THE KAHN PRECIPITATION TEST FOR SYPHILIS

DEMONSTRATION

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THIS paper will discuss the author's precipitation test for syphilis as demonstrated before the Laboratory Section of the fifty-second annual meeting of the American Public Health Association at Boston on October 10, 1923. By courtesy of the editors of this JOURNAL, we are able to include some of the results of standardization studies carried out since the demonstration and fully discussed in another paper.[1] The following are included: (1) a simplified and standardized procedure for the preparation and titration of antigen, (2) some improvements in the details in carrying out the routine test, (3) a special quantitative procedure applicable particularly to the study of the serologic effect of antisyphilitic treatment, (4) a more standardized technic for the test with spinal fluids.

I. GLASSWARE AND APPARATUS REQUIRED FOR THE TEST *

All glassware employed in the test must be chemically clean and neutral.

1. *Test tubes:* Standard antigen dilution tubes are 5.5 cm. in length and 1.5 cm. in diameter.

Standard tubes for performing the test are 7.5 cm. in length and 1 cm. in diameter.

2. *Test tube racks:* Racks should be of such construction as to permit vigorous shaking of the tubes.

3. *Pipettes:* 10 c.c. pipettes marked in 0.1 c.c. quantities. 1 c.c. pipettes marked in 0.01 c.c. quantities. 0.2 c.c. pipettes marked in 0.001 c.c. quantities.

4. *Shaking machine:* May be of any construction which will hold the test tube racks employed.

5. *Inactivating bath* (*56° C.*) as well as centrifuge and centrifuge tubes may be of any make which will be found convenient in the particular laboratory.

II. REAGENTS EMPLOYED IN THE TEST

The three ingredients entering into the test are (1) Antigen, (2) Serum, and (3) Physiologic Salt Solution.

1. ANTIGEN †

Method of Preparation:

The method of antigen preparation has recently been standardized with a view of eliminating several of the variable elements inherent in the method previously used.

The unit amount of powdered beef heart used for preparing antigen is 25 gms. and it is always extracted in a 250 c.c. Erlenmeyer flask. The ether extraction consists of four ether "washings" of the powdered muscle at ten minute intervals with 100, 75, 75 and 75 c.c. ether, respectively. The subsequent alcohol extraction is carried out for three days at room temperature (21° C.). Twenty-five gms. of powdered beef heart will yield about 75 c.c. antigen. If the preparation of larger amounts is desired, as many 25 gm. quantities are employed as needed.

Powdered Heart Muscle.—About 400 gms. of heart muscle are cut out from at least three fresh beef hearts and passed four times through a meat grinder. The ground material is spread into a thin layer on a porcelain platter or glass plate and dried by means of one or two revolv-

* Glassware and apparatus for the test may be procured from the Chicago Glass Products Company, Chicago, Ill.

† The Michigan Department of Health furnishes antigen at a minimum cost.

ing fans. After six or eight hours, when the exposed surface is relatively dry, the material is turned over and drying continued over night. When the layer of beef heart is in the form of a dry plate, it is broken up into small pieces and drying continued until the material is brittle and easily breakable. The material is now ground into powder form by means of a mortar or coffee grinder which is used for no other purpose.

Powdered beef heart is now obtainable on the market from the Digestive Ferments Company, Detroit. It is prepared essentially as outlined above, except on a large scale—as many as seventy-five beef hearts entering into the preparation of a given lot. This assures a higher degree of uniformity than can be obtained with three beef hearts. Because of this uniformity element, the market product is used in this laboratory almost exclusively.

Extraction of Powdered Muscle with Ether.—Twenty-five gm. of powdered beef heart are placed in a 250 c.c. Erlenmeyer flask and 100 c.c. ether (anesthesia) added. The flask is shaken from time to time for an interval of ten minutes after which the ether is filtered off. The filtration process may be hastened somewhat by applying gentle pressure to the beef heart by means of a spatula. Filtration is completed when pressure with the spatula does not cause drops of ether to pass through the funnel.

