WASSERMANN REACTION

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SOME PRACTICAL POINTS IN CONNECTION WITH THE WASSERMANN REACTION.

By

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It is a fact generally known to medical men who are in the habit of sending specimens of blood to the pathologist for a Wassermann report that the report may come back marked with the letters "A.C." which indicate that this particular blood has given an anti-complementary result. The medical man also knows that this can be easily remedied by collecting another specimen of blood from that patient and having the test repeated. Though to-day such anti-complementary results do not occur so often as they used to, every practitioner having learned to collect the blood specimens in clean, sterile test-tubes, yet at times for one reason or another not always possible to unravel, the serum will turn out anti-complementary.

By anti-complementary action of a serum (especially in connection with the complement-fixation for syphilis) is meant that particular phenomenon in which the fixation of the complement of the haemolytic system takes place in the presence of a serum without the extract. Various external agents and conditions, e.g., acids, alkalis, bacterial infection of the serum, heat, etc., are known to exert an action on the complement in the direction of diminishing or destroying its activity. Up to the present we are unable to state just why these various agents act in this particular

fashion.

In this communication I have selected for discussion and for experiment a few of those factors which in a practical way come to the forefront in performing the Wassermann reaction, and it is my intention to deal with the following conditions:-

1. Antigen in high doses;
2. Fat in the serum;
3. Fat-solvents and haemolysing agents, water, and also tinct. iodi;
4. Overheating of the sera;
5. Old sera and sera infected with bacteria;
6. Complement fixation by precipitates;
7. Action of diarsenol on the serum.

Antigen in High Doses:

When we titrate an extract to be used in the Wassermann reaction our object is simultaneously to fulfil a number of conditions: (1) we must find out that special dose which with a known positive luetic serum gives a well defined, sharp, positive complement-fixation; (2) we must assure ourselves that the same dose shall give, with a known normal serum, a complete haemolysis; (3) the extract by itself, i.e., without serum, must also in that dose give a complete haemolysis.

Having found this dose, we must also leave a margin to provide against absorption of a certain small amount of complement by the extract and by the serum of the patient. It is nowadays an

agreed convention to use of the original dose of antigen found (the so-called "anti-complementary dose") one half or according to others even a one-fourth part of it. However a considerable number of pathologists still use that dose which just fulfils the requisites specified above, in this case increasing the danger of having a non-specific fixation of complement. Though, with an appropriate and well adjusted haemolytic system the reaction in this case becomes a very sensitive one, capable of revealing the presence of very small quantities of antibodies in the patient's serum, yet if by any chance the serum of the patient absorb a certain amount of complement, the result may be a positive Wassermann reaction or an anti-complementary one. The positive reaction, taking place in the front row of test-tubes, will originate from a certain quantity of complement being absorbed by the extract and another quantity by the serum. The test-tube of the second row, with the serum alone will just show a trace of inhibition, so that we should read it as negative; and the same may happen by overlooking the control test-tube containing the extract by itself. This danger is even worse than if a mere anti-complementary action should take place, because in the latter case we should discard the result. The danger of using extracts bought on the market, without first titrating them with the haemolytic system used by the pathologist will, in virtue of the high doses of extract present, give rise to anti-complementary results, and a large number of sera will also be taken as giving positive results. Only the most scrupulous attention in titrating the extracts and using a large set of controls, will eliminate this grave source of error.

Sera Containing Fat:

The disturbing effect of fat (as well as of lipoid solvents) in the serum has recently been stressed by Oertel[1], who leans to the opinion that such influence may produce a positive Wassermann reaction. In the instructions issued by certain Provincial and Municipal authorities for the guidance of practitioners in collecting samples of blood for the Wassermann test a caution is inserted against taking the blood just after a meal.

In another communication[2] I have recently shown that the presence of fat in the serum produces only an anti-complementary result. Using a fatty serum, we know that in both rows of test-tubes, viz., in that containing the serum as well as that containing the extract, fixation of the complement occurs. This obviously anti-complementary result may, it is true, be claimed as obscuring the Wassermann reaction. If, however, after the serum has stood for a time, one pipettes out the clear fluid which lies under the meniscus of fat, such serum gives a perfectly straightforward negative reaction. With a little patience and care it should be quite possible for a pathologist to overcome this particular hindrance to the reaction, thus saving the patient another puncture of the vein.

