野口英世著　Journal of Experimental Medicine　所収論文

A HOMOHEMOLYTIC SYSTEM FOR THE SERUM DIAGNOSIS OF SYPHILIS.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 20, 1918.)

There is little doubt that the elimination of the use of guinea pig complement from the serodiagnostic procedure is a great advance towards the simplification of this important reaction, for that element has to be used while it is perfectly fresh, necessitating the maintenance of guinea pigs in the laboratory. Attempts to preserve the activity of the complement, either by means of desiccation[1] or hypertonization,[2] have been only partially successful, and it deteriorates in a comparatively short time. The best preservative for guinea pig complement is that recommended by Rhamy,[3] who found that sodium acetate in appropriate concentration keeps the complement active for several weeks.

Some years ago Hecht[4] proposed the utilization of the natural anti-sheep hemolysin and complement in the fresh serum of patients, but the difficulty in this method lies in the fact that in some sera there is no natural hemolysin or too little to produce hemolysis, even in the control tubes without the antigen. On the other hand, some sera contain a considerable excess of the natural anti-sheep hemolysin. Granting that the insufficiency of the natural anti-sheep hemolysin can be remedied by adding an adequate quantity of a non-syphilitic

[1] Noguchi, H., On the influence of the reaction and of desiccation upon oposonins, *J. Exp. Med.*, 1907, ix, 455; Serum diagnosis of syphilis, and the butyric acid test for syphilis, Philadelphia and London, 1st edition, 1910.

[2] Austin, F. D., A new method for preserving complement for making the Wassermann or Noguchi blood-test, *J. Am. Med. Assn.*, 1914, lxii, 868.

[3] Rhamy, B. W., Preservation of complement. A preliminary report, *J. Am. Med. Assn.*, 1917, lxix, 973.

[4] Hecht, H., Eine Vereinfachung der Komplementbindungsreaktion bei Syphilis, *Wien. klin. Woch.*, 1909, xxii, 338.

serum containing enough hemolysin, the test still calls for the washed sheep corpuscles. In other words, having eliminated the use of guinea pig serum as complement, we still have to depend upon the sheep corpuscles for an indicator of hemolysis.

In ordinary times there should be no difficulty in obtaining the guinea pig complement or sheep blood corpuscles, and it would be immaterial whether one used the anti-sheep hemolytic system or the anti-human hemolytic method as advocated by the writer[5] and accepted by many serologists, including those in the United States Army and Navy. Perhaps it may not be amiss to call attention here to the fact that the anti-human hemolytic system of the writer is not limited in its use to unheated sera but applies equally to inactivated sera, the only differences between the examination of fresh and inactivated sera being that only 0.02 cc. of the former is required, instead of 0.08 to 0.1 cc. of inactivated specimens, and that in the case of inactivated sera not only the acetone-insoluble fraction of tissue lipoids, but also any properly titrated alcoholic extracts may be used as the antigen.[6] With fresh sera emphasis has been placed on the fact that the crude alcoholic extracts or those alcoholic extracts to which cholesterol has been added are to be avoided, as in this combination there is apt to occur a non-specific proteotropic complement fixation.[7] It is therefore essential that only the acetone-insoluble fraction of tissue lipoids should be used in combination with fresh sera. With these points in view there should be no confusion as to the extent of applicability of the test to fresh and inactivated sera.[6]

There is another possibility, namely that by eliminating the use of the guinea pig complement from the anti-human hemolytic system the technique and the material for the serodiagnosis of syphilis can be greatly simplified. That this is the case is shown below. Efforts to introduce similar methods have already been made by Tscher-

[5] Noguchi, A new and simple method for the serum diagnosis of syphilis, *J. Exp. Med.*, 1909, xi, 392.

[6] Noguchi, Serum diagnosis of syphilis, and luetin reaction, together with the butyric acid test for syphilis, Philadelphia and London, 3rd edition, 1912.

[7] Noguchi, On non-specific complement fixation, *Proc. Soc. Exp. Biol. and Med.*, 1909–10, vii, 55.

nogubow,[8] Emery,[9] Butler and Landon,[10] Myer,[11] and Thompson,[12] all employing human complement. A brief review of these methods will be given later.

Principle of the Method.

It is well known that complement is present in every fresh serum and that the quantity may sometimes vary. In the serodiagnosis of syphilis, whether by the anti-sheep system of Wassermann or the anti-human system of the writer, the serum of the guinea pig is chosen because of its richness in complement and also because guinea pig complement is probably more readily fixed[13] by the antigen-antibody combination than the sera of other animals, such as the horse, rabbit, sheep, pig, etc.[14, 15] As to the complement in fresh human sera, there are not many data regarding its action upon human corpuscles. It is understood that human sera exert no hemolytic action upon human corpuscles, especially when there is no isohemolysin. But upon the addition of a sufficient quantity of the anti-human hemolytic amboceptor the complement dissolves the human corpuscles just as guinea pig complement does when added to the suspension of human corpuscles and the specific anti-human hemolytic amboceptor.

[8] Tschernogubow, N. A., Ein vereinfachtes Verfahren der Serumdiagnose bei Syphilis, *Deutsch. med. Woch.*, 1909, xxxv, 668.

[9] Emery, W. d'E., Clinical bacteriology and hæmatology for practitioners, Philadelphia, 4th edition, 1912.

[10] Butler, C. S., and Landon, W. F., A technic for the absorption test for syphilis using human complement, *U. S. Nav. Med. Bull.*, 1916, x, 1.

[11] Myer, S. B., A complement-fixation test for syphilis using human complement, *U. S. Nav. Med. Bull.*, 1917, xi, 175.