The moist beef heart is now returned to the original 250 c.c. extraction flask. This may be done by first transferring the beef heart from the funnel to a sheet of white paper and breaking the material up with a spatula into pieces small enough for the mouth of the flask. Seventy-five c.c. ether are now added and the mixture again shaken for a ten-minute interval and filtered as above.

After the second filtration of ether, the beef heart is transferred to the flask for a third time and 75 c.c. ether again added. The mixture is shaken for a ten-minute interval and again filtered as described above.

The moist beef heart is now transferred for the fourth and last time to the flask and 75 c.c. ether added, the mixture shaken for ten minutes and filtered. After gentle pressure with a spatula does not cause drops of ether to pass through the funnel—the endpoint employed in each of the four ether filtrations—the beef heart is transferred to a sheet of white paper and dried either at room temperature or at 37° C. The drying usually requires from ten to fifteen minutes. When no ether odor is detectable, the beef heart is ready for extraction with alcohol. This extraction may be carried out in the same flask used for the ether extractions, provided the flask is entirely freed from ether odor.

Extraction of Powdered Muscle with Alcohol.—The ether extraction being completed, the dry powdered muscle is weighed and placed in a 250 c.c. flask. Usually there will be twenty-three gms. or less of the powder due to the loss during the ether extraction. Five c.c. of 95 per cent alcohol are added per gm. of powder. The flask is shaken for ten minutes and extraction allowed to continue at room temperature (21° C.) for three days without shaking. At the end of this period, the mixture is shaken for five minutes and filtered. The filtrate is kept in the dark at room temperature as stock antigen solution.

Cholesterinization of Alcoholic Extract.—A given amount of alcoholic extract—likely to be used in a month or two—is cholesterinized by adding 6 mg. of chemically pure cholesterin per c.c. of extract. The cholesterin is dissolved by rotating the flask in a water bath at 37° C. When all the cholesterin has been dissolved, the antigen is filtered to remove impurities and allowed to stand one day. It is then ready to be titrated or standardized for the test.

It is well to emphasize in connection with antigen preparation that the ether and alcohol employed should be of high purity and that the latter should be 95

per cent. At one time we unknowingly used 80 instead of 95 per cent alcohol with misleading results.

Tinfoil-covered corks have been found most satisfactory as stoppers for flasks used in antigen preparation and storing. Rubber as well as cork stoppers give off soluble elements into the alcohol which modify the final product.

Method of Antigen Titration:

The aim of the titration of antigen for this test is to find the minimum amount of physiologic salt solution to use with antigen which will result in an antigen-salt solution precipitate that is soluble on further addition of salt solution. The titration is carried out in the presence of salt solution and not in the presence of serum. solution and not in the presence of serum, although the latter may be used as a check on the titration, if desired.

One c.c. amounts of cholesterinized antigen are added to each of five standard antigen dilution tubes (5.5 cm. length and 1.5 cm. diameter). To five similar tubes are added the following amounts of physiologic salt solution, respectively: 0.8, 0.9, 1.0, 1.1 and 1.2 c.c. Each salt solution tube is emptied into a given anti gen tube and, without waiting to drain the salt solution, the mixture is immediately poured back and forth five or six times to permit thorough mixing. Each of the five antigen dilutions will show the presence of a definite precipitate. The character of these precipitates, however, will vary according to the quantity of salt solution used. Some of the precipitates will be found to be stable and will not dissolve when mixed with salt solution; other precipitates again will readily dissolve in salt solution.

