A SIMPLE QUANTITATIVE PRECIPITATION REACTION FOR SYPHILIS *

PRELIMINARY COMMUNICATION

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The precipitation method proposed in this paper is based on the employment of syphilitic serum with alcoholic extract antigens of heart muscle. In this regard it is similar to the precipitation reactions of Meinicke,¹ Sachs and Georgi² and Dreyer and Ward (sigma reaction).³ The test proposed, however, differs from each of these reactions in essential phases. Among these are the preparation of the antigens and their proper dilution for the tests, as well as the concentration of serum and antigen in the tests. As a result of these and other technical factors, the test possesses the following salient features:

1. The diluted antigen possesses considerable stability. This dispenses with the necessity of diluting fresh antigen with salt solution before using it in the tests.

2. The strongly positive serums show, in most cases, spontaneous precipitation, and the test as a whole is completed after three hours' incubation in the water-bath.

3. The end-results can be read with relative ease. Only in the case of a doubtful positive reaction does one need to apply oneself, so to speak, to try to recognize a precipitate. The flocculations in the positive reactions are so heavy that even one who has had no experience in observing precipitates will not have the slightest difficulty in recognizing them. And with regard to the weakly positive reactions, a little experience will enable one to see the precipitates with ease.

THE FLOCCULATION REACTIONS OF MEINICKE, SACHS AND GEORGI AND DREYER AND WARD (SIGMA REACTION)

Meinicke Reaction.—In studying the Meinicke reaction, we have been fortunate in having the services of Mr. J. L. Landau, for many years serologist in Finger's Clinic, Vienna, who in that place has had considerable experience with this reaction. The tests were carried out precisely as indicated in the author's Third Modification.⁴ The

* From the Bureau of Laboratories, Michigan Department of Health.

1. Meinicke, E.: Berl. klin. Wchnschr., No. 4, p. 83, 1918; München. med. Wchnschr. **65**:1379, 1918.

2. Sachs, H., and Georgi, W.: Med. Klin., No. 33, p. 805, 1918.

3. Dreyer, G., and Ward, H. K.: Lancet **1**:956 (May 7) 1921.

4. Meinicke, E.: München. med. Wchnschr., No. 33, p. 932, 1919.

antigen was prepared from horse heart in the following manner: Fat-free heart muscle was finely ground and dried at from 50 to 55 C. Ether was added to the dried material in the proportion of nine parts to one, and shaken for one hour. The ether was then filtered off and the heart muscle dried at 37 C. Alcohol (96 per cent.) was then added to the dried material in the proportion of nine to one, shaken from time to time for one day and filtered. The filtrate was permitted to stand for several days, when it was ready for use.

Accordingly, the antigen titration was carried out with alcohol and distilled water, as presented in Table 1.

TABLE 1.—ANTIGEN TITRATION WITH ALCOHOL AND DISTILLED WATER

Tube	Antigen, C.c.	96 Per Cent. Alcohol, C.c.	Distilled Water C.c.	Results: After 1 Hour, Each Tube Having Received 3.5 C.c. Distilled Water
1	0.4	0.1	0.25	Precipitation present
2	0.3	0.2	0.25	Very slight precipitation present
3	0.2	0.3	0.25	Opalescent but no precipitate
4	0.1	0.4	0.25	Weakly opalescent, no precipitate

Tube 3 showed a degree of opalescence which suggested the optimum combination of extract and alcohol to be used in the tests.

In the preparation of antigen for a number of tests, a given amount was mixed with half its quantity of distilled water and permitted to stand for one hour. After this period, seven times the quantity of 2 per cent. sodium chlorid solution was added and the mixture was ready for use. (Thus, 5 c.c. of antigen was mixed with 2.5 c.c. of distilled water, and after one hour, 35 c.c. of 2 per cent. salt solution was added). For a given test, 0.8 c.c. of antigen and 0.2 c.c. of inactivated serum were employed. Readings were made after twenty-four hours' incubation in the thermostat at 37.5 C. and checked by another reading after twenty-four hours at room temperature.

