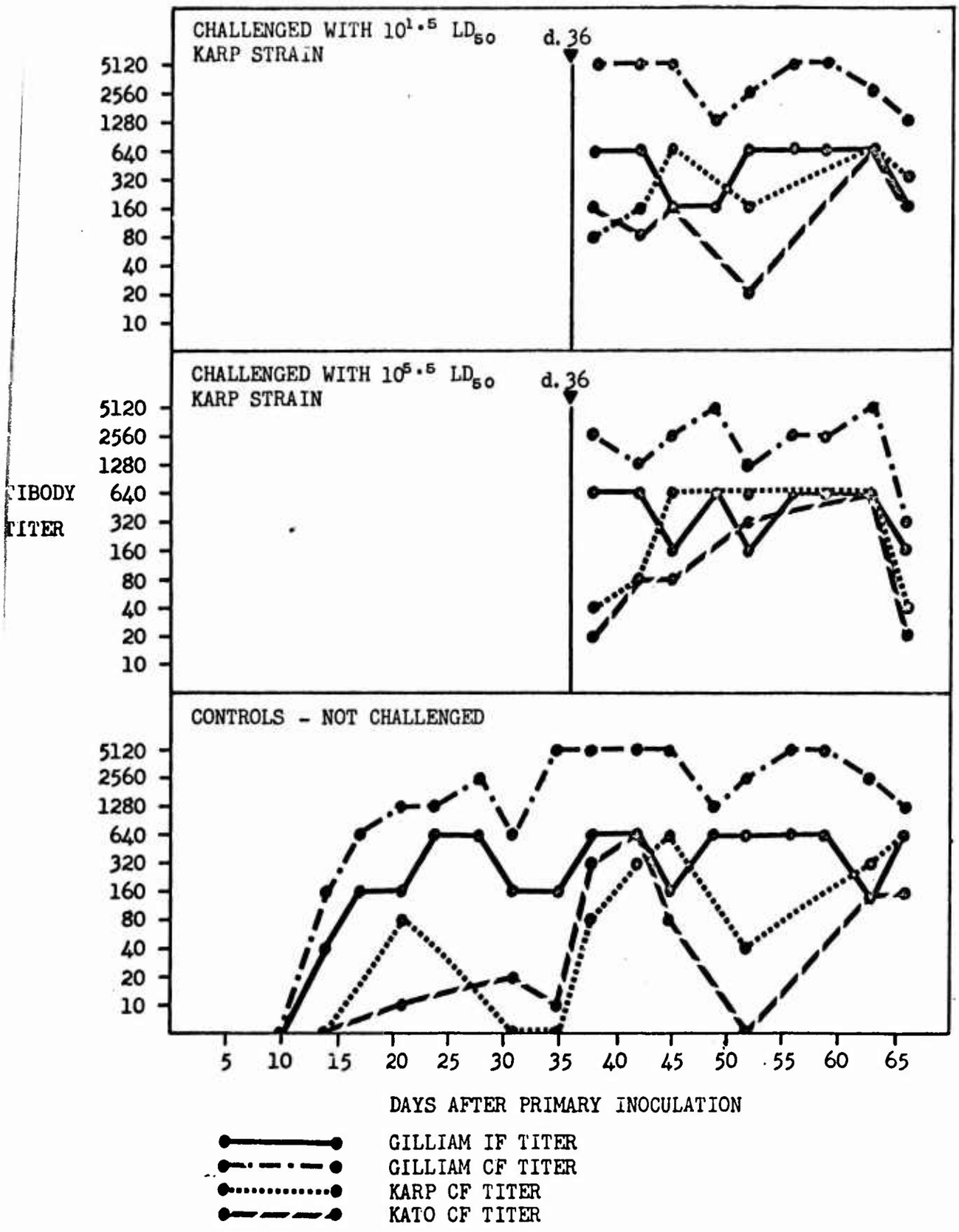
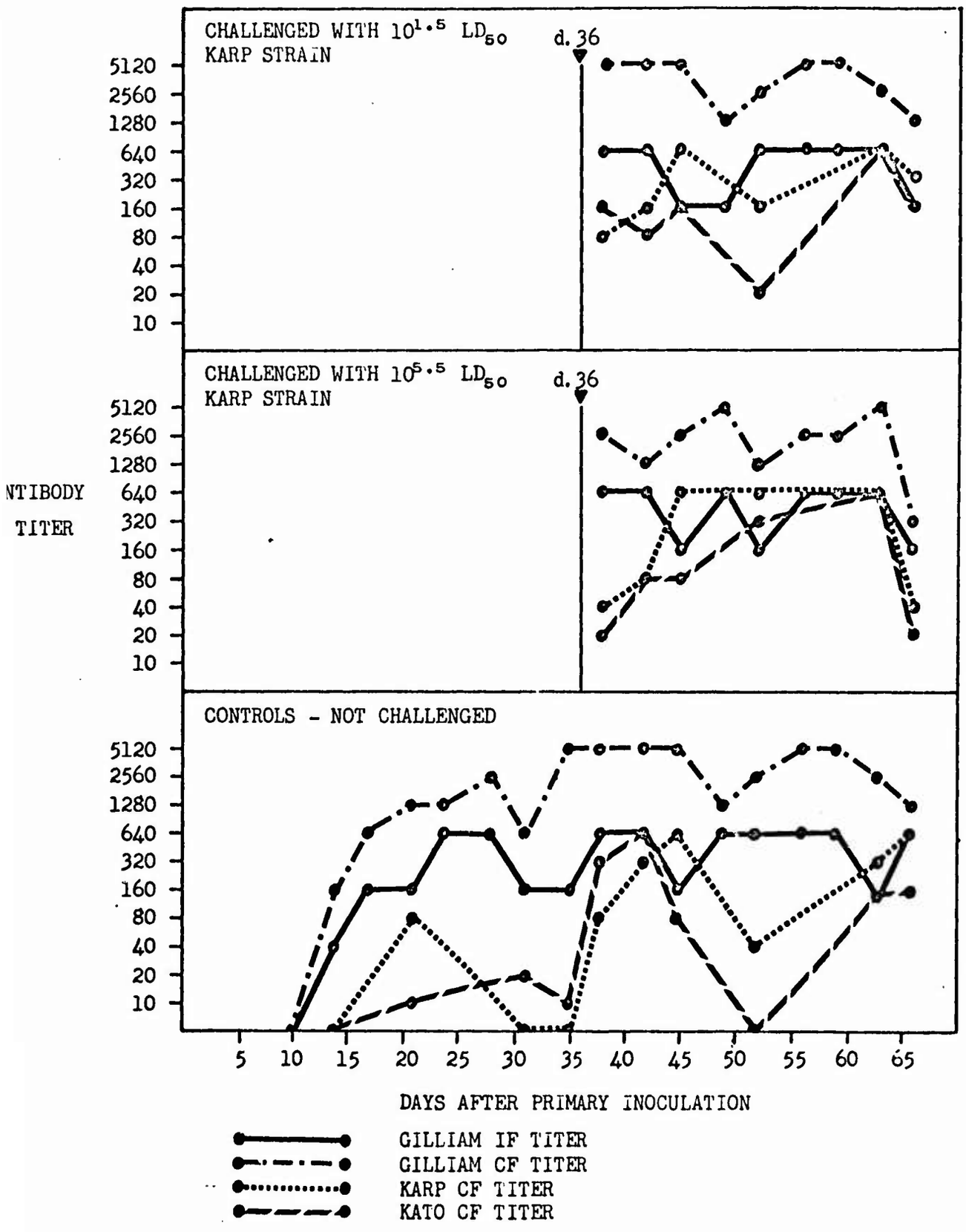


FIGURE 8

ANTIBODY RESPONSE OF MICE FOLLOWING SUBCUTANEOUS INOCULATION OF $10^{2.6}$ ID_{50} GILLIAM STRAIN AND AFTER SUBSEQUENT CHALLENGE WITH THE KARP STRAIN



that after exposure to the heterologous strain a booster response would develop preventing death but not infection. There were no differences in the rate of heterologous antibody production or the ultimate titers attained following challenge which were distinguishable from the antibody response of unchallenged controls. Additional studies are in progress to determine if the type of immunoglobulin associated with the heterologous response following primary infection differs from that found after challenge.

2. Effects of Newer Antimicrobials on Rickettsiae. There has been little effort to improve upon the antimicrobials available for the treatment of rickettsial diseases since first chloramphenicol and, subsequently, the tetracyclines were found to be highly effective therapeutic agents. Indeed, only rarely have the newer antibiotics been tested for an effect on rickettsiae. This apparent indifference to rickettsial diseases on the part of the pharmaceutical industry can be accounted for by the low incidence of these infections in the United States, as well as in most parts of the world, and the lack of evidence that rickettsiae can develop resistance to the available antibiotics.

The concentrations of chloramphenicol and the tetracyclines attained with the usual therapeutic dosages are rickettsiostatic. Recovery of the patient with a rickettsial disease depends on the development of natural immunity while clinical signs and symptoms are suppressed and the growth of the micro-organisms is limited. Because of the high relapse rate in scrub typhus, antibiotics must be given continuously, or on an interrupted schedule, until the 12th or 14th day after onset of illness even though the patient is usually afebrile and clinically much improved 36 to 48 hours after treatment has begun. Limited experience with Rocky Mountain spotted fever and murine typhus has shown that similar relapses occur when antibiotic therapy is discontinued too early. In addition to extending the period of hospitalization or medical care, the prolonged use of broad spectrum antibiotics can have undesirable side effects.

A therapeutic agent which is rickettsiocidal would markedly reduce the time required for treatment and prevent relapses. Furthermore, it might prevent the development of latent infections in certain cases of epidemic typhus which result in late recrudescences (Brill-Zinsser Disease), thereby eliminating the sole, or at least the major interepidemic reservoir of this disease.

Early this year a project was started to determine the effect of the newer antibiotics on Rickettsia tsutsugamushi. Strains of this rickettsia were selected because scrub typhus is the most important military rickettsial disease problem at this time. A screening method was devised which determines the direct effect of the antibiotic on the viable rickettsiae. A suspension of scrub typhus organisms was incubated at 37° C for 2 hours in an aqueous solution containing 10, 50, and 250 μg/ml of the antibiotic to be tested. The lowest concentration

approximated the blood levels attainable during therapy. Following incubation, the LD_{50} and ID_{50} titers of the suspension were determined by intraperitoneal inoculation of decimal dilutions of the suspension into 14 to 16 gram white mice. An incubated control suspension without antibiotic was titrated in mice in an identical manner. The direct effect of the antibiotic was manifested as a significant reduction of the LD_{50} titer of the rickettsial suspension with antibiotic from that of the control suspension. The effect was considered rickettsiocidal when the LD_{50} and ID_{50} titers were reduced equally. The Karp strain of R. tsutsugamushi was used for the screening test because the LD_{50} and ID_{50} titers of yolk sac suspensions of this organism are identical.

It was originally thought that a stabilizer such as bovine serum albumin (BSA) would be necessary to prevent excessive loss of activity of the rickettsial suspension during the 2 hours incubation at 37° C. Such indeed proved to be the case as a 1×10^{-4} dilution of a suspension of Karp strain R. tsutsugamushi in Snyder I diluent lost more than $10^{3.7}$ mouse LD_{50} after only one hour incubation, while after addition of BSA with a final concentration of 1%, there was less than a 10-fold loss of activity after 2 hours incubation at 37° C.

However, it was later found that crude yolk sac suspension provided a similar protective effect. When a 1×10^{-2} suspension in Snyder I diluent only was incubated 2 hours at 37° C, the loss of activity was negligible. Thereafter, all trials were carried out with a 1:100 dilution of the seed suspension without significant loss of activity during incubation.

Work in eggs is being done using the standard method developed in this laboratory. Briefly, this consists of inoculating sets of eggs with a rickettsial suspension after injection of different quantities of the antibiotic to be tested. Effectiveness is measured as prolongation of the mean day of death of the set of eggs as compared to a control set without antibiotic.

Since the effects of some of the tetracyclines on rickettsiae had been investigated in the past, these antibiotics were used as reference drugs. Synthetic penicillins, certain anti-viral agents, anti-tuberculous agents, broad-spectrum and gram-negative spectrum agents, and a miscellaneous set of other drugs including anti-malarials are being investigated. Much of the data is still pending the outcome of the screening trials. However, Table II summarizes the data available at this time. It shows the reduction in the mouse LD_{50} titer of the Karp strain rickettsial suspension following 2 hours incubation at 37° C with the 3 concentrations of antibiotic as compared to the control suspension LD_{50} titer after the same incubation without antibiotic. The LD_{50} titer of the control suspensions after incubation ranged from 7.2 to 8.4. The ID_{50} titer reductions are not given because, aside from the tetracyclines, the reductions produced by the other antibiotics were variable, small, and seemingly insignificant.

TABLE II

DIRECT EFFECT OF ANTIBIOTICS ON KARP STRAIN OF
RICKETTSIA TSUTSUGAMUSHI

ANTIMICROBIAL	REDUCTION OF LD_{50} TITER FROM CONTROL (CONCENTRATION OF ANTIMICROBIAL)		
	10 MCG/ML	50 MCG/ML	250 MCG/ML
CHLORTETRACYCLINE	2.68	4.07	≥ 6.93
DOXYCYCLINE	1.58	3.68	4.34
OXYTETRACYCLINE	——*	1.62	≥ 5.05
AMPICILLIN	——	——	0.10
IDOXURIDINE	0.67	0.47	——
ISONIAZID	1.83	0.86	0.96
ETHIONAMIDE	1.00	0.85	1.95
AMANTADINE	——	0.06	2.64
NOVOBIOCIN	0.02	——	2.02
KANAMYCIN	——	0.28	0.52
AMPHOMYCIN	0.27	0.24	1.27

* INDICATES NO DIFFERENCE OR SLIGHT POSITIVE DIFFERENCE FROM CONTROL SUSPENSION LD_{50}

3. Recovery of a Chlamydia from a Patient in Thailand. In the course of studies being conducted by the Thai Component, SEATO Medical Laboratory, Bangkok, to investigate the presence and extent of rickettsial diseases in Thailand, an agent (TH 4043) was recovered from the blood of a patient which was highly lethal for mice. The organism could not be identified as a rickettsia, and failed to grow on bacteriological media. After storage for 5 months, the agent was successfully re-isolated from the patient's acute phase blood clot. Spleens from the 14th and 19th mouse passages were sent to the Department of Rickettsial Diseases, WRAIR, for further studies.

a. Patient's Clinical Course. A hill tribe boy attending school in Chieng Rai (Northwest Thailand) was seen in the Outpatient Department of the District Hospital in January, 1967, after having had high fever and a cough for 3 days. At the time of examination, a fever of 99.6° F, angular stomatitis, and inflamed oral cavity was noted and the illness was clinically diagnosed as influenza with avitaminosis. Streptomycin, sulfadiazine, aspirin, and vitamins were given for 3 days. On the 4th day, the patient had a rash extending over his whole body which lasted for 3 days and then completely subsided. Because there was no basic improvement in his physical condition, he returned to the hospital and was given chloramphenicol which resulted in a dramatic response.

Blood and serum drawn at the time the patient first appeared at the hospital (3rd day of disease) were frozen on dry ice and shipped to the laboratory in Bangkok for isolation studies. Adult mice inoculated intraperitoneally with the blood died within 3 to 7 days. Suspensions of spleens from infected mice constituted the inoculum for subsequent passages. Serum from guinea pigs inoculated with the agent was negative in complement fixation tests with Typhus, Spotted Fever Group and Q Fever antigens. Immunity-challenge experiments in mice indicated the microorganism was not Scrub Typhus.

b. Recovery of the Agent at WRAIR. A suspension of the 14th passage mouse spleens received at WRAIR was inoculated intraperitoneally into 12 adult mice. All of the mice developed ruffled fur and diarrhea on the 2nd or 3rd day and 9 mice died on the 4th day. Autopsy of the 3 surviving mice revealed no gross pathological lesions. Examination of impression smears of the spleens stained with Giemsa initially suggested the possible presence of two intracellular microorganisms; i.e., a very small discrete pinpoint body, and a larger irregular shaped inclusion with a nondescript boundary. Later, observation of the growth of the agent in cell culture indicated that the 2 bodies seen were different stages of the developmental cycle of a single organism. A suspension of the spleens was inoculated into 12 more mice, constituting the 16th mouse passage. Five mice which were sick on the 2nd day were sacrificed and a 20% spleen suspension was inoculated intraperitoneally into guinea pigs and into the yolk sac of 7 day-old embryonated eggs. The guinea pigs had fever ($\geq 104^{\circ}$ F) from day 2 through day 6-7. They were exsanguinated 28 days

after inoculation to obtain convalescent serum. The majority of the chick embryos were dead on the 4th day, at which time a 20% suspension of yolk sac tissue was prepared and stored in 1 ml amounts at -65° C to serve as early egg passage seed material. The suspension contained $\geq 10^{6.0}$ 50% egg lethal doses (LD_{50}) and $10^{7.1}$ mouse LD_{50}. A similar suspension prepared from the 2nd egg passage had an LD_{50} titer of $10^{-8.6}$ in mice. All infected mice died between the 2nd and 9th day after inoculation of 0.2 ml intraperitoneally. Macchiavello-stained smears of infected yolk sacs of living chick embryos harvested when the majority of the embryos had died revealed numerous minute red elementary bodies.

c. Serological Studies. A complement-fixing antigen prepared from a suspension of infected yolk sacs by differential centrifugation with diethylaminoethyl cellulose (DEAE) and ether extraction titered 1:16 with 2 to 4 units of 2 different psittacosis convalescent human serum pools and a lymphogranuloma venereum (LGV) positive control serum.

Indirect immunofluorescent tests using smears of infected yolk sac as the antigen and known positive psittacosis sera also were positive, thus, identifying the TH 4043 agent as a member of the psittacosis-LGV-trachoma group.

The only sera available from the patient were the 3 day acute phase specimen and another obtained 5 months after his illness. In complement fixation tests employing the TH 4043-DEAE antigen, as well as a commercial Psittacosis-LGV antigen, the acute phase serum titered < 1:5 and the 5 month serum titered 1:5.

Of interest is the difference in antibody levels detected in the serum obtained from the TH 4043 infected guinea pigs when tested with the TH 4043-DEAE antigen and the commercial Psittacosis-LGV diagnostic antigen. (See Table III.)

The difference in antibody titers of the guinea pig sera obtained with the 2 antigens cannot be explained at this time. It is possible, however, that the TH 4043 agent contains an antigen which is not present in the commercial antigen.

d. Chlamydia Group Identification. Methods have not been developed to definitively identify a specific type species in this large group of agents which share common antigens. Currently, the Chlamydia are divided into Group A and Group B. Within the former, designated TRIC, are included the etiologic agents of trachoma, inclusion conjunctivitis, LGV and mouse pneumonitis. These agents are sulfadiazine-sensitive and form compact round or oval intracytoplasmic inclusions or microcolonies which produce glycogen. Included in Group B are the agents of psittacosis, meningopneumonitis, human and feline pneumonitis, guinea pig and bovine conjunctivitis and avian strains. All of these strains display diffuse intracytoplasmic inclusions, do not produce glycogen and are sulfadiazine insensitive (except for the 6 BC psittacosis strain, which is sulfadiazine sensitive, but does not produce glycogen).

TABLE III

SEROLOGIC IDENTIFICATION OF TH 4043

SERUM	COMPLEMENT-FIXING ANTIBODY TITERS WHEN TESTED WITH TWO UNITS OF THE FOLLOWING ANTIGENS	
	COMMERCIAL PSITT—LGV	TH 4043-DEAE
TH 4043 - GP2(2)*	< 5**	10
GP2(5)*	< 5	≥ 80
GP9(5)*	< 5	20
TH 4043 - GP #440	< 5	40
·559	< 5	20
SITT. HUMAN POOL 3	16	20
4	128	≥ 80
THAI PATIENT		
ACUTE*	< 5	< 5
CONVAL.*	5	5

* RECEIVED FROM THAI COMPONENT, SEATO

** RECIPROCAL OF SERUM DILUTION

The TH 4043 agent was tested for its sensitivity to sulfadiazine and ability to produce glycogen. The drug test was done by inoculating each of one group of 7 day-old embryonated eggs via the yolk sac route with 1 mg of sodium sulfadiazine. Ten-fold serial dilutions of TH 4043 seed material then were inoculated into the sulfadiazine-treated eggs as well as into a comparable group of eggs which received buffered saline without sulfadiazine. The embryos were candled daily for 13 days at which time the effect of the drug on the LD_{50} titer of the infectious suspension was determined. For purposes of taxonomic differentiation, susceptibility to sulfadiazine (1 mg per egg) is defined as a 100-fold or more reduction in the egg LD_{50} titer of a suspension of chlamydiae containing initially 10^6 LD_{50} or more. The titer of the TH 4043 seed material in the sulfadiazine-treated eggs was $10^{-8.9}$ as compared to $10^{-9.1}$ in the control eggs. Thus, it was concluded that the TH 4043 agent is sulfadiazine-insensitive.

The formation of glycogen by chlamydiae is a transient phenomenon that occurs from the first through the third day of growth and is detected by staining infected cells with iodine. Intracytoplasmic clusters of chlamydiae, or microcolonies containing glycogen, appear brown against a light tan background, while non-glycogen-forming microcolonies cannot be distinguished from other cellular material. The optimal time for staining is about 48 hours after infection.

In order to determine if the TH 4043 agent produces glycogen, coverslip cultures of HeLa cells and 14pf cells (a continuous line of normal rat fibroblasts) were infected with about 10^5 egg LD_{50} of seed material diluted in a medium of M199 containing 20% horse serum and no penicillin or streptomycin. The presence of penicillin will inhibit glycogen production. At 40 and 48 hours after infection, coverslips containing infected cells as well as coverslips of uninfected control cells were fixed in absolute methanol for 4 hours. They were then immersed in a 1:1 mixture of 5.0% iodine in 100% ethyl alcohol and aqueous 5.0% potassium iodide for 4 hours, afterwhich they were mounted on microscope slides in 50% glycerine and examined at 430 X magnification. No differences were noted between infected and uninfected cells indicating that glycogen was not produced. Examination of other infected cultures stained by the Macchiavello method after 40 and 48 hours showed that about 15-20% of the cells contained bright red microcolonies containing elementary bodies, as well as larger reticulated inclusions which stained red or blue.

The insensitivity of the TH 4043 agent to sulfadiazine and the failure to produce glycogen in intracytoplasmic microcolonies indicate that the agent belongs to the B Group of Psittacosis-type agents.

4. Field Use of the Indirect Immunofluorescent Test for Scrub Typhus. In the Annual Progress Reports, WRAIR, 1 July 1961 - 30 June 1962, and 1 July 1962 - 30 June 1963, the use of indirect immunofluorescence (IF) for the specific serologic diagnosis of scrub typhus was reported and

its applicability as a test procedure for screening sera for detection of past R. tsutsugamushi infections was described. In order to make the technique practical for use in field laboratories, a screen test was developed, which permitted examination of larger numbers of specimens and conserved reagents. Methods of packaging and conditions of storage of antigens were evaluated. (See Annual Progress Report, WRAIR, 1 July 1965 - 30 June 1966.)

During the latter months of 1966, all reagents necessary to perform the scrub typhus IF tests were shipped to requesting U. S. military laboratories in Southeast Asia. Each initial shipment contained antigens in the form of acetone-fixed smears of the screening polyvalent antigen, KGKt, as well as slides of each of the definitive test antigens (strains Karp, Gilliam and Kato). Laboratories which are equipped and experienced in the handling of infectious material were sent frozen viable suspensions of the 3 antigens for preparation of their own antigen smears. In addition, normal yolk sac diluent for dilution of sera, positive and negative control sera, fluorescein-labeled antihuman globulin, and buffered glycerin mounting medium were sent along with a manual of explicit instructions for performing the test. The shipment contained enough reagents for about 3 months use and since that time stocks have been replenished upon request.

The U. S. Army Medical Research Unit (WRAIR) Vietnam was supplied first. During 1967 and 1968, shipments were made regularly also to the 9th Medical Laboratory and to the U. S. Naval Medical Research Unit, No. 2 in Taipei, Taiwan. Although scrub typhus was an important medical problem of the French Military Forces during the Indochina War, only rare cases of this disease were being recognized and reported among U. S. Military personnel during 1966 and 1967. The etiology of Fevers of Unknown Origin (FUO) which constitute a serious problem in U. S. personnel is being investigated in the southern portion of South Vietnam by the 9th Medical Laboratory and in the northern area by the U. S. Naval Medical Research Unit, No. 2, as well as in other laboratories in the area. The use of the scrub typhus immunofluorescence test has revealed that scrub typhus is an important cause of febrile disease. Only fragmentary results have been received from these 2 laboratories. However, the available information is presented in Table IV. In both laboratories approximately 16% of the Fevers of Unknown Origin were diagnosed serologically as scrub typhus. Unofficial reports from the field laboratories confirm the value of the immunofluorescent test and have not indicated any major problems or deficiencies.

