THE COMPLEMENT FIXATION TEST IN THE DIAGNOSIS OF VIRUS INFECTIONS OF THE CENTRAL NERVOUS SYSTEM

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The application of the complement fixation reaction to the study of central nervous system virus infections has been complicated by the fact that infected brain or cord tissue is usually the only source of antigen. Brain tissue extracts have the property of being anticomplementary and can be used only when they are diluted and some of their components removed. Such procedures have resulted in a decrease of the final antigenic titre.

Howitt (1), although stating that the margin between specificity and non-specificity is narrow, reported specific complement fixation reactions with the viruses of equine encephalomyelitis, lymphocytic choriomeningitis, and St. Louis encephalitis. Several other workers have reported contradictory results with rabies virus. Greval (2) and Havens and Mayfield (3), using sheep and guinea pig hyperimmune sera respectively, obtain protocols sufficiently clear-cut to permit evaluation of the specificity of the test. Because of the practical importance of a satisfactory complement fixation test for the diagnosis of central nervous system virus infections, renewed attempts have been made in this laboratory to resolve the complicating factors. The result of these attempts to the present time will now be described in detail,[1] together with a simple technique for carrying out complement fixation tests with brain virus antigens. Finally, the application of this test to the diagnosis of human virus encephalitides is reported.

Materials and Methods

Viruses.—Rabies, St. Louis encephalitis, Japanese B encephalitis, lymphocytic choriomeningitis, Eastern equine encephalomyelitis, Western equine encephalomyelitis, louping ill, spontaneous encephalomyelitis of mice (Theiler's disease), and a mouse strain of human poliomyelitis (Armstrong) have been studied. These viruses are propagated in this laboratory by intracerebral mouse passage; rabies virus is propagated also by intracerebral passage in dogs, rabbits, and guinea pigs.

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[1] Preliminary statements have been published in *Science* (4) and reported also at the Forty-first Annual Meeting of the American Association of Pathologists and Bacteriologists, held in New York in April, 1941 (5).

Antigens.—Brain and sometimes the brain and cord are used as antigen. They have been prepared in several ways in an effort to eliminate anticomplementary effects. The different methods will be compared later; meanwhile, the procedure which we finally adopted as routine will be described. Fresh, infected brain tissue is weighed, ground up in a mortar, and emulsified in ten times its weight of a diluent consisting of 0.85 per cent saline containing 2 per cent normal, inactivated guinea pig serum. This emulsion is kept in the ice box for 20 hours and then centrifuged in a horizontal centrifuge at 2500 R.P.M. for ½ hour. The supernatant is then frozen and thawed five times in a dry ice-alcohol mixture, whereupon a flocculate appears. Finally it is centrifuged in a small angle head centrifuge for 1 hour at approximately 3500 R.P.M. After the addition of merthiolate in a dilution of 1:10,000, the supernatant is stored in the ice box ready for use. Antigens prepared in this manner are usually employed undiluted. Their titre varies according to the virus and the species of animal from which the virus derives, from 1:8 to 1:128. They have not proved anticomplementary when kept for periods of 2 months; on further aging, however, they have tended to become anticomplementary. By means of this method good antigens have been obtained from all viruses except mouse passage poliomyelitis (Armstrong strain) and occasional strains of rabies street virus. In these latter instances the virulence of the virus from which the antigen has been prepared has been low,—0.03 cc. of the 10^{-3} dilution intracerebrally, at best; whereas all other viruses, including those of most strains of street rabies, have titred higher,—rabies street, 10^{-4}, spontaneous encephalomyelitis of mice (Theiler), strain GVII, 10^{-5}, St. Louis encephalitis, Japanese B encephalitis, lymphocytic choriomeningitis, and louping ill, 10^{-6} or 10^{-7}, rabies fixed, 10^{-7}, and Eastern and Western equine encephalomyelitis, 10^{-8}.

Immune Sera.—Hyperimmune sera have been obtained by injecting dogs, mice, rabbits, and guinea pigs with homologous infected brain tissue. In general the following procedure has been followed in the case of mice: A first injection of 0.5 cc. of a 10^{-4} brain emulsion is given subcutaneously; after 1 week, intraperitoneal injections of 0.5 cc. are given at 4 to 6 day intervals, starting with a dilution of 10^{-4}, following this with a dilution of 10^{-3} and then 10^{-2}, and repeating the last injection from three to five times. The number of injections has varied from five to ten and the length of the period of immunization from 20 to 30 days. The titre of virulence of the viruses used for immunization is determined at frequent intervals, as noted above.

This course of vaccination has proved fatal to a number of mice, especially when animals too young have been employed; however, by employing mice 3 to 4 months old at the time immunization is started, the proportion of deaths can be reduced to 10 per cent or less. In order to maintain a constant supply of immune sera, the mice were kept under immunization by repeated intraperitoneal injections of infected brain emulsions while they lived, sometimes as long as 6 months (repeated bleedings from the heart proved fatal in the end in most cases). These subsequent injections were administered at intervals longer than those of the initial immunization period, usually every 10 or 15 days. The sera contained complement-fixing antibodies whenever tested, although the highest titre seemed to be reached at the 4th or 5th week; thereafter it diminished gradually.

Rabies hyperimmune sera in guinea pigs were obtained by means of five to ten intraperitoneal injections of infected brain tissue in dilutions from 10^{-4} to 10^{-2}, 1 cc. being administered each time; in rabbits from eight to fifteen injections of the same dilutions in 2 cc. volumes were employed. All injections were given about 1 week apart.

