

HEMAGGLUTINATION WITH ARTHROPOD-BORNE VIRUSES*

BY JORDI CASALS, M.D., AND LENORA V. BROWN,† M.D.

(From the Laboratories of the Division of Medicine and Public Health, The Rockefeller Foundation, New York)

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In the course of a study conducted by investigators in the Division of Medicine and Public Health of The Rockefeller Foundation on the serological relationships and the epidemiology of certain arthropod-borne virus infections, it was thought that application of the hemagglutination (HA) test might be useful in elucidating some of the problems.

Specific hemagglutination (HA) with certain neurotropic viruses was first reported by Hallauer (1), Lahelle and Horsfall (2), and Olitsky and Yager (3). In the arthropod-borne group, Hallauer (4) stated that he had obtained an agglutinating antigen from viscerotropic but not from neurotropic strains of yellow fever virus. It was, however, Sabin and his associates (5-10) who showed the existence of HA with arthropod-borne viruses generally, among them dengue Types 1 and 2, Japanese B, St. Louis, Russian Far Eastern, Uganda S, West Nile, and Western equine encephalitis (WEE). Chanock and Sabin (8-10) described in detail the optimal conditions for carrying out the tests, emphasizing the proper choice of erythrocytes as to both species and age of the donor animals, and a satisfactory method for removing non-specific inhibitory substances from sera. Furthermore, they showed the existence of cross-reactions among different viruses and their antisera. MacDonald (11), using newborn-mouse brain as the source of material, later prepared a hemagglutinin from Murray Valley encephalitis virus.

In a previous communication by the present authors (12), a new method was described for the preparation of hemagglutinins for chick erythrocytes with the following viruses: dengue Types 1 and 2, Ilhéus, Japanese B, Ntaya, West Nile, and yellow fever.

It is the purpose of the present paper: (a) to describe an extension of the results of the earlier work to other viruses in this group; (b) to show that with the methods developed, two types of hemagglutinins have been observed; and (c) to note that on the basis of the behavior of their agglutinins, as well as from the results of HA inhibition tests, a number of arthropod-borne viruses

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can be classified into two sharply different groups, designated A and B. Group A consists of Eastern equine encephalitis (EEE), Semliki Forest, Sindbis, Venezuelan equine encephalitis (VEE), and WEE viruses; Group B includes dengue Types 1 and 2, Ilhéus, Japanese B, louping ill, Ntaya, St. Louis, Russian Far Eastern, Uganda S, West Nile, and yellow fever viruses.

Materials and Methods

Viruses.—The viruses tested for their capacity to show HA are listed in Table I. Some of the viruses are not of the arthropod-borne group; however, they were included in this

TABLE I
Viruses Tested for Capacity to Yield Hemagglutinins

| Virus and reference to source | Strain | No. of passages in mice | | Virus and reference to source | Strain | No. of passages in mice | |
|-------------------------------|-----------------------|-------------------------|---------|-------------------------------|--------------------|-------------------------|---------|
| | | Adult | Newborn | | | Adult | Newborn |
| Anopheles A (13) | | 166 | 2 | Sindbis (25) | AR 339 | 0 | 10 |
| California (14) | BFS-283 | ? | 5 | Uganda S (26) | | 21 | 2 |
| Dengue Type 1 (15) | Hawaiian | 75 | 2-6 | VEE (27) | | ? | 2-4 |
| Dengue Type 2 (16) | New Guinea B | 0 | 46 | West Nile (28) | B 956 | 30 | 1 |
| EEE (17) | Rockefeller Institute | Over 200 | 2-6 | West Nile (29) | Egypt 101 | 0* | 5-8 |
| Ilhéus (18) | | 25 | 2-6 | WEE (30) | McMillan | 6-10 | 6-8 |
| Japanese B (19) | Nakayama | 100-200 | 2-10 | WEE (31) | North Dakota | 1-2* | 1-2 |
| Ntaya (20) | | 10* | 4-6 | Wyeomyia (13) | | 204 | 1-2 |
| Poliomyelitis Type 2 (21) | MEF1 | 20 | 140 | Yellow fever (32) | Asibi | 0* | 2-4 |
| Rabies (22) | Pasteur | 199* | 2 | Yellow fever (33) | French neurotropic | 260 | 1-5 |
| Russian Far Eastern (23) | | 10-20* | 1 | Yellow fever (34) | JSS | 5-10 | 1-5 |
| St. Louis (8) | Parton | 21 | 2-4 | Yellow fever (34) | Ogbomosho | 5-10 | 1-5 |
| Semliki Forest (24) | | 7 | 2-4 | Yellow fever (35) | 17 D | 0* | 1-5 |

* For passages in other animal species, see text.

study to investigate the possibility of the methods applying to other agents, and also to serve as a control.

Table I gives ascertainable data concerning the number of passages in mice for each virus. From the time of their isolation or shortly thereafter, the viruses for the most part had been maintained in adult mice in this or other laboratories. Exceptions were dengue Type 2, Sindbis, and the Egypt 101 strain of West Nile, all of which had no passages in adult mice. Ntaya after propagation in adult mice was passed through chick embryos 7 times, then through newborn mice. Rabies virus had been maintained in rabbits for about 1,500 passages when received in this laboratory, at which time adult mouse passages were initiated. The passages reported for Russian Far Eastern virus are those known to have taken place in this country; the previous history of this virus is not known. The Egypt 101 strain of West Nile virus was propagated in chick embryos for 5 passages. The North Dakota strain of WEE had been through 11 passages in guinea pigs prior to injection into mice. Of the yellow fever

strains, Asibi had been maintained in monkeys for an undetermined number of passages and 17 D had been propagated in chick embryo tissue culture. The passages in newborn mice as they appear in Table I were in addition to the previous passages in adult mice or other species, or in tissue cultures.

All the viruses could be readily passaged in newborn mice with the exceptions of the dengue viruses (15, 16) and poliomyelitis Type 2 (21). Antigens for the HA tests were prepared with central nervous system (CNS) tissue of the newborn-mouse passages.

Mice.—The newborn mice used in preparation of the HA antigens were raised in this laboratory and belonged to a strain of Swiss mice free of Theiler's virus. As a rule, mice were inoculated when 3 or 4 days old. However, with viruses such as EEE and WEE which have a short incubation period, the animals were injected when 7 or 8 days old, and with viruses having a longer period, when 1 or 2 days old. The procedure for harvesting CNS tissue has been described (36). It is to be noted here that, in general, the HA antigens used in these tests were not prepared from freshly harvested tissue, but rather from tissue that had been held at -20°C . for as much as several weeks.

Preparation and Preservation of Antigens for HA Tests.—The method used in preparing the extracts to be employed in the HA tests—referred to hereafter as antigens—is the one used for the preparation of complement-fixing antigens (36); in fact, the same preparations were used interchangeably. Essentially, the procedure comprised extracting the infected CNS tissue twice with 20 volumes of acetone, once with a mixture of equal parts of acetone and ether, and twice again with 20 volumes of ether. After evaporation of the ether, the extracted tissue was resuspended in physiological saline in a proportion of 1 gm. of the original tissue and 2 ml. of saline; the supernatant fluid, after centrifugation at about 9,000 *g* for one hour, constituted the antigen. The antigen thus represented a 33 per cent suspension of CNS tissue in saline; in this paper, the titers of hemagglutinins are expressed in terms of dilution of this 33 per cent suspension—designated undiluted antigen—not in terms of dilution of CNS tissue. It is to be noted also that no buffering system was added to the antigens while they were being prepared or upon completion; their pH was between 7.2 and 7.5.

Antigens are now preserved routinely at -20°C . in lyophilized form. Under these conditions the titer has remained unchanged for months; lyophilization itself may occasionally result in a moderate loss of titer. This mode of preservation has not as yet been tried with Uganda S and yellow fever antigens. Antigens have also been preserved at -20°C . in the frozen state with no appreciable loss of titer when so kept for several months; a given sample was thawed not more than 3 or 4 times, since repeated freezing and thawing could result in a loss of titer.