In addition to supplying the scrub typhus IF test reagents, the Department of Rickettsial Diseases has performed serologic tests with serum specimens submitted by the U. S. Army Medical Research Unit (WRAIR) Vietnam in support of their special investigations into the etiology of infectious diseases. In the current reporting period, 157 paired specimens were tested by complement fixation employing Typhus, Spotted Fever

Group and Q Fever antigens. During the period January to April 1967, specimens were collected from 65 U. S. personnel at the 24th and 93rd Evacuation Hospitals as well as from 11 indigenous soldiers. From June to November 1967, 81 paired specimens were obtained from U. S. personnel in IV Corps. No serologic evidence of current or past murine typhus, tick typhus or Q fever was found among this group of patients.

The results of the scrub typhus IF tests carried out by the U. S. Army Medical Research Unit (WRAIR) Vietnam since 1966 are not available. However, increases in antibody titer, diagnostic of scrub typhus, were found in 5 of 81 cases from IV Corps which were tested at WRAIR.

TABLE IV

SUMMARY OF RESULTS OF SCRUB TYPHUS IF TESTS ON SERA FROM CASES WITH FEVERS OF UNKNOWN ORIGIN IN VIETNAM

REPORTING LABORATORY	TIME OF TESTING	NUMBER PATIENTS	NUMBER POSITIVE	PER CENT POSITIVE
9TH MEDICAL LABORATORY	SEP - DEC 67	424	68	16
U.S. NAVAL MEDICAL RESEARCH UNIT, NO. 2	JAN - SEP 67	400	63	16

5. Rickettsia canada: A New Member of the Typhus Group. Studies carried out to confirm the occurrence of Rocky Mountain spotted fever in nature in the Richmond area of Ontario, Canada, resulted in the isolation of a new species of rickettsia belonging to the Typhus Group from Haemaphysalis leporispalustris, (McKiel, J.A., Bell, E.J. and Lackman, D.B. Canad. J. Microbiol. 13:503, 1967). The presence of complement-fixing antigens in this rickettsia which are common to R. prowazekii and R. mooseri was shown by cross reactions of antisera prepared in guinea pigs, rabbits and hamsters. Tests with mouse antisera, which were species-specific, indicated that the agent was not identical with epidemic or murine typhus rickettsiae. The lack of identity also was confirmed by toxin neutralization tests with convalescent sera from guinea pigs, hamsters, rabbits and mice. The authors have proposed the name Rickettsia canada, n. sp. for the new rickettsia.

The Department of Rickettsial Diseases, WRAIR, requested the new rickettsia from the Rocky Mountain Laboratory, Hamilton, Montana.

Because of a deficiency in sufficient volumes of infected yolk sac seed material, 9 female Dermacentor andersoni ticks, which had been inoculated with 0.03 ml of a 50% yolk sac suspension, were shipped.

It is known that spotted fever rickettsiae in ticks are reactivated if the tick has a blood meal, or if it is incubated at 37° C for 48-72 hours. Attempts were made to feed the R. canada-infected ticks on guinea pigs but none of the arthropods attached over a 4 day period. Six of the ticks were then taken and treated individually by soaking each in 25 ml of 1:10,000 merthiolate. Following 2 successive washings in 5 ml sterile distilled water containing 300 units penicillin and 60 mcg streptomycin per ml, each tick was ground in 3 ml of buffered saline containing the same concentration of antibiotics. Each tick suspension was inoculated into the yolk sac of 7 seven-day-old embryonated eggs via the yolk sac route. One set of eggs was grossly contaminated with bacteria; 2 lines were negative for the presence of an infectious agent; and rickettsiae were recovered from the eggs inoculated with the 3 other tick suspensions. Two serial passages were made with selected yolk sacs containing numerous rickettsiae as determined by examination of Macchiavello-stained smears. A 20% suspension of 3rd passage yolk sacs was prepared and stored in 1 ml aliquots at -65° C. This seed suspension contained $\geq 10^{8.0}$ egg LD_{50} and $\geq 10^{8.3}$ guinea pig ID_{50} as determined by the presence of complement-fixing antibodies in serum collected 28 days after inoculation. The agent is virtually avirulent for guinea pigs; only 2 of 8 animals inoculated with a 10^{-2} dilution exhibited fever (104° F) on the 3rd day. No scrotal reactions were noted. Similarly, mice remained asymptomatic following intraperitoneal inoculation. However, intravenous inoculation of the seed material resulted in the death of mice within 18 hours. Death is attributed to a toxic factor associated with viability of the rickettsiae and is caused by profound alterations in endothelial permeability with extravascular loss of plasma. The toxic mouse LD_{50} of the R. canada suspension was 1:107. R. mooseri and R. prowazekii also elicit a lethal toxic effect for mice when administered in sufficiently large doses.

Soluble-type group antigens were prepared from yolk sacs infected with R. canada. In CF tests, the antigens showed cross reactions with epidemic and murine typhus immune guinea pig sera; but the antibody titers were usually 4 to 8-fold lower than were found in the homologous antigen-antibody system. Similarly, the titer of antibody in R. canada immune serum was usually slightly higher with the R. canada antigen than with a murine or epidemic typhus soluble antigen. Future studies include preparation of highly purified suspensions of these 3 strains of rickettsiae for use as specific complement-fixing antigens and for rickettsial agglutination tests.

The presence and extent of R. canada in the United States are not known. In the studies on the Ecology of Rocky Mountain Spotted Fever conducted by the Department of Rickettsial Diseases, WRAIR, and the

Virginia State Department of Health (See Annual Progress Report, July 1965 - June 1966), a number of wild animal sera were found to contain Typhus Group antibodies. Since there is no epidemic typhus in the U. S., the question was raised as to whether there might be a wild animal reservoir for murine typhus. With the recognition of a 3rd species belonging to the Typhus Group of rickettsiae, it is possible that the antibodies detected in the wild mammals were, indeed, due to R. canada infection. Of particular interest was the incidence of typhus antibodies found in the sera of flying squirrels (Glaucomys volans) collected in 1963-1965. In the positive animals, the titers ranged from 1:4 to ≥ 1:32 and were found primarily in the squirrels collected in the fall, winter and spring months. The sera of 13 flying squirrels collected near Montpelier, Virginia, in March and April of 1968 were recently tested. Seven of the sera had Typhus Group antibodies ranging in titer from 1:8 to ≥ 1:32. Attempts are currently being made to capture additional flying squirrels. They will be shipped to the Department of Rickettsial Diseases, bled and tested for the presence of typhus antibodies. If a serum is positive, the animal will be sacrificed and attempts made to recover the agent from its tissues.

6. Q Fever Vaccine Study. Under a program sponsored by the Commission on Rickettsial Diseases, Armed Forces Epidemiology Board, the Department of Rickettsial Diseases is participating with other governmental departments and academic institutions to evaluate the relative effectiveness of Q fever phase I and phase II vaccines. During the past year the Department of Biological Research, WRAIR, in conjunction with the U. S. Army Medical Unit, Fort Detrick, has attempted to prepare seed material which would be suitable for production of phase I vaccine. The new seed material had to be devoid of all known egg and guinea pig adventitious agents. The potency of the vaccine derived from the seed had to be comparable to the Henzerling strain phase I vaccine proved to be effective in previous experimental trials. The responsibility of the Department of Rickettsial Diseases was to characterize the phase composition of the seed material and vaccine.

It was previously reported that as little as a single dose of 30 μg of Henzerling phase I, Lot 1 Vaccine fully protected volunteers 30 weeks after inoculation against aerosol challenge of 3,000 guinea pig intraperitoneal ID_{50} of the AD strain of C. burneti. (See Annual Report, WRAIR, 1 July 1965 - 30 June 1966.)

The complete passage history of the Henzerling strain comprising the phase I, Lot 1 Vaccine, from initial isolation from man, is as follows: GP-6:EP-21:GP-1:EP-5; i.e., 6 successive passages in guinea pigs, followed by 21 serial passages in embryonated eggs, after which one guinea pig passage was made and then 5 successive passages in eggs. The concentration of organisms in the vaccine, which was equivalent to a 5% suspension on the basis of original yolk sac weight, was estimated to be 48.7 μg/ml. The phase composition of the vaccine was determined by complement fixation

tests of fractions recovered from cesium chloride gradients after high-speed centrifugation. (See Annual Report, WRAIR, 1 July 1964 - 30 June 1965.) The phase I complement-fixing antigen titer of the vaccine, concentrated about 120-fold, was ≥ 1:64 and phase II reactivity was present only in the undiluted suspension (titer = 1:1). Unfortunately, end points were not obtained in tests performed at that time. The phase I titer of the vaccine reconstituted for use was 1:2 and it is unlikely that the titer of the concentrated suspension recovered from the gradients would have titered higher than 1:128. On this basis it is assumed that the relative concentration of phase I to phase II in the Henzerling phase I, Lot 1 Vaccine was about 128 to 1. (Table V) In tests on other vaccines density gradient centrifugation did not dependably separate the 2 phases and the procedure was not used to characterize the phase composition of the new Henzerling seeds and vaccines. In order to detect both phases and define their relative proportions at different passage levels, highly concentrated purified suspensions of Q fever organisms were prepared from formalized yolk sac homogenates by the use of differential centrifugation in M/1 KCl solution (Ormsbee, R.A., J. Immunol. 88:100-108, 1962) and extensive ether extraction. The dry weight of the organisms in the final suspensions was calculated from a comparison of the absorption at 670 mu in a Beckman spectrophotometer with that of a standard suspension of C. burneti made up to 200 μg/ml. The phase I and II content of the Q fever suspensions was determined by titration of the antigenic reactivity of the suspension with both an immune guinea pig serum pool containing only phase II antibody and another immune guinea pig serum pool containing both phase I and phase II antibody. The phase I antigen titer was determined in the presence of 8 units of phase I antibody, and the phase II antigen titer in tests with 8 units of phase II antibody.

The passage history and the results of complement fixation tests of the Henzerling strain suspensions are summarized in Table V. All passages beyond the GP-6:EP-22 level were made in guinea pigs and embryonated eggs certified to be free of known adventitious agents. The first effort to develop a phase I Henzerling vaccine seed from the GP-6:EP-22 strain, which was predominantly in phase II, was not successful. After one guinea pig passage and 4 egg passages a product (GP-6:EP-22:GP-1:EP-4, Feb 67) was obtained that had only 8 times more phase I reactivity than phase II. In order to increase the phase I content, the strain at this passage level was again inoculated into guinea pigs. A single spleen was used to initiate the egg propagation and after 3 successive passes in eggs the proportion of phase I to phase II more closely approximated that of the original phase I, Lot 1 Vaccine. The suspension recovered from GP-6:EP-22:GP-1:EP-4:GP-1:EP-3, August 67 had a phase I to phase II content of 64:1. Following one more passage in eggs, the relative proportion of phase I:II decreased to 32:1. Another trial confirmed that in order to obtain an acceptable vaccine it would be necessary to limit the number of terminal egg passages to 3. The appropriate seed suspensions were prepared and a highly satisfactory pilot phase I vaccine was produced.

TABLE V

PHASE COMPOSITION OF Q FEVER HENZERLING STRAIN SEED AND VACCINE

PASSAGE LEVEL[+]	RICKETTSIAL CONCENTRATION (MG/ML)	COMPLEMENT-FIXING ANTIGEN TITER PHASE I & II ANTISERUM	PHASE II ANTISERUM	PHASE PROPORTION I:II
GP-6:EP-21:GP-1:EP-5 PHASE I LOT 1 VACCINE JUL 63	N.D.[++]	≥ 1:64	1:1	128:1
GP-6:EP-22:GP-1:EP-4 FEB 67	N.D.	1:32	1:4	8:1
GP-6:EP-22:GP-1:EP-4: GP-1:EP-3 AUG 67	2.4	1:128	1:2	64:1
GP-6:EP-22:GP-1:EP-4: GP-1:EP-4 (S-56) SEP 67	2.4	1:128	1:4	32:1
GP-6:EP-22:GP-1:EP-4: GP-1:EP-3 (S-62) OCT 67	3.6	1:512	1:8	64:1
GP-6:EP-22:GP-1:EP-4: GP-1:EP-3 PILOT PHASE I VACCINE MAY 68	6.2	1:1024	1:8	128:1

[+] GP = SUCCESSIVE PASSAGES IN GUINEA PIGS

EP = SUCCESSIVE PASSAGES IN EMBRYONATED EGGS

[++] NOT DONE

The relative phase I:phase II content of the vaccine (GP-6:EP-22:GP-1: EP-4:GP-1:EP-3; May 68) was 128:1, and the estimated concentration of rickettsiae in the vaccine, which was equivalent to a 5% suspension on the basis of original yolk sac weight was 106 μg/ml.

It is evident from this experience that the conversion of Q fever strains from phase I to phase II during the course of propagation in embryonated eggs does not occur with predictable regularity.

Summary and Conclusions.

1. Scrub Typhus Vaccine Development.

a. Studies have continued with 5 strains of R. tsutsugamushi recovered in Thailand which were antigenically distinctive from the established prototype Gilliam, Kato and Karp strains. Difficulties have been encountered in obtaining the concentrated yolk sac suspensions of these agents required for production of complement-fixing antigens. Antigenic characterizations are not complete but data obtained thus far indicate that each strain represents a new antigenic type. When the purity and antigenic individuality of the candidate prototypes are confirmed, investigations will be undertaken to determine if it will be necessary to incorporate all 8 prototypes in a polyvalent vaccine in order to protect against scrub typhus strains encountered in nature.

b. Investigations are in progress to determine the immunological basis of the protection afforded mice by infection with one strain of scrub typhus against subsequent lethal challenge with an antigenically different strain. The results of experiments thus far have not revealed any significant differences in the rate of heterologous complement-fixing antibody production, or in the ultimate titers attained, in convalescent animals following challenge which are distinguishable from unchallenged controls.

2. A rickettsiocidal antibiotic would be a valuable addition to the therapy, and perhaps the prevention, of rickettsial diseases. Feasible methods of testing antibiotics against rickettsiae are available. Preliminary testing with a screening method shows only the tetracyclines in large concentrations to be rickettsiocidal. Further work, using both the screening test in mice and an in vivo method in embryonated eggs, is underway. Additional antibiotics to be tested are: paromomycin, griseofulvin, polymyxin, erythromycin, viomycin, methisazone, DDS, hetacillin, coumermycin A_1, cephaloridine, and others as they become available.

3. A transmissible agent, which is highly lethal for mice and embryonated eggs, was recovered in Thailand from the blood of a patient whose illness was associated with fever and rash. Serological studies by the Department of Rickettsial Diseases, WRAIR, identified it as a member of the psittacosis-LGV-trachoma group of agents (Chlamydia). It is insensitive to sodium sulfadiazine and does not form glycogen, thus, placing it in the B Group of psittacosis-like agents.

4. The immunofluorescent test employing reagents supplied by the Department of Rickettsial Diseases, WRAIR, is being used by medical laboratories in South Vietnam for serologic diagnosis of scrub typhus. Results of tests reported by the 9th Medical Laboratory and the U. S. Naval Medical Research Unit, No. 2, thus far reveal that about 16% of the patients with Fevers of Unknown Origin are infected with R. tsutsugamushi. Approximately 6% of the illnesses investigated by the U. S. Army Medical Research Unit (Vietnam) during the period June to November 1967 were diagnosed serologically as scrub typhus.

5. The Department of Rickettsial Diseases has acquired a new species of rickettsia which belong to the typhus group and has been designated as R. canada, n. sp. It possesses antigens common to epidemic and murine typhus rickettsiae, but can be differentiated by toxin neutralization tests and by the use of species-specific mouse sera in complement fixation tests. Studies are in progress to determine if the typhus antibodies detected in certain wild animal sera can be attributed to R. canada infections.

6. The Department of Biological Research, WRAIR, in conjunction with the U. S. Army Medical Unit, Fort Detrick, has developed a Q fever Henzerling vaccine seed which is devoid of known egg adventitious agents and can be used to produce a phase I vaccine comparable to the experimental vaccine shown previously to be highly effective in prevention of disease.

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RESEARCH AND TECHNOLOGY RESUME

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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 MENINGOCOCCAL INFECTIONS AND MYCOPLASMA INFECTIONS IN MILITARY FORCES.

(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 HISTORIES, CLINICAL MATERIALS, ETC. FROM CASES OF ABOVE DISEASE ENTITIES. FIELD STUDIES ON PROPHYLACTIC REGIMENS, SPREAD AND PERSISTENCE OF MENINGOCOCCI IN RECRUIT POPULATIONS - AIR SAMPLING OF TRANSMISSION OF ACUTE RESPIRATORY DISEASE AGENTS.

(U) PROGRESS - JUL 67 THRU JUN 68 MENINGOCOCCAL POLYSACCHARIDES FROM GROUP A AND C ORGANISMS HAVE BEEN PREPARED, PURIFIED, CHARACTERIZED AND TESTED IN ANIMALS FOR SAFETY AND IMMUNOGENICITY. ANTIBODIES WHICH AGGLUTINATE POLYSACCHARIDE SENSITIZED ERYTHROCYTES HAVE BEEN EVALUATED IN NATURAL INFECTIONS (CASES CARRIERS) AND IN VACCINATED VOLUNTEERS AND HAVE BEEN DEMONSTRATED TO BE GROUP SPECIFIC, THE SERUM BACTERICIDAL TEST SHOWS MORE ANTIGENIC CROSSREACTIVITY BETWEEN GROUPS AND THOSE ANTIBODIES MAY BE RELATED TO IMMUNITY FROM DISEASE. EPIDEMIOLOGIC STUDIES, BOTH AIR SAMPLING AND NASOPHARYNGEAL CARRIER SURVEYS, HAVE SHOWN THAT MENINGOCOCCAL TRANSMISSION MOST OFTEN IS BY DIRECT CONTACT WITH LARGE DROPLETS AND IS NOT RELATED TO BED POSITION IN THE BARRACKS. THE BIOCHEMICAL ACTIVITIES, NUCLEIC ACID COMPOSITION, AND SEROLOGIC IDENTITY OF REPRESENTATIVE STRAINS OF NEARLY ALL MYCOPLASMA SPECIES AND OF L-PHASE VARIANTS REPRESENTING DIVERSE GENERA OF BACTERIA HAVE BEEN DETERMINED. AVAILABILITY OF THIS FUNDAMENTAL DATA NOW MAKES POSSIBLE THE RELIABLE AND RAPID IDENTIFICATION OF ISOLATES FROM CLINICAL MATERIAL AND FACILITATES INVESTIGATION OF THE ROLE OF A WIDE VARIETY OF THESE AGENTS IN DISEASE. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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Project 3A014501B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 168, Bacterial diseases

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Description.

The reported studies were undertaken with the long range goal of controlling bacterial infections of military importance. Investigations of mycoplasma and bacterial variants were mainly at the level of identification, classification and development of serologic techniques which are necessary before it will be possible to ascertain the etiological role of these agents in human disease.

The approach to the study of meningococcal diseases has been broad. Purification and characterization of meningococcal polysaccharide antigens have been tied in closely with serologic investigations of host response to infection, both disease and the carrier state. Meningococcal transmission has been investigated by carrier surveys in recruits and civilian populations. Air sampling studies have been carried out with the aim of identifying those factors responsible for dissemination by carriers.

Progress.

1. Investigations on mycoplasmas and L-phase variants of bacteria.

Previous WRAIR Annual Reports (1966, 1967) have described various approaches for characterizing, distinguishing, and identifying mycoplasmas and L-phase variants of bacteria. These studies have been continued employing methods described in the earlier reports.

a. Molecular genetic studies on mycoplasmas and L-phase variants of bacteria.

The % G+C (molar ratio of guanine + cytosine x 100/adenine + thymine + guanine + cytosine of the DNA) of strains of most of the recognized species of Mycoplasma was determined by McGee et al. (Ann. N. Y. Acad Sci., 143:21, 1967). However, only a few "type" strains of Mycoplasma species were included in that study. For characterization of a given species, the characteristics of the type (or representative) strain of the species are required. Therefore, determinations have been made of the base composition of 23 additional strains, 19 of which are type or representative strains and four of which may represent new species. To date, the Tm (meltingtemperature) from which the % G+C can be calculated has been determined for these species. The Tm results are given in Table 1.

It will be noted that with three exceptions (M. neurolyticum, M. arthritidis, and M. salivarium) the Tm's determined in 1968 are, on the average, 0.66° lower than the Tm's determined in 1966 on other strains of the same species. This would be equivalent to approximately 1.3% G+C lower for the 1968 series than for the 1966 series. Since a 2% difference in G+C represents the limit of accuracy of this determination, these results, obtained by two different investigators in this laboratory, in different years, using different strains of each species, illustrate the high degree of consistency in results obtained, as well as the constancy of base composition of strains within a species. In order to obtain a valid base-line for calculating the % G+C of the 1968 series of organisms, a standard curve, using organisms for which the % G+C has been determined by chemical analysis, is now being obtained. The organisms being employed for the standard curve and the reported values for their % G+C are: Neisseria meningitidis, Nel5, (50.5) - Escherichia coli, K12, (50.1) - Proteus mirabilis, 9, (39.3) - Bacillus cereus, ATCC #12137, (36.0) - Mycoplasma gallisepticum, S6, (33.3) - and perhaps several others.

One of the characteristics that distinguishes mycoplasmas from bacteria is their relatively low G+C ratio. The thermal denaturation method for determining Tm decreases in accuracy when the G+C ratio reaches 25% or below. Since most of the mycoplasma species have G+C ratios in the range of 23-30%, other methods for confirming their base composition are desirable. One such alternative method is that of Hirschman and Felsenfeld (J. Mol. Biol. 16:347, 1966) based on hyperchromic spectral analysis. A modification of this method is currently being evaluated and has been tested on two species of bacteria and on 11 species of Mycoplasma. Results to date appear to be reproducible, and fairly good correspondence between G+C ratios by the two methods has been obtained. Of nine strains compared, five gave similar G+C ratios by the two methods, two gave approximately 2% G+C higher by spectral analysis and two gave approximately 2-3% G+C lower by spectral analysis.

Table 1. Thermal denaturation data on DNA of Mycoplasma strains studied by different investigators.

Mycoplasma species	ATCC No.	Strain	Tm+	Investigator**
arthritidis	19611*	Preston	82.97 ± 0.1	Ref. 1
	14152	Campo	84.05 ± 0.1	Ref. 2
	13988	H606	84.12 ± 0.5	Ref. 2
bovigenitalium	19852*	PG11-B2	82.58 ± 0.1	Ref. 1
	14173	PG11	83.01 ± 0.3	Ref. 2
bovirhinis	19884*	PG43	80.38 ± 0.1	Ref. 1
canis	19525*	PG14	82.43 ± 0.3	Ref. 2
felis	23391*	CO	80.26 ± 0.1	Ref. 1
fermentans	19989*	PG18	81.81 ± 0.2	Ref. 1
	15474	GII	82.14 ± 0.1	Ref. 2
gallinarum	19708*	Fowl	81.06 ± 0.3	Ref. 1
	15319	PG16	81.99 ± 0.1	Ref. 2
gallisepticum	15302	S6	83.74 ± 0.1	Ref. 1
			84.17 ± 0.5	Ref. 2
gateae	23392*	CS	81.71 ± 0.1	Ref. 1
granularum	19168*	BTS39	83.38 ± 0.1	Ref. 2
histotropicum	23115*	Sabin C	82.02 ± 0.3	Ref. 1
hominis, type 1	23114*	PG21	82.03 ± 0.1	Ref. 1
	14027	4387	82.56 ± 0.4	Ref. 2
	15056	H34	82.79 ± 0.1	Ref. 2
hyorhinis	17981*	BTS7	81.88 ± 0.2	Ref. 2
iners	19705*	PG30	82.01 ± 0.1	Ref. 1
laidlawii	23206*	PG8	83.69 ± 0.1	Ref. 1
	14089	Laidlaw A	84.40 ± 0.5	Ref. 2
	23217	PG9	83.42 ± 0.1	Ref. 1
	14192	Laidlaw B	84.30 ± 0.4	Ref. 2
maculosum	19327*	PG15	81.58 ± 0.3	Ref. 2
neurolyticum	19988*	Sabin A	80.72 ± 0.1	Ref. 1
	15049	KSA	79.79 ± 0.3	Ref. 2

Table 1. (Continued)

Mycoplasma species	ATCC No.	Strain	Tm+	Investigator**
orale, type 1	23714*	CH19299	81.44 ± 0.2	Ref. 1
	15539	823B	81.52 ± 0.4	Ref. 2
orale, type 2	23636*	CH20247	80.69 ± 0.1	Ref. 1
	23204	DC1600	81.43 ± 0.2	Ref. 2
pneumoniae	15531*	FH	86.74 ± 0.2	Ref. 1
	15377	Bru	87.38 ± 0.1	Ref. 2
pulmonis	19612*	Ash	81.48 ± 0.1	Ref. 1
	14267	Kon	81.97 ± 0.3	Ref. 2
	23554	Negroni	82.38 ± 0.5	Ref. 2
salivarium	23064*	PG20	82.99 ± 0.1	Ref. 1
	23557	Manire	81.90 ± 0.4	Ref. 2
spumans	19526*	PG13	81.79 ± 0.1	Ref. 1
	15146	PG13-old	82.42 ± 0.2	Ref. 2
sp. (dog)	23462	PG24	82.04 ± 0.1	Ref. 1
sp. (goat)	15718	C3OKS1	81.90 ± 0.1	Ref. 1
sp. (human)	15497	Navel	80.97 ± 0.2	Ref. 1
sp. (sheep)	23254	67-166	81.17 ± 0.1	Ref. 1

+ Melting temperature: Results of three thermal denaturation curves with standard deviation of mean.

