8 July 1950

Lt. Col. Frank L. Bauer  
Armed Forces Epidemiological Board  
Office of The Surgeon General  
Room 2745, Main Navy Building  
Washington 25, D. C.

Dear Colonel Bauer:

Enclosed herewith is the Semi-Annual Progress Report No. 1 of the V & R Commission covering the period 1 January – 30 June 1950.

This report has been assembled in this office according to Dr. Paul's instructions left with me before his departure.

Sincerely yours,

Anne D. Sheldon  
Administrative Assistant  
to Dr. Paul

cc: Dr. MacLeod  
Dr. Sabin
WAR DEPARTMENT
Office of The Surgeon General

Semi-Annual Progress Report No. 1

Date 6 July 1950

Period covered: 1 January–30 June 1950

Director of Commission:
John R. Paul, M.D.

Subject: Commission on Virus and Rickettsial Diseases

I. General

Hepatitis Research Team (Dr. J. W. Colbert, Jr., Clinical Director), 98th General Hospital, EUCOM, Germany.

The Hepatitis Research Team has been working in connection with the "Center for Hepatitis Patients" at the 98th General Hospital in Munich. Dr. James W. Colbert, Jr., has continued as Director during the past six months, 1 January – 30 June; and in connection with the clinical work, the virus laboratory of the Team is now operating. A number of the studies in clinical investigation on hepatitis patients covering the work of the past two years has been assembled in a series of papers published as a Symposium of Acute Hepatitis which has appeared in the American Journal of Medicine, May issue, 1950.

Epidemiological investigation. Although it has been an accepted conclusion by observers in Germany that the high rate of hepatitis prevailing among U.S. troops in EUCOM is a result of direct personal contact among Germans and German households, there is also a growing accumulation of evidence to indicate that serum hepatitis accounts for a fair percentage of these patients. This evidence is based on: 1) the clinical types of hepatitis seen among U.S. troops, 2) on the seasonal characteristics which indicate two waves of prevalence, one in the autumn and one in the early winter, a characteristic which is not particularly indicative of infectious hepatitis alone, and 3) it has now been definitely shown in one rather crucial experiment that serum hepatitis is present among troops on the basis of experimental work on human volunteers (See report by Evans). A second experiment with equivocal results (See also Evans' report) rather points to the fact that serum hepatitis may have been present in the materials used but does not prove it. It has seemed of fundamental importance to attempt to establish this point by further work. Obviously measures to control these two diseases (or two aspects of the same disease) should be made from two angles if both IH and SH virus are being disseminated among this military population.

In the virus laboratory in Germany, attempts to isolate hepatitis virus have also been carried out. To date, these have been negative and include attempts to inoculate eggs, to transmit hepatitis to young pigs, hamsters and suckling mice, and demonstrate interference between hepatitis virus and Lansing poliomyelitis virus and ectromelia virus in mice. Renewed efforts have been made to produce formalized, ultracentrifuged concentrates of acute hepatitis sera and fecal samples as antigens, presumed to contain hepatitis virus, which might be useful in skin tests on patients. The serum antigens used to date have given reactions in patients comparable to those seen in healthy controls.

Work on the virus of Teschen disease (porcine encephalomyelitis), a virus which is prohibited from being imported into the United States, is being actively pursued in Europe. Three strains of virus have been established in pigs.
Infection has been induced orally, intranasally and intracerebrally; a detailed study of the histologic alterations in the central nervous system is being made in collaboration with local pathologists. Although this is not finished, it can be stated that the anatomical distribution of the lesions in the brains of pigs, the only animal known as yet to be susceptible to infection by this virus, is very wide-spread suggesting a type of encephalomyelitis seen perhaps more often in rabies or louping ill. Hyperimmune serum against Teschen virus has been prepared in pigs and monkeys; cross-immunization experiments are in progress in which a variety of encephalomyelitic viruses are being employed.

On the clinical side (the report of Dr. J. W. Colbert, Jr.), attempts to determine the clinical effect of aureomycin in patients with viral hepatitis have been made. Twenty-four patients were given 1 gram of this antibiotic every six hours by mouth for ten days; four additional patients were given supplementary intravenous aureomycin in amounts of 100 mg. q6h for 10 days. No significant differences in the course of the disease were evident in the treated patients as compared with a group of untreated similarly observed. Chloromycetin in doses of 50 mg. per kilogram body weight initially and 500 mg. every six hours thereafter has been given to 8 patients for 10 days without any apparent effect on the course of the disease.

The use of the liver biopsy in certain patients with hepatitis to investigate relationships between histologic alterations and functional status has given interesting results. It has not been possible to predict from the observations of the histologic alterations whether or not functional capacity of the liver as determined by liver function tests is impaired. If the capacity to remove dye from the blood in the bromasulfalin test was impaired, histologic alterations were always present.

Studies on the effect of exercise on the convalescent rate in hepatitis have been carried out in convalescent patients.

A follow-up study of patients who had hepatitis 2 years ago in Germany is now underway. Biopsy has been made in many of these patients showing only occasional mild periportal fibrosis with cellular infiltration. No evidence of chronic progressive hepatitis was seen.

ACTH has been given to 5 patients with acute viral hepatitis. ACTH appears to have a definite effect upon the course of the serum bilirubin. In all cases the serum bilirubin promptly fell. In two cases after stopping ACTH for a short time the serum bilirubin rose with exacerbation of the symptoms of the disease. Readministration of ACTH to one of these patients was followed by a prompt remission of symptoms and fall in serum bilirubin. The other patient is being followed without readministration of ACTH.