Sera.—The sera used in the present study were obtained from mice, guinea pigs, and monkeys (*Macacus rhesus*). The number of injections of formalized or active virus given to an animal was, for mice, 5 to 7; this constituted a hyperimmune serum. Guinea pigs received fewer injections, either 1 or from 3 to 4; their sera were designated immune sera. Monkeys, on the other hand, were given only 1 injection of active virus, which resulted in either febrile response or circulating virus or both, followed by recovery; these sera were therefore marked as convalescent. Thus it was considered that with the different sera—hyperimmune, immune, and convalescent—it might be possible to detect any serological crossings as well as viral specificity.

Mouse sera were prepared against 22 viruses or strains, following a course of vaccination and bleeding to which close attention was paid in order to have comparable results. Groups of 60 mice, 5 to 7 weeks old, were immunized as follows: all injections were 0.2 ml. in volume, given intraperitoneally, and consisted of a 10 per cent suspension of infected newborn-mouse CNS tissue. The first 2 injections given on the 1st and 3rd days respectively were of virus to which formalin in final concentration of 0.5 per cent had been added, and which had been

kept at 4°C. for 1 week at least. The remaining injections, given on the 10th, 15th, and 20th days, were of active virus; the mice were bled from the heart under ether anesthesia on the 28th or 30th day. Often on the 45th day a 6th injection of active virus was given, and the mice were rebled 1 week later. The serum pools were kept at -20°C. These sera were tested for and found to have complement-fixing antibodies in all cases, including those that failed to react in the HA inhibition system, as will be discussed later.

Some of the guinea pig immune sera were obtained through the courtesy of Dr. J. A. Kerr and were prepared as described by him (37), through repeated injections of virus. For Ilhéus, Japanese B, St. Louis, and Sindbis viruses, immune sera were obtained by bleeding animals that had received a single intracerebral injection of newborn-mouse CNS virus in dilution 10^{-1} (38). These animals failed to show any signs of illness, including fever, yet developed antibodies.

Monkey sera were derived from animals bled at intervals during the course of convalescence and recovery after an experimental infection; these sera were kept lyophilized at 4°C. or at -76°C. For each animal a series of sera was available, comprising a sample taken before infection and several taken at different times after infection, extending in some cases over a period of 1 year.

Diluents.—Glass-distilled water was used in all phases of the test, except for preparation of the antigens. As diluent for the chick erythrocytes, a 0.9 per cent solution of sodium chloride was used. The diluent for the antigens and sera was a 0.9 per cent solution of sodium chloride containing a phosphate buffer system 0.1 M designated "standard diluent." Actually 0.1 M solutions were prepared of Na_2HPO_4 and KH_2PO_4 in 0.9 per cent sodium chloride; by mixing adequate amounts of the two, the pH range from 6 to 8 was covered, determinations being made in a Beckman pH meter. The ionic strength of the standard diluent was therefore 0.3 N. As will be seen later, the final mixture in the tubes of a given test had an ionic strength of approximately 0.22 N; this concentration of electrolyte did not result in any apparent damage to the erythrocytes, as judged by adequate controls, yet it enhanced the sharpness and stability of the precipitating patterns or shields and, especially with the antigens derived from Group A, produced more clearly defined titration end-points and often higher titers. When phosphate buffer 0.02 M in physiological saline was used (9), the results of the tests were in close agreement with those secured using the more concentrated buffer although, in general, they were somewhat less well defined. When a pH lower than 6.0 was required, usually between 5.5 and 6.0, phthalate buffer (9) was used; the pH ranges below 6.0 and above 8.0 have not, however, been investigated extensively.

Preparation of Sera for HA Inhibition Tests.—A method was developed for removing non-specific inhibitors from sera to be used in the HA inhibition tests. The procedure, now routine, consists of filtering the sera through Seitz pads in the following manner: of a given serum, an amount ranging from 0.4 ml. to 0.8 ml. was diluted to 1:10 in standard diluent at pH 7.0. Two Seitz E-K pads were put in a filter casing and 10 ml. of the same diluent was passed through at 5 pounds positive pressure; when the fluid was exhausted, the diluted serum was introduced in the filter and allowed to go through slowly by using a positive pressure of 1 to 2 pounds. The filtered sera no longer retained non-specific inhibitors, and were stored at -20°C. until used. The sera thus treated had a pH about 7.0 and could therefore be used directly with those antigens which required this pH; *i.e.*, Group B. But with antigens requiring a pH 6.4, Group A, an adjustment was made by adding to the serum thus filtered a sufficient amount of a 10 per cent solution of HCl; this amount was found to be 0.03 ml./ml. of the diluted serum. In this laboratory filtration could be carried out simultaneously on 12 sera by means of a manifold pressure outlet. It was found that in the course of a regular work-day a person, while still otherwise engaged, could prepare 48 sera with a yield of 6 to 7 ml., which was a sufficient quantity for 12 to 14 titrations at an initial dilution of 1:10.

Erythrocytes.—The method described by Chanock and Sabin (8) was rigidly adhered to. One-day-old chicks were bled from the heart under ether anesthesia using Alsever's solution as a preservative as well as anticoagulant; the proportion of blood to Alsever's solution was 1 to 4. The blood was not used until after it had remained in the refrigerator at 4°C. for 48 hours; then it was used for the next 5 or 6 days. When needed, a sufficient amount of blood was centrifuged and the sedimented cells were washed 3 times and resuspended in physiological saline at a concentration of 0.25 per cent; of this suspension, cooled at 1°C., 0.5 ml. per tube was used in the tests.

The use of other erythrocytes, whether from older chicks or from other animal species, man or sheep, was not generally satisfactory and therefore abandoned.

Performance and Reading of Tests.—All tests were conducted in tubes measuring 75 by 12 mm., specially tooled so as to have a uniform inside bottom surface.

Titration of antigen. A new lot of antigen was titrated in the following manner: in each of a series of 12 to 16 tubes was measured 0.5 ml. of standard diluent at pH 6.4 or 7.0, depending on the virus; the tubes were placed in an ice-water bath at 1°C. and when chilled, the antigen was diluted in increasing twofold dilutions beginning at 1:4 or 1:8. Then 0.5 ml. of the red cell suspension was added to each tube; the tubes were shaken and put in a cold room at 4°C. for the viruses of Group B or in a water bath at 37°C. for those of Group A; for yellow fever antigens the incubation was done at room temperature (22°C.).

When investigating new viruses or strains for their ability to induce HA, titrations as just described were carried out at pH 5.6, 6.0, 6.4, 6.8, 7.0, and 7.6; for each pH a titration was set up in triplicate, one being incubated at 37°C., another at 22°C., and the third at 4°C. In all cases adequate control tubes were included, with erythrocyte suspension alone in the same final volume.

With the exceptions noted later, the pattern of agglutination was as illustrated by Chanock and Sabin (8); namely, positive agglutination consisted of a smooth deposit or shield of erythrocytes covering the entire bottom surface of the tube; a negative result was seen as a button of sedimented cells covering only the lowermost portion of the bottom. No agglutination was recorded as 0 and complete agglutination as ++; intermediate degrees were represented as + when there was a shield surrounded by a light ring of cells, and ± for a lesser degree of reaction. The reading of a test could be done in 2 hours when the incubation was at 4°C., sooner when incubated at 22°C. or 37°C. At any time thereafter during the next 24 hours or even longer, the pattern of agglutination remained practically unchanged. The titer of an antigen was given by the highest dilution showing a + agglutination or better; this amount of antigen represented 1 unit. In tubes containing the higher concentrations of antigen, 1:4 to 1:128, there was often a tendency toward slippage and folding over of the sheet of erythrocytes, as already observed (8); this did not interfere with the reading of the test.