* Type or representative strain of species.

** Ref. 1: Williams, C. O. et al., 1968, unpublished data.
Ref. 2: McGee, Z. A. et al., 1966, unpublished data.

Studies on the genetic relatedness of parent bacteria and their L-phase variants have been continued using Streptococcus faecalis, strain G-K parent, and its stable L-phase variant, G-K-L. (These organisms were kindly supplied by Dr. L. B. Guze.) The agar column hybridization method employed by McGee et al. (Ann. N. Y. Acad. Sci., 143:21, 1967) was used. The results in terms of per cent relative homology between parent coccus and L-phase variant are given in Table 2.

Table 2. Agar column homology data on S. faecalis, parent, G-K, and L-phase variant, G-K-L.

Radioactive "Donor" DNA	"Recipient" DNA in Agar	% Labeled DNA Bound	% Relative Homology
G-K	G-K	28.08	100
G-K	G-K-L	43.44	155
G-K	Blank agar	0.20	0
G-K-L	G-K-L	33.31	100
G-K-L	G-K	25.42	76
G-K-L	Blank agar	0.13	0

The actual per cent binding between both homologous and heterologous phases of the organism was somewhat less than expected. The relative per cent homology between labeled G-K and non-labeled G-K-L was, however, excessively high and probably indicates that some non-specific binding took place.

The relationship between G-K and G-K-L was reexamined using the elution profile method for DNA homology determination (Falkow and Citarella, J. Mol. Biol., 12:138, 1965). The results are given in Table 3. By this method the actual per cent binding between both homologous and heterologous phases was in the range expected. However, the excessively high relative per cent homology between labeled G-K and non-labeled G-K-L was again encountered and reinforced the impression that non-specific binding had occurred.

Comparing these results with results obtained previously (WRAIR Annual Report, 1967) in homology studies on S. faecalis (?), parent F24-P, and L-phase variant F24-L, it is apparent that with both sets of strains the percentage of binding was considerably higher when the parent coccus was used as the radioactive donor and the L-phase as the recipient than vice versa. The over-all results indicate there was at least as much, and probably somewhat more, homology between the two phases of the G-K set than there was between the two phases of the F24

Table 3. Elution profile homology data on S. faecalis, parent, G-K, and L-phase variant, G-K-L.

Radioactive "Donor"DNA	"Recipient" DNA in Agar	% Labeled DNA Bound	% Relative Homology
G-K	G-K	65.76	100
G-K	G-K-L	81.24	124
G-K	Blank agar	1.94	0
G-K-L	G-K-L	55.98	100
G-K-L	G-K	31.18	55
G-K-L	Blank agar	0.90	0

set of organisms. However, the nature of the differences in nucleic acid sequence of the parents when compared with their respective L-phase variants is not yet known.

Studies of the biochemical activities of S. faecalis strains G-K and G-K-L and of S. faecalis (?) strains F24-P and F24-L have shown that in each case the L-phase organism was quite similar to its respective parent. However, the two parent strains were not similar to each other. G-K appeared to be a strain of S. faecalis, whereas F24-P appeared to be a strain of S. faecium. The genetic relatedness of these two sets of strains was, therefore, examined, as well as the relationships among a number of other streptococcal strains that had been employed in various studies in this laboratory. The results are shown in Table 4. It is apparent that all these streptococcal strains have very similar G+C ratios (36.6 - 39.3%), and yet there is very little or no genetic relatedness among them. The highest percentage relative homology obtained between different strains was 20% between S. faecalis G-K and S. faecium (?) F24-P, supporting the impression gained from biochemical characterizations that these two are not members of the same species.

Thus, a number of genetic studies are being performed in an attempt to devise a system for identifying mycoplasma strains and for relating L-phase variants to the bacterial-phase of the respective strains. Further work is necessary in comparing the various techniques before deciding which are the most precise and convenient.

b. Biochemical characterization of L-phase variants.

An L-phase organism is a bacterial variant that has a defective or absent cell wall and that differs from the classical bacterium morphologically, culturally and, to a greater or lesser extent, physiologically. Morphological and cultural characteristics of L-phase variants of different bacterial species are closely similar. For this reason it has been impossible to use these characteristics for distinguishing the species of variant. Diagnostic biochemical tests for identification of these L-phase variants have also been unavailable, because modified procedures suitable for use with the variants have not been developed. Also, very few determinations have been made to date concerning retention by L-phase variants of the biochemical capabilities of the parent bacterial phase.

Such lack of adequate diagnostic procedures for use with L-phase variants has allowed three diagnostic problems to persist. The first is the inability to distinguish L-phase variants of different bacterial species when present in mixed culture. Such mixtures could arise either from concurrent isolation of L-phase variants of different species or from contamination of a previously pure culture. The second problem is the inability to relate an L-phase organism to a particular bacterial phase organism. Classic bacteria occasionally appear in L-phase cultures as a result of reversion of the L-phase or of contamination of

Table 4. Agar column homology tests for relatedness among streptococcal strains.

Radioactive "Donor" DNA	% G+C of "Donor"	"Recipient" DNA in Agar	% G+C of "Recipient"	% Labeled DNA Bound	% Relative Homology	Source of Data**
S. (faecium ?) F24-P	38.1 ± 0.3	S. (faecium ?) F24-P	38.1 ± 0.3	59.3	100	Ref. 1
S. faecalis G-K	36.6 ± 0.3	S. (faecium ?) F24-P	38.1 ± 0.3	5.8	20	Ref. 2
S. (faecium ?) F24-L	37.9 ± 0.3	S. (faecium ?) F24-P	38.1 ± 0.3	32.8	57	Ref. 1
S. faecalis G-K-L	36.6 ± 0.2	S. (faecium ?) F24-P	38.1 ± 0.3	3.4	9	Ref. 2
S. (faecalis ?) D-Campo-L	39.2 ± 0.3	S. (faecalis ?) D-Campo-L	39.2 ± 0.3	44.6	100	Ref. 3 Ref. 2
S. (faecalis ?) D-Campo-L	39.2 ± 0.3	S. (faecium ?) F24-P	38.1 ± 0.3	5.9	11	Ref. 3 Ref. 2
S. (faecalis ?) D-Campo-L	39.2 ± 0.3	S. (faecium ?) F24-L	37.9 ± 0.3	1.8	2	Ref. 3 Ref. 2
S. (faecalis ?) D-Campo-L	39.2 ± 0.3	S. sp. MG 9	39.3 ± 0.2	4.5	8	Ref. 3 Ref. 2
S. sp. MG 9	39.3 ± 0.2	S. sp. MG 9	39.3 ± 0.2	50.2	100	Ref. 3
S. sp. MG 9	39.3 ± 0.2	S. (faecium ?) F24-P	38.1 ± 0.3	3.3	0	Ref. 1
S. sp. MG 9	39.3 ± 0.2	S. (pyogenes ?) Bruno-L	37.2 ± 0.1	3.5	0	Ref. 3 Ref. 2

Table 4. Agar column homology tests for relatedness among streptococcal strains - (continued).

Radioactive "Donor" DNA	% G+C of "Donor"	"Recipient" DNA in Agar	% G+C of "Recipient"	% Labeled DNA Bound	% Relative Homology	Source of Data**
S. (faecium ?) F24-P	38.1 ± 0.3	S. (pyogenes ?) Bruno-L	37.2 ± 0.1	1.9	2	Ref. 1 Ref. 2
S. (faecium ?) F24-L	37.9 ± 0.3	S. (pyogenes ?) Bruno-L	37.2 ± 0.1	3.2	5	Ref. 1 Ref. 2
S. pyogenes Richards-L	36.8 ± 0.1	S. (pyogenes ?) Bruno-L	37.2 ± 0.1	ND*	ND	Ref. 2

* ND = Not determined.

** Ref. 1: Wittler, R. G., McGee, Z. A., Williams, C. O., Burris, C., Cohen, R. L., and Roberts, R. B. 1968, p.333. In: Microbial Protoplasts, Spheroplasts and L-Forms, editor L. B. Guze, Williams and Wilkins Co., Baltimore.

Ref. 2: Williams, C. O. et al., 1968, unpublished data.

Ref. 3: McGee, Z. A., Rogul, M., and Wittler, R. G., 1967, Ann. N. Y. Acad. Sci., 143:21.

the culture. If the bacterium arose by reversion, the L-phase could be indirectly identified by using classical diagnostic techniques to identify the revertant. If, however, the bacterium arose by contamination, such an indirect identification would yield an erroneous result. The third problem is the inability to identify the species of a non-reverting L-phase variant.

With these problems in mind the present study was undertaken. It had three facets; namely, (1) development of rapid, simple and reliable procedures for determining biochemical capabilities of L-phase organisms; (2) use of these procedures for determining whether L-phase variants retained the diagnostically significant capabilities of the bacteria from which they were derived; and (3) determination of suitable combinations of these test procedures to solve each of the three problems discussed above.

Routine biochemical diagnostic test procedures were selected and were modified for use with the fastidious L-phase variants. One modification, common to all the procedures, was enrichment of the test media with 10% (v/v) horse serum (inactivated at 60 C for 30 min) and 0.5% (w/v) Oxoid yeast extract. The tests included those for the breakdown of 24 carbohydrates including: adonitol, arabinose, cellobiose, dulcitol, fructose, galacose, glucose, glycerol, inositol, inulin, lactose, maltose, mannitol, mannose, melibiose, melizitose, raffinose, rhamnose, salicin, sorbitol, sorbose, sucrose, trehalose, and xylose. Reduction of tetrazolium and tellurite, as well as tolerance to tellurite, methylene blue, NaCl, and bile was tested. Also, hydrolysis of arginine, urea, and aesculin; production of catalase, oxidase, and phosphatase; tolerance to ethyl hydrocuprein; and production of acetylmethylcarbinol from glucose were included. Finally, the methyl red test and tests for phenylalanine deamination, oxidation or fermentation of glucose, nitrate reduction, and indole production were used.

The results of the tests for tolerance to tellurite, methylene blue, NaCl, and bile indicated that most of the L-phase variants were significantly less tolerant to these substances than the bacterial phases. Therefore, these tests were not considered to have diagnostic value for use with L-phase variants.

Otherwise, the results demonstrated that L-phase and revertant bacterial phase organisms could retain the biochemical capabilities of their parent bacterial organisms. The L-phase variants of Proteus mirabilis 9, Neisseria meningitidis 47 VI, N. meningitidis 55 III, Streptococcus faecalis G-K, S. pyogenes Richards, and Staphylococcus aureus ATCC retained all the significant capabilities of the bacterial phases from which they were induced. The revertant bacterial phases of N. meningitidis 47 VI, N. meningitidis 55 III, and Streptococcus sp. D-TC retained all of the significant metabolic capabilities of the L-phase variants from which they were derived.

The reactions for the bacterial and L-phases of the other strains, however, did not agree so closely. Results for the L-phases of S. faecalis F-24, Corynebacterium sp. D Campo B, S. aureus Smith, Streptococcus sp. Bruno, and Streptobacillus moniliformis differed somewhat from those of their respective bacterial phases, but were similar enough to indicate that the two phases of each strain were closely related. The L-phases of Streptococcus sp. Bruno and S. moniliformis were isolated independently of the respective bacterial phases, therefore, the differences between the biochemical capabilities of the two phases were probably due to differences existing between the bacterial phase used in the test and the bacterial phase from which the L-phase was actually derived. On the other hand, the differences between phases of S. faecalis F-24, Corynebacterium sp. D Campo B, and S. aureus Smith arose by selection of mutants or by contamination after the L-phases were derived from their respective bacterial phase parents.

Differences between the L-phases of Corynebacterium sp. D-5, Corynebacterium sp. D Campo A, and Gaffkya tetragena Gil and their respective bacterial phases were more extreme. Reactions for the two phases of Corynebacterium sp. D-5 indicated that they were both corynebacteria but only distantly related. Results for Corynebacterium sp. D Campo A suggested that the variant was a contaminant derived from a group D streptococcus rather than from a corynebacterium, and results for G. tetragena Gil indicated that the variant was a mycoplasmal contaminant. The conclusions for Corynebacterium spp. D-5 and D Campo A were supported by molecular genetic data obtained in this laboratory, and those for G. tetragena Gil were supported by serological growth inhibition data also obtained in this laboratory.

The final set of results included those for Proteus sp. 18-L, Proteus sp. 52-L, and Salmonella sp. Sal-L. The reactions for the three variants were identical, indicating that they were cultures of the same strain rather than different strains.

It was evident from the results that biochemical characteristics could be used to relate an L-phase variant to its presumed parent, for differentiating an L-phase variant of one species from that of another, and for identifying the species of an unknown, non-reverting L-phase variant.

The biochemical characteristics of the members of P. mirabilis 9, N. meningitidis 47 VI, and N. meningitidis 55 III sets were essentially the same, and those of Corynebacterium sp. D Campo A were considerably different. These results indicated that the L-phase variants of the first three strains were directly related to the bacterial phases, and the variant of the last set was not even closely related to the bacterial phase. These conclusions were confirmed by molecular genetic data. Results for the S. faecalis F-24 set, however, were not so clear-cut. The biochemical characteristics of both phases indicated that the L-phase was closely related to the bacterial phase, whereas molecular genetic data, obtained in this laboratory, indicated that the L-phase was

not so closely related to the bacterial phase. It was obvious from this example, therefore, that biochemically established relationships between L-phase variants and their presumed bacterial phase parents must be confirmed by other methods.

The second problem, that of differentiating L-phase variants of separate species and even separate strains within the same species, could clearly be resolved by the use of biochemical characteristics alone. Results demonstrated that variants of the different genera, species within the genera Streptococcus and Corynebacterium, and even strains of the species S. faecalis and S. aureus could be distinguished solely on the basis of different biochemical capabilities.

Resolution of the final problem, that of identifying an unknown, non-reverting L-phase variant, was not possible for all the variants on the basis of biochemical characteristics alone. Of the various L-phase organisms, the biochemical capabilities of which were consistent with those of their parents and representative of the species, only the variant phase of P. mirabilis 9 was distinctive enough to be identified solely on the basis of these characteristics. Although the characteristics of some of the other L-phase variants were typical for their species, the absence of typical morphological and cultural characteristics rendered their unequivocal identification impossible. It is apparent, however, that biochemical characteristics can be of great value for identification of all L-phase organisms when used in combination with other tests, such as those utilizing molecular genetic or serologic techniques.

The results have demonstrated, therefore, that biochemical test procedures can be adapted for use with L-phase organisms; that diagnostically significant biochemical capabilities of the parent bacterial phase organisms were retained by the L-phase variants as well as by the revertant bacterial phase organisms tested; and that suitable combinations of biochemical procedures could be established by which the three diagnostic problems could be approached and in many cases resolved.

c. Biochemical characteristics of Mycoplasmataceae.

Type and representative strains of 23 species of Mycoplasma and five unidentified strains of Mycoplasma have been characterized in terms of 12 biochemical activities. The reactions appear to be relatively constant for strains within a given species, and most species appear to be distinctive in the pattern of their reactions in the tests employed. These studies are nearing completion and will be reported in full at a later time.

d. Type culture collection of mycoplasmas and L-phase variants of bacteria.

The combined efforts of The American Type Culture Collection and Walter Reed Army Institute of Research have resulted in the

establishment of a collection of Mycoplasmataceae and L-phase variants of bacteria that is unique for its size and quality and that continues to increase its acquisitions. At the present time 34 Mycoplasma species are recognized; the collection contains 24 of these species. Three species are barred from the USA by import regulations, so that only seven species remain to be included in the collection. All of the cultures received last year from the Wellcome Research Laboratories' collection of type Mycoplasma species have been submitted to the ATCC during the current year.

The mycoplasma collection consists now of 60 different strains. Ten strains were received from investigators this year. In the near future seven additional strains, which are stored at WRAIR, will be submitted to the ATCC.

Sixteen strains of L-phase variants, 13 bacterial parent strains and 8 revertants from the L-phase are in the ATCC. In addition to these 10 L-phase strains, 6 bacterial parents and 6 bacterial revertants are to be submitted in the coming year.

The number of requests for both mycoplasmas and L-phase organisms has greatly increased this year. ATCC supplied 296 vials of mycoplasmas or L-phase organisms to investigators all over the world. Forty vials were sent out as exchange orders (no charge) to 11 investigators at WRAIR or other military installations.

Routine storage consists of 90 lyophilized (manifold type) vials and 20 vials stored in liquid nitrogen. Due to the increased number of vials lyophilized per acquisition and due to better methods of preservation and storage, only 750 lyophilized vials needed to be replenished during this year.

Of the mycoplasma strains in the collection, 50 per cent have been adapted to and are now propagated in 70 parts Bacto heart infusion broth plus 20 parts horse serum and 10 parts fresh yeast extract. This medium has been found satisfactory for growth and storage of the majority of strains and will gradually replace other media still in use. Initially, two or three passages of all strains are made in the medium recommended by the submitting investigator before adapting his strains to the heart infusion broth-horse serum-fresh yeast extract medium.

All mycoplasma cultures are now being submitted to ATCC in a concentration of 40 fold. It has been found through the joint efforts of ATCC and WRAIR that the storage of "hard to lyophilize" strains is greatly enhanced, if a final concentration of 12 per cent sucrose is added to the broth suspension. Previously there was a 4 log drop in colony count, whereas with this new method there is only a 1 log drop after lyophilization.

Colony counts, viability, and purity tests were carried out on 112 vials of mycoplasmas after preservation of seed stocks and on 20 cultures before preservation. Twelve vials of L-phase organisms, parent, and revertant bacteria were tested for viability. Another in the series of long term viability tests will be conducted this year on 14 strains of mycoplasma that have been in storage at ATCC for five years.

Growth inhibition (GI) tests were performed on 145 vials of mycoplasmas. These included 18 strains presently in ATCC of which 10 are identified species. In addition, GI tests were run on a number of unidentified mycoplasmas (not in ATCC) from human and animal sources. Tests were also run on hyperimmune mycoplasma serum from various sources including several biological houses. Thus, the GI test is used routinely as one of the tools for identification of all strains employed by members of this section in their various projects.

The following biochemical tests are being carried out routinely on the mycoplasma strains in the collection: acid production from dextrose, hydrolysis of arginine, reduction of tetrazolium, tellurite, and methylene blue, production of film and spots, and hemolysis of sheep red blood cells. At present 20 different strains of mycoplasma in the collection have been reexamined, and the stability of their biochemical characteristics verified by these tests. Selected appropriate tests for L-phase organisms will likewise be employed in the future.

To assure the purity and validity of all strains used in the various projects of this section, the strains are grown from ATCC vials, and propagated in suitable quantities by the bacteriologist responsible for the culture collection. These well-grown cultures are then turned over to other investigators of this section for use in particular projects. Thus, large quantities of 23 strains of mycoplasma and L-phase organisms were grown for DNA extraction and large quantities of eight mycoplasmas were grown for complement fixation antigens. At times a large batch of growth will be divided among different members of the section for simultaneous biochemical, genetic, and serologic investigations of a particular strain. Thus, strain and cultivation variations are eliminated from the results.

e. Effect of M. hominis on chromosomes of leukocytes in culture.

Since 1965 several reports have appeared linking infections of tissue cultures of human cells by mycoplasmas with abnormalities of the cells' chromosomes. Such abnormalities have included bizarre morphological variations, minute breaks in chromosomal continuity, and the addition of an extra chromosome. These observations, along with the finding of a high percentage of positive results for M. hominis antibody in women of the gestational age group which produces the greatest numbers of mongoloid babies, have given rise to the question of whether chronic infection of the genitourinary tract with M. hominis could be the cause

of sporadic mongolism, and could explain certain "outbreaks" of mongolism that have been noted historically. The present study was undertaken to explore the possibility of a relationship between mycoplasma infection and one of the clinical trisomic syndromes.

M. hominis, type 1, strain PG21 was the organism employed for this study. The leukocytes used were obtained from 11 females (15-26 years of age) and two males (5 and 30 years of age) all in good health. Plasma of all donors was tested for antibodies to M. hominis by the complement fixation test and was found to be negative. The methods employed were derived from those reported by Fogh et al. 1965, Paton et al. 1965, and others.

A series of leukocyte cultures were inoculated with viable M. hominis, incubated, processed, and subjected to analysis of chromosome count, morphology, and rate of mitotic activity. Replication of the mycoplasma inoculum was confirmed by the production of typical colonies on agar at the end of incubation. Antibiotics were not used.

A total of 21 infected and 13 control leukocyte cultures were satisfactory and were analyzed for mitotic index (number of mitoses in 1000 cells) and chromosome morphology. The mitotic index varied from 0 to 25 in both infected and control cultures and appeared unrelated to inoculum size or replication. No abnormal chromosome morphology was observed in any of the cultures.

These experiments demonstrated that variations occurring in leukocyte cultures are considerable and that studies of this type require rigid controls. Nevertheless, M. hominis infection did not produce chromosomal changes under the conditions utilized.

f. Studies on mycoplasma infections in man and animals.

(1) Study of M. pneumoniae infection in a family group.

The father, FBR, a 31 year old Army Medical Officer, became ill with frontal headache and malaise on 25 February developing hacking cough and myalgia on the following day. After an episode of night sweats and chills he was hospitalized 28 February with clinical and X-ray evidence of right middle lobe pneumonia. An acute serum from the fourth day of illness was negative at 1:4 dilution for M. pneumoniae antibodies. The patient's fever reached a peak of 102.5°F., with a lytic response on the sixth day of illness, ten hours after starting erythromycin. Intermediate PPD and skin tests for fungi were negative. After an eight day hospitalization the patient was discharged to complete a 14 day course of erythromycin. The right middle lobe pneumonia gradually cleared over the next two months, while the patient remained asymptomatic. A convalescent serum on 28 March, four weeks after the acute, had a C.F. antibody titer of 512 with M. pneumoniae antigen.

On 18 March, FWR, the nine year old son of FBR, became ill with bronchitis and fever of 102°F. Chest X-ray showed increased bronchovascular markings only. After four days of fever and progressive cough the child was placed on a ten day course of erythromycin. Lysis of fever occurred within 18 hours from starting the antibiotic. An acute serum from 23 March was negative at 1:4 dilution for M. pneumoniae C.F. antibodies; convalescent serum on 4 April reacted at 1:128.

Another son, JAR, six years old, became ill 21 March with cough and fever. His X-ray was similar to the older brother's, showing increased bronchovascular markings. No antibiotics were given. The initial serum of 23 March was negative at 1:4 for complement fixing antibodies. A convalescent specimen drawn on 4 April was positive at titer 64.

ALR, the mother and only remaining family member, had severe headache on 21 March without respiratory symptoms. Serum from 23 March and 4 April were negative at 1:4 dilutions for M. pneumoniae complement fixing antibodies.

Additional specimens will be submitted to determine the duration of time that antibodies remain present in significant titer after convalescence.

(2) Study of M. pulmonis infection of laboratory rats.

A number of rats (Walter Reed/WCF) used in malaria transmission experiments by the Department of Medical Zoology (WRAIR), developed arthritis during the course of the experiments. Two arthritic rats, experimentally infected with Plasmodium berghei, were autopsied and blood, joint, liver, and spleen cultured. Mycoplasmas were isolated from all specimens. Two of the isolates, one from blood and the other from knee joint of one of the rats, were examined biochemically and serologically (growth-inhibition) and identified as M. pulmonis. This species has not previously been known with certainty to cause arthritis in rats, although it is a common respiratory pathogen. (M. arthritidis and M. histotropicum have been the species associated in the past with rodent arthritis.) Cultures of the M. pulmonis isolates were tested for pathogenicity by intraperitoneal injection into normal and malaria-infected rats, and in both sets of animals produced arthritic symptoms.

Antigens were prepared from the blood and joint isolates of M. pulmonis and tested by CF against known anti-M. pulmonis serum and against other serums from spontaneously and experimentally infected rats. These antigens reacted strongly with all the test serums but not with controls (see Table 5).

