when run in antihemagglutination tests with a standard NDV-immune serum from the Bureau of Animal Industry. Inoculated mice developed encephalitis suggestive of Newcastle disease in fowls. Viruses from infected mouse brains, produced Newcastle disease when inoculated intracerebrally into 2-week-old chicks. The agents transmitted were resistant to freezing and thawing, penicillin, and streptomycin. The ease with which 2 different strains of NDV are transmissible in the brains of suckling mice may have value in investigations of the immunology and other aspects of Newcastle disease. Further passages, now in progress, suggest that weanling and older mice are susceptible to NDV following preliminary passages already described.

Summary. Suckling mice of the Swiss albino strain are susceptible to Newcastle disease of fowls following intracerebral inoculation. This has been demonstrated by 10 consecutive passages of the California and of an Australian strain of NDV.

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Unique Physico-Chemical Properties of Japanese B Encephalitis Virus Hemagglutinin.* (17861)

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The discovery of the phenomenon of hemagglutination with the influenza viruses(1,2) has provided an important new tool for the study of certain basic properties of a number of viruses(3,4). No such hemagglutinins, however, could be demonstrated for the neuronotropic viruses affecting the human nervous system. Recently, the phenomenon has been found to occur with the virus variously known as the "Columbia S.K.," "M.M.," encephalomyocarditis (EMC), or Mengo encephalomyelitis(5-7) and with the GDVII strain of mouse encephalomyelitis(8). During a visit to the University of Leiden (Holland) in September, 1949, Prof. J. D. Verlinde informed one of us (A.B.S.) that he obtained the same results with the Nakayama strain of Japanese B encephalitis as with the "S.K.-M.M." virus, and that by hemagglutination inhibition tests it was indeed not possible to distinguish these viruses. Olitsky and Yager(6), however, shortly thereafter in reporting their own studies with the "S.K.-M.M." virus indicated that they obtained only negative results with the Japanese B encephalitis virus and a number of other viruses affecting the human nervous system. In an attempt to clarify this situation it was discovered that the virus of Japanese B encephalitis was indeed associated with a specific hemagglutinin. Its properties, however, were found to be so different from all others previously described that it became evident that Verlinde and DeBaan(7) were dealing not with the Japanese B encephalitis virus, originally obtained from this laboratory, but with

* Aided by the Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board.

† Captain, M.C., U.S.A., on active duty.

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the "S.K.-M.M." virus with which an unfortunate accidental contamination must have occurred.‡

The original positive results in this laboratory were obtained with centrifuged, unbuffered saline extracts of infected mouse brains and 0.25% suspensions of either sheep or chick cells, incubated at room temperature (over 22°C) or 37°C. Agglutination of the erythrocytes was evident only in the sedimentation pattern, the affected cells settling out in a shield over the entire round bottom of the tube, while the unaffected cells sedimented in a compact button as described by Salk(9) for the influenza viruses. Further experience with this system indicated, however, that the erythrocytes obtained from the same chicks at various periods after hatching or from different sheep not infrequently yielded negative or very poor results. Regular and reproducible results were obtained only with erythrocytes from chicks bled during the first 24 hours after hatching. No hemagglutination was obtained with human type "O" or *Macacus rhesus* erythrocytes. Extracts similarly prepared from uninfected mouse brains or from brains infected with the viruses of St. Louis (SLE), Western equine (WEE), Eastern equine (EEE), or Russian spring-summer (RSSE) encephalitis, or with the EMC (encephalomyocarditis) virus, yielded negative results with chick cells, which in simultaneous tests gave positive hemagglutination with the standard Nakayama and the more recently recovered Okinawa(10) and Korea(11) strains of Japanese B encephalitis virus.