The terramycin program is not fully completed but partial analysis of the results does not reveal any remarkable effects.

II. Dr. W. McD. Hammon, DA-49-007-MD-29 (University of Pittsburgh, Pittsburgh, Pa.)

1. ISOLATION OF VIRUS FROM TOKYO MOSQUITOES OF 1949

From 100 female Culex tritaeniorynchus collected on 13 August 1949 from Ueno Park in Tokyo, a virus has been isolated. This has been tentatively identified as Japanese B by complement fixation test. Isolation was effected directly from the mosquito suspension by inoculation of both baby and three-week old mice.
From a tube containing 2 female Aedes niveus (a rare species), one collected on 6 August in Ueno Park, Tokyo and the other on 16 August in Grant Heights, Tokyo, another strain of virus appears to have been isolated. Preliminary tests also indicate that this is probably a strain of Japanese B. This isolation was effected only by baby mouse inoculation and only one mouse became ill (6th day). Attempts will be made to repeat this for confirmation.

These mosquitoes were collected (August 6-16) during the period just before the explosive onset of human cases in the Tokyo area (last week of August). All mosquitoes have now been tested, although a few lots are being repeated because of suspicious results. No other virus has been isolated. During a similar period of time in 1948, preceding the outbreak in Okayama, 9 strains were isolated from C. tritaeniorhynchus.

These tubes of frozen mosquitoes came from Tokyo in a poorly insulated metal container which contained no dry ice on arrival in San Francisco and which failed again to hold dry ice during the next 48 hours. This possibly explains why more isolations were not made from the mosquito collections of this critical period of time.

2. **mites from wild bird nests in tokyo, 1949**

About half (23,661) of the 58,000 mites collected have now been tested. No virus has been isolated. The remaining mites are now being tested.

3. **move to pittsburgh**

Work in the San Francisco laboratory was terminated on January 28, at which time viruses and sera were packed for shipment to Pittsburgh. Within the next few days special Army-owned equipment was moved to Fort Baker for temporary storage prior to shipment to the Pittsburgh laboratory. The new laboratories at the University of Pittsburgh are now in operation, although none of the equipment stored at Fort Baker has been forwarded yet.

4. **kern county, California, encephalitis studies**

The total of cases diagnosed serologically (C.F. and neutralization) in 1949 has now increased to 8 St. Louis and 5 Western equine. Three additional probables can be listed for St. Louis type but inadequacy of specimens has made it impossible to obtain conclusive results. Complement fixation continues to be a more effective means of detecting a clear-cut rise in titer to the St. Louis virus, while failure to obtain a rise to Western equine virus C.F. antigen during a reasonable period of time continues to render the neutralization test somewhat more dependable for use in infections with this virus. It is felt that for most dependable results both types of test should be performed. It should be pointed out, however, that the St. Louis complement fixation test alone, using the Webster III strain for antigen as we currently do, does not permit certain differentiation between St. Louis and Japanese B encephalitis should both be present in the same area. Since, as mentioned in the next section, there is some question about the presence of Japanese B virus in California, we are now repeating the tests on those sera of patients diagnosed on the basis of the C.F. test with low titers of neutralizing antibodies.
5. **POSSIBLE ISOLATION OF JAPANESE B VIRUS FROM CALIFORNIA MOSQUITOES**

From *Aedes dorsalis* of Kern County, California, collected in the summer of 1949, a virus was isolated which was not identified until after the move to Pittsburgh. This appears to be Japanese B virus on the basis of neutralization tests, but vaccination challenge tests are still in an incompletely stage. Since the Nakayama strain of virus was used once, although in separate rooms and by different persons, at the time this isolation was made in San Francisco it is impossible to state with absolute certainty that a contamination could not have occurred. While we believe it improbable, a thorough biological comparison between the two viruses is being undertaken and is partially complete. One apparent difference has appeared which is being repeated. Hamsters, as in the case with most laboratory strains of Jap B virus, are not susceptible to inoculation by the intraperitoneal route with the Nakayama strain (dilutions 10^{-1} and 10^{-2}), while the possible new virus has killed hamsters from 10^{-2} through 10^{-4} by the i.p. route. It is recalled that we were somewhat critical of the Okinawa strain isolation, since its behavior in mice was so parallel to that of the Nakayama strain until we demonstrated significant differences in their behavior in hamsters. A separate report on this possible isolation and virus comparisons is being prepared and should be ready in two to three weeks.

6. **WILD BIRD SERUM SURVEY IN JAPAN**

A group of three men have orders to proceed to Tokyo to the 406th Medical General Laboratory to make a summer and fall survey of neutralizing antibodies in wild birds to Japanese B encephalitis. Malaria smears will be made on the same birds and mosquito dissections will be made to determine the vectors of avian malaria, a disease known to be present in Japan. This may give some highly significant information on the feeding habits of certain species of mosquitoes suspected as vectors of Japanese B encephalitis. The members of this field crew are: Dr. H. Elliott McClure who formerly made a three-year bird survey for us in California; Ronald Reuther who assisted Dr. McClure in California and worked on the encephalitis team in Tokyo last year; Clark Johnson who has spent the last two summers in our San Francisco laboratory.