With some preparations, particularly those derived from dengue viruses, the pattern of agglutination was at times of a coarse nature as though large clumps of red cells had fallen to the bottom of the tube. With viruses of Group A, the reaction was of a somewhat different type in that it consisted of both agglutination and hemolysis; ordinarily with the higher concentrations of high-titered antigens, hemolysis was complete, then became less marked as the amount of antigen decreased, and in the highest dilutions approaching the end-point, none was apparent. The reading of the test in the last case was done by observing and recording completeness of reaction, whether agglutination or hemolysis.

HA inhibition tests with serum: Tests to investigate serum inhibition of HA were carried out in the following manner: an ampule of lyophilized antigen was rehydrated, care being taken in this and all subsequent steps to hold the antigen, whether diluted or undiluted, at 1°C. Using standard diluent at pH 6.4 or 7.0, depending on the virus, a portion of the

antigen was brought to a dilution which from previous experience could be expected to yield between 8 and 16 units. This diluted antigen was then titrated twice to make up the preliminary titrations—once immediately and then 10 minutes later. While awaiting the result of the titrations, the sera to be tested were diluted in serial twofold dilutions in standard diluent at the same pH at which the antigen was being titrated. The first dilution of serum was usually 1:10, rarely 1:5; the number of dilutions was, as a rule, not fewer than 6 tubes per serum. The amount used in each dilution was 0.25 ml. Dilution of the sera was done at room temperature and when completed, the racks were placed in a cold room until the remaining reagents were added.

As soon as the titer of the antigen had been determined, the diluted sera were placed in an ice-water bath at 1°C.; red cell suspension was added (10) in amounts of 0.5 ml. per tube. Then from the preliminary titrations was deduced the dilution of antigen which in 0.25 ml. would contain 8 units; a sufficient amount was prepared from the same ampule of antigen used for the preliminary titrations. Of the diluted antigen 0.25 ml. was added to each tube, and the tubes were shaken and incubated at the required temperature. A sample of the diluted antigen that had been added to the tubes was then titrated; almost invariably its titer was found to be identical with that shown in the preliminary titrations. When the number of tubes in a test was greater than 150, the test was divided into 2 or more sections, each containing no more than that number of tubes, and each treated as a different test in that different batches of diluted antigen were prepared from the same original sample and each titrated at the end of its run. The entire test was then placed at either 4°C. or 37°C., or at 22°C. in the case of yellow fever antigens.

Serum inhibition of HA was also carried out in a similar manner, with a constant dilution of serum and increasing dilutions of antigen.

EXPERIMENTAL

Stability and General Properties of Hemagglutinating Antigens.—Extracts capable of agglutinating 1-day-old chick erythrocytes have been prepared from the following viruses: dengue Type 1, dengue Type 2, EEE, Ilhéus, Japanese B, Ntaya, St. Louis, Sindbis, Uganda S, VEE, West Nile (Egypt 101), WEE, and yellow fever (Asibi, French neurotropic, and JSS strains). Antigens deriving from dengue Type 2 virus have usually been poor, *i.e.* of low titer and giving a coarse pattern of HA; in serum inhibition tests the antigen was unsatisfactory and not used extensively. Antigens from dengue Type 1 were similarly unsatisfactory in 3 out of 8 different preparations. The remaining antigens have been easily prepared on repeated occasions, yielding clear-cut titrations and serum inhibition tests.

Antigens held frozen at $-20^{\circ}\text{C}.$, or lyophilized and kept at either -20° or $4^{\circ}\text{C}.$, showed no appreciable loss of titer for periods of time extending to 9 months, the longest period tested. If held at $4^{\circ}\text{C}.$ in undiluted fluid form, no loss of titer was noted in 48 hours with samples of West Nile (Egypt 101) and dengue Type 1. With none of the undiluted antigens was there any detectable loss of titer when kept for 2 hours at $1^{\circ}\text{C}.$

The keeping properties described above refer to the antigens in undiluted form; since high dilutions were often necessary for the tests it became desirable to examine their stability in diluted form and at different pH values.

TABLE II
Reaction and Stability of Hemagglutinating Antigens at Different pH Values

pH of antigen dilutions and time tested in hrs.

| Antigen* | pH of antigen dilutions and time tested in hrs. | | | | | | | | | | | | | | | | | | | |
|--------------------------|---|-------|-------|-------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|-------|
| | 6.4 | | | | 6.8 | | | | 7.0 | | | | 7.2 | | | | 7.6 | | | |
| | 0 | 1 | 2 | 4 | 0 | 1 | 2 | 4 | 0 | 1 | 2 | 4 | 0 | 1 | 2 | 4 | 0 | 1 | 2 | 4 |
| Dengue Type 1 | 4,096† | 512 | 256 | 256 | 4,096 | 2,048 | 2,048 | 2,048 | 1,024 | 1,024 | 1,024 | 2,048 | 1,024 | 1,024 | 1,024 | 2,048 | 0 | 0 | 0 | 0 |
| Dengue Type 2 | 32 | 0 | 0 | 0 | 512 | 64 | 64 | 16 | 256 | 32 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ithaus | 16,384 | 512 | 256 | 32 | 16,384 | 4,096 | 4,096 | 2,048 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 8,192 |
| Japanese B | 8,192 | 2,048 | 1,024 | 512 | 32,768 | 32,768 | 16,384 | 16,384 | 32,768 | 32,768 | 16,384 | 16,384 | 16,384 | 32,768 | 8,192 | 2,048 | 512 | 0 | 0 | 0 |
| Niaya | 512 | 64 | 0 | 0 | 1,024 | 512 | 512 | 128 | 2,048 | 1,024 | 512 | 512 | 2,048 | 1,024 | 1,024 | 512 | 2,048 | 2,048 | 2,048 | 1,024 |
| St. Louis | 4,096 | 2,048 | 2,048 | 1,024 | 16,384 | 8,192 | 8,192 | 8,192 | 16,384 | 8,192 | 8,192 | 8,192 | 16,384 | 16,384 | 8,192 | 16,384 | 8,192 | 8,192 | 8,192 | 8,192 |
| West Nile (Egypt 101) | 1,024 | 256 | 128 | 64 | 4,096 | 1,024 | 512 | 512 | 4,096 | 2,048 | 1,024 | 1,024 | 4,096 | 2,048 | 1,024 | 1,024 | 4,096 | 2,048 | 1,024 | 1,024 |

* Antigens maintained in 1:16 dilution.

† Throughout Tables II to V, the figures indicate the titer of antigen. Thus 4,096 means that the titer of antigen was 1:4,096; 512 means a titer of antigen of 1:512; etc. A zero indicates that there was no reaction in dilution 1:16.

In Table II are reported experiments in which each antigen was diluted to 1:16 in standard diluent at the indicated pH; this dilution, held at 1°C., was titrated after different periods of time. The diluent for the titration was in each case at the same pH as that of the initial 1:16 dilution. It can be seen that, for the antigens tested, the range of reaction extended from pH 6.4 to 7.2 and even 7.6; and that at pH 7.0, which may not necessarily be the optimum in terms of titer, all antigens with the exception of dengue Type 2 were active in high titers and stable for periods ranging from 1 to 4 hours.