When the phosphate-buffered saline (pH 7.6) used by Olitsky and Yager(6) was substituted for the unbuffered saline, we also obtained only negative results with the Japanese B virus. Preliminary tests indicated that in certain concentrations phosphate, citrate, borate and arsenate ions prevented hemagglutination, while oxalate, fluoride, tartrate and sulfate ions did not. It was found, however, that pH, rather than the ions present, was the determining factor in hemagglutination with this virus. The reaction occurred only in the narrow zone between pH 6.2 and 7.1, the optimum being between pH 6.5 and 6.8. Saline buffered with 0.01 M collidine prepared according to Gomori(12) or with 0.01 M borate yielded results very similar to those obtained with 0.01 M phosphate. The pH zone was narrower within this range and the activity very much lower when all the dilutions were first prepared in unbuffered 0.9% NaCl and the buffers of different pH added afterwards than when the dilutions were made directly in the buffered solutions of 0.9% NaCl. Hemagglutinin titers in unbuffered saline varied with the age of the preparation and various other factors, to be described elsewhere, but the marked enhancement of titer (from 1:60 to 1:5,120 shown in Fig. 1) occurred only when the dilutions of appropriately prepared extracts were made in the buffer and not when they were first made in saline and adjusted to the optimum pH and buffer concentration afterwards. It is also noteworthy that this enhancing action of appropriate pH and buffer concentration is obtained regularly only with erythrocytes of newly hatched chicks, and irregularly, not as well or not at all with those of chickens or sheep. Suitable experiments have shown that the hemagglutinin is not rapidly destroyed at a pH directly to the acid or the alkaline side of the required zone but rather that it does not combine with the erythrocyte receptors except in the narrow pH zone indicated above. Hemagglutination cannot be reversed merely by resuspension in solutions of a pH outside the required zone.

Continued experience with extracts prepared from mouse brains infected with the virus of Japanese B encephalitis yielded highly variable and occasionally negative results which suggested that the infected mouse brain

‡ In a recent personal communication Prof. Verlinde indicated that he agrees with this conclusion.

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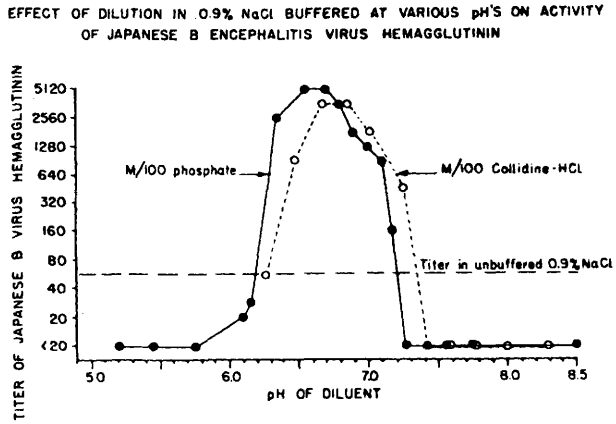

FIG. 1.

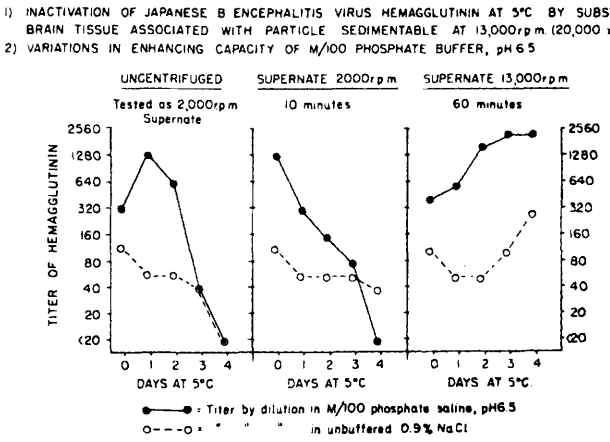

FIG. 2.

suspension was a highly complex, dynamic mixture in which a variety of reactions influencing the ultimate activity of the hemagglutinin were in progress. A systematic investigation of hemagglutinin activity in various fractions of a 20% suspension of infected mouse brains in unbuffered saline at various time intervals after preparation yielded information which permitted the separation of the hemagglutinin in a form which remained stable at 4 to 6°C for many weeks. Two findings require special mention: (1) that 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) inactivates the hemagglutinin at about 5°C in 3 to 4 days; (2) that the 13,000 r.p.m. supernate increases in activity after storage at about 5°C for several days and then remains stable for 8 weeks or longer (Fig. 2). Based on these studies, the following optimum method for preparing Japanese B encephalitis virus hemagglutinin was devised.