Fat Solvents, Distilled Water and Iodine:

Fat solvents such as ether or alcohol are possible sources of contamination in taking specimens of blood. They are used for disinfecting the skin; hypodermic needles are also frequently stored under alcohol. Again iodine, whether wet or dry, may gain entrance

to a collected specimen of blood, while distilled water is another reagent which may readily gain entrance to collected blood.

For the study of the anti-complementary action of these substances I set up a series of bloods of man, of dog, of calf, of hog and of sheep. All the bloods having been defibrinated, 5 c.c. of each kind was put into each of twelve test-tubes so that there were 60 test-tubes in all, 12 for each blood and each containing 5 c.c. of blood. In the series of test-tubes containing any particular variety of blood I put an increasing number of drops of the reagent selected for investigation; thus, supposing that the effect of alcohol were being investigated, into No. 1 test-tube I put one drop of alcohol, into No. 2 test-tube 2 drops of alcohol, and so until I reached No. 10 test-tube with 10 drops. Test-tubes No. 11 and No. 12 contained 15 and 30 drops of alcohol respectively. For each reagent 60 serparate experiments on 5 different bloods were thus carried out. To secure uniformity of the drops I used an ordinary fountain-pen filler, calibrated in such a way that at room temperature 17 drops of alcohol was equal in volume to 1/2 c.c. The tubes of blood so treated were plugged with cotton wool and left standing at room temperature for 12 hours; they were then centrifuged and the serum was pipetted into sterile test-tubes and inactivated for thirty minutes at 55°C. After the inactivation it was noticed that all test-tubes marked 11 and 12 and containing respectively 15 and 30 drops of the different reagents were, owing to the large amount of haemoglobin dissolved, converted into a thick paste of a dark chocolate colour. As they did not contain any free serum with which to carry out the Wassermann reaction they were discarded. The other sera, though rich in dissolved haemoglobin,

were still fluid, though, owing to the quantity of reagent added, the last of the series, e.g., Nos. 9 and 10, had a syrupy consistency.

Having subjected all the sera so treated to the Wassermann test, I obtained the following results. Chloroform, ether, alcohol and water added to the various bloods (sheep's blood alone excepted) in an amount varying from one to ten drops gave, both with the alcohol-cholesterin extract and without any extract, no anti-complementary results. They were absolutely negative, though the test-tubes containing sera treated with the large amounts of reagent were slow in giving haemolysis.

As a rule the tincture of iodine gave with all kinds of blood, in amounts up to 4 to 5 drops, completely negative results. When tinct, iodi is added in amounts higher than this the blood used in the haemolytic system was practically all haemolysed; nevertheless, at the bottom of each of these test-tubes one noticed after twelve hours a dark sediment, which the microscope showed to be red cells. Thus with more than 5 drops of tinct. iodi there was in both rows of test-tubes a slight anti-complementary reaction.

In the case of sheep's blood all test-tubes containing the various added substances showed a slight inhibition of haemolysis. This phenomenon may undoubtedly be placed in relation with the fact that anti-sheep amboceptor was used in the research and that in the presence of the immune serum an invisible precipitate had formed.

The conclusion to which I have come is that in taking blood from a patient and using an ordinary hollow needle (which

may hold about four drops of alcohol or of water) it would make little difference to the test if one should by chance forget to empty the needle before use, provided 10 c.c. of the patient's blood is withdrawn. A similar statement may be made with regard to tincture of iodine, but the risk of massive contamination with this substance is considerably less than in the case of alcohol or water.

Overheating of the Entire Blood and of the Sera:

In the paper referred to I have shown that if entire blood is heated even as high as 60°C. for two hours and the serum then tested by the Wassermann reaction, no anti-complementary action results. Heating of the blood or of the separate serum to a higher degree of temperature and for a protracted period will, by coagulating action on the proteins of the serum, give rise to an anti-complementary action. This latter effect however does not come into consideration in a practical way because in carrying out the Wassermann test inactivation of the serum takes place at 55° to 56°C.

