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Application of author's precipitation test to spinal fluids.

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This paper will give a resumé of the author's test for syphilis as applied to serum[1] and an outline of the application of the test to spinal fluids.

I. ANTIGEN

1. *Preparation of Alcoholic Extract:* Beef heart muscle is ground, dried and powdered in a manner usually employed for antigen preparation.[2] About 50 gm. of powdered muscle are placed in a 500 cc. Erlenmeyer flask and about 250 cc. ether added and shaken. This is extracted over night at icebox temperature. The ether is filtered off and about 150 cc. fresh ether added to the powdered muscle. Extraction is further carried on over night in the icebox. After 3 or 4 ether extractions, the supernatant ether is practically free from coloring matter, when the extraction is completed. The ether is then filtered off and the powdered muscle is dried at room temperature until free from ether odor. The ether extracts are either discarded or the ether redistilled.

The dried material is weighed; placed in a 500 cc. Erlenmeyer flask, and 5 cc. of 95 per cent alcohol added per gram of ma-

1 The following are more recent discussions on this text: Kahn, R. L., Rapid Precipitation Phase of the Kahn Test for Syphilis, *J. Am. Med. Assn.*, 1923, lxxxi, 88. Holmes, Janet A., The Kahn Precipitation Test for Syphilis, *J. Am. Med. Assn.*, 1923, lxxxi, 294; Strumia, M. M., A Study of Serum Flocculation Reactions in Syphilis with Special Reference to the Meinicke, Sachs-Georgi, Kahn and Vernes Reactions, *Arch. Dermatol. and Syphil.*, 1923, viii, 50; Yagle, E. M., and Kolmer, J. A., The Kahn Precipitation Reaction in Leprosy, *Arch. Dermatol. and Syphil.*, 1923, viii, 183; Detweiler, H. K., The Value of the Kahn Precipitation Test for Syphilis, *J. Am. Med. Assn.*, 1923, lxxxi, 815; The Kahn Precipitation Test in a Public Health Laboratory, *Public Health J.* (Canada), 1923, xiv, 464; Anderson, J. F., and Fisher, E. E., A Comparison of the Wassermann and Kahn Tests in Three Hundred and Twenty-nine Cases, *N. Y. Med. J.*, 1923, cxviii, 490; Fox, J. C., Jr., and Sanderson, E. S., Observations on the Kahn Precipitation Reaction for Syphilis, *Am. J. Syphil.*, 1923, vii, 687.

2 Powdered beef heart is now obtainable on the market from the Digestive Ferments Company, Detroit, Mich.

terial. This alcohol extraction is carried out for about 10 days at icebox temperature with daily shaking. After this period, the extract is filtered off and kept in the dark as stock solution.[3]

2. *Cholesterinization of Alcoholic Extract:* A given amount of extract (enough for about a month's use) is measured into an Erlenmeyer flask and cholesterin added to it in the proportion of 6 mg. per cc. The cholesterin is dissolved by warming the flask in the water bath and rotating. The cholesterinized extract is then filtered to free it from impurities, when it is ready for use in the tests.

3. *Titration of Cholesterinized Extract:* This is to determine proper proportion of antigen and saline to mix for the tests. The smallest amount of saline which, added to antigen, produces a precipitate capable of going back into solution upon further addition of saline, represents the desired amount to mix with antigen for use in the tests.

Method: Five 1 cc. quantities of antigen are measured into small test tubes of about 5 cc. capacity. To five similar tubes are added 0.6, 0.75, 0.9, 1 and 1.2 cc. saline, respectively. The saline in each case is poured into the antigen tube and the mixture immediately poured back and forth several times. A heavy precipitate will be present in each of the tubes.

A preliminary test of the solubility of these precipitates is carried out as follows: A small quantity, such as 0.05 cc. of each of the precipitates is measured into five clean tubes and saline (from 0.3 to 0.5 cc.) is added to each of these tubes. The precipitates in the tubes containing 1 cc. quantities of antigen and 0.6 or 0.75 cc. of saline are usually insoluble in saline; the precipitate in the tube containing 0.9 cc. saline may appear questionable, whereas the precipitates in the remaining tubes are usually soluble in saline.