Mice inoculated intracerebrally with 10% virus suspension are bled and sacrificed when they are almost prostrate (dead mice are not used) and a 20% suspension of the brains, washed free of blood, in unbuffered, 0.9% NaCl is prepared by grinding with alundum in a mortar. The Waring blendor is not used because severe agitation is deleterious to the hemagglutinin. The brains are not suspended in buffered saline of optimum pH because this results in extraction of larger amounts of inhibitor or inactivating sub-

stances. After storage of the uncentrifuged brain suspension at 4 to 6°C for not less than 3 hours and not more than 12 hours, it is centrifuged either at 2,000 r.p.m. for 10 minutes or preferably at 13,000 r.p.m. in the cold for 10 minutes. The supernatant liquid is then centrifuged at 13,000 r.p.m. for 1 hour in the cold room. The 13,000 r.p.m. supernate is then stored in an ordinary refrigerator at 4 to 6°C. Although high activity is often demonstrable immediately, the maximum titer of hemagglutinin may not appear for 3 to 10 days. The Nakayama, Okinawa and Korea strains of Japanese B encephalitis have yielded similar results with this method of preparation. Extracts of brains from uninoculated mice or from mice inoculated with the EMC, WEE, EEE, SLE or RSSE viruses prepared and stored in the same manner have given completely negative results with chick cells.

The marked instability of *various dilutions* of this hemagglutinin at temperatures above 4 to 6°C is especially noteworthy. The results shown in Fig. 3 are typical for the inactivation observed when a series of dilutions prepared in 0.01 M phosphate saline, pH 6.5, are stored at different temperatures for 30 or 60 minutes before addition of the 0.25% chick erythrocyte suspension. Glycine in 0.25% to 1% concentration in phosphate-buffered saline, pH 6.5, without significantly affecting the pH depressed the titer and did not alter the stability of the diluted hemagglutinin at room temperature. Rapid freezing at about –80°C (solid CO_2 and alcohol) and thawing, as well as lyophilization, have a different effect on the 20% uncentrifuged brain suspension and the 13,000 r.p.m. supernate. The uncentrifuged suspension retains its activity after a single freezing and thawing but loses it completely after being frozen and thawed 5 times in rapid succession, as well as after lyophilization. The 13,000 r.p.m. supernate, on the other hand, loses all of its activity after a single freezing and thawing, but retains approximately 50% of its activity after lyophilization. Mere bubbling of nitrogen gas through a 1:80 dilution of hemagglutinin in saline buffered at pH 6.8 with 0.01 M borate, kept in an ice bath, was enough to inactivate 75% of it in 5 minutes and 96% in 20 minutes, without significantly altering the pH.

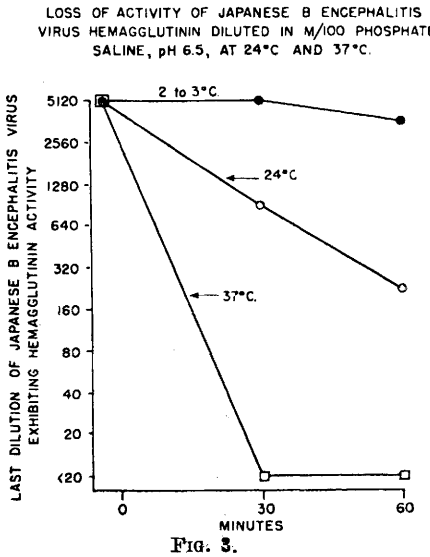

FIG. 3.

The fact that the 13,000 r.p.m. supernates exhibited high activity suggested that the particle size of much of the Japanese B encephalitis hemagglutinin was less than 100-150 mμ. Centrifugation of the 13,000 r.p.m. supernates on the Spinco preparative, vacuum ultracentrifuge§ for 1 hour removed 75% of the hemagglutinin from the entire supernatant liquid at 21,740 r.p.m. (average 27,950xG), and practically 100% at 31,440 r.p.m. (average 58,300xG). The hemagglutinin would thus appear to be a macromolecular substance not smaller than the infective particle of Japanese B encephalitis virus, which by gradocol membrane filtration was previously found to be in the range of 15-22 mμ. However, several gradocol membrane filtration tests with hemagglutinin diluted in 0.01 M phosphate buffered saline, pH 6.5, or in unbuffered saline yielded data which were not in agreement with those obtained by centrifugation. The filtrates through membranes with an average pore diameter (A.P.D.) of 260 mμ or greater contained hemagglutinin

§ We are indebted to Drs. J. E. Smadel and J. Warren of the Army Research and Graduate School for putting this centrifuge at our disposal, and to Dr. Warren and Capt. M. Weil, M.C., for their help with the tests.

while those from membranes with an A.P.D. of 200 mμ or less did not. One cannot be certain from the available data that the infectious particle and the hemagglutinin are identical, although they suggest that much of the hemagglutinin may exist in an aggregated or polymerized form which is larger than the infectious particle. This larger component may either represent the precursor or other modified form of the infectious particle, or a specific product of infection unrelated to it.

