A STANDARDIZED METHOD OF PERFORMING THE WASSERMANN REACTION

Adopted by Public Health, Municipal and Hospital Laboratories in Massachusetts

By

WILLIAM A. HINTON, M.D.,
Boston, Mass.

From the Department of Preventive Medicine and Hygiene of the Harvard Medical School and the Wassermann Laboratory of the Massachusetts Department of Public Health

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THE Bordet-Gengou phenomenon as applied by Wassermann, Nisser and Bruck in 1906 to the serodiagnosis of syphilis and as modified by many others since this time is now almost universally used. The numerous Wassermann methods have in general established the clinical specificity of complement inhibition by syphilitic serums and cerebrospinal fluids. Since the test is chiefly used by clinicians as an aid in the diagnosis and treatment of syphilis, there should be, as far as possible, a standard basis for interpretation. Nevertheless, very little progress has been made toward the choice of a uniform method of executing this important reaction. The following method is being published at this time because of its adoption by the large public health, municipal and hospital laboratories* in Massachusetts. It has served our purpose best because it is simpler than most methods, none of which has given consistently better results when simultaneously compared. It has been compared with the original Wassermann technic and with many of its

*At a meeting held in March, 1918, the following institutions were represented and adopted the method described: Massachusetts General Hospital, Boston; Dr. J. Homer Wright, Pathologist. Peter Bent Brigham Hospital, Boston; Dr. I. Chandler Walker, Associae in Med. Boston Board of Health; Dr. Philip Castleman, Bacteriologist. Sias Laboratory, Boston; Dr. Francis Slack, Director. Worcester Board of Health; James C. Coffey, Executive Officer. Brockton Board of Health; G. E. Bolling, City Bacteriologist and Chemist. Boston Laboratories (Private); L. W. Lee, Director. A Private Laboratory, Boston; A. R. G. Booth. Boston City Hospital, Boston; Dr. Frank B. Mallory, Pathologist. Westboro State Hospital, Westboro; Dr. Solomon C. Fuller, Pathologist. Base Hospital, Camp Devens; Dr. Leslie Spooner (in charge of Laboratory). Homeopathic Hospital, Boston; Dr. W. J. Watters, Pathologist. Boston Dispensary, Boston; Dr. Wm. A. Hinton, Pathologist.

modifications. During the past seven years extensive opportunity has been afforded to study its reliability. The material has been drawn chiefly from institutional cases where other laboratory tests and where the clinical facilities for diagnosing syphilis have been unusually good.

GLASSWARE AND APPARATUS

Before considering the preparation of reagents and the technic of their titration, it is well to describe briefly such apparatus as contributes to the accuracy of the method.

All glassware should be of good quality and sufficiently thick to prevent easy breaking. Jena glass and other expensive kinds add nothing to the accuracy of the method.

Test tubes 120 mm. long, 18 mm. in diameter, and 1 mm. thick are suitable for the titrations and the tests. Test tubes 100 mm. long, 12 mm. in diameter, and 1 mm. thick, are especially convenient for serums. For the preparation of reagents, wide-mouth, thick, clear, colorless, glass bottles with a large base, and of 200 to 500 c.c. capacity are especially adapted. Pipettes which have thick tips should be selected so that they will not break easily when the tips strike the bottom of the reagent bottles. Convenient sizes of pipettes are 0.5 c.c., 1.0 c.c., 2.0 c.c., 5.0 c.c. and 10 c.c. each graduated in tenths of a cubic centimeter. It is best that the graduation should extend no lower than 1 cm. from the tip and that the graduated portion of each pipette measure no less than 20 cm. long.

All new glassware should be boiled in dilute nitric acid (2.0 c.c. concentrated nitric acid to one liter of tap water) and then thoroughly washed in cold running tap water until every trace of acid has been removed. Glassware used in the test and titrations should be thoroughly cleansed as soon after use as possible with cold running tap water, so as to remove protein material, and then dried in a hot air oven. From time to time glassware should be placed in a cleaning solution consisting of two parts of potassium bichromate, three parts of crude sulphuric acid, and twenty-five parts of water, where it should remain for twelve hours or more and then be rinsed in hot running tap water until all traces of acid are removed. Frequent cleaning in this way will prevent the accumulation of proteins upon the surface of the glassware. In general, it may be said

that all glassware should be chemically clean. It is not always necessary, however, to sterilize it except when specified in the technic of the method.

Heavy brass wire test tube racks holding 40 tubes (being four tubes wide and ten tubes long) are convenient for the tests and titrations. The space for each tube should be about 3/4 inch square. These racks should be partitioned about one inch from the bottom, as well as on the top, so that the tubes will not be displaced by handling and shaking. Test tube racks of similar construction and material (except that the partitions should be about ¼ inch square) are used to hold the serums during inactivation and subsequent manipulations. These racks, however, should have sufficient capacity for the maximum number of specimens to be tested at one time. Further, it is best that these racks hold ten tubes or some multiple of ten. This will avoid errors and confusion when the serums are being pipetted for the Wassermann reaction.

