

2. Title: Arbovirus Infections in Man and Experimental Animals

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Objectives:

a) To develop improved virologic and serologic methods for diagnosis and study of arbovirus diseases in Thailand.

b) To determine the antigenic relationships and study the biologic properties of arboviruses of medical importance.

c) To obtain information on the sequential changes in the immunoglobulin response of man and experimental animals to single and multiple dengue infection and to relate these observations to disease processes.

d) To study the etiology of human cases of encephalitis and determine the importance of arboviruses and other viruses as causative agents.

Description:

A plaque reduction neutralization test for dengue virus antibody using agar overlay method with LLC MK-2 tissue culture was tested for use in antigenic classification of dengue viruses. Using antisera made in monkeys by a single subcutaneous injection of prototype viruses, the specificity of the serum was tested by cross neutralization tests with prototype strains and a group of dengue virus strains isolated in Bangkok were tested against this reference antisera.

The immunoglobulin response of monkeys to dengue infection was investigated by collecting serial serum specimens after experimental infection, fractionating the sera by density gradient centrifugation and gel-filtration chromatography, and testing serum fractions for antibody. Ig-G and Ig-M antibodies were identified by 2-mercaptoethanol sensitivity and immunoelectrophoresis.

Serum from hemorrhagic fever cases was studied by similar methods to determine the nature of the immunoglobulin response following dengue infection in man.

Comparative studies were done to determine the relative sensitivity of tissue culture systems and suckling mice for isolation of Japanese encephalitis and Chikungunya viruses.

Cases of human CNS disease referred from Chiangmai, Korat and Bangkok Hospitals were studied by serologic and virologic methods. Serologic tests for JE, dengue chikungunya, herpes, mumps and LCM were done and when suitable specimens were obtained, isolation of enteroviruses was attempted using primary monkey kidney tissue culture and suckling mice.

Autointerference produced by chikungunya virus when inoculated into BS-C-1 cells has been studied from several aspects. Attempts were made to determine the nature of the autointerfering substance by physico chemical methods. The role of interferon in the production of the autointerference phenomenon was studied.

Progress:

Plaque Reduction Neutralization Test: A plaque reduction neutralization test (PRNT) for dengue antibody is presently being used extensively in this laboratory in a variety of studies. The method described below was used for all tests unless otherwise stated.

The LLC-MK2 strain* of continuous rhesus monkey kidney was serially propagated in a growth media consisting of Medium 199 with 20% calf serum, 15% tryptose phosphate broth and antibiotics. One oz. prescription bottles were seeded with 4ml of growth medium containing 100,000 cells per ml. Confluent monolayers were obtained by the fourth day. Use of four day old cultures produced the best results. Media for the first agar overlay consisted of 1% Noble agar, 0.02% DEAE dextran, 5% inactivated calf serum and antibiotics, pH was adjusted to 8.3 with NaHCO_3 . For the second overlay, calf serum and NaHCO_3 were omitted and neutral red (final conc. 1:10,000) added.

Diluent for virus and serum dilutions consisted of Medium 199 plus 5% inactivated fetal bovine serum with pH adjusted to 8.3 with NaHCO_3 . Serial 2 or 4 fold dilutions of serum were made and equal amounts of diluent containing an estimated 50 plaque forming units (pfu) of dengue virus per 0.15 ml added. Virus-serum mixtures were incubated for 1 hour at 37°C. Cell sheets were washed with Hank's BSS (pH 8.3) and 0.3 ml of virus-serum mixtures applied. After allowing virus adsorption for 1½ hours at 37°C cell sheets were again washed with Hank's BSS and 4 ml of agar overlay added. Cultures were incubated at 37°C for 7 days and the 2nd overlay containing neutral red dye added. Plaque counts were made 24 hours after application of the 2nd overlay.

Three replicate bottles were used for each serum dilution and control. Fifty percent plaque reduction end points were determined by plotting on log-probit paper after the method of Cutchins.

The method described above has been found to be satisfactory for use with mouse adapted reference dengue virus strains types 1 through 6 as well as over 20 strains recently isolated in tissue culture and/or suckling mice. A similar procedure is used for Japanese encephalitis and Chikungunya viruses, the only change necessary is to shorten the incubation time from 7 days to 3 days.

Dengue Virus Identification. Antigenic variation and serologic cross reactions make classification of dengue virus isolates a difficult problem. Previous experience by this and other laboratories with various typing methods has made it apparent that methods which require high titered virus seeds are not completely satisfactory for many strains, especially those strains isolated in tissue culture which have low infectivity titers and low mouse pathogenicity.

The system presently under investigation in this laboratory utilizes a plaque reduction neutralization test and monkey antiserum prepared by a single subcutaneous infections of live dengue virus in antibody-free Macacus cynomologi monkeys.

Results of cross-neutralization tests with standard reference strains of dengue viruses are shown in table 2. Types 1 through 4 are readily distinguishable by this system. Types 2 and 5 cannot be differentiated since reciprocal titers are in the same range. Types 1 and 6 appear to be distinguishable by the relatively low titer of dengue 1 antiserum with dengue 6 virus although their close relationship is apparent. Until the extent of antigenic variations within the dengue 1, 6 and dengue 2, 5 groups are better defined, newly isolated strains will merely be placed in the group and more refined classification will be deferred.

* Obtained from Dr. Robert Hull, Eli Lilly Corp.

Further attempts to improve the specificity of this typing method are being made. One experiment on the effect of serum-virus incubation time is recorded in table 3. Results indicate that a shorter incubation time decreases the heterologous reaction but also lowers the homologous titer significantly.

Table 4 summarize the results of virus typing by PRNT of 15 dengue strains isolated from HF cases in Bangkok. The titers of the reference antiserum with these strains are generally lower than the titers obtained against the high mouse passage prototype strains. However, the reactions are sufficiently specific to classify these strains accurately. Dengue-3 antiserum cross has a low titered cross reaction with the majority of the strains in the 2, 5 and 1, 6 groups, however, the type 3 strains react only with the type 3 antisera.

Four type 3 strains isolated in successive years from 1963 to 1966 all appear to be similar in their antigenic reactions with these antisera. In addition, plaque morphology of these strains in LLC MK-2 cell culture shows a significant uniformity. All produce plaques approximately 3 mm. in diameter with clear centers and hazy borders and with little variation of plaque size.

The AP-18 strain of dengue-3 virus isolated from East Pakistan in 1964 appears similar to the Thai strains. With AP-18 virus the dengue-3 (H-87) antiserum titer was 1:270 and the titers of the antisera to the other five dengue reference strains was less than 1:10.

The strains in the type 1, 6 and 2, 5 complexes show some evidence of antigenic variation. Strain No. 10572 appears to be more closely related to type 1 while strains No. 12900, 14580 and 22448 appear to be more closely related to type 6. In the 2, 5 complex, strains 21868 and 11340 appear to differ from 20833 and 20731. A more detailed comparison of these strains using cross neutralization tests is in progress in an attempt to define the antigenic variation within these types and to assess the effects of mouse passage and tissue culture passage on the antigenic structure.

Dengue Antibodies in Monkeys. The immunologic response of monkeys to experimental dengue-2 virus infection was studied to determine the time of appearance and specificity of 7S and 19S antibody fractions. Three monkeys, Macacus cynomologi, were injected subcutaneously with 1.2×10^2 pfu of dengue-2 virus. The virus used was a Bangkok strain (3360-62) in 3rd BS.C-1 passage. Following inoculation, sera were collected periodically and tested for hemagglutination inhibiting (HI) antibody and neutralizing (N) antibody.

HI tests on serum and serum fractions were done in microtiter using a modification of Casal's method. Sera to be tested for HI antibody and serum fractions to be tested for HI and N antibody were heat inactivated (56° , 30 min.) and extracted with cold acetone prior to testing. Neutralization tests were done by the plaque reduction method in LLC MK-2 cell culture. The N and HI antibody titers of whole serum at various times following infection are shown in Figs. 1, 2 and 3. Monkeys A-21 and A-37 had no HI or N antibody prior to infection or for the first 6 days post inoculation; and presumably, had no previous antigenic experience with dengue virus. Monkeys A-39 had low titered antibody prior to infection and a rapid response indicating previous exposure to a dengue or closely related agent.

Sera were fractionated by ultracentrifugation for 18 hrs. at 100,00 G on a 10 to 40% sucrose density gradient and fractions collected by the drop method. Results of antibody studies are given in tables 5 to 7.

At 6 days post inoculation, only monkey A-39 had detectable antibody and all HI and N activity was found in the upper fractions indicating the antibody was entirely 7S. At 14 days, two separate zones of antibody activity were found in the sera of all three animal. Both the lower (19S) zone and upper (7S) zone had HI and N activity. At 60 days the 19S antibody had entirely disappeared.

To confirm the identity of the antibody found in the two separate zones of the density gradient, aliquots of each fraction were treated with 2-mercaptoethanol (ME) and HI activity compared with an

TABLE 2

Neutralizing Antibody Titers of Monkey Antisera to Reference Strains of Dengue Viruses.