Rabies hyperimmune sera in dogs have been difficult to obtain; three dogs were kept under immunization for a period of 3 months by means of weekly 5 cc. injections of infected brain tissue in a dilution of 10^{-2} before they showed any complement-fixing antibodies in their blood, and then only in low titre.

Sera from mice were obtained by bleeding from the heart under ether anesthesia and pooling the blood samples; the sera from other animals were drawn either from the heart or from one of the veins and always tested individually. For reasons to be discussed later, temperatures of inactivation for the different sera have been established as follows: for guinea pig sera, 56°C., for mouse sera, 60°C., and for rabbit and dog sera, 65°C. All sera were heated for 20 minutes. When inactivated at 65°C. the sera were usually diluted 1:2 or 1:3 in order to prevent coagulation—which may occur with undiluted serum at this temperature.

Data on the smallest number of injections necessary to induce complement-fixing antibodies, the promptness of their appearance, and their persistence in serum are not included herein; we have limited ourselves in this paper to a report of observations on the presence and specificity of the complement fixation reaction.

Complement.—Fresh guinea pig serum constituted the complement. It was titrated in the presence of the antigens, as indicated later, and 2 units were used in the test.

Hemolytic System.—Sheep red blood cells and rabbit anti-sheep hemolysin constituted the hemolytic system. A 3 per cent suspension of packed cells in a volume of 0.25 cc. plus 3 M.H.D. of hemolysin, also in a volume of 0.25 cc., was used. The cells and hemolysin were mixed ½ hour before use in the test.

Reaction.—The reaction was carried out in the following manner. 0.25 cc. of antigen, 0.25 cc. of serum in serial twofold dilutions, and 0.5 cc. of complement diluted to contain 2 units were incubated in the ice box for 18 hours and then left at room temperature for ½ hour. The hemolytic system, consisting of 0.5 cc. of sensitized sheep cells, was then added. The total volume in each tube was thus 1.5 cc. The tubes were next incubated in a water bath at 37°C. for ½ hour and the reaction was read. Complete hemolysis was expressed as 0, absence of hemolysis as 4 plus, with ±, 1, 2, and 3 plus indicating intermediate degrees of hemolysis. The titre of the serum was taken as the last dilution giving a 2 plus or better fixation.

Development of a Specific Complement Fixation Test with Brain Antigens

The technique outlined above was developed after considerable experimentation to eliminate anticomplementary effects of the antigens, non-specific reactions between antigens and sera, and anticomplementary effects of sera. The various steps will now be described in detail.

Anticomplementary Power of the Antigens.—Determination of the anticomplementary power of an antigen, necessarily the first and most important

step in all complement fixation tests, is even more essential in the case of brain extract antigens since their pronounced anticomplementary effect is well known, especially following long incubation of the first phase at low temperature. For this reason particular attention has been paid to this point.

In general the different methods to eliminate anticomplementary power have consisted either in the use of high dilutions of brain tissue or in extraction of the brain tissue with fat solvents, since the lipoids in the brain seem to be mainly responsible for the anticomplementary action. Of course these methods must be such that in reducing the anticomplementary action to a minimum the antigenicity remains unchanged.

In our hands, 10 per cent, 5 per cent, and 1 per cent brain emulsions in saline or distilled water merely centrifuged in the horizontal centrifuge have exhibited anticomplementary effect; if the brain tissue was further diluted this inhibitory effect was eliminated but then the antigenicity of the preparation was either very low or non-existent. Consequently the method of bringing the brain tissue to high dilutions proved unsatisfactory.

We have tried the technique described by Howitt (1), in which the brain tissue is dried from the frozen state and this procedure followed by ether extraction. Although the resultant antigens were not anticomplementary, their antigenicity was low in all cases and usually no better than 1:2 to 1:4.

The supernates of brain emulsions, when spun in the ultracentrifuge at speeds varying from 10,000 to 30,000 R.P.M. for 1 hour and especially if filtered through a Seitz pad, showed no anticomplementary power and were used to advantage. Filtration through a Seitz pad dispensed successfully with all non-specific effects, although it did reduce the antigenicity to a variable degree.

Filtration of a brain emulsion through a Seitz filter, either directly without centrifugation or following centrifugation in the horizontal centrifuge, proved to be very unsatisfactory; the rate of filtration was extremely slow and the filtrate of very low antigenicity or entirely devoid of it.

From a comparison of these different methods of preparing brain tissue antigens we have determined that the technique described under materials and methods gives the highest antigenic titre without anticomplementary effects and at the same times dispenses with constant use of the ultracentrifuge. The freezing and thawing of the brain emulsion are done not so much to disrupt the cells and extract more antigen as to produce marked flocculation, thus making subsequent centrifugation in the angle head centrifuge more effective.

As shown by Bedson and Bland (6) long incubation of the first phase at low temperature is preferable to incubation at 37°C. for ½ or 1 hour to detect complement-fixing antibodies associated with some virus infections. This has also been our experience and on this account prolonged incubation in the ice box has been adopted. However, this treatment has the disadvantage of increasing the anticomplementary power of the antigens. Hence it has been found neces-

sary to determine the titre of the complement in the presence of the antigens at the beginning and at the end of the period of incubation of the first phase in order to estimate more precisely the titres obtained.

Titration of the complement and determination of the anticomplementary power of the antigens were carried out in the following manner.