The solubility in salt solution of each of the five antigen dilution precipitates is tested as follows: 0.05, 0.025 and 0.0125 c.c. amounts, respectively, of each antigen dilution are pipetted with a 0.2 c.c. pipette graduated in 0.001 c.c. into three tubes (7.5 cm. in length and 1 cm. diameter). These small quantities are pipetted in each case to the bottom of the tubes. 0.15 c.c. quantities of physiologic salt solution are now added to each tube. The rack is shaken vigorously for two minutes after which 0.5 c.c. salt solution is added to each tube and observation made as to whether or not the original antigen dilution precipitate has gone back into solution. The antigen dilution tube containing the smallest amount of salt solution in proportion to antigen, having a precipitate which goes back into solution in salt solution, as shown by this three tube test, represents the endpoint of this titration and determines the proportion in which antigen is to be mixed with salt solution in the performance of the tests.

The accompanying table gives an outline of a typical antigen titration.

TABLE 1.

TYPICAL ANTIGEN TITRATION FOR TEST WITH SERUM

Antigen Dilution Series	1	2	3	4	5
Antigen + Salt Solution c.c.	1 + .8	1 + .9	1 + 1.0	1 + 1.1	1 + 1.2
Result of Dilution	Heavy precipitate in each antigen dilution				
Scheme Used in Testing Solubility of Precipitate in Each Antigen Dilution	Tube No. 1 2 3 *Antigen Dilution c.c. .05 .025 .0125 Salt Solution c.c. .15 .15 .15 Tubes are shaken 2 minutes and 0.5 c.c. salt solution added to each. All are observed for precipitates.				
Solubility of Precipitate as Determined by Three-Tube Test	Precipitate Not Soluble	Precipitate Not Soluble	Precipitate Soluble	Precipitate Soluble	Precipitate Soluble
Standard Antigen Dilution			Antigen + minimum amount of salt solution giving precipitate which dissolves in salt solution		

*Each antigen dilution is allowed to stand 30 minutes after mixing antigen and salt solution before solubility test is made.

2. SERUM

The serums are separated from the clots by centrifugation in the usual manner, pipetted off and inactivated for 30 minutes at 56° C. The main point regarding serums is that they be free from red cells, fibrin and particles of any kind. No difficulty is encountered in this test with milky or chylous serums or with moderately hemolyzed specimens. Only such hemolyzed specimens which are not fit for a Wassermann test are not fit for a precipitation test.

3. PHYSIOLOGIC SALT SOLUTION

Salt solution is prepared by dissolving 8.5 gm. of chemically pure sodium chloride per liter of distilled water and filtering. Although sterility of this solution is not essential, the same type of chemical cleanliness usually employed in quantitative chemical work is required.

III. THE ROUTINE TEST WITH SERUM

The routine test consists of three tubes containing three different proportions of serum and antigen dilution in accordance with the following outline:

Tube No.	1	2	3
Serum: Antigen Dilution	3:1	6:1	12:1
Antigen Dilution c.c.	.05	.025	.0125
Serum c.c.	.15	.15	.15

Preparation of Standard Antigen Dilution.—It is well when carrying out a number of tests to inactivate the serums and set up and number the tubes before diluting antigen with salt solution for the tests. The antigen dilution is so standardized as to necessitate its use within a half hour after mixing antigen with salt solution. In this laboratory, with over 200 routine tests per day, enough antigen dilution for 60 or 80 tests is usually prepared at one time—two experienced workers pipetting antigen dilution and serum for about 80 tests in less than twenty minutes.

One c.c. antigen diluted with one c.c. salt solution gives sufficient antigen dilution for about 18 tests. Two or three c.c. antigen may be diluted with corresponding amounts of salt solution by utilizing the same standard antigen dilution tubes.

Procedure: One c.c. antigen is measured into an antigen dilution tube. An amount of salt solution usually approximating that of the antigen, according to the titre of the antigen, is measured into a similar tube. The salt solution is poured into the antigen and, without waiting to drain the tube, the mixture is immediately poured back and forth five or six times to insure thorough mixing. For uniformity, this antigen dilution is permitted to stand ten minutes at room temperature before pipetting.

As a preliminary control of the antigen dilution, 0.05 c.c. is measured into a test tube, 1 c.c. salt solution added and the tube shaken vigorously for ten or fifteen seconds. The mixture should appear opalescent.