TABLE III
Stability at pH 7.0 of Hemagglutinating Antigens in Dilutions

| Antigen | Dilution and time tested in hrs. | | | | | | | | | | | |
|--------------------------|----------------------------------|--------|--------|-------|--------|-------|-------|-------|--------|-------|-------|-------|
| | 1:16 | | | | 1:64 | | | | 1:256 | | | |
| | 0 | 1 | 2 | 4 | 0 | 1 | 2 | 4 | 0 | 1 | 2 | 4 |
| Dengue Type 1 | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 |
| Japanese B | 16,384 | 16,384 | 16,384 | 8,192 | 8,192 | 8,192 | 8,192 | 4,096 | 16,384 | 8,192 | 8,192 | 4,096 |
| Ntaya | 1,024 | 512 | 512 | 32 | 512 | 512 | 512 | <64 | 1,024 | 512 | 256 | 256 |
| St. Louis | 16,384 | 16,384 | 8,192 | 8,192 | 16,384 | 8,192 | 2,048 | 2,048 | 8,192 | 8,192 | 1,024 | 1,024 |
| West Nile (Egypt 101) | 2,048 | 2,048 | 1,024 | 512 | 2,048 | 1,024 | 512 | 128 | 2,048 | 1,024 | 512 | <256 |

TABLE IV
Stability of Hemagglutinating Antigens Held at High Dilutions for Short Intervals

| Antigen | pH | Reciprocal of antigen dilution | Min. | | |
|---------------|-----|--------------------------------|--------|--------|--------|
| | | | 0 | 5 | 10 |
| EEE | 6.4 | 500 | 4,000 | 4,000 | 4,000 |
| VEE | 6.4 | 400 | 12,800 | | 12,800 |
| WEE | 6.4 | 200 | 1,600 | | 1,600 |
| Dengue Type 1 | 7.0 | 128 | 4,096 | 4,096 | 4,096 |
| Dengue Type 2 | 7.0 | 30 | 240 | 240 | 120 |
| Ilhéus | 7.0 | 1,600 | 25,600 | 25,600 | 25,600 |
| Japanese B | 7.0 | 2,000 | 32,000 | 32,000 | 32,000 |
| Uganda S | 7.0 | 250 | 2,000 | | 1,000 |

In view of the titers shown by most antigens, a 1:16 dilution was in general considered a highly concentrated solution. Experiments were next carried out to determine the stability of antigens when held at 1°C. in dilutions higher than 1:16. The tests were done only at pH 7.0, as the results so far gathered showed that at this pH the antigens studied were markedly active. Each antigen was diluted 1:16, 1:64, and 1:256 in standard diluent and titrated at the end of the intervals stated in Table III.

The results in Table III show that at the end of 1 hour even when held as a 1:256 dilution, the activity of the preparations remained similar to that shown at zero time; in fact, the titer was unchanged or diminished by only one dilution. With increasing time the titers of the antigens diminished; but even at the end of 4 hours there was an appreciable titer in most instances. For some of the antigens, dilution 1:256 was close to the end-point, containing only 4 units.

Finally, the titrations reported in Table IV were preliminary titrations done in conjunction with serum inhibition tests.

The results shown in Table IV are reported as evidence that even in dilutions containing only 8 to 16 units, most antigens retained their titer unchanged for 10 minutes. This information was important in performing serum inhibition tests, as 10 minutes was more than adequate for adding antigen to a block of 150 tubes.

It appears from the reported experiments that preparations could be secured from the indicated viruses which, when diluted and held at 1°C., were stable over a somewhat wide pH range, extending generally over 1 unit. They were particularly stable at pH 7.0 for viruses of Group B, and at pH 6.4 for those of Group A. This stability was found to hold even with dilutions of antigen close to the end-point.

Antigens were not particularly stable at 22° or 37°C. when in the fluid state. No extensive investigation was carried out, however, at these temperatures.

Division of Hemagglutinating Antigens into Two Distinct Groups.—By testing antigens at 4° or 22°C., it was possible to demonstrate the presence of HA with a number of arthropod-borne viruses; those of the equine encephalitis group, however, failed to show any reaction. On further investigation, it was decided to test the effect of higher temperature of incubation in addition to that of pH.

Antigens of practically all the viruses listed in Table I were titrated simultaneously at different pH values, from 5.6 to 7.6, and at 4°, 22°, and 37°C. Some of the results are shown in Table V, in which, for the sake of brevity, results at 22°C. are not given as they were essentially similar to those at 4°C. The only differences were that at the latter temperature the titers were as a rule one or more dilutions higher. With yellow fever antigens, on the other hand, the pattern was more stable and the pH range slightly wider at 22°C.

In Table V it can be seen that antigens derived from EEE, VEE, and WEE reacted only when incubated at 37°C., with the exception that when there was a reaction at 4°C., the titer was exceedingly low compared with that of the same preparation at 37°C. On the other hand, viruses such as dengue, Japanese B, and yellow fever reacted best at 4°C. At 37°C. reaction was either not detected, as with dengue Types 1 and 2, Ntaya, Uganda S, and yellow fever, or if present, its titer was lower—at pH 7.0, considerably lower—than at 4°C., as was the case with Japanese B, St. Louis, and West Nile viruses. Sindbis virus is the only agent thus far encountered which gave a similar titer at 4° and 37°C. Also the influence of pH on the HA reaction and the connection between it and temperature of incubation was noted. Thus, the antigens agglutinating at 37°C. did so at pH 5.6–6.4, but not at pH 7.0 or higher. Viruses requiring temperatures of 4° or 22°C. reacted better at a higher pH. Reactions began to appear at pH 6.0 and extended in all cases tested beyond pH 7.0, usually 7.2–7.4; with some viruses the range of reaction extended to