Antisera

| Viruses | D-1 | D-2 | D-3 | D-4 | D-5 | D-6 |
|---------------------|--------------------|------------------|-------------------|-----|------|-----|
| Dengue-1 (Hawaii) | 200 ⁽¹⁾ | 0 ⁽²⁾ | 90 | 10 | 0 | 260 |
| Dengue-2 (N.G. "C") | 0 | 1400 | 30 | 13 | 1700 | 0 |
| Dengue-3 (H-87) | 0 | 0 | 350 | 0 | 0 | 0 |
| Dengue-4 (H-241) | 0 | 0 | 14 | 150 | 0 | 0 |
| Dengue-5 (TH-36) | 0 | 2500 | NT ⁽³⁾ | NT | 2500 | NT |
| Dengue-6 (TH-Sman) | 40 | 0 | NT | NT | 0 | 240 |

(1) Reciprocal of 50% plaque reduction titer

(2) 0 = less than 1:10

(3) NT = not tested.

TABLE 3

Effect of Serum-Virus Incubation Time at 37° C on Homologous and Heterologous Neutralizing Antibody Titers of Dengue-3 Antiserum.

| <u>Time</u> | <u>Virus</u> | |
|-------------|-----------------|-----------------|
| | <u>Dengue-3</u> | <u>Dengue-1</u> |
| 10 min. | 85* | < 10 |
| 30 min. | 120 | < 10 |
| 60 min. | 330 | 40 |

* Reciprocal of 50% plaque reduction titer

TABLE 4

Results of Dengue Virus Identification by Plaque Reduction Neutralization Tests with Monkey Antisera.

| Strain No. | Year isolated | (1) host and passage | Reference Antisera | | | | | | Dengue type |
|------------|---------------|-------------------------|--------------------|-------|--------------------|-----|-------|-----|-------------|
| | | | D-1 | D-2 | D-3 | D-4 | D-5 | D-6 | |
| 648 | 1663 | sm-7 | 0 ⁽²⁾ | 0 | 150 ⁽³⁾ | 0 | 0 | 0 | 3 |
| 14670 | 1964 | tc-3, sm-1 | 0 | 0 | 140 | 0 | 0 | 0 | 3 |
| 10286 | " | tc-3, sm-1 | | 600 | 50 | 10 | | 0 | 2,5 |
| 16681 | " | tc-3 | | 500 | 90 | 0 | | 0 | 2,5 |
| 12900 | " | sm-3 | 40 | 0 | 40 | 0 | 0 | 120 | 1,6 |
| 10044 | " | sm-3 | | > 640 | 60 | 20 | | 0 | 2,5 |
| 14580 | 1965 | tc-3 | 100 | 20 | 35 | 22 | 15 | 150 | 1,6 |
| 21868 | " | tc-3 | 0 | 190 | 50 | 0 | 100 | 0 | 2,5 |
| 20833 | " | sm-7 | 0 | > 500 | 10 | 0 | > 500 | 0 | 2,5 |
| 20731 | " | sm-10 | 0 | > 500 | 20 | 0 | > 500 | 0 | 2,5 |
| 14962 | " | sm-3 | 0 | 0 | 160 | 0 | 0 | 0 | 3 |
| 11340 | " | sm-7 | 0 | 350 | 0 | 0 | 90 | 0 | 2,5 |
| 10572 | " | sm-2, tc-1 | 400 | 0 | 25 | 0 | 0 | 50 | 1,6 |
| 21153 | 1966 | sm-3 | 0 | 0 | 200 | 0 | 0 | 0 | 3 |
| 22448 | " | sm-2 | 100 | 0 | 13 | 0 | 0 | 190 | 1,6 |

(1) sm-suckling mice, tc-tissue culture (BSC-1 or LLC MK-2)

(2) 0 = less than 1:10

(3) reciprocal of 50% plaque reduction titer

TABLE 5

Antibody Titers of Sucrose Density Gradient Fractions, Monkey No. A-21, 14 and 60 days after Dengue-2 Infection.

| Fraction No. | 14 days | | 60 days | |
|--------------|------------------|------------------|----------|---------|
| | HI titer | N titer | HI titer | N titer |
| 1 | 0 ⁽¹⁾ | 2 ⁽²⁾ | 0 | 0 |
| 2 | 80 | 250 | 0 | 0 |
| 3 | 160 | 160 | 0 | 0 |
| 4 | 80 | 130 | 0 | 0 |
| 5 | 40 | 0 | 0 | 5 |
| 6 | 80 | 5 | 80 | 500 |
| 7 | 320 | 100 | 80 | 250 |
| 8 | 80 | 5 | 20 | 20 |
| 9 | 40 | 10 | 20 | 2 |
| 10 | 0 | 0 | 0 | 0 |

(1) reciprocal of titer vs 8 units of dengue 2 antigen (0=10)

(2) reciprocal of 50% plaque reduction titer vs dengue 2 virus

TABLE 6

Antibody Titers of Sucrose Density Gradient Fractions. Monkey No. A-37, 6, 14 and 60 Days after Dengue-2 Infection

| Fraction No. | 6 days | | 14 days | | 60 days | |
|--------------|------------------|-------------------|----------|------------------|----------|---------|
| | HI titer | N titer | HI titer | N titer | HI titer | N titer |
| 1 | 0 ⁽¹⁾ | ND ⁽³⁾ | 0 | 0 ⁽²⁾ | 0 | 0 |
| 2 | 0 | ND | 20 | 80 | 0 | 0 |
| 3 | 0 | ND | 320 | 1000 | 0 | 0 |
| 4 | 0 | ND | 80 | 60 | 0 | 0 |
| 5 | 0 | ND | 0 | 0 | 20 | 100 |
| 6 | 0 | ND | 20 | 180 | 80 | 290 |
| 7 | 0 | ND | 80 | 40 | 40 | 360 |
| 8 | 0 | ND | 40 | 120 | 0 | 0 |
| 9 | 0 | ND | 0 | 0 | 0 | 0 |
| 10 | 0 | ND | 0 | 0 | 0 | 0 |

- (1) reciprocal of titer vs 8 units of dengue 2 antigen (0 = 10)
- (2) reciprocal of 50% plaque reduction titer vs dengue 2 virus
- (3) not done