A serologic study of rats used by the Department of Medical Zoology was then undertaken. These included 20 rats with or without malaria infection and with or without arthritic symptoms. The serums were tested by CF for antibodies to M. arthritidis (Campo) and M. pulmonis (Kon). There were no positive reactions with M. arthritidis

Table 5. Comparison by complement fixation test between mycoplasma isolates from rat and known mycoplasma species.

		Antigen			
		M. pulmonis Kon	*M. sp*; isolate (knee)	*M. sp*; isolate (blood)	*M. arthritidis* Campo
Serum designation	Source of serum	Titer			
Rat 3	Spontaneous infection rat	128	256	128	< 4
Rat 34	Experimental infection rat	32	256	128	< 4
Rat 42	Experimental infection rat	128	128	64	< 4
Rat 43	Experimental infection rat	128	256	128	< 4
Rat 4	Normal rat	< 4	< 4	< 4	< 4
Rat 20	Normal rat	< 4	< 4	< 4	< 4
Anti-pulmonis Kon	Rabbit	128	256	256	16
Anti-pulmonis Negroni	Rabbit (commercial)	256	512	256	ND*
Anti-arthritidis Campo	Rabbit	< 4	< 4	< 4	512

* Not determined.

antigen but 12 positive reactions with M. pulmonis antigen. The titers ranged from 4 to 256.

The serums of several spontaneously infected rats were also tested by CF against M. histotropicum antigen but yielded only partial fixation at dilutions of 1:4 and 1:8.

Finally, in an effort to determine the source of the infection, the serums from another series of 90 rats were tested by CF against M. pulmonis (Kon) antigen. Included were rats with and without malaria, with and without spontaneous or experimental mycoplasma infection, and controls. Correlation between the presence of arthritic symptoms and a serum titer against M. pulmonis was very high.

Complete results of this study will be reported later. However, the impression gained so far is that the stock rat colony is not endemically infected with M. pulmonis. Animals used for the malaria transmission studies appear to be the only ones infected with Mycoplasma and the source may originally have been one mycoplasma-infected-malaria-blood-passage specimen.

Several passages of the malaria strain through mice combined with tetracycline treatment of infected rats resulted in prevention of the mycoplasmal arthritis.

g. Serological studies with mycoplasma.

Mycoplasmal antigens have been prepared in concentrations of 10^{11} to 10^{12} viable organisms per ml. For use in CF tests these antigens are heated at 56°C for two hrs and diluted 1:50 to 1:100 in saline.

The metabolic inhibition test for antibody has been adapted for use in this laboratory with M. pneumoniae and M. gallisepticum.

Preparation of antisera in rabbits.

A comparison of various routes of inoculation was performed:

(a) IV-IP route

1 ml I.V. and 1 ml I.P. once weekly x 3.
Rest 2 weeks, then 1 ml I.V.
Bleed 7-10 days later.
For I.P. inoculation mix antigen with Freund's incomplete adjuvant.

(b) Subcutaneous (S.C.) (back)

Antigen mixed with Fruend's incomplete adjuvant.
Two ml of mixture given S.C. in thoracic area and also lumbar.
Repeat 7-10 days later.
Day 23 - 1 ml. given I.V.

(c) Foot pad (F.P.)

Use equal amounts of antigen and Freund's.
Inoculate 1 ml into each foot pad.

The results of CF antibody tests are shown in Table 6. It can be seen that the F.P. method of immunization is as good as the other routes and has the advantage of requiring less antigen and only one injection day. Currently these reference sera are being tested by the metabolic inhibition test.

CF tests on human sera for clinical diagnostic work are being performed frequently with M. pneumoniae. As a greater number of mycoplasmal antigens are prepared they will be utilized in clinical studies.

2. Studies of meningococcal meningitis.

a. Prevalence of meningococcal serogroups.

From 16 May 1967 to 8 May 1968, 305 meningococcal strains have been submitted from other laboratories for confirmation or assistance in identification. These are shown in Table 7. All but those labelled miscellaneous were derived from cases of systemic infection (blood or CSF isolate) in military personnel or dependents. Two hundred and forty-six (83%) of these strains were resistant to sulfadiazine at a level of 0.1 mg%. This represents an increase in resistant strains over the proportion observed in 1966. Once again group A strains were rare, a single strain being submitted from a military patient hospitalized in Europe. Group C sulfadiazine resistant organisms accounted for 162 of the 281 cases (58%). It can be seen from the table that this serogroup was the predominant virulent strain in all but one U. S. Army area from which only six isolates were submitted. Thus, the pattern which began to emerge last year at Fort Dix became widespread.

Table 8 shows the total experience of this laboratory since 1964. The shift from group B sulfa resistant to group C sulfa resistant strains began in 1966 and has steadily increased in 1968.

b. Carrier surveys.

Meningococcal carrier surveys were undertaken throughout the year on several populations in order to compare the frequency of the carrier state, the serogroups being carried and the level of transmission. They were also utilized for the purpose of supporting other aspects of the program by collection of strains and serum.

These surveys showed a number of interesting points (Tables 9, 10 and 11):

(1) In recruits, initial throat cultures were positive in from 4 to 31

Table 6. CF antibody titer on rabbit antisera.

Antigen used	Route	Day after inoculation								
		0	7	14	21-24	28-30	36-38	42-45	50	70
M. gallisepticum A-1 Agent	F.P.	16		1024	2048	4096		2048	2048	1024
	S.C.	16		256	512	4096	4096	4096	2048	1024
M. pneumoniae FH strain	F.P.	16	1024	1024	2048	2048	4096	4096		
	S.C.	16	256	512	1024	2048	4096	4096		
	IV-IP	16		1024	2048	2048	2048	2048		
M. pulmonis	F.P.	16	512	512	1024	2048	4096	1024		
M. hominis	F.P.	16		1024	2048	8192	8192	8192	4096	
M. arthriditis	F.P.	16	2048	4096	4096	4096	8192	8192	4096	

Table 7. Meningococci strains received at WRAIR for confirmation - 16 May 1967 through 8 May 1968.

Army Area	Sensitive							Resistant							Area Totals
	A	B	C	Bo	29E	Other	Total	A	B	C	Bo	29E	Other	Total	
I	0	4	2	2	0	1 Rough	9	0	2	82	0	0	0	84	93
II	0	4	0	1	0	135-III	6	0	0	20	0	0	0	20	26
III	0	8	5	4	0	0	17	0	11	38	0	0	0	49	66
IV	0	1	0	0	0	0	1	0	3	1	0	0	0	4	5
V	0	2	1	1	0	0	4	0	1	9	0	0	0	10	14
VI	0	2	1	0	0	0	3	0	4	11	0	0	0	15	18
Europe	1	5	1	0	1	135-III	9	0	0	1	0	0	0	1	10
Sub Total	1	26	10	8	1	R=1 135-III=2	49	0	21	162	0	0	0	183	232
Misc.	1	3	1	1	1	135-III=3	10	1	1	1	0	0	135-III	4	14
Totals	2	29	11	9	2	R=1 135-III=5	59	1	22	163	0	0	135-III=1	187	246

Table 8. Meningococci associated with systemic disease.

Serogroups	1964		1965		1966		1967		1968*		Summary Totals			
	S**	R***	S	R	S	R	S	R	S	R	S	R	All	% R
A	5	0	5	0	1	0	1	0	0	0	12	0	12	0
B	82	155	71	86	67	164	21	33	12	16	253	454	707	64
C	21	3	22	2	14	26	13	75	7	144	77	250	327	76.5
WRAIR-Boshard	2	0	3	1	8	5	9	5	7	0	29	11	40	26.8
WRAIR-29E	1	0	1	0	0	0	0	0	1	0	3	0	3	0
WRAIR-135					2	0	20	0	1	0	5	0	5	0
Totals	111	158	102	89	92	195	46	113	28	160	379	715		
	269		191		287		159		188		1094			
% R		58.7		46.6		67.9		71.6		85				65.3

* 1968 data through 8 May 1968 only.

** S = sulfa sensitive (0.1 mg%); R = resistant to 0.1 mg%.

Table 9. Meningococcal carrier surveys - Fort Dix, New Jersey, 1967-1968.

Company	Date	Week of training	No. pos. / No. tested	% Pos.	% of strains sulfa resistant	Serogroup					Non typable
						B	C	Bo	29E	135	
A-4-3	9 Oct 67	1st	10/48	21	20	4(1)	2(1)	-	-	-	4
	28 Nov 67	8th	21/38	55	0	4	5	7			5
A-1-3	28 Nov 67	1st	2/50	4	0	-	1	-	-	-	1
	4 Jan 68	4th	45/135	33	9	16(2)	11(2)	4	2	-	12
	10 Jan 68	5th	53/129	41	21	29(6)	9(4)	7(1)	3	-	5
	16 Jan 68	6th	61/133	46	26	8(4)	16(10)	6	1	-	30(2)
	25 Jan 68	7th	80/123	65	40	8(4)	31(25)	5	1	-	35(3)
B-1-3	13 Feb 68	1st	15/49	31	20	5(2)	3(1)	2	2	-	3
	26 Feb 68	3rd	32/46	70	63	4(2)	22(18)	2	1	-	3
	2 Apr 68	8th	37/44	84	89	-	33(32)	1	1	-	2(1)
D-2-3	13 Feb 68	1st	12/51	24	25	-	5(2)	1	2	-	4(1)
	26 Feb 68	3rd	29/46	63	83	-	23(23)	2(1)	1	-	3
	2 Apr 68	8th	44/49	90	84	-	37(33)	1	5(4)	-	1
E-5-3*	10 Mar 68	5th	162/195	83	87	53(50)	84(82)	10(4)	-	1(1)	14(3)
	2 Apr 68	8th	25/28	89	96	8(7)	17(17)	-	-	-	-

* 3 cases of meningococcal systemic infection in this Company.

Table 10. Meningococcal carrier surveys - Fort Benning, Georgia, 1968.

Company	Date	Week of training	No. pos. / No. tested	% Pos.	% of strains sulfa resistant	Serogroup					Non-typable
						B	C	Bo	29E	135	
D-7-2	9-10 Feb*	0	16/139	11.5	13	3(1)	1	1	-	-	11(1)
	6 Mar	3rd	79/224	35	38	8(1)	31(25)	40(4)	-	-	-
	2 Apr	7th	106/154	69	30	6	67(28)	22	1	1(1)	9(3)

* San Juan AFES - 6 NT were lactose positive.

Table 11. Meningococcal carrier surveys - Pediatric Clinic, Walter Reed General Hospital, 1967-68.

Date	No. pos. / No. tested	% Pos.	Carriers by age group: 1-3 mos.	4-6 mos.	7-12 mos.	13-23 mos.	2-4 yrs.	5-7 yrs.	8-10 hrs.	11-13 yrs.	14-17 yrs.
19-29 Sept. 1967	13/137*	9.5	0/36	0/4	0/7	1/5	5/19	6/29	0/16	1/21	-
6 Feb-6 Mar 1968	20/140**	14	1/32	1/11	2/15	3/16	9/29	2/12	0/10	2/15	-
Oct 67 WRAIR families	Children*** 7/36	19	0/1	-	0/1	0/2	3/10	2/5	2/8	0/4	0/5
	Parents 1/24	4									

* Only 1/13 groupable (B)
8/13 Lactose pos.
1/9 sulfa resistant

** Parents 4/42 = 9.5%

Serogroups	A	B	NT
	2*	5	17
Lactose	(1)	(2)	(14)

*Did not produce A substance

*** Only 1 groupable (B-sulfa resistant)
All lactose negative

per cent of the men. Group C, sulfadiazine resistant strains were predominant after Jan 1968 in all companies studied (eastern United States). These group C strains spread very rapidly in most of the units and were associated with many cases of meningitis (see above).

(2) Carrier rates in pediatric populations and in adults living in civilian communities were similar to those in newly arrived Army inductees. Meningococcal strains isolated from children and their parents and from Puerto Rican inductees were quite different in several characteristics from the highly transmissible organisms found in recruit camps. The majority were nontypable; often agglutinating spontaneously in saline, many fermented lactose in addition to dextrose and maltose. Group C sulfadiazine resistant organisms were not found in the pediatric populations studied. Meningococcal carriers were infrequent in children under one year of age and were common in the age group two to seven years.

(3) Group A organisms continued to be absent from both civilian and recruit populations. The two possible group A strains isolated (Ped. Clinic, Feb-Mar 68) were found not to produce the group A specific polysaccharide when tested by haptene inhibition.

c. Transmission of meningococci.

Factors which affect the spread of meningococci among Army recruits are poorly understood. Previous studies by this laboratory have shown that there is no correlation between acute respiratory disease rates or adenovirus infections and meningococcal carrier rates. Furthermore, the type of barracks housing (new brick buildings with two and eight man rooms versus old, wooden, open bay cantonment-type structures housing 25 men on each floor) did not affect meningococcal carrier rates.

In the present studies, analysis of meningococcal transmission amongst men who slept in the same room was made in two basic training companies more than a year apart.

Company G-2 (Nov-Dec 1966) consisted of four platoons of 44-50 men each. The fourth platoon suffered two cases of meningitis the week prior to initiation of surveys (5th and 8th weeks of BCT). Bunk placing of each man was determined and the results of nasopharyngeal carrier surveys are shown in Table 12.

Total carrier rates for meningococci were about the same in both new and old barracks at the time of the first survey and rose to the same degree three weeks later. The main difference between the two barracks was that sulfadiazine sensitive type B organisms accounted for only 16 per cent of isolates in Platoons 1, 2 and 3 (new barracks) but comprised 50 per cent of strains recovered from men in old barracks in the Dec 6th survey. Although two cases of meningitis due to group B sulfadiazine sensitive meningococci occurred in men bunking on the second floor of the old barracks, total carrier rates as well as the proportions of group B sulfa sensitive organisms were similar on both floors, suggesting

Table 12. Results of meningococcal carrier surveys in Company G-2.

Type of quarters			No. of rooms	Date 17 Nov 1966		Date 6 Dec 1966		No. of sq ft foor space/man
New Barracks	2 man rooms		19	5/29 17%	(2B)**	15/32 47%	(2B)	81*
(Platoons 1,2,3)	7-8 man rooms	1st floor	3	4/20	(1B)	16/23	(4B)	66
		2nd floor	6	8/34	(2B)	19/42	(2B)	66
		3rd floor	6	17/35	(3B)	38/44	(6B)	66
				29/89 33%	(6B)	73/109 67%	(12B	
	Total (3 platoons)			34/118 28.8%	(8B) 23.5%	88/141 62.3%	(14B) 15.9%	
Old Barracks	1st floor			5/19	(2B)	10/19	(5B)	72
(Platoon 4)	2nd floor***			4/22	(1B)	16/27	(8B)	66
	Total			9/41 22.0%	(3B) 33.3%	26/46 56.5%	(13B) 50%	

* 3 rooms had 56, 56 and 65 sq ft.

** ( ) = No. of sulfadiazine sensitive group B meningococci.

*** Two cases of meningitis (group B, sulfa sensitive) occurred in this group.

that bed distance from a known case bore no direct relationship to nasopharyngeal acquisition. Average floor space per man in old barracks was 72 sq ft on first floor and 66 sq ft on second floor.

Analysis of carriers in new barracks showed variations in transmission in rooms sleeping seven-eight men (Table 12). For example, in three rooms on the first floor, carrier prevalence increased from 4/20 (25 per cent) on Nov 17 to 16/23 (70 per cent) on Dec 6. Six such rooms on the second floor showed rates of 8/34 (24 per cent) and 19/42 (45 per cent) and six rooms on the third floor had rates of 17/35 (49 per cent) and 38/44 (86 per cent). The men in these rooms had, on the average, 66 sq ft of floor space. With only a few exceptions each platoon was quartered on a different floor (a third of the men in 1st platoon occupied two man rooms on the second floor).

In the two man rooms carrier rates were lower, 5/29 (17 per cent) and 15/32 (47 per cent) on November 17 and December 6, respectively. Five of these rooms showed one man to be a carrier on November 17. Of these, men from four rooms were included in the December 6 survey and showed no acquisition by the other occupant in three instances and acquisition of a strain different from that of the index carrier in the fourth room. Only two rooms of the remainder showed two men positive on December 6 and none of these roommates were carrying organisms of the same serotype. Thus, little or no transmission occurred between roommates in two man rooms.

Of 15 rooms occupied by seven or eight men each, Group B sulfadiazine sensitive strains were isolated from carriers in seven different rooms, but a density of three B sensitive carriers was found in only one room (307) (Table 13). Therefore, in these eight man rooms, group B sensitive strains did not show a high transmissibility but dissemination of meningococci was greater than in two man rooms. Data concerning secondary attack rates on 6 December for meningococci present initially in index carriers in the rooms are shown in Table 14. It can be seen that more acquisitions were derived outside the room than within.

Table 14. Secondary attack rates in seven-eight rooms in a three week period (G-2).

51 noncarriers (29 index carriers)	
13 (26%)	acquired an index strain
	(also, 2 index carriers converted to another index strain)
15 (29%)	acquired a new strain
	(also, 6 index carriers acquired a new strain
2	acquired a nontypable strain.

These results show that the percentage of carriers of meningococci of all types in the 4th platoon (the origin of the two cases) did

Table 13. Carrier status of men sleeping in eight man rooms.

Room No.	Meningococcal isolations		
	Nov. 17	Dec. 6	Serotypes isolated
103	1/8*	6/8	2 B-R, 1 B, 1 C-R, (B + Bo-R), (C + B-R)
104	2/7	5/8	2 B, B-R, C, NT
105	1/5	5/7	2 NT, B-R, Bo-R, C-R
202	2/6	3/7	B, C-R, NT
203	2/5	2/6	B, NT
205	0/5	3/7	Bo, B-R, C-R
206	2/5	5/8	3 B-R, (B-R + C), NT
207	2/7	4/6	4 C-R
208	0/6	2/8	C, +
302	3/7	6/8	B, B-R, C, Bo, NT, +
303	1/4	8/8	4 C-R, 3 Bo, B-R and C
305	1/4	5/7	3 Bo, C-R, B-R and C
306	6/7	6/7	4 C-R, Bo, Bo-R
307	5/6	7/7	3 B, 2 Bo, C, C-R
308	1/7	6/7	2 Bo, B-R, C, (B + C-R), (B + Bo-R)

* No. positive/No. tested

B-R = group B, sulfadiazine resistant; NT = nontypable;
+ = meningococcus, serotyping not performed.

( ) mixture of two serotypes.

not differ significantly from that found in the other three platoons. Group B sulfadiazine sensitive meningococci similar to the strains associated with the two meningitis cases were distributed amongst all four platoons in small numbers at the time of the first survey but accounted for 50 per cent of carriers in the 4th platoon at the time of the final survey. Thus, strain virulence was associated with a high transmissibility in this circumstance.

The bunk spacing data suggest that although the platoon (48-50 men) is the unit most basic to meningococcal transmission, the nearness of the bunk to a known meningococcal carrier or meningitis patient did not correlate with acquisition of the meningococcus in the old open-bay barracks where 20 to 28 men shared one large room. In the eight bunk rooms of the new style barracks, transmission reached a peak level (67 per cent) which was slightly greater than that of the old type barrack (57 per cent). The two man rooms contrasted with the other types of sleeping arrangements in that total carrier rate was lower (47 per cent) and spread of a strain from one man to his roommate was not found.

Although differences in square feet of floor space per man might explain some of these findings (i.e., the two man rooms had the greatest amount of floor area per man and the lowest carrier rate) the variations in carrier rates between different eight man rooms (Table 13), all of which had similar floor space per man, would tend to diminish the significance of floor space considerations in meningococcal transmission.

Company A-1-3 was a small company consisting of three platoons of 47-48 men. This company began training during the week of 28 Nov 1967, was interrupted by a two week leave period for the Christmas holidays and returned to training on 1 Jan 1968. The men were quartered in new style barracks being dispersed in rooms with two or seven bunks each. Each platoon occupied one floor of the building but shared a common lounge, telephone booths and laundry room on the first floor.

Meningococcal carrier surveys were performed during the first week of training (only 50 men tested - two positive) and then weekly from 4 Jan 1968 through 25 Jan 1968. Carrier rates increased from 33 per cent to 65 per cent between these dates (4th and 7th weeks of BCT). Group C sulfa resistant strains and nontypable sulfa sensitive strains spread to about the same extent during this time.

Analysis of the spread of meningococci among men bunking in the same room was carried out in several ways. Carrier status of occupants of two man rooms at the time of the final survey (7th week of basic training) is shown in Table 15; that of seven man rooms is presented in Table 16. It is apparent that men sleeping in the same room very often carried meningococci of different serogroups. If the nontypable organisms are tabulated as "similar" (to index strains) then the ratio of rooms containing "similar" carriers to those with "different" carriers is 11:11 or about 50 percent (data from both tables combined).

Table 15. Carrier status in two man rooms.

Both men positive	(a)	same strain	1 room
	(b)	different strains	2 rooms
	(c)	uncertain typings	1 room
Incomplete data			4 rooms

Table 16. Meningococcal carrier status in seven man rooms.

	No. of carriers per room							
Strains isolated	1	2	3	4	5	6	7	Totals
3 or more different	-	-	3	1	0	0	1	5
At least 2 different	-	0	0	0	1	1	1	3
3 or more similar	-	-	0	3	1	0	0	4
Identification uncertain	-	1	3	1	0	0	0	5
Total rooms studied	1	1	6	5	2	1	2	18

Table 17 presents data concerned with transmission within a room over a three week period. Spread from index carriers (positive at first testing) to roommates occurred in only four rooms; index strains did not spread to other men in seven rooms. If those rooms in which nontypable strains made analysis uncertain are considered as showing positive evidence for spread then 11 rooms would show transmission of index strains to at least one man. For comparison, 11 rooms showed the introduction of a different serogroup.

Table 17. Spread of meningococci to roommates (seven man rooms) over three week period.

Spread of index strains to	1 man	3 rooms
	3 men	1 room
No spread of index strains		7 rooms
Uncertain (nongroupable strains)	1 man	5 rooms
	2-3 men	2 rooms
Other strains introduced		11 rooms

Secondary attack rates were calculated as shown in Table 18. Index carrier organisms spread to only eight per cent of men (25 per cent if NT strains are considered the same as index strains). Twenty-five per cent of susceptible roommates acquired a strain different from that of index carriers in each room. Thus, these data indicate that meningococcal acquisitions occurred independent of bunk arrangements.

Table 18. Secondary attack rates in seven man rooms in a three week period.

77 noncarriers (41 index carriers)

6 (8%) acquired organism similar to that carried by a roommate

19 (25%) acquired a new strain unlike that of a roommate

13 (17%) acquired a NT strain

If all NT strains are considered similar to index carriers then 19 (25%) acquired a strain similar to roommate.

Personal observations confirmed by discussions with individuals who have undergone Army basic training indicate that recruits have as much or more direct contact with other members of their platoon

as with their roommates.

Air sampling tests performed last year indicated that droplets larger than five microns in diameter are produced in much greater quantity by carriers than true droplet nuclei, which would favor direct contact spread rather than true airborne transmission. Thus, for purposes of controlling transmission, opportunities for contact will have to be decreased. Since the significant contacts occur within the platoon rather than amongst roommates, measures aimed at increasing floor space between bunks or increasing ventilation in sleeping quarters will not be expected to decrease transmission. Other places of close contact within the platoon and common facilities where contact with men from other training units occurs must be examined for the possibility of interrupting transmission.

Air sampling for meningococci: Previous studies in this laboratory have shown that meningococci are dispersed by coughing, each individual producing rather constant numbers of viable organisms with each cough over a period of three days. Individuals varied in the number of meningococci expelled, from none to more than 1000 per cough, but the factors responsible for the differences were not apparent. In that study all ten persons tested had symptomatic respiratory disease but only four had a concomitant adenovirus infection. These four men were not expelling more meningococci than those without adenovirus infection.

The present studies were an attempt to study several factors which might be related to dispersal; namely, duration of the carrier state and presence or absence of respiratory disease. Two different populations were studied: (1) laboratory personnel and (2) Army recruits.

Methods: The Litton Large Volume Air Sampler (LVS) was that used previously and was modified by the addition of a preimpactor which trapped particles over 15 microns in size. Mueller-Hinton agar containing lincocin and polymixin was used in the preimpactor and colonies were counted on the solid medium after incubation. This differed from last year when a gelatin medium was used and was melted and then filtered through a .45 micron Millipore filter, colonies being counted on the surface of the filter. The fluid phase medium was that previously described; it also contained the selective antibiotics and was filtered as above. Two to four representative colonies from each sample were picked and studied by gram stain, oxidase reaction, carbohydrate fermentation, sulfadiazine sensitivity and serum typing for comparison with nasopharyngeal isolates from the same subject. Each cough's aerosol production was sampled for one minute at 10,000 liters/minute. Relative humidity (RH) was measured by a sling psychrometer or a Serdex recorder (Bacharach Industrial Instrument Co., Pittsburgh, Pa.).