TABLE V
Influence of Temperature of Incubation and pH on Hemagglutination

| Antigen | 4°C. | | | | | | | | | | 37°C. | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|------------------------------------|-------|--------|---------|--------|--------|--------|--------|-------|-----|-----|-------|-----|-----|-----|-----|-----|------------------------------------|-------|--------|---------|--------|--------|--------|--------|-------|---|---|---|---|---|---|---|---|------------------------------------|-------|--------|---------|--------|--------|--------|--------|-------|---|---|---|---|---|---|---|---|------------------------------------|-------|--------|---------|--------|--------|--------|--------|-------|--|--|--|--|--|--|--|--|------------------------------------|-------|--------|---------|--------|--------|--------|--------|-------|--|--|--|--|--|--|--|--|------------------------------------|-------|--------|---------|--------|--------|--------|--------|-------|--|--|--|--|--|--|--|--|------------------------------------|-------|--------|---------|--------|--------|--------|--------|-------|--|--|--|--|--|--|--|--|------------------------------------|-------|--------|--------|--------|--------|--------|--------|-------|--|--|--|--|--|--|--|--|------------------------------------|-------|--------|--------|--------|--------|--------|--------|-------|--|--|--|--|--|--|--|--|------------------------------------|---|--------|--------|--------|--------|--------|--------|--|--|--|--|--|--|--|--|--|------------------------------------|---|--------|--------|--------|--------|--------|--------|--|--|--|--|--|--|--|--|--|------------------------------------|--|--------|--------|--------|--------|--------|--------|--|--|--|--|--|--|--|--|--|------------------------------------|--|-------|--------|--------|--------|--------|--------|--|--|--|--|--|--|--|--|--|
| | pH | | | | | | | | | | pH | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | 5.6 | 6.0 | 6.4 | 6.6 | 6.8 | 7.0 | 7.2 | 7.6 | 5.6 | 6.0 | 6.4 | 6.6 | 6.8 | 7.0 | 7.2 | 7.6 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| EEE | 128 | 0* | 512 | | | 0 | | | | | | | | | | | VEE | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | WEE | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Sindbis | | 512 | 512 | 0 | 0 | 0 | 0 | | | | | | | | | | Dengue Type 1 | 4,096 | 4,096 | 8,192 | 8,192 | 8,192 | 8,192 | 0 | | | | | | | | | | Dengue Type 2 | 0 | 0 | 0 | 0 | 128 | 128 | 0 | | | | | | | | | | Japanese B | 4,096 | 16,384 | 131,072 | 65,536 | 65,536 | 65,536 | 8,192 | | | | | | | | | | Niaya | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 1,024 | | | | | | | | | | St. Louis | 8,192 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 8,192 | 4,096 | | | | | | | | | Uganda S | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 2,048 | | | | | | | | | | West Nile (Egypt 101) | 0 | 0 | 0 | | | | | | | | | | | | | | Yellow fever (Asibi) | | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | | | | | | | | | | Yellow fever (French neuro-tropic) | | 1,024 | >1,024 | >1,024 | >1,024 | >1,024 | >1,024 | | | | | | | | | |
| VEE | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | WEE | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Sindbis | | 512 | 512 | 0 | 0 | 0 | 0 | | | | | | | | | | Dengue Type 1 | 4,096 | 4,096 | 8,192 | 8,192 | 8,192 | 8,192 | 0 | | | | | | | | | | Dengue Type 2 | 0 | 0 | 0 | 0 | 128 | 128 | 0 | | | | | | | | | | Japanese B | 4,096 | 16,384 | 131,072 | 65,536 | 65,536 | 65,536 | 8,192 | | | | | | | | | | Niaya | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 1,024 | | | | | | | | | | St. Louis | 8,192 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 8,192 | 4,096 | | | | | | | | | Uganda S | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 2,048 | | | | | | | | | | West Nile (Egypt 101) | 0 | 0 | 0 | | | | | | | | | | | | | | Yellow fever (Asibi) | | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | | | | | | | | | | Yellow fever (French neuro-tropic) | | 1,024 | >1,024 | >1,024 | >1,024 | >1,024 | >1,024 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| WEE | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Sindbis | | 512 | 512 | 0 | 0 | 0 | 0 | | | | | | | | | | Dengue Type 1 | 4,096 | 4,096 | 8,192 | 8,192 | 8,192 | 8,192 | 0 | | | | | | | | | | Dengue Type 2 | 0 | 0 | 0 | 0 | 128 | 128 | 0 | | | | | | | | | | Japanese B | 4,096 | 16,384 | 131,072 | 65,536 | 65,536 | 65,536 | 8,192 | | | | | | | | | | Niaya | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 1,024 | | | | | | | | | | St. Louis | 8,192 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 8,192 | 4,096 | | | | | | | | | Uganda S | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 2,048 | | | | | | | | | | West Nile (Egypt 101) | 0 | 0 | 0 | | | | | | | | | | | | | | Yellow fever (Asibi) | | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | | | | | | | | | | Yellow fever (French neuro-tropic) | | 1,024 | >1,024 | >1,024 | >1,024 | >1,024 | >1,024 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Sindbis | | 512 | 512 | 0 | 0 | 0 | 0 | | | | | | | | | | Dengue Type 1 | 4,096 | 4,096 | 8,192 | 8,192 | 8,192 | 8,192 | 0 | | | | | | | | | | Dengue Type 2 | 0 | 0 | 0 | 0 | 128 | 128 | 0 | | | | | | | | | | Japanese B | 4,096 | 16,384 | 131,072 | 65,536 | 65,536 | 65,536 | 8,192 | | | | | | | | | | Niaya | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 1,024 | | | | | | | | | | St. Louis | 8,192 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 8,192 | 4,096 | | | | | | | | | Uganda S | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 2,048 | | | | | | | | | | West Nile (Egypt 101) | 0 | 0 | 0 | | | | | | | | | | | | | | Yellow fever (Asibi) | | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | | | | | | | | | | Yellow fever (French neuro-tropic) | | 1,024 | >1,024 | >1,024 | >1,024 | >1,024 | >1,024 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Dengue Type 1 | 4,096 | 4,096 | 8,192 | 8,192 | 8,192 | 8,192 | 0 | | | | | | | | | | Dengue Type 2 | 0 | 0 | 0 | 0 | 128 | 128 | 0 | | | | | | | | | | Japanese B | 4,096 | 16,384 | 131,072 | 65,536 | 65,536 | 65,536 | 8,192 | | | | | | | | | | Niaya | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 1,024 | | | | | | | | | | St. Louis | 8,192 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 8,192 | 4,096 | | | | | | | | | Uganda S | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 2,048 | | | | | | | | | | West Nile (Egypt 101) | 0 | 0 | 0 | | | | | | | | | | | | | | Yellow fever (Asibi) | | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | | | | | | | | | | Yellow fever (French neuro-tropic) | | 1,024 | >1,024 | >1,024 | >1,024 | >1,024 | >1,024 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Dengue Type 2 | 0 | 0 | 0 | 0 | 128 | 128 | 0 | | | | | | | | | | Japanese B | 4,096 | 16,384 | 131,072 | 65,536 | 65,536 | 65,536 | 8,192 | | | | | | | | | | Niaya | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 1,024 | | | | | | | | | | St. Louis | 8,192 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 8,192 | 4,096 | | | | | | | | | Uganda S | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 2,048 | | | | | | | | | | West Nile (Egypt 101) | 0 | 0 | 0 | | | | | | | | | | | | | | Yellow fever (Asibi) | | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | | | | | | | | | | Yellow fever (French neuro-tropic) | | 1,024 | >1,024 | >1,024 | >1,024 | >1,024 | >1,024 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Japanese B | 4,096 | 16,384 | 131,072 | 65,536 | 65,536 | 65,536 | 8,192 | | | | | | | | | | Niaya | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 1,024 | | | | | | | | | | St. Louis | 8,192 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 8,192 | 4,096 | | | | | | | | | Uganda S | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 2,048 | | | | | | | | | | West Nile (Egypt 101) | 0 | 0 | 0 | | | | | | | | | | | | | | Yellow fever (Asibi) | | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | | | | | | | | | | Yellow fever (French neuro-tropic) | | 1,024 | >1,024 | >1,024 | >1,024 | >1,024 | >1,024 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Niaya | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 1,024 | | | | | | | | | | St. Louis | 8,192 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 8,192 | 4,096 | | | | | | | | | Uganda S | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 2,048 | | | | | | | | | | West Nile (Egypt 101) | 0 | 0 | 0 | | | | | | | | | | | | | | Yellow fever (Asibi) | | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | | | | | | | | | | Yellow fever (French neuro-tropic) | | 1,024 | >1,024 | >1,024 | >1,024 | >1,024 | >1,024 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| St. Louis | 8,192 | 16,384 | 16,384 | 16,384 | 16,384 | 16,384 | 8,192 | 4,096 | | | | | | | | | Uganda S | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 2,048 | | | | | | | | | | West Nile (Egypt 101) | 0 | 0 | 0 | | | | | | | | | | | | | | Yellow fever (Asibi) | | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | | | | | | | | | | Yellow fever (French neuro-tropic) | | 1,024 | >1,024 | >1,024 | >1,024 | >1,024 | >1,024 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Uganda S | | 1,024 | 1,024 | 2,048 | 2,048 | 2,048 | 2,048 | | | | | | | | | | West Nile (Egypt 101) | 0 | 0 | 0 | | | | | | | | | | | | | | Yellow fever (Asibi) | | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | | | | | | | | | | Yellow fever (French neuro-tropic) | | 1,024 | >1,024 | >1,024 | >1,024 | >1,024 | >1,024 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| West Nile (Egypt 101) | 0 | 0 | 0 | | | | | | | | | | | | | | Yellow fever (Asibi) | | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | | | | | | | | | | Yellow fever (French neuro-tropic) | | 1,024 | >1,024 | >1,024 | >1,024 | >1,024 | >1,024 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Yellow fever (Asibi) | | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | >8,192 | | | | | | | | | | Yellow fever (French neuro-tropic) | | 1,024 | >1,024 | >1,024 | >1,024 | >1,024 | >1,024 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Yellow fever (French neuro-tropic) | | 1,024 | >1,024 | >1,024 | >1,024 | >1,024 | >1,024 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

* A zero signifies lowest dilution either 1:8 or 1:16; otherwise figures have same meaning as previously.

pH 7.6. From its pH requirements it can be seen that Sindbis virus was similar to the equine encephalitis viruses.

It was noticed that if titrations of antigens from viruses such as dengue or Ntaya—which were negative at 37°C.—were shaken and then kept at 4°C., no reaction occurred; it was assumed that incubation at 37°C. resulted in destruction of the antigen in 2 hours. On the contrary, in negative HA with the equine encephalitis group of antigens that had been held at 4°C., shaking and then holding at 37°C. yielded a positive reaction with a titer equal or almost equal to that given by the antigen on direct incubation at 37°C. It was assumed that either a combination of antigen and erythrocytes had occurred at 4°C. and higher temperature was needed for detection of HA, or else that the antigen had retained its titer practically unchanged at 4°C. and combined later at 37°C.