SERUM HEMAGGLUTINATION-INHIBITING (HI) AND NEUTRALIZING (N) ANTIBODY FOLLOWING DENGUE - 2 INFECTION

FIGURE 1

**MONKEY
NO A-21**

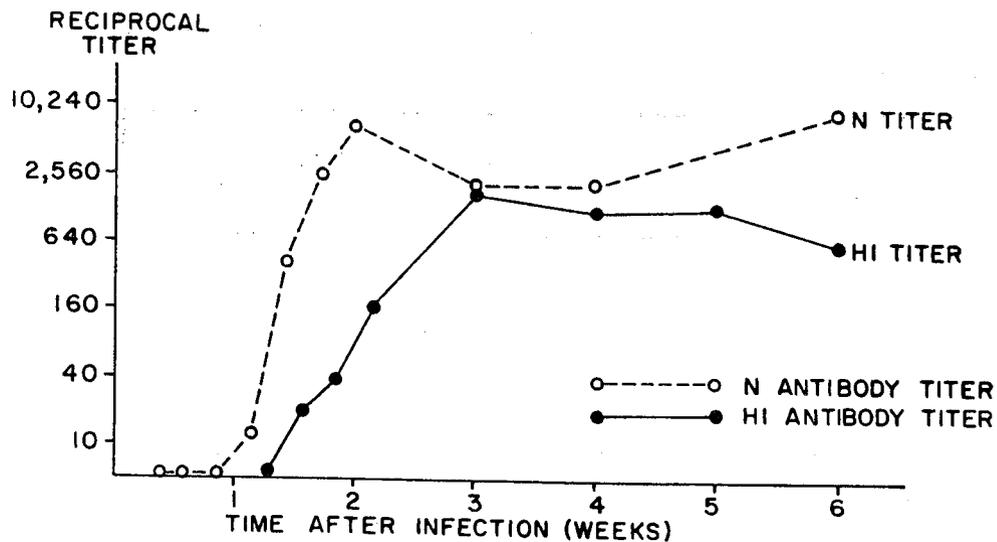


FIGURE 2

**MONKEY
NO A-37**

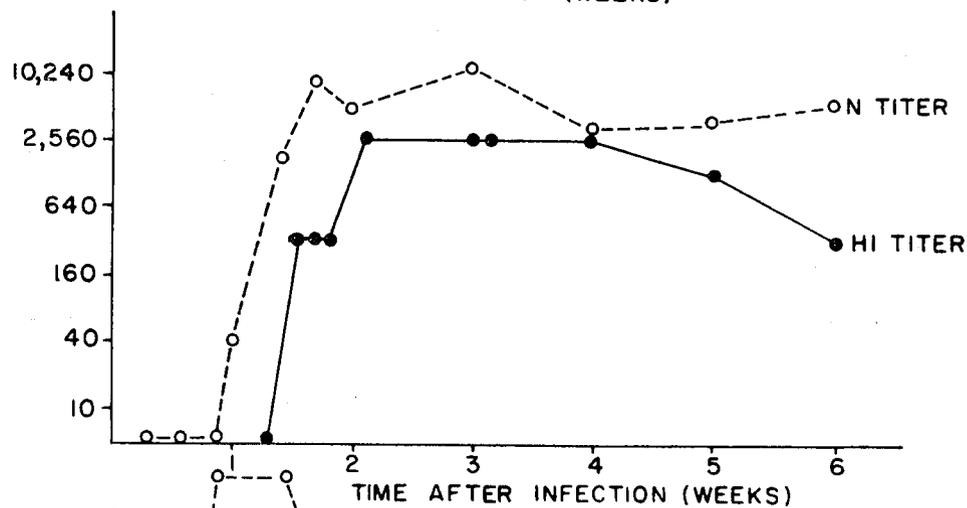


FIGURE 3

**MONKEY
NO A-39**

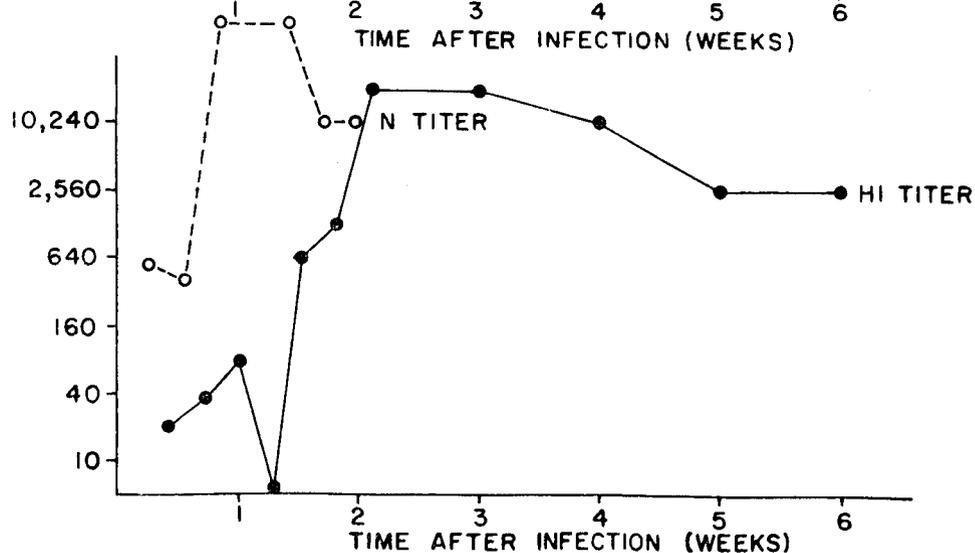


TABLE 7

Antibody Titers of Sucrose Density Gradient Fractions. Monkey No. A-39, 6, 14 and 60 Days after Dengue-2 Infection.

| Fraction No. | 6 days | | 14 days | | 60 days | |
|--------------|------------------|------------------|----------|---------|----------|---------|
| | HI titer | N titer | HI titer | N titer | HI titer | N titer |
| 1 | 0 ⁽¹⁾ | 0 ⁽²⁾ | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 20 | 640 | 0 | 0 |
| 4 | 0 | 0 | 40 | 50 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 15 | 320 | 480 | ND | ND |
| 7 | 40 | 10 | 2560 | 640 | 320 | 300 |
| 8 | 320 | 700 | 5280 | 640 | 640 | 640 |
| 9 | 160 | 90 | 640 | 640 | 80 | 150 |
| 10 | 40 | 0 | 80 | 20 | 0 | 10 |

(1) reciprocal of titer vs 8 units of dengue 2 antigen (0= 10)

(2) reciprocal of 50% plaque reduction titer vs dengue 2 virus

untreated sample. The results, shown in figures 4 to 10, indicate that the antibody in the lower fractions (2 through 5) is entirely destroyed by ME while the antibody in the upper fractions is unaffected. Gel chromatography on Sephadex G-200 of the 14 day sera confirmed the presence of 19S, ME sensitive antibody and 7S, ME resistant antibody. These results are shown in figures 12 to 14.

Immunoelectrophoresis of pooled, concentrated fractions from the gel chromatography showed the presence of an Ig-M precipitin band and absence of an Ig-G band in the early fractions containing only ME sensitive antibody. Conversely the later fractions containing only ME resistant antibody showed an Ig-G band and no Ig-M band.

Similarly, immunoelectrophoretic studies showed that the sucrose density gradient fractions No. 2, 3 and 4 contained Ig-M and fractions No. 6, 7, 8 and 9 contained Ig-G.

The experiments described above indicate that dengue infection in monkeys produces transient Ig-M antibodies which disappeared within 60 days post infection. In the absence of previous dengue infection, the HI and N antibody titers rose rapidly between 7 and 14 days post infection. On day 14, in the two monkeys (A-21, A-37) with "primary" infections, the HI antibody titers of the 19S and 7S components were approximately equal. In the monkey (A-39) with a "secondary" infection the HI and N antibody on day 6 consisted entirely of 7S globulins. On day 14 a 19S component was present but the 19S antibody titers were low compared to the 7S titers and also lower than the 19S titers seen in the other two animals.

Further studies are in progress to determine more precisely the Ig-G Ig-M antibody levels following infection and to determine the relative specificity of these two major immunoglobulins.

Effect of Kaolin Extraction on 19S Antibody. The routine method previously in use in this laboratory for arbovirus diagnostic and epidemiologic studies used kaolin treatment of sera to remove non specific inhibitors prior to HI testing. Recent information via personal communication from the Yale Arbovirus Research Laboratory suggested that kaolin extraction has an adverse effect on 19S antibody.

Experiments to test this observation were carried out by centrifuging monkey antisera known to contain 19S HI antibodies against dengue on a sucrose density gradient. The effects of kaolin extraction and cold acetone extraction were compared on aliquots of fractions containing 19S, mercaptoethanol sensitive antibody or 7S, mercaptoethanol resistant antibody.

Results are given in table 8. Kaolin treatment removed the 19S HI antibody and activity had little or no effect on 7S antibody.

Immunologic Response to Dengue Infection in Man. Plaque reduction neutralization tests (PRNT) and sucrose density gradient centrifugation of serum were used to study the nature of the immune response in patients with dengue or dengue hemorrhagic fever. Sera from patients from whom dengue viruses had been isolated and typed were tested for neutralizing antibody to prototype dengue viruses types 1 to 4. Sera from hemorrhagic fever patients was centrifuged in a sucrose density gradient and the fractions tested for HI antibody against dengue antigens. Antibody was tested for sensitivity to 2-mercaptoethanol. Pooled fractions from the 19S zone (fraction 2, 3, 4) and the 7S zone (fractions 6, 7, 8, 9) were tested for N antibody by PRNT against dengue types 1 to 4.

Results of N antibody tests on 3 serum pairs and 4 convalescent sera are given in table 9. In patients with no dengue antibody in the acute serum specimen, indicating no previous exposure to dengue, the N titers were relatively low (less than 1:650) and the homologous titers were significantly higher than heterologous titers. In such cases a specific diagnosis could be made in retrospect without virus isolation. Previous dengue exposure resulted in extremely high titers and broad cross reactivity. The difference in magnitude between primary and secondary responses is striking.

**EFFECT OF MERCAPTO-ETHANOL ON HI ANTIBODY
IN SUCROSE DENSITY GRADIENT FRACTIONS.**

FIGURE 4

**MONKEY NO. A 21
14 DAYS POST
INFECTION WITH
DENGUE-2**

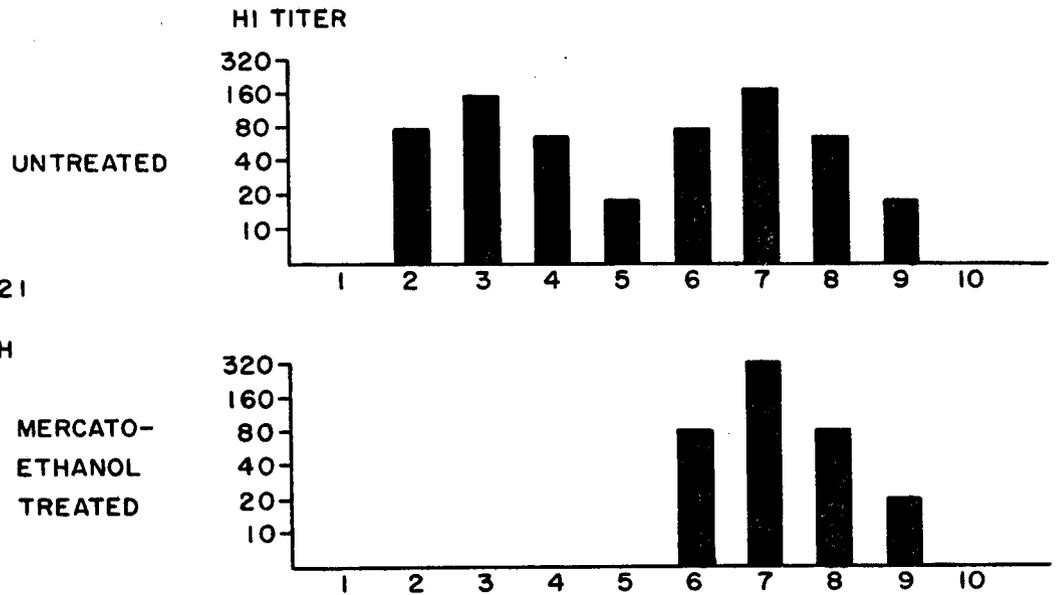
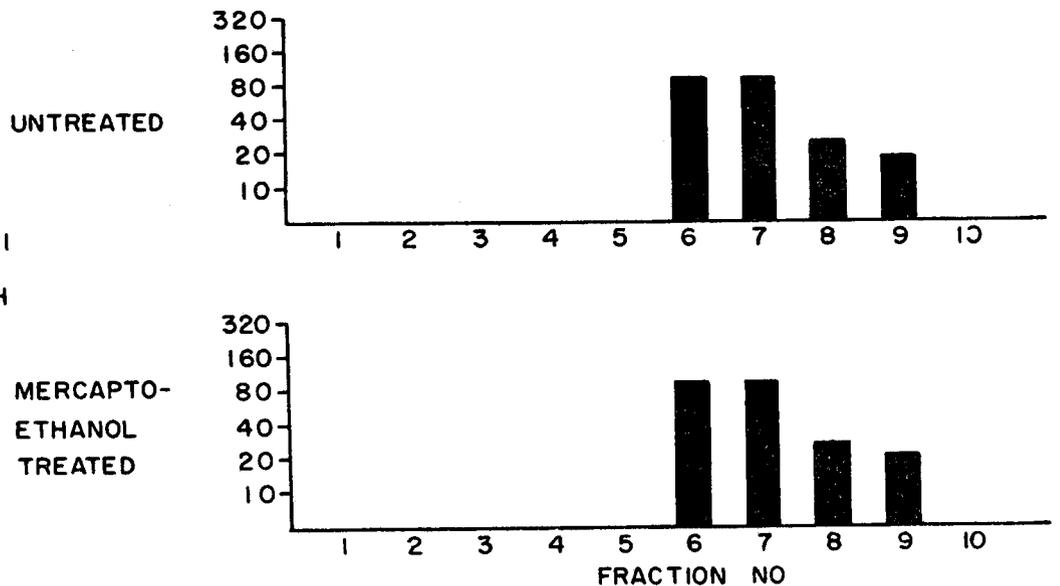