Pipetting of Antigen Dilution.—The antigen dilution is always pipetted to the bottom of the tubes. The 0.05 c.c. amounts may be pipetted with a 1 c.c. pipette in which 0.05 c.c. graduations are indicated with a wax pencil. For the 0.025 and 0.0125 c.c. amounts of antigen dilution, 0.2 c.c. pipettes are employed and the proper markings may also be indicated with a wax pencil. The antigen dilution should be mixed frequently during the pipetting period to assure a uniform mixture. Due to the possibility of evaporation of the small amounts of antigen dilution used in the test, it is necessary to pipette the serum within several minutes after the antigen dilution has been pipetted. If a worker desires to run forty precipitation tests, for example, it is better to pipette antigen dilution and serums for ten tests at a time, than to pipette the antigen dilution for all the tests first and then follow with the serums.

Pipetting of Serum.—The 0.15 c.c. amounts of each serum are added to the antigen dilution by means of a 1 c.c. pipette graduated in 0.01 c.c. In pipetting these amounts of serum it is not necessary to lower the pipette to the bottom of

the tube. As soon as the serums have been added for ten tests, or less, the rack is shaken sufficiently to insure thorough mixing of the serum with antigen dilution.

Shaking of Tests.—After the serums have been mixed with the antigen dilution, the tests are shaken for a two minute interval. A shaking machine is of the utmost importance for this purpose, particularly in the examination of comparatively large numbers of specimens at a given time.

Effect of Incubation.—Although the final results may be read immediately after the shaking period, different workers have observed that a 15 minute incubation period in the water bath at 37° C. produces sufficient clumping of the precipitates to make the reading of the results easier, particularly in the case of weak reactions. In studying the effect of 15 minute incubation on this test, no tendency for false positive reactions has been observed, and on the basis of easier reading we consider the employment of this incubation period of some advantage.

Addition of Salt Solution and Reading of Results.—After the shaking of the tests as well as after the 15 minute incubation period, if employed, the serum-antigen mixtures appear uniformly cloudy. In order to render the negative reactions clear and thus simplify the reading of results, 0.5 c.c. salt solution is added to each tube. The tests should be read immediately after the addition of salt solution as an occasional weak precipitate may go back into solution on standing.

Readings are best made in front of a window with a darkened background. The negative serums appear opalescent and readily distinguishable without lifting the tubes from the racks. The strongly positive serums show heavy precipitates which are also easily read directly.

Only the tubes showing weak reactions need to be removed from the rack and examined individually. Each tube is lifted from the rack above the eye level, slanted to spread the fluid into a thin layer and examined for a precipitate.

In this laboratory, precipitation tests are read at night with the same ease as during the day. Light is furnished by a 300-watt daylight bulb in an overhead indirect fixture.

NOTE: If it is desired to make a check reading of the results at some later period, it is well to keep the tests at icebox temperature. Practically all positive reactions will be slightly stronger at the second reading. Occasionally, however, even at icebox temperature, a serum which gave a —, +, ++ at the first reading may be found to be negative during the second reading. We have observed these reversible precipitation reactions especially in early primary and in highly treated cases. The reading made immediately after the addition of 0.5 c.c. salt solution to each tube is the standard reading and is always the one reported.

The Control System.—1. Antigen Control. When pipetting antigen dilution for a series of tests, the last set-up of three regular antigen amounts receives 0.15 c.c. salt solution instead of serum and is read with the regular tests. All three tubes should show freedom from a precipitate.

2. Serum Controls. In the case of each positive reaction, the serum used in the test is examined for particles that might give the appearance of a specific precipitate.

3. Positive and Negative Controls. One or more such controls are included with each series of tests.

Interpretation of Results.—A definite precipitate suspended in a clear medium is considered a complete reaction and is read four plus. Proportionally weaker reactions are read three, two and one plus, respectively. The final result in each test is the average finding of the three tubes. Thus, if the precipitation reaction is four plus in each of the three tubes, the final result is four plus. If the reaction is —, +++, ++++, the final result is two plus. A number of typical reactions with this method and the final result in each case, are illustrated in Table 2.