III. Dr. A. B. Sabin, MD 400 (Children's Hospital Research Foundation, Cincinnati, Ohio).

1 January - 31 March:

**Japanese B Encephalitis Virus Hemagglutinin.** Continued systematic studies on the unique physico-chemical properties of this hemagglutinin and its receptor have yielded information which resulted in the preparation of stable hemagglutinin of high activity (titers ranging from 1:2,000 - 1:10,000) and the development of a simple serological test for the detection of infections with the virus of Japanese B encephalitis. The following new findings deserve special mention:

1. Infected mouse brain suspension contains a substance associated with particles sedimentable at approximately 20,000xG (13,000 r.p.m. for 1 hour on the Sorvall SS-1 centrifuge in the cold room) which inactivates the hemagglutinin at about 5°C in 3 to 4 days.
2. The 13,000 r.p.m. supernate increases in activity after storage at about 50°C for 3 to 10 days and then remains stable in the fluid state for 8 weeks or longer.

3. Combination between hemagglutinin and its receptor on the chick erythrocytes occurs only in the narrow pH zone of 6.2 to 7.1, the optimum being between pH 6.5 and 6.8. Dilution of the hemagglutinin in 0.01 M inorganic or organic buffers of optimum pH results in an enhancement of titer which can be as much as 100-fold. This enhancement does not occur when the dilutions are first made in unbuffered saline and immediately thereafter adjusted with the buffers.

4. The reaction between hemagglutinin and receptor is of the equilibrium type in accord with the mass law. A certain minimum number of receptors on each erythrocyte must combine with hemagglutinin for agglutination to occur. Accordingly, the hemagglutination titer decreases in a constant ratio as the number of erythrocytes in the mixture is increased. Reversal of hemagglutination was accomplished in accord with these laws.

5. No receptor destroying enzyme was found to be associated with this hemagglutinin, and the enzymes of Vibrio cholerae were without effect on it.

6. The hemagglutinin in the 13,000 r.p.m. supernate was found to be most unstable under the following conditions:

   a) In 1:20 and greater dilution, the titer drops approximately 95% in 60 minutes at room temperature and to less than 1:20 in 30 minutes at 37°C.

   b) A single freezing and thawing results in complete inactivation, although it proved possible to preserve 50% of the activity by suitable lyophilization.

   c) Bubbling of an inert gas (nitrogen) through the solution rapidly inactivates the hemagglutinin. Similarly, the severe agitation which occurs in a Waring blender, must be avoided in the preparation of the extract.

7. The inhibitor in normal serum was found to react with the hemagglutinin, the inhibition titer being directly proportional to the concentration of hemagglutinin when the hemagglutinin is diluted in appropriately buffered saline but not in unbuffered saline. The evidence further suggested that the inhibitor in the serum was either identical with or closely similar to the receptors on the erythrocytes, and it proved possible to reverse the agglutination of erythrocytes which had combined with small amounts of hemagglutinin, by adding appropriately diluted normal serum.

8. Many attempts to get rid of the inhibitor in normal serum (including periodate and various enzymes) were unsuccessful, until a variety of lipid solvents were tested. Chloroform proved to be best, and the mere shaking of fluid serum with chloroform removed the normal inhibitor but left the specific antibody.

9. Preliminary tests with this simple chloroform extraction technique on "normal" sera from Americans and Japanese and on acute and convalescent sera from 3 Americans who contracted Japanese B encephalitis in Korea in 1946, showed that the method can be used for diagnostic and possibly also for survey purposes (see attached table).
### Table I

**Separation of Normal Inhibitor from Specific Antibody in Human Sera by Extraction with Chloroform**

<table>
<thead>
<tr>
<th>Source of serum tested</th>
<th>Inhibition titer* vs. 6 units of Japanese B hemagglutinin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated serum</td>
</tr>
<tr>
<td>Pool of 2 normal American children</td>
<td>160</td>
</tr>
<tr>
<td>Normal American adult — R. L.</td>
<td>320</td>
</tr>
<tr>
<td>&quot; &quot; — A. W.</td>
<td>640</td>
</tr>
<tr>
<td>American patient 2**— 4 days after onset</td>
<td>640</td>
</tr>
<tr>
<td>&quot; &quot; — 15 &quot; &quot;</td>
<td>320</td>
</tr>
<tr>
<td>&quot; &quot; — 4 &quot; &quot;</td>
<td>640</td>
</tr>
<tr>
<td>&quot; &quot; — 13 &quot; &quot;</td>
<td>640</td>
</tr>
<tr>
<td>&quot; &quot; — 4 &quot; &quot;</td>
<td>160</td>
</tr>
<tr>
<td>&quot; &quot; — 10 &quot; &quot;</td>
<td>640</td>
</tr>
<tr>
<td>&quot; &quot; — 25 &quot; &quot;</td>
<td>320</td>
</tr>
<tr>
<td>Japanese adults from Sapporo</td>
<td>SA-2E</td>
</tr>
<tr>
<td>No history of encephalitis</td>
<td>SA-7E</td>
</tr>
<tr>
<td>No neutralizing antibodies for</td>
<td>SA-8E</td>
</tr>
<tr>
<td>Japanese B virus</td>
<td>SA-9E</td>
</tr>
<tr>
<td>Japanese adults from Okayama</td>
<td>OK-E5</td>
</tr>
<tr>
<td>No history of encephalitis</td>
<td>OK-E7</td>
</tr>
<tr>
<td>Antibodies for Japanese B virus</td>
<td>OK-E9</td>
</tr>
<tr>
<td>present, suggesting inapparent</td>
<td>OK-E10</td>
</tr>
<tr>
<td>infection in the past</td>
<td></td>
</tr>
</tbody>
</table>

* Highest original dilution of serum (0.25 ml) added to hemagglutinin (0.25 ml) and 0.25% suspension of chick erythrocytes (0.5 ml) which prevented hemagglutination.

