

## A MODIFICATION OF THE TECHNIC OF THE WASSERMANN REACTION \*

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The "Wassermann" blood test for syphilis is now generally recognized as a valuable aid to the clinician, not only in the diagnosis of the disease, but often as an indicator of the effect of treatment. It is not, perhaps, so generally appreciated, however, that the test, in order to be of real value, must be so carried out that a "positive" reaction will be obtained only in syphilis and in certain other conditions that are readily distinguished from syphilis. No one, thus far, has succeeded in so improving the efficiency of the test that in every case of syphilis with apparent lesions a positive reaction is obtained; so that as a diagnostic means a negative "Wassermann" reaction offers no guarantee of the absence of luetic infection; but properly performed, the test, when it results *positively*, must be expected to indicate either syphilis or certain other easily recognized conditions, such as leprosy, malaria, frambesia, paroxysmal hemoglobinuria and probably lead-poisoning.

It follows, therefore, that no modification of the original technic of the Wassermann test can be adopted if, with its use, any positive reactions are encountered in diseases other than those mentioned; and this policy would have to be adhered to even if the modifications proposed promised a great increase in the number of positive reactions obtained in known cases of syphilis.

A number of grave defects attach to the technical procedure originally recommended for the performance of Wassermann test, all of which are due to the impurities contained in the originally prescribed "antigen" preparations (i. e., the carbolized watery extract or the alcoholic extract of the liver of a syphilitic fetus). These impurities may interfere in three ways with the performance of the test: (1) by exerting a directly anticomplementary influence during the period of fixation; (2) by exerting a hemolytic influence on the blood corpuscles that are used as indicator of the reaction; and (3) by causing a pseudoreaction with the serum of individuals who are neither syphilitic nor subject to any of the other conditions that are mentioned above.

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The first two of these three disturbing actions of the impurities contained in the fetal liver extracts are avoided by selecting for test that quantity of extract which, in double amount, is neither hemolytic nor anticomplementary. The use of this quantity of extract, however, is attended with the third disturbing action and for the elimination of that source of error it has been found necessary to heat the sera to be examined for one-half hour at 50 C.<sup>1</sup>

One of the earliest modifications of the "Wassermann" technic was the substitution of an alcoholic extract of normal organs—for example, the heart muscle of the guinea-pig—for the extracts of syphilitic fetal liver. The most extensive studies of both syphilitic and non-syphilitic organ extracts in their "antigenic" capacity were made by Noguchi, who showed that the "antigenic" function of all the extracts resides solely in the lipid fraction, and that the process of separating the lipoids from the extracts eliminates to a large extent the disturbing impurities.

On the basis of this important discovery Noguchi recommended the use of the isolated lipoids; i. e., the ether-soluble, acetone-insoluble fraction of an alcoholic organ extract as "antigen." Noguchi at first believed that when the isolated lipoids are used as "antigen" in the Wassermann test the sera to be examined could be used without the usual heating at 50 C.; but other workers<sup>2</sup> who employed the isolated lipoids found that these also, when used according to the original prescription of the Wassermann technic—i. e., as regards quantity—gave spurious positive reactions with unheated sera. For this reason, as well as on account of the practice of many workers of storing the sera for several days before examining them (during which time they often become anticomplementary), it is customary, also when the isolated lipoids are used as "antigen," to heat the sera.

Our own experiences with the isolated lipid "antigen," which are described in detail elsewhere,<sup>3</sup> have convinced us that *when this "antigen" preparation is used according to the method originally prescribed for the performance of the Wassermann test, its one great advantage over the original organ extract is in part sacrificed and a new source of error is introduced.*

The sole advantage of the isolated lipoids over the original organ extracts as an "antigen" preparation lies in the availability of the "antigenic" substances in the former preparation for use in *much greater quantity* than is possible with the latter preparation. This advantage has, however, a two-fold application. We have found, in the first place, that in a considerable proportion of luetic cases—usually

1. In some laboratories higher temperatures (54 to 56 C.) are employed.

2. See article by MacRae, Eisenbrey and Swift, THE ARCH. INT. MED., 1910, vi.

3. Zeitschr. f. Immunitätsf., 1912, xiv, p. 139.

those exhibiting lesser lesions or those that have received only mercurial treatment—a positive reaction is obtained when a larger quantity of the isolated lipoids is used, but the test results negatively with smaller quantities; i. e., with quantities that represent the amount of “antigenic” substance that is used in the originally recommended procedure.