A preliminary titration of the complement was done by placing in test tubes increasing amounts, from 0.05 cc. to 0.5 cc., of fresh guinea pig serum in a dilution of 1:30 and completing the volume to 1 cc. in each tube with saline. Then the hemolytic system was added and the whole incubated at 37°C. for ½ hour. This gave a preliminary titre of the complement. Following this preliminary titration the anticomplementary power of the antigens was determined in duplicate.

Two sets of tubes, each set having as many series of six tubes as there were antigens plus an extra series for saline alone, were set up. Increasing amounts of diluted complement, from 0.05 cc. to 0.5 cc. were placed in each series of tubes, completing the volume to 0.75 cc. in each tube with saline. Then 0.25 cc. of a given antigen was added to each tube of the series of six, in both sets, a series in each set being reserved for saline instead of antigen. The first set of tubes was incubated at 37°C. for ½ hour; the second incubated in the ice box for 18 hours along with those of the test proper. After incubation at 37°C. the first set of tubes received the hemolytic system and it was again incubated at 37°C. for another ½ hour period. This latter titration gave the titre of the complement in the presence of the antigens and determined the amount of complement to be used in the final test. Moreover, it revealed any gross anticomplementary action that might have taken place in the antigens. The second set received the hemolytic system at the end of the 18 hour incubation period.

The real value of the procedure described above was determined by the incubation of the tubes of second titration at low temperature in conjunction with the test proper, for it disclosed not only anticomplementary properties in some antigens that appeared suitable on first titration but at the same time indicated the actual amount of free complement present in the tubes when the hemolytic system was added. This second titration replaced the routine antigen control tube and provided more accurate information. After repeated trials we now know that antigens prepared as described above do not show any anticomplementary effect at the end of an 18 hour incubation period. The titre of the complement is usually the same and sometimes even better than that in the tubes with saline and no antigen.

Table I presents in detail the result of one experiment in which the anticomplementary power of several antigens was determined. After incubation at 37°C. for ½ hour, the titre of the complement in the series with saline alone was represented by 0.10 cc. Of all antigens tested only No. 4 was anticomplementary in the gross and had to be discarded; this was a 10 per cent crude brain emulsion centrifuged at low speed with no further treatment. The remaining antigens, namely, 1 per cent and 5 per cent brain emulsions

centrifuged at low speed, an antigen prepared according to the method described by Howitt, and three antigens prepared according to our method, were not anticomplementary. After incubation in the ice box for 18 hours, however, the titre of the complement in saline was 0.13 cc. Preparations 2 and 3 which at 37°C. were not anticomplementary proved to be strongly anticomplementary after standing in the ice box 18 hours, as in the case of antigen 4 which had al-

TABLE I

Titration of Complement in Presence of Several Antigens

Material employed as antigen	Incubation of first phase											
	½ hr. at 37°C.						18 hrs. in ice box (2°C.)					
	Amount of complement in dilution of 1:30											
	0.20 cc.	0.16 cc.	0.13 cc.	0.10 cc.	0.08 cc.	0.065 cc.	0.20 cc.	0.16 cc.	0.13 cc.	0.10 cc.	0.08 cc.	0.065 cc.
No. 1. No antigen: saline	0	0	0	0	1	3	0	0	0	±	1	2
No. 2. Mouse brain 5 per cent. Centrifuged 500 R.P.M. for 5 min.	0	0	0	0	1	2	3	4	4	4	4	4
No. 3. Mouse brain 1 per cent. Centrifuged 500 R.P.M. for 5 min.	0	0	0	0	1	2	1	2	3	4	4	4
No. 4. Dog brain 10 per cent. Centrifuged 2000 R.P.M. for 10 min.	0	0	3	4	4	4	4	4	4	4	4	4
No. 5. Dog brain 10 per cent. Centrifuged 15,000 R.P.M. for 45 min.	0	0	0	0	±	1	0	0	0	±	1	3
No. 6. Mouse brain 10 per cent—frozen and thawed. Swedish centrifuge for 1 hr.	0	0	0	0	±	1	0	0	0	0	±	1
No. 7. Dog brain 10 per cent—frozen and thawed. Swedish centrifuge for 1 hr.	0	0	0	0	±	1	0	0	0	±	1	3
No. 8. Dog brain 1 per cent—Howitt's method	0	0	0	0	±	1	0	0	0	±	1	2

0 indicates complete hemolysis.
4 " no hemolysis.
±, 1, 2, and 3 indicate intermediate degrees of hemolysis.

ready proved anticomplementary at 37°C. Antigens prepared in accordance with our method, together with that of Howitt, did not become anticomplementary.

The use of antigens such as Nos. 2 and 4 in a complement fixation test would not be suitable because of their anticomplementary action; that of No. 3 would be inadvisable even though the antigen control tube showed hemolysis because its specific action would be too close to the non-specific zone to be of any great significance. Titration in the ice box gave a better estimate of the reaction than the antigen control tube. In the final specific tests we have used 2 units of complement as indicated by the titration at 37°C. When there are several

antigens in one test, as may often by the case, there may be differences in the titre of the complement as given in the presence of each antigen. These differences are generally not referable to more than one tube in the series and in such cases the 2 units of complement are computed from the antigen giving the lowest titre—an amount equivalent to $2\frac{1}{4}$ or $2\frac{1}{2}$ units with the remaining antigens.