The final observation of the solubility of these precipitates in saline is made one-half hour after originally mixing antigen and saline. The antigen-saline proportion containing the smallest amount of saline which gives a precipitate still soluble at the end of this half-hour period is the proportion to be used in the test.

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[3] Rubber stoppers should be avoided in connection with antigen—and corks should preferably be covered with tin foil.

TYPICAL ANTIGEN TITRATION

Tubes	1	2	3	4	5
Antigen cc.	1	1	1	1	1
Saline cc.	0.6	0.75	0.9	1.0	1.2
The saline is poured into the antigen in each case and the resulting mixture immediately poured back and forth several times.					
Results	Pptate	Pptate	Pptate	Pptate	Pptate
The solubility of each precipitate is tested (immediately and a half hour after mixing saline with antigen) by placing a small amount, such as 0.05 cc., into a tube and adding about 0.5 cc. salt solution.					
Final Results after half-hour	Pptate	Pptate	Pptate?	No Pptate	No Pptate

According to this titration, tube No. 4 (1 cc. antigen + 1 cc. saline) contains the smallest amount of saline in proportion to antigen which will produce a precipitate capable of going back into solution in saline. This particular antigen, therefore, should be mixed for the tests in the proportion of 1 cc. + 1 cc. saline.

An important factor in mixing antigen with salt solution is that the latter should come in contact with the former with reasonable rapidity. This explains why the saline is added to the antigen by pouring it from another tube rather than from a pipette. When employing a small amount of antigen for the titration, such as 0.5 cc. or less, the saline should preferably be added from a 1 cc. pipette which must, however, permit a fair sized stream.

It is recommended that an antigen be re-titrated at periods of four to six weeks.

II. THE TEST WITH SERUM

1. *Dilution of Antigen for the Tests.* This should be carried out just before using in the test. The antigen-saline mixture will usually not keep longer than one-half hour.

(a) A given amount of cholesterinized antigen is measured into a small tube.

(b) An equal amount of saline is measured into another small tube (based on titration).

(c) The saline is poured into the antigen tube and the mixture is immediately poured back and forth several times. This mixture is now ready for use. (If 0.5 cc. or less of antigen is employed, the saline is added with a pipette.)

2. *The Test Proper.* The antigen-saline mixture and serum are added to three tubes in the proportion of 1:3, 1:6 and 1:12.

The following outline is based on quantities employed in this laboratory. The antigen mixture is always pipetted first and to the bottom of the tubes.

Tubes	1	2	3
Antigen-Saline Mixture cc.	.05	.025	.0125
Serum (heated 30 min. at 56° C.) cc.	.15	.15	.15

3. *Completion of Tests.*

(a) After adding serum to the antigen-saline mixtures, the racks are shaken vigorously for one minute.

(b) The tests are now ready to be recorded. To simplify reading, an amount of saline is added to each tube, sufficient to render the negatives so clear that they can be read without being removed from the racks. From 0.3 to 0.5 cc. saline will usually suffice. This should be added immediately before reading.

(c) The results should be read in front of a window with a darkened background. Each tube suggesting a positive reaction is lifted above the eye level, slanted to spread the fluid into a thin layer and examined for a precipitate.

(d) Readings are recorded on the basis of one, two, three and four plus, depending upon the distinctness of the precipitate. The final result is the average reading of the three tubes. Any given reaction will show quantitatively fewer particles in the tubes containing the smaller quantities of antigen, but, if the precipitation is complete in all three tubes, it should be read four plus in spite of the quantitative difference in the number of particles.

(e) A check reading of the tests should be made, preferably by another worker. If it is necessary for the same worker to make this reading, a better check will probably be obtained if made at a later period. In such a case, the racks should be kept in the icebox until the check reading is made.

NOTE: Studies on the effect of incubation on this test indicate that, if carried out after the shaking period and before the addition of saline, 15 minutes to an hour (or even longer) at 37° C. does not produce falsely positive reactions.