FIGURE 5

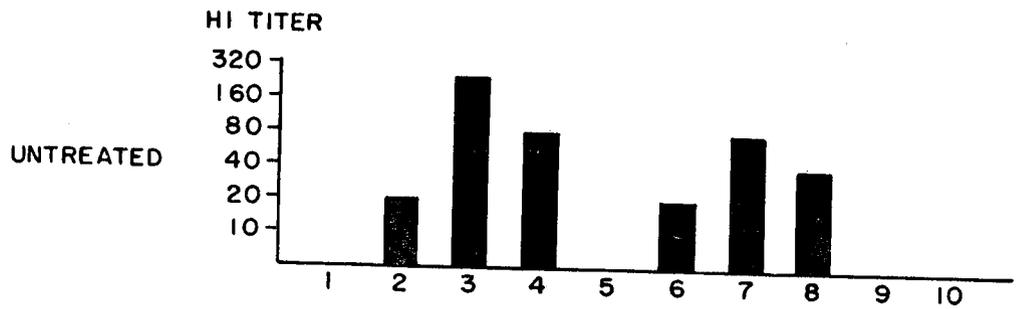
**MONKEY NO. A 21
60 DAYS POST
INFECTION WITH
DENGUE-2**



EFFECT OF MERCAPTO-ETHANOL ON HI ANTIBODY IN SUCROSE DENSITY GRADIENT FRACTIONS.

FIGURE 6

MONKEY NO. A 37
14 DAYS POST
INFECTION WITH
DENGUE-2



MERCAPTO-
ETHANOL
TREATED

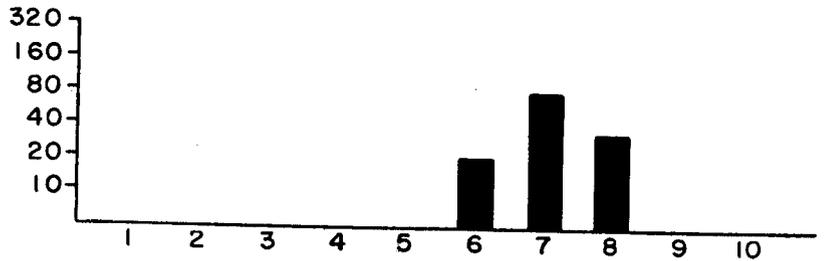
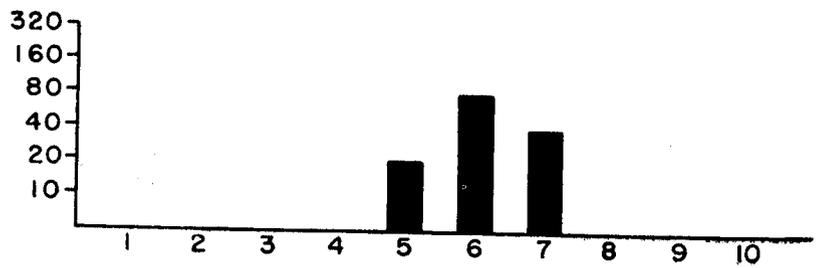


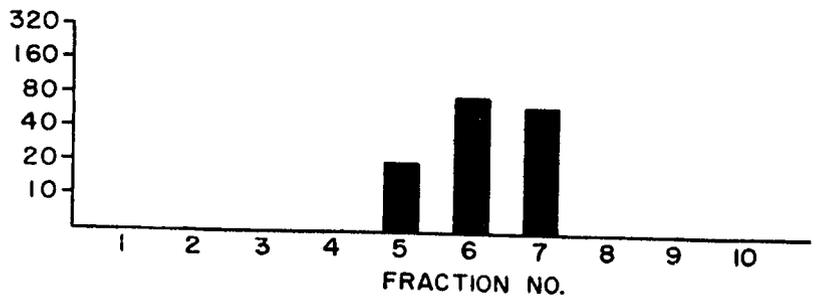
FIGURE 7

MONKEY NO. A 37
60 DAYS POST INFECTION
WITH DENGUE-2

UNTREATED



MERCAPTO-
ETHANOL
TREATED



**EFFECT OF MERCAPTO-ETHANOL ON HI ANTIBODY
IN SUCROSE DENSITY GRADIENT FRACTIONS.**

FIGURE 8

**MONKEY NO. A39
6 DAYS POST
INFECTION WITH
DENGUE -2**

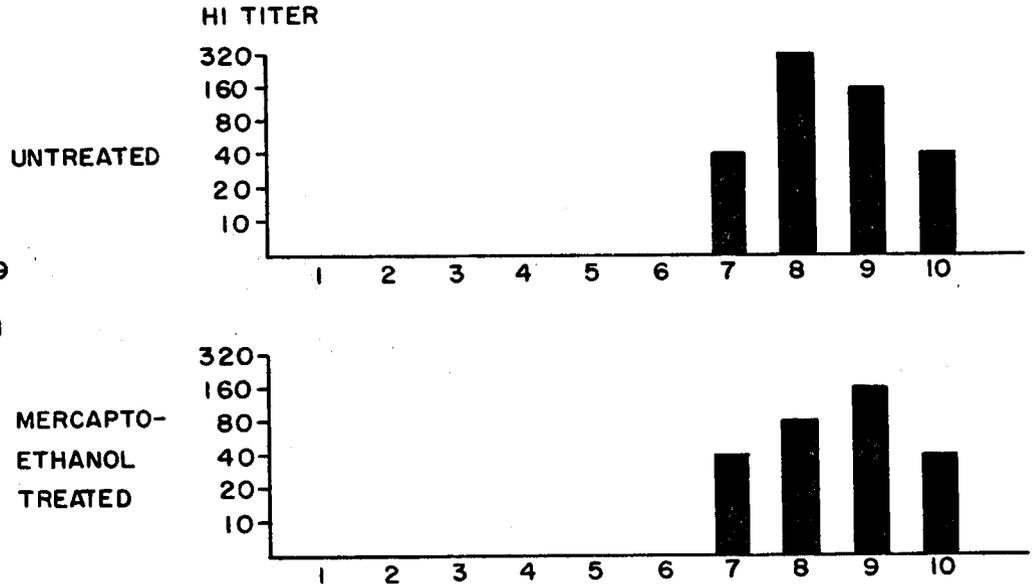
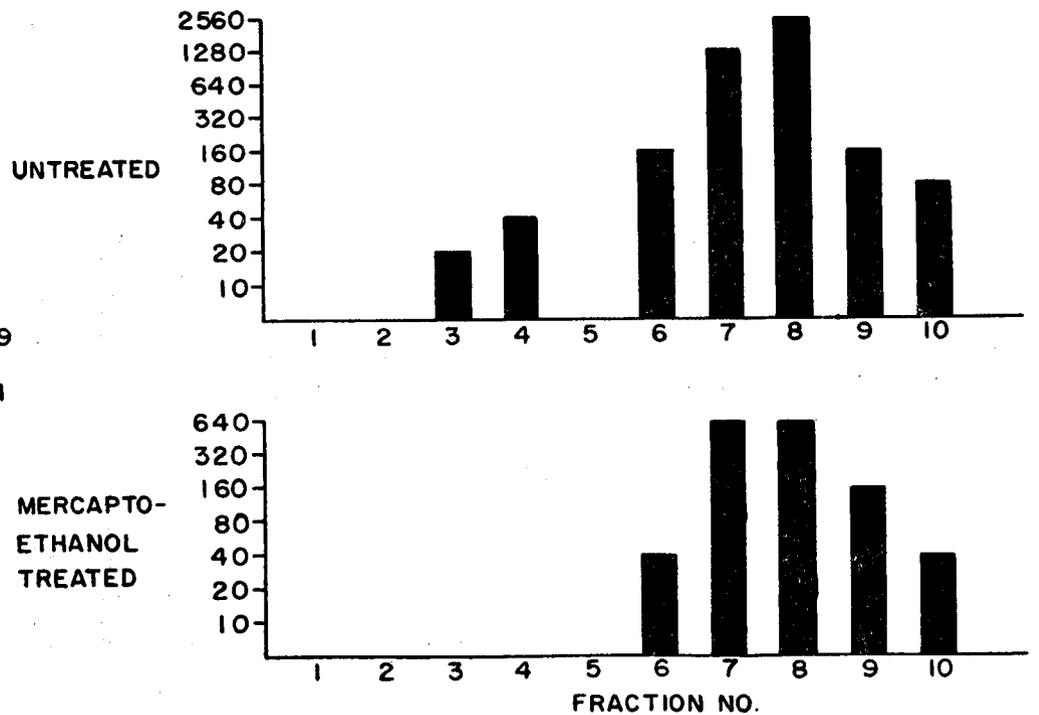


FIGURE 9

**MONKEY NO. A39
14 DAYS POST
INFECTION WITH
DENGUE -2**



EFFECT OF MERCAPTO-ETHANOL ON HI ANTIBODY IN SUCROSE DENSITY GRADIENT FRACTIONS.