For the purpose of incubation it is essential to employ a water-bath containing enough water to prevent appreciable lowering of temperature when 300 to 500 tubes are immersed. Incubation temperature should not vary more than one degree centigrade from 37° C. For inactivation a water-bath should be used which maintains a temperature of from 55° to 56° C. Higher temperatures than 56° C., even if maintained for only a short time, are very detrimental.

PREPARATION OF REAGENTS

Antigen.—This reagent is prepared from fresh human heart muscle (less than twenty-four hours postmortem, if possible). The connective tissue and fat are removed from the heart with scissors. The heart muscle is then cut into pieces of about 1 c.c., weighed, and the weight recorded. These pieces are kept for one or two weeks in a tightly covered mason jar containing sufficient 95 per cent alcohol to cover them. The hardened pieces of heart tissue are then removed from the alcohol and ground as finely as possible with an ordinary meat grinder. The alcohol used to harden the pieces of heart muscle and an additional quantity of 95 per cent alcohol should be added to the ground heart tissue to make the proportion of heart weight to total alcoholic volume equal 1:10; that is, 100 grams of heart tissue will require a total of 1000 c.c. of 95

per cent alcohol. This alcoholic suspension of ground heart tissue is allowed to stand in the incubator at 37° C. for from two to three weeks. Daily shaking by hand is required. About 100 c.c. of the alcoholic extract, free from heart fragments and sediment, is decanted and saturated with cholesterin. This saturation can be done most easily by adding about 0.7 gm. of cholesterin to 100 c.c. of the alcoholic extract and incubating at 37° C. for from twelve to eighteen hours. The cholesterinized heart extract is then allowed to stand at 20° C. in a water-bath for five or six hours. If cholesterin crystallizes out, the extract is saturated; otherwise, the procedure is repeated until saturation occurs. After saturation, the excess of cholesterin should be removed by filtering through ordinary filter paper. To the clear filtrate an equal amount of the filtered uncholesterinized alcoholic extract of heart muscle should be added. This makes the *stock antigen*. It will keep indefinitely at room temperature, but should not be kept in the refrigerator because most of the cholesterin will recrystallize. For routine tests, the stock antigen is freshly diluted with physiologic salt solution. (See Procedure under Table IV.)

Complement.—This is obtained from guinea pig's blood. From 8 c.c. to 15 c.c. of blood may be secured by cutting the carotid artery or preferably, by aspirating 6.0 c.c. to 10 c.c. of blood from the heart of a full grown guinea pig. An 18 or 19 gauge needle attached to a 10 c.c. Luer syringe may be used for this operation which most of the guinea pigs survive. If the guinea pigs are bled from the heart, the interval of bleeding should not be less than three weeks. No less than three guinea pigs should be bled for making any test or titration and the blood obtained pooled (mixed). The pooled blood is allowed to stand for one to two hours at 37° C. to hasten the separation of the serum. The serum is then withdrawn and centrifuged to remove the corpuscles. The clear serum, which contains complement, is diluted with physiologic salt solution to make a 10 per cent solution; that is, one part of guinea pig's serum plus nine parts of physiologic salt solution. The guinea pig's serum, diluted, (complement) should be kept in a refrigerator to keep it cool and to prevent exposure to intense sunlight because heat and sunlight effect its strength. Experience has shown that complement prepared and used on the same day gives the most dependable results.

Guinea pigs which are fed on any mixed ration consisting largely of carrots appear to yield the most uniform complement.

Washed Sheep's Corpuscles.—These are prepared from freshly obtained, defibrinated blood. The blood may be obtained by thrusting a 12 gauge needle or trochar into the external jugular vein of a sheep and allowing from 25 c.c. to 100 c.c. of blood (as required) flow into a thick glass bottle containing glass beads. A rubber tourniquet should be placed around the sheep's neck before the puncture to make the blood flow more rapidly. If a sheep is not kept for this purpose, blood may be obtained from a local abattoir. The blood must be constantly shaken for at least five minutes to prevent clotting. It is then strained through thin sterile gauze into 50 c.c. or 100 c.c. centrifuge tubes. In order to remove the serum four or five volumes of physiologic salt solution are added to the blood in the centrifuge tubes. These are centrifuged and as much of the supernatent fluid as possible is poured off. The corpuscles are washed three times in this way. Finally enough physiologic salt solution is added to make the volume of washed cells equal to that of the defibrinated blood originally used. This suspension of corpuscles in salt solution is called washed sheep's corpuscles and will keep for from three to five days in the refrigerator. The suspension is unsuitable for use when it shows spontaneous hemolysis. A standardized 5 per cent suspension of washed sheep's corpuscles—called standardized cells—is used in the test and titrations.