Non-Specific Reactions of Normal or Immune Sera.—Our immune sera were obtained with few exceptions by injection of infected homologous brain tissue. As shown by Witebsky and Steinfeld (7), injection of heterologous brain tissue gives rise, at least in certain species, to organ-specific antibodies which rule out the use of such sera for complement fixation tests in which brain extracts are used as antigens. But even when homologous brain tissue was used for the production of hyperimmune sera, a major difficulty arose in our work because of the property in sera from some species of reacting in a non-specific manner with brain tissue extracts, a reaction similar to that described by Takenomata (8) for bacterial cultures and filtrates, and by Mackie and Finkelstein (9) for a number of substances such as cholesterol, peptone, diluted alcohol, etc.

Takenomata showed that the serum of normal rabbits has the property of fixing complement in the presence of bacterial cultures and filtrates; that the reaction was more pronounced when the first phase was incubated at 37°C. rather than at 0°C., and that this property of the sera could be destroyed by heating at 62°C. Mackie and Finkelstein described a similar property for sera from normal individuals of several species. Such sera could fix complement in the presence of cholesterol, peptone, alcohol, and other substances, whereas sera from other species either did not possess this property to equal extent or lacked it entirely. This power of fixing complement was in general destroyed by heating the sera at 60°C. and in most instances was greatly reduced by heating at 56°C.

As noted above, antigens which were not anticomplementary could be obtained from brain tissue. However, as a result of tests for complement-fixing antibodies on sera from different animal species, it soon became apparent that so much non-specificity was present that the reaction was rendered valueless. Sera from mice, rabbits, dogs, and human beings exhibited, to a variable extent, the property of reacting with brain extracts and gave a complex which bound complement.

Table II shows the extent of this non-specific reaction. Normal sera and immune sera from several different species were tested against antigens prepared according to the standard method. The column under homologous antigens shows the result of the reaction between the immune sera and the antigens containing the same virus as that used for immunization of the animals. The column under heterologous antigens gives the result with one of the unrelated antigens. Three rabbit sera, one normal, another rabies immune, and the third Eastern equine encephalomyelitis immune, reacted with

both antigens. Although the immune sera showed a higher titre with the corresponding antigen, the degree of non-specificity was too high to be of any diagnostic use. On the other hand, neither the sera alone nor the antigens were anticomplementary. Mouse rabies immune, Eastern equine encephalomyelitis immune, and Japanese B immune sera also exhibited a degree of non-specific reaction but not as high as that shown by rabbit sera. Two human convalescent sera, Eastern equine encephalomyelitis and St. Louis encephalitis,

TABLE II

Non-Specific Complement Fixation Shown by Sera from Different Animal Species When Tested with Mouse Brain Extracts as Antigens

Sera inactivated at 56°C. for 20 minutes

Sera	Homologous antigens							Heterologous antigens							No antigen: saline			
	Sera in dilutions																	
	1:3	1:6	1:12	1:24	1:48	1:96	1:192	1:3	1:6	1:12	1:24	1:48	1:96	1:192	1:3	1:6	1:12	1:24
Rabbit normal	—	—	—	—	—	—	—	4	4	2	±	0	0	0	0	0	0	0
" Eastern equine encephalomyelitis immune	4	4	4	4	4	3	0	4	4	4	3	±	0	0	0	0	0	0
Rabbit rabies immune	4	4	4	4	4	3	0	4	4	4	3	1	±	0	0	0	0	0
Mouse Eastern equine encephalomyelitis immune	4	4	4	2	0	0	0	2	±	0	0	0	0	0	0	0	0	0
Mouse rabies immune	4	4	4	4	1	0	0	3	1	0	0	0	0	0	0	0	0	0
" Japanese B immune	4	4	4	3	1	0	0	4	3	0	0	0	0	0	0	0	0	0
Human St. Louis convalescent	4	4	4	±	0	0	0	4	4	±	0	0	0	0	0	0	0	0
Human Eastern equine encephalomyelitis convalescent	4	4	4	4	4	2	0	4	4	4	2	0	0	0	0	0	0	0
Guinea pig rabies immune	4	4	4	4	4	2	0	0	0	0	0	0	0	0	0	0	0	0
No serum; control of antigens	0	—	—	—	—	—	—	0	—	—	—	—	—	—	0	—	—	—

— indicates not tested.

Other footnotes as in Table I.

reacted with both antigens but not when saline was substituted for the antigens. Finally, guinea pig serum was the only one that gave an entirely specific reaction.

It was found possible to eliminate this non-specific reaction by employing as antigen a brain emulsion filtered through a Seitz pad, following centrifugation at high speed. But because of the reduction in titre of the antigen thus obtained, and especially to eliminate constant use of the ultracentrifuge, a different method was applied; namely, subjecting the sera to be tested to the action of heat (4, 5).

Sera from various animal species as well as from human convalescents were heated for periods of 20 minutes at temperatures of 56°C. (the usual temperature of inactivation which destroys complement present in the sera), 60°C., and 65°C. In some instances higher temperatures—70°C. and 75°C.—were also applied. When temperatures of 65°C. or higher were employed, the sera were ordinarily diluted to avoid

TABLE III

Effect of Heating Sera at Different Temperatures on Specific and Non-Specific Complement-Fixing Antibodies