Cooling of the Blood Serum:

Through the kindness of Professor Tait, Dr. Cassidy and of Mr. Britton of this laboratory some specimens of blood of deeply cooled cats and woodchucks (Arctomys marmotta) were placed at my disposal. The animals had been cooled down in some cases to 5°C. for periods varying from 2 to 5 hours before the blood was withdrawn. The sera of such bloods did not chow any anti-complementary property.

Old Sera and Sera Infected with Bacteria:

A large number of human and of animal sera were used in this case, and I tried the Wassermann reaction on them at different intervals, the strictest precautions being taken against artificial contamination. Having examined these sera, in some cases after a month, in other cases after longer periods, I found that most of those which had given a negative Wassermann reaction on first testing continued to give a completely negative reaction. Only a few of them, and a good many of the original positive sera, were in various degree anti-complementary. In my opinion, if the sera are kept sterile and in the ice chest, especially if they are negative, they will keep negative for a longer period than positive sera. At the same time it is always a safe practice when using old sera to heat them again to 55°C. for 30 minutes before using. This will always prevent any anti-complementary action.

Infected sera, especially if old, are always strongly anti-complementary. I purposely infected a few sera with some of the ordinary pathogenic microorganisms (Staphylococcus aureus was used as a rule) and left the blood sera so contaminated in an open Petri dish for 24 hours. I then collected the serum in test-tubes and inactivated them. These sera, compared with the non-infected specimens, were turbid and cloudy, and although already partially anti-complementary on the same day as that on which they were infected, were strongly so after a day or two. The anti-complementary action caused by bacteria is practically impossible

to eliminate by filtration or other mechanical means, and the only safe way is to get another specimen of blood.

If the blood which had been collected in a test-tube was left in the ice chest without plugging until the evening or even until the day after the day of collection, no anti-complementary action was noticed. This absence of result is due no doubt to the bactericidal action of the serum. In spite of these results the best method is always to use scrupulous asepsis, collecting the blood in sterile tubes and plugging well.

Anti-Complementary Action of Precipitates:

This phenomenon was first found by Gengou(3) and then confirmed by Moreschi(4) and by Gay(5). It takes place in the process of immunisation of an animal, especially when we want to obtain an amboceptor against a special blood for use in the haemolytic system of the Wassermann reaction. If the red cells used in the process of immunisation are not well washed and the serum not completely removed, we obtain, in addition to a haemolytic serum against the cells used, an immune body against the serum itself (precipitin). Suppose we use now in our haemolytic system such a mixed or impure amboceptor and suppose the red cells employed in the system also contain a certain amount of serum, instead of obtaining a complete haemolysis of the cells we get a partial haemolysis. This is due to the fact that the small amount of serum (precipitinogen) still attached to the red cells will, in presence of the amboceptor containing precipitin-immune

serum, give rise to a precipitate, which as a rule is invisible. This precipitate, absorbing a certain amount of complement, prevents a complete haemolysis of the red cells. Such anti-complementary action is very grave, especially when, as is the practice of some pathologists, the amboceptor against human red cells is obtained by employing the method just described.

The positive results claimed to have been obtained by some pathologists with sera that have given negative results in the hands of others are, perhaps in most cases, attributable to the intrusion of this grave source of error. The surest way to eliminate the possibility of this mistake is to use only an anti-sheep and never an anti-human amboceptor.

Influence of Diarsenol:

Having noticed that specimens of blood taken soon after an intravenous injection of diarsenol were reported as giving an anti-complementary action, I thought it worth while to investigate this particular point more carefully. The apparatus used at the Montreal General Hospital for injecting salvarsan is the usual gravity apparatus. It consists of a large glass tube (capacity 60 c.c.) to the lower attenuated part of which two feet of rubber tubing is attached. After the interposition of a glass window at the distal end, a second short piece of rubber tubing serves to connect the apparatus to the needle. This injection apparatus,

containing a few c.c. of NaCl solution free of air-bubbles, is by the insertion of the needle into one of the veins of the bend of the elbow, put in communication with the circulatory fluid of the patient. When one has assured oneself that the fluid runs into the vein, the requisite amount of diarsenol solution (0.1 gram to 20 c.c. of NaCl solution) is poured into the glass receptacle. Just before all the solution is passed into the vein, a few more c.c. of saline solution are poured into the apparatus for the purpose of washing out the walls of the apparatus and of the vein. Then by lowering the apparatus below the point of insertion of the needle (which still remains in situ) and after having, by inversion of the glass receptacle, discarded the first few c.c. of blood mixed with saline and diarsenol solution, one collects about 10 c.c. of blood.