Standardized Cells.—A 5 per cent suspension of washed sheep's corpuscles is not always uniform because a greater or lesser clot may be formed during the process of defibrinating the sheep's blood. In order to employ a suspension of uniform strength it is necessary to have a standard for comparison. A color standard may be made by adding red ink and a small amount of methylene blue to 0.5 per cent carbolized tap water. This colored mixture is so adjusted as to give a concentration of color equivalent to 0.5 c.c. of a carefully* and accurately prepared suspension of washed sheep's corpuscles, which have been hemolyzed by the addition of 1.5 c.c. of tap water. After this color standard has been properly adjusted it will keep indefinitely. Each new suspension of cells should be standardized. Two small homeopathic vials of about the same diameter as the tubes employed in the test and titrations are

*This applies particularly to the prevention of partial clotting during the process of defibrinating the blood.

suitable for the comparison. In order to do this 5.0 c.c. of washed sheep's corpuscles are added to 95 c.c. of physiologic salt solution. Five-tenths of a cubic centimeter of this suspension is accurately pipetted into one of the vials, 1.5 c.c. of tap water is added and the vial shaken by hand until the cells are hemolyzed. This solution of hemolyzed cells is then compared with 2 c.c. of the color standard. If the intensity of color is not the same as that of the standard, the suspension should be adjusted to make it so. A suspension prepared in this way is called *standardized cells*. In practice it is often necessary to use 6 c.c. or 7 c.c. washed sheep's corpuscles with 95 c.c. of physiologic salt solution.

Amboceptor.—Amboceptor is obtained from rabbit's blood and is prepared by injecting washed sheep's corpuscles into the peritoneal cavity of a full grown rabbit at three or four day intervals. Freshly obtained and prepared washed sheep's corpuscles must be used for each injection. The amount of the first injection is about 7.0 c.c., the second 14 c.c., the third 21 c.c., and the fourth about 28 c.c. On the eighth or ninth day after the last injection the rabbit is bled from the ear, after moistening its surface with xylol, to obtain from 2 to 3 c.c. of blood. This blood is allowed to clot, and clear serum is removed and heated in a water-bath at 55° C. for one-half hour, in accordance with the preparation of stock amboceptor (q. v.). This heated immune rabbit's serum (*amboceptor*) is titrated immediately according to Table I, to see whether it is sufficiently strong to be used in the Wassermann reaction. It is inconvenient to use an amboceptor with a titre less than 1:1000. If the amboceptor is strong enough for convenient use, 40 to 60 c.c. of blood is obtained from the rabbit's heart and delivered in a sterile, covered bottle. For bleeding, a large syringe and an 18 or 19 gauge needle should be used. On the other hand, should the titre be less than 1:1000, a fifth intraperitoneal injection of 35 c.c. of washed sheep's corpuscles is immediately made. After a period of five days from the fifth injection 40 to 60 c.c. is obtained from the heart and treated as described below. After an intervening rest of from four to six weeks and upon being reimmunized, the same rabbit may be used repeatedly for the production of amboceptor.

Stock amboceptor is prepared from the immune rabbit's blood according to the following method: place the bottle containing the 40 c.c. to 60 c.c. of immune rabbit's blood in a water-bath at 37° C.

for from one-half to three-quarters of an hour to hasten the separation of the serum. All of the serum is withdrawn with a pipette and delivered into a sterile 100 c.c. centrifuge tube and stoppered with a sterile cork. It is then centrifuged to remove the corpuscles and distributed in 2 c.c. to 5 c.c. quantities into small sterile test tubes. The tubes containing the serum are heated to 55° C. for one-half hour and corked with sterile paraffined stoppers. This heating is done to prevent bacterial growth. Each of the tubes contains *stock amboceptor* which should be titrated, according to Table I, before it is used. Stock amboceptor keeps indefinitely in a refrigerator. Bacterial contamination may effect its role in hemolysis but rarely does.

Sensitized Cells.—These are prepared by mixing equal parts of standardized cells and dilute amboceptor. (See discussion under Table I.) This mixture is incubated in a water-bath at 37° C. for one-half hour to sensitize the cells. One cubic centimeter of a freshly prepared mixture is used in the Wassermann reaction.

Physiologic Salt Solution.—This is prepared by adding 8.5 grams of sodium chloride (c.p.) to 1000 c.c. of distilled water. It is better (but not absolutely necessary) to sterilize it by boiling or autoclaving.

PREPARATIONS OF SERUMS AND SPINAL FLUIDS FOR TESTING

The blood is preferably withdrawn from the patient with a Luer syringe, immediately delivered into a test tube, and allowed to stand at room temperature until it clots. After the blood has clotted it should be gently shaken to loosen the clot from the sides of the tube, and then placed in a refrigerator. The serum should be poured, not later than the third day, into a small test tube and inactivated at 55° C. for one-half hour. This is chiefly to destroy the complement, but it also inhibits bacterial growth. If the serum is not clear because of the presence of corpuscles, it should be centrifuged, and the clear serum poured off for inactivation. If the amount of serum is small, it is better to withdraw it with a medicine dropper which has a long tip. Only one such dropper is necessary, provided it is very carefully washed with salt solution at least three times before using it for another serum. If the serum is not tested for two or more days after inactivation, it should be preserved by the addition of 5 per cent carbolic acid in physiologic salt solution

in the proportion of ten parts of serum to one part of the carbolic acid solution. If one wishes to test the specimen the day it is obtained from the patient, it should be placed in a water-bath at 37° C. for one-half to three-quarters of an hour to hasten the separation of the serum, centrifuged, and the serum withdrawn and inactivated.