** These are unvaccinated American adults who contracted Japanese B encephalitis in Korea in 1946. For ease of comparison, the same numbers are used as in the communication in which these cases are reported (12).

*** The inhibition titers in 6 of the 8 Japanese sera tested are distinctly lower than any encountered in American sera tested thus far.

† It has proved possible to reduce these normal inhibitory titers to less than 5 by adjusting the chloroform-treated serum to pH 6.6 with 0.01 N HCl.
10. Another finding, whose significance cannot as yet be fully evaluated, is the specific inhibitory action of ZnSO₄ in minute amounts — 0.03 to 1.92 micrograms per unit of hemagglutinin, depending on the dilution. It was found that the zinc combined with the hemagglutinin in an equilibrium type of reaction, which could be reversed with reactivation of the hemagglutinin by the addition of H₂S or by dilution. FeSO₄, MnSO₄ and MgSO₄ were without effect; HgCl₂ and CuSO₄, which could be tested only in 1/80,000 concentration, were also without effect. ZnSO₄ had no inhibitory effect on influenza virus hemagglutinin.

11. Although this work was started because of Verlinde's personal and then published communication that Japanese B encephalitis and the "S.K.-M.M.-EMC-Mengo" virus behaved similarly in hemagglutination and indeed could not be distinguished from one another by hemagglutination inhibition tests, it is now clear that the two viruses have totally different properties and that no relationship is demonstrable between the two by hemagglutination inhibition. It is also evident that the Japanese B virus used by Verlinde had unfortunately become contaminated with the M.M. virus in his laboratory, a conclusion in which Verlinde now concurs. I am grateful to Prof. Verlinde because if this unfortunate accident had not occurred in his laboratory, I would not have embarked on this study.

Tests on Possible Interference between the Viruses of Japanese B Encephalitis and Poliomyelitis. Certain epidemiologic observations of Gen. Crawford F. Sams, MC, in Japan made it desirable to test the possibility that inapparent infection with Japanese B virus may modify the effects of infection with poliomyelitis virus. The Okinawa strain of Japanese B virus, which produces a high incidence of inapparent infection and a high degree of immunity after subcutaneous injection in adult mice, was injected in a group of 200 mice. At intervals of a few minutes, 1, 2, 3, 4, 7, and 10 days thereafter different groups received an intracerebral injection of 30 to 50 LD₅₀ of the Lansing strain of poliomyelitis virus, and the incidence and time of appearance of paralysis and death were compared with that in a group of 50 untreated mice which received the same dose of Lansing virus. No evidence of interference or enhancement was found. Furthermore, mice which were proved to be immune to an intracerebral injection of Japanese B virus, were found to have no immunity to 30 to 50 LD₅₀ doses of Lansing virus.

1 April - 30 June

Japanese B Encephalitis Virus Hemagglutinin. Further studies in this field were concerned with the practical application of this phenomenon for the following purposes:

a) diagnosis of acute infection
b) detection of inapparent infection for epidemiologic survey purposes
c) test for immunogenic capacity of vaccines

In all of these categories only orienting studies were carried out, realizing full well that additional adjustments and modifications in technique and criteria might prove necessary when the work is done on a large scale.

The optimum technique tentatively selected for the hemagglutination inhibition test with human sera involves the following:
1) The serum, heated at $56^\circ$ C for 30 min., is shaken with 4 volumes of chloroform for 2 min., and centrifuged at 2,000 r.p.m. for 45 min. The supernatant extracted serum is adjusted to approximately pH 6.5 as follows: 1 part undiluted serum + 2 parts N/100 HCl + 2 parts of 0.01 M phosphate buffered saline, pH 6.5. This pH adjustment was found necessary to obtain titers of 1:15 or less in American sera without antibody. However, for certain oriental sera (with presumably less protein) this adjustment yielded too acid a solution (which, in turn, nonspecifically inhibited hemagglutination to titers as high as 1:10), and it was necessary to eliminate the N/100 HCl. Further dilutions in all instances were made in 0.01 M phosphate saline, pH 6.5.

2) Although the highest titers were obtained with 1.5 units of hemagglutinin, it appeared advisable to use 3 to 6 units of a preparation of highest potency, i.e., 1:2560 to about 1:10,000 units per 0.5 ml. (based on original dilution before addition of 0.5 ml. of 0.25% chick RBC). The hemagglutinin must be kept in ice-water before addition to diluted sera, and RBC added immediately after the two are mixed.

3) The test, kept at room temperature, must be read within 45 to 60 minutes, because of the tendency of the agglutinated RBC to "slip" and for the typical "positive" pattern to disappear in the presence of "high" protein concentrations contained in sera diluted 1:5 to 1:80.

By means of this "adjusted" technique tests on 12 sera from people in the Cincinnati area yielded 10 with titers of less than 1:15 and 2 titers of 1:15. Similarly, of 12 oriental sera, without neutralizing antibody for Japanese B virus, 7 had titers of < 5, 4 titers of 1:15, and 1 questionable at 1:15 and 1:10. However, among 6 prevaccination sera from Army Medical School personnel there were 2 with titers of < 5, 2 with titers of 10, one with 1:20, and one with 1:40. Since no other data are available on the 4 sera with titers of 1:10 or over, it is not possible to tell whether they might be due to antibodies for the St. Louis encephalitis virus or the Japanese B virus. It should also be noted that 2 investigators in this laboratory, who apparently had acquired apparent infections while working with the Japanese B virus before 1940 and in 1942 respectively, still have sera with hemagglutination inhibition titers of 1:320.