As we have just indicated, there is another way in which the availability of the isolated lipid “antigen” for use in relatively large quantities can be applied to advantage in carrying out the Wassermann test. In determining the reacting strength of immune sera by means of the Bordet-Gengou technic—which is the prototype of the Wassermann technic—it is customary to estimate the relative values of such sera according to the smallest amount of the respective antigen with which, under like conditions, complete complement fixation is produced. Since the mechanism of the Bordet-Gengou reaction and that of the Wassermann reaction are undoubtedly identical, it must be possible to apply, in the latter test, the same principle of quantitative comparison that is used in the former.

This plan was suggested a number of years ago by Neisser, Bruck and Schucht, but in the absence of any further publication on the matter by these authors it may be assumed that their attempt in this direction was not successful.

Their failure can be easily explained.

An examination of their published protocols discloses the fact that, owing to the admixture of the usual impurities in their standard “antigen” preparation, which was an alcoholic extract of the liver of a syphilitic fetus, they were limited to the use of only eight times the smallest amount of “antigen” with which, in cases of untreated secondary syphilis, complete complement fixation could be produced. With an “antigen” preparation limited to such a narrow working range, it was not possible to apply the quantitative method of the Bordet-Gengou test.

The isolated lipid “antigen,” on the other hand, can be safely used in a quantity that is at least 500 times the minimum complete fixing unit—as determined in cases of untreated secondary syphilis—and with this preparation, therefore, the quantitative technic of the Bordet-Gengou test becomes directly applicable to the Wassermann test.<sup>4</sup>

If then, as is commonly done, the isolated lipid “antigen” is employed for the Wassermann test in only one quantity—i. e., one-half of the largest amount that, by itself, is neither hemolytic nor anti-

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4. It must be emphasized here that whether the reacting strength of luetic sera as determined according to the minimum fixing quantity of “antigen” does or does not always coincide with the degree of severity of the syphilitic process that exists at the time of the examination, the result of such a determination does give a true index of the concentration of the reacting substances in the sera.

complementary—the advantage of the quantitative method of the Bordet-Gengou test is sacrificed.

But the sacrifice of the quantitative technic is not the only objection that must be urged against the application of the Wassermann prescription to the use of the isolated lipoid “antigen.” We have mentioned the experience of MacRae, Eisenbrey and Swift, who found that even with the isolated lipoid “antigen,” unless the sera to be examined are heated, “positive” reactions are sometimes obtained in many diseases other than syphilis.<sup>5</sup>

The practice of those who use the isolated lipoid “antigen” of heating the sera to be examined in order to avoid such spurious positive reactions, greatly exaggerates a new source of error—one that is not encountered when the smaller quantities of the “antigenic” substances are used, as under the original Wassermann technic—which attaches to the use of the isolated lipoid “antigen.” The source of error referred to is the familiar prezone phenomenon of wanting reaction, which occurs in the testing of specific antisera when either of the two reacting substances, antigen and antibody, is combined with the other in a quantity that is in excess of a definite proportion.

The prezone phenomenon that is sometimes produced in the performance of the Wassermann test is due to the use of a relative excess of the “antigenic” substances. It is much more frequently met with when the sera to be examined are heated, but it has been observed by us in twelve instances in which the sera had not been heated.

The source of error presented by the prezone phenomenon occurring in the Wassermann test can be easily eliminated by carrying out each test with descending quantities of the “antigen,” so that it is not necessary, only on account of this possible fallacy, to omit the heating of the sera. That the custom of heating the sera should be discarded when it is possible is, however, evident in view of the well-known fact that heating considerably reduces the reacting power of luetic sera.

As a matter of fact, the heating of the sera *can* be dispensed with, for we have found an equally certain and much simpler way to accomplish the end for which the heating was usually performed, namely, to avoid the spurious “positive” reactions of diseases other than syphilis. We have observed, in a series of several hundred examinations, that whereas with unheated sera 0.2 c.c. of a 1 to 10 dilution of our 2 per cent. lipoid solution often gives complete fixation in many conditions other than syphilis, no such spurious reaction is produced by 0.1 c.c. of that dilution of the lipoids. With one exception all of the fixations produced with the latter

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5. Notwithstanding this serious complication these writers conclude that “the Noguchi method, using *active* serum, gives the most sensitive reaction in syphilis.” We have already pointed out the fallacy of this view.

quantity of the isolated lipoid "antigen" have been obtained in cases of known syphilis. That one exception was in a case of paroxysmal hemoglobinuria, in which disease the original Wassermann technic produces regularly a "positive" result."