Although the preparation of serums is very simple in practice, experience is necessary to develop technic which will give reliable results. A large proportion of anticomplementary serums usually indicates that they are improperly prepared.

Spinal fluids are not inactivated before testing. If they are to be kept for several days, they should be preserved by the addition of 5 per cent carbolic acid solution in the proportion of ten parts of the spinal fluid to one part of the carbolic acid solution and kept in the refrigerator.

TITRATION OF REAGENTS AND THE WASSERMANN REACTION

After the reagents have been prepared, it is necessary to determine their quantitative relationships by titration. The following outline gives the steps in this procedure:

1. The amboceptor should be titrated according to Table I.
2. The test for anticomplementary and natural hemolytic properties should be made according to Table III. As a preliminary procedure for this titration "the complement titre" should be obtained according to Table II (Rows I and V only are used in this preliminary titration).
3. The Wassermann reaction should be performed according to Table IV after having performed the entire titration according to Table II as a preliminary step.

In the tables which follow, each square represents a space in the rack containing a test tube into which the reagents are pipetted according to directions.

PROCEDURE *for table I.*

I. Prepare:

1. Cells—a standardized 5 per cent suspension of washed sheep's corpuscles in physiologic salt solution.
2. Complement—a 10 per cent solution of guinea pig's serum in physiologic salt solution.
3. Amboceptor.—This is prepared from (stock amboceptor) by diluting with physiologic salt solution in the following manner: Pipette very ac-

Table I

Titration of Amboceptor

Row I To show that each reagent has no hemolyzing effect; therefore, no tube in this row should show hemolysis.		(1:500)									
	Amboceptor	0.5 c.c.	.....	.....							
	Complement	.....	0.5 c.c.	.....							
	Cells	0.5 c.c.	0.5 c.c.	0.5 c.c.							
	Salt Solution	1.5 c.c.	1.5 c.c.	2.0 c.c.							
		(1:500)	(1:1000)	(1:1500)	(1:2000)	(1:2500)	(1:3000)	(1:3500)	(1:4000)	(1:4500)	(1:5000)
Row II To determine the unit of amboceptor.	Amboceptor	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
	Complement	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
	Cells	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
	Salt Solution	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.

Diagram of Dilutions of Amboceptor

Amboceptor diluted 1:100	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.
Physiologic salt solution	0.4 c.c.	0.9 c.c.	1.4 c.c.	1.9 c.c.	2.4 c.c.	2.9 c.c.	3.4 c.c.	3.9 c.c.	4.4 c.c.	4.9 c.c.
Resulting dilution of amboceptor	1:500	1:1000	1:1500	1:2000	1:2500	1:3000	1:3500	1:4000	1:4500	1:5000

curately 0.1 c.c. of the stock amboceptor (see page 8) into a test tube, and add 9.9 c.c. of 0.85 per cent salt solution. This makes a dilution of 1:100. From the dilution of 1:100 the following dilutions are prepared according to the diagram below. A separate test tube and a separate pipette should be used for the preparation of each dilution and each should be vigorously shaken before pipetting. (See Diagram, p. 11.)

II. Pipette in the following order according to Table I:
1. Amboceptor with a 1.0 c.c. pipette,
2. Complement with a 5.0 c.c. pipette,
3. Cells with a 5.0 c.c. pipette, and,
4. Salt solution with a 10 c.c. pipette.

A separate pipette should be used for each reagent and it should be placed in a flat pan half full of cool tap water after use. This applies to every pipetting procedure.

III. Incubate in water-bath one hour at 37C.

IV. Read the results of the titration.

V. Make a final titration by employing amboceptor dilutions which differ by 100 between the limits of complete and partial hemolysis. Suppose the tubes containing a dilution of 1:1000 shows complete hemolysis and the tube which contains the 1:1500 dilution shows partial hemolysis; then a final titration should be made by employing amboceptor dilutions of 1:1000, 1:1100, 1:1200, 1:1300, 1:1400 and 1:1500. The technic is otherwise a repetition of the above procedure. When the preliminary titre is above 1:4000 it is better to dilute two to three cubic centimeters of the stock amboceptor so that its strength will be about 1:2000, and then make a final titration as above directed. Amboceptor so diluted gradually loses its potency and therefore, should be carefully watched to discover any slight change in its hemolyzing power.