I tested specimens of blood of 100 patients before and after injection of diarsenol and obtained in two cases an anti-complementary result in the blood received immediately after injection. Consequently I decided to do some more precise experiments on the blood of normal individuals, of syphilitic and of animals (calf, sheep and hog).

In the first preliminary experiments I added increasing amounts of fresh diarsenol solution of the same stock as is used for the treatment of patients to a series of test-tubes containing in each case 5 c.c. of defibrinated blood. The amount added varied from 3 drops up to 3 c.c. The blood so treated I left for twelve hours in the ice chest, whereupon they were centrifuged, while the

sera after separation and inactivation were subjected to the Wassermann test. All sepcimens of sera turned out negative, though those containing larger quantities of diarsenol solution were very slow and near the point of becoming anti-complementary. Curious to relate, the hog serum thus treated gave repeatedly a true positive fixation of the complement and no anti-complementary action.

I then carried out numerous tests by making up fixed percentages of diarsenol solutions in the serum (viz. 0.1, 0.05, 0.025 and 0.0125, etc.), using the same cholesterinized extract as for the Wassermann test. I was surprized to obtain a marked anti-complementary result with the first dilutions. The action may just possibly be due to the presence of alkali in the diarsenol solution; this is however unlikely, for repeated tests on fresh, on heated and on old diarsenol solutions showed a gradual shifting from a strong anti-complementary reaction to a negative Wassermann reaction. When the heated or old solution of diarsenol gives rise to a gray precipitate and the supernatant fluid from a yellow canary colour is changed into a practically colourless fluid, then it gives an entirely negative reaction.

The presence in the serum of fresh solution of diarsenol in a proportion of 0.025% may give rise to an anti-complementary action. However if care be taken to discard the first c.c. of fluid from the apparatus this proportion will never be attained and the danger of an anti-complementary action will be eliminated.

Before concluding may I be permitted to say that in spite

of all attempts to substitute for the somewhat cumbrous Wassermann test a more direct and less complicated procedure, no reaction as yet proposed can compare in reliability with the full Wassermann test? In another communication(6) I have dealt with the comparative value of the Sachs-Georgi(7) and of the Dreyer-Ward(8) flocculation reaction in relation to that of the Wassermann test, my finding being that the Dreyer-Ward is distinctly superior to the Sachs-Georgi, but that neither is equal to the Wassermann itself. It is unfortunate that the Wassermann test is so ringed around with pitfalls, but, given a reasonable measure of care on the part of the pathologist, these can in almost all cases be surmounted.

CONCLUSIONS.

1. Extracts in high doses may give rise to anti-complementary action. The necessity of titrating the extracts as often as possible and of using a dose one half of the "anti-complementary dose" is emphasized.

2. If present in large quantities, fat also gives an anti-complementary action. This is remedied by removal of the fat previous to the test.

3. Alcohol, ether, chloroform, water and tincture of iodine, in the proportion with which they can reasonably come into contact with the blood in the taking of blood specimens, cannot cause an anti-complementary action.

4. Even if heated to 60°C. sera or bloods used in the

Wassermann test will not give rise to anti-complementary action.

5. Sera infected with bacteria, especially if long infected, give an anti-complementary action. Negative old sera as a rule are less liable to give anti-complementary action than old positive sera. The best advice is to heat them to 55°C. before testing them.

6. Anti-human amboceptor so frequently given an anti-complementary action through precipitin-formation that it is best to resort only to anti-sheep amboceptor.

7. Collecting of blood for the Wassermann test from a patient who has just received an injection of diarsenol does not have any influence on the Wassermann reaction. If however the blood is received in the same apparatus as is used for injection it is absolutely necessary to discard the first small amount of blood received.