28

FIGURE 10

MONKEY NO. A 39
60 DAYS POST
INFECTION WITH
DENGUE-2

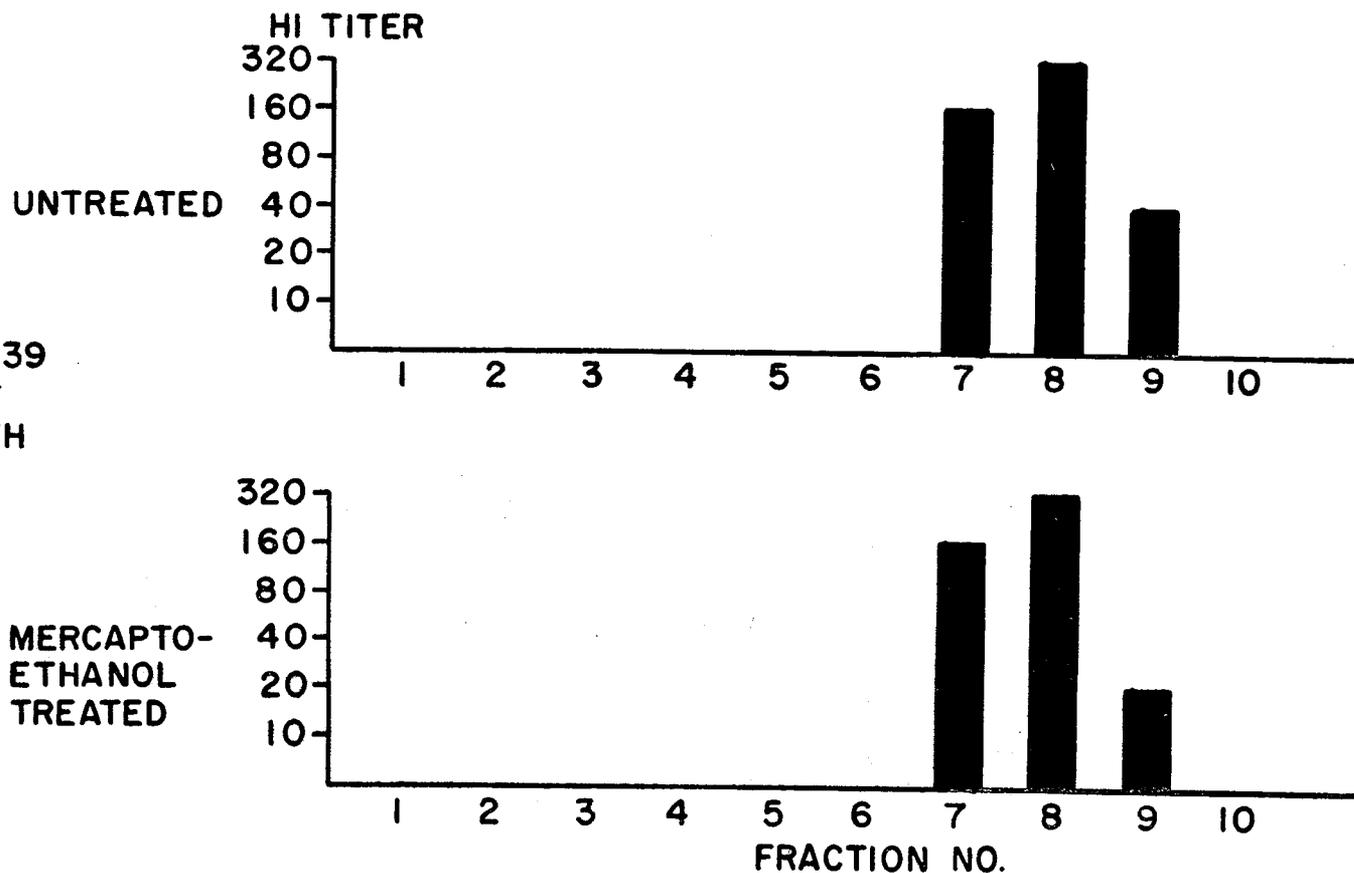


FIGURE II

**SEPHADEX G-200 FRACTIONATION OF SERUM
MONKEY NO. A-21, 14 DAYS AFTER DENGUE-2 INFECTION.**

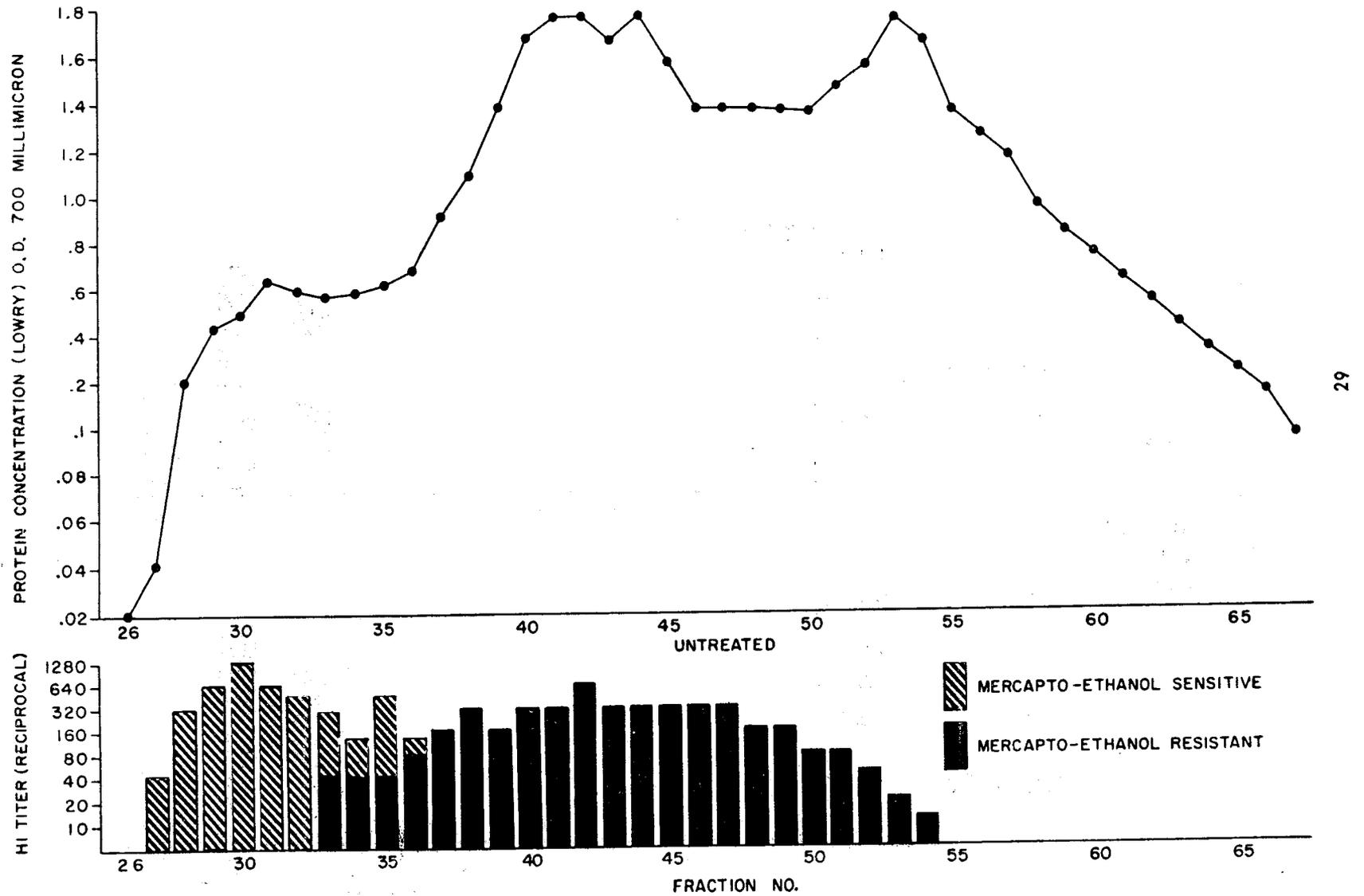


FIGURE 12

**SEPHADEX G-200 FRACTIONATION OF SERUM
MONKEY A-37, 14 DAYS AFTER DENGUE-2 INFECTION.**

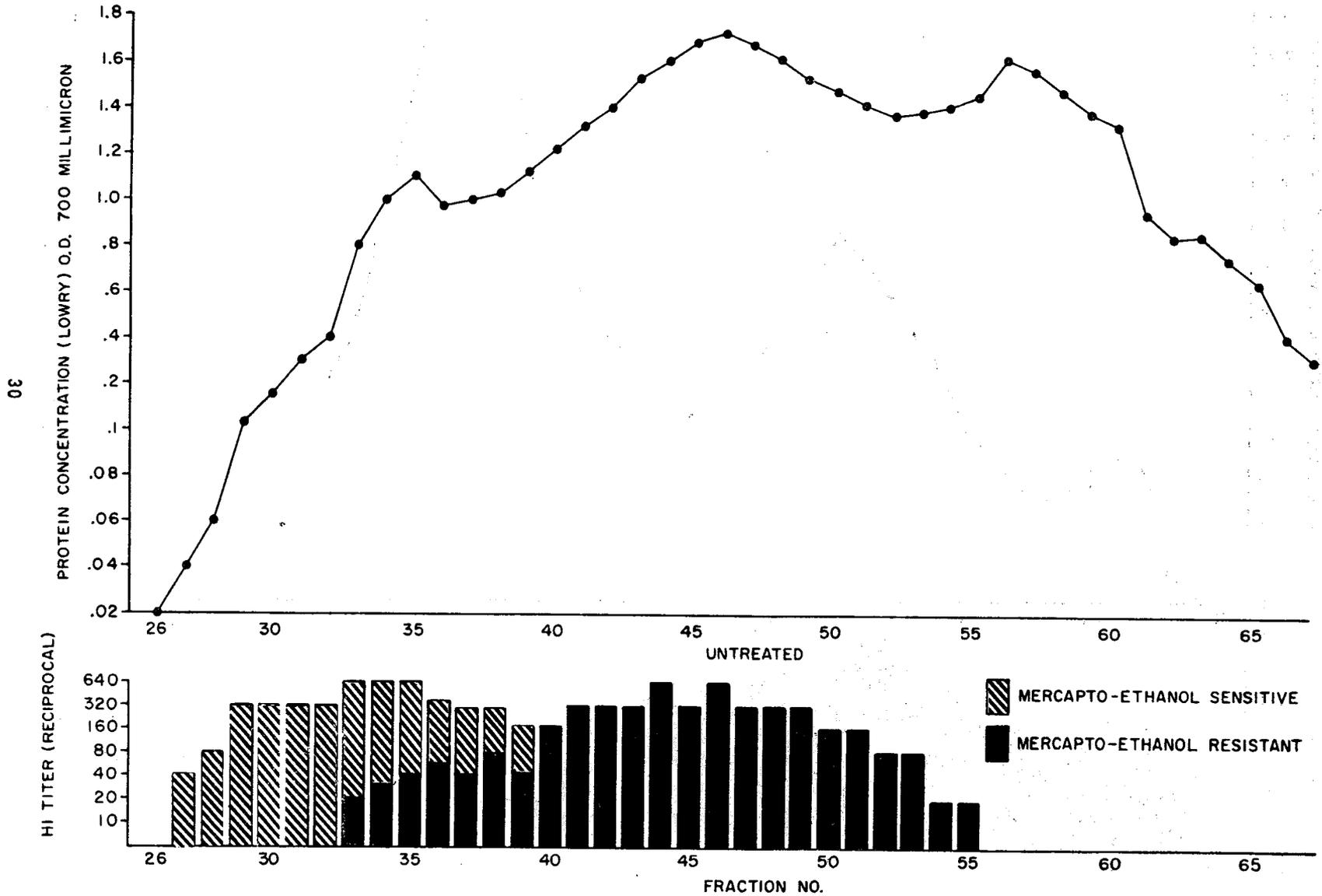


FIGURE 13

**SEPHADEX G-200 FRACTIONATION OF SERUM
MONKEY NO. A-39, 14 DAYS AFTER DENGUE-2 INFECTION**

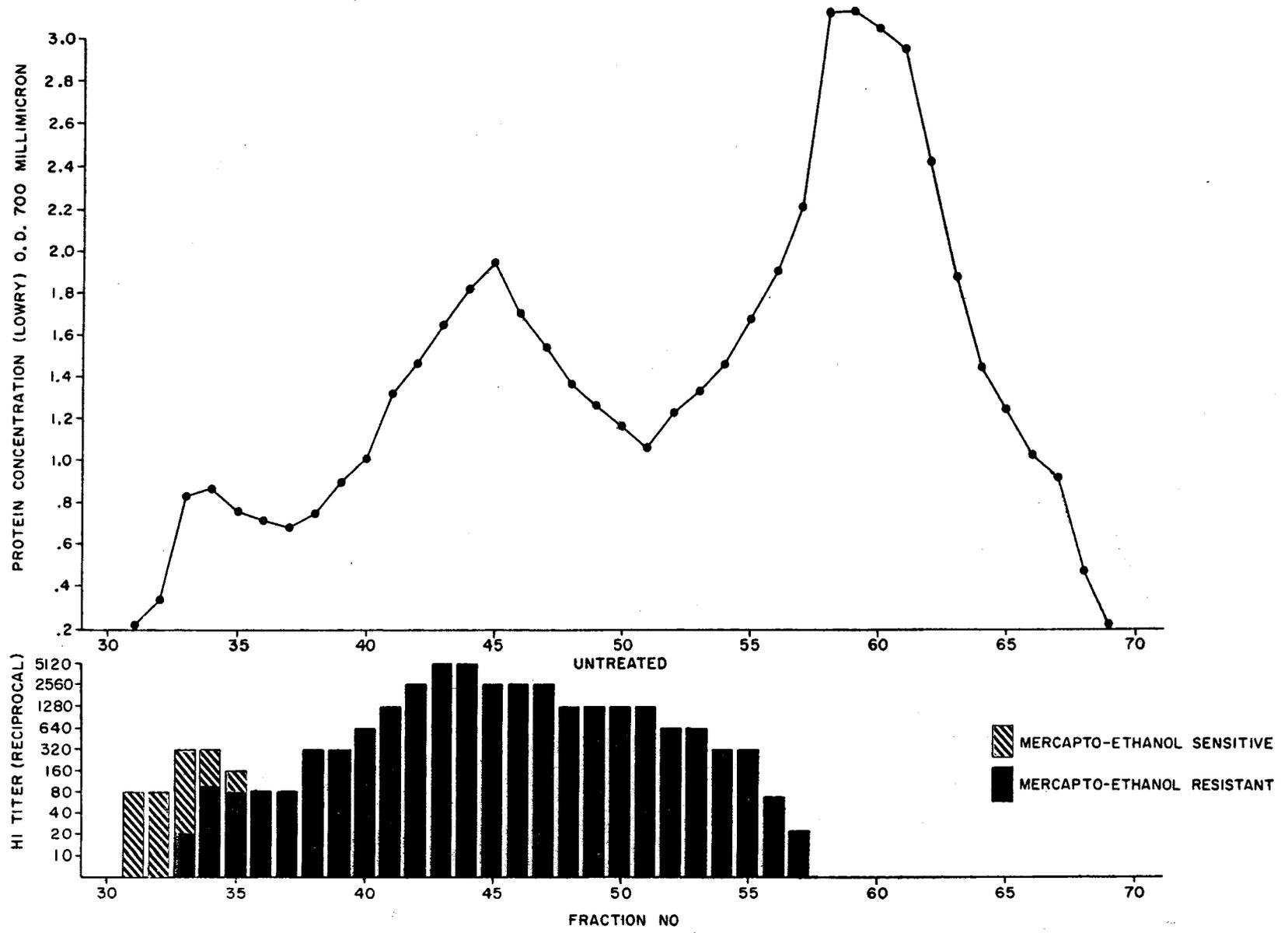


Table 8

Effect of Acetone and Kaolin Treatment on 19S and 7S HI Antibodies (1)

| Fraction ⁽²⁾ No. | Acetone treated | | Kaolin treated | |
|--------------------------------|------------------|------------|----------------|------------|
| | Control | ME treated | Control | ME treated |
| 1 | 0 ⁽³⁾ | 0 | 0 | 0 |
| 2 | 40 | 0 | 0 | 0 |
| 3 | 40 | 0 | 0 | 0 |
| 4 | 20 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 |
| 6 | 40 | 40 | 0 | 0 |
| 7 | 80 | 80 | 80 | 80 |
| 8 | 40 | 40 | 20 | 20 |
| 9 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 |

- (1) Monkey antiserum 14 days after dengue-2 infection
- (2) Sucrose density gradient fraction
- (3) Reciprocal HI titer vs 8 unit dengue-2 antigen 0=10

Density gradient fractionation has been done on 32 sera from 14 hemorrhagic fever cases. Results from 6 representative cases are shown in table 10. The first 3 cases represent "primary" dengue infections i. e. with no previous exposure to dengue. HI and CF antibody titers were low in these patients and no antibody was present in the acute serum. In these cases 19S HI antibody was detected in sera collected between the 3rd and 19th day of illness. The 19S antibody was reduced by 2-mercaptoethanol. A total of 6 cases were studied in which 19S antibody was present. In 2 cases, the 19S antibody was present on the 3rd and 10th day but was not detected in the 3rd serum specimen on day 17 or 18. In 4 cases, 19S antibody persisted until at least 18 days after onset of illness. In a single instance, 19S antibody was found on day 3, prior to the appearance of 7S antibody. In all other sera containing 19S HI antibody, 7S HI antibody was also present.