Sera	Non-specific reaction with heterologous antigen. Sera heated for 20 min. at temperature:					Specific reaction with homologous antigen. Sera heated for 20 min. at temperature:				
	56°C.	60°C.	65°C.	70°C.	75°C.	56°C.	60°C.	65°C.	70°C.	75°C.
Rabbit 1, normal	1:16*	1:8	0							
" 2, rabies immune	1:12	1:6	0	0	0	1:96	1:96	1:48	1:24	0
" 3, " "	1:32	1:32	0			1:64	1:64	1:32		
" 4, Eastern equine encephalomyelitis immune	1:24	1:24	0			1:96	1:96	1:48		
Rabbit 5, Western equine encephalomyelitis immune			0					1:4		
Mouse, lymphocytic choriomeningitis immune	1:3	0				1:48	1:48			
Mouse, rabies immune	1:6	0	0			1:24	1:12	0		
" Japanese B encephalitis immune	1:6	0	0			1:24	1:24	0		
Human, No. 1, louping ill convalescent	0	0				1:16	1:16			
Human, No. 2, Eastern equine encephalomyelitis convalescent	1:48	1:24	0			1:96	1:96	1:48		
Human, No. 3, St. Louis encephalitis convalescent	1:16	1:8	0			1:32	1:16	1:8		
Human, No. 4, Wassermann-positive	1:48	1:12	0							
Guinea pig, rabies immune	0	0	0			1:24	1:12	1:6		
Dog, rabies immune	1:32	1:8	0			1:64	1:32	1:16		

* Highest dilution of serum giving a 2 plus or better reaction: first dilution, 1:1, 1:2, or 1:3.

coagulation. Table III presents a summary of the results of several tests. This table shows the highest dilution of serum giving a 2 plus or better reaction with the homologous and a heterologous antigen. The first dilution of the sera was 1:1, 1:2, or 1:3. Rabbit sera gave a non-specific reaction in dilution as high as 1:32; this reaction remained practically unchanged at 60°C. but disappeared at 65°C. On the contrary, the reaction with the homologous antigen persisted with only slight decrease at 65°C.; at 70°C. there were still antibodies left, although of lower titre. It was only following heating at 75°C. that the sera were no longer active. With mouse sera the non-

specific reaction present at 56°C. disappeared at 60°C., whereas the specific reaction persisted practically unchanged at 60°C. and even at 62°C. in some cases. Antibodies in the mouse sera were lost at 65°C. Some of the human sera did not give a non-specific reaction at 56°C. and others did. This non-specific reaction was especially noted in Wassermann-positive sera, although others, such as Nos. 3 and 4, which were not Wassermann-positive, likewise showed a non-specific reaction at 56°C. and 60°C., but not at 65°C. The specific reaction shown by the human sera was not altered by heating at 60°C. and 65°C. Whether there was a great loss of titre after heating at 65°C. is difficult to say because of the fact that the reaction was masked in part by the non-specific effect; the drop in titre shown by some sera, for example No. 3, may be more apparent than real. A dog rabies immune serum reacted similarly to rabbit sera. And finally, with guinea pig immune sera there was never a non-specific reaction following inactivation at 56°C.; the specific antibodies were still present at 60°C., with accompanying loss of titre; they were usually no longer present at 65°C. and never at 68°C.

These observations indicate that there is a margin sufficiently wide between the temperature capable of destroying elements in the serum responsible for the non-specific reaction and that materially affecting specific antibodies. By observing this varying resistance to heat it has been possible to obtain a specific reaction. As indicated above, temperatures of inactivation have been established at 56°C. for guinea pig sera, 60°C. for mouse sera, and 65°C. for rabbit and dog sera, all sera being heated for 20 minutes.

For human sera a temperature of 56°C. has seemed to be sufficient in most cases; but in order to eliminate slight degrees of non-specificity a temperature of inactivation of 60°C. has been adopted except in cases of Wassermann-positive sera or those sera that for some unknown reason give a non-specific reaction at this temperature. In such cases the temperature of inactivation has been 65°C. If enough serum is available, the best procedure is to carry out the reaction with the serum in duplicate, inactivating one sample of serum at 60°C. and the other at 65°C.

Anticomplementary Power of the Sera.—Some rabbit, dog, and human sera, especially when kept in the ice box for some time, may have an anticomplementary effect of their own, that is, they may fix complement in the absence of antigen. This power may be present when the serum is diluted as much as 1:4 or 1:6 but heating at 60°C. or 65°C. destroys the anticomplementary effect of the serum.

Results of Newly Developed Complement Fixation Test with Hyperimmune Sera

Hyperimmune Sera from Mice.—Mouse hyperimmune sera prepared as described above were tested against homologous and heterologous antigens. The result of a single test is summarized in Table IV.

Each serum was tested against each one of the antigens. The test was done in duplicate, one set with sera being inactivated at 56°C., the other set with sera at

60°C. Only the reaction at 60°C. has been reproduced here: Its specificity is complete. There was no cross-reaction in this test between the St. Louis No. 3 virus and the Japanese B strains; the serological relationship between these two viruses has been investigated by Webster (10) by means of the neutralization test with the result that they have been shown to be serologically different even though cross-neutralization not exceeding 1 to 10 M.L.D. may be present. Whether there is any slight degree of cross-reaction in the complement fixation test is being further investigated, although if present, it would appear to be very insignificant. The serum of lymphocytic

TABLE IV

Complement Fixation Test with Mouse Immune Sera Inactivated at 60°C. for 20 Minutes. Mouse Brain Antigens