Tests on 14 oriental sera, possessing neutralizing antibodies for the Japanese B virus, without, however, any history of encephalitis, exhibited the following titers:

<table>
<thead>
<tr>
<th>Titer of:</th>
<th>1:320</th>
<th>1:40</th>
<th>1:20</th>
<th>1:10</th>
<th>&lt; 1:10</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Sera:</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

It is perhaps noteworthy that the titer of 1:320, occurred in a 2 year old child from Okinawa. Accordingly, it was desirable to know what happens to the titers after a lapse of years in patients who have had clinical encephalitis. We were fortunate in being able to locate 2 of the Americans who contracted Japanese B encephalitis in Korea in 1946, on whom we had a number of sera at intervals up to 53 days after onset, and to obtain additional specimens 3 1/2 years after onset.
The tests on these sera are especially significant since both patients were removed from the area after their illness and had no further exposure to this virus. The results of a simultaneous test on all the samples were as follows:

<table>
<thead>
<tr>
<th>Time after Onset</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 days</td>
<td>320</td>
<td>80</td>
</tr>
<tr>
<td>7 &quot;</td>
<td>320</td>
<td>...</td>
</tr>
<tr>
<td>13 - 15 &quot;</td>
<td>640</td>
<td>320</td>
</tr>
<tr>
<td>20 - 22 &quot;</td>
<td>640</td>
<td>320</td>
</tr>
<tr>
<td>28 - 30 &quot;</td>
<td>320</td>
<td>320</td>
</tr>
<tr>
<td>39 - 41 &quot;</td>
<td>320</td>
<td>320</td>
</tr>
<tr>
<td>52 - 53 &quot;</td>
<td>320</td>
<td>160</td>
</tr>
<tr>
<td>3 1/2 years</td>
<td>40</td>
<td>80</td>
</tr>
</tbody>
</table>

These preliminary data indicate that both inapparent and clinically apparent infections can give rise to titers in the range of 1:320, and that the hemagglutination inhibition antibody persists for many years although in lower titer. However, in order to be able to correlate titer of antibody with "recency" of infection, it would be necessary to study a considerable population with inapparent and apparent infection during an epidemic, to determine the character of antibody response.

Before this test can be applied to animal sera for diagnostic or epidemiologic survey purposes, it will be necessary to work out for each species the optimum method for removing the "normal" hemagglutination inhibitor. Thus, while chloroform extraction was incapable of removing the normal inhibitor from mouse serum, acetone precipitation, with extraction of the precipitate by additional acetone, yielded serum proteins free of inhibitory capacity.

A preliminary test on 3 laboratory workers without antibody for the Japanese B virus indicated that the one individual who developed neutralizing antibody following administration of chick embryo vaccine also developed hemagglutination inhibition antibody to a titer of 1:320, while the other two developed neither antibody after the vaccine. The sera of six individuals (sent by Dr. Warren of the Army Medical School), which exhibited high titers of neutralizing antibody following booster injections of vaccine, all had hemagglutination inhibition antibody: 2 had titers of 1:20, one a titer of 1:40, two titers of 1:80, and one a titer of 1:160. In carrying out these tests it became apparent that all those inoculated with chick embryo vaccine developed hemagglutinins for chick or chicken RBC, in titers of 1:40 to 1:80, which had to be removed by absorption. Furthermore, it was important that the chloroform extraction be carried out after the absorption with chicken RBC. Otherwise the extracted serum proved capable of removing receptor substance from the RBC used for absorption and again became inhibitory.

Loss of Hemagglutinating Capacity after Prolonged Passage of Japanese B Virus in Mice. The Nakayama strain of the virus currently in use at the Army Medical School was originally derived from that passaged in Cincinnati. However, the A.M.S. strain has had at least 150 more passages in mice than that in Cincinnati. It was of interest, therefore, that the A.M.S. strain was found to be completely without hemagglutinating capacity. Neutralization tests and complement fixation tests carried out at the A.M.S. and in Cincinnati showed that both strains...
were identical by these tests, but differed in their capacity to produce hemagglutination. Hemagglutination tests with 6 additional strains of Japanese B encephalitis virus, 4 of them recently isolated by the 406th Medical General Laboratory, and the Kalinina and Matsunaga strains obtained from Japanese laboratories, all yielded hemagglutinins possessing the same properties, although not the same titers, as those obtaining for the Cincinnati, Nakayama virus. Accordingly it appeared reasonable to conclude that prolonged passage of the Nakayama strain at the A.M.S. was responsible for its loss of hemagglutinating capacity. This was an important principle, since if it obtained for other neurotropic viruses it might explain our inability to demonstrate hemagglutinins with strains that have had too many laboratory passages.

Demonstration of Hemagglutinins for Certain Other Neurotropic Viruses. Utilizing chick, sheep, rhesus and human type "O" erythrocytes and diluents buffered at pH 5.5, 6.5, 7.5 and 8.5, attempts were made to demonstrate hemagglutinins with the SLE, WEE, EEE, West Nile, Russian Spring–Summer encephalitis and poliomyelitis viruses.