TABLE 2.
INTERPRETATION OF RESULTS OF ROUTINE TEST WITH SERUM.

Tube No.		1	2	3	Average of Reaction in the Three Tubes = Final Result
Serum: Antigen Dilution Antigen Dilution c.c. Serum Undiluted c.c.		3:1 .05 .15	6:1 .025 .15	12:1 .0125 .15	
	Reaction No.				
	1	++++	++++	++++	++++
	2	+++	++++	++++	
	3	++	++++	++++	
	4	+	++++	++++	+++
Some	5	—	++++	++++	
	6	—	+++	++++	
Typical	7	—	++	++++	++
	8	—	+	++++	
Reactions	9	—	—	++++	
	10	—	—	+++	+
	11	—	+	++	
	12	—	±	++	
	13	—	—	++	±
	14	—	±	+	

Correlation of Results with Clinical Findings.—When the average result with this test is four plus, it will usually be found that we are dealing with untreated or little treated syphilis. When the result is less than four plus, the clinical condition is usually early primary or intensively treated syphilis. As a general rule, the average result corresponds with a cholesterinized antigen Wassermann except that the precipitation test is possibly more specific—due to the elimination of the hemolytic system.

In reporting the results with this test to physicians, it is well to give the actual precipitation findings in each of the three tubes as well as the final results. This gives the physician a more correct picture of the serologic condition of the patient than the average result alone.

Clinical studies carried out in conjunction with the Department of Dermatology and Syphilology of the University of Michigan Medical School indicate that this precipitation test is highly specific for syphilis. Tuberculosis, scarlet fever and other febrile diseases which occasionally give falsely positive reactions with the Wassermann test appear to give no such reactions with this method. Pregnancy also gives no false reactions. Yaws, yellow fever and pellagra, which give occasional false Wassermann reactions, are still to be studied with this method.

While strong reactions give every indication of being associated with syphilitic infection, weak reactions—particularly when the average finding is one plus—appear to have the same diagnostic significance as weak Wassermann reactions. Needless to say, as is true with any laboratory method, the finding with this test should be considered but one factor in the diagnostic syndrome in a given case.

IV. SPECIAL QUANTITATIVE TEST WITH SERUM

The routine test being quantitative in only a limited degree, a special quantitative procedure has been devised for the purpose of determining the relative potency of serums in terms of a unit system.

The Unit Reaction.—In the quantitative procedure, a constant amount of standard antigen dilution is employed with varying dilutions of serum. In the derivation of the unit reaction, 0.0125 c.c. antigen dilution and 0.15 c.c. undiluted

serum are the unit quantities employed. If complete precipitation is obtained when 0.0125 c.c. antigen dilution is used with 0.15 c.c. serum, only in undiluted form, the reaction represents four reacting units. If precipitation is complete with certain dilutions of serum, then the potency of the serum in terms of reacting units (S) is equal to four times the maximum dilution of the serum giving complete precipitation (D). The potency of any serum may be expressed by the formula

$$S = 4\ D$$

Thus, if 1:10 represents the highest dilution of serum capable of giving complete precipitation, the serum contains 4 x 10 or 40 reacting units. If 1:60 represents the highest dilution in which a serum gives a complete reaction, the serum contains 4 x 60 or 240 units. Similarly the potency of any serum may be determined.

In the quantitative procedure only complete precipitation reactions are considered in determining the concentration of reacting units in a given serum. The interpretation of intermediary reactions must be left to the judgment of individual workers. A reaction giving the appearance of a three plus might be included among complete reactions, while weaker reactions are considered negative. The results of the quantitative procedure, furthermore, are always expressed in units and not in plus signs. By thus adhering to the use of plus signs in connection with the routine test with serum and of units in connection with the special quantitative test, confusion of the results with the two procedures will be avoided.