The employment of 2 per cent. salt solution is claimed by Mienicke to give the optimum flocculations with positive serums and no flocculations with negative ones. This author claims further that his antigen, owing to the preliminary ether extraction, is practically free from cholesterin and in this regard differs in principle from the Sachs and Georgi reaction, in which cholesterin is employed. According to Hull and Faught,⁵ however, the Sachs and Georgi reaction may be carried out with noncholesterinized as well as cholesterinized antigens.

The Sachs and Georgi Reaction.—Sachs and Georgi make their antigen in the following manner: Ground (wet) heart muscle is mixed with alcohol in the proportion of 1 : 5. After extraction, 100 c.c. of antigen is mixed with 200 c.c. of alcohol and 13.5 c.c. of 1 per cent.

5. Hull, T. G., and Faught, E. E.: J. Immunol. 5:521 (Nov.) 1920.

solution cholesterin in alcohol. This antigen, diluted 1:5 with salt solution, is ready for use. In the actual test 1 c.c. of 1:10 inactivated serum diluted with physiologic solution of sodium chlorid was mixed with 0.5 c.c. of antigen. This was incubated for twenty-four hours in the thermostat, followed by twenty-four hours' incubation at room temperature (Gaeltgens).⁶

The Meinicke and Sachs and Georgi reactions were carried out in this laboratory, side by side with the Wassermann test. The last was performed in duplication with an alcoholic and cholesterinized antigen. The procedure of this test is discussed elsewhere.⁷ Altogether, 300 precipitation tests were performed with each procedure. Our results approximate those of Stern,⁸ who found 88 per cent. check with the Wassermann test. We also seemed to have experienced at different times what Miss Stern calls the "negative phase." It will be recalled that, for an interval of three weeks, this observer obtained extremely weak precipitations, so weak indeed that they were hardly perceptible. We observed these weak reactions on some days without being able to explain their presence. Our results indicated that neither the Sachs and Georgi nor the Meinicke reaction could profitably be carried out as a routine procedure in conjunction with the Wassermann test. The prolonged incubation periods and the numerous subsequent contaminations in these reactions, furthermore, rendered the reading of the results quite undependable.⁹

The Dreyer and Ward Sigma Reaction.—In contradistinction to the Meinicke and the Sachs and Georgi reactions, which are qualitative in nature, this precipitation test is quantitative. The care and precision with which this method has been worked out by the authors is, in my opinion, most commendable. Whether or not, however, the test will have wide application remains to be seen. The numerous and precise technical details which this reaction demands may possibly hinder its acceptance on a large scale.

These workers employ an acetone insoluble antigen of calf's heart. The patient's serum is used in nine different dilutions. The tests are read after seven hours' incubation in the water-bath, and the strength of the reaction is based on the highest dilution showing the presence of a precipitate. We have not had any practical experience with this procedure.

6. Gaeltgens, W.: München. med. Wchnschr., No. 33, p. 933, 1919.

7. Kahn, R. L.: J. Lab. & Clin. M. **6**:153 (Dec.) 1920; *ibid.* **6**:218 (Jan.) 1921; *ibid.* **6**:579, 1921; Am. J. Pub. Health **11**:410 (May) 1921; Optimum Conditions of Fixation of Complement in Wassermann Test, Arch. Derm. & Syph. **4**:358 (Sept.) 1921; Kahn, R. L., and Olin, R. M., Jr.: J. Infect. Dis. **29**:630, (Dec.) 1921.

8. Stern, M.: Ztschr. f. Immunitats. forsch. u. exper. Therap. **32**:167, 1921.

9. Compare, for example, remarks of Dreyer and Ward, Footnote 3, p. 961.

THE PROPOSED METHOD

To begin with, an attempt was made to find the reason for the long incubation periods necessary in the Meinicke and Sachs and Georgi reactions before the appearance of the precipitate. It appeared that there might possibly be some element in the antigen-serum mixture which delayed precipitation. It appeared furthermore that if it be true, as is generally believed, that the precipitate consists largely of antigen lipoids,¹⁰ then one should aim at greater antigen concentration than that employed in the three foregoing procedures. This surmise was found true, after a large number of different antigens were tested. It was observed that a cholesterinized concentrated antigen will frequently give spontaneous precipitations with syphilitic serums, whereas a weak antigen, although cholesterinized to the same degree, will show far weaker precipitates with the same serums after prolonged incubation in the thermostat. It was furthermore observed that the comparatively large amount of salt solution used in the Sachs and Georgi and sigma reactions tend to delay precipitations. Table 2 illustrates this point.