Russian Spring–Summer Encephalitis Virus. A hemagglutinin became demonstrable at pH 7.5 only with sheep RBC at room temperature (26°C), but not at 4°C, which differentiates it from the hemagglutinin of the encephalomyocarditis — MM — Columbia S.K. virus. The pattern of agglutination was unique in that only part of the RBC formed a shield around a central button. The specificity of this reaction was indicated by the fact that anti-RSSE guinea-pig serum had a hemagglutination inhibition titer of 1:160, as compared with a titer of 1:20 for normal guinea pig serum; there was no difference in inhibitory activity of normal rabbit as compared with WEE and Jap. B immune rabbit sera. Chloroform extraction did not remove the normal inhibitor in this case. The RSSE hemagglutinin is relatively unstable on storage, even at 4°C, and much more work remains to be done on its basic properties before it can be used for practical purposes.

West Nile Virus. In the preliminary survey with different RBC and diluents of varying pH indicated above, the freshly prepared mouse brain extract yielded hemagglutination at room temperature only with chick and sheep RBC at pH 7.5, thus differing from the Japanese B hemagglutinin which gives negative results at pH 7.5. Subsequent tests showed that the effective pH range for West Nile virus was between 7.0 and 7.5 with an optimum at about pH 7.3. As the West Nile virus preparations were stored in the refrigerator, negative results were obtained with chick RBC, while the sheep RBC continued to yield positive hemagglutination. While many of the basic properties are still poorly understood and remain to be worked out, a peculiar phenomenon was discovered in the interaction of stored West Nile virus and chick RBC. Thus when the 2 components were allowed to interact at room temperature (25° C) and the RBC were left to sediment at 25° C, negative results were obtained. When the virus, diluent, and RBC were all cold to begin with (20–4°C), and allowed to sediment at 4°C, the results were also completely negative. But when the reagents were all at 25°C and the sediment was allowed to take place at 4°C, hemagglutination occurred with titers as high as 1:2560. This may be summarized as follows (remembering that it is true only for stored virus and chick RBC):
<table>
<thead>
<tr>
<th>Temperature of reagents at time of mixing</th>
<th>Temperature for subsequent sedimentation of RBC</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>25° C</td>
<td>25° C</td>
<td>Negative</td>
</tr>
<tr>
<td>25° C</td>
<td>4° C</td>
<td>Positive</td>
</tr>
<tr>
<td>2°-4° C</td>
<td>4° C</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Other tests also indicated that while the low temperature was needed for the final positive result, the higher temperature was needed for initial combination between the hemagglutinin and the RBC. Normal human and rabbit sera contain inhibitor which, as in the case of the Japanese B virus, can be removed by shaking with chloroform. Anti-West Nile serum exerted specific hemagglutination inhibition after chloroform extraction. Both human and rabbit anti-Japanese B sera contained high titers of hemagglutination inhibition antibody for the West Nile virus, indicating that the common antigen possessed by these viruses plays an important role in the hemagglutination reaction. Much more work remains to be done on the basic properties and stability of the West Nile virus hemagglutinin.

St. Louis Encephalitis Virus. The original work done with the Webster No. 3 strain which has had "hundreds" of mouse brain passages since its isolation in 1933, gave completely negative results with the various RBC and diluents of different pH. When prolonged passage was found to abolish the hemagglutinating capacity of the Nakayama strain of Japanese B virus, the tests were repeated with 3 different recently isolated strains of SLE virus, namely: "Winkler" (3 to 6 passages from human case), "Parton" (passages 2 and 3 from human case) and "MP 126" (passage 2 from pool of Culex tarsalis). Hemagglutination with chick and sheep RBC effective at about pH 6.3 to 6.7 at room temperature was demonstrated in low titer especially well with the "Parton" strain, with sheep cells more than with chick cells in the case of the "Winkler" strain, and only slightly so with sheep RBC only in the case of strain "MP 126." The preparations proved to be very unstable even after storage at 4° C for short periods of time. However, with both the "Parton" and "Winkler" strains, it was possible to demonstrate specific inhibition of hemagglutination with SLE antisera, and a high degree of crossing with Japanese B antisera. Much more work remains to be done on the basic properties of the SLE virus hemagglutinin before it can become useful for practical purposes.

Western and Eastern Equine Encephalitis Viruses. Since the original negative survey tests were carried out with strains of virus which had been continuously passaged in mice for almost 20 years, the work has been repeated thus far with 3 WEE and 2 EEE freshly isolated strains. Two of the 3 WEE strains gave negative results and one (North Dakota strain from the Amary Medical School) gave suggestively positive results with chick and sheep cells at pH 5.46 (0.01 M phosphate buffer) at room temperature in titers not exceeding 1:20. This has not yet been checked for serological specificity. The 2 recently isolated EEE strains yielded negative results, but further work with additional strains and modifications of technique are indicated with this group of viruses.

Poliomyelitis. Although negative results were obtained with the mouse-passaged Lansing virus, reproducible hemagglutination was obtained at 37° C, pH 5.4-6.2 (0.01 M phosphate buffer) with human type "A" and "O" cells but not with
chick or sheep cells when early monkey passage material of several recently recovered strains was used. However, while normal monkey cords were negative under the same conditions, the hemagglutination could not be inhibited by specific antiserum. Further work with the poliomyelitis group of viruses is also indicated.

Capt. Edward L. Buescher collaborated in this work. John Wallace, M.Sc., and Harold Nolting, B. S., provided technical assistance, and Miss Maria J. Clark, B.A., administrative and secretarial assistance.