DISCUSSION

The largest dilution showing complete hemolysis contains the unit. The unit of amboceptor, therefore, may be defined as the quantity of heated immune rabbit's serum which hemolyzes 0.5 c.c. of a standardized 5 per cent suspension of washed sheep's corpuscles in the presence of 0.5 c.c. of 10 per cent guinea pig serum (complement). Two units of amboceptor are used in the other titrations and in the Wassermann reaction. For convenience, the rabbit's immune serum is diluted with physiologic salt solution so that 0.5 c.c. contains two units. The method of making such a dilution is shown in the following illustration: Suppose that the tubes containing dilutions greater than 1:1200 show gradually increasing inhibition of hemolysis, the amboceptor should be used in a dilution of 1:600 which is prepared by very accurately pipetting 0.1 c.c. of the stock amboceptor into a flask or bottle of 100 to 300 c.c. capacity and adding 60 c.c. of physiologic salt solution and then thoroughly shaking. This latter solution is called *dilute amboceptor*, 0.5 c.c. of which is used in the test and in the subsequent titrations. Dilute amboceptor may weaken in two to four days after its preparation even if kept in the refrigerator.

TABLE II

COMBINED TITRATION OF COMPLEMENT, AMBOCEPTOR AND ANTIGEN

Row I To show that each reagent has no hemolyzing effect. Therefore, no tube in this row should show hemolysis.	Complement	1.0 c.c.	.....	.....						
	Amboceptor	.....	0.5 c.c.	.....						
	Cells	0.5 c.c.	0.5 c.c.	0.5 c.c.						
	Salt Solution	1.0 c.c.	1.5 c.c.	2.0 c.c.						
Row II To show that *dilute antigen C* has been properly prepared. Then it has little or no anticomplementary effect.	Complement	0.5 c.c.	0.4 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
	Amboceptor	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.4 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.
	Cells	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
	Antigen C	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
	Salt Solution	0.5 c.c.	0.6 c.c.	0.7 c.c.	0.8 c.c.	0.9 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.
Row III To show that *dilute antigen B* has been properly prepared. Then it has little or no anticomplementary effect.	Complement	0.5 c.c.	0.4 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
	Amboceptor	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.4 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.
	Cells	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
	Antigen B	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
	Salt Solution	0.5 c.c.	0.6 c.c.	0.7 c.c.	0.8 c.c.	0.9 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.
Row IV To show that *dilute antigen A* has been properly prepared. Then it has little or no anticomplementary effect.	Complement	0.5 c.c.	0.4 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
	Amboceptor	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.4 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.
	Cells	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
	Antigen A	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
	Salt Solution	0.5 c.c.	0.6 c.c.	0.7 c.c.	0.8 c.c.	0.9 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.
Row V To determine the unit of complement.	Complement	0.5 c.c.	0.4 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
	Amboceptor	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.4 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.
	Cells	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
	Salt Solution	1.0 c.c.	1.1 c.c.	1.2 c.c.	1.3 c.c.	1.4 c.c.	0.6 c.c.	0.7 c.c.	0.8 c.c.	0.9 c.c.

PROCEDURE for table 2

I. Prepare:

1. Cells.—A standardized 5 per cent suspension of washed sheep's corpuscles in physiologic salt solution.
2. Complement.—A 5 per cent solution made by diluting 12 c.c. of the 10 per cent guinea pig's serum with 12 c.c. of physiologic salt solution.
3. Dilute Amboceptor.—Diluted stock amboceptor, 0.5 c.c. of which contains two units.
4. Dilute antigen.—For technic of its preparation (see Procedure I under Table IV).

II. Pipette in the order indicated in Table II:

1. Five per cent complement with a 2 c.c. pipette,
2. Dilute amboceptor with a 5 c.c. pipette,
3. Dilute antigen with a 5 c.c. pipette,
4. Cells with a 5.0 c.c. pipette and,
5. Physiologic salt solution with a 10 c.c. pipette.

III. Incubate in water-bath for one-half hour at 37°C.

IV. Read the results of the titration.

DISCUSSION

This titration is always made immediately before testing specimens by the Wassermann reaction (Table IV).

The tube in Row V containing the smallest amount of complement that causes complete hemolysis contains the unit. Two units or an equal amount of 10 per cent complement is used in the test. For example—suppose 0.4 c.c. is the smallest amount of the 5 per cent guinea pig serum that gives complete hemolysis, then 0.4 c.c. of the 10 per cent solution should be used in the test.

For convenience the 10 per cent guinea pig serum is diluted with salt solution so that 0.5 c.c. contains two units of complement. Such a solution is called *dilute complement*. In this example where the complement titre is 0.4 c.c. the 10 per cent guinea pig serum is again diluted by the addition of one part of physiologic salt solution to each four parts of the 10 per cent guinea pig serum to make it have the concentration of *dilute complement*. The hemolytic system is suitably adjusted for the Wassermann reaction when the tube in Row V containing 0.3 c.c. of amboceptor is completely hemolyzed and the one containing 0.2 c.c. is moderately hemolyzed. With correct technic the degree of hemolysis will be proportional to the amount of complement in the first five tubes of each row. Guinea pig serum which gives a complement titre of less than 0.3 c.c. or greater than 0.5 c.c. usually does not give dependable results.