The last 3 cases in table 10 represent "secondary" dengue infections and in these cases no 19S HI antibody was detected. The 7S HI titers were consistently higher than those seen in the primary infections. Eight cases were studied in which very high HI and CF titers in whole serum indicated a secondary response. No. 19S HI antibody was detected in any of the 8 cases.

Neutralizing antibody was detectable in the 19S fractions when HI titers were greater than 1:40. N antibody in 19S fractions was low titered (1:10 to 1:50) and type specific. The apparent lack of sensitivity of the PRNT compared to the HI test was due to dilution factors and the larger serum volumes required for the PRNT.

Virus Isolation Methods: The challenge virus resistance (CVR) method in BS-C-1 cells has been shown previously to be more sensitive than suckling mice for some strains of dengue virus and at least equally sensitive for other strains. Recent attempts at isolation of Japanese encephalitis (JE) virus from sutopsy specimens done simultaneously in BS-C-1 cells primary Hamster kidney cells, and one day old mice indicate that the BS-C-1 system is superior. Results from two cases are summarized in table II. The validity of each of these isolations was confirmed by reisolation. Suckling mouse isolation attempts were carried through 2 blind passages. CVR in BS-C-1 cells became positive on the first or second passage.

Comparative titration of low passage JE and Chikungunya seed viruses (table 12) confirm the high sensitivity of BS-C-1 cells to JE virus and provide satisfactory evidence that this single system is highly satisfactory for isolation of the arboviruses known to cause human illness in Thailand.

Table 9. Dengue Neutralizing Antibody in Human Serum

| <u>Resum No.</u> | <u>Day of Virus</u> | | <u>HI⁽¹⁾</u> | <u>Neutralizing</u> | | <u>Antibody⁽²⁾</u> | | <u>Previous Exposure</u> |
|------------------|---------------------|----------------------|-------------------------|---------------------|------------|-------------------------------|------------|--------------------------|
| | <u>Disease</u> | <u>Isolated</u> | <u>titer</u> | <u>D-1</u> | <u>D-2</u> | <u>D-3</u> | <u>D-4</u> | |
| 10109 | 2 | D-2 | 0 | < 10 | < 40 | < 10 | < 10 | |
| 10110 | 23 | | 20,408 | 400 | 600 | 50 | 30 | YF, ? JE |
| 13212 | 4 | D-1 | 0 | < 10 | < 10 | < 10 | < 10 | none |
| 13818 | 26 | | 320 | 150 | 40 | < 10 | < 10 | |
| 16327 | 4 | D-1 | 80 | 160 | 70 | < 10 | < 10 | |
| 17020 | 19 | | 20,480 | 8,500 | > 2,560 | 500 | 4 | dengue |
| 16495 | 18 | (D-2) ⁽³⁾ | 80 | 25 | 650 | 210 | 25 | none |
| 16928 | 17 | (D-2) | 640 | > 640 | ≥ 2,560 | ≥ 2,560 | 60 | dengue |
| 16929 | 15 | (D-2) | 2,560 | > 640 | 2,200 | 100 | 75 | dengue |
| 10246 | 17 | (D-1) | 80 | 160 | 70 | < 10 | < 10 | none |

(1) reciprocal titer vs 8 units dengue 1 antigen

(2) reciprocal of 5% plaque reduction titer vs indicated prototype virus

(3) viruses () isolated from acute serum

Table 10

Dengue HI Antibody Titers of Sucrose Density Gradient Fractions of Serum from Hemorrhagic Fever Cases.

| Case No. | Dry of disease | HI titer ⁽¹⁾ | Fraction No. | | | | | | | | | | |
|----------|----------------|-------------------------|--------------|----|-----|----|----|------|------|------|-----|----|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| HFI-363 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 3 | 160 | 0 | 10 | 40 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 18 | 640 | 0 | 0 | 0 | 0 | 0 | 0 | 40 | 40 | 0 | 0 | 0 |
| HFI-547 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 10 | 160 | 0 | 0 | 20 | 80 | 20 | 160 | 320 | 80 | 40 | 0 | 0 |
| | 17 | 640 | 0 | 0 | 0 | 0 | 20 | 320 | 320 | 80 | 10 | 20 | 0 |
| HFI-735 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 10 | 320 | 40 | 80 | 160 | 40 | 0 | 20 | 80 | 80 | 40 | 0 | 0 |
| | 19 | 640 | 0 | 40 | 80 | 10 | 0 | 40 | 160 | 160 | 40 | 10 | 0 |
| HFI-555 | 4 | 80 | 0 | 0 | 0 | 0 | 0 | 20 | 20 | 0 | 0 | 0 | 0 |
| | 12 | 5120 | 0 | 0 | 0 | 0 | 40 | 1280 | 1280 | 160 | 40 | 0 | 0 |
| TH-415 | 3 | 2560 | 0 | 0 | 0 | 0 | 0 | 0 | 80 | 0 | 0 | 0 | 0 |
| | 10 | 20480 | 0 | 0 | 0 | 0 | 20 | 320 | 640 | 1280 | 160 | 40 | 0 |
| HFI-165 | 3 | 160 | 0 | 0 | 0 | 0 | 0 | 40 | 20 | 0 | 0 | 0 | 0 |
| | 16 | 10240 | 0 | 0 | 0 | 0 | 20 | 160 | 640 | 80 | 20 | 20 | 0 |

(1) titer of whole serum

(2) reciprocal of titer, 0 = <10

Table 11. Virus Isolation from Autopsy Specimens

| <u>Case No.</u> | <u>Specimen</u> | <u>BS-C-1</u> | <u>Isolation Results</u> <u>HK cells</u> | <u>Suckling mice</u> |
|-----------------|-------------------|---------------|---|----------------------|
| CAU 507 | brain (frontal) | JEV | negative | negative |
| " " | brain (parietal) | JEV | " | " |
| " " | brain (occipital) | JEV | " | " |
| " " | heart | negative | " | " |
| TH 2022 | CSF | negative | not done | " |
| " " | brain | " | " " | " |
| " " | brain | JEV | " " | " |
| " " | brain | negative | " " | " |

Table 12. Titrations of low passage, tissue culture strains of JE and Chikungunya viruses

| <u>Host</u> | <u>JE⁽¹⁾</u> | <u>JE⁽²⁾</u> | <u>Chik⁽³⁾</u> |
|----------------|-------------------------|-------------------------|---------------------------|
| Suckling mice | 4.7 ⁽⁴⁾ | 4.0 | 5.5 |
| BS-C-1 (CPE) | — | — | 6.3 |
| BS-C-1 (CVR) | 5.0 | 5.2 | — |
| LLC MK-2 | — | — | 4.0 |
| Hamster Kidney | — | 4.2 | 5.7 |
| Hela | — | 0 | 4.5 |

(1) No. 20872

3rd BS-C-1 passge.

(2) No. 20875

" " "

(3) No. 12908

1st " "

(4) negative log₁₀/0.1 ml

Sensitivity of LLC-MK-2 Cells to Arboviruses. The sensitivity of LLC-MK-2 cells to several arboviruses was tested using a plaque count method and tube cultures. Medium used in tube cultured was M199 with 5% calf serum and antibiotics. Medium for plaque bottles was BME with 5% calf serum, antibiotics and 1% Noble agar with DEAE dextran.

Titration of several standard strains of arboviruses are tabulated below (table 8) and compared with suckling mouse titrations of the same seed.

Table 8.

Comparative Titrations of Mouse Adapted Arboviruses in Mice and LLC MK-2 Tissue Culture

| <u>Virus</u> | <u>Suckling Mouse LD₅₀</u> | <u>Virus Titer⁽¹⁾ TCD₅₀</u> | <u>PFU</u> | <u>Plaque size (mm.)</u> |
|--------------------------|---------------------------------------|---|------------|--------------------------|
| JE (sm 14) (Nakayama) | 9.5 | 7.5 | 8.6 | 2.0 |
| Chik (sm 182) (Ross) | 8.7 | 7.5 | 7.3 | 2.5 |
| Sindbis | 9.5 | 7.3 | 7.3 | 2.0 |
| Akabane (sm 19) | 9.5 | 6.5 | 7.3 | 2.0 |
| TP 21 | 7.5 | No CPE | 8.1 | 2.5 |
| Dengue 1 (Haw., sm 70) | 7.5 | partial CPE | 6.7 | 1.5 |
| Dengue 2 (N.G.C., sm 26) | 8.1 | " | 6.9 | 2.0 |
| Dengue 3 (H-87, sm 21) | 7.5 | " | 6.5 | 3.0 |
| Dengue 4 (H-241, sm 25) | 6.3 | " | 9.3 | 2.5 |

(1) Negative logarithm per 0.1 gm of brain

Viral Central Nervous System Disease. Diagnostic studies have confirmed the etiology of 11 cases of encephalitis. One case was due to herpesvirus, one due to chikungunya virus and the remaining 9 cases due to JE. Two of the JE cases were diagnosed by isolation of virus from brain tissue at autopsy. One case was from Korat, the other from Phanomsarakam. A presumptive diagnosis of JE by serology was made in an additional 5 cases, but, because of inadequate specimens, the diagnosis in these cases is questionable.

The serologic studies on 8 JE cases are presented in table 14. In Thailand where cross reactions with other B group arboviruses makes serologic diagnosis of JE infection a difficult problem, the plaque reduction neutralization test is a useful adjunct, providing an efficient and accurate method of measuring specific antibody.