Cough sampling of recruits was performed in an unused room in the barracks which measured 1316 cubic feet in size. All personnel involved in collecting samples were tested daily and found to be free of meningococci in the nasopharynx. The LVS was washed with alcohol and

distilled water after each test sample; the room was ventilated by opening the windows and door to a cross draft. Occasionally, samples were taken in larger rooms. Three minute control samples were taken each day prior to testing the first subject and were uniformly negative. LVS isolates were identified in but one trial in which the subject had no meningococci recovered from a throat swab. These isolates were the same serogroup as that found in the throat of the previous test subject. Review of the experimental notes indicated that in this one circumstance the room ventilation had been omitted.

Chronic carriers: Four laboratory personnel known to be meningococcal nasopharyngeal carriers for more than three months were tested for dispersal in a small room in the laboratory. None had respiratory symptoms during the period of study. As many as five determinations were made over a period of seven to eight weeks as shown in Table 19. The first three individuals were low grade dispersers although occasional coughs produced 14-16 colonies in the LVS fluid. J. Dav. was tested only twice over a two day interval and was a moderate disperser (25 and 13 colonies). During these studies ambient temperature was fairly high (77-82°F) and relative humidity varied on different days (18-69%).

Army recruits: Twenty-five individuals from Company A-1-3, Fort Dix, New Jersey, were tested on one or more occasions. Based upon frequent nasopharyngeal surveys (see above) it was possible to document the approximate time of acquisition of the carrier state. Table 20 indicates the duration of carriage for each man when first tested for dispersal.

Table 20. Duration of carrier state in individuals tested for meningococcal dispersal.

Duration	Roster numbers of subjects
>8 weeks	101, 147
>9 days but <8 weeks	113, 127, 145, 214, 240, 331, 332, 341
12 to 18 days	126, 217, 225
>6-7 days but <13 days	105, 106, 133, 215, 229, 303, 306, 342
<7 days	301, 304, 324, 325

Two successive coughs were tested on five subjects (Table 21) and on the basis that variability was minimal and the first cough usually produced the larger number of recoveries, a single cough was utilized for the remaining tests.

Variation of dispersal by the same subject in two or more tests over a period of eight days was not great, as shown in Table 22. For this and subsequent analyses degrees of dispersal were arbitrarily assigned (see footnote Table 22). Ten of 19 showed no significant

Table 19. Meningococcal dispersal by chronic carriers.

Carrier duration	Sample No.	Date	N.P. swab		Aerosol isolates from coughs: Fluid	Aerosol isolates from coughs: Impactor	RH	Temp(F)
E. Will. >3 mo.	9	12/13/67	6 col.	B+C	16	0	35%	80
					0	1		
	12	12/18/67	4+	B	1	3	41%	81
					0	0		
	14	1/8/68	9 col.	Ro	8	2	18%	78
					15	0		
	87	1/31/68	4+	B	0	0	61%	82
	90	2/1/68	4+	B	1	0	69%	82
R. Witk. >3 mos.	11	12/18/67	4+	B	5	0	41%	80
					3	1		
	16	1/8/68	4+	Ro	5	2	20%	78
					14	0		
	92	2/1/68	1+	Ro	0	0	69%	82
J. Wilc. >3 mo.	10	12/13/67	31 col.	C	1	0	33%	82
					2	0		
	13	12/27/67	3+	C	3	0	24%	78
					0	0		
	15	1/8/68	3+	C	0	0	20%	77
					0	0		
	88	1/31/68	4+	C	0	0	61%	82
	91	2/1/68	2+	B+C	0	0	69%	82
J. Dav. >8 mo.	89	1/31/68	3+	B	25	0	61%	82
	93	2/1/68	3+	B	13	0	69%	82

Table 21. Correlation of first and second coughs from one carrier.

Trial number	Roster number	No. of Mgc dispersed			
		1st cough		2nd cough	
		fluid	impactor	fluid	impactor
24	145	50	6	7	0
23	214	12	0	3	0
18	240	0	1	2	0
20	133	57	3	22	9
22	215	8	20	0	0

Table 22. Extent of variation between tests in subjects having two or more tests.

No. of degrees* of difference	Roster No.
0	101, 332, 127, 126, 217, 225, 106, 306, 301, 303
1	105
2	147, 113, 331, 215, 324
3	341, 229, 342

* No. col.: 0-12 13-25 26-50 51-100 101-200
Degree: 1 2 3 4 5

viability; eight of 19 varied two or three degrees. Raw data on several of these individuals are shown in Table 23 to demonstrate that even these variations were not striking.

Table 23. Variability in dispersal.

Roster	Duration of carriage	Date	Trial No.	LVS isolates: Fluid	LVS isolates: Impactor	URI	RH %	Serogroup
342	> 6 d. but >12 d.	1/22	47	152	5	1+	37	group C sulfa-resist.
		1/23	59	119	3		52	
		1/24	70	17	0	-	32	
		1/25	79	16	27	-	36	
324	>2 d. but <8 d.	1/18	26	30	3	-	36	group C sulfa-resist.
		1/22	45	103	13	-	37	
		1/23	51	144	9		48	
		1/24	75	82	1	-	30	
		1/25	85	41	50	-	36	
229	> 6 d. but <12 d.	1/22	44	53	3	2+	37	Non-typable 29E
		1/24	71	6	0	1+	32	

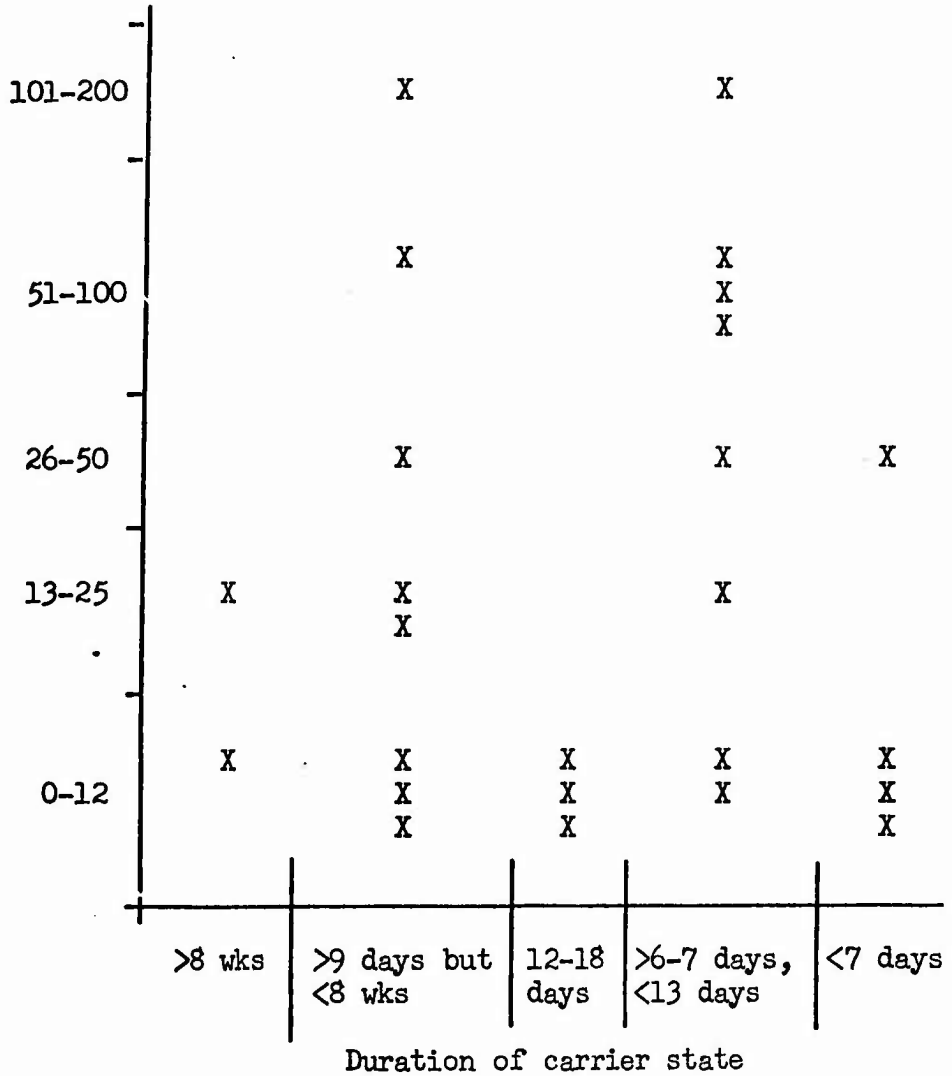
In general, poor dispersers remained poor; great dispersers did not become poor ones over the time period studied.

The relation of the duration of carrier state and degree of dispersal is presented in Table 24. For this table only the first test on each man was used. Although these data are not conclusive because of the small number of subjects in the chronic group, they do not suggest that recent acquisition of the carrier state by itself is directly correlated with greater shedding.

The effect of the pathological state of the respiratory tract upon meningococcal transmission was studied. Symptomatic respiratory disease was present in half of the men but was very mild in each. Throat cultures for viral studies were obtained the first time air sampling was performed yet only one yielded an agent, herpes simplex (Trial 22), when tested in human embryonic kidney tissue culture.

The relationship of ARD and number of organisms dispersed is shown in Table 25. From these results it is apparent that there exists no direct relationship between the two parameters. Previous studies last year had encompassed only men with ARD symptoms; a similar range of

Table 24. Relation of duration of carrier state to dispersal of meningococci.



dispersal was noted then.

Table 25. Relation of number of organisms dispersed to presence of acute respiratory disease.

No. of organisms	No. of individuals with-	
	No ARD	ARD
0- 12	7	5
13- 25	1	0
26- 50	1	3
51-100	1	3
101-200	1	1

Serotype of organism: Organisms of several different serogroups were identified in throat swabs of dispersers. LVS isolates, with a single exception, were identical in serogroup and sulfadiazine resistance level to the subject's throat isolate. Nongroupable organisms were carried by 10 of the 25 tested individuals; group C sulfa resistant strains accounted for nine others; Bo, B, B-R and C serogroups were carried by the remainder. Table 26 shows that no serogroup was dispersed to a greater degree than the others. Carrier rates in the company at this time showed that 20 per cent of the men had group C sulfa resistant organisms (25/123) and 28 per cent carried nontypable sulfadiazine sensitive strains (35/123). Thus, the group C and sulfa resistance characteristics themselves are not the reason for the high transmission of these strains seen in recruits this season.

Table 26. Comparison of meningococcal serogroup and number of organisms dispersed.

	No. or organisms			
0-12	13-25	26-50	51-100	101-200
5 NT	1 CR	2 NT	2 NT	1 NT
5 C-R	1 C	1 B-R	2 C-R	1 C-R
2 Bo				
1 B				
1 B-R				

NT = nontypable

C-r = group C, sulfadiazine resistant

d. Chemoprophylaxis of meningococci.

A method has been developed for screening in vitro the susceptibility of meningococci to a variety of anti-folate compounds both alone and in combination with sulfadiazine (SD). Stock solutions of the compounds are added to Mueller-Hinton medium to achieve the desired concentrations, organisms are applied to the solidified medium by a replicate plating technique (50-100 organisms) and the presence or absence of growth is recorded after 24 hours incubation.

After determination of the minimum inhibitory concentration (MIC) of each compound alone, one-tenth of this amount was added to medium containing varying amounts of SD (0.5 to 100 mcg/ml) and the combination compared to SD alone for SD MIC. The result is expressed as the ratio M.I.C. SD/M.I.C. SD + new drug (1/10). Thus, if SD alone is inhibitory at 5 mcg/ml and the combination is inhibitory at SD concentration of 0.5, the ratio equals 10.

Early trials with a small number of anti-folate compounds showed that trimethoprim (TMP) was the best, giving a ratio of 10. Since TMP and sulfadiazine have been tested in vivo with only marginal beneficial effects on eradication of the nasopharyngeal carrier state (WRAIR Ann. Report 1966) compounds which produce ratios greater than 10 are being sought.

e. Physicochemical and immunological properties of meningococcal group specific polysaccharides.

The group A and the group C polysaccharides have been isolated by a new method employing the cationic detergent hexadecyl trimethyl ammonium bromide (cetavlon). To an overnight culture of meningococci grown in modified Frantz medium was added cetavlon to a concentration of 0.1 per cent. This causes the formation of a precipitate which was collected by centrifugation and was washed with water. The sediment was then extracted three or four times with 0.9 M calcium chloride solution. Ethanol was added to the pooled calcium chloride extracts to a final concentration of 25 per cent, resulting in the precipitation of nucleic acids which were removed by spooling on a glass rod and centrifugation. The polysaccharide was then precipitated in an impure form by raising the ethanol concentration to 80 per cent. This precipitate was washed with absolute ethanol, acetone and di-ethyl ether, and vacuum dried. The powder was dissolved in 0.1 M neutral sodium acetate and centrifuged at 100,000 X G for 3 hrs. The clear supernate was then shaken or homogenized with chloroform to remove protein. After removal of protein was accomplished the polysaccharide was precipitated by the addition of four volumes of ethanol. The precipitate was dissolved in about 30 ml of saturated neutral sodium acetate and reprecipitated with ethanol to ensure that it be isolated as the sodium salt. The polysaccharide was dissolved in 30 ml of water, centrifuged at 100,000 X G for 2 hrs, ethanol precipitated, washed with ethanol and acetone, and vacuum dried. The yields averaged about 10 mg per liter of culture.

Group A and group C polysaccharide prepared by this method almost always contained less than one per cent nucleic acid and less than one per cent protein. Nucleic acid was determined by ultraviolet spectroscopy and protein was determined by the method of Lowry employing bovine serum albumin as a standard.

The molecular weights of several preparations of group A and group C polysaccharide were estimated by G 200 Sephadex gel filtration through a column calibrated with dextrans of known molecular weights. Both the A and C polysaccharide were excluded by the Sephadex G 200 indicating that their molecular weights were in excess of 200,000. The purification using the cationic detergent, therefore, allows isolation of these antigens in high molecular weight form which heretofore has not been possible. The molecular weights of several preparations of A and C polysaccharide prepared by methods described in the previous annual report were less than 50,000. The reason the new method achieves this is not known, but it is probable that the detergent inactivates hydrolytic enzymes produced by the meningococcus.

The method has also been applied to group B organisms and it has been possible to isolate a polysaccharide which contains 80 per cent sialic acid. However, it should be noted that it is not yet known whether this material is analogous to the group A and the group C antigen in defining all organisms possessing it as belonging to group B.

The A and C polysaccharide were found to be suitable for sensitizing red cells which could then be used for passive hemagglutination tests. Human O, Rh negative cells, which had been fixed with one per cent glutaraldehyde, were employed and found to be very useful both because of their stability and because human sera to be assayed for the presence of antibodies did not require prior absorption with red cells. Sensitization was accomplished by incubating the washed cells for 30 min. in the presence of purified antigen and a temperature of 37°C. The hemagglutination test was found to be highly sensitive and specific. Eight hyperimmune rabbit sera prepared against three different strains of group A organisms reacted with cells sensitized with A polysaccharide in dilutions as high as 1/20,000 and failed to react with cells sensitized with C polysaccharide. Eight rabbit sera prepared against three different group C organisms reacted with C sensitized cells in titers as high as 1/5,000 and failed to react with A sensitized red cells. However, for as yet unknown reasons, rabbit hyperimmune sera prepared against group B organisms will sometimes react with A and C sensitized cells in dilutions as high as 1/80.

The hemagglutination of red blood cells sensitized with either A or C polysaccharide can be inhibited with soluble A or C polysaccharide. Thus, a very sensitive and specific assay exists for the presence of these group specific antigens. Concentrations of antigen of 0.1 μg/ml are easily detectable. This test will be useful to define the optimum cultural conditions and strains of organisms to be used for production of the group specific antigens. Furthermore, it can be used

for typing unknown isolates submitted to this laboratory. The technique at present consists of suspending the growth from an agar plate in about 1 ml of saline and removing the organisms by centrifugation. The supernates are diluted by the Microtiter technique in hemagglutination plates. A dilution of antiserum about 4-fold less than its hemagglutination titer is added to each well and the antigen and antibody are allowed to react for 15 mins. Then appropriately sensitized red cells are added. A negative reaction indicates that the organism did contain the antigen in question and hence belongs to that group. Twelve group A and 20 group C organisms have been typed both by the regular slide agglutination and by the hemagglutination inhibition method. In the occasional instances where at first there was disagreement between the two techniques it has been possible to show that the traditional slide agglutination method had been in error.

The hemagglutination test has also been employed to study the serological response of patients with meningococcal disease. Thirty-three patients with group C meningococcal disease all to a greater or lesser extent formed antibodies against the group C polysaccharide whereas 15 patients with group B meningococcal disease did not. The maximum hemagglutination titers were reached within a week following onset of the disease and then a decrease occurred over the next week. Sera beyond four weeks are not available at this time. The data are summarized in Table 27 wherein are indicated the median reciprocal hemagglutination titer and the range observed at the time of admission, at about one week, two weeks and four weeks following onset of the disease.

Table 27. Meningococcal antibody in patients with meningitis due to group C organisms.

Weeks after onset of disease	Reciprocal hemagglutination titer	
	median	range
0	< 2	0 to 8
1	750	32 to 8000
2	128	16 to 1000
4	128	8 to 2000

Only one set of sera was available from a case of group A meningitis in a child. The sera were kindly donated by Dr. Daniel Ivler. The initial titer was 1/27 and the second serum had a titer of 1/2200 with A sensitized red cells.

A specific polysaccharide has been isolated from serogroup Bo, a "new" meningococcal group associated with about four per cent of cases of meningitis or septicemia studied in this laboratory (see above). Hyperimmune rabbit antisera containing precipitin antibodies have been

prepared against standard and "new" strains of meningococci. Utilizing extraction methods similar to those described for group A and C polysaccharides (above) a polysaccharide containing sialic acid has been recovered from Bo cultures and has been partially purified. By comparison of antisera following absorption with intact organisms and using immunoelectrophoretic migration of the antigen, the specificity of the Bo polysaccharide was established.

f. Serum bactericidal activity against Neisseria meningitidis in carriers.

Bactericidal activity of serum, in the presence of excess complement, is a sensitive indicator of antibody to Gram-negative bacteria. In addition to detecting antibody in vitro, the test measures a property of antibody which is operative in vivo and which presumably is important in host resistance to bacteremia. In the present experiments the complement-dependent bactericidal activity of human serum was used to test the role of the meningococcal carrier state as an immunizing process.

The bactericidal reaction-mixture consisted of equal parts diluted human serum and bacterial suspension (10^4 organisms/ml). Human complement was added in lieu of diluent where indicated. The complement source was normal human serum which had no bactericidal activity against the meningococcal strain being tested. The reaction mixture was incubated at 37°C for 30 min and colony counts were performed to test the efficiency of killing.

Tables 28 and 29 record the titers of bactericidal activity of paired sera obtained from recruits in their first half of basic training. Only data from recruits who became nasopharyngeal carriers of meningococci during the test period are included. Sera from each recruit were reacted against his own (homologous) carrier-strain of meningococcus.

The results show that many acquired carriers of N. meningitidis develop significantly increased titers of bactericidal activity against the homologous meningococcal strain. Correspondingly, there is an increase in titer of specific IgG, IgM and IgA antibodies to the homologous meningococcus as determined by immunofluorescence.

Although not all carriers of a newly-acquired strain of N. meningitidis developed increased titers of antibody during the study, results in Table 28 indicate that a significant immune response can occur within two weeks of acquisition of the nasopharyngeal strain. Indeed, among some laboratory personnel at WRAIR, significant immune responses were detectable both by bactericidal activity and immunofluorescence within seven days of acquisition of the meningococcal strain. It is conceivable, therefore, that those recruits in Table 28 who failed to develop a detectable immune response became carriers of the meningococcus less than a week before the second serum sample was obtained. This

Table 28. Bactericidal activity of human sera* against homologous nasopharyngeal strains*** of Neisseria meningitidis.

Recruit No.	Serogroup of meningococcus	Bactericidal titer of serum***		Added complement
		Day 1	Day 13	
302	C	4	16	-
304	C	8	>32	-
307	C	4	8	-
319	C	4	4	-
320	C	16	16	-
342	C	8	16	-
345	C	4	8	-
306	C	4	>64	+
329	C	4	16	+
335	C	4	4	+
337	C	32	>64	+
338	C	4	>64	+
339	C	16	16	+

* D-2-3 Company, Fort Dix, N. J., 1968.

** Acquired by recruit between 1st and 13th day of study. Isolated from recruit on 13th day of study.

*** Reciprocal of highest serum dilution killing greater than 50 per cent of test organisms. Complement added to titrations where indicated (+).

Table 29. Bactericidal activity of human sera* against homologous nasopharyngeal strains** of Neisseria meningitidis.

Recruit No.	Serogroup of meningococcus	Bactericidal titer of serum***	
		Day 1	Day 13
5	C	4	16
30	C	4	8
32	Bo	4	16
45	Bo	<4	8
48	Bo	4	16
61	B	4	16
64	B	4	32
65	C	4	8
106	Bo	4	32
123	Bo	4	32

* D-7-2 Company, Fort Benning, Ga., 1968.

** Acquired by recruit between 1st and 24th day of study.
Isolated from recruit on 24th day of study.

*** Reciprocal of highest serum dilution killing greater than 50 per cent of test organisms.
No exogenous complement added.

interpretation is supported by data in Table 29 in which 24 days elapsed between bleedings. Here, all recruits who became carriers of meningococci (serogroups B, C and Boshard) developed increased titers of bactericidal antibody to the homologous strain.

In addition to formation of bactericidal antibodies to the homologous strain of N. meningitidis, acquired carriers also form cross-reacting antibodies to heterologous meningococcal strains. Table 30 records the change in bactericidal activity of sera from recruits who became carriers of meningococci (serogroups B, C and Boshard) during a 35 day study period. Weekly nasopharyngeal cultures were taken so that the approximate date of acquisition of the meningococcus is known. It is clear from the data that, in each instance, there was increased bactericidal activity against heterologous strains of meningococci in serogroups identical to and/or different from that of the carrier strain. It is also seen that the immune response occurred within 14 days of acquisition of the carrier strain and in recruit #155 within seven days of acquisition.

As in studies with homologous strains of meningococci, the development of bactericidal activity against heterologous strains of meningococci was reflected in the appearance of specific IgG, IgM and IgA antibodies as determined by indirect immunofluorescence. Recruits who did not become meningococcal carriers during the study showed no change in pattern of bactericidal activity or specific immunofluorescence against the test strains.

The dynamics of the cross-reactive immune response to the meningococcal carrier-state were studied more closely in recruits who were cultured semi-weekly and bled weekly for seven weeks. Results in Table 31 show that the peak titer to the heterologous test strain (serogroup A) was reached 10-14 days after conversion from a carrier-negative to a carrier-positive state (recruits #1, 3, 9, 21, 25). This titer was maintained for the duration of the study. Recruit #18 was a meningococcal carrier during the entire study and maintained a constant, elevated bactericidal titer throughout. In contrast, recruit #41, also a carrier for the seven weeks of study, showed an initial increase in bactericidal titer suggesting that he had acquired the meningococcal strain shortly before the study began. Recruit #48 converted from a carrier-positive to a carrier-negative state at the end of the first week of study. However, his elevated cross-reactive bactericidal titer remained constant over the ensuing six weeks. Recruit #14, who remained carrier-negative during the study, showed neither increase nor decrease in bactericidal titer during the seven weeks of observation.

In summary, the results indicate that the meningococcal carrier state is an immunizing process and that some of the antibody formed cross-reacts with heterologous strains of N. meningitidis of diverse serogroups.

Table 30. Bactericidal activity of human sera* against heterologous strains** of Neisseria meningitidis.

Recruit No.	Nasopharyngeal strain: Serogroup	Nasopharyngeal strain: Day acquired	Bactericidal activity*** of serum against: A-1 Day 1	A-1 Day 35	B-11 Day 1	B-11 Day 35	C-11 Day 1	C-11 Day 35
4	C	21-27	-	+	-	+	+	+
58	C	21-27	-	+	-	+	+	+
61	C	14-20	-	+	+	+	+	+
90	C	14-20	+	+	-	+	+	+
96	C	10-14	-	+	-	+	+	+
130	Bo	3	-	+	+	+	+	+
166	C	21-27	-	+	-	+	+	+
208	C	10-14	-	+	+	+	+	+
219	C	21-27	-	+	-	+	-	+
155	Bo	28-35	-	+	+	+	-	+
2	Non-carrier		-	-	-	-	+	+
63	Non-carrier		-	-	-	-	+	+
84	Non-carrier		-	-	-	-	+	+
148	Non-carrier		-	-	+	+	+	+
199	Non-carrier		-	-	-	-	-	-

* I-2 Company, Fort Dix, N. J., 1967.