[12] Thompson, L., Complement fixation in syphilis, with a preliminary report of a new technic, *Am. J. Syph.*, 1917, i, 555.

[13] Noguchi, H., and Bronfenbrenner, J., Variations in the complementary activity and fixability of guinea pig serum, *J. Exp. Med.*, 1911, xiii, 69.

[14] Noguchi, Non-fixation of complement, *Proc. Soc. Exp. Biol. and Med.*, 1909–10, vii, 14.

[15] Noguchi and Bronfenbrenner, The comparative merits of various complements and amboceptors in the serum diagnosis of syphilis, *J. Exp. Med.*, 1911, xiii. 78

The only difference between the action of the human and the guinea pig complement lies in the fact that the former requires more anti-human hemolytic amboceptor to render it active against the human corpuscles than the latter. The relative lytic values of the human and the guinea pig complement are shown in the following experiments.

Comparative Complement Values of the Human and the Guinea Pig Sera Relative to the Anti-Human Hemolytic Amboceptor and Human Corpuscles.

0.04 cc. of guinea pig complement was put in each of a number of tubes, together with 1 cc. of a 1 per cent suspension of human corpuscles. Varying quantities of the anti-human hemolytic amboceptor (immunized rabbit) were added to the tubes and the results read after 30 minutes at 37°C. in a water bath thermostat. The results are shown in Table I. The experiment shows the titer of the anti-

TABLE I.

Titration of the Anti-Human Amboceptor with Guinea Pig Complement.

Amount of serum to each tube.*	Anti-human hemolytic immune serum.	
	No. 633 (rabbit).	No. 634 (rabbit).
cc.		
0.01	Complete hemolysis.	Complete hemolysis.
0.007	" "	" "
0.005	" "	" "
0.004	" "	" "
0.003	" "	" "
0.002	" "	" "
0.0015	" "	" "
0.001	" "	" "
0.0007	" "	" "
0.0005	Partial hemolysis.	" "
0.0004	No "	Partial hemolysis.
0.0003	" "	No "
0.0002	" "	" "
0	" "	" "

* Each tube contained guinea pig complement 0.04 cc. and 1 per cent human corpuscle suspension 1 cc.

human hemolytic immune serum No. 633 to have been 0.0007 cc. and that of No. 634, 0.0005 cc. in the presence of 0.04 cc. of guinea pig complement. 0.1 cc. of the guinea pig serum alone was itself somewhat hemolytic.

From the results recorded in the first part of Table II it appears that in the presence of 0.1 cc. of a fresh human serum at least 0.005

TABLE II.

Titration of the Anti-Human Amboceptor with Human Complement.

Serum 3 + amboceptor.*	Result.
cc. *cc.*	
0.1 + 0.1	Complete hemolysis.
0.1 + 0.05	" "
0.1 + 0.03	" "
0.1 + 0.02	" "
0.1 + 0.01	" "
0.1 + 0.005	" "
0.1 + 0.003	Considerable "
0.1 + 0.002	No "
0.1 + 0.001	" "
0.5 + 0.02	Complete hemolysis.
0.3 + 0.02	" "
0.2 + 0.02	" "
0.1 + 0.02	" "
0.05 + 0.02	Slight hemolysis.
0.02 + 0.02	No "

* Human serum as complement. 1 per cent human corpuscle suspension 1 cc. in each tube.

cc. of the anti-human amboceptor No. 633 was needed to produce complete hemolysis; that is, about seven times the amount required in the case of guinea pig complement. In other words, the activity of human complement is nearly one-seventh that of the guinea pig serum.

In the second part of the table it is shown that 0.05 to 0.02 cc. of the same fresh human serum was unable to cause complete hemolysis even in the presence of 0.02 cc. of the amboceptor No. 633, which is an equivalent of thirty minimal lytic doses when calculated on the basis of 0.04 cc. of the guinea pig complement. It is evident that

the amount of amboceptor required to dissolve the human corpuscles in the presence of human complement is many times that necessary with guinea pig complement.

The next point is to determine whether or not average fresh human serum contains enough complement to produce complete hemolysis. Upon this fact depends the possibility of utilizing the human com-

TABLE III.

Quantitative Relation between Human Complement and Anti-Human Amboceptor.

Serum No. (48 hrs. old).*	Human serum 0.1 cc.		Human serum 0.05 cc.	
	Amboceptor 0.005 cc.	Amboceptor 0.01 cc.	Amboceptor 0.005 cc.	Amboceptor 0.01 cc.
1	Considerable hemolysis.	Complete hemolysis.	No hemolysis.	Complete hemolysis.
2	No hemolysis.	No hemolysis.	" "	No hemolysis.
3	Considerable hemolysis.	Complete hemolysis.	" "	Complete hemolysis.
4	" "	" "	" "	" "
5	" "	" "	" "	" "
6	Complete hemolysis.	" "	" "	" "
7	Considerable hemolysis.	" "	" "	" "
8	No hemolysis.	No hemolysis.	" "	No hemolysis.
9	" "	" "	" "	" "
10	Considerable hemolysis.	Complete hemolysis.	" "	Complete hemolysis.
11	Almost complete "	" "	" "	" "
12	Considerable "	" "	" "	" "
13	Complete hemolysis.	" "	" "	" "
14	Considerable hemolysis.	" "	" "	" "
15	" "	" "	" "	" "
16	" "	" "	" "	" "
17	" "	" "	" "	" "
18	" "	" "	" "	" "
19	" "	" "	" "	" "
20	" "	" "	" "	" "

* Each tube contained 1 per cent washed human corpuscle suspension 1 cc.

plement in the serodiagnosis of syphilis. Twenty sera were tested for this purpose. All except three of them (Nos. 2, 8, and 9, Table III) contained sufficient complement in 0.05 to 0.1 cc. to cause complete hemolysis in the presence of 0.01 cc. of the anti-human amboceptor No. 633. No hemolysis occurred, however, in any tube containing 0.005 cc. of the amboceptor and 0.05 cc. of human serum.