It is to be recalled here also that with the antigens of the equine encephalitis group a further manifestation of reaction was observed between the hemagglutinin and the red cell suspension; in that group the reaction was a combination of agglutination and hemolysis, as already described. With the antigens from the dengue-Japanese B-yellow fever group, no such activity was seen at 4°C.; when, however, a reaction was positive at 37°C., as with Japanese B virus, hemolysis similar in pattern, although less complete than that shown by the viruses in Group A, was observed.

To sum up, the viruses that have thus far given positive HA could be assembled, on the basis of temperature and pH requirements, in two groups: Group A comprising EEE, VEE, WEE, and Sindbis (see later); and Group B consisting of dengue Types 1 and 2, Ilhéus, Japanese B, Ntaya, St. Louis, Uganda S, West Nile (Egypt 101 strain), and yellow fever (Asibi, JSS, and French neurotropic strains).

Treatment of Sera in Order to Eliminate Non-Specific Inhibitors.—Sera deriving from representatives of different animal species, when tested in HA inhibition tests with the arthropod-borne viruses, have been found to possess non-specific inhibitors in high titer; hence the necessity for devising methods to remove such inhibitors. Chanock and Sabin (10) advocated treatment of sera with organic solvents, particularly acetone which was found effective against inhibitors for several viruses. Their method could be easily reproduced; however, when the extension of our work required the use of large numbers of sera, the acetone method was not entirely practical. In attempts to improve on this method, and also to secure additional information concerning properties of the inhibitor, the following trials for its removal were made: acetone precipitation (10), chloroform extraction (5), precipitation with 1:3 saturated ammonium sulfate, filtration through graded collodion membranes, adsorption in ion exchange resins, dialysis against phosphate buffer 0.1 and 0.02 M in physiological saline, filtration through glass filters, filtration through Seitz pads, and adsorption with powdered asbestos. Sera were treated as just indicated, either subsequent to heating at 56° or 60°C. for half an hour or without heating. These tests led finally to the adoption of the present procedure of filtration of the unheated sera through Seitz pads as the method of choice.

In Table VI is given the result of one experiment in which normal mouse serum was subjected to different procedures.

It can be seen that the titer of the non-specific inhibitor in heated or unheated serum was 1:5,120 or higher. Of the methods used, dialysis, filtration through sintered glass, or filtration through collodion membranes with an average pore diameter of 303 $m\mu$ failed to remove the inhibitor; filtration through a collodion membrane with an average pore diameter of 69 $m\mu$ wholly removed the inhibitor from heated serum and only partially from unheated serum. Finally, both treatment with acetone and Seitz filtration removed the inhibitor completely, better from unheated than from heated serum. In this

TABLE VI
Removal of Normal Mouse Serum Inhibitor for St. Louis Encephalitis Virus

| Treatment of Serum | | Reciprocal of dilution of serum | | | | | | | | | |
|--------------------|----------------------------------|---------------------------------|----|----|----|-----|-----|-----|-------|-------|-------|
| | | 10 | 20 | 40 | 80 | 160 | 320 | 640 | 1,280 | 2,560 | 5,120 |
| Heated at 56°C. | None | 0* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Acetone | ± | + | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| | Dialysis | ± | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Filtration, Seitz | ± | + | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| | Filtration, glass | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ± |
| | Filtration, collodion 303 $m\mu$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + |
| | Filtration, collodion 69 $m\mu$ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Not heated | None | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| | Acetone | + | + | + | ++ | ++ | ++ | ++ | ++ | ++ | |
| | Dialysis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| | Filtration, Seitz | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | |
| | Filtration, glass | ± | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| | Filtration, collodion 303 $m\mu$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | |
| | Filtration, collodion 69 $m\mu$ | 0 | 0 | 0 | 0 | 0 | 0 | + | ++ | ++ | |

* See text for interpretation of signs in Tables VI to VIII; in all of these tables, 4 units of antigen were used.

test, the antigen was used in dilution 1:1,024; actual titrations showed that the amount of antigen in the test was equivalent to 4 units.

It was next necessary to determine whether or not those procedures which had removed the non-specific inhibitor from normal mouse serum had any action that might result in removing or in any way affecting the specific antibody in mouse serum (Tables VII and VIII).

In Table VII it can be seen that an unheated, hyperimmune mouse anti-serum against St. Louis virus had the same titer of inhibition of HA following Seitz treatment as it did after acetone treatment. Serum heated at 56°C. for 30 minutes, and then treated either with acetone or by Seitz filtration, gave similar titers although somewhat lower than those obtained with unheated serum. Finally, filtration through a 69 $m\mu$ collodion membrane resulted in almost complete elimination of the antibody when the serum had been previously heated, but it was not so effective with the unheated serum. This type

of filtration could not, therefore, be used as a means of preparing sera for inhibition tests.

TABLE VII
Hemagglutination Inhibition of St. Louis Virus Antigen by Hyperimmune Mouse Serum Treated to Remove Non-Specific Inhibitor

| Treatment of serum | | Reciprocal of dilution of serum | | | | | | | | | |
|--------------------|------------------------------|---------------------------------|----|----|----|-----|-----|-----|-------|-------|-------|
| | | 10 | 20 | 40 | 80 | 160 | 320 | 640 | 1,280 | 2,560 | 5,120 |
| Heated at 56°C. | Acetone | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | ++ | ++ |
| | Filtration, Seitz | 0 | 0 | 0 | 0 | 0 | 0 | ± | + | ++ | ++ |
| | Filtration, colloidion 69 mμ | 0 | 0 | ± | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Not heated | Acetone | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | ++ |
| | Filtration, Seitz | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | ++ |
| | Filtration, colloidion 69 mμ | 0 | 0 | 0 | 0 | 0 | 0 | ± | + | ++ | ++ |

TABLE VIII
Hemagglutination Inhibition of West Nile (Egypt 101) Virus Antigen by Monkey Convalescent Serum Treated to Remove Non-Specific Inhibitor

| Time of bleeding | Treatment of serum | Reciprocal of dilution of serum | | | | | | | | | |
|-------------------------|--------------------|---------------------------------|----|----|----|-----|-----|-----|-------|-------|----|
| | | 10 | 20 | 40 | 80 | 160 | 320 | 640 | 1,280 | 2,560 | |
| Before infection | Acetone | + | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| | Filtration, Seitz | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| | Asbestos powder | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 34 days after infection | Acetone | 0 | 0 | 0 | 0 | 0 | ± | + | ++ | ++ | |
| | Filtration, Seitz | 0 | 0 | 0 | 0 | 0 | 0 | + | ++ | ++ | |
| | Asbestos powder | ± | 0 | 0 | 0 | 0 | 0 | + | ++ | ++ | |

Non-specific inhibitors could also be removed by Seitz filtration from monkey sera (Table VIII). In one of the experiments, serum derived from a monkey, before and after infection with West Nile (Egypt 101 strain) virus, was treated by the acetone and Seitz filtration methods and also by adsorption with powdered asbestos. The results would seem to indicate that the removal of inhibitors by Seitz filtration was more a phenomenon due to adsorption of elements in the serum by the material of the filter than one of simple filtration.

When sera were filtered through Seitz pads as a 1:5 dilution, similarly satisfactory results were obtained, except that occasionally the reaction in the tube containing that dilution of serum did not offer a clear reading.