** Strains of N. meningitidis from cases of meningitis temporally and geographically distant from the present study group. Serogroup indicated by letter in code name (e.g. A-1 = serogroup A).

*** (-) = no killing at 1:4 serum dilution.
(+) = 100 per cent killing at 1:4 serum dilution.
No exogenous complement added.

Table 31. Bactericidal activity of human sera* against a heterologous strain (A-1) of Neisseria meningitidis.

Recruit No.	Parameters measured	Day of study 1	3	7	10	14	17	21	24	28	31	35	38	42	45	49
1	Carrier status**	-	-	-	-	-	-	Bo	Bo	Bo	Bo	Bo	Bo	Bo	Bo	Bo
	Bactericidal titer***	<4		<4		<4		<4		16		16		16		16
3	Carrier status	-	-	-	-	-	Bo	Bo	Bo	Bo	Bo	Bo	Bo	Bo	Bo	Bo
	Bactericidal titer	16		16		16		16		64		64		64		64
9	Carrier status	-	-	-	-	-	-	-	-	-	-	Bo	Bo	Bo	Bo	Bo
	Bactericidal titer	<4		<4		<4		<4		<4		<4		32		32
18	Carrier status	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
	Bactericidal titer	64		64		64		64		64		64		64		64
21	Carrier status	-	-	-	-	-	-	-	-	-	C	C	C	C	C	C
	Bactericidal titer	4		4		4		4		4		4		32		32
25	Carrier status	-	-	-	-	B	B	B	B	B	B	B	B	B	B	B
	Bactericidal titer	4		4		4		16		16		16		16		16
41	Carrier Status	Bo	Bo	Bo	Bo	Bo	Bo	Bo	Bo	Bo	Bo	Bo	Bo	Bo	Bo	Bo
	Bactericidal titer	16		64		64		64		64		64		64		64
48	Carrier status	B	B	B	-	-	-	-	-	-	-	-	-	-	-	-
	Bactericidal titer	64		64		64		64		64		64		64		64
14	Carrier status	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Bactericidal titer	4		4		4		4		4		4		4		4

* I-3 Company, Fort Dix, N. J., 1966

** (-) = negative culture for meningococci; other notations - serogroup isolated (e.g. Bo = Boshard).

*** Reciprocal of highest serum dilution killing greater than 50 per cent of test organisms (A-1). Exogenous complement added to all titrations.

3. Laboratory infection with diphtheria.

While injecting a suspension of a stock culture of Corynebacterium diphtheriae into a rabbit a laboratory worker accidentally stuck the needle tip into her finger. She immediately "milked" the finger to produce bleeding and later that day was given oral penicillin and a booster dose (0.5 ml) of combined tetanus-diphtheria toxoid (1 Lf diphtheria toxoid). That evening she felt chilly and feverish and by the next morning the right middle finger was swollen and red. There was also a red streak running up the arm to the axilla where a small tender lymph node was palpable.

Following admission to the hospital she was treated with intravenous penicillin and 80,000 units of diphtheria antitoxin (horse). Except for transient urticaria there were no untoward effects of treatment and her fever was gone in six hours. The local cellulitis and lymphangitis receded in a few days but a small 2-3 mm. vesicle at the site of penetration on the finger became black and surrounded by an area of decreased sensation. The eschar resolved within two weeks but the area of hypesthesia persisted for several months. Electrocardiograms and neurological examinations showed no manifestations of diphtheria toxicity.

Blood culture obtained the day after the accident, and after several doses of oral penicillin, yielded a corynebacterium which, as yet, is not characterized as to toxin production. Serum antitoxin levels will also be tested. Search of immunization records revealed that diphtheria toxoid had been administered in 1958 and 1959.

Summary and Conclusions.

Identification and classification of mycoplasma and L-phase variants of bacteria have been pursued by studies of nucleic acid composition and homology, as well as by their biochemical activities and serologic reactions. Antigens and antisera have been prepared and are being used in serological studies of clinical problems.

Meningococcal studies have shown that group C sulfadiazine resistant organisms have now become the predominant strains causing disease in military populations. Strains of this group have been found in carrier surveys in recruits in several east coast camps. Group A strains were exceedingly rare again this year. A large proportion of strains isolated from the pharynges of normal children has been found to ferment lactose, a property not found in spinal fluid and blood isolates.

The transmission of meningococci, studied by carrier surveys and air sampling, appeared to be random within a platoon of recruits rather than directly related to bunking arrangements. Studies of dissemination from carriers using a large volume air sampler attempted to determine the factors responsible for dispersal. These showed that the presence of symptomatic respiratory disease and the serogroup of the organism carried were not important factors. The role of the duration of the carrier

state could not be conclusively determined.

The group A and C polysaccharides have been purified and characterized and have been used to develop a specific hemagglutination. The Boshard serogroup also appears to possess a specific polysaccharide. Meningococcal isolates can be grouped by their ability to specifically inhibit a positive HA test.

Bactericidal antibody develops in most meningococcal carriers. Unlike the findings with the HA test, antibody cross-reactive with other serogroups of N. meningitidis can be detected.

A case of diphtheritic lymphangitis occurred in a laboratory worker following accidental inoculation. Treatment with penicillin and antitoxin was curative.

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RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA CA6447	CSGRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 01 07 67	REV 0 MAN 0	NA	6A	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61145011 3AC145C1071C 00 170

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) ZOONOTIC DISEASES

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
010100 MICROBIOLOGY 005900 ENVIRONMENTAL BIO 003500 CLINICAL MEDICINE	06 62	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
C. IN-HOUSE	a. NUMBER NA b. TYPE NA c. DATE NA d. AMOUNT NA	PRIOR FY 68	12	250
		CURRENT FY 69	12	250

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21. TECHNOLOGY UTILIZATION: NA

22. COORDINATION: NA

23. KEYWORDS: ZOONOSES, LABORATORY ANIMALS, GENETICS, EPIDEMIOLOGY, LEPTOSPIRAS, ANIMAL VIRUSES.

24

(U) TECH OBJECTIVE - TO DEFINE AND STUDY ZOONOSES AND INFECTIOUS DISEASES OF ANIMALS OF REAL OR POTENTIAL MILITARY SIGNIFICANCE. TO STUDY DISEASES OF LABORATORY ANIMALS USED IN MILITARY MEDICAL RESEARCH TO PROVIDE SUITABLE TEST ANIMALS, TO MINIMIZE INFECTION HAZARDS AND TO ENSURE SAFETY AND PURITY OF BIC PRODUCTS OF ANIMAL ORIGIN. SPECIFIC STUDIES INCLUDE- BIOLOGICAL CHARACTERISTICS OF LEPTOSPIRAS, ETIOLOGY OF ACUTE RESPIRATORY DISEASE IN DOGS, ETIOLOGY OF FRANK AND LATENT MICROBIAL INFECTIONS OF LABORATORY PRIMATES AND RODENTS, AND ROLE OF MICROBIAL INFECTIONS IN EARLY NON-SPECIFIC IRRADIATION DEATHS IN MICE.

25

(U) APPROACH- CONVENTIONAL EPIDEMIOLOGIC AND MICROBIAL TECHNICS WERE USED. NEW CANINE CELL CULTURES WERE DEVELOPED FOR VIRUS STUDIES. PROCEDURES FOR THE PRESERVATION OF LEPTOSPIRA WERE DEVELOPED.

(U) PROGRESS - JUL 67 THRU JUN 68 LEPTOSPIRA WERE PRESERVED BY CONTROLLING THE RATE OF FREEZING IN A GLYCEROL MEDIA AND STORAGE IN LIQUID NITROGEN VAPOR. KNOWN AND NEW VIRAL AGENTS OF DOGS WERE CHARACTERIZED AND COURSE OF INFECTION STUDIED. A NEW VIRUS ISOLATED FROM MILITARY DOGS APPEARS TO BE A MEMBER OF THE PICODVA VIRUS GROUP. STUDIES ON THE IMMUNOLOGY OF CANINE HERPES STRAINS HAVE BEEN COMPLETED. PARAINFLUENZA SV5 VIRUS INFECTIONS OCCUR IN MILITARY DOGS WITH RESPIRATORY DISEASE THRU OUT THE YEAR. THE DOGS ARE INFECTED WITH SV5 USUALLY WITHIN TWO MONTHS OF ENTRY INTO SERVICE. A FORMALIN KILLED PARAINFLUENZA SV5 VACCINE HAS BEEN PRODUCED AND IS BEING EVALUATED IN HAMSTERS AND DOGS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

26

TEXT NOT REPRODUCIBLE

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)	35.

DD FORM JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 Identical to NASA Form 1122)

Project 3A014501B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 170, Zoonotic diseases

Investigators.
Principal: A. D. Alexander, Ph.D.
Associate: CPT Stanley S. Green, MSC; L. B. Evans

Description.

This Work Unit deals with diseases of animals that have or may have potential military significance. Studies encompass: basic biological characteristics of infectious agents, their mode and course of infection, diagnosis, treatment, and control measures. Current studies relate to leptospirosis, melioidosis.

Progress.

1. Preservation of Leptospiras by Liquid Nitrogen Freezing.

Suitable methods for preserving leptospiras were sought to enable standardization of infectivity doses in experimental infections of animals used in drug screening and other studies, and to provide a more effective and simple method for maintaining stock cultures. Long-term studies on the preservation of a pathogenic strain of canicola by liquid N_2 freezing were initiated in May 1966 (WRAIR Annual Reports 1966, 1967). In cooperation with American Type Culture Collection (ATCC), observations on the viability and virulence of the stored canicola cultures have now been made over a two-year period. Samples were removed from the liquid N_2 refrigerator, rapidly thawed at 37°C, and diluted serially by 10-fold increments. The concentration of viable organisms was determined by direct microscopic counts in a Petroff-Hausser counting chamber, and by cultural tests in Fletcher's medium. Titration for virulence was done in weanling hamsters employing 5 hamsters per dilution. Hamsters were inoculated i.p. with 0.5 ml dose. Summary of findings to date is shown in t e 1.

The disparate findings in counts and virulence titers at various test times may reflect differences in growth promoting properties of different culture lots, differences in relative susceptibility of hamsters used at respective test intervals, possible variations in the treatment and handling of cultures (e.g., thawing procedure), and operator variations. Notwithstanding these variations, it was apparent that a decrease in viability and virulence titers in the range of 1 to 2 logs occurs at time of freezing, but was not altered further after storage over a 2 year observation period.

Table 1. Viability and Virulence of a Culture of Serotype canicola after Freezing and Storage in Liquid Nitrogen.

Determination	Pre-treatment	Values after Freezing and Storage				
		1 day	1 month	7 month	1 year	2 year
Microscopic count/ml	$1.25 \times 10^{8.0}$	$8.12 \times 10^{6.0}$	$1.12 \times 10^{7.0}$	$1.14 \times 10^{7.0}$	$1.5 \times 10^{7.0}$	$2 \times 10^{7.0}$
Viable count by culture/ml	$10^{7.8}$	$10^{6.1}$	$10^{6.0}$	$10^{6.25}$	$10^{4.6}$	$10^{6.0}$
LD_{50} hamsters	$10^{-7.25}$	$10^{-5.9}$	$10^{-4.4}$	$10^{-5.3}$	$10^{-4.9}$	$10^{-6.25}$
LD* hamsters	$10^{-7.50}$	$10^{-6.5}$	$10^{-6.0}$	$10^{-5.5}$	$10^{-5.5}$	$10^{-6.0}$

*Lowest dose producing death.

On the basis of these findings, the ATCC deemed it practicable to stock leptospiral cultures in their collection. Arrangements were made to deposit in the ATCC collection 17 strains commonly used in diagnosis and 9 selected virulent strains. To date, 12 diagnostic and 9 virulent strains have been sent to ATCC where they have been processed and stored. The stored cultures have been tested for viability at 14 or 23 days after storage. The concentration of cultures prior to freezing as determined by direct microscopic counts ranged from 1 to 9 X 10^8 per ml. At test time after N_2 storage, the decrease in viable organisms as determined by counts in Petroff-Hausser chamber did not exceed one log. However, by cultural tests, the decrease in viability among diagnostic (avirulent) strains was 1 to 3 logs; among virulent strains there was a remarkable 3 to 7 log (10^{-1} to 10^{-4} dilutions) decrease in cultural viability for all but one of the strains. However, all of the virulent strains elicited frank leptospiral infections in hamsters at 10^{-5} to 10^{-7} dilutions. The difference in viability titers by cultural and animal inoculation could not be explained. Repeat examinations are planned.

To determine if metabolic by-products in frozen whole cultures affected viability of leptospiras, comparative tests were done on 38, 62, and 86 hour cultures of a virulent bataviae strain and on aliquots of these cultures whose cells were centrifuged and resuspended in fresh media. After freezing and storage in liquid N_2 for 24 hours, the recovery of viable leptospiras and the virulence titration in hamsters of respectively paired preparations were the same.

Since it is apparent from findings to date that liquid N_2 can be used for storage of leptospiras to maintain their viability and/or virulence, 91 cultures have been rapidly frozen with 10% glycerol as a cryoprotectant and stored in the Division of Veterinary Medicine. More cultures will be eventually maintained in the frozen state in liquid N_2 vapor.

2. Serological Studies-Leptospirosis.

Serological studies were continued on strains isolated by Middle American Research Unit (MARU), Canal Zone, during the course of an epidemiologic survey at a loci associated with leptospiral infections in troops. Fourteen of a total of 35 strains isolated and characterized were studied during the period of this report. A summary of the source and culture-typing findings on the 35 isolates is shown in Table 2.

Strains represented by L-137 were first placed in the grippotyphosa group but were subsequently identified to be serotype canalzoni. Three strains could not be related to any serogroup and may be new serological types. Strain L-40 has been extensively tested. It is a new serotype, sufficiently distinct to warrant placement in a separate serogroup.

At the request of the Mysore Veterinary College, India, sera randomly collected from 88 animals and 6 human beings were tested for leptospiral antibodies. Positive findings were seen in 4 of 5 goat, 1 of 3 sheep, all of 4 dog, 11 of 52 bovine and 20 of 23 swine sera. All six human samples and single specimens from a bird and guinea pig were negative. There was a selective host-distribution of predominant serotype reaction, pomona in goats and swine, canicola in dogs, and canicola and hebdomadis serotypes in cattle. The findings provided evidence that leptospirosis occured in Mysore, India.

Occurence of sporadic deaths among animals in the Barbary Ape (Macaca sylvana) colony at the Washington Zoo was investigated by Veterinary Pathology Section of AFIP. Serotype icterohaemorrhagie was isolated from liver and kidney tissues submitted to this Division from one of the fatal cases. Leptospiras were recovered by direct culture in medium containing 5-Fluorouracil and by hamster inoculation procedures. Conventional media inoculated with the same material contained contaminating micro-organisms. Leptospiral antibodies were demonstrated in 1 of 4 apparently normal Barbary apes in the colony. Frank cases of leptospirosis in monkeys have rarely been observed. The cultural findings affirmed the usefulness of 5-Fluorouracil medium for direct isolation of leptospiras from contaminated media.

Table 2. Source and Culture Typing Findings on 35 Isolates from Canal Zone

Serogroup	Representative Strains	No. of Isolates	Source
cynopteri	L-892	(8)	spiny rat-5, spiny pocket mouse-1, opossum-2
	L-137	(3)	spiny rat-3
bataviae	L-759	(3)	opossum-1, cotton rat-1, spiny pocket mouse-1
	L-920	(5)	spiny rat-3, opossum-2
	L-727	(1)	spiny rat-1
hebdomadis	L-348	(1)	man
	L-792	(4)	spiny rat-4
pyrogenes	L-542	(1)	man
	L-792	(1)	spiny rat
	L-1036	(2)	spiny rat-1, cotton rat-1
pomona	L-2025	(1)	spiny rat
	L-1031	(1)	spiny rat
new group	L-40	(1)	spiny rat
undetermined	L-55	(1)	spiny rat
	L-915	(1)	spiny rat
	L-942	(1)	opossum

Summary and Conclusions.

1. Preservation of Leptospiras by Liquid Nitrogen Freezing.

Observations were continued on the long-term preservation of leptospiras by liquid N_2 freezing and storage. After a two year period of storage the viability and virulent titers of a leptospiral strain remained the same as those elicited the first day after treatment and storage. Additional observation on selected screening antigen and virulent strains served to affirm the usefulness of liquid N_2 for long-term preservation of leptospiras. Storage of WRAIR stock cultures in liquid N_2 has been initiated.

2. Serologic Studies--Leptospirosis.

Serological characterization studies were done on 35 leptospiral strains obtained from soldiers and various mammals trapped at a locus in Canal Zone where cases occured in troops. The isolates gave 16 different cross-reactive patterns with diverse serotype antisera. Four of the strains, each with distinct patterns, could not be related to known serogroups. The remainder could be classified in the following serogroups: bataviae, hebdomadis, pyrogenes, pomona, and cynopterus. The continued disclosure of the presence of unique serological types points up the limitation of currently recommended agglutination tests for detection of antibodies and need for greater utilization of genus-specific serological procedures.

Publications.

Z. A. McGee, M. Rogul, and R. G. Wittler. Molecular genetic studies of relationships among mycoplasma, L-forms and bacteria. Ann. N. Y. Acad. Sci. 143: 21-30, 1967.

RESEARCH AND TECHNOLOGY RESUME

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4. DATE OF SUMM.	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
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10a. CURRENT NUMBER/CODE
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10b. PRIOR NUMBER/CODE

11. TITLE
(U) DEVELOPMENT OF BIOLOGICAL PRODUCTS

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01100 MICROBIOLOGY	05 53	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT			18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
	a. NUMBER	b. DATE				
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	b. TYPE NA	d. AMOUNT	NA	CURRENT FY 69	6	190

19. GOVT. LAB./INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
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	TEL 202-576-3209 TYPE DA

21. TECHNOLOGY UTILIZATION	22. COORDINATION
PUBLIC HEALTH CIVIL DEFENSE	NA NA

23. BIOLOGICAL ASSAY, BIOLOGICAL EFFECTIVENESS, BIOLOGICAL PRODUCTS, CHOLERA VACCINE, EEE VACCINE, FREEZE-DRYING, PLAGUE VACCINE, Q FEVER VACCINE, VACCINES, SHIGELLA VACCINE.

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 EXISTING BIOLOGICAL PRODUCTS TO INCREASE EFFECTIVENESS AND REDUCE REACTIVITY, TO AFFORD GREATER STABILITY, AND TO MINIMIZE LOGISTIC REQUIREMENTS.

(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 ACHEIVED BY APPLICATION OF MODERN FREEZE-DRYING AND PACKAGING TECHNIQUES.

(U) PROGRESS - JUL 67 THRU JUN 68 INVESTIGATIONS HAVE CONTINUED ON SEVERAL NEW AND MODIFIED BIOLOGICALS - 1. APPLYING PRODUCTION METHODS DEVELOPED IN THIS LABORATORY STABLE LIVE PLAGUE VACCINES WERE PREPARED FROM SEVERAL ATTENUATED STRAINS OF THE PLAGUE BACILLUS. THESE VACCINES ARE CURRENTLY BEING EVALUATED IN ANIMALS PRIOR TO EVALUATION IN MAN. 2. TECHNIQUES HAVE BEEN DEVELOPED FOR ELIMINATING A FACTOR IN FRESH GUINEA PIG SERUM WHICH INHIBITS THE FULL EXPRESSION OF Q FEVER COMPLEMENT-FIXING ANTIBODIES, THUS PERMITTING THE TRUE EVALUATION OF THE INFECTIVITY OF RICKETTSIAL SUSPENSIONS AND THE IMMUNOGENICITY OF Q FEVER VACCINES. 3. METHODS HAVE BEEN DEVELOPED FOR THE PREPARATION OF HIGH TITER EEE HEMAGGLUTINATING ANTIGENS IN CHICK EMBRO FIBROBLAST CELL CULTURES. 4. PROCEDURES HAVE BEEN WORKED OUT FOR THE PILOT-SCALE PRODUCTION OF CHOLERA CULTURE FILTRATES WITH HIGH CHOLERAGEN ACTIVITY. 5. EXPERIMENTAL LOTS OF MENINGOCOCCAL POLYSACCHARIDE WERE PREPARED FOR HUMAN EVALUATION. 6. EXPERIMENTAL LOTS OF FREEZE-DRIED LIVE ATTENUATED SHIGELLA VACCINES WERE PREPARED FOR EVALUATION IN ANIMALS AND MAN. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		ER	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT
35. EST. FUNDS (In Thousands)	36.

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Project 3A014501B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 171, Development of biological products

Investigators.
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Associate: Sanford Berman, PhD; Patricia L. Altieri, BS; Arthur White, MS; Albert Groffinger; Calvin Powell, MS; Doria Dubois, BS; Richard A. Finkelstein, PhD.

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. Plague.

Laboratory studies on the development of a stable living plague vaccine, prepared with the attenuated EV-76 strain of Pasteurella pestis, have continued during the past year.

a. Long term surveillance of the stability of freeze-dried EV-76 (Saigon strain) vaccine suitable for human use (Lot 7, prepared in Nov. 1965) was continued during this period. A summary of the results of periodic titrations of the number of viable organisms in samples stored at different temperatures is recorded in Table I.

TABLE I

Stability of Viable Count, Freeze-Dried EV-76 Vaccine, Lot 7

	Storage Temperature				
Time	-20°C	+4°C	+22°C	+37°C	+45°C
0	5.9×10^9				
1 week	7.4×10^9	5.9×10^9		1.0×10^9	3.0×10^7
2 weeks	3.8×10^9	5.2×10^9	2.0×10^9	1.3×10^8	9.0×10^4
5 weeks	4.5×10^9	2.7×10^9	1.9×10^9	1.8×10^5	
12 weeks	9.1×10^9	3.7×10^9	6.3×10^8	9.0×10^2	
23 weeks	10.0×10^9	6.0×10^9	4.5×10^8		
41 weeks	10.8×10^9	5.7×10^9	3.1×10^7		

TABLE I
(Cont'd)

Time	Storage Temperature -20°C	+4°C	+22°C	+37°C	+45°C
50 weeks	4.9×10^9	1.5×10^9	1.3×10^7		
75 weeks	6.4×10^9	8.1×10^8	$<1.0 \times 10^5$		
95 weeks	8.4×10^9	1.2×10^9			
125 weeks	4.5×10^9	9.2×10^7			

These results demonstrate that the number of viable organisms in the freeze-dried vaccine is stable for at least 125 weeks when stored at -20°C, and for 41 weeks when stored at +4°C. At higher storage temperatures significant loss in viability occurs within two weeks.

b. The immunogenicity and toxicity for man of the freeze-dried EV-76 (Saigon strain) vaccine was tested during this period in limited trials carried out at Ft. Detrick and at the University of California School of Medicine. Although the vaccine was well tolerated, the volunteers failed to elicit a satisfactory F-1 antibody response.

Therefore, two new attenuated EV-76 strains of the plague bacillus, the Devignat strain (obtained from Ft. Detrick, Md.) and the 51-F strain (obtained from Dr. K. F. Meyer, University of California School of Medicine, San Francisco) were investigated to determine their suitability as candidate strains for a living vaccine for human use. Laboratory studies indicate that these strains have growth and freeze-drying characteristics like those of the Saigon strain (Annual Report, 1967). Consequently, freeze-dried seed preparations and vaccines suitable for human use have been produced with the new strains. The new vaccines are currently being evaluated in animals prior to consideration for use in man.

2. Q Fever.

a. Previously reported studies have revealed the presence of an inhibitor which masks the full expression of Q fever complement-fixing antibodies in the freshly collected blood sera of Ft. Detrick Hartley strain guinea pigs convalescing from an infection with Coxiella burneti, Nine Mile Strain, phase 2, EP-88 (Annual Report 1967). The effect of this inhibitor is reduced by storage of the sera at 4°C, -20°C or -70°C and is eliminated by acetone or kaolin extraction.

Since the complement-fixation (CF) test is widely used to evaluate the infectivity of rickettsial suspensions and the immunogenicity of rickettsial vaccines, an erroneous evaluation may be obtained if the CF test is performed on untreated fresh sera.

Consequently, additional studies have been carried out to determine the nature of this inhibitor and to provide a simple and practical method for treating large numbers of sera to permit an accurate measure of the CF antibody content.

Heat treatment of the fresh sera at 56°C for 30 minutes, the conditions generally employed for the inactivation of complement, has no effect on the inhibitor. However, adjustment of the pH of the sera to increase the alkalinity renders the inhibitor sensitive to heat. The results of a CF test on the fresh sera of guinea pigs, collected 28 days post-infection with the EP-88 strain and diluted 1:4 in triethanolamine buffered saline (TBS) at several pH levels prior to inactivation, are given in Table II.