Many experiments on the nature of the combination between the hemagglutinin and the chick erythrocytes suggest that hemagglutinin (H) combines with receptors (R) on the erythrocytes in an equilibrium type of reaction which may be expressed as:

$$H_x + R_y \rightleftharpoons H_xR_y$$

The data suggest that the number of R on a single erythrocyte combined with H varies with the concentration of H and the number of erythrocytes in the mixture, and that a minimum number of R on each cell must be combined with H to result in hemagglutination. Thus, in a solution properly buffered for optimum dispersion of H and combination with R, the hemagglutinin titer diminishes in a constant ratio as the concentration of erythrocytes increases (Fig. 4). When a series of tubes containing varying amounts of erythrocytes agglutinated by decreasing amounts of hemagglutinin (H) are repeatedly shaken at room temperature or at 37°C, the agglutination is reversed first in the mixtures with the smallest amount of H, while in the mixtures containing the largest concentration of H reversal occurs when the number of erythrocytes is large but not when it is small (at least not during a period of 48 hours). There is no evidence that the Japanese B encephalitis hemagglutinin is associated with a receptor destroying enzyme as is the case with influenza and certain other viruses. The reversal of agglutination here is due rather to the rapid destruction of unbound hemagglutinin (H) at room temperature and 37°C with the result that the equilibrium in the above reaction is more in the direction of dissociation of H_xR_y with the liberation of free "R" groups on the erythrocytes. The erythrocytes with the smallest number of R originally combined thus revert to their original state first. That the receptors (R) are unaffected in this reaction is evident from the fact that the erythrocytes are readily reagglutinated by the addition of more hemagglutinin (H). It may be worth noting here that in several simultaneous experiments, the enzyme or enzymes of *Vibrio cholerae*, as was expected, destroyed the receptor for the influenza virus on chick erythrocytes, but were without effect on the receptor for the hemagglutinin of Japanese B encephalitis virus.

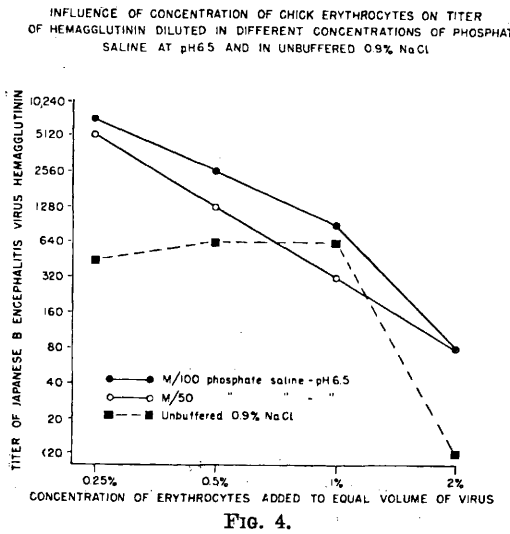

FIG. 4.

Normal human, rabbit, monkey and mouse sera were found to be capable of inhibiting hemagglutination by the Japanese B encephalitis virus. The inhibition in higher dilutions was due neither to the buffering capacity of the serum nor to any dialyzable component, and was only partly reduced by boiling for 15 minutes. The inhibitor does not act on the erythrocytes but appears rather to combine with the hemagglutinin. When the hemagglutinin is diluted in appropriately buffered saline, the inhibition titer is directly proportional to the concentration of hemagglutinin (Fig. 5); this proportionality cannot be demonstrated with hemagglutinin diluted in unbuffered saline. The available data furthermore suggest that the principal inhibitor in normal serum may represent cell receptor (R) in solution or a substance hav-