There was considerable, and in some cases complete hemolysis in the tubes containing the same amount of the amboceptor but 0.1 cc. of the human serum.

For comparison these twenty sera were also tested upon sheep corpuscles (1 cc. of a 1 per cent suspension) for their natural anti-sheep hemolysin. The results obtained show that 0.05 cc. and 0.1 cc. both dissolved the sheep corpuscles in all except the three specimens (Nos. 2, 8, and 9) which failed to dissolve the human corpuscles in the presence of anti-human amboceptor. This means that there were at least three sera out of twenty which did not contain sufficient complement.

Out of 1,250 specimens of fresh human sera so far examined, 1,157 contained enough complement to produce complete hemolysis of 1 cc. of a 1 per cent suspension of human corpuscles in a dose of 0.1 cc., with the addition of 0.01 cc. of anti-human amboceptor (one unit), within a period of 20 to 30 minutes at 37°C. in a water bath thermostat. 72 specimens caused a partial hemolysis, and 21 no hemolysis.

Additional amboceptor in doses of from one-half to one ordinary minimal hemolytic unit to the partially hemolyzed tubes produced complete hemolysis on a further incubation of 15 minutes or longer. In the case of the sera, however, which showed no complementary action in the first combination, the addition of an extra quantity of the amboceptor caused only a tardy partial hemolysis or no hemolysis. From the serological standpoint we encounter at least three groups of human sera, those which contain the average amount of complement, those which contain a subnormal quantity (hypocomplementosis), and those which contain no complement (acomplementosis). Occasionally one meets with a fourth group in which the activity of the complement is unusually strong (hypercomplementosis). It is highly important to take these facts into account in testing human sera by the Bordet-Gengou reaction or the Wassermann reaction in syphilis.

The next step was devised in order to determine whether the insufficiency of complement could not be supplemented by the addition of an adequate quantity of fresh active serum. That this is easily accomplished was soon proved.

Mode of Utilization of Human Complement for the Serodiagnosis of Syphilis.

The experiments discussed above made it evident that in the majority of fresh human sera there is sufficient complement to cause a complete and prompt hemolysis of human corpuscles in the presence of an adequate quantity of the specific anti-human hemolytic amboceptor. Moreover, in cases where there is not enough complement, an active human serum may be added as a supplement. In fact, we are now in a position to produce complete hemolysis, and from this point it is only another step to test the presence or absence of a complement-fixing principle in a given specimen of human serum. One merely measures out a definite amount of the suspected serum into two tubes and then adds to one of the two an adequate amount of the antigen suspension. Both tubes are incubated for 30 minutes at 37°C. in a water bath; then the human corpuscular suspension and the anti-human hemolytic amboceptor are introduced into both tubes and the contents are well mixed by shaking. The tubes are once more incubated for 30 minutes at 37°C., and then after another 30 minutes or so at room temperature the result is read. It is necessary to shake the tubes two or three times during the incubation. No result should be taken as final unless the control tube without the antigen shows complete hemolysis. If hemolysis is incomplete at the end of the period indicated, adequate modification, which will be described later, must be made.

Procedure for the Examination of Human Sera Not More than 48 Hours Old.

It must be made clear in the beginning that specimens should be examined as soon as practicable, preferably within 24 hours after they are withdrawn from the patients. After 48 hours, even when the specimens are kept in the refrigerator, the complement gradually disappears from the serum. Sera kept for more than 3 days in a refrigerator must be tested by a special technique, to be given later. Specimens tinged deeply with hemoglobin give unsatisfactory results and should be rejected. Table IV indicates the amounts of reagents to be used in the test for fresh human sera and other details regarding it. The entire set of tubes should be duplicated for each specimen.

TABLE IV.

Procedure for Examining Fresh Human Sera.

Tube.	1st step.	2nd step.	3rd step.	4th step.	Final step.
Determinative tube (front row).	Patient's serum (fresh) 0.2cc. Antigen 0.1 " 0.9 per cent saline solution 1 "	First incubation at 37°C. for 30 min. in water bath or 1 hr. in air thermostat.	Both tubes receive 0.1 cc. of anti-human amboceptor, representing 1 hemolytic unit, and 0.1 cc. of 10 per cent human corpuscular suspension. Total volume 1.5 cc. Contents are well mixed by shaking.	Second incubation at 37°C., same as first, except that tubes are shaken three times during the period.	Reading of results after tubes have stood 30 min. at room temperature.
Control tube (back row).	Patient's serum (fresh) 0.2 " Antigen omitted. 0.9 per cent saline solution 1.1 "				

Positive and Negative Controls.

As in any other serodiagnostic procedure, each serum tested must be accompanied by a positive and a negative serum in order to control the reliability of the reagents. In a well appointed laboratory, where many tests are being made daily or every other day, the necessary positive and negative sera will be furnished by the tests of the previous occasion.

Varieties of Irregular Reactions and Their Adjustment.