*Instead of heating the sera, therefore, we avoid the spurious or "pseudoreactions" by using about half the prescribed amount of the isolated lipoid "antigen," which, as we have already stated, is 500 times the minimum fixing quantity for secondary syphilis.*

#### SUMMARY

The substance of the preceding discussion may be more briefly presented as follows:

1. Only the lipoid fraction of organ extracts possesses the "antigenic" properties requisite for the performance of the Wassermann blood test for syphilis (Noguchi).

2. On account of the large admixture in the originally recommended "antigen" preparations (i. e., the carbolized watery extract and the simple alcoholic extract of the liver of a syphilitic fetus) of substances not possessing "antigenic" properties, the quantitative limits within which the "antigenic" substances are available for the Wassermann test, are very narrow—4 to 8 units. When such extracts are used for the test the sera must be heated, otherwise positive reactions are obtained in many diseases besides syphilis. The reacting strength of luetic sera is considerably reduced by heating.

3. The quantitative limits within which the isolated lipoid "antigen" is available for the Wassermann test, are relatively wide—at least 500 units—so that with this preparation positive reactions can be obtained with luetic sera with which the test would result negatively if only the relatively small amount of "antigen" available under the original Wassermann procedure was used; furthermore, on account of the greatly extended working-range of the isolated lipoid "antigen" preparation the usual quantitative method of determining the reacting strength of specific antisera according to Bordet-Gengou can be employed.

4. When the isolated lipoid "antigen" preparation is used according to the original Wassermann prescription, "positive" reactions are often obtained with active sera in many diseases other than syphilis. These spurious or "pseudoreactions" can be entirely avoided, without heating the sera, by not using more than about half of the prescribed amount of the "antigen" preparation; i. e., one-fourth instead of one-half of the largest quantity of the preparation that by itself is neither hemolytic nor anticomplementary.

5. When the isolated lipoid "antigen" preparation is used in descending quantities in the Wassermann test, the prezone phenomenon is some-

times observed, which is due to the use of an excess of the "antigenic" substances. This source of error is met with in a considerable proportion of heated luetic sera, and occasionally even when the sera are not heated; it can be avoided only by the use, in each test, of descending quantities of the "antigen" preparation.

#### PROPOSED TECHNIC

*Antigen.*—The most convenient source of suitable lipoids is the heart muscle of the ox. The muscular tissue is dissected out from the heart of a recently slaughtered ox, and, after being finely comminuted in a meat grinder, is shaken well with about ten volumes of 95 per cent. alcohol. The alcoholic extract is filtered after five to ten days and evaporated nearly to dryness with the use of an electric fan. The sticky residue is extracted with ordinary ether and the ether extract again evaporated with the use of the fan, supplemented finally with gentle warming. The resulting residue is then extracted with water-free ether in small quantity and the clear solution obtained by centrifugation is mixed with five volumes of acetone. After thorough shaking the fluid is poured off the waxy precipitate, which is then shaken several times with fresh portions of acetone. The stock solution is a filtered 2 per cent. solution of the lipoids in pure methyl alcohol. For each series of tests a fresh emulsion is made by diluting one part, by volume, of the stock solution up to ten parts with physiological salt solution (Ringer's). Of this 0.2 per cent. emulsion, 0.4 c.c. has never produced the slightest hemolysis when mixed with 0.1 c.c. of a 5 per cent. suspension of sheep's blood, nor is that amount anticomplementary with 0.01 c.c. of guinea-pig's serum (indicator 0.1 c.c. of 5 per cent. sensitized sheep's blood).

Some lipid preparations from ox's heart muscle are not capable of functioning as "antigen" in the Wassermann test. Of four such preparations made from four different ox-hearts, one was completely lacking in "antigenic" property. The other three were efficient, and equally so, in that capacity. The "antigenic" property of our first preparation has remained unchanged for more than one year.

In the interest of economy of materials, all the reagents entering into the Wassermann test may be used in one-tenth of the usual quantities. Under this condition we have found that with cases of untreated secondary syphilis, the minimum fixing amount of ox-heart lipoids is frequently as little as 0.0002 c.c. of a 0.2 per cent. emulsion, that is, 0.0000001 g.

Some of the sera that have been obtained more than twenty-four hours previous to the examination are found to have become anticomplementary, that is, 0.4 c.c. or less, of the diluted serum inhibits by itself the complementary function of 0.1 c.c. of guinea-pig's diluted serum.