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SUPPOSED DISTURBING FACTORS IN THE WASSERMANN REACTION

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THOUGH the Wassermann reaction has been criticized as nonspecific, it remains, up to the present, the only reaction of approved reliability in the diagnosis of syphilis, provided frambesia tropica and leprosy (*forma tuberosa*) can be excluded, in which diseases it also gives a positive reaction.

There are some other pathologic conditions in which with the Wassermann reaction positive results have been, from time to time, recorded, e.g., psoriasis, malaria, pellagra, tuberculosis, pneumonia, scarlet fever, and fever in general. With other observers, I am of the opinion, that these, however, are false interpretations, due in all likelihood to technical faults in the carrying out of the test. With scrupulous attention to technic there is no doubt that the Wassermann reaction in such cases invariably gives negative results.

There remain one or two other conditions in which anomalous results have been obtained with this reaction. Though I have failed to find any accounts of detailed work on the subject, in some textbooks of pathology and of biochemistry it is asserted that chloroform and ether anesthesia, likewise the presence of fat in the serum after ingestion of a fatty meal, may give rise to results which simulate a luetic infection. So long as such exceptional cases stand on record we are obviously unable to claim the Wassermann reaction as exclusively pathognomonic of luetic infection, and it is of importance that the observations should be controlled with a view either to confirmation or the reverse. The object of this present research has been to investigate these anomalous cases.

TECHNIC

The technic employed in the investigation was that in use in Wassermann's laboratory in 1910, with the addition of certain modifications which since then have received general approval. On the whole, the method of

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Harrison[1] has been adhered to, except that the hand method was preferred to the dropping method and in every case 0.5 c.c. was used, bringing the total volume to 2.5 c.c. So far as possible, all titrations and the preparation of material were carried out under my own supervision in the laboratory.

The following notes apply to the reagents used:

1. *Amboceptor Against Sheep's Erythrocytes.*—This was prepared in the physiology department. The titre was between 1:3000 and 1:5000. Amboceptors with a titre inferior to 1:1000 were discarded; 4 M.H.D. were used.

2. *Sheep's Blood.*—This was obtained from the local abattoir as nearly sterile as possible. The corpuscles were washed four times with a constant number of revolutions, and in each case for a similar period of time.

3. *Complement.*—This consisted of the fresh serum of two guinea pigs, sacrificed on the night previous to the experiment. 2 M.H.D. of complement was used after titration by itself and with antigen.

4. *Extracts.*—In every case three different extracts were used in one and the same experiment, viz.: (a) a commercial guinea pig heart extract with cholesterol (Burroughs, Wellcome & Co.); (b) a guinea pig heart extract prepared according to the method of Fildes and McIntosh,[2] to which at the time of use a 1:100 alcoholic solution of cholesterol was added; (c) acetone insoluble, alcohol soluble, calf's heart extract, prepared according to the method of Bordet and Ruelens,[3] to which at the time of use a 1 per cent alcoholic solution of cholesterol was added. The extracts were repeatedly titrated beforehand with three rows of test tubes, one containing luetic serum, the second normal serum, the third saline solution. For the titration several different luetic and normal sera were used.

5. *Sera.*—The sera to be tested were obtained from animals by the method of heart puncture, the blood being received through aseptic apparatus into sterile test tubes. The clear serum, expressed after coagulation of the blood was inactivated for thirty minutes at 55° C., and then kept in the ice chest in readiness for the test, being warmed to 55° C. just before the test. The saline solution, 0.9 per cent NaCl, was sterilized in the autoclave under 20 pounds pressure. The quantity of each substance used was brought up to 0.5 c.c. with NaCl solution.

In the main test, before incubating for half an hour, previous to the addition of the sensitized sheep's cells, I let the sera, with extract and complement, stand at room temperature (16 to 20° C.) for half an hour.

All glassware used was cleansed with a hot solution of sulphuric acid and bichromate, then with sodium bicarbonate solution, followed by ordinary tap water and distilled water. After drying it was sterilized in the usual way by enclosure for thirty minutes in an oven at 120° C.