PROCEDURE for table 3

I. Prepare:

1. Cells.—A standardized 5 per cent suspension of washed sheep's corpuscles in physiologic salt solution.
2. Dilute complement—10 per cent guinea pig serum diluted so that 0.5 c.c. contains 2 units. Before setting up this titration, determine the complement unit according to Table II, using Rows I and V only.

TABLE III

TITRATION FOR THE NATURAL HEMOLYTIC AND ANTICOMPLEMENTARY PROPERTIES OF ANTIGEN

Row I To determine the natural hemolytic properties of antigen. Usually no tube in this row shows hemolysis.	Antigen Complement Cells Salt Solution	1.0 c.c. 0.5 c.c. 0.5 c.c. 0.5 c.c.	0.8 c.c. 0.5 c.c. 0.5 c.c. 0.7 c.c.	0.6 c.c. 0.5 c.c. 0.5 c.c. 0.9 c.c.	0.4 c.c. 0.5 c.c. 0.5 c.c. 1.1 c.c.	0.2 c.c. 0.5 c.c. 0.5 c.c. 1.3 c.c.	0.1 c.c. 0.5 c.c. 0.5 c.c. 1.4 c.c.	0.05 c.c. 0.5 c.c. 0.5 c.c. 1.45 c.c.
Row II To determine the largest amount of antigen that does not inhibit hemolysis. Usually tubes containing 0.4 c.c. or more antigen show inhibition of hemolysis.	Antigen Complement Salt Solution	1.0 c.c. 0.5 c.c.	0.8 c.c. 0.5 c.c. 0.2 c.c.	0.6 c.c. 0.5 c.c. 0.4 c.c.	0.4 c.c. 0.5 c.c. 0.6 c.c.	0.2 c.c. 0.5 c.c. 0.8 c.c.	0.1 c.c. 0.5 c.c. 0.9 c.c.	0.05 c.c. 0.5 c.c. 0.95 c.c.
Row III To show the amount of turbidity due to cholesterin and other lipoidal substances contained in antigen.	Antigen Hemolyzed cells Salt Solution	1.0 c.c. 1.5 c.c.	0.8 c.c. 1.5 c.c. 0.2 c.c.	0.6 c.c. 1.5 c.c. 0.4 c.c.	0.4 c.c. 1.5 c.c. 0.6 c.c.	0.2 c.c. 1.5 c.c. 0.8 c.c.	0.1 c.c. 1.5 c.c. 0.9 c.c.	0.05 c.c. 1.5 c.c. 0.95 c.c.

3. Dilute amboceptor—diluted stock amboceptor, 0.5 c.c. of which contains 2 units. (See discussion under Table 1.)
4. Antigen—a dilution of the stock antigen prepared by taking 3.0 c.c. of stock antigen and adding 12.00 c.c. of physiologic salt solution drop by drop and shaking well after the addition of each drop.
5. Sensitized cells—a mixture consisting of 5.0 c.c. of a standardized suspension of washed sheep's corpuscles and 5.0 c.c. of dilute amboceptor.
6. Hemolyzed cells.—These are prepared by adding 10.0 c.c. of tap water to 5.0 c.c. of the standardized 5 per cent suspension of washed sheep's corpuscles and shaking until the cells are hemolyzed.

II. Pipette:
1. Antigen—(diluted one plus four as above described) with a 2 c.c. pipette.
2. Complement with a 5 c.c. pipette,
3. Standardized cells with a 5 c.c. pipette in Row I,
4. Hemolyzed cells with a 10 c.c. pipette in Row III, and,
5. Physiologic salt solution with a 10 c.c. pipette.

III. Incubate in water-bath at 37°C. for 10 minutes.

IV. Pipette 1.0 c.c. of sensitized cells with a 10 c.c. pipette, into each tube in Row II.

V. Incubate one hour longer.

VI. Read results of titration.

DISCUSSION

Cholesterinized antigens cause a turbidity proportional to the amount of antigen. This turbidity should not be mistaken for the inhibitory effect of the antigen upon the complement. Compare Rows II and III in order to determine the strictly anticomplementary effect of the antigen.

An available antigen contains no natural hemolytic properties either in one-half the amount or in twice the amount to be employed in the test, while the degree of anticomplementary effect is variable. A maximum of one-half the largest amount causing no inhibition may be used in testing unknown serums and spinal fluids.

The only way to standardize an antigen either qualitatively or quantitatively is by testing its inhibitory properties against a large number of known positive and negative specimens of blood and spinal fluid.

PROCEDURE for table 4

I. Prepare enough of the following reagents to perform the necessary titrations and tests for the entire day.
1. Cells—a standardized 5 per cent suspension of washed sheep's corpuscles in physiologic salt solution.
2. Dilute complement—10 per cent guinea pig serum titrated according to Table II, and diluted with physiologic salt solution so that 0.5 c.c. contains two units.
3. Dilute amboceptor—diluted stock amboceptor 0.5 c.c. of which contains two units.