Reference has already been made to the possible deficiency of complement in certain specimens of fresh human sera. With these hypocomplementary sera hemolysis in the control tubes without antigen proceeds slowly and remains incomplete at the end of the second incubation. To these sets of tubes, both the determinative and the control, an additional hemolytic amboceptor unit is introduced in order to reinforce the hemolytic activity of the complement. In cases in which hemolysis is marked only one-half the unit is added, but when hemolysis is slight a whole unit of the amboceptor is needed. The tubes should then be put in another rack and subjected to further incubation until complete hemolysis occurs in the control tubes (without the antigen). 15 minutes or longer may be required. Specimens which fail to hemolyze or in which hemolysis is incomplete, even with the additional amboceptor should be tested again by adding to them a quantity of fresh serum which has been shown to contain an average complement and at the same time to be devoid of any syphilitic fixation substance (negative serum). In these cases 0.1 cc. of the complementary serum is used with 0.2 cc. of the acomplementary serum. The mixture is then tested in the same way that any fresh serum is tested.

Procedure for the Examination of Human Serum More than 48 Hours Old.

As previously stated, specimens of human serum which have stood in a refrigerator longer than 48 hours are inconstant in their complementary activity, and many are markedly deficient. As a rule

sera not more than 72 hours old which have been kept constantly in the refrigerator at 4–6°C. still contain enough complement to make the test possible. It is best, however, to inactivate all sera whose complementary activity is no longer a certain factor, supplementing them with active human complement from negative fresh sera. If inactivation is complete (55°C. for 30 minutes), the sera are rendered free of the remnant of their native complement, and what is added later is of known and uniform quantity. 0.1 cc. of the complement serum (previously tested) is added to 0.2 cc. of the inactivated serum and the mixture then tested like any fresh serum. Table V gives the details of the procedure.

Procedure for the Examination of Cerebrospinal Fluids.

This procedure is comparatively easy and gives an entirely satisfactory result. It differs from that used for inactivated sera only in one respect, that of the quantity of the specimen used, which may vary from 0.2 to 0.5 cc. No inactivation is required, as the cerebrospinal fluid contains no complement, and 0.1 cc. of active negative human serum (previously tested) is added as complement (Table VI).

A Method of Preserving the Complement of Fresh Human Serum by Means of Sodium Acetate.

Rhamy[3] first observed that guinea pig complement remains active for a long time when mixed with sodium acetate in a strength of approximately 6 per cent of the acetate in the mixture. He recommends mixing 4 parts of the complement with 6 parts of a 0.9 per cent sodium chloride solution containing 10 per cent sodium acetate.

Human complement can also be kept active for some time by adding sodium acetate in a similar proportion. Specimens of human serum which have been mixed with the acetate can be satisfactorily tested after several days without the aid of complement from another source, since the complement remains active for at least 4 days at room temperature (18°C.). Therefore any serum which cannot be examined within 48 hours may be mixed with the acetate saline solution while perfectly fresh. If a good refrigerator is not accessible the specimen

TABLE V.

*Procedure for Examining Inactivated Human Sera.**

Tube.	1st step.	2nd step.	3rd step.	4th step.	Final step.
Determinative tube (front row).	Patient's serum (inactivated) 0.2 cc. Active negative serum 0.1 cc.† Antigen 0.1 " 0.9 per cent saline solution 0.9 cc.	First incubation for 30 min. in water bath or 1 hr. in air thermostat.	Anti-human amboceptor 1 unit in 0.1 cc. of 10 per cent human corpuscular suspension, 0.1 cc. Total volume 1.5 cc. Contents are well mixed.	Second incubation same as first except that tubes are shaken three times during incubation.	Reading of results within 30 min. after removal of tubes from incubator.
Control tube (back row).	Patient's serum (inactivated) 0.2 cc. Active negative serum 0.1 cc.† Antigen omitted. 0.9 per cent saline solution 1 cc.				

* This applies also to unheated sera which have too little complement from the beginning or have lost complement on standing. Old sera are often anticomplementary and for such specimens only 0.1 cc. is indicated.

† A second complete test of this serum should accompany the test of the serum to which it is added as complement.

TABLE VI.

Procedure for Examining Cerebrospinal Fluid.

Tube.	1st step.	2nd step.	3rd step.	4th step.	Final step.
Determinative tube (front row).	Cerebrospinal fluid 0.2 cc.* Active negative serum 0.1 cc.† Antigen 0.1 " 0.9 per cent saline solution 0.9 cc.	First incubation same as in Tables IV and V.	Anti-human amboceptor 1 unit in 0.1 cc. of 10 per cent human corpuscular suspension, 0.1 cc. Total volume 1.5 cc. Contents are mixed by shaking.	Second incubation same as first except that contents of tubes are shaken three times during incubation period.	Reading of results within 30 min. after removal of tubes from incubator.
Control tube (back row).	Cerebrospinal fluid 0.2 cc. Active negative serum 0.1 cc.† Antigen omitted. 0.9 per cent saline solution 1 cc.				

* Graduated quantities of from 0.2 to 0.5 cc. may be used in certain cases, the amount of saline solution being so adjusted as to make the total volume 1.3 cc.

† A second complete test of this serum should accompany the test of the serum to which it is added as complement.

may always be acetated, since the presence of the acetate does not interfere with the test (Rhamy).

The acetate may be used as preservative for the serum in either of two ways. A sterile solution, containing 0.9 per cent sodium chloride and 10 per cent sodium acetate, may be mixed in equal parts with the serum after separation from the clot; or 0.5 cc. of a sterile concentrated acetate solution (50 per cent) in 0.9 per cent saline solution may be placed in a graduated centrifuge tube and the blood drawn from the patient directly into the tube up to the 5 cc. mark, the whole being then shaken thoroughly. Coagulation takes place as usual, and the clot is just as firm as in a control tube in which 0.5 cc. of saline solution is added to the same amount of blood. There is no disturbing effect from the presence of the acetate in the serum, either when it is fresh, or when it has stood for several days. The serum acetate mixture may be kept in the refrigerator, where it will retain complement activity longer than at room temperature; in the latter, activity is preserved for several days. Once the acetate serum has become inactive by long standing, it can still be tested by adding fresh human complement.