I. DOES CHLOROFORM OR ETHER INFLUENCE THE WASSERMANN REACTION?

Chloroform, ether, and other narcotics are said to produce an increased amount of free lipins in the blood, and the assumption is that these lipins in a nonsyphilitic serum account for the positive Wassermann reaction. In this connection, two lines of investigation were employed: (1) the effect of

chloroform and ether added directly to the blood *in vitro;* (2) the effect of general anesthesia with chloroform and with ether.

The former question may be dealt with first. Specimens of defibrinated blood (of sheep, of cats and of dogs) were collected in large sterile test tubes and connected with a flask containing chloroform or ether, as the case might be, through which flask a current of air was made to pass by means of an electric pump. In this way the anesthetic vapor came in direct contact with the blood, which, at regular intervals, was thoroughly shaken. It was later found that if a test tube containing ether or chloroform was placed inside a stoppered Erlenmeyer flask, the bottom portion of which contained the defibrinated blood, hemolysis rapidly occurred, *pari passu* with absorption by the blood of the volatile anesthetic. By this latter device all splashing and frothing are avoided, interaction between the blood and the anesthetic vapor taking place silently and quietly. After a certain time, which varied from ten minutes to one hour, the blood was centrifuged. The clear serum, red owing to the presence of dissolved hemoglobin, was pipetted into a sterile test tube, and inactivated for thirty minutes at 55° C.; in this process the serum, if containing a large amount of hemoglobin, acquired a dark chocolate color. It was then tested by the Wassermann reaction.

The result of application of the anesthetic directly to the blood can be stated in a clear and categorical fashion. Thirty samples of blood (ten of dog, ten of cat, and ten of sheep) were treated with chloroform. In no case did the slightest sign of a positive reaction appear. An identical statement, word for word, applies to the investigation with ether, and to the results therewith obtained. Should any one desire to repeat this experiment, it is only fair to point out that occasionally, and particularly when the amount of hemolysis, through the long delayed action of the chloroform or of the ether, is large, the serum gives an *anticomplementary* reaction, which, however, can be readily distinguished from a positive Wassermann result.

The second series of experiments, which from the practical point of view is of most importance, was carried out on a large number of animals, mostly dogs and cats. The animals, having received no food for at least twelve hours previously, were in some cases injected subcutaneously with 0.5 c.c. of a 2 per cent solution of morphine per kilo, one hour before being anesthetized; in other cases they were put under anesthesia without any morphine. The animals were kept under complete anesthesia for two and a half hours, and the blood, 5 to 10 c.c. at a time, was drawn by heart puncture every fifteen minutes, with aseptic precautions, from the circulation. In cats and in guinea pigs the amount of blood thus obtained was naturally less than in dogs. Specimens of blood from the same animal were in this way secured at different intervals. The serum was prepared in the usual way, and then inactivated for thirty minutes at 55° C.

As in the previous set of experiments, the results in this series were uniformly negative. Once or twice the same serum tested with three different extracts gave a doubtful result (– +) with one of the antigens. As the doubtful reading occurred in each case with the same extract, and as this

extract by itself was found to give a trace of inhibition of hemolysis, it was considered legitimate and in accordance with recognized canons to interpret the result as negative.

II. DRUGS OTHER THAN GENERAL ANESTHETICS

Owing to the provision of a mammalian operative class for students in this department, it was possible to carry out experiments on an unusually large number of animals. Furthermore, it was possible to test the blood of anesthetized animals after injection of various substances such as morphine, atropine, caffeine, amyl nitrite, adrenaline, pituitary extract and secretine. In these cases the blood was obtained either directly from the heart or from the carotid.

As before, the results were wholly and entirely negative. It is of some slight interest to know that the injection of these drugs does not affect the result of the Wassermann reaction. In particular, morphine[4] is a narcotic which might conceivably be considered as exercising an effect on blood similar to that of ether, viz., of increasing the amount of circulating lipins.