Outline of Quantitative Test.—The quantitative procedure is applicable only to strongly positive serums, since only such serums are capable of giving precipitation reactions after dilution. A routine test, therefore, is always made before employing the quantitative test with a given serum.

Eight tubes are employed in this test, each tube containing 0.0125 c.c. standard antigen dilution with 0.15 c.c. quantities of serum dilutions ranging from 1:1 to 1:60.

The serum dilutions may be prepared as follows:

1:1 = undiluted serum
1:5 = 0.2 c.c. undiluted serum + 0.8 c.c. salt solution
1:10 = 0.6 c.c. 1:5 dilution + 0.6 c.c. salt solution
1:20 = 0.2 c.c. 1:10 dilution + 0.2 c.c. salt solution
1:30 = 0.2 c.c. 1:10 dilution + 0.4 c.c. salt solution
1:40 = 0.1 c.c. 1:10 dilution + 0.3 c.c. salt solution
1:50 = 0.1 c.c. 1:10 dilution + 0.4 c.c. salt solution
1:60 = 0.1 c.c. 1:10 dilution + 0.5 c.c. salt solution

NOTE: If it is desired to make more exact determinations of the number of reacting units in certain cases, serum dilutions in between those already indicated may be included. Serums of high potency, which give complete precipitation in a 1:60 dilution may be diluted still further until a negative reaction is obtained.

The antigen dilution is prepared as for the routine test and pipetted in 0.0125 c.c. quantities to the bottom of the tubes in the usual manner. This is followed by 0.15 c.c. amounts of the various serum dilutions, in order, beginning with the undiluted serum. The rack is shaken for two minutes, 0.5 c.c. salt solution added to each tube, and the results read and recorded. If a fifteen minute incubation period at 37° C. is employed, it should be carried out as suggested in connection with the regular test, namely, after the shaking period and prior to the addition of salt solution.

Table 3 gives some typical reactions with the quantitative procedure.

Correlation of Quantitative Procedure with Routine Serum Test.—Since the last proportion of serum and antigen dilution of the routine test is the basis for determining the reacting unit, the results of the routine procedure can be readily interpreted in terms of reacting units. The three types of reactions in the routine

TABLE 3.

SOME TYPICAL REACTIONS WITH THE QUANTITATIVE PROCEDURE.

Serum: Salt Solution	1:1	1:5	1:10	1:20	1:30	1:40	1:50	1:60	Four times the maximum dilution of serum giving precipitation (4D)	Serum potency in terms of reacting units (S)
Serum Dilution c.c.	.15	.15	.15	.15	.15	.15	.15	.15		
Antigen Dilution c.c.	.0125	.0125	.0125	.0125	.0125	.0125	.0125	.0125		
Serum No.										
1	++++*	—	—	—	—	—	—	—	4 x 1	4
2	++++	++++	—	—	—	—	—	—	4 x 5	20
3	++++	++++	++++	—	—	—	—	—	4 x 10	40
4	++++	++++	++++	++++	—	—	—	—	4 x 20	80
5	++++	++++	++++	++++	++++	—	—	—	4 x 30	120
6	++++	++++	++++	++++	++++	++++	—	—	4 x 40	160
7	++++	++++	++++	++++	++++	++++	++++	—	4 x 50	200

*++++ = Complete precipitation reaction.

procedure of especial interest in this connection are:

	Standard Antigen Dilution c.c.		
	.05	.025	.0125
Reaction	Undiluted Serum c.c.		
No.	.15	.15	.15
1	—	—	++++
2	—	++++	++++
3	++++	++++	++++

The first reaction, in which the third tube only gives a four plus, represents four reacting units. The second, in which a four plus results from the use of double the amount of antigen dilution, indicates that the serum in a dilution of 1:2 would give complete precipitation with 0.0125 c.c. antigen dilution, thus representing approximately eight reacting units. On the same basis, the third reaction indicates that the serum would give complete precipitation in a dilution of 1:4 and therefore represents at least sixteen reacting units.