The method of mixing clear serum with the saline acetate solution is much to be preferred to the direct mixing of the blood and concentrated acetate solution.

Preparation of Reagents.

Although the details with regard to the reagents used in conducting the present test are essentially the same as those described in earlier publications[6, 16] dealing with the anti-human heterohemolytic system (the use of guinea pig complement with anti-human amboceptor and human corpuscles), it nevertheless seems desirable to summarize them briefly here. Antigen, anti-human hemolytic amboceptor, and a suspension of human blood corpuscles are the reagents required to test the patient's serum.

Antigen.—The preparation, titration for its antigenic properties, and preservation of the antigen are described in other publications dealing with the subject in minute detail.[6] Suffice it to say that the acetone-insoluble fraction of tissue

[16] Noguchi and Bronfenbrenner, Biochemical studies on so-called syphilis antigen, *J. Exp. Med.*, 1911, xiii, 43.

lipoids is recommended, 0.1 cc. of a 1: 10 emulsion with 3 per cent methyl alcohol solution (stock) and 0.9 per cent saline solution being used for each test. No preparation which hemolyzes in 0.4 cc. or interferes with complement in the same dose should be used. The antigen should be effective in doses of 0.02 cc. of the emulsion. In the actual test at least five antigenic units are employed in order that no positive reaction may be overlooked. The methyl alcohol stock solution remains active indefinitely at room temperature, and a saline emulsion of 1: 10 strength may be made up at any time. The latter, when kept in a refrigerator, remains unchanged for several days, although it is best to make a fresh emulsion on the day that tests are to be made.

Amboceptor.—The production of the anti-human amboceptor is one of the most important parts of the present method. Rabbits, when immunized with thoroughly washed human corpuscles in sufficiently large quantities, yield a powerful serum, which is able to produce complete hemolysis of 1 cc. of a 1 per cent suspension of human corpuscles in doses of from 0.01 to 0.005 cc. This point will be discussed at greater length later. The usual amount of this reagent used in the test is 0.1 cc., which is so made as to represent one hemolytic unit in the presence of 0.1 cc. of fresh human serum and against 1 cc. of the 1 per cent corpuscular suspension, hemolysis becoming complete within 20 to 30 minutes at 37°C. in a water bath or 1 hour in an air thermostat.

Corpuscular Suspension.—0.1 cc. of a 10 per cent suspension is best suited for the purposes of the test. The suspension is prepared as follows: 1 part of the washed corpuscles from any individual (it is convenient to use corpuscles obtained from one of the patients being bled for test serum) is mixed with 9 parts of 0.9 per cent saline solution. A few cubic centimeters of blood are drawn into a centrifuge tube containing an equal volume of sodium citrate solution (2 per cent in 0.9 per cent saline solution) and the mixture is repeatedly centrifuged with changes of saline solution until there is no more serum in the supernatant fluid. The final sediment of corpuscles is suspended in a quantity of saline solution equal to the original quantity of the blood. The corpuscular suspension thus prepared should be used when fresh and when not in use should be kept in a refrigerator, where it can be preserved for a period of about 72 hours. A suspension older than 72 hours should not be used, however, because the use of corpuscles with diminished resistance to hemolysis may cause the masking of a possible weak positive reaction.

Utilization of the Patient's Corpuscles.—For any well equipped laboratory the preparation of a corpuscular suspension of a definite concentration by the above method offers no difficulty. There may be occasions, however, for example on board ship or in field hospitals, when no centrifuge is available. Under these conditions the preparation of a uniform corpuscular suspension for an entire set of tests is not possible, and to meet this sort of emergency the utilization of the patient's own corpuscles for testing his serum is recommended. As has already been emphasized, the serum of the patient to be examined must be perfectly fresh and the corpuscles which can be liberated by gently stirring the clot are correspondingly fresh and can be used as the indicator of hemolysis. The fol-

lowing technique gives the best result: 0.4 cc. of the fresh clear serum from the tube containing the coagulated blood is measured out and put into one of the two tubes used for the test. 2 cc. of 0.9 per cent saline solution are added, making a total volume of 2.4 cc. After putting the remaining clear serum into a tube for future use, the clot is gently shaken, by means of a medium sized pipette, to liberate enough corpuscles to tinge the serum dilution to the desired color value (suspension). The standard of suspension aimed at is 1 per cent of corpuscles, and after a certain amount of practice no difficulty is experienced in detecting the difference between a 1 per cent and a 1.5 per cent suspension by this means. A 1 per cent suspension is an opaque fluid with a yellowish red hue. As the concentration of the suspension increases, the red becomes more predominant over the yellow tint. Having made a mixture of 0.4 cc. of the fresh serum and approximately a 1 per cent corpuscular suspension in a total of 2.4 cc., one divides this quantity into two equal portions by measuring out 1.2 cc. into the second tube of the set. One of the two tubes receives the antigen 0.1 cc., and the other receives the saline solution 0.1 cc., and serves as the control without the antigen. Both are incubated for 30 minutes in a water bath or 1 hour in an air incubator. 0.1 cc. of the anti-human amboceptor, representing one hemolytic unit, is then added to both tubes, and after thorough mixing the tubes are again incubated, as in all other procedures.

When the test is done in this way, the corpuscles are, of course, present from the beginning, instead of being added after the first incubation simultaneously with the amboceptor, as in procedures in which corpuscles from other patients are used. But in any procedure the corpuscles may be introduced from the beginning and the final result remain the same.