Antigens	Sera									
	St. Louis encephalitis No. 3	Japanese B encephalitis No. 2604	Japanese B encephalitis No. 17	Japanese B encephalitis No. 12	Lymphocytic choriomeningitis	Eastern equine encephalomyelitis	Louping ill	Rabies (street)	Rabies (fixed)	No serum
St. Louis encephalitis No. 3	1:32*	0	0	0	0	0	0	0	0	0
Japanese B encephalitis No. 2604	0	1:32	1:32	1:64	0	0	0	0	0	0
Japanese B encephalitis No. 17	0	1:32	1:64	1:128	0	0	0	0	0	0
Japanese B encephalitis No. 12	0	1:32	1:128	1:128	0	0	0	0	0	0
Lymphocytic choriomeningitis	0	0	0	0	1:128	0	0	0	0	0
Eastern equine encephalomyelitis	0	0	0	0	0	1:64	0	0	0	0
Louping ill	0	0	0	0	0	0	1:64	0	0	0
Rabies (street)	0	0	0	0	0	0	0	1:32	1:8	0
Rabies (fixed)	0	0	0	0	0	0	0	1:16	1:8	0
No antigen	0	0	0	0	0	0	0	0	0	—

* Highest dilution of serum giving a 2 plus or better reaction: first dilution, 1:4.

choriomeningitis-immune mice has a high titre of complement-fixing antibodies which is in contrast to the uniform absence of neutralizing antibodies in the blood o these animals. Louping ill immune serum has a high titre of antibodies. Finally, rabies fixed and street viruses react with each other.

In another test, presented in Table V, two additional viruses were studied for their capacity to elicit complement-fixing reactions, the Western equine encephalomyelitis and the mouse spontaneous encephalomyelitis viruses. The sera were used in this test undiluted and in increasing twofold dilutions, the temperature of inactivation being 60°C. for 20 minutes. With the viruses of Eastern equine encephalomyelitis and Western equine encephalomyelitis there was no cross-reaction. The GVII strain of mouse spontaneous encephalomyelitis gave a high titre serum.

Hyperimmune Sera from Guinea Pigs.—Guinea pigs have been used to obtain rabies immune serum. Thus far seven animals have been immunized with the result that all except one have shown antibodies in high titre in dilutions varying from 1:48 to 1:382. Some of them required no more than three or four injections to give a positive serum. When tested against a normal brain antigen or against an unrelated antigen (and this is done in every individual test), guinea pig serum has always given a specific reaction following inactivation at 56°C.

Hyperimmune Sera from Rabbits.—Rabies, Eastern and Western equine encephalomyelitis immune sera have been prepared in rabbits. The Eastern and Western equine encephalomyelitis sera were adapted originally for neutralizing antibodies by inoculation of mouse rather than rabbit infected brain

TABLE V

Complement Fixation Test: Mouse Immune Sera Inactivated at 60°C. for 20 Minutes. Mouse Brain Antigens

Antigens	Sera		
	Eastern equine encephalomyelitis	Western equine encephalomyelitis	Spontaneous encephalomyelitis of mice
Eastern equine encephalomyelitis	1:4*	0	0
Western " "	0	1:16	0
Spontaneous encephalomyelitis of mice (Theiler's disease)	0	0	1:64

* Highest dilution of serum giving a 2 plus or better reaction: first dilution, 1:1.

tissue. Only two injections of a 1 to 1,000 suspension were given to each of two rabbits, 2 cc. each time. The animals received no further treatment and after 1 year their sera were tested for complement-fixing antibodies, although it may be added that a specific reaction was not expected on account of the injection of heterologous (mouse) brain virus. These two sera, when tested in dilutions of 1:2 or 1:3, have given an occasionally non-specific reaction, even following inactivation at 65°C., probably as a result of the presence of some mouse protein antibody; in higher dilutions the reaction has been specific at all times. In Table VI are given the results of cross-reactions between the two sera and a rabies immune serum at three different temperatures of inactivation.

The Western equine encephalomyelitis serum was tested undiluted and in serial twofold dilutions against Western and Eastern equine encephalomyelitis and rabies antigens. The titre of this serum was very low, 1:4, although it was completely specific. The Eastern equine encephalomyelitis immune serum inactivated at 65°C. had a titre of 1:48 with the homologous antigen, but in a dilution of 1:3 it reacted

also with rabies antigen. Finally, the rabies immune serum at 65°C. reacted only with the rabies antigen.

The production of rabies immune sera in rabbits has not been an easy task. In most instances no antibodies were present in the first 4 or 5 weeks of immunization, by which time from five to seven injections had been given. A total of nine rabbits have been immunized with the Pasteur strain of rabies virus propagated in the same species.

Table VII gives the titre of all sera as obtained in a single test. The immunization of the individual rabbits had been started at different times, so that at the time of

TABLE VI

Rabbit Immune Sera. Complement Fixation Tests Following Inactivation for 20 Minutes at Different Temperatures

Antigens	Sera inactivated at temperature of:						
	56°C.		60°C.		65°C.		
	Eastern equine encephalomyelitis immune	Rabies immune	Eastern equine encephalomyelitis immune	Rabies immune	Eastern equine encephalomyelitis immune	Rabies immune	Western equine encephalomyelitis immune
Eastern equine encephalomyelitis.....	1:96*	1:24	1:96	1:12	1:48	0	0
Western equine encephalomyelitis.....	—	—	—	—	0	0	1:4
Rabies.....	1:24	1:96	1:24	1:96	1:3	1:48	0

Eastern and Western equine encephalomyelitis rabbit immune sera were obtained by injecting mouse infected brain.

* Highest dilution of serum giving a 2 plus or better reaction.