TABLE 2.—APPARENT INHIBITORY EFFECT OF NORMAL SALT SOLUTION ON THE PRECIPITATION OF SYPHILITIC SERUM AND ANTIGEN

	Serum 1						Serum 2						Serum 3					
Serum, C.c.	0.3	0.2	0.1	0.3	0.2	0.1	0.3	0.2	0.1	0.3	0.2	0.1	0.3	0.2	0.1	0.3	0.2	0.1
Antigen, C.c.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Salt solution, C.c.	..	..	..	0.6	0.7	0.8	..	..	..	0.6	0.7	0.8	..	..	..	0.6	0.7	0.8
Results after 1 hr. incubation in the water bath*.....	cp	p	wp	d	—	—	cp	cp	p	p	d	—	cp	cp	p	d	—	—

* In this line, cp indicates complete precipitation; p, almost complete precipitation; wp, weak precipitation; d, doubtful precipitation; —, negative.

Whether or not the delay in precipitation is due to the salt or the dilution element is at present being investigated in this laboratory. Our preliminary findings, however, indicated that the mixing of serum with concentrated antigen, using as little salt solution as possible, would give the best results. The practical difficulty which we had to combat was the false precipitations which followed when the antigen was too highly concentrated.

The Antigen.—One of the requirements regarding antigen is that the heart muscle be dried previous to extraction with alcohol. The usual procedure of preparing antigen with wet heart muscle does not give a sufficiently concentrated extract for this method. We have further observed that crude alcoholic extracts carried out with dried muscle do not give satisfactory results. These extracts seem to possess a

10. Epstein, E., and Paul, F.: Arch. f. Hyg. **90**:98, 1921; Scheer, K.: München med. Wchnschr. No. 2, p. 43, 1921; Niederhoff, P.: München med. Wchnschr., No. 11, p. 330, 1921.

marked tendency for precipitation when mixed with physiologic salt solution of sodium chlorid. Two antigens suggested themselves: an acetone insoluble prepared from dried heart muscle (Noguchi¹¹), and one freed from ether extractives, recently described by Neymann and Gager.¹² We are not as yet ready to report on our experiences with the Noguchi antigen, and the discussion in this paper will be limited to the latter antigen. Its source was pig and beef hearts. The preparation is as follows: The hearts are freed from fat, fibers and blood vessels and are ground and dried. The dried heart is extracted during several days with liberal amounts of ether, using four or five fresh ether quantities. The extraction is completed when the supernatant ether is freed from coloring matter. The ether is then filtered off and the heart tissue dried at room temperature until no odor of ether is detectable. To 10 gm. of dried material is then added 50 c.c. of absolute alcohol, and the mixture is extracted about ten days in the icebox. The alcohol is then filtered off and an amount of fresh alcohol corresponding to that filtered off is added and permitted to extract for several months. Both alcoholic filtrates may be used as antigens.

Cholesterinization of Antigens.—Four hundred mg. of cholesterin is added to 100 c.c. of alcoholic antigen. The mixture is dissolved by warming and rotating, and then filtered through a small filter paper. The antigen is now ready for use. This proportion of cholesterin to antigen is an essential element of this procedure.

Dilution of Antigen for Tests.—In determining the proper dilution of antigen, the following factors are of utmost importance: The diluted antigen must be free from a precipitate and must remain free from it on standing. In a general way, the smallest amount of salt solution should be employed to bring about these ends. In most cases, two and one-half parts of salt solution rapidly added to one part of antigen will give the desired results. In some cases, three parts of salt solution has to be added. We have never had to dilute an antigen more than three parts saline to one part antigen. The correct antigen will be slightly milky but opalescent.