IV. Dr. W. P. Havens, Jr., MD 403 (Jefferson Medical College, Philadelphia, Pennsylvania).

(a) In conjunction with the Commission on Liver Diseases, determinations of the capacity of patients with chronic hepatic disease to elaborate antibody to diphtheria toxoid are being made. Nineteen patients with chronic viral hepatitis or Laenneque's cirrhosis have been studied and a wide variability of response is evident. Preliminary observations suggest that the capacity to make antibody (to diphtheria toxoid) persists in the presence of severe hepatic disease and hypoalbuminemia. In general, those patients with the largest amounts of serum globulin make the greatest amount of antibody. The effect of administration of ACTH on the level of antibody is being studied.

The serums of 90 patients in various phases of viral hepatitis (largely in the first two weeks) were tested for the presence of C-reactive protein, and the results were uniformly negative.

Drs. J. R. Paul and W. P. Havens, Jr., made a trip to Germany between 9 February and 4 March 1950 for the purpose of inspecting the work done at the Hepatitis Center at the 98th General Hospital, Munich. A report of the findings and recommendations was submitted to the Office of the Surgeon General 11 March 1950.

(b) During the next six months, it is planned to continue the study of production of antibody and the effect of ACTH upon it. In addition, it is hoped to initiate attempts to adapt hepatitis virus to embryonating eggs and certain laboratory animals. The possibility of obtaining volunteers through the assistance of Dr. Mirick at Johns Hopkins Medical School is again under consideration.

V. Dr. J. C. Snyder, MD 402 (Department of Bacteriology, School of Public Health, Harvard University, Boston, Massachusetts.

Brill's Disease. With the cooperation of the staff of the Beth Israel Hospital a screening test is being studied for the detection of subjects who might be candidates for Brill's disease. A rickettsial agglutination test using highly purified suspensions of epidemic rickettsiae has shown a surprisingly large number of positive sera in low titers among foreign-born patients; comparisons with complement fixation tests are in progress. When we have developed a satisfactory and reliable method to indicate those subjects who definitely have specific epidemic typhus antibodies in low titer, we then will attempt the isolation of rickettsiae from such subjects for various procedures including sternal puncture to secure bone marrow.

A group of 59 foreign-born persons has now been listed from the recent admissions to the Beth Israel Hospital. These persons have been admitted to and discharged from the hospital for miscellaneous disorders. All have antibodies to
to epidemic typhus antigen by complement fixation as well as rickettsial agglutination tests. Arrangements are being made to follow these subjects by serologic tests at suitable intervals, and to obtain specimens for possible isolation of *R. prowazekii* when conditions permit.

Fuller's membrane technique has been used for a careful study of the effect of temperature and humidity on the longevity of normal lice as well as the development of experimental epidemic typhus in these insects. Some of the lice survived nearly a month after an infecting meal, finally succumbing to a massive rickettsial infection. The incidence of bacterial contamination in female lice taken one to three days after molting from the third instar stage was negligible, whereas male lice of the same age had a high rate of bacterial infection. This experiment will be repeated and the results will be applied to titrations of viability of rickettsiae using the body louse as the experimental animal.

Further experiments have been carried out with various tissue extracts for the enhancement of survival of typhus rickettsiae. Metabolic studies are continuing. Comparisons have been made of the relative effectiveness of aureomycin, chloramphenicol, and terramycin in chick embryos, mice, and cotton rats. The antirickettsial activity of terramycin is very similar to that of aureomycin and chloramphenicol.

Dr. E. S. Murray left for Yugoslavia on June 10 to obtain field data from typhus zones in which he worked during 1945.

Dr. H. S. Fuller's insect experiments are continuing. The optimum conditions for use of human body lice as experimental animals for rickettsiae are being worked out.

VI. Dr. J. R. Paul, MD 376 (Yale University, New Haven, Connecticut).

**Hepatitis**

A. Volunteer experiments: Two experiments attempting transmission of hepatitis to volunteers, ages 21 to 25, have been completed and the results are summarized in Table II. In experiment 1, one sample of serum (J.W.) produced jaundice in 3/3 volunteers, and the other sample produced no jaundice in three volunteers. While the incubation period in the jaundiced group was long (56-89 days), 2/3 experienced mild and transient symptoms consisting of malaise, slight fever, and a tender liver three weeks after inoculation. Similar mild and transient symptoms were also noted at the same time in 2/3 of the volunteers who never developed definite jaundice.

In experiment 2 several features are worthy of comment: (1) no volunteer developed definite evidence of hepatitis although one had an elevated serum bilirubin without symptoms; (2) two out of three volunteers in each of the three groups tested developed symptoms suggestive of mild hepatitis without jaundice and without marked changes in liver function tests; (3) such symptoms occurred from 41 to 64 days after oral administration of serum, a period beyond the expected incubation period of infectious hepatitis. Such observations emphasize the problem of what can be considered a "take" in a given volunteer.