In order to remove this anticomplementary property it is necessary to heat such sera for one-half hour at about 50 C. For the exact determination of the fixing strength of a luetic serum, it is, therefore, important that the test be made within twenty-four hours after the blood has been taken, so that the necessity of inactivation may be avoided. If, as we have always done, the reagents entering into the Wassermann test are all used in one-tenth of the usual quantities, the amount of the patient's serum required for such examination is correspondingly reduced; so that sufficient blood for the test can be easily obtained from the ear or finger. Such small amounts (2 c.c.) are best defibrinated and centrifugated at once. The serum can then be removed with a pipet and is ready for immediate examination.

*Complement.*—This function is supplied by 0.1 c.c. of a 1 to 10 dilution of fresh guinea-pig's serum in physiological salt solution. Whenever this serum is naturally hemolytic for sheep's blood corpuscles, the natural hemolysin is to be removed by the cold separation method—one to two hours at 0 C. in contact with washed sheep's corpuscles.

*Indicator.*—Two per cent. sheep's blood corpuscles sensitized with two to three minimum hemolytic doses of immunized rabbit's serum are used as indicator. To each tube is added 0.25 c.c. of this suspension, representing 0.1 c.c. of the usual 5 per cent. suspension.

When the test was carried out according to the plan that we have described, we were not able to observe any essential difference in the results that could be due to differences, within the limits given above, in the degree of sensitization of the blood corpuscles that were used as indicator of the reaction. The sensitization should be so adjusted to the complementary activity of the guinea-pig's serum that not more than 0.025 c.c. of that serum diluted 1 to 10 will be required, and that not less than 0.0125 c.c. will be able completely to hemolyze 1 unit (0.25 c.c. of a 2 per cent. suspension) of the sensitized blood cells.

In the practical performance and reading of the Wassermann test we proceed as follows:

1. All the reagents entering into the reaction of fixation can be most conveniently diluted 1 to 10 with physiological salt solution. The sensitized sheep's blood suspension may be 2 per cent. (unit=0.25 c.c.) or 5 per cent. (unit=0.1 c.c.).
2. Five different amounts of a 0.2 per cent. emulsion of ox-heart lipoids are to be combined with 0.2 c.c. of the patient's diluted serum and 0.1 c.c. of the diluted guinea-pig's serum. The five amounts of antigen are 0.1, 0.05, 0.02, 0.01 and 0.001. A series of examinations with non-luetic sera should be made for the purpose of determining the lower limit of pseudo-reaction.
3. If complete fixation of complement occurs with all of the five quantities of "antigen" or with only the smallest two or three quantities, we report a "strongly positive" reaction; if with 0.001 c.c. hemolysis has not been prevented, but fixation has occurred with all the other combinations or with the lesser one or two quantities of "antigen," we report a "positive" reaction; if hemolysis is not prevented with 0.001 nor with 0.01 c.c., but fixation occurs with any of the other amounts of the "antigen," we report a "weakly positive" reaction.

We have never met with an occasion for reporting a "doubtful" reaction.

We have confirmed the observations of Jacobsthal and of Guggenheimer, that with some luetic sera a positive Wassermann reaction can be obtained when the "incubation" for fixation is carried out at ice-box temperature, whereas the test as usually performed results negatively. The reverse of this, also, has been found, in some instances, to be true.

In view of these facts it is necessary to perform each test in duplicate series, one for fixation at ice-box temperature, and the other for fixation at 37 C. The period of incubation for fixation at low temperature may be twelve to eighteen hours (conveniently over night).

In the table are given examples of the results that we have obtained with our technic, together with our "reading" of them.

TABLE GIVING EXAMPLES OF THE RESULTS OF THE WASSERMANN REACTION OBTAINED UNDER AUTHORS' TECHNIC, CLASSIFIED ACCORDING TO REACTING STRENGTH OF THE SERA

Indicator 0.25 c.c. of 2 per cent sheep's corpuscles. In each tube 0.02 c.c. of patient's serum + 0.01 c.c. of guinea-pig's serum.

—————0.2 per cent. Emulsion of Ox-Heart Lipoids—————

0.1 c.c.	0.05 c.c.	0.02 c.c.	0.01 c.c.	0.001 c.c.	
0	0	0	0	0	} Strongly positive reactions.
++++	++	0	0	0	
++++	++++	++	0	0	
0	0	0	0	++++	} Positive reactions
++++	++	0	0	++++	
++++	++++	++	0	++++	
0	0	0	++++	++++	} Weakly positive reactions.
0	0	+	++++	++++	
0	++	++++	++++	++++	
++++	0	0	++	++++	
++++	+++	+	+	++++	

++++ = complete hemolysis; +++ = very strong hemolysis; ++ = partial hemolysis; + = slight hemolysis.