Tschernogubow[8] once proposed the use of a suspension of the patient's blood directly diluted in saline solution in a ratio of 1 drop to 1 cc. of saline. This was to serve as the source of complement, corpuscles, and, if present, the fixing substance. By careful scrutiny, however, one soon discovers the enormous disproportion among the various elements concerned. For example, the amount of serum probably present in 1 drop of blood, which would perhaps be 0.07 cc., would be approximately 0.035 cc. at most (about one-sixth the amount used in the writer's system), while the corpuscular suspension approaches 7 per cent (seven times the concentration in the writer's system). The amount of syphilitic antibodies present would be too small to make possible the detection of a weak positive reaction, and the minuteness of complement present in such a mixture precludes any practical possibility. Even an enormous amount of the amboceptor fails to complete hemolysis of such a concentrated

suspension of the corpuscles. Moreover, the mixture forms a loose gelatinous fibrin, involving the whole contents, making it impossible to stir by shaking. When the first fibrin is removed, a second may form on further standing. Such a method cannot be used, and Tschernogubow himself soon abandoned it.

Emery[9] employs active serum with the cholesterolized alcoholic extract of heart muscle, disregarding the possibility of obtaining a false positive fixation with certain non-syphilitic sera. The amount of the patient's serum is minute, but the concentration of the corpuscles (20 per cent of the firmly packed sediment after centrifugation) is almost six times that used in the method being proposed. There is no economic gain in Emery's method, therefore, since the amount of amboceptor required is no less than that used in the proposed method. Moreover, the manipulation of minute quantities of various factors by Wright's capillary technique requires a considerable degree of skill as compared with the ease with which regular graduated pipettes can be handled.

Butler and Landon[10] and Myer[11] inactivate the patient's serum before the test and add fresh negative human serum from a non-syphilitic individual as complement. They recommend the use of sensitized human corpuscles and the acetone-insoluble tissue lipoids (Noguchi). Their method is decidedly more rational than Emery's and seems to have given satisfactory results in 300 cases so far reported.[16] They also employ Wright's capillary technique.

Thompson[12] uses fresh human serum, with preliminary titration of each specimen for its complement activity. This procedure seems to be unessential, since the majority of specimens already contain enough complement. The amount of corpuscles used is 0.2 cc. of a 2 per cent suspension. A stronger concentration would give a more distinct reaction.

Results of Practical Application of the Test.

The writer has been able, up to the present time, to examine 1,331 specimens of blood and 52 cerebrospinal fluids.[17] Of 1,118 specimens of sera from these sources 517 gave a positive and 601 a negative

[17] I wish to express my appreciation to the members of the staffs of several hospitals through whose cooperation this work was made possible.

reaction, the results conforming to those reported by the serological departments of the various hospitals. Of 132 specimens from psychiatric cases, 54 were from general paralysis cases, and all except 2 gave a strongly positive reaction. Among other psychoses, including 75 cases of dementia præcox, 3 of alcoholic psychosis, 3 of imbecility, 3 of senile psychosis, 6 of arteriosclerosis, 1 of manic-depressive insanity, and 1 paranoic condition, there were only 2 positive reactions, these occurring among the dementia præcox cases. The reactions with 81 inactivated sera agreed with those obtained by others with the same material. 20 cerebrospinal fluids from cases of general paralysis gave a strongly positive reaction, while 32 specimens from other non-syphilitic cases showed a negative reaction. The statement will perhaps bear repeating that of 1,250 fresh human sera complement was deficient in 93 specimens, which had to be examined either by means of additional amboceptor or by supplying active human complement from fresh negative sera. This special adjustment with fresh sera is one which demands particular attention on the part of serologists adopting the method.

Quantitative Consideration of the Complement Fixation Test.

In an ideal method for the serum diagnosis of syphilis every ingredient should be separately controllable by accurate titration. This is possible with the anti-human heterohemolytic system (Noguchi), in which the amount of guinea pig complement is accurately measured and added to a definite quantity of serum, whose native complement plays no part in the reaction, or has been removed by inactivation, and in which the antigen, anti-human amboceptor, and corpuscles are equally definite, no factor being present which can give rise to a quantitative disturbance. The homohemolytic system, upon critical examination, will be seen also to be capable of equal accuracy. The possible sources of error and the methods of adjusting them are discussed below.

The Frequency and Extent to Which a Positive Reaction May Be Masked by an Excess of Complement.—Only 2 per cent of several hundred specimens examined in this study were found to contain an extra unit of complement activity. None contained three units.

An excess of complementary activity may cause a positive serum to give a weakly positive or even a negative reaction, but only when the so called antibody content of the serum is less than one fixing unit. For example, a specimen containing half of an antibody unit in the presence of two complement units may give a negative result. No change in the reaction can occur, however, from an excess of less than two complement units in the presence of $1\frac{1}{2}$ units of syphilitic antibody. Error from this source is therefore extremely rare; and the reaction cannot be completely negative when the specimen contains more than one antibody unit.

TABLE VII.

Relation between Various Quantities of Human Complement and of Antibody.