— = not tested.

bleeding for the test they had received differing numbers of injections and their immunization had been in progress for varying lengths of time. The number of injections received by each rabbit as well as the time elapsed from the beginning of immunization are shown in the table. Here it may be seen that all reactions at 56°C. and 60°C. were non-specific. Following inactivation of the sera at 65°C., six rabbits gave a specific reaction; another rabbit, No. 4, gave a negative reaction, and rabbits 5 and 9 a non-specific one. The sera of these two latter rabbits were tested at even higher temperatures of inactivation and also with antigens filtered through a Seitz pad, with negative results: The reaction remained non-specific. These animals had been immunized with tissue that had remained in the ice box for a period of 5 to 7 days, while in most other cases the infected tissue used for immunization was fresh. This possible explanation is being studied further.

In general, with respect to hyperimmune sera from eleven rabbits the different resistance of the specific and non-specific antibodies to heat was consistent.

Hyperimmune Sera from Dogs.—The complement fixation reaction with dog sera has been difficult because of the presence of non-specific antibodies similar to those encountered in rabbit sera and also because of the hemolysins for sheep cells that may occur spontaneously. Moreover, dogs do not seem to

TABLE VII

Rabbit, Rabies Immune Sera. Result of Complement Fixation Test Following Inactivation of Sera for 20 Minutes at Different Temperatures

Sera			Temperature of inactivation of sera								
			56°C.			60°C.			65°C.		
Rabbit No.	No. of injections	Period of immunization	Rabies antigen	Heterologous antigen	No antigen	Rabies antigen	Heterologous antigen	No antigen	Rabies antigen	Heterologous antigen	No antigen
		mos.									
1	31	12	1:48*	1:24	0	1:48	1:24	0	1:24	0	0
2	8	2	1:24	1:24	0	—	—	—	1:12	0	0
3	15	6	1:12	1:12	0	1:6	0	0	1:3	0	0
4	11	5	1:24	1:24	0	1:6	1:6	0	0	0	0
5	11	5	1:96	1:96	0	1:96	1:96	0	1:24	1:48	0
6	9	4½	1:24	1:24	0	1:12	1:12	0	1:6	0	0
7	9	4½	1:48	1:24	0	1:48	1:24	0	1:24	0	0
8	9	4½	1:24	1:24	0	1:24	1:6	0	1:12	0	0
9	9	4½	1:96	1:96	0	1:96	1:96	0	1:48	1:24	0

* Highest dilution of serum giving a 2 plus or better reaction: first dilution, 1:3.

TABLE VIII

Dog, Rabies Immune Sera. Result of Complement Fixation Test Following Inactivation of Sera at 65°C. for 20 Minutes. Mouse Brain Antigens

Antigens	Dog 2-26, serum in dilutions:						Dog 2-31, serum in dilutions:						Dog 2-32, serum in dilutions:					
	1:2	1:4	1:8	1:16	1:32	1:64	1:2	1:4	1:8	1:16	1:32	1:64	1:2	1:4	1:8	1:16	1:32	1:64
Rabies, mouse.....	—	4	1	0	0	0	4	4	4	4	3	0	—	4	4	1	0	0
Japanese B encephalitis, mouse..	—	0	0	0	0	0	0	0	0	0	0	0	—	0	0	0	0	0
No antigens.....	—	0	0	0	0	0	0	0	0	0	0	0	—	0	0	0	0	0

— = not tested.

Other footnotes as in Table I.

respond to the immunizing injections as readily as do guinea pigs or rabbits. Three dogs have been under immunization treatment for a period of nearly 6 months, their sera being tested at frequent intervals. The first definite specific reaction was obtained at 3 months after approximately twenty injections had been given.

Table VIII shows the result of a test with the sera of the three dogs. The sera were inactivated at 65°C. for 20 minutes following dilution to 1:2. The reaction with

rabies antigen gave titres of 1:4, 1:32, and 1:8 respectively, whereas there was no reaction with the unrelated antigen (Japanese B encephalitis).

Results of Newly Developed Complement Fixation Test with Sera from Cases of Central Nervous System Virus Infections

Animal Sera.—Mice intramuscularly infected with 0.01 cc. of rabies street virus did, in some instances, have complement-fixing antibodies coincident with the development of paralysis. Also mice intracerebrally inoculated with the virus of spontaneous encephalomyelitis (Theiler), that developed paralysis but survived for 30 days, showed antibodies in their blood in titres from 1:2 to 1:8. Finally, preliminary studies with the virus of St. Louis encephalitis indicate that, following a single 0.5 cc. intraperitoneal injection of virus in a dilution of 1:100, about one-third of the mice have antibodies as early as the the 6th day and all mice have antibodies in fairly high titre by the 13th day.

The sera of dogs receiving injections of 0.5 cc. of street virus in a dilution of 1:20 into the neck muscles (11) have been tested, thus far with negative results.

Human Sera.—Smadel, Baird, and Wall (12) showed that in a proportion of human cases of lymphocytic choriomeningitis complement-fixing antibodies were present; that they appeared early in the disease or the first stages of convalescence; later they disappeared and were substituted by neutralizing antibodies. This seems to be the only instance in which complement-fixing antibodies in the blood of human beings have been described in connection with any of the diseases herein considered. Accordingly we applied our test to human cases of central nervous system virus encephalitides which had previously been diagnosed on the basis of neutralization tests. Thus far, few such cases have been available and most of these old ones.[2]

Tests with human sera are summarized in Table IX. All cases of Eastern and Western equine encephalomyelitis and louping ill tested gave a positive specifice reaction; the persistence of louping ill antibodies in the blood of an individual 8 years following infection is striking. Only recent cases of St. Louis encephalitis and lymphocytic choriomeningitis have shown complement-fixing antibodies.