Chikungunya virus was isolated from a fatal case of encephalitis and pneumonitis in a 1½ year old girl. The patient had died after 2 days of high fever. Clinical manifestation included convulsion, coma and respiratory distress. Cerebrospinal fluid showed pleocytosis. Gross pathological study revealed brain swelling, edema of the meninges and congestion of the lungs. Histopathologic study showed encephalitis with neuronal necrosis, and cellular infiltration as well as interstitial pneumonitis.

Chikungunya virus, antigenically identical to other Thailand strains was isolated from brain and lung tissue. Virus titers measured by plaque method in LLC-MK-2 cell culture were 7×10^3 pfu and 3×10^4 pfu per gram of tissue for brain and lung respectively.

Table 14. Serologic Studies on Japanese Encephalitis Cases.

| Case No. | Location | | JE Serology | | |
|----------|---------------|-------|-------------|-----|------|
| | | | HI | CF | N |
| CAU-504 | Phanomsarakam | acute | 80 | 0 | 3 |
| | | conv. | 640 | 32 | 200 |
| TH-2088 | Chiengmai | acute | 80 | 0 | 640 |
| | | conv. | 320 | 8 | 640 |
| TH-2089 | Chiengmai | acute | 40 | 0 | 15 |
| | | conv. | 320 | 0 | 200 |
| TH-2090 | Chiengmai | acute | 1,280 | 16 | 500 |
| | | conv. | 1,280 | 32 | 4000 |
| TH-2091 | Chiengmai | acute | 0 | 0 | 10 |
| | | conv. | 80 | 0 | 700 |
| TH-2177 | Korat | acute | 320 | 4 | 90 |
| | | conv. | 320 | 256 | 500 |
| TH-2234 | Bangkok | acute | 0 | 0 | ND |
| | | conv. | 320 | 8 | ND |
| TH-2301 | Chachoengsao | acute | 320 | 4 | 300 |
| | | conv. | 320 | 16 | 300 |

Chikungunya Autointerference. Previous studies at WRAIR and in this laboratory established the occurrence of a marked autointerference effect with certain strains of chikungunya virus.

Extensive investigation in both laboratories showed that several low mouse passage Bangkok strains produce autointerference (AI) in both suckling mice and BS-C-1 tissue culture. High mouse passage African strains produce AI in BS-C-1 cells but not in mice. The most marked AI is produced by suckling mouse brain seed virus harvested late in the course of the infection i.e. at 48 to 72 hrs, when the mice are moribund. Seed virus harvested 24 hours after infection may have the same infectivity titer (approx. $10^{7.5}$) but no AI is produced.

The substance producing the AI has the following characteristics:

- a. Sediments at 100,000 xg
- b. Trypsin resistant
- c. RNA-ase resistant
- d. Destroyed at pH-2
- e. Destroyed by ether

Thus, this substance is not interferon and is a large particle with physico-chemical characteristics similar to the infectious virus.

The mechanism of the autointerference is as yet unknown but recent experiments indicate that the autointerfering particle (AIP) acts by rapid induction of interferon production. Table 15 shows the relationship of virus production, interferon production and cytopathic effect (CPE) following infection of BS-C-1 cell cultures with chikungunya virus (Ross strain smp. 180) at varying dilutions.

Table 15. Results of Infection of BS-C-1 Cells with Chikungunya Virus.*

| Dilution of inoculum | Time of CPE (4+) | day harvested | virus titer | interferon titer |
|----------------------|------------------|---------------|-------------|------------------|
| 10^{-1} | 2 days | 2 | $10^{5.5}$ | 1:32 |
| 10^{-3} | 7 days | 3 | $10^{5.0}$ | 1:16 |
| 10^{-5} | 3 days | 3 | $10^{7.5}$ | 1:2 |

* Infectivity titer in BS-C-1 cells= $10^{7.5}$ TCD₅₀/0.1 ml

From these results it appears that at the 10^{-1} dilution, a large amount of interferon was produced and virus production moderately suppressed but the overwhelming virus dose (virus-cell multiplicity of 10) caused early CPE. At the 10^{-3} dilution, interferon was produced in large amounts, virus production suppressed, and CPE markedly delayed. At the 10^{-5} dilution the autointerfering particles were diluted out and very little interferon produced; large amounts of infectious virus were produced and CPE was complete on the third day. The above experiment was done three times with essentially identical results.

It appears that the effect on the cell morphology and the amounts of infectious virus and interferon produced, depends on the relative amounts of infectious virus and autointerfering particles in the inoculum. The interaction of these variables is highly complex and further experiments are in progress to test the hypothesis that rapid induction of endogenous interferon production is the mechanism by which the non-infectious autointerfering particle acts to produce autointerference.

Separation of the infectious virus from the AI particle was attempted by density gradient centrifugation using both sucrose and cesium chloride gradients. The results (summarized in table 16 and 17) are not conclusive, but do tend to indicate that the infectious virus is heavier than the AI particles. In both systems the peak virus activity was demonstrated in lower fractions than the peak AI activity. In addition, in those fractions in which the infectivity titer was low and the AI titer remained high, the AI observed was complete, showing complete suppression at all dilutions below the end point. This is in contrast to the uncentrifuged material where the AI is not seen at the 10^{-1} dilution and CPE is not completely suppressed but merely delayed.

Further attempts to attain complete separation of the AIP from infectious virus will be made using improved centrifugation and fraction collecting techniques.

Table 16. Cesium Chloride Density Gradient Centrifugation⁽¹⁾ of Chikungunya Seed Virus

| <u>Fraction Number</u> | <u>Infectivity Titer⁽³⁾</u> | <u>Autointerference Titer⁽⁴⁾</u> |
|------------------------|--|---|
| 1 (bottom) | 2.0 | 0 |
| 2 | 4.5 | 0 |
| 3 | 6.0 | 1.0 |
| 4 | 6.5 | 2.0 |
| 5 | 7.0 | 2.0 |
| 6 | 5.5 | 2.0 |
| 7 | 5.5 | 3.0 |
| 8 | 6.0 | 3.0 |
| 9 | 6.0 | 3.0 |
| 10 (top) | 6.0 | 3.0 |

(1) 100,000 x g for 42 hours

(2) Ross strain smp 180, Infectivity $10^{7.0}$ TCD₅₀, AI titer $10^{3.0}$

(3) Negative log of TCD₅₀/0.1 ml

(4) Negative log of highest dilution showing complete suppression of CPE

Table 17. Sucrose Density Gradient Centrifugation⁽¹⁾ of Chikungunya Seed Virus⁽²⁾

| <u>Fraction Number</u> | <u>Infectivity Titer⁽³⁾</u> | <u>Autointerference Titer⁽⁴⁾</u> |
|------------------------|--|---|
| 1 (bottom) | 4.5 | 0 |
| 2 | 6.0 | 0 |
| 3 | 6.5 | 0 |
| 4 | 5.0 | 0 |
| 5 | 6.0 | 1.0 |
| 6 | 5.5 | 2.0 |
| 7 | 5.5 | 2.0 |
| 8 | 5.5 | 2.0 |
| 9 | 6.0 | 0 |
| 10 (top) | 6.0 | 0 |

(1) 10 to 40% sucrose gradient, 100,000 x g for 18 hrs

(2) Ross strain smp 180, Infectivity $10^{8.0}$, TCID₅₀, AI titer $10^{3.0}$

(3) Negative log of TCD₅₀/0.1 ml

(4) Negative log of highest dilution showing complete suppression of CPE

Summary :

The technique used for plaque reduction neutralization tests with dengue viruses is described. A study on antigenic classification of dengue viruses using monkey antisera and the PRNT is reported. This method is useful for typing newly isolated dengue virus strains and appears superior to previous methods.

The immunoglobulin response of monkeys to experimental infection with dengue-2 virus was studied. Subcutaneous injection of live virus produces antibodies of the Ig-M and Ig-G types. Ig-M antibody has both HI and N activity, it appears within two weeks after inoculation and disappears within two months. Ig-G HI and N antibody persisted for the duration of the study. In one animal with evidence of previous exposure to a dengue virus the earliest detectable antibody rise consisted of Ig-G antibody and the Ig-M antibody response was of smaller magnitude than in the animals experiencing a first antigenic exposure to dengue virus.

Neutralizing antibody following natural dengue infection in man was measured by the PRNT. Following a primary infection the neutralizing antibody was relatively specific. In patients with previous exposure to dengue virus (es) the N antibody titers reached very high titers and broad cross reactions were observed. Serum fractionation studies revealed a 19S antibody (HI and N) following primary dengue infections, however, in those cases studied which had a secondary type antibody response no 19S antibody was detected.

Studies on monkey antiserum indicated that kaolin extraction of serum to remove non-specific inhibitory substances prior to HI testing also removes the 19S antibody activity.

The challenge virus resistance method using BS-C-1 cell culture has been found to be more sensitive than suckling mice for the primary isolation of JE virus from autopsy material.

Japanese encephalitis cases from Bangkok, central, northern, and northeastern Thailand were diagnosed. JE virus was isolated from brain tissue in two fatal cases. Chikungunya virus was isolated from brain and lung tissue of a fatal case of encephalitis.

Studies on autointerference of chikungunya virus in tissue culture are reported. Evidence is presented that suggests interferon production is associated with the mechanism of autointerference. Ultracentrifugation studies indicate that a non-infectious particle slightly less dense than infectious virus causes autointerference.