TABLE IV

THE WASSERMANN REACTION

	REAGENTS	POSITIVE SERUM FOR CONTROL	DOUBTFUL SERUM FOR CONTROL	NEGATIVE SERUM FOR CONTROL	EACH SERUM FOR WASSERMANN TEST	EACH SPINAL FLUID FOR WASSERMANN TEST	ANTIGEN CONTROLS To show that twice the amount of antigen used in the reaction is not anticomplementary. Therefore, hemolysis should be complete in each tube.	
Row I Serum Controls*	Serum Comp-sal-mixt.	0.2 c.c. 1.0 c.c.	0.2 c.c. 1.0 c.c.	0.2 c.c. 1.0 c.c.	0.2 c.c. 1.0 c.c.	1.0 c.c. 1.0 c.c.		
Row II Tests with Antigen C.	Serum Comp-sal-ant. C. mixt.	0.1 c.c. 1.0 c.c.	0.1 c.c. 1.0 c.c.	0.1 c.c. 1.0 c.c.	0.1 c.c. 1.0 c.c.	0.5 c.c. 1.0 c.c.	Dilute Antigen C.	0.5 c.c. 1.0 c.c.
Row III Tests with Antigen B.	Serum Comp-sal-ant. B. mixt.	0.1 c.c. 1.0 c.c.	0.1 c.c. 1.0 c.c.	0.1 c.c. 1.0 c.c.	0.1 c.c. 1.0 c.c.	0.5 c.c. 1.0 c.c.	Dilute Antigen B.	0.5 c.c. 1.0 c.c.
Row IV Tests with Antigen A.	Serum Comp-sal-ant. A. mixt.	0.1 c.c. 1.0 c.c.	0.1 c.c. 1.0 c.c.	0.1 c.c. 1.0 c.c.	0.1 c.c. 1.0 c.c.	0.5 c.c. 1.0 c.c.	Dilute Antigen A.	0.5 c.c. 1.0 c.c.

*To show that twice the amount of each serum or spinal fluid used in the Wassermann reaction is not anticomplementary, therefore, hemolysis should be complete for each specimen that gives a satisfactory test.

4. Dilute antigen.—Calculate the amount of stock antigen required for the day's test and for the combined titration of complement, amboceptor and antigen (see Table II). Pipette this amount of stock antigen into a glass bottle or flask, add the four parts of physiologic salt solution drop by drop (shaking well after the addition of each drop) and again dilute with physiologic salt solution so that each 0.5 c.c. of the resulting mixture contains the amount of antigen used in the Wassermann reaction. Dilute antigen should be freshly prepared for each day's test.

5. Complement-saline-antigen-mixture.—This is prepared as follows: Mix enough of the dilute antigen A with an equal amount of dilute complement to perform the day's test. In like manner make complement-saline-antigen-mixture for antigens B and C.

CALCULATIONS FOR THE PREPARATION OF COMPLEMENT-SALINE-ANTIGEN-MIXTURE

FOR 35 SPECIMENS

Tubes to contain antigen A:
1. for titration of Table II.. 9 tubes
2. for 35 specimens .....35 tubes
3. for working margin..... 6 tubes

Total number required.....50 tubes

50 x 0.15 antigen A..... 7.5 c.c.
50 x 0.35 salt solution.....17.5 c.c.

Total amt. dilute antigen A..25.0 c.c.

In this example it is assumed that experience has shown that 0.15 c.c. of the one plus four dilution of this particular stock antigen A contains the correct amount of inhibitory property for one specimen. To make 7.5 c.c. of this one plus four antigen as needed for the 50 tubes, take 1.5 c.c. of stock antigen A and add 6.0 c.c. of physiologic salt solution drop by drop as previously directed.

Likewise it has been found that 0.1 c.c. of this particular antigen B and 0.075 c.c. of this particular antigen C. (the one plus four dilutions) contain the proper amount of antigenic property. The calculations which follow for the preparation of the antigens are otherwise similar.

50 x 0.1 antigen B..... 5.0 c.c.
50 x 0.4 salt solution.....20.0 c.c.

Total amount of dilute antigen B..... 25.0 c.c.

50 x .075 antigen C..... 3.75 c.c.
50 x 0.425 salt solution.....21.25 c.c.

Total amount dilute antigen C.....25.00 c.c.

To calculate the number of tubes to contain dilute complement, multiply the number of tubes (50 in this example) by four so that there will be enough for each of the three antigens and for the serum controls. (See Table IV.) Then multiply this number (200) by the complement titre 0.4 since it is assumed that four-tenths of a cubic centimeter of this particular complement contains two units as determined by titration according to Table II.

200 x 0.4 complement.....80.00 c.c.
200 x 0.1 salt solution .....20.00 c.c.

Total amt. dilute complement..100.00 c.c.