The following is a simple method of determining the proper dilution of a new antigen: Five tenths cubic centimeter quantities of antigen are measured in four good-sized test tubes; 0.75 c.c. of salt solution is poured rapidly into the first tube, 1 c.c. into the second, 1.25 c.c. into the third and 1.5 c.c. into the fourth. The two substances are mixed well immediately on adding the saline in each case, and the tubes are allowed to stand for several hours or overnight at room temperature. That tube in which the antigen is somewhat milky but

11. Noguchi, Hideyo: *Serum Diagnosis of Syphilis*, Ed. 2. Philadelphia, J. B. Lippincott Company, 1911.

12. Neymann, C. A., and Gager, L. T.: *J. Immunol.* **2**:573 (Oct.) 1917.

opalescent and absolutely free from a precipitate may be considered as containing the proper proportions of antigen and salt solution to be used in the regular tests. Needless to say, such an antigen should be tested with at least six normal and six syphilitic serums before it is employed in the tests.

This diluted antigen has kept in our laboratory for two weeks at room temperature, in the dark, with practically no change in its antigenic properties. We believe that this antigen will keep for much longer periods, but have not had any practical experience beyond this time. We would advise gently mixing the antigen by rotation before using it in the tests, as in some cases there is an apparent precipitation on standing—which, however, immediately disappears on slight shaking. No antigen should be employed which shows a sediment on standing.

The Serum.—The serum must be inactivated. In this regard, our procedure differs from that of Sachs and Georgi, wherein active serums may be employed (Hull and Faught)⁵. Thirty minutes at 56 C. is sufficient for inactivation. A serum which has been kept overnight after inactivation should be reactivated for about ten minutes before being used in the tests. The serum also must be clear. Serums when permitted to stand for some hours have a tendency to show faint precipitations. These, however, in most cases permit light to pass through them and can be easily distinguished from the true precipitates which are whitish and opaque. Serums showing slight hemolysis do not seem to interfere with the precipitation reaction.

Amounts of Serum and Antigen Used in the Tests.—The quantitative relations between serum and antigen are at present being studied in this laboratory. Those quantities which have given us the best results and which we recommend are 0.3 c.c. of serum mixed with 0.1 c.c. of antigen. The strongly positive serums show, as a rule, marked precipitations in 0.1 c.c. quantities as well. In the case of weaker positives, however, 0.3 c.c. is often required to elicit a precipitate. Three-tenths cubic centimeter quantities of clear inactivated serum are pipetted in agglutination or small Wassermann tubes; to each tube is added 0.1 c.c. of antigen; it is then shaken gently and incubated. We do not recommend a serum or antigen control. We believe that neither are controls in a sense that they control anything. On the other hand, we do insist that a small number of tests are not dependable unless accompanied by a considerable number of normal and syphilitic serums. We recommend that the test be run in duplication, employing with each serum two antigens obtained from different sources.

Amount of Spinal Fluid Used in the Tests.—We have employed quantities of both 1 c.c. and 1.5 c.c. of spinal fluid with good results. We recommend, wherever sufficient fluid is available, the employment

of both of these quantities with 0.1 c.c. of antigen. All other phases of the reaction are the same as with serums.

The Method of Incubation.—In accordance with our findings, the water-bath at 37.5 C. represents the optimum temperature of incubation for this reaction. The incubator at 37.5 C. is perhaps the next best mode of incubation. Needless to say, a strongly positive serum will react quite well at room temperature and even at icebox temperature. The weaker serums, however, require the water-bath temperature for optimum results.

The Reading of Results.—As stated in the foregoing, the strongly positive serums react spontaneously. After the antigen is added to the serum and placed in the water-bath, clouding will be observed immediately, and a definite precipitate will be visible in from three to five minutes. However, in order that the laboratory worker may not be obliged continuously to watch the formation of precipitates, the tests are read after one hour and three hours' incubation, respectively. After the first hour, those tubes showing marked precipitations are recorded as strongly positive. These, in most cases, correspond with quantitative Wassermann findings, giving results considerably above four plus. The final reading is made at the end of three hours' incubation. Only those tubes showing varying degrees of cloudiness need be closely observed. Those tubes which appear clear can, in practically every case, be considered negative without further observation. The tubes showing marked precipitation, excluding those which have been recorded at the end of one hour as strongly positive, are considered positive. These correspond to four plus and three plus reactions in the Wassermann test. Those showing weak precipitations are read as weakly positive, corresponding to two plus in the Wassermann test. Those showing very weak precipitations are considered doubtful, corresponding to one plus and plus-minus in the Wassermann test.