Syphilitic serum (active).		Fresh negative human serum as complement.						
Amount.	No. of units of antibody.	0.1 cc.	0.125 cc.	0.15 cc.	0.2 cc.	0.25 cc.	0.3 cc.	0.4 cc.
cc.								
0.008	0.5	+++	+++	++	++	+	+	−
0.016	1.0	++++	++++	++++	++++	+++	+++	+
0.024	1.5	++++	++++	++++	++++	++++	+++	++
0.032	2.0	++++	++++	++++	++++	++++	++++	+++
0.04	2.5	++++	++++	++++	++++	++++	++++	++++
0.048	3.0	++++	++++	++++	++++	++++	++++	++++
0.064	4.0	++++	++++	++++	++++	++++	++++	++++

Table VII records experiments in which the relation between various quantities of human complement and of antibody was determined. The amount of anti-human amboceptor used was that which produced complete hemolysis of 1 cc. of a 1 per cent suspension of human corpuscles in the presence of 0.1 cc. of fresh human serum (one complement unit) within 20 to 30 minutes at 37°C. (water bath). Table VII shows that the addition of quantities of the human complement ranging from 0.1 to 0.2 cc. did not materially change the ++++ reaction when combined with 0.016 cc. of syphilitic serum. The reactions became somewhat weaker, however, when 0.15 and 0.2 cc. of the complement were used with 0.008 cc. of the serum. When the amount of the syphilitic serum was increased to 0.024 to 0.032 cc. or more, the reaction was ++++ against 0.3 cc. of the complement, and

0.04 cc. of serum prevented hemolysis in the presence of 0.4 cc. of complement. The reduction in the degree of positive reaction caused by the variations of 0.1 to 0.2 cc. of the human complement is insignificant and has no serious effect upon the ultimate result when the serum contains more than $1\frac{1}{2}$ antibody units. The apparent disproportion of complement in the routine amount of 0.2 cc. of the fresh serum is well balanced by the antibody content, which is four to five times that of the same serum inactivated. For that reason only 0.1 cc. of human serum is added to 0.2 cc. of inactivated serum. This proportion is similar to that of the guinea pig complement and inactivated serum used in the Wassermann system and in the anti-human heterocomplement system (Noguchi). But the addition of 0.2 cc. of the human complement does not mask a strongly positive reaction (++++).

The Possibility of Interference by Negative Serum in Complement Fixation in the Homohemolytic System.—The use in the homohemolytic system of fresh negative serum as complement when there is no complement or too little in the specimen to be examined, while entirely analogous to the use of fresh guinea pig serum as complement for an inactivated patient's serum, may rouse apprehension as to the possibility of reduction in the degree of fixation by this comparatively large amount of human complement, owing to its indifferent serum constituents. The experiments recorded in Table VIII, in which human and guinea pig complement were studied in parallel series, show that the addition of an inactivated negative serum to a syphilitic serum does not cause any so called complementoid blocking of the complement fixation of any significance, and that no error can result from this source.

All the other experiments of this sort with syphilitic sera gave similar results. It was found, however, that a syphilitic serum containing less than one-half an antibody unit caused less inhibition in the tubes to which more than 0.3 cc. of the inactivated negative human serum had been added, but never completely masked the reaction. There was no appreciable difference in the tubes containing 0.1 or 0.2 cc. of the inactivated serum and that containing none. The addition, however, of inactivated guinea pig serum (56°C.) to a syphilitic serum produces marked weakening of the fixation reaction, and even com-

TABLE VIII.

Effect of Inactivated Negative Human Serum upon the Complement Fixation.

	With human complement.	Results.	With guinea pig complement.	Results.
With inactivated syphilitic serum.	Syphilitic serum (56°C.) containing 1 antibody unit 0.2 cc. Human complement (active serum) 0.1 " The same + negative human serum (56°C.) 0.1 " The same + negative human serum (56°C.) 0.2 " The same + negative human serum 56°C.) 0.3 " The same + negative human serum (56°C.) 0.4 "	All gave complete fixation, regardless of the addition of the inactivated negative serum.	Syphilitic serum (56°C.) containing 1 antibody unit 0.2 cc. Guinea pig complement 40 per cent 0.1 " The same + negative human serum (56°C.) 0.1 " The same + negative human serum (56°C.) 0.2 " The same + negative human serum (56°C.) 0.3 " The same + negative human serum (56°C.) 0.4 "	All gave complete fixation, no interference being observed from the addition of the inactivated negative human serum.
With active syphilitic serum.	Active syphilitic serum, containing 4 antibody units 0.2 cc. The same + negative human serum (56°C.) 0.1 "	Complete fixation in all.		

TABLE VIII—*Concluded.*

	With human complement.	Results.	With guinea pig complement.	Results.
With active syphilitic serum.	The same + negative human serum (56°C.) 0.2 cc. The same + negative human serum (56°C.) 0.3 " The same + negative human serum (56°C.) 0.4 "	Complete fixation in all.		

plete blocking when more than 0.3 cc. of that serum is added to one syphilitic antibody unit. This confirms earlier observations.[18]

Result of the Presence of an Excess of Amboceptor in the Complement Fixation Test.—The disturbance resulting from an excess of amboceptor in any complement fixation test has been repeatedly pointed out and is recognized by impartial workers as inherent in the anti-sheep hemolytic system. Removal by absorption of the natural anti-sheep amboceptor from each specimen of serum prior to test has been advocated, but for obvious reasons is impracticable when several dozen specimens must be examined at one time.

In Table IX are recorded the results obtained in parallel series of tests,[19] the anti-sheep system with guinea pig complement being used in one series, and the anti-human homocomplement system in the other. These experiments confirm earlier observations[20] that a

[18] Noguchi and Bronfenbrenner, The interference of inactive serum and egg-white in the phenomenon of complement fixation, *J. Exp. Med.*, 1911, xiii, 92.

[19] These tests were carried out by Major Felix R. Hill, Captain George L. Schadt, and Lieutenant Ralph R. Simmons in this laboratory.

[20] Noguchi, Die quantitative Seite der Serodiagnostik der Syphilis, *Z. Immunitätsforsch., Orig.*, 1911, ix, 715.

positive serum containing 1 antibody unit can become completely negative when 4 amboceptor units are used, or 3 antibody units with 20 amboceptor units. Table IX shows that 1 antibody unit is made negative by 6 amboceptor units and 3 antibody units by 10 amboceptor units with the Wassermann system, while with the anti-human homocomplement system nearly twice the amount of amboceptor is required to produce the same effect.