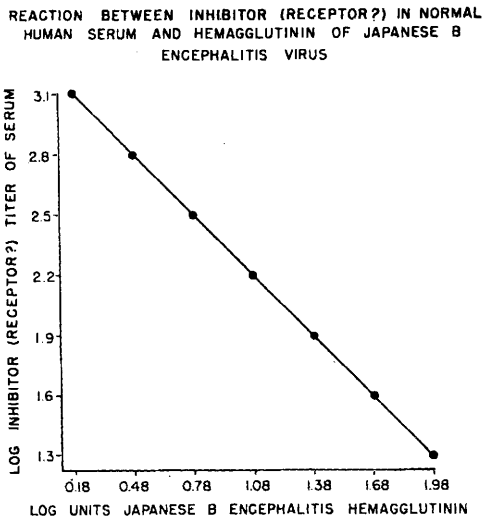

FIG. 5.

ing an affinity for the same combining groups on H. Thus, by the addition of diluted normal serum (free R?) it is possible to remove some of the H from the R on the erythrocytes and to reverse the agglutination of cells which had combined with a small amount of H (2 to 8 units). Although it was found that hyperimmune animal sera and sera from human beings recently recovered from Japanese B encephalitis generally possessed higher inhibitory titers than normal sera, the existence of a specific antibody for this hemagglutinin was established by experiment. After preliminary bleeding, one group of mice was given a series of inoculations of normal mouse brain suspension, another mouse brains infected with the virus of Western equine encephalitis (WEE), and a third with Japanese B encephalitis virus. The inhibitory titers of the preinoculation and postinoculation sera were identical in the groups which received normal mouse brain or the WEE virus, but increased 4 to 8-fold in the mice inoculated with the virus of Japanese B encephalitis. Neither normal sera nor hyperimmune Japanese B virus sera were capable of inhibiting the hemagglutination of sheep erythrocytes by the EMC (encephalomyocarditis, M.M., Columbia S.K., Mengo encephalomyelitis) virus, thus providing additional evidence that Verlinde and DeBaan(7) were not working with Japanese B encephalitis virus when they reported that these viruses could not be distinguished from one another by hemagglutination inhibition tests.

However, since the inhibitory titers of normal sera were high and variable, the hemagglutination inhibition test could not be used as a simple indicator of infection with the Japanese B encephalitis virus until some means could be found of removing the normal inhibitor without affecting the specific antibody. Periodate and several strong proteases had no effect on the normal inhibitor. The lecithinase in highly potent, purified *Clostridium perfringens* toxin, in the presence of calcium, changed only the heat stability of the normal inhibitor permitting its destruction by boiling. Extraction with lipid solvents (ether, benzene, chloroform, ethylene chloride) and precipitation and extraction with acetone or alcohol-ether mixtures were tried to test the hypothesis that the inhibitor (or receptor) might be a phospholipid or lipoprotein. It proved possible to remove the normal inhibitor to varying extent by these methods from rabbit serum and human serum but only partially from mouse and monkey serum. Simple shaking of 1 ml of fluid human or rabbit serum in 4 to 12 ml of chloroform yielded the best results with the removal of approximately 95% of the normal inhibitor, but leaving the specific antibody in seemingly undiminished titer. The results obtained with a number of normal human sera and the acute and convalescent sera of 3 unvaccinated Americans who contracted Japanese B encephalitis in Korea(11), shown in Table I, indicate that the simple chloroform extraction technique makes it possible to use the hemagglutination inhibition test for diagnostic purposes and as an indicator of previous infection with the virus of Japanese B encephalitis.

During the course of investigations on the possible role of iron-binding substances as inhibitors it was found that partially purified preparations derived from cultures of *Clostridium histolyticum* and *Clostridium lentoputrescens* (but not from *Clostridium perfringens*)‖ were capable of inhibiting hemagglutination by the Japanese B virus in final dilutions as high as 1:1,000,000 after boiling

TABLE I.
Separation of Normal Inhibitor from Specific Antibody in Human Sera by Extraction with Chloroform.

