Dr. J. Casals,
The Rockefeller Institute,
66th Street and York Avenue,
New York, New York.

My dear Casals:

This is a last hour letter before I leave tonight which I am anxious
to get off to you while the material and issues are still fresh in my mind.
As I told you over the telephone about a week ago, I have spent the last two
weeks ironing out certain wrinkles in the complement fixation test. I
believe that what I found should be added to the directions that you have already
published for carrying out the test if other people are not to run into the same
difficulties that I did. I shall try to organize the difficulties that I en-
countered as follows:

1. It is misleading to have in the directions the simple statement
that the titer of complement is to be determined as for the Wassermann reaction.
The exact amount of complement to be used in the test I have found to be one of
the most important features with sera from human cases of encephalitis as dis-
tinguished from work with very potent hyperimmune animal sera. For example:
I have found that certain antigens may show no trace of anti-complementary action in
the preliminary titration after incubation at 37° C. for 1/2 hour and yet will
be found to be highly complementary in the test after incubation for 18 hours
in the refrigerator and then 1/2 hour at room temperature. I know that you
have run into this phenomenon with mouse brain extracts and published it in
your first communication. However, I have encountered it with reconstituted
lyophilized antigens sent to me both by yourself and by Sharp & Dohme, as well
as with freshly made antigens prepared according to your directions. I am con-
vinced, therefore, that final directions for complement fixation tests must
contain the explicit statement that before any antigen can be used it must be
established that the titer of complement determined with it after incubation
at 37° for 1/2 hour is practically the same as the titer obtained after incuba-
tion for 18 hours in the refrigerator and then 1/2 hour at room temperature.
Good antigens satisfied this requirement, as you have shown.

The difficulties that I found with the lyophilized and freshly pre-
pared antigens mentioned above were solved as follows:

a) After reconstituting the lyophilized antigens there was a certain
amount of insoluble or poorly soluble material which could only partly be re-
moved by centrifugation on the horizontal centrifuge. The horizontal centrifuge
reconstituted lyophilized antigen exhibited the anticomplementary property after
refrigerator incubation, but not after the 37° incubation. When, however, such
a reconstituted antigen was centrifuged on the Swedish angle centrifuge for about
one hour, it was thoroughly clarified and fulfilled the requirements for a good
antigen in that the same titer of complement was obtained with it, both at 37°
and refrigerator incubation.
b) The difficulty that I found with antigens prepared according to your directions was chiefly in the part that says that the centrifuged brain suspension should be frozen and thawed 5 times and that a certain amount of flocculation usually appears during the process. You will recall that I told you that when I carried out the procedure according to those directions, I did not get the precipitation you mentioned. I may say that I carried out the freezing and thawing in glass bottles and I have a feeling that, perhaps, that may be a feature. However, the thoroughly clarified angle centrifuged supernatant liquid thus prepared exhibited the property of being anticomplementary upon refrigerator incubation, but not at 37° C. for 1/2 hour.

It was possible to remove this anticomplementary property by repeating the freezing and thawing another six times in lusteroid tubes (and I think that the use of lusteroid tubes may be necessary) during which process very heavy flocculation occurred. The angle centrifuge supernatant liquid resulting from this was then a perfect antigen giving excellent results in the complement titrations at 37° as well as after prolonged refrigerator incubation. I think that the lesson that I learned from this is that the directions should stress that definite flocculation must appear during the course of the freezing and thawing and that this process must be carried out not just five times, but as long as it may be necessary to remove all the material which will flock out until it is no longer anticomplementary after prolonged refrigerator incubation.

2. Another point of importance in using the complement fixation test with human sera from clinical cases of encephalitis is the importance of not exceeding two units of complement in the test. In your original paper you pointed out that when several antigens are used slightly different titers of complement may be obtained with each and that the amount of complement to be used is determined by the one giving the lowest titer, thus increasing the number of units of complement for some of the antigens used to as much as 2-1/2 units. Now, I have found that when 2-1/2 units are used you get quite good fixation with hyperimmune sera and with very potent human convalescent sera, particularly those obtained later than three weeks after onset of the disease. However, one can miss distinct positive specific reactions with that amount of complement. I, personally, now prefer to use a dose of complement as close to 2 units as possible, but if anything slightly under it in the range of 1.5 to 2. I have data which show specific complement fixation with that amount of complement and yet negative results with 2-1/2 units. I would personally stress this point in the directions for carrying out the test.

3. There is just one other point that I think is worth stressing now that the number of units of antigen used in the test must satisfy a certain minimum standard, by that I mean that the antigen should have a definite minimum standard of potency. I appreciated the importance of this in a series of tests in which I used freshly prepared antigen from my Japanese B Okinawa strain and some of the reconstituted lyophilized antigen which you were good enough to send me. In simultaneous tests your antigen titered against a standard amount of immune serum had a titer of 1:8, whereas my freshly prepared antigen had a titer of 1:16. This difference was enough to give me positive results with the freshly prepared Okinawa antigen and negative results with your antigen in certain of the human sera, particularly those obtained early after onset. I think that antigens that have a titer of only 1:8 can be used, but that the re-
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results obtained with them will not be as good as with one of higher potency. I am also under the impression that something might be done to improve the lyophilization of the antigen so that it could be reconstituted without loss of potency and without need for further angle centrifugation. I know that you have reported that with the WRA lyophilized antigen after a period of 2 months you recover full potency and apparently did not have to resort to further centrifugation. However, such good results are not apparently obtained as a rule.

Finally, may I say that I think the complement fixation test as you have worked it out is one of the finest aids in the serological diagnosis of the virus encephalitides. I have just completed a large series of neutralization and complement fixation tests with sera not only from the military cases of encephalitis, on Okinawa, but also with sera from Okinawan natives without any history of encephalitis and I know that you will be interested to see the results when we have an opportunity to meet the next time. I would like to say here, however, that for practical purposes of diagnosis, I would personally prefer to use only the complement fixation test with all the wrinkles removed from it. Of particular importance, I think, is my finding that the sera of Okinawan natives without histories of encephalitis and possessing very high titers of neutralizing antibodies, as a rule gave no complement fixation. This means that in Japanese B encephalitis a complement fixation titer greater than 1:4 (of the original serum) is very likely an index of recent infection. Under the circumstances when only a single serum specimen can be obtained and a late one at that, I should think that the diagnosis of recent infection could almost be made if a high titer of complement fixing antibodies were found.

I expect to come East on March 13th for the federation meetings and I hope to have an opportunity to go over some of this material with you personally.

With kindest regards to Peter Olitsky and yourself.

Sincerely yours,

Albert B. Sabin, M.D.

cc.: Dr. John R. Paul
Dr. William McD. Hammon