4. *Controls.*

(a) Antigen Controls: After mixing antigen with saline for the tests, a small amount, such as 0.05 cc., of the mixture is measured into a tube and 0.5 to 1 cc. of saline added. There should be no sign of precipitation. Also, when pipetting antigen for a series of tests, the last set-up is used with saline instead of serum and is read with the regular tests.

(b) Serum Controls: All serums should be observed for precipitates prior to their use in the tests.

(c) Positive and Negative Controls: Three positive and three negative controls should be used with each set of tests.

III. THE TEST WITH SPINAL FLUID

Although this precipitation test gives good results with strongly reacting spinal fluids when used in the same proportion as serum, the test lacks sensitiveness when used with moderately and weakly positive fluids. It was believed that better results could be obtained by precipitating and subsequently concentrating the proteins—and therefore the reacting substances—of the fluid. Various methods of protein precipitation are still under investigation in this laboratory. Recently, Herrold[4] suggested precipitating the globulins of spinal fluid with ammonium sulfate in connection with his ring modification of the writer's test.[5] This ammonium sulfate precipitation method, when applied to our test, has given good results. The procedure to be outlined involves: First, precipitating the globulins in the spinal fluid with ammonium sulfate and concentrating this precipitate in saline; second, testing this concentrated solution for the presence of syphilitic reacting substances by means of the regular precipitation test.

Procedure: The spinal fluid is mixed with saturated ammonium sulfate (C. P.) to produce 40 per cent saturation. Thus, 3 cc. of fluid are mixed with 2 cc. ammonium sulfate; 4.5 cc. fluid with 3 cc., etc. This mixture is allowed to stand for about an hour at room temperature. It is then centrifuged at high speed for 10 minutes and the supernatant fluid poured off as completely as possible. The last drop adhering to the lip of the tube can be removed by means of filter paper. The resulting precipitate will be found suspended in a small drop of fluid. This precipitate is then dissolved in an amount of saline equal to one-tenth the volume of spinal fluid used in the test. (A precipitate from 3 cc. of fluid, for example, is dissolved in 0.3 cc. saline.) The saline should be added to the bottom of the tube to prevent washing

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[4] Herrold, R. D., The Precipitation Test for Syphilis of Concentrated Arachnoid Fluid and Serum, *J. Am. Med. Assn.*, 1923, lxxxi 203.

[5] Herrold, R. D., A Ring or Contact Precipitation Test for Syphilis, A Modification of the Kahn Test, *J. Am. Med. Assn.*, 1923, lxxix, 957.

down traces of ammonium sulfate from the inner wall. The globulin solution formed is now ready to be tested for specific precipitation with the antigen-saline mixture described above.

In carrying out the test, two tubes are employed with different proportions of antigen and concentrated fluid. Tube 1 receives 0.01 cc. antigen-saline mixture followed by 0.1 cc. of concentrated spinal fluid. Tube 2 receives 0.005 cc. antigen mixture with also 0.1 cc. of fluid. The antigen should be measured with a 0.1 cc. pipette graduated in 0.01 cc. and the respective amounts deposited at the bottom of the tubes.

After adding the fluid to the antigen, the racks are vigorously shaken for 1 minute. To simplify reading, each tube receives 0.2 cc. saline, and the tests are read and recorded. The final result is the average reading of the two tubes.

The controls are: (1) Antigen control, same as with serum test. (2) Concentrated fluid, to be observed for the presence of a precipitate prior to its use in the test. (3) In view of the fact that an ammonium sulfate solution of sufficient concentration is capable of producing a precipitate with antigen, the following special control should be added to each series of tests. To 3 cc. saline are added 2 cc. ammonium sulfate to make a 40 per cent saturation. This is mixed and poured off in the same manner as the supernatant fluid of the spinal fluid-ammonium sulfate mixture is poured off after centrifugation. To the minute amount remaining in the tube is added 0.3 cc. saline and the resulting solution tested with antigen in the same proportions as the concentrated spinal fluid. (4) Positive and negative controls should accompany each series of tests.

This method compares favorably with the Wassermann test. Clinical observations with reference to specificity and sensitiveness will be reported in another place.