Mix 25 c.c. of the dilute antigen A with 25 c.c. of dilute complement. This makes 50 c.c. of complement-saline-antigen mixture (abbreviated: comp-sal-ant.

mix.) which is enough for the day's test. Likewise prepare complement-saline-antigen mixtures from stock antigen B and C. The remaining 25 c.c. of dilute complement is for use in Row I.

6. Complement-saline-mixture—equal parts of dilute complement and physiologic salt solution.
7. Sensitized cells—equal parts of standardized 5 per cent suspension of washed sheep's corpuscles and dilute amboceptor which have been incubated together for one-half hour at 37°C.

II. Pipette:

1. The serums with a 0.5 c.c. pipette and spinal fluids with a 1.0 c.c. pipette graduated in tenths in the amounts indicated in the table. Use a separate pipette for each specimen. In practice it is often necessary to use half quantities spinal fluid and of each reagent in testing it because the amount of spinal fluid submitted for examination will not admit of the full amounts as indicated in the table. Important: Carefully inspect each tube to be certain that it contains the proper serum or spinal fluid. This may be done by matching the color of each specimen in the test with that of the serum or spinal fluid in the inactivating rack.
2. Five tenths of a cubic centimeter of the dilute antigen A with a 10 c.c. pipette into the antigen control tube Row IV.
3. Five tenths of a cubic centimeter of dilute antigen B with a 10 c.c. pipette into the antigen control tube, in Row III.
4. Five tenths of a cubic centimeter of dilute antigen C with a 10 c.c. pipette into the antigen control tube, Row II.
5. One cubic centimeter of the complement-saline-antigen mixture (prepared from the stock antigen A) with a 10 c.c. pipette into each of the tubes in Row IV.
6. One cubic centimeter of the complement-saline-antigen mixture (prepared from stock antigen B) with a 10 c.c. pipette into each of the tubes in Row III.
7. One cubic centimeter of the complement-saline-antigen mixture (prepared from stock antigen C) with a 10 c.c. pipette into each of the tubes in Row II.
8. One cubic centimeter of complement-saline mixture with a 10 c.c. pipette into each tube in Row I.

III. Shake each rack containing the tubes so that the contents will be thoroughly mixed.

IV. Incubate at 37°C. for forty minutes.

V. Pipette 1.0 c.c. of sensitized cells with a 10 c.c. pipette into each tube.

VI. Shake the racks again to mix contents in tubes.

VII. Incubate at 37°C. for one hour.

VIII. Read the results. Complete or moderate inhibition of hemolysis with antigen A, B, and C, equals positive. Slight inhibition of hemolysis (75 per cent or more the cells hemolyzed) with antigen A and B, together with slight or no hemolysis with antigen C. equals doubtful. Complete hemolysis with all antigens equals negative.

DISCUSSION

Antigen A and Antigen B should be prepared according to direction given on page 600.* Each is obtained from a different human heart. Each should inhibit to the same degree in the presence of 0.1 c.c. of any positive serum or 0.5 c.c. of any positive spinal fluid. It is not necessary that the fixing amounts be equal, for example in the above illustration, 0.15 c.c. of antigen A, has the same fixing power as 0.1 c.c. of antigen B. It is essential to use two antigens in this way because faulty technic in preparing the dilutions of the antigens or in pipetting the serums or spinal fluids are easily detected by this comparison.

Antigen C is prepared from guinea pigs' hearts which have been allowed to stand in 95 per cent alcohol for two months or more, otherwise the method of preparation is the same as for the human heart antigen. Antigen C as employed in the State Wassermann Laboratory is more sensitive than either antigens A or B, and is therefore, useful in selecting the presumptive positives from the negatives. As a preliminary test, each specimen is prepared and set up according to Row II only (no serum control is necessary for this preliminary test). Since antigen C is more sensitive than the other two antigens, those specimens which show inhibition of hemolysis are presumptively positive. Such specimens are selected, placed in a separate rack, and in the afternoon are not only retested with antigen C, but also tested with antigens A and B, the whole test being exactly as indicated in Table IV. Complement prepared from the same guinea pigs' serum is used in the preliminary test and in the Wassermann reaction as given in Table IV.

CONCLUSION

The reaction as carried out by this method is reported and interpreted as follows: POSITIVE indicates syphilis, except very rarely in acute febrile conditions such as malaria and pneumonia. NEGATIVE does not exclude syphilis. In dealing with obscure conditions, less than three negatives has little diagnostic significance. DOUBTFUL suggests syphilis. It is, therefore, advisable to have three or more specimens submitted in such a case. A persistently or predominatingly doubtful reaction usually indicates syphilis. UNSATISFACTORY means that the test was unsuccessful either because of the condition of the specimen or because of some difficulty with technic.†

*Standardized antigens and standardized ambocepter are furnished to any laboratory in Massachusetts to perform the Wassermann test according to this method.

†Hemolyzed specimens of blood, bacterially or chemically contaminated specimens frequently give unreliable results even if they are not anticomplementary. The presence of bacterial or chemical contamination is usually indicated by a purplish, brownish or other unusual discoloration of the mixture after the test has been incubated for the last time.