B. "Gamma globulin": The turbidometric determination of substances with the solubility characteristics of gamma globulin by the ammonium sulfete technique has been carried out on the serum of volunteers inoculated in experiment 1, which
<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Inoculum*</th>
<th>Route</th>
<th>No. of Men inoculated</th>
<th>Result**</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Serum #1</td>
<td>Oral and Parenteral</td>
<td>3</td>
<td>3/3</td>
<td>Clinical and laboratory evidence of jaundice in all three with incubation periods of approximately 56, 66, and 89 days.</td>
</tr>
<tr>
<td></td>
<td>Serum #2</td>
<td>Oral and Parenteral</td>
<td>3</td>
<td>0/3</td>
<td>No clinical or laboratory evidence of hepatitis.</td>
</tr>
<tr>
<td>2</td>
<td>Serum #3</td>
<td>Oral</td>
<td>3</td>
<td>0/3</td>
<td>A. Elevated serum bilirubin (2.16 mgm percent) at 44 days. No signs or symptoms except for tender liver at 64 days.</td>
</tr>
<tr>
<td></td>
<td>Serum #4</td>
<td>Oral</td>
<td>3</td>
<td>0/3</td>
<td>B. Continuing vomiting, abdominal distress, malaise from 45-60 days after inoculation. No definite clinical or laboratory evidence of hepatitis.</td>
</tr>
<tr>
<td></td>
<td>Serum #6</td>
<td>Oral</td>
<td>3</td>
<td>0/3</td>
<td>C. No signs or symptoms, or laboratory changes.</td>
</tr>
</tbody>
</table>

* These sera were obtained from patients with acute hepatitis in the Hepatitis Research Center, Munich, Germany.

** Numerator = no. of men who developed definite jaundice; Denominator = no. of men inoculated. A, B, and C refer to individual volunteers in each group.
produced long incubation period hepatitis in 3 volunteers. Variations in values for this test beyond the upper limit of normal (1.25 gms/100 cc) were uncommon, even during the period of clinical jaundice. Fluctuations up to and slightly beyond this normal value were observed not only at the time of icterus but also during the incubation period. This latter change occurred 14-21 days after inoculation during the period of transient symptoms, and a questionable increase was also seen at about 40 days. Similar patterns were also found in the serum of volunteers who did not develop definite icterus, i.e., peaks at approximately 3 weeks and 9 weeks after inoculation. The reliability and reproducibility of this test in our hands is not sufficiently well established to place undue emphasis on these results.

C. Experiments in chimpanzees: Four young chimpanzees were inoculated intravenously and intramuscularly with serum shown in volunteer experiments to contain a long incubation period icterogenic agent. Serum for intramuscular injection was incorporated in an adjuvant consisting of paraffin oil, Falba, and heat killed TB bacilli. All chimp developed a slow-healing sterile abscesses at the site of intramuscular injection one month after inoculation. None of these animals developed clinical or laboratory evidence of jaundice over an observation period of over 100 days, nor did significant alteration in ammonium sulfate turbidity units occur in their sera.

D. Chick embryo experiments: Several samples of sera from patients in the acute phase of hepatitis, one of which was known to be icterogenic in humans, have been passed in embryonated eggs by the amniotic or allantoic routes without producing histologic evidence of hepatitis in chick liver tissue. Concentrates of allantoic fluid of the 8th passage of known icterogenic serum have failed to fix complement in the presence of convalescent sera from volunteers infected with this strain and have failed to alter human Rbc so that they are agglutinated by these convalescent sera.

E. Electron microscope studies: The possible effect of disease, particularly hepatitis, and virus particles on the human red blood cell is being investigated. By means of a replica technique, details of the surface structure of erythrocytes and materials attached thereto are being studied.

This work involves essentially 3 phases, which are being conducted concurrently: (i) the determination of the characteristics of normal blood when examined by this technique, (ii) the effects of various diseases, and (iii) the alterations produced by addition of viruses to nonlysed blood cells.

Blood smears from a group of laboratory personnel, hospital patients, two groups of volunteers in hepatitis transmission experiments, and a group of hepatitis patients are being studied in the electron microscope.

At present four classes of red cells are recognized in whole blood smears, by this technique. They are (1) normal cells, (2) crenated cells, (3) cells with a rough surface, and (4) cells having unusual particles or outlines on their surface.

In addition, normal red cells have been treated with suspensions of influenza virus (PR8 strain). The particulate bodies which seem to adhere to the surface of the cells have a diameter within the ranges of sizes for influenza virus.
F. Immunological studies of several types have been carried out with special emphasis on use of the serum pool known to contain the agent of SH. These experiments have been negative or inconclusive and include the following: a) interference in newborn mice between hepatitis and Coxsackie virus (Easton strain), b) similar interference experiments carried out in chick embryos using Newcastle disease virus, c) "sensitization" of human Rbc by acute phase hepatitis sera and testing for agglutination with convalescent sera, d) immunization of rabbits with Rbc exposed to hepatitis sera and testing this serum.

Newcastle Disease: (Dr. F. W. A.

An epidemiological and serological study has been carried out in an attempt to evaluate the possible importance of NDV in producing systemic disease in man. The following observations have been made: of 94 individuals interviewed who had intimate contact on poultry farms with chickens ill with NDV, none had illnesses serious enough for hospitalization and minor illnesses were unrelated in time to the occurrence of NDV in the chickens. Fifteen of 33 sera from these individuals inhibited the hemagglutination of NDV in a titer of 1:16 or more but the occurrence of this antihemagglutinin was unrelated to the occurrence of minor illnesses, and a similar incidence of this antibody was observed in a group of 100 control sera studied. A heat labile substance capable of neutralizing several logs of NDV in chick embryos was found in these sera as well as in normal rabbit and normal monkey serum. A heat stable neutralizing antibody was not demonstrated in significant titer. Preliminary studies of the NDV antihemagglutinin in these sera suggest that it may represent an atypical immunological response to mumps infection. It is concluded that in the group studied, NDV was not responsible for systemic infections.

Respectfully submitted,

John R. Paul, M.D.
Director

JRP:mmr
PUBLICATIONS

1 January - 30 June 1950