Source of serum tested		Inhibition titers* vs 6 units of Japanese B hemagglutinin	
		Untreated serum	Chloroform-treated
Pool of 2 normal American children		160	10§
Normal American adult—L.		320	10
" " " —W.		640	10
American patient 2†— 4 days after onset		640	320
15		320	320
" " 3 4 " " "		640	20
13 " " "		640	320
" " 4 4 " " "		160	20
10 " " "		640	320
25 " " "		320	320
Japanese adults from Sapporo.	SA—2E	40‡	10§
No history of encephalitis	SA—7E	160	10
No neutralizing bodies for	SA—8E	20	10
Japanese B virus	SA—9E	320	<10
Japanese adults from Okayama.	OK—E5	40	40
No history encephalitis.	OK—E7	80	40
Neutralizing antibodies for Japanese	OK—E9	40	20
B virus present, suggesting inapparent infection in the past	OK—E10	40	20

* 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 (11).

‡ 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.

for 15 minutes. The effect of these preparations was shown to be on the hemagglutinin and not on the receptors of the erythrocytes. Further purification of these substances by Dr. A. A. Tytell indicated that the inhibitor was associated with certain nucleic acid or protein components which were different from the proteinase. During the course of this work it was also found that in suitably buffered solution diethyl dithio carbamate (in a final concentration of about 1:2,000) was capable of inhibiting the hemagglutination, while the other, simple organic substances, 8-hydroxyquinoline and α, α'dipyridyl which also bind heavy metals, although not as well in the presence of proteins, were without effect in 20 times greater concentration.

In studying the effects of various metallic ions, it was discovered that $ZnSO_4$ in minute concentration (from 0.03 to 1.92 μg per unit depending on dilution) was capable of inhibiting hemagglutination by the Japanese B virus hemagglutinin, while $FeSO_4$ $MnSO_4$ and $MgSO_4$ were without effect. Bichloride of mercury and copper sulfate, beyond M/70,000, because in greater concentration they produced hemagglutination or hemolysis by themselves, were without effect on the hemagglutination produced by the virus. It was found that the inhibitory concentrations of $ZnSO_4$ (from M/4,000 to M/28,000 depending on the concentration of hemagglutinin) were without effect on the erythrocytes, but reacted rather with the hemagglutinin. The influenza virus hemagglutinin proved to be unaffected by

|| We are indebted to Dr. A. A. Tytell of the Department of Biochemistry for putting these preparations at our disposal.

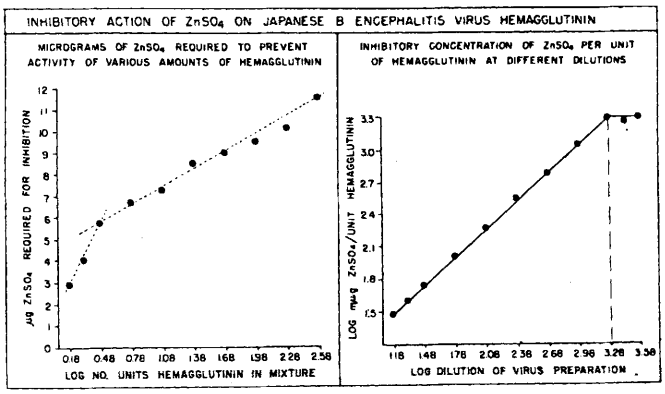

FIG. 6.

M/4,000 $ZnSO_4$. The reaction between $ZnSO_4$ and the Japanese B virus hemagglutinin (H) was found to be of the equilibrium type, which may be expressed as:

$$H_x + Zn_y \rightleftharpoons H_xZn_y$$

It proved possible to reverse this reaction by dilution and by the careful removal of Zn from the mixture with H_2S, which also indicated that the hemagglutinin was not denatured in the process. The amount of $ZnSO_4$ required to inhibit varying amounts of H was proportionately considerably greater when H was in highly dilute solution than when it was more concentrated (Fig. 6). It could also be shown that different amounts of $ZnSO_4$ were required to inhibit the same amount of H contained in different volumes, which may either be due to a disaggregation, "unfolding," depolymerization or dissociation of H upon dilution resulting in the appearance of more of the groups which combine with zinc, or to some other change in the kinetics of the reaction. All the experiments with zinc were carried out in 0.01 M collidine buffer (pH 6.5) or 0.01 M borate buffer (pH 6.8).