The tendency of many serums spontaneously to precipitate on standing was a source of much difficulty in the early part of this work. The serum precipitates are different in many ways from those produced by mixing serum with antigen. The former appear to be crystalloid in character and permit light to pass through them, while the latter, being colloidal in nature, are whitish and opaque in appearance. It was nevertheless found difficult on different occasions to distinguish, at the final reading of the tests, between doubtful reactions and so-called serum precipitates. To overcome this difficulty the following procedure is employed:

At the end of the three hours' incubation period, a preliminary reading is made, particular attention being paid to those tubes showing clouding as distinguished from those which show the clear opalescence

of serum. Then to each tube, twice its content is added, i. e., 0.8 c.c. physiologic solution of sodium chlorid, and mixed. The precipitates in the positive tubes are so pronounced that this high dilution does not in any way interfere with the ease with which they could previously be seen. On the other hand, those which showed faint precipitations, and caused speculation as to whether they were doubtful or negative reactions, show after dilution, in practically every case, no trace of precipitate. In other words, the precipitates in the doubtful reactions must be sufficiently pronounced to withstand a dilution of twice the amount of that contained in the original test.

COMPARISON WITH WASSERMANN TESTS

This precipitation test is now being run daily in the Michigan health department laboratory on all serums and spinal fluids sent in for a Wassermann test. It is as yet not being reported to the physician, but we feel that our Wassermann reports possess a greater degree of reliability, owing to the high percentage of checks with the precipitation test. We find this test more sensitive than the Wassermann test in hereditary syphilis and in treated cases of this disease. This, however, is not judged from unquestionable clinical histories but rather from the Wassermann blanks filled out by physicians.

TABLE 3.—COMPARISON OF RESULTS OF WASSERMANN AND PRECIPITATION TESTS ON 1,119 SERUMS

	Wassermann	Precipitation		
		Positive	Doubtful	Negative
227 positive* tests gave.....		213	7	7
90 doubtful tests gave.....		40	20	30
802 negative tests gave.....		14	21	767

* Positive means strongly as well as weakly positive.

This table shows that of 1,119 serums examined:

1. Two hundred and thirteen were positive by both methods; twenty were doubtful by both methods; 767 were negative by both methods; total, 1,000 specimens checked by both methods, showing agreement of 89.36 per cent.
2. Seven positive Wassermann reactions were doubtful in the precipitation test; forty doubtful Wassermann reactions were positive; thirty doubtful Wassermann reactions were negative; twenty-one negative Wassermann reactions were doubtful; the total ninety-eight specimens may be considered as showing relative agreement (8.75 per cent.).
3. Fourteen negative Wassermanns gave weakly positive precipitation reactions; seven weakly positive Wassermanns gave negative precipitation reactions; total, twenty-one specimens did not check (1.87 per cent.).

4. In not a single instance did a serum giving a four plus or a three plus Wassermann reaction show a negative precipitation, or a serum showing a strongly positive, or positive precipitation give a negative Wassermann result.

PRACTICAL VALUE OF THE PROPOSED TEST

In my opinion, this simple precipitation test should prove a valuable check on the Wassermann test. The importance of detecting syphilis is such that two tests should prove highly desirable. This disease is different from typhoid fever or diphtheria, for example. The patient does not recover from it in a sense that he recovers from the two other diseases. There are, indeed, those who believe that syphilis is not completely curable. May it not be possible that this belief has been evolved as a result of pronouncing syphilitic patients cured on the basis of apparent freedom from clinical symptoms and repeated negative Wassermann reactions, when in truth, lesions might still persist in a latent form? A test, therefore, which is more sensitive than the Wassermann, as the proposed precipitation reaction appears to be, should prove a valuable aid in the proper diagnosis of syphilis.

It is hoped that the simplicity and ease with which the test can be carried out will attract many workers to investigate it in conjunction with clinical studies.