The phenomenon just described assumes practical importance in any system in which are used foreign blood corpuscles for which human serum normally contains varying amounts of natural hemolytic amboceptor, in which case there is a possibility of an excess of amboceptor in the test. It does not occur in an anti-human hemolytic

TABLE IX.

Reversion of Reaction through Excess of Amboceptor.

No. of units of amboceptor.	Syphilitic antibody 1 unit.		Syphilitic antibody 3 units.		Syphilitic antibody 10 units.	
	Wassermann anti-sheep system.	Anti-human homocomplement system.	Wassermann anti-sheep system.	Anti-human homocomplement system.	Wassermann anti-sheep system.	Anti-human homocomplement system.
1	++++	++++	++++	++++	++++	++++
2	++++	++++	++++	++++	++++	++++
3	++	++++	+++	++++	++++	++++
6	−	+++	+	++++	++++	++++
10	−	−	−	++	++++	++++
20	−	−	−	−	+++	+++
40	−	−	−	−	+	+

TABLE X.

Titration of Natural Anti-Sheep Amboceptor in Human Serum.

Sera.	No. of specimens examined.	No. of units of anti-sheep amboceptor in 0.2 cc. of human serum (56°C.), titrated with guinea pig complement.										
		None.	< 1	1	2	3	4	5	6	7	8	>10
Syphilitic sera	190	19	21	42	21	25	26	17	8	4	3	4
Non-syphilitic sera	111	3	15	25	31	10	12	8	5	1		1
Normal sera	25	1	3	5	4	3	1	2	2	3	1	
Total	326	23	39	72	56	38	39	27	15	8	4	5

system, whether guinea pig complement or human complement is used, and especially when the patient's own corpuscles are employed for each specimen. Data which have been collected concerning the amount of natural anti-sheep amboceptor in 326 specimens of human serum are given in Table X.

Tables IX and X explain the possibility of a reduced or reversed reaction with certain syphilitic sera in the anti-sheep system. The frequency and extent of error from this source are much greater than those due to slight variations in complement content, which are readily amenable to quantitative adjustment.

Quantitative Estimation of the So Called Syphilitic Antibody.—If necessary, any strongly positive serum can be titrated by the homocomplement system, the procedure being the same as that recommended for the anti-human heterocomplement system; namely, that of keeping all the other ingredients constant and determining the smallest quantity of the serum which will give complete fixation. For this purpose the serum may be inactivated before titration. To a number of tubes containing 0.1 cc. of the fresh human complement (previously titrated) and the standard antigen (usually contained in 0.1 cc. of a suitable dilution) are added varying amounts (ranging from 0.01 to 0.2 cc.) of the positive human serum (inactivated) to be titrated, and the mixture is made up to 1.3 cc. in each tube. After an incubation at 37°C. for 30 minutes in the water bath or 1 hour in an air incubator, the corpuscle suspension (0.1 cc.) and one unit of amboceptor (0.1 cc.) are added, and a second incubation follows. The smallest quantity of serum which produces complete fixation is taken as one antibody unit.

SUMMARY.

The elimination of the foreign complement and corpuscles from the test for the serodiagnosis of syphilis has been attempted, and the results so far obtained are very satisfactory. Instead of using guinea pig complement, fresh human serum was utilized for the source of complement for the production of hemolysis upon the human corpuscles in the presence of an adequate amount of the specific anti-human amboceptor (prepared in rabbits). Usually 0.1 cc. of fresh

human serum contains enough complement to hemolyze 1 cc. of a 1 per cent suspension of human corpuscles, but the amount of anti-human amboceptor required in this combination is about five to seven times that necessary when guinea pig complement (0.04 cc.) is used. It has also been shown that when a given human serum contains insufficient complement, an adequate quantity (0.1 cc. is usually enough) of another fresh negative serum may be added to supplement it. However, one rarely encounters this group of sera. Inactivated human sera can also be examined by utilizing human complement from another source (the serum must be fresh, active, and negative).

The only drawback to the present method is the comparatively large amount of anti-human hemolytic amboceptor required. It is estimated that 30 to 40 cc. of the anti-human hemolytic immune serum, from one rabbit, of high potency—say 0.005 cc.—would be enough to examine about 3,000 to 4,000 cases (0.01 cc. for each case), whereas if guinea pig complement were used the same amount would cover about 15,000 tests (0.002 cc. for each case). In a large hospital or in the Army there should be no difficulty in preparing any amount of the anti-human hemolytic amboceptor. For example, material for 100,000 tests could be prepared within 1 month in less than 100 rabbits. The amboceptor serum can be used in the fluid state, or, if the titer is high, impregnated into filter papers.

Special attention should be called to the fact that to obtain a powerful anti-human hemolytic amboceptor five to six intraperitoneal injections of corpuscles, thoroughly washed (until there is no trace of serum in the supernatant fluid), in doses of 5, 7, 10, 10, 10, and possibly another 10 cc. of the concentrated suspension (restored to the original volume of the blood) are required.[21] The bleeding may be done by the 9th or 10th day. The animals may be kept after bleeding for the production of more amboceptor by subsequent injections of the washed corpuscles.

Finally, it should be emphasized that only the acetone-insoluble fraction of tissue lipoids of required standards (Noguchi) should be used when utilizing the human complement in the fixation test.

[21] Intravenous injections of 3, 3, 4, and 4 cc. of the corpuscular suspension every 4 or 5 days also give excellent results.