TABLE II

Effect of Inactivation at Various pH Levels on CF Titers of Sera of Guinea Pigs Infected with C. burneti, Nine Mile Strain, Phase II (EP-88)

	Sera diluted 1:4 in Triethanolamine Buffered Saline at			
Serum No.	pH 7.4	8.0	8.5	9.0
1	-	-	-	8
2	-	-	8	16
3	-	-	4	8
4	-	-	-	-
5	-	-	8	8
6	-	-	16	16
7	-	-	-	4
8	-	-	-	-
9	-	-	-	-
10	-	16	32	32

Thus, all of the sera were negative when diluted in TBS at pH 7.4 prior to inactivation (the standard procedure for performing the CF 52 test of Fife & Kent). However, seven of the ten sera showed specific CF antibodies when diluted in TBS at pH 9.0 prior to inactivation.

A comparison of the CF titers obtained with "untreated" sera, sera diluted in TBS at pH 9 prior to inactivation, and kaolin-extracted sera is given in Table III.

TABLE III

Q Fever CF Test on Fresh Guinea Pig Sera
(Collected 28 days post-infection with *C. burneti*, EP-88)

Infective Dose:	10^{-8}				10^{-9}				10^{-10}		
GP#	pH 7.4*	pH 9.0**	Kaolin***	GP#	pH 7.4	pH 9.0	Kaolin	GP#	pH 7.4	pH 9.0	Kaolin
1	-ℓ	4	-	21	-	16	20	41	-	-	-
2	-	-	-	22	-	16	20	42	-	8	20
3	-	32	40	23	-	-	-	43	-	-	-
4	-	-	-	24	-	-	-	44	-	-	10
5	-	8	20	25	-	-	-	45	-	-	-
6	8	16	20	26	-	-	-	46	-	8	20
7	-	-	-	27	-	-	-	47	-	8	10
8	-	-	-	28	-	-	-	48	-	4	10
9	-	16	20	29	-	4	10	49	-	-	-
10	-	-	-	30	-	8	10	50	-	-	-
11	-	32	20	31	-	8	10	51	-	-	-
12	-	4	10	32	-	-	-	52	-	-	-
13	-	8	20	33	-	-	-	53	-	4	-
14	-	-	-	34	-	4	-	54	-	-	-
15	-	8	20	35	-	-	-	55	-	-	-
16	-	16	20	36	-	8	10	56	-	-	-
17	32	32	40	37	-	16	20	57	-	4	-
18	-	-	-	38	-	8	10	58	-	16	20
19	16	32	40	39	-	-	-	59	-	-	-
20	-	8	20	40	ND	ND	ND	60	-	8	10
Conv/Total	3/20	13/20	12/20		0/19	9/19	8/19		0/20	8/20	7/20

ID_{50} pH 7.4 = < 8.0 pH 9.0 = 9.0 Kaolin = 8.8

* Initial Dilution 1:4 in TBS pH 7.4 prior to inactivation.
** Initial Dilution 1:4 in TBS pH 9.0 prior to inactivation.
*** Kaolin extracted (by method of Clarke and Casals) prior to inactivation. Initial dilution 1:10.
ℓ - = < 4 for pH 7.4 and pH 9.0 and < 10 for Kaolin.

by liver cells in culture (Sandström, Acta Soc. Med. UPSALIENSIS, LXXI, No. 1 - 2, 14, 1966), has been shown to inactivate arboviruses (Theiler, Proc. Soc. Exp. Biol. & Med., 1957, 96, 380).

Primary chick embryo fibroblast cultures were prepared from 9 - 11 day old embryos by Porterfield's method (Nature, 183: 1069, 1959). Virus was assayed by the plaque technique, with counts being made after 48 - 72 hours incubation at 37°C. Table IV summarizes the results of viral assays in the three types of cell culture preparations.

TABLE IV

Plaque Production by EEE Virus Grown in Cell Monolayers Prepared from Intact, Eviscerated and Hepatectomized Chick Embryos

Virus Dilution ($-\log_{10}$)	Preparation of Embryos*					
	Intact		Eviscerated		Hepatectomized	
	No. plaques	Mean	No. plaques	Mean	No. plaques	Mean
9.0	60,62,70	64	110,110,100	107	105,101,106	104
9.3	32,27,23	27	52,47,41	47	49,46,39	45
9.6	10,14,14	13	29,36,30	32	30,31,26	29
9.0	61,51,63	58	115,101,104	106	112,110,112	108
9.3	27,34,32	31	45,50,46	47	52,43,48	47
9.6	12,13,12	12	32,28,33	31	31,32,26	30

* Each preparation consisted of the pooled tissues from 15 embryo torsos.

These results show a similarity in the numbers of plaques observed in the eviscerated cell preparation and those observed in the hepatectomized cell preparation. On the other hand, the plaque counts were consistently lower in the intact embryo torso cell preparation than in the eviscerated and hepatectomized embryo cell preparations. It would appear, therefore, that cells derived from the liver of 9 - 11 day old chick embryos inhibit plaque formation by EEE virus within 48 - 72 hours after infection, resulting in about a 40% reduction in the number of plaques. This reduction in plaque counts on the monolayers derived from non-eviscerated embryos is greater than was expected from the number of liver cells present in these monolayers, suggesting that the inhibition is due to some diffusable substance produced by the liver cells rather than to specific liver cell rejection. Further evidence for this hypothesis is found in Table V which shows the effect on the plaque count of a soluble liver extract and that of a similar extract from other soft tissues of the chick embryo.

The results show that the simple procedure of preparing the initial 1:4 dilution of the serum in TBS at pH 9.0 prior to inactivation permits the detection of the specific CF antibody responses which are present in many of the sera but which are not manifested under the conditions usually employed for the performance of the complement-fixation test. In addition, this procedure appears to be as effective as the kaolin-extraction method for removing the inhibitor, and is far simpler and more practical for use with large numbers of guinea pig sera.

b. In cooperation with the U. S. Army Medical Unit, Ft. Detrick, an effort was made to prepare Henzerling strain seed material free of all detectable adventitious agents for use in the production of phase 1 Q fever vaccine for human use.

The standard Henzerling EP-22 seed material (predominately in phase 2) was converted to phase 1 by a series of passages through one guinea pig, four embryonated eggs, another guinea pig, and finally two embryonated eggs. The guinea pigs and eggs had been thoroughly tested and found to be free of adventitious agents. The guinea pig passages also served to eliminate the avian agents which undoubtedly were present in the original Henzerling seed preparation.

Sufficient material was obtained from the pooled yolk sac membranes of the final egg passage to provide approximately 2000 vials of seed material. This seed preparation, designated "Henzerling strain, EP-2, phase 1" is currently being characterized.

3. Eastern Equine Encephalomyelitis.

During this period experimental studies with EEE virus in chick embryo fibroblast cell cultures were continued. This system is being used as a model to determine the practical value of various modifications to usual tissue culture procedures for the production and evaluation of viral vaccines and antigens.

a. During the processing of chick embryos for the preparation of cell culture monolayers, some laboratories remove only the head and the extremities, while others also eviscerate the embryo torsos. Since evisceration adds another step to the procedure, it would be desirable to eliminate this step from the processing of cells used for the titration of virus and for the pilot scale production of vaccines and antigens, unless there is adequate compensation in terms of more accurate counts or increased virus yields. Therefore, a series of experiments were performed to compare the growth of EEE virus on chick cell monolayers derived from eviscerated and from non-eviscerated chick embryos. In addition, cells were also prepared from hepatectomized embryos since sodium deoxycholate, secreted

TABLE V

Effect of Soluble Liver Extract and Hepatectomized Embryo Extract on EEE Plaque Production

Liver Extract		Hepatect. Embryo Extract	
Diln. of Extract	Mean Plaque No.	Diln. of Extract	Mean Plaque No.
10^0	8	10^0	110
10^{-1}	36	10^{-1}	110
10^{-2}	43	10^{-2}	107
10^{-3}	81	10^{-3}	107

Since EEE virus is a small (35 - 50 mu) RNA virus containing approximately 45% lipid in its envelope, the inhibition observed may be due to the effect of the bile acids produced by the liver cells on the lipids of the virus.

Thus, the use of hepatectomized or eviscerated chick embryos for the preparation of cell monolayers for use in the evaluation of EEE virus yields by the plaque technique is indicated. Additional studies are currently being carried out to determine whether the use of eviscerated embryos for the preparation of monolayers for the production of vaccine or antigen will result in a significant increase in the virus yield.

b. Hemagglutinating (HA) antigens for the arboviruses are usually prepared from the infected brains of suckling mice and are extracted by an acetone-ether procedure such as that described by Casals and Brown (J. Exper. Med., 1954, 99, 429). Many investigators have attempted to obtain HA antigens from tissue culture fluids, but the titers obtained have been considerably lower than those obtained from the mouse brain preparations.

During the course of the preparation of experimental EEE vaccines in chick embryo fibroblast cell cultures, the culture fluids were sampled periodically after infection and were tested for infective virus titer (in chick cell cultures) and for HA titer (with goose red blood cells). The results of a typical titration are given in Table VI below.

TABLE VI

Infectivity and Hemagglutinin Titers of EEE Infected Chick Cell Culture Fluids

Post-Infection Time (hrs)	Infectivity Titer ($-\log_{10}$)	HA Titer (1:)
2	1.0	<5
6	4.0	<5
18	9.5	1280
24	8.5	1280
96	2.5	1280
120	<1.0	1280

Thus, within 18 hours post-infection, both the infectivity and hemagglutinin titers had reached their maximum levels. Upon further incubation, the infectivity titer dropped but the HA titer was maintained at the maximum level.

Comparable high titered hemagglutinins were obtained from monolayer cultures prepared from either $2X10^6$ or $6X10^6$ chick cells per ml, with Medium 199 + 5% fetal bovine serum or Gey's Tris Medium + 5% fetal bovine serum as the growth medium for the cells. However, prior to infection, the monolayers were washed and a maintenance medium without serum was added.

The stability of the hemagglutinin in 18 hour culture fluids was determined by placing samples in storage at 4°C, 37°C and 56°C and testing these periodically for HA activity. The results to date are summarized in Table VII.

TABLE VII

Stability of EEE Hemagglutinin in 18 Hour Chick Cell Culture Fluids

Time	Storage Temperature 4°C	37°C	56°C
6 hours	1280*	1280	1280
1 day	1280	1280	<5
2 weeks	1280	1280	<5
4 weeks	1280	<5	<5
8 weeks	1280	<5	<5
11 weeks	1280	<5	<5

* Reciprocal of titer.

The results indicate that the hemagglutinin is stable for at least 11 weeks when stored at 4°C, and for at least 2 weeks when stored at 37°C. At 56°C, however, the maximum titer is maintained for at least 6 hours, and no activity is measurable at 24 hours.

4. Cholera.

Experimental studies on the development of pilot plant scale methods for the production of choleragen-containing Vibrio cholerae culture filtrates were continued during this period.

Earlier attempts to produce 10 liter quantities of choleragen-containing filtrates in Syncase medium, used by Finkelstein to produce choleragen in agitated shallow-layer (225 ml in a one liter flask) cultures (Jour. Inf. Dis., 1964, 114: 203), were unsuccessful. On the other hand, good yields of choleragen were obtained in aerated 10 liter volumes of a medium consisting of 3% casamino acids, 0.1% sucrose, plus salts (Annual Report 1967).

Further attempts were made to utilize the Syncase medium for larger scale production since it was felt that the alteration of the growth medium would affect the purification of the choleragen by Finkelstein's method, due to the presence of other metabolic products or medium constituents.

In experiments designed to determine the effect of the rate of aeration on the production of choleragen in Syncase medium, it was found that as the rate of aeration was increased to the level required for choleragen production (30 - 40 liters of air per min.) the growth of the vibrio was reduced, due to a lowering of the temperature of the culture below the optimum. This condition was corrected by preheating the air before it entered the medium and by placing the culture container in a 37°C water bath. The results of a typical experiment, carried out under controlled conditions of temperature, are given in Table VIII.

TABLE VIII

Choleragen Production in 10 Liters of Syncase Medium

Sampling Time (hrs)	Viable Count (per ml)	Choleragen Activity*
Aerated Culture (10 L Volume)		
20	1.15×10^9	1600
21	1.22×10^9	1600 ± 3200
24	1.09×10^9	3200

TABLE VIII
(Cont'd)

Sampling Time (hrs)	Viable Count (per ml)	Choleragen Activity*
Shake Culture (225 ml Volume)		
21	1)	3200
	2)	1600

* Reciprocal of dilution of filtrate which produces a typical reaction in the skin of adult rabbits.

The results indicate that with controlled temperature of the air flow, the choleragen yield from an aerated 10 liter volume of Syncase medium was equal to that obtained with 225 ml of the Syncase medium aerated on a reciprocal shaking machine. The filtrates obtained were highly active in producing cholera-like symptoms when fed to infant rabbits or young dogs.

5. Shigella.

a. The Department of Biologics Research has continued to provide freeze-drying support to Dr. S. B. Formal of the Department of Applied Immunology, WRAIR, in his work on the development and evaluation of a live, attenuated oral Shigella vaccine.

During the past year a new candidate strain, Shigella flexneri 2a, strain 24570X130, was isolated by Dr. Formal and studies with this strain were initiated to investigate several factors which previously have been shown to have an effect on the recovery of viable organisms through the freezing and freeze-drying processes. The results of the effect of the suspending medium and the temperature of freezing are summarized in Table IX.

TABLE IX

Effect of Harvesting Medium and Temperature of Freezing on Recovery of Viable Shigella flexneri 2a (strain 2457X130)

Harvesting Fluid			
BHIB*		SPG & HSA**	
Freezing Temperature (°C)	Recovery (%)	Freezing Temperature (°C)	Recovery (%)
-20	17	-20	55
-40	26	-40	64
-60	40	-60	61

* BHIB = Brain Heart Infusion broth.

** SPG & HSA = A medium consisting of 8.2% sucrose, 0.01 M phosphate, 0.07% monosodium glutamate and sufficient Human Serum Albumin to a final concentration of 2.5%.

The results indicate that better recovery rates are obtained when the organisms are harvested in SPG & HSA medium than in BHIB. The temperature of freezing does not affect recovery rates as significantly as does the suspending medium.

b. Similar recovery results were obtained with a streptomycin-dependent strain of S. flexneri 2a (SD-Mel) submitted by Dr. Formal. A lot of freeze-dried vaccine, suitable for human use, was prepared from this strain and has been provided to Dr. Formal for evaluation in animals and man.

6. Meningococcus.

a. Facilities, equipment and personnel of the Department of Biologics Research were made available for the preparation of purified polysaccharide from cultures of Neisseria meningitidis by the method worked out by CPT Emil C. Gotschlich of the Department of Bacteriology, WRAIR. One lot of polysaccharide from a group A strain and two lots from a group C strain were prepared, suitable for human use. These preparations are currently being evaluated.

b. Studies have been initiated on the development of pilot scale methods for the preparation of the meningococcal polysaccharides.

Summary and Conclusions.

1. Laboratory investigations on the freeze-dried live attenuated plague vaccine have continued. Surveillance of the stability of the freeze-dried EV-76 vaccine has demonstrated that there is no loss in viability after storage at -20°C for at least 125 weeks and after storage at +4°C for 41 weeks. Limited trials in humans indicate that the Saigon strain fails to elicit a satisfactory F-1 antibody response. Consequently, vaccines prepared with two other EV-76 isolates are being evaluated in animals prior to consideration for use in man.

2. A simple and practical method has been developed for eliminating a factor in fresh guinea pig serum which inhibits the full expression of Q fever complement-fixing antibodies. The standard Q fever vaccine seed strain (Henzerling, EP-22, predominantly in phase 2) was converted to phase 1 by passage through guinea pigs and embryonated eggs free of adventitious agents, resulting in a phase 1 seed preparation suitable for the preparation of Q fever vaccines for human use.

3. Laboratory studies on the EEE virus-chick cell monolayer system have demonstrated that there is a factor derived from the liver cells of the embryo which inhibits plaque formation by the EEE virus. Methods have been developed for the preparation of high titer EEE hemagglutinating antigen in chick embryo fibroblast cell cultures.

4. Procedures have been worked out for the pilot-scale production of choleragen in Finkelstein's Syncase medium.

5. Experimental freeze-dried live attenuated Shigella vaccines, made with two new strains of S. flexneri, were prepared for evaluation in animals and man.

6. Experimental lots of meningococcal polysaccharide were prepared for human evaluation. Studies have been initiated on the development of pilot scale methods for the preparation of the polysaccharide.

Publications.

Berman, S., Lowenthal, J. P., Sorrentino, J. V. and White, A. B. A Safety Test for Eastern Equine Encephalomyelitis Vaccine. Applied Microbiol., 1967, 15: 968-969.

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24.

(U) TECH OBJECTIVE - METHOD DEVELOPMENT AND IMPROVEMENT FOR SERORECOGNITION OF MICROBIAL INFECTIONS. PROCEDURES EVALLATED 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.

(U) APPROACH- TECHNICS SUCH AS CF AND FA ARE UTILIZED IN CONJUNCTION 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.

(U) PROGRESS - JUL 67 THRU JUN 68 FURTHER TECHNICAL IMPROVEMENTS OF SAFA PROCEDURE ACHIEVED AND APPLICATION EXTENDED. SAFA TEST FOR TUBERCULOSIS NOW USED TO STUDY ANTI-BODY RESPONSE IN EXPERIMENTALLY INFECTED MONKEYS AND TO EVALUATE POTENTIAL OF THE TECHNIC FOR MONITORING TB IN SUB-HUMAN PRIMATE COLONY - THREE SKIN TEST NEGATIVE, INFECTED MONKEYS DETECTED TO DATE. ANTIBODY PATTERNS AND HYPERSENSITIVITY REACTIONS IN GUINEA PIGS RECEIVING H. CAPSULATUM ARE BEING USED TO INVESTIGATE IMMUNE RESPONSE IN HISTO-PLASMOSIS, FINDINGS VALUABLE IN INTERPRETING DIAGNOSTIC TESTS RESULTS. STUDIES USING EXPERIMENTAL MOUSE SYPHILIS MODEL TO INVESTIGATE CELLULAR LEVEL IMMUNE RESPONSE TO T. PALLIDUM INFECTION ARE IN PROGRESS, PARTICULAR ATTENTION BEING GIVEN TO ETIOLOGY AND RELATIONSHIP OF REAGINIC AND TREPONEMAL ANTIBODIES. IMPROVEMENT OF IHA TEST FOR AMEBIASIS ACHIEVED BY TECHNICAL INNOVATIONS WHICH STABILIZE AND EXTEND STORAGE LIFE OF SENSITIZED ERYTHROCYTES. STUDIES ON FURTHER PURIFICATION AND EVALLATION OF SERODIAGNOSTIC ANTIGENS FROM PARASITIC, MYCOTIC AND TREPONEMAL DISEASE AGENTS ARE CONTINUING. SPECIFICITY OF RAPID CARD TEST FOR CHAGAS-DISEASE FURTHER INCREASED BY ADJUSTING LECITHIN CONTENT OF ANTIGEN, EARLY FIELD STUDIES ANTICIPATED. THESE EXAMPLES HAVE DIRECT APPLICATION TO DEVELOPMENT AND IMPROVEMENT OF SERODIAGNOSTIC CAPABILITIES AND TO BASIC INVESTIGATIONS ON IMMUNE RESPONSE TO INFECTIOLS AGENTS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 29 JUNE 1968.

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Project 3A014501B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 01, Communicable Diseases and Immunology

Work Unit 172, Sero-recognition of microbial infections

Investigators.
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Description.

This task is concerned with the mechanisms and patterns of immune responses. In vitro and in vivo methods are used to study host response to antigens. In vitro studies involve the development, improvement, and evaluation of procedures for detection of host antibodies. The studies also entail isolation, purification, and identification of antigens by chemical and serological methods. In vivo studies include: (1) investigations on the ability of antigens to stimulate serologically detectable antibodies, (2) cellular level immune response to microbial infection, and (3) production of specific antisera by infection and/or 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. Soluble antigen fluorescent antibody (SAFA) tests for serodiagnosis of infectious diseases. Details of the progressive technical development and improvements of the SAFA procedure have been presented in previous reports on this Work Unit (WRAIR Research & Development Reports, 1966, 1967). The potential value of the procedure for serodiagnosis is indicated by its present application in numerous diverse infectious diseases. In addition to the studies described in this report, other investigators in WRAIR and the NIH are employing the SAFA test for serodiagnosis of schistosomiasis, meningococcosis, malaria, and a variety of viral diseases. Still further applications of the technic are anticipated. Although the basic technic has been well established, experience has shown that minor modifications, particularly with respect to the procedure for fixing the antigen to the test disc, may be necessary for certain antigen-antibody systems. The technical innovations described in this report illustrate the general approach used to improve the efficacy of the SAFA test for a given disease.

a. Tuberculosis. Tuberculosis long has been a serious problem among sub-human primates maintained in captivity for extended periods, and the problem progressively has become more critical because of the increased use of monkeys, chimpanzees, etc. as experimental hosts in research. The currently recommended palpebral intradermal tests using

Koch's O.T. antigen have been of some value in detecting infection in such animals. Nevertheless, it is recognized that this procedure also has certain serious limitations. The I.D. tests may be negative during the early stages of infection because of delayed development of hypersensitivity, and the reactions usually revert to negativity during the terminal stages of untreated disease. In addition, the procedure does not differentiate between current and past infection, nor does it provide a reliable index of the efficacy of therapy. Various investigators have been unsuccessful in attempts to use complement fixation, hemagglutination, hemagglutination-lysis, and gel precipitin technics for serodiagnosis of tuberculosis. These procedures either showed a high incidence of false positive reactions or exhibited a level of sensitivity far too low to be of practical serodiagnostic value.

Efforts have been made to overcome these problems by employing the SAFA technic with purified antigens derived from M. tuberculosis cultures. Human tuberculosis was used as the model in the development of methodology since sera and detailed clinical histories from documented human cases were readily available. Details of the technic that evolved and results of a preliminary evaluation of the procedure were presented in the previous report on this Work Unit (WRAIR Research & Development Report, 1967). Twelve documented cases of tuberculosis were selected for these studies. A pretreatment serum was collected from each individual at the time of admission to the hospital and additional specimens were drawn at monthly intervals throughout the course of hospitalization. In brief, the SAFA test results were as follows: The majority of these patients exhibited evidence of anergy and only 4 were reactive in SAFA tests on the pretreatment specimens. However, a significant rise in antibody levels occurred following the initiation of therapy and usually reached maximum levels within 60-90 days. Three of the patients that were seronegative on admission became reactive after the initiation of therapy. Five of the group remained nonreactive throughout the course of observation. Three of the latter had far advanced cavitary TB and apparently remained in an anergic state. Of the remaining 2 nonreactors, one patient was transferred 42 days after initiation of therapy and specimens therefore were not collected during the period of maximum antibody production (60-90 days). The final patient giving no reaction in the SAFA was shown to be infected with a photochromogenic M. kansasii rather than typical M. tuberculosis. These findings, considered in conjunction with the observations that very few sera from healthy individuals or persons with diseases other than TB reacted in the SAFA test, suggested that reactions in the SAFA procedure were highly indicative of current or recent active tuberculosis. Therefore, it was believed that the SAFA test for TB had excellent potential for monitoring early tuberculosis in sub-human primate colonies and investigations were initiated to evaluate this aspect.

In view of the inherent dangers associated with the handling and care of sub-human primates infected with readily transmitted infectious agents, particularly when proper facilities are not available, initial evaluation of the SAFA procedure with sera from experimentally infected tuberculous animals was not undertaken. However, sera have

been collected from all monkeys undergoing normal quarantine during the past 6 months and tested in the SAFA procedure with the "A" and "C" protein antigens used in the earlier studies on human tuberculosis. The findings were compared with the results of the standard intradermal test using O.T. antigen. These studies are being conducted in collaboration with members of the Department of Laboratory Animals, WRAIR, and thus far a total of 605 monkeys have been evaluated.

During the initial phase of these investigations, studies were conducted to determine whether repeated skin tests with the O.T. antigen stimulated the production of antibodies that reacted in the SAFA test. Fortunately, this did not occur. Animals receiving weekly, biweekly, or monthly skin tests for a period of 4 months all remained seronegative in the SAFA test. It was apparent, therefore, that the skin tests required in the standard screening procedures probably would not influence the results obtained with the SAFA tests.

To gain definitive information concerning the advantages and limitations of the ID and SAFA tests, and to minimize the possibility of an outbreak of the disease among initially healthy animals, it was agreed that any monkey giving a reaction in either test would be immediately sacrificed and necropsy performed. Among the 605 monkeys examined to date, 14 gave reactions with one or both of the antigens in the SAFA procedure but were skin test negative. Gross necropsy revealed that 4 of these animals had active tuberculosis. On the other hand, 15 of the group gave positive intradermal reactions but were nonreactive in the SAFA test. Three of the latter were shown to have tuberculosis. It was apparent from these limited data that there was little if any correlation between the results obtained with the ID and SAFA tests. Obviously, some cases would have gone undetected if either test had been used alone. On the other hand, the two tests complemented each other and when used together, considerably reduced the risk of overlooking an active infection.