V. THE TEST WITH SPINAL FLUIDS

The test with spinal fluids requires preliminary concentration of the reacting substances in these fluids with a saturated solution of ammonium sulphate.* This in turn necessitates a special antigen titration. The test consists of one proportion of concentrated fluid and antigen dilution and is carried out in duplication.

Titration of Antigen for Spinal Fluid Test.—This titration differs from the antigen titration for the test with serum in that the solubility of the precipitates in the various antigen dilutions is tested with an ammonium sulphate solution approximating 10 per cent, instead of physiologic salt solution. This is necessary because the concentrated spinal fluid to be tested for specific reacting substances contains approximately 10 per cent ammonium sulphate.

Five 1 c.c. quantities of standard antigen are measured into five standard antigen dilution tubes. Into five similar tubes are measured 1.1, 1.2, 1.3, 1.4 and 1.5 c.c. physiologic salt solution, respectively. Each of the saline amounts is poured into an antigen tube and the mixture, in each case, immediately poured back and forth several times. These five antigen-saline dilutions contain precipitates and, after twenty minutes' standing, the solubility of these precipitates is tested with an ammonium sulphate solution prepared by mixing 1 c.c. of saturated solution with 9 c.c. normal saline. These tests are carried out in duplication employing 0.01 c.c. amounts of the five antigen dilutions with 0.15 c.c. quantities of the approximately 10 per cent ammonium sulphate solution. The proportion of ammonium sulphate solution to antigen dilution is 15:1. The antigen dilutions are pipetted to the bottom of the tubes with a 0.2 c.c. pipette graduated in 0.001 c.c. The ammonium sulphate solution is then added, the tubes shaken for two minutes and 0.2 c.c. saline

* Merck's Reagent Ammonium Sulphate has been found to give good results.

added to each tube to render easier the reading of the results. The dilution of antigen and saline which contains the smallest amount of saline in proportion to antigen, giving a precipitate which is soluble in ammonium sulphate solution represents the titre of the antigen for spinal fluids. A typical titration is given in Table 4.

TABLE 4.

TYPICAL ANTIGEN TITRATION FOR USE WITH SPINAL FLUID.

Antigen Dilution Series	1	2	3	4	5
Antigen + Salt Solution c.c.	1 + 1.1	1 + 1.2	1 + 1.3	1 + 1.4	1 + 1.5
Result of Dilution	Heavy precipitate in each antigen dilution				
Scheme Used in Testing Solubility of Precipitate in Each Antigen Dilution	Tube No. 1 2 *Antigen Dilution c.c. .01 .01 10% Sol. Ammonium Sulphate c.c. .15 .15 Tubes are shaken 2 minutes and 0.2 c.c. salt solution added to each. All are observed for precipitates.				
Solubility of Precipitate	Precipitate Not Soluble	Precipitate Not Soluble	Precipitate Not Soluble	Precipitate Soluble	Precipitate Soluble
Standard Antigen Dilution				Antigen + minimum amount of normal saline giving precipitate which dissolves in 10% ammonium sulphate sol.	

*Each antigen dilution is allowed to stand 20 minutes after mixing antigen and salt solution before solubility test is made.

Concentration of Fluid with Ammonium Sulphate.—Three c.c. of spinal fluid, previously rendered free from cellular material by centrifugation, are pipetted into a centrifuge tube and 2 c.c. of saturated solution ammonium sulphate added (Herrold). This is mixed, permitted to stand for one hour at room temperature and centrifuged at high speed for about fifteen minutes—a period usually sufficient to completely throw down the globulins. The supernatent fluid is poured off as completely as possible, the last drop adhering to the lip of the tube being taken up with filter paper. The globulin precipitate is found to be suspended in a small drop of ammonium sulphate solution. Three-tenths c.c. of salt solution (one-tenth of the original amount of spinal fluid used) is now added to the tube. This is added by lowering the pipette into the tube and running the saline down in the center so as to avoid washing down ammonium sulphate from the inner wall of the tube. The precipitate immediately dissolves and the resulting solution is tested with antigen dilution in a manner to be described.