III. FAT DIGESTION

It is well known that the blood normally contains only a small percentage of fat. After a fatty meal, however, it may contain so large an amount that the fat actually rises to the surface of the serum like cream.[5] "In the dog the percentage of fat in the blood is remarkably constant under normal conditions. After a fatty meal the increase of fat begins in about an hour and reaches its maximum in about six."[6] A series of sera collected from fasting dogs was compared with other series of sera of the same animals obtained at different periods after a fatty meal. The two sets of sera showed macroscopically a very striking difference. While the sera of the fasting animals were absolutely clear, those taken during digestion showed

TABLE I

RESULTS OF THE WASSERMANN REACTION ON SERA OBTAINED AFTER FATTY MEALS PREVIOUS TO SEPARATION OF FAT

		I	II	III	IV
TEST TUBE NO.	SERUM .1	GUINEA PIG HEART AND CHOLESTEROL	GUINEA PIG HEART AND CHOLESTEROL (B. & W.)	CALF HEART AND CHOLESTEROL	NaCl SOLUTION
1	Ser. 24	++	++	+	+
2	" 26	+	+	−+	+
3	" 28	−+	+	−+	+
4	" 30	+	+	+	+
5	" 32	++	++	++	++
6	" 34	+	+	+	+
7	" 37	+	++	+	+
8	" 40	+	+	+	+
9	" 42	−+	−+	−+	+
10	" 49	−+	+	+	+
11	" 51	−+	−+	+	+
12	U. S. S. 14	+++	+++	++	−
13	N. S. 19	−	−	−	−
14	NaCl—Ext.	−	−	−	−

a turbid and milky appearance; after standing, the fat rose in a layer to the surface. All the sera were inactivated for thirty minutes at 55° C. The Wassermann reaction was tested both immediately after inactivation, when the globules of fat were still equally distributed throughout the serum, and at a later stage when, after prolonged sojourn of the serum in the ice chest, the fat had risen to the surface leaving the serum clear.

The results in this case are best exhibited by means of Table I, which applies to sera containing naturally admixed fat.

The sera marked 1 to 11 were taken from dogs during fat digestion; No. 12 is an undoubted syphilitic serum from a hospital patient; No. 13 is again a serum from a nonsyphilitic patient taken during fasting; No. 14 is a simple mixture of NaCl solution and extract. As a control all the sera were tested against a 0.9 per cent solution of NaCl, as shown in column IV. It will be noted that all the fat-containing sera gave virtually the same results with the NaCl solution as with the various extracts; in other words, all these sera show an anticomplementary action. We are thus forced to the conclusion that the asserted positivity of fats contained in serum is no true Wassermann reaction.

That the anticomplementary action in question is due to the fat, can be proved by testing the serum after mechanical removal of the fat. Table II shows the results of such a control set of experiments in which the fat-denuded serum is seen to give uniformly negative results.

TABLE II

RESULTS OF WASSERMANN REACTION ON SAME SERA AS IN TABLE I, AFTER SEPARATION OF THE FAT

		I	II	III	IV
TEST TUBE NO.	SERUM .1	GUINEA PIG HEART AND CHOLESTEROL	GUINEA PIG HEART AND CHOLESTEROL (B. & W.)	CALF HEART AND CHOLESTEROL	NaCl SOLUTION
1	Ser. 24	–	–	–	–
2	" 26	–	–	–	–
3	" 28	–	–	–	–
4	" 30	–	–	–	–
5	" 32	–	–	–	–
6	" 34	–	–	–	–
7	" 37	–	–	–	–
8	" 40	–	–	–	–
9	" 42	–	–	–	–
10	" 49	–	–	–	–
11	" 51	–	–	–	–

IV. FEVER

Fever is another factor which in treatises on pathology and on immunology is stated to disturb the regular outcome of the Wassermann reaction, causing an inhibition of hemolysis and thus masking a syphilitic infection.

In its bearing on the Wassermann test a decision on this question is of the same practical importance as a decision on the influence of anesthesia or of the recent ingestion of a meal. Official instructions issued from provincial and from municipal laboratories for the guidance of practitioners tak-

ing samples of blood for the test, frequently warn against taking the blood "after alcohol or general anesthesia," "after a meal," or "if the patient has a high temperature."