The unique properties of the Japanese B virus hemagglutinin discovered in this investigation indicate the lines along which the search for similar hemagglutinins may profitably be made in nervous tissue infected with the poliomyelitis and other encephalitis viruses. What is most important to remember is that the physico-chemical properties and biophysical state of the hemagglutinin may be totally different from that of the infectious particle, and that each virus may have its own narrow zone of pH for combination with receptor, and each be associated with its own special variety of inhibitors and inactivators. Utilizing chick, sheep, rhesus and human type "O" erythrocytes and diluents buffered at pH 5.5, 6.5, 7.5 and 8.5, a preliminary survey has been made of other neurotropic viruses and specific hemagglutinins have already been found for the West Nile and Russian Spring-Summer encephalitis viruses. The West Nile hemagglutinin became demonstrable with chick and sheep erythrocytes at pH 7.5 and the RSSE hemagglutinin only with sheep erythrocytes at pH 7.5, and both of these hemagglutinins gave negligible or negative reactions at pH 5.5, 6.5 and 8.5. The other properties of these hemagglutinins are now under investigation and appear to be different from those reported here for the hemagglutinin of the Japanese B virus.

Summary. A specific hemagglutinin was recovered from mouse brains infected with the virus of Japanese B encephalitis, and it was found to possess properties different from all other viral hemagglutinins described thus far. Stable preparations of high potency were obtained after removal of an inactivating substance by differential centrifugation. The hemagglutinin combined with the receptors on the erythrocytes in a very narrow pH zone, the optimum being between 6.5 and 6.8. Marked enhancement of activity was obtained on dilution in certain buffers of optimum pH. No receptor destroying enzyme was found either in the infected mouse brains or in

cultures of *Vibrio cholerae,* which destroyed the receptors for influenza virus. $ZnSO_4$ in minute amounts inhibited the hemagglutinin of the Japanese B virus but not that of influenza virus. The inactive zinc-hemagglutinin complex was reactivated by dilution or removal of zinc with H_2S. The inhibitor present in normal human serum was removed by shaking with chloroform, while the specific hemagglutination inhibiting antibody was unaffected, thus providing a simple test for diagnosis of infection with the Japanese B virus. Specific hemagglutinins, with properties different from that of the Japanese B virus, have also been found for the West Nile and Russian Spring-Summer encephalitis viruses.

Addendum. Since this paper was submitted for publication, work carried out in association with Drs. J. Warren and M. Weil of the Army Medical School, indicated that prolonged passage of the Nakayama strain of Japanese B encephalitis virus in mice resulted in complete loss of its hemagglutinating activity. Tests on 6 additional strains of Japanese B encephalitis virus, with relatively few passages in mice, revealed that all possessed a hemagglutinin similar to that described here for the Nakayama, Okinawa and Korea strains. Since the SLE, WEE, and EEE strains of virus we had used, had all had hundreds of mouse passages for many years, we repeated the work with freshly isolated strains of these viruses and have succeeded in demonstrating a specific hemagglutinin for chick and sheep cells at pH 6.5 to 7.0 with 3 strains of SLE, but thus far not with WEE or EEE. This work will be reported in detail in forthcoming publications.

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A Method for the Determination of Sodium Ferrocyanide at Low Concentrations in Body Fluids.* (17862)

GEORGE S. HUSSON (Introduced by Kendrick Hare)

From the Department of Pediatrics, University of Buffalo, and the Statler Research Laboratory, Children's Hospital of Buffalo, N. Y.

Gersh's(1) histochemical studies in the mammalian kidney on the distribution of ferrocyanide established its exclusive glomerular excretion. Renal clearances of ferrocyanide have been measured in the dog with plasma concentrations from 60-200 mg%. In one study the ferrocyanide was determined as Prussian blue(2); in a second as ferricyanide(3). These methods are accurate only at these high plasma concentrations. When human subjects were used, nephrotoxic reactions were encountered even with plasma concentrations of 3.3 to 43.6 mg%. The analyses were performed by hydrolysis and recovery of HCN(4). This time consuming method has lowered the working range of ferrocyanide concentrations, but in order to avoid the possible danger of toxic reactions, we have devised an accurate method sensitive enough to measure sodium ferrocyanide concentrations of 5-15 mg%. By the acid mixture described below, ferrocyanide is oxidized to ferricyanide and allowed to react with ferrous sulfate to form Turnbull's blue. The per cent transmittance is measured at 700 millimicra in the Coleman, Jr. spectrophotometer. The calibration curves show that the reaction follows Beer's law, and do not vary significantly from day to day. The color is

* Aided by a grant from the National Heart Institute, United States Public Health Service.

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