A comprehensive collaborative study with investigators at the US Army Medical Unit, Ft. Detrick, recently was initiated and should provide a basis for more critical evaluation and comparison of the ID and SAFA tests for tuberculosis. These studies are designed primarily to investigate various aspects of the natural transmission of tuberculosis among sub-human primates. In addition, it will provide specimens and clinical information ideally suited for evaluating the serological and immunological response in this disease. Thirty monkeys will be divided in two groups, one to receive therapy either before or after exposure, and the other to remain untreated. Each animal will be carefully monitored throughout the course of the experiment. Skin tests will be performed and serum samples drawn prior to exposure, and at biweekly intervals during the first two months following exposure. Surviving animals will be similarly tested at monthly intervals for four additional months. Complete necropsies will be performed on all animals when they expire or are sacrificed at the termination of the study. The results of these investigations will be given in a subsequent report on this Work Unit.

b. Histoplasmosis. Initial studies on the use of the SAFA procedure for the serodiagnosis of histoplasmosis were described in the previous report on this Work Unit (WRAIR Research & Development Report, 1967). Concentrated histoplasmin was fractionated by passage through a Sephadex G-200 gel column and the eluates containing the components shown to give the greatest serologic activity in the SAFA test were combined. A high level of sensitivity was obtained in a procedure wherein the antigen was fixed to the test disc by treatment with ethanol and the standard SAFA test employed. Considerable cross reactivity, however, was observed with sera from individuals with other mycotic diseases (coccidioidomycosis and blastomycosis). Results of recent studies suggested that the specificity of the reaction could be significantly improved by certain modifications of the basic SAFA technic. These technical innovations included: 1) use of 5% gluteraldehyde rather than acidified ethanol as antigen fixative, 2) employment of 2% rather than 1% Tween 80 in the serum diluent, and 3) increasing the incubation time of the primary reaction (i.e., the reaction of the test disc with patient's serum) from 45 minutes at room temperature to 18 hours at 3°C. Preliminary tests with the modified procedure showed excellent sensitivity with homologous sera, and a marked reduction of cross reactivity with sera from diseases other than histoplasmosis. The modified procedure currently is being evaluated with sera submitted for routine serodiagnosis and the results compared with those obtained with the standard complement fixation, precipitation and latex agglutination tests.

2. Serodiagnosis of American trypanosomiasis (Chagas' disease). Chagas' disease currently is considered to be the major health problem in practically all Central and South American countries. On the basis of recent surveys in endemic and presumably nonendemic areas, it is estimated that 5-7 million people now have either acute or chronic Chagas' disease. Moreover, transmission of the infection in urban areas that heretofore had been essentially free from the disease has markedly increased during recent years because of transfusions with blood from donors with unrecognized chronic Chagas' disease. Since serologic methods provide the only practical means for detecting the disease, particularly in individuals with asymptomatic chronic infection, many investigators currently are engaged in studies on the development and improvement of serodiagnostic methods. Investigations in this Department have led to the isolation and purification of two highly specific, sensitive complement fixing antigens derived from Trypanosoma cruzi. These consisted of a somatic protein antigen isolated from cultured lyophilized T. cruzi, and an exoantigen recovered from the liquid phase of the culture medium. Results of comprehensive evaluations of the antigens have been presented in previous reports on this Work Unit (WRAIR Research & Development Reports, 1960, et seq.). In addition to continued research and development along these lines, this Laboratory is a participant in a Study Group organized by the Pan American Health Organization to evaluate antigens and technics currently in use, and to recommend a standard procedure for the serodiagnosis of Chagas' disease. A comprehensive evaluation of 11 complement fixing antigens is being undertaken.

a. Development and evaluation of a rapid card test for Chagas' disease. The technical intricacies and the requirement for overnight incubation seriously limit the suitability of the complement fixation procedure for use in small field laboratories and blood banks. The need for a simple, rapid and sensitive serodiagnostic procedure was evident and stimulated interest in investigations on the development of a rapid card test for Chagas' disease. Details of the methodology that was devised and results of preliminary serological evaluation of the test were given in previous reports on this Work Unit (WRAIR Research & Development Reports, 1966, 1967). In brief, the technic consisted of adsorbing the T. cruzi exoantigen on lecithin-cholesterol crystals and suspending the sensitized particles in a saline diluent containing EDTA and choline chloride. Activated charcoal was added to the emulsion to permit visual reading of the reactions without magnification. Unheated serum or plasma could be used and the tests were performed on the disposable cards developed for the RPR Card Test for Syphilis. The sensitivity of the Card Test compared favorably with that of the CF test. However, it was observed that both tests were nonreactive with 4 (15%) of the 23 sera from xenodiagnosis positive donors. Since the Card Test was designed primarily for use as a screening proceudre, it was believed that the sensitivity should be further increased, even at the expense of some specificity, to minimize the risk of false negative results. Further studies revealed that the sensitivity could be increased by altering the lecithin concentration in the emulsion. However, the anticipated loss of specificity was observed with the more sensitive antigens. The latter was particularly evident in tests with syphilitic sera; approximately 20% of the nonchagasic syphilitic sera were reactive. In addition, 9% of the healthy donors also reacted. Studies now are in progress to determine whether the desired sensitivity can be maintained and the nonspecific reactivity reduced by using a particular fraction of the exoantigen isolated by filtration through a Sephadex G-200 gel column. Preliminary results have been encouraging and it is anticipated that the Card Test soon can be subjected to comprehensive evaluation in field studies in an endemic area.

b. Complement fixing antigens from cultured T. cruzi. In continued efforts to further improve the serodiagnostic tests for Chagas' disease, a variety of serologically active antigens have been obtained from T. cruzi cultivated in conventional culture media and more recently in tissue culture. Investigations on the latter are being conducted in collaboration with LTC Bryce Walton of the Middle America Research Unit, Panama. The purified somatic protein antigen and the exoantigen obtained from cultured T. cruzi both have shown a high level of specificity in comprehensive evaluations. However, serum titers obtained with these antigens generally are relatively low even in acute infections. The possibility that these antigens might fail to detect antibody in individuals with asymptomatic chronic Chagas' disease therefore must be considered. It has also been suggested that the antigenic composition of the culture form organisms may be different from the tissue or blood form, and that the culture form trypanosomes might not yield the best antigens for detecting antibodies produced during infection. Until

recently, the developmental forms of the trypanosome that occur in the blood and tissues of an infectedmammalian host (i.e., trypanosome and leishmania forms) could not be obtained in quantities sufficient for the preparation of antigens. However, this problem was overcome by the development of methods for the cultivation of T. cruzi in tissue culture. It was observed that the developmental cycle of the trypanosomes in tissue culture simulated that which occurred in the infected host, and large numbers of either the trypanosome or leishmania form of the organism could be obtained simply by harvesting the organisms at the appropriate time. In addition, the overlays of these cultures contained serologically active antigens that were designated "exoantigens." In preliminary studies on the leishmania form exoantigen, tests were performed on a group of sera from individuals with chronic Chagas' disease and the results compared with those obtained with the standard antigens prepared from organisms cultivated on conventional culture medium. In general, the sensitivity of the leishmania form exoantigen compared favorably with that of the standard antigens and in two instances yielded significantly higher titers. These studies are being continued and extended to include investigations on somatic leishmania form antigens and the somatic and exoantigens derived from the trypanosome form organisms.

c. Development of a hemagglutination test for Chagas' disease. Although complement fixation tests using the standard purified protein or exoantigens described above have been shown to be highly specific for Chagas' disease, a question has been raised concerning the ability of the tests to detect antibody in individuals with asymptomatic chronic disease. Since hemagglutination tests generally are more sensitive than other serologic procedures, studies were initiated to investigate the feasibility of using the standard T. cruzi antigens in an indirect hemagglutination test procedure. It was observed that the somatic protein antigen could be adsorbed to tanned sheep erythrocytes. Optimal sensitization was achieved by incubating the tanned cells at 37°C for 15 minutes with antigen diluted 1:4 in phosphate buffer (pH 6.4). A 2% suspension of the sensitized cells was prepared in PBS containing 3% gamma-globulin-free human serum as a stabilizing agent. Tests were performed in Microtiter plates by combining 25µl volumes of serially diluted serum with 25µl of sensitized cells and incubating at room temperature for 1 hour. Agglutination patterns then were read and interpreted in accordance with criteria normally used for hemagglutination procedures. Preliminary tests on sera from 5 selected cases of Chagas' disease indicated that the hemagglutination test was considerably more sensitive than the complement fixation procedure. Even though only 1 hour at room temperature was allowed for the reaction to occur in the HA test, serum titers were 16 to 64-fold greater than those obtained in the CF test incubated 18 hours in the cold. In parallel tests with 20 normal sera, no reactions were observed with the 1:4 or higher dilutions. To further evaluate the serodiagnostic potential of the new procedure, HA tests currently are being conducted on all sera submitted for Chagas' disease serology and the results compared with those obtained with the standard complement fixation procedure.

Efforts to employ the T. cruzi exoantigen in HA tests were unsuccessful. The exoantigen could not be adsorbed on tanned or untreated sheep erythrocytes regardless of the incubation time or temperature employed. Investigations along these lines therefore have been discontinued.

3. Studies on the immunogenic properties of Trypanosoma cruzi exoantigen. Preliminary findings on the immunogenic properties of T. cruzi exoantigen were presented in previous reports on this Work Unit (WRAIR Research & Development Reports, 1966, 1967). Mice immunized with the antigen generally survived longer than the nonimmunized controls following challenge with a virulent strain of T. cruzi. As was noted in the previous report, arrangements were made with Dr. Paul Thompson of Parke, Davis & Co. to conduct the comprehensive experiments necessary to obtain statistically significant findings. These experiments have been completed and although the immunized mice tended to live somewhat longer than the controls after challenge, the difference was not sufficient to be statistically significant. No further studies along these lines are being considered at this time.

4. Experimental mouse syphilis: Immunological and physiological responses in mice infected with Treponema pallidum. Laboratory strains of the house mouse (Mus musculus) have been used for many years as a potential experimental model for syphilis. The literature on this subject (reviewed by Gueft & Rosahn, Am. J. Syph., 32 : 59, 1948; and by Willcox & Guthe, Bull. WHO, 35, (Suppl.) 169, 1966) is unusually large and is indicative of the interest stimulated by the availability of highly inbred mouse strains, the economy and convenience of working with small mammals, and the desire to find a species other than man or rabbit which manifests clinical and pathological evidence of syphilis. It is now well established that mice will harbor a primary infection with the human derived Nichols strain of T. pallidum for the life of the infected animal. However, there is no overt septicemia and although virtually every tissue (including brain) contains virulent treponemes, no chancres have been observed, either at the site of primary infection or at any other site. Furthermore, serologic tests for reagin in sera of infected mice are uniformly nonreactive. Thus, the mouse offers distinct advantages in studies designed to discover the precipitating factors of symptomatology following infection, and provides a model for demonstrating that viable, infective, actively mitotic treponemata do not incite the symptoms of syphilis through the production of endo- or exotoxins alone.

a. Establishment of a colony of inbred mice. Twenty males and 40 females of each of the highly inbred C57BL/6J and A/J strains of mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. In addition, a small colony of BALB mice was procurred from the WRAIR vivarium. The three strains are being maintained by strict brother-sister matings to preserve homozygosity and to localize residual heterozygotes, as well as heterozygotes arising from continual mutation, into sublines with each strain. The first two strains (C57BL/6J

and A/J) are "histocompatible"; that is, intrastrain tissue grafts are permanently accepted (except male-female intrastrain grafts) and heterozygosity has not been detected at any of the genetic loci which prescribe transplantation antigens. The degree of isogenicity of the BALB strain has not yet been established. The colony now contains more than 600 mice and is in its fifth generation.

b. Antibody production in experimental mouse syphilis. The consistent lack of reagin production by mice infected with T. pallidum reported by many investigators (Gueft & Rosahn, op. cit.) was confirmed in 18 BALB male and female adult mice injected intraperitoneally with 10^6 motile Nichols strain treponemata. Cardiolipin microflocculation (CMF) tests were uniformly negative during more than 5 months of testing. These serum samples also were tested in the Treponema pallidum Immobilization (TPI) test. Results of the latter are summarized in Table 1.

Table 1

TPI results of serums taken from Balb mice injected with 10^6 T. pallidum

Time after injection	% immobilization	Interpretation
Before injection	0	Not reactive
25 days	16	Weakly reactive
16 weeks	87	Reactive
23 weeks	85	Reactive

From the results obtained in the TPI tests, it is clear that the consistent lack of detectable serological response in tests employing lipoidal antigens, a characteristic of mouse syphilis, is not due to an inherent deficiency in antigen recognition or antibody response, but rather to the test system employed. It is important to note that the mouse serves as a host for persistent treponemal infection with simultaneous production of immobilizing antibody, but without detectable formation of reagin. The typical infection in the mouse is characterized by very low numbers of spirochaetes. In fact, detection of the organisms by direct methods is very difficult and inoculation of rabbit testes with putatively infected tissue is frequently the only method for demonstrating the presence of the spirochaete. Thus, the presence of only low numbers of organisms, coupled with the production of immobilizing antibody throughout a period of more than 5 months (cf. Table 1), suggests that active antibody production removes the spirochaetes that are not sequestered in "immunologically priviledged" sites, and that continued antibody formation is maintained at a high level through antigenic stimulus by organisms (or their subcellular immunogens) that escape such sites. A fluorescein-conjugated rabbit anti- G

globulin preparation recently was acquired and all future sera in these studies will be tested in parallel in a modification of the Fluorescent Treponemal Antibody (FTA) test as well as the TPI.

c. Attempts to induce splenomegaly in perinatal mice with a standard inoculum of treponemata. It has been established that the spleen of a perinatal mouse rapidly undergoes hyperplasia following perenteral injection of soluble antigen or foreign lymphoid cells. In view of the lack of overt symptoms in syphilitic mice, it was postulated that quantitation of organ enlargement could provide a useful measure of the host's response to treponemal infection. Studies were initiated to determine whether the spleen weights of animals injected perenterally with viable treponemata abnormally increased. Accordingly, five litters of C57BL/6J mice, ranging 2-10 days in age, were assigned completely at random to one of three experimental groups. Group 1 received the treponeme diluent alone, whereas Group 2 received two million motile, virulent spirochaetes. A portion of the treponemal inoculum was injected intratesticularly into a rabbit; the animal developed an orchitis 7 days after inoculation, thus confirming the infectivity of the inoculum. The third group of mice received heat-inactivated treponeme inoculum to provide an index of the organ changes mediated by a noninfectious inoculum. All animals were weighed prior to inoculation and daily until they were sacrificed on the 6th day. The spleen, kidneys, and thymus of each animal were removed and weighed separately. An "organ index" was calculated as the organ weight divided by the body weight recorded on day 6. Analysis of variance of the accumulated data showed that there was no significant difference between any of the three groups. It was apparent, therefore, that injection of 2 million treponemata induced no splenomegaly or caused no gross change in the weight of the thymus or kidney.

d. Weight-gain analysis of perinatal mice. A variety of etiologic agents have been shown to cause either transient or progressive weight loss in perinatal mice. These include foreign lymphoid cells, cortisol acetate, echo virus, and several species of bacteria. In further efforts to find a suitable test system for studying mouse syphilis in vivo, 12 litters of perinatal C57BL/6J and BALB mice were used to determine whether injection with infectious treponemata caused a change in body weight. Such a change, if demonstrable, could be submitted to statistical analyses and a variety of sophisticated experimental designs could be employed to study the kinetics of weight change as a function of time and numbers of spirochaetes. Each litter was divided between a control and experimental group using a table of random numbers. The control animals received 5×10^6 heat-inactivated spirochaetes intraperitoneally; the experimental littermates received the same number of motile, virulent organisms. Body weights were recorded before injection and at intervals of several days thereafter. Statistical analysis of the data indicated no significant body weight loss or increase when compared with the littermate controls. These mice currently are 2 months old and apparently are healthy. Their sera will be tested for the presence of treponemal

antibodies at bi-monthly intervals. The lack of significant weight loss, particularly in the youngest litters, is in contrast to the findings reported by Festenstein, et al (Clin. Exp. Immunol., 2 : 311, 1967). These investigators claimed that newborn and perinatal rabbits developed progressive acute weight loss following infection with T. pallidum, and suggested that acquired immunological tolerance had been produced, leading to overwhelming septicemia and resulting in wasting and death. Further studies in this laboratory will be directed toward the use of large numbers of treponemata for inducing tolerance in newborn mice.

e. Studies on antibody production elicited by T. pallidum emulsified in complete Freund's adjuvant. A suspension of virulent treponemata was emulsified in complete Freund's adjuvant for 15 minutes using the conventional double-syringe technic. Immediately after preparation of the emulsion, microscopic examination revealed no motile treponemes. One-tenth ml of the emulsion containing ca. 5×10^6 organisms was injected subcutaneously into each of two sites on the backs of 4 male and 4 female adult BALB mice. CMF and TPI tests on sera obtained before injection, on the 26th day, and 7 weeks after injection were uniformly nonreactive. These findings suggest that use of the subcutaneous site for inoculation or the absence of motile, mitotic organisms (or perhaps both) was responsible for the failure to develop detectable antibody. It is noteworthy that the same number of motile organisms administered systemically in Nelson's basal medium produced relatively high antibody titers detected in the TPI test (see b above).

f. Kinetic studies of the antibody response to T. pallidum. The experiment cited in b. above showed that considerable amounts of antibody are produced in adult mice inoculated with 10^6 motile treponemata. Attention therefore was given to detailed studies on the kinetics of antibody production as a function of time, using a variety of spirochaete doses, and considering the kinetics of the primary as well as the anamnestic response (if the latter occurs). Accordingly, 13 cages, each containing 5 adult C57BL/6J mice (irrespective of sex), were assigned at random to 3 experimental groups. The first cage of mice received 0.1 ml of Nelson's basal medium only. The mice in 6 cages (2nd experimental group) received doses of treponemata ranging from 10^8 to 10^3 organisms in decreasing log increments. Animals in the remaining 6 cages, constituting the third experimental group, received the same dosages used in the second group except that the spirochaetes were heat-inactivated at 56°C for 40 minutes before injection. All inoculations were given intraperitoneally. Each animal was marked for identification and will be bled and tested individually for the presence of treponemal antibody 3-4 weeks after inoculation. All three experimental groups then will receive 10^6 viable spirochaetes and will be bled and tested in parallel FTA and TPI tests at bi-monthly intervals thereafter. These experiments will permit investigation of a number of factors: 1) determination of the smallest dose of treponemata required to stimulate detectable antibody in the primary response; 2) the capacity of heat-inactivated

and presumably nonmitotic spirochaetes to sensitize recipients; 3) determination of the smallest dose that will sensitize mice to a second challenge; 4) the molecular class(es) of antibody produced during the primary and secondary challenge; and 5) the possible onset of resistance to superinfection (also known as premunition or symptom immunity).

g. Future studies. The excellent antibody response to T. pallidum infection in the mouse, coupled with the fact that symptomatology is nonexistent, suggests that immunosuppression of prospective recipients might permit manifestation of clinical disease such as that reported in perinatal rabbits by Festenstein, et al (op. cit.). Two different preparations of the most potent immunosuppressant known, anti-lymphocyte serum (Levey & Medawar, Proc. Nat. Acad. Sci., 56 : 1130, 1966), have been produced in rabbits in this laboratory. Preliminary tests revealed that 0.05 ml of this reagent (ALS) was fatal to 28/29 perinatal C57BL/6J mice. These findings illustrated the potency of the reagent and indicated the need to determine the nonfatal dose that will produce immunosuppression. Adult mice will be treated with ALS and then challenged with virulent T. pallidum in an effort to determine whether clinical symptomatology can be provoked through immunosuppression. Newborn mice also will be altered immunologically with ALS, as well as by thymectomy, in an attempt to create a physiological condition more compatible with treponemal mitosis. In addition, studies on acquired immunological tolerance in newborn mice inoculated with subcellular treponemal extracts and with living organisms are planned.

5. Development of serodiagnostic tests for lupus erythematosus. Previous studies on the development of serodiagnostic tests for systemic lupus erythematosus (SLE) have been summarized in previous reports on this Work Unit (WRAIR Research & Development Report, 1966, 1967). The complement fixation and soluble antigen fluorescent antibody (SAFA) tests have been shown to complement each other when used together for serodiagnosis. Evidence to date suggests that the two tests do not react with precisely the same antibodies even though the same antigen is used in both procedures. Approximately 95% of patients with SLE give reactions in the CF and/or SAFA tests. It has been suggested that the possibility of SLE be considered in the clinical evaluation of any patient giving a reaction in either test. On the other hand, individuals with autoimmune diseases other than SLE show a much lower incidence of reaction in the two tests. Consideration was given to the possibility that the antibody titers developed in these other diseases often may remain below the levels detectable in the CF and SAFA tests. Studies, therefore, were undertaken to determine whether the serodiagnostic capabilities could be improved by employing the more sensitive conglutination reaction. The horse complement and bovine conglutinin required for the reaction have been obtained and the system standardized using the calf thymus nucleoprotein antigen. Conglutination tests now are being performed on all sera submitted for serologic tests for SLE or other autoimmune disease. Results will be compared with those obtained with the standard CF and SAFA tests when sufficient data have been acquired.

Summary and Conclusions.

1. The soluble antigen fluorescent antibody (SAFA) procedure has continued to show excellent diagnostic potential and the technic now is being employed for the serodiagnosis of a variety of parasitic, bacterial, mycotic, and viral diseases. Technical innovations have been introduced to improve the serodiagnostic value of the SAFA test in certain diseases.

a. Studies on the use of the SAFA test for serodiagnosis of tuberculosis have been extended to include the capability of monitoring the infection in sub-human primate colonies. Findings indicate that the SAFA and intradermal tests complement each other and both procedures are being used in the monitoring program. Evidence of anergy was noted in the SAFA tests on humans with advanced disease. However, anergy did not appear to seriously interfere with the detection of antibody in early tuberculosis.

b. Technical innovations have improved the specificity and sensitivity of SAFA test for histoplasmosis. Use of gluteraldehyde rather than acidified ethanol for the antigen fixative, increase of the concentration of Tween 80 used in the serum diluent, and extension of the primary incubation time to 18 hours were responsible for the noted improvements of the procedure. The modified technic is being evaluated with sera submitted for routine serodiagnosis and the results compared with those obtained in the standard test procedures.

2. Efforts to further improve serodiagnostic tests for Chagas' disease have been continued. Studies have been conducted along the following lines:

a. Investigations on improvement of the sensitivity and specificity of the rapid card test for Chagas' disease are nearing completion. Adjustment of the lecithin content of the antigen and the use of a purified exoantigen fraction appear to have achieved the desired results. It is anticipated that the card test soon can be subjected to comprehensive evaluation in field studies.

b. Successful cultivation of T. cruzi in tissue culture has made it possible to collect large numbers of the developmental forms of the trypanosome that occur in the blood and tissues of an infected mammalian host (i.e., trypanosome and leishmania forms). In view of the possibility that the antigen composition of the various developmental forms may differ, it was postulated that antigens derived from leishmania form organisms might be superior to the usually employed crithidia form antigens for detecting antibody in asymptomatic chronic Chagas' disease. Preliminary studies on an exoantigen from the leishmania form cultures have shown promise along these lines and the investigations are being continued.

3. Comprehensive evaluation of the immunogenic properties of T. cruzi exoantigens has been completed. Although initial studies

indicated that the antigen might confer protection against challenge with virulent trypanosomes, and thus have potential value as a vaccine, differences of the survival times of the immunized and control animals were not statistically significant. The studies, therefore, have been discontinued.

4. Studies on the physiological and immunological responses in mice infected with T. pallidum have been initiated. Mice were considered to provide an ideal laboratory model for studying the basic mechanisms of the immune response in syphilis since the treponemes become widely disseminated throughout the tissues, but no clinical symptoms appear. Moreover, no reaginic antibody can be detected even though treponemal antibodies are produced in relatively large amounts. Highly inbred, homozygous strains of mice are being used in these investigations to facilitate in vivo studies on the basic mechanisms and kinetics of immune response. Technics are being developed to elucidate the kinetics of primary and secondary immune responses and to identify the classes of antibody associated with each. Attempts will be made to alter the immunological status of the host in a manner that will permit development of the classical symptoms exhibited in rabbit and human syphilis.

5. Studies on the improvement of serodiagnostic tests for systemic lupus erythematosus and other autoimmune diseases have been continued. More than 95% of the lupus patients tested reacted in the CF and/or SAFA tests. However, the incidence of reactivity was considerably lower among patients with autoimmune diseases other than lupus. Attempts are being made to improve the diagnostic [illegible]abilities for these latter diseases by employing the more sensitive conglutination procedure.

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