TABLE IX
Hemagglutination Inhibition with Mouse Hyperimmune Sera

| Serum | Antigen | | | | | | | | | | | |
|-----------------------|---------------|------------|--------------|--------------|------------|--------------|------------|-----------|------------|------------|------------|--------------|
| | Group A | | | | Group B | | | | | | | |
| | EEE | Sindbis | VEE | WEE | Dengue 1 | Ilhéus | Jap. B | Ntaya | St. L. | Ug. S | W.N. (E) | Yf |
| EEE | 2,560* | 40 | 20 | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Semliki Forest | 40 | 40 | 40 | 20 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sindbis | | 640 | | 320 | | | 0 | | | 0 | | |
| VEE | 640 | 10 | 5,120 | 20 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| WEE | 320 | 320 | 160 | 1,280 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Dengue Type 1 | 0 | 0 | 0 | 0 | 320 | 80 | 160 | | 160 | 80 | | |
| Dengue Type 2 | 0 | 0 | 0 | 0 | 40 | 20 | 10 | 20 | 40 | 80 | 40 | 20 |
| Ilhéus | 0 | 0 | 0 | 0 | 640 | 1,280 | 320 | 640 | 640 | 640 | 640 | 320 |
| Japanese B | 0 | 0 | 0 | 0 | 320 | 640 | 640 | 640 | 320 | | 320 | 320 |
| Ntaya | 0 | 0 | 0 | 0 | | 40 | 40 | 80 | 80 | 80 | 80 | 160 |
| Russian Far Eastern | 0 | | 0 | 0 | 40 | 80 | 20 | 80 | 80 | 80 | 40 | 40 |
| St. Louis | 0 | | 0 | 0 | 640 | 160 | 40 | 160 | 640 | 640 | 160 | 320 |
| Uganda S | 0 | | 0 | 0 | 160 | | | | | 160 | | |
| West Nile (Egypt 101) | 0 | 0 | 0 | 0 | 320 | 160 | 160 | 320 | 320 | 320 | 640 | 160 |
| Yellow fever (Asibi) | | | | | | 0 | 0 | 0 | 20 | | 10 | 1,280 |
| Yellow fever (JSS) | 0 | | 0 | 0 | 40 | | | | | 80 | | |

* Throughout Tables IX to XI, the figures signify the reciprocal of the highest dilution of serum giving complete inhibition of hemagglutination; a zero indicates no inhibition at 1:10 dilution. In all of these tables, 8 units of antigen were used.

As a result of all these investigations a method was developed which removed non-specific inhibitors from sera deriving from all species tested—man, monkey, guinea pig, and mouse—for all viruses under consideration. The method described in the section on Materials and Methods resulted in no loss of specific antibody titers.¹

Results of Hemagglutination Inhibition Tests.—In this section will be described results of HA inhibition tests done with sera from mice, guinea pigs, and monkeys. The studies with human sera are to be reported at a later date.

¹ Another simple and effective method of removing non-specific inhibitors from sera without affecting the antibody titer has been developed by Dr. D. H. Clarke. It is based on adsorption of sera with bentonite and will be described in detail at a later date.

In all cases a sufficient amount of serum was prepared to allow for testing the same sample against all, or as many as possible, of the available antigens. The first dilution of serum was 1:10. On each separate test from 40 to as many as 100 sera were run against 8 units of antigen.

Mouse sera: A summary of some of the tests conducted with mouse hyperimmune sera is presented in Table IX.

Table IX shows that: (a) antisera against a number of viruses fall into two groups which coincide with the antigen Groups A and B already described; (b) within each group a marked degree of cross-reaction was apparent; and (c) at no time was there any cross-reaction between a serum from one group and an antigen from the other.

The cross-reactions in Group B were of such magnitude that the heterologous reaction often was of the same titer as the homologous one; for example, Japanese B antiserum gave approximately the same titer against all antigens in Group B, although it failed to react with any of the antigens in Group A. The only exception was yellow fever antiserum, Asibi strain, which had a high titer against a yellow fever antigen, yet reacted slightly or not at all with the remaining antigens in Group B. In Group A, the cross-reactions were not, as a rule, as pronounced as in Group B; thus VEE antiserum, with a titer of 1:5,120 against its own antigen, gave titers of only 1:10 and 1:20 against two other members of the group and 1:640 against EEE. It is further noticeable that the titers of the sera were generally higher in Group A than in Group B. Hyperimmune antisera for viruses for which HA was not available were also included in these tests; as a result it could be seen that Semliki Forest virus was related to the viruses in Group A, while Russian Far Eastern virus cross-reacted with the agents in Group B.

Several samples of normal mouse serum, not shown in Table IX, were included, as were sera from mice hyperimmunized with other viruses, following a method of immunization identical with the one used for the preparation of the active sera; these viruses were Anopheles A, California BFS 283, Coxsackie Types 1 and 2 (muscular tissue used for the immunization), herpes, poliomyelitis Type 2, and Wyeomyia. At no time did any of these sera show the slightest inhibitory effect on any of the hemagglutinins.

Guinea pig sera: The preparation of antisera from guinea pigs differed from that of antisera from mice in that fewer injections of the immunizing agents were given and the samples tested were from individual animals rather than from pools.

Table X gives a summary of tests which amply confirm the findings with mouse hyperimmune sera concerning the existence of two distinct groups of viruses, with the members of each group interrelated. Table X also reveals a louping ill serum reacting with antigens of Group B, though not with those of Group A. Finally, samples of serum from 6 different normal guinea pigs, as well as samples of serum from animals immunized with Anopheles B, Bunyamwera, Bwamba, California, GD VII, Mengo, and rabies, failed to inhibit agglutination by any of the antigens.

Monkey sera: Monkeys were injected once with active virus and bled during

convalescence; from each animal there was available one sample of serum taken prior to injection and one or more at intervals following recovery. The results of the tests with sera from some of the monkeys are shown in Table XI.

Tests with antigens of Group A are not tabulated since all results were negative. Antibodies inhibiting HA developed following infection with dengue Types 1 and 2, St. Louis, West Nile (Egypt 101 strain), and yellow fever

TABLE X
Hemagglutination Inhibition with Guinea Pig Immune Sera

| Guinea pig No. | Serum Virus | Antigen | | | | | | | | | | | |
|----------------|-----------------------|---------|---------|-----|-----|----------|--------|--------|-------|--------|-------|----------|----|
| | | Group A | | | | Group B | | | | | | | |
| | | EEE | Sindbis | VEE | WEE | Dengue 1 | Ilhéus | Jap. B | Ntaya | St. L. | Ug. S | W.N. (E) | Yf |
| 1 | EEE | 160 | 160 | 10 | 40 | 0 | | | | | | 0 | |
| 2 | Semliki Forest | | | 40 | 40 | | | | | | | 0 | |
| 3 | Sindbis | 10 | 320 | 0 | 40 | 0 | 0 | 0 | | | | 0 | |
| 4 | Sindbis | | 160 | | 40 | | | 0 | | | | 0 | |
| 5 | Sindbis | | 160 | | 20 | | | 0 | | | | 0 | |
| 6 | VEE | 80 | 80 | 640 | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | WEE | 0 | | 0 | 20 | 0 | | | | | | 0 | |
| 8 | Ilhéus | 0 | 0 | 0 | 0 | 160 | 320 | 320 | | 320 | 160 | | |
| 9 | Japanese B | 0 | 0 | 0 | 0 | 40 | 40 | 80 | | 80 | 40 | | |
| 10 | Louping ill | 0 | | 0 | 0 | 40 | | 20 | | | 40 | 40 | |
| 11 | Russian Far Eastern | | | | | 40 | 40 | 40 | 80 | 160 | | 80 | 80 |
| 12 | Russian Far Eastern | 0 | | 0 | 0 | 10 | 40 | 10 | 10 | 10 | | 20 | 20 |
| 13 | St. Louis | 0 | 0 | 0 | 0 | 20 | 40 | 80 | | 160 | 40 | | |
| 14 | West Nile (Egypt 101) | | | | | | 20 | 20 | 40 | 40 | | 80 | 20 |
| 15 | West Nile (Egypt 101) | | | | | 20 | 20 | 20 | 20 | 40 | | 160 | 20 |

viruses. There was a degree of cross-reaction which is, however, less pronounced here than with the other animal sera tested. In monkey 4, antibodies were detectable almost 1 year after infection. Monkeys infected with different strains of yellow fever developed antibodies promptly with high titers and a minimum of cross-reaction. Finally, in addition to the 8 samples of normal monkey serum shown, an additional 16 normal monkeys possessed sera that failed to inhibit HA by any of the antigens tested.