Preparation of Antigen Dilution.—Antigen is diluted with salt solution as described under the test with serum, using the proportion indicated by the special titration method. The antigen dilution is allowed to stand for ten minutes before use in the test.

Pipetting of Antigen Dilution.—For each fluid to be tested, 0.01 c.c. of antigen dilution is pipetted to the bottom of each of two tubes, using a 0.2 c.c. pipette.

Pipetting of Concentrated Fluid.—Concentrated spinal fluid in 0.15 c.c. quantities is added to each of the tubes containing the antigen dilution.

Shaking of Tests.—After the concentrated fluids have been mixed with the antigen dilution, the tests are shaken for a two minute period.

Incubation.—The tests may be read immediately after the shaking period. As in the case of serums, however, a fifteen minute incubation period in the water bath may be employed, if desired.

Addition of Salt Solution and Reading of Results.—Immediately before reading the tests, 0.2 c.c. salt solution is added to each tube in order to render the negatives clear and reading easier.

The results are read as described under the test with serum.

The Control System.—The controls are of particular importance in the spinal fluid test in view of the fact that certain concentrations of ammonium sulphate are capable of giving precipitates with standard antigen dilution. The control of particular importance is carried out as follows: Three c.c. normal saline are added to a centrifuge tube and 2 c.c. of a saturated solution of ammonium sulphate thoroughly mixed with it. The tube is now emptied, in the same manner as the spinal fluid tube is emptied after centrifugation, and 0.3 c.c. salt solution added. We thus have here a concentration of ammonium sulphate approaching that in the concentrated globulin solution. A regular test performed with 0.15 c.c. of this control ammonium sulphate solution and 0.01 c.c. standard antigen dilution should show no precipitation.

An antigen dilution control, 0.01 c.c. mixed with 0.15 c.c. salt solution, is also included with the regular tests. Furthermore, every concentrated fluid to be tested is examined to establish total freedom from foreign particles. Finally, positive and negative spinal fluid controls should accompany each series of tests.

Interpretation of Results.—Reactions are read on the basis of four, three, two and one plus, respectively, depending upon the distinctness of the precipitates. Since the two tubes used for testing each fluid are duplicates, the reactions in the two should be identical.

VI. SUMMARY

1. *Routine Test with Serum.*—This test is quantitative to a limited degree—consisting of three tubes, each containing different proportions of serum and antigen dilution. The final result is the average of the precipitation findings in the three tubes, and is expressed in plus signs. The test is of value both in the diagnosis of syphilis and as a check on treatment. It may be used for this purpose independently of any other laboratory procedure provided (1) the workers performing it have had both training and experience in bacteriology and immunity and that (2) each test is performed in duplication.

2. *Special Quantitative Procedure.*—This procedure is highly quantitative in character, consisting of eight tubes, each containing a constant amount of antigen dilution with different dilutions of serum. The results with this procedure are always expressed in reacting units and are in this way distinguished from the results with the routine test which are expressed on a plus sign basis. This procedure enables one to determine the relative number of reacting substances in syphilitic serum and is of especial value when it is desired to study the quantitative effect of specific treatment on the number of these reacting substances.

3. *Test with Spinal Fluids.*—In this procedure, the globulins of the fluid are precipitated with ammonium sulphate and the precipitate subsequently dissolved in a minimum amount of salt solution—thereby concentrating the reacting substances of the fluid. This concentrated fluid is tested with antigen dilution in the usual manner. The test is essentially qualitative, its quantitative character being limited to the relative intensity of the precipitate formed in one proportion of concentrated fluid and antigen dilution. The results with this method appear to conform with the findings of a conservative Wassermann test.

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