In addition, several other human sera have been tested, among them six sera from patients with St. Louis encephalitis from the 1933 and 1937 epidemics, three from patients with lymphocytic choriomeningitis, one of them infected

[2] The sera from human cases have been kindly supplied by the following persons: Dr. W. Lloyd Aycock, Dr. Margaret G. Smith, Dr. G. O. Broun, Dr. Charles D. May, Dr. Morgan B. Hodskins, Dr. Horace L. Hodes, Dr. Richard E. Shope, Dr. Sherman F. Gilpin, Dr. Roy F. Feemster, Dr. R. V. Platou, Dr. Albert B. Sabin, Dr. Walter O. Klingman, Dr. Charles F. Branch, Dr. Thomas Henley, Dr. Joseph Johnson, Dr. Herald R. Cox, and Dr. Joseph L. Lilienthal, Jr. To all we express our gratitude.

5 years previous to the present bleeding, and the other two recent ones; and finally, about fifteen sera from patients with indefinite central nervous system symptomatology, none of which has, to our knowledge, been diagnosed. These sera have been tested against Eastern and Western equine encephalomyelitis,

TABLE IX

Complement Fixation Tests with Human Sera

Sera				Antigens (mouse brain)						
No.	Diagnosis of disease by neutralization test	Interval between disease and bleeding for complement fixation test	Temperature of inactivation for 20 min.	Louping ill	Lymphocytic choriomeningitis	Eastern equine encephalomyelitis	Western equine encephalomyelitis	St. Louis encephalitis	Japanese B encephalitis	No antigen: saline
			°C.							
1	Eastern equine encephalomyelitis	2 yrs.	60		0	1:8*			0	0
2	" "	2 "	65	0	0	1:48		0		0
3	" "	2 "	65		0	1:8		0		0
4	" "	2 "	60		0	1:32		0		
5	" "	2 "	65		0	1:2		0		0
6	Western equine encephalomyelitis	3 "	65		0	0	1:8	0		
7	Western equine encephalomyelitis (?)	?	60		0	0	1:4	0		0
8	Western equine encephalomyelitis	2 yrs.	60		0	0	1:1	0	0	
9	Louping ill	8 "	60	1:16	0	0			0	0
10	" "	4 "	60	1:4	0	0		0	0	0
11	Lymphocytic choriomeningitis	2 wks.	60	0	1:4	0		0	0	0
12	" "	3 "	60	0	1:1	0		0	0	0
13	" "	2–4 wks.	60		1:1		0	0		
14	St. Louis encephalitis	4 mos.	60			0	0	1:1		
15	" "	3 "	65		0	0		1:8		0
16	" "	3 "	60		0	0		1:1		0
17	" "	?	60				0	1:4		

* Highest dilution of serum giving a 2 plus or better reaction: first dilution, 1:1 or 1:2.

St. Louis encephalitis, lymphocytic choriomeningitis, and some against louping ill antigens, all with negative results.

COMMENT

The etiological diagnosis of the human encephalitides remains a difficult one. Thus far the clinician has had to depend on methods that are either unsatisfactory or else cannot be used as routine. Isolation of virus from the

cerebrospinal fluid or blood has been accomplished only in a few cases of lymphocytic choriomeningitis and, perhaps, Japanese B encephalitis. In general, though, virus is recoverable only from the central nervous system, thus providing only a postmortem diagnosis. In most of the cases in which a diagnosis has been possible, it has been brought about through the medium of the cumbersome neutralization test. The neutralization test has two disadvantages: In the first place, neutralizing antibodies do not appear in general until after convalescence is well under way so that the diagnosis is a delayed one. In the second place, routine use of the neutralization test is hardly possible in a diagnostic laboratory because of the complicated procedure involved.

The complement fixation test as described above appears eminently practical. The antigens are stable for a considerable period of time and the test procedure is, in general, a familiar one. Lastly, no animals are required and the result is apparent within a few hours.

SUMMARY AND CONCLUSIONS

A specific complement fixation test can be obtained in various central nervous system virus infections by using as antigens emulsions of infected brain tissue, freezing and thawing the brain emulsion, and then centrifuging it in an angle head centrifuge at 3500 R.P.M. for 1 hour. The method has proved reliable in the case of rabies, St. Louis encephalitis, Japanese B encephalitis, lymphocytic choriomeningitis, Eastern equine encephalomyelitis, Western equine encephalomyelitis, louping ill, and spontaneous encephalomyelitis of mice (Theiler's disease).

The specificity of the reaction, regardless of the virus involved, requires different temperatures of inactivation of the sera according to animal species: 56°C. for guinea pig, 60°C. for mouse, and 65°C. for rabbit and dog sera, all heated for 20 minutes. For human sera a temperature of inactivation of 60°C. also for 20 minutes has been adopted; at this temperature the reaction is in general specific.

Complement-fixing antibodies in high titre were found in the sera of rabbits, guinea pigs, mice, and dogs immunized with rabies virus.

Complement-fixing antibodies were present in high titre in sera drawn from two persons 8 years after an attack of louping ill, from five persons $2\frac{1}{2}$ years after an attack of Eastern equine encephalomyelitis, and from two persons $2\frac{1}{2}$ years after Western equine encephalomyelitis. In cases of St. Louis encephalitis and lymphocytic choriomeningitis, complement-fixing antibodies have been found shortly following infection but not after long periods.

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