Recapitulation of the results with animal sera: From the results observed with animal sera, hyperimmune as well as convalescent, it is concluded that an HA inhibition reaction could be demonstrated which was specific in the sense that

group antibodies were detected as well as antibodies against the agent with which the animal had been vaccinated or infected. With hyperimmune mouse sera, particularly in Group B, the homologous and heterologous reactions were

TABLE XI
Hemagglutination Inhibition with Monkey Convalescent Sera

| Serum | | | Antigen | | | | | | | |
|--------------------|--------------------------|--|---------------|-------------|-----------|------------|-----------|----------|-------------|-------|
| Mon- key No. | Virus | Bleeding Time after in- jection | Den- gue 1 | Ilh- éus | Jap. B | Nta- ya | St. L. | Ug. S | W.N. (E) | Yf |
| | | <i>days</i> | | | | | | | | |
| 1 | Dengue Type 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 42 | 160 | 20 | 20 | 40 | 20 | | 80 | 40 |
| | | 156 | 160 | 20 | 20 | 40 | 40 | | 20 | 10 |
| 2 | Dengue Type 2 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 109 | | 10 | 0 | 10 | 20 | 0 | 20 | 10 |
| | | 130 | | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| | | 334 | | 10 | 10 | 10 | 10 | | 10 | 10 |
| 3 | St. Louis | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 |
| | | 41 | | 0 | 10 | 0 | 40 | | 10 | 10 |
| 4 | West Nile (Egypt 101) | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 |
| | | 34 | 40 | 40 | 40 | 80 | 80 | | 320 | 40 |
| | | 88 | 20 | 0 | 10 | 10 | 20 | | 80 | 10 |
| | | 118 | 20 | 10 | 10 | 20 | 20 | | 80 | 10 |
| | | 145 | 20 | 0 | 10 | 20 | 10 | | 40 | 10 |
| | | 307 | 10 | 0 | 10 | 20 | 10 | | 40 | 10 |
| 5 | Yellow fever (Asibi) | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 |
| | | 19 | | 40 | 20 | 40 | 40 | | 80 | 640 |
| 6 | Yellow fever (JSS) | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 |
| | | 41 | | 20 | 20 | 10 | 20 | | 20 | 640 |
| 7 | Yellow fever (JSS) | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 |
| | | 41 | | 20 | 20 | 20 | 40 | | 20 | 160 |
| 8 | Yellow fever (Ogbomosho) | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 |
| | | 13 | | 80 | 10 | 40 | 80 | | 80 | 1,280 |

of almost the same titer. The consistent lack of inhibition by the large number of normal sera, and by sera from animals immunized with viruses other than the ones in Groups A and B, was taken as evidence that the inhibitions of HA described here were not due to a non-specific inhibitor such as that shown to be present in normal, untreated sera.

DISCUSSION

The work of Sabin and his associates showed the possibility of using HA and HA inhibition tests for the study of the arthropod-borne viruses. As reported here, that work has now been elaborated and extended to other viruses, particularly in the equine encephalitis group. Moreover, it has been possible to introduce a certain degree of systematization and uniformity in the methods for detection of HA, with the result that all tests can now be done with a minimum of easily controlled variables. There is then available an additional tool for the investigation of this large and complex group of viruses, particularly the investigation of their antigenic relationships.

Over the past 15 years information has been gathered showing that by neutralization, complement-fixation, and cross-resistance tests, certain immunological relationships can be established among certain viruses in this group. Smithburn (39) showed a relationship to exist between Japanese B, St. Louis, and West Nile viruses; Casals and Webster (40) showed that louping ill and Russian Far Eastern viruses were closely connected, almost identical, agents. Sabin (41) found by complement fixation that the viruses of dengue Type 1, dengue Type 2, Japanese B, West Nile, and yellow fever had common antigenic constituents. Havens *et al.* (42) reported the crossing of EEE with WEE virus by complement-fixation tests. As might be expected, improved techniques resulted in uncovering serological crossings which with less sensitive methods either were missed or if reported led to controversy. Sabin emphasized the usefulness of high-titered antigens in his work on complement fixation; and in this laboratory and that of The Rockefeller Institute for Medical Research, the development of highly potent complement-fixing antigens extracted with acetone-ether has led to the discovery of additional crossings among arthropod-borne viruses which had not been hitherto detected (43).

These observations fit in well with the concepts discussed by Olitsky and Casals (44), Theiler (45), and Sabin (46) concerning the probable existence of a large group of arthropod-borne viruses having common antigenic constituents, and the practical consequences that this fact might have in epidemiological studies.

The work reported here brings forth clear-cut serological crossings among arthropod-borne viruses by the use of the HA test, and the separation of a number of them into two completely distinct groups. Whether or not this classification corresponds to deeper differences in the nature or essential properties of the viruses involved remains to be seen. It seems apparent at this time, however, that such a sharp grouping might be used to advantage in the systematization of the viral agents.

In the HA tests no crossing was noted between the viruses in Group A and those in Group B. Whether this applies only to the results of HA tests, or to neutralization and complement-fixation tests as well, is still to be thoroughly investigated. Smithburn (47), for example, found that louping ill immune serum neutralized EEE virus; also that Ntaya antiserum neutralized to some

extent Anopheles A virus. Such results are at variance with the observations reported here, and they may be proof that the particular test used influences the type of relationships elicited. That this may well be the case seems to be partly borne out by parallel studies using neutralization and complement-fixation tests now being conducted in this laboratory on the same specimens of sera. It would appear that HA inhibition shows the greatest serological overlap, complement fixation less, and neutralization least. A recent reinvestigation, however, of neutralization tests with certain of the arthropod-borne viruses resulted in serological relationships of a degree hitherto unrecognized. Thus, several dengue Type 1 monkey convalescent sera neutralized 100 LD₅₀ of St. Louis virus (48).

The advantage of the serological overlap by HA tests is that it facilitates the grouping of the arthropod-borne viruses and their immune sera, since even sera of only moderate homologous titer are apt to exhibit cross-reactions; the disadvantage is that no definite etiology can be established in epidemiological surveys. In the latter case when sera were tested against numerous antigens of a related group, the difference in the antibody titer for the presumed etiological agent and for the related viruses was apt to be only one or two dilutions, which is too small to be definite. Furthermore, there is the possibility that the detected antibodies might have been originated by the action of an incitant, still undiscovered, but belonging to one of the two groups here described. Where the HA inhibition test may be of use in epidemiological work is in preliminary screening; that is, by testing sera against one or two antigens of Groups A and B.

CONCLUSIONS

Through the use of acetone and ether extraction of brain tissue from newborn mice infected with certain arthropod-borne viruses, it has been possible to demonstrate hemagglutinins for chick erythrocytes associated with the following viruses: dengue Type 1, dengue Type 2, Eastern equine encephalitis, Ilhéus, Japanese B, Ntaya, St. Louis, Sindbis, Uganda S, Venezuelan equine encephalitis, West Nile (Egypt 101 strain), Western equine encephalitis, and yellow fever (viscerotropic and neurotropic strains).

On the basis of the temperature and pH required for reaction, the viruses can be assembled in two groups: A—those that require 37°C. and a pH of about 6.4, comprising Eastern, Venezuelan, and Western equine encephalitis and Sindbis viruses; and B—those that require either 4° or 22°C. and a pH of about 7.0, comprising dengue Types 1 and 2, Ilhéus, Japanese B, Ntaya, St. Louis, Uganda S, West Nile, and yellow fever viruses.

A method of eliminating non-specific inhibitory substances present in sera was developed. The method consists essentially of filtration through Seitz pads.

Extensive serological crossings were found among viruses of each group,

while antisera of one group failed consistently to cross-react with antigens of the other.

Antisera deriving from animals immunized with certain viruses for which no hemagglutinins could be developed by the present method, reacted with members of either one or the other group. Thus Semliki Forest virus would appear to belong to Group A, and Russian Far Eastern and louping ill viruses to Group B.

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