Two methods were adopted to study this question. In one case whole blood from man and from animals was heated *in vitro* before the application of the test; in the other case bloods from patients with high fever were subjected to the Wassermann test.

a. *Heated Blood.*—Thirty c.c. of fresh defibrinated blood each of man, calf, sheep, dog and cat were in each case equally distributed in three test tubes, so that each test tube contained 10 c.c. of blood. The fifteen test tubes were put in a water-bath at 60° C. (about 140° F.). After thirty minutes one test tube of each specimen was taken out, after sixty minutes a second specimen of each was removed, while the remainder were removed after two hours. They were all centrifuged at room temperature and the clear serum collected in sterile test tubes. All the sera were then in routine fashion inactivated at 55° C. for thirty minutes, then placed in the ice chest for an hour and finally, on the same day, tested by the Wassermann reaction. The results were uniformly negative.

b. *Sera of Fever Patients.*—By arrangements with hospital authorities in Montreal, I was privileged to obtain blood from a number of patients with high temperatures. The cases selected for examination were of heterogeneous nature and included pneumonias, septicemias, surgical infections, etc., only such cases being selected as had no history of syphilitic infection. The temperature at the time of taking the blood specimen ranged between 101° and 105° F. These results again were wholly negative.

V. CONTROL BY MEANS OF THE Σ REACTION

Lately Dreyer and Ward[7] have proposed "A Simple Quantitative Serum Reaction for the Diagnosis of Syphilis," which, for shortness, they call the "Σ reaction." This reaction, similar in principle to that of the Sachs and Georgi[8] reaction, is based on flocculation produced by the serum of a luetic individual when such serum is put in contact with an acetone insoluble, alcohol soluble extract as prepared by Bordet and Ruelens.

I have for some time been engaged in testing this reaction, not quantitatively but qualitatively, on a large number of luetic and normal sera (see the next succeeding paper of the series).

I have carried out the Σ reaction on a considerable number of sera of animals subjected to chloroform and ether anesthesia; I have made parallel series of observations with the Wassermann and with the Σ reaction on sera of animals obtained during fat digestion, and have made corresponding parallel observations on sera of animals injected with laboratory drugs, and on sera of fever patients; indeed, almost all the sera used in this research were controlled by the Σ reaction. The two sets of investigations were carried out quite independently, comparison of results being made only after the full tests with any given serum were completed. The fact that the sera gave identical results with both reactions seems to me to provide independent

confirmative evidence of what I stated before, viz.: that if a serum does not contain syphilitic "reagins," then chloroform, ether, fat digestion, or fever are powerless to convert an otherwise negative into a positive reaction.

CONCLUSIONS

1. Chloroform and ether, whether added directly to a mammalian, including human blood, or taken up by the blood in the process of anesthesia, are absolutely without influence upon the Wassermann reaction.

2. Sera removed from animals during fat digestion, and therefore containing large quantities of fat, disturb the results of the Wassermann reaction in the direction of simulating a positive reaction. Such apparently positive results are not, however, really so, the influence of the fat being merely anticomplementary; for, the serum by itself, without the extract, gives the same seemingly positive results. If the fat is removed from the serum previously to the test, or if the clear serum is pipetted from the bottom of the test tube after the fat has floated to the surface, the serum gives a wholly negative reaction.

3. The following drugs have been tried as to their possible influence on the Wassermann reaction: Morphine, atropine, caffeine, amyl nitrite, adrenaline, pituitary extract and secretine. They gave negative results.

4. Blood heated *in vitro* to 60° C. and blood from fever patients gave a negative result.

5. All results of 1, 2, 3 and 4 above have been independently controlled by the Σ reaction of Dreyer and Ward and with identical findings.

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FLOCCULATION REACTIONS AS SUBSTITUTES FOR THE WASSERMANN TEST

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FLOCCULATION REACTIONS AS SUBSTITUTES FOR THE WASSERMANN TEST*

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SOON after the application by Wassermann[1] of the complement-fixation for syphilis, numerous observers with the aim of reaching the same results by simpler methods tried to apply in the case of syphilis, methods already approved in other fields of immunity, e.g., the precipitation and flocculation tests. Fornet[2] early proposed his "ring test," which consists in the formation of a whitish ring at the zone of contact of a luetic with a paretic serum *in vitro*. Porges and Meyer,[3] Elias, Neubauer, Porges and Salomon,[4] and others, using the same principle, tried to obtain similar results by mixing 0.2 c.c. of a 1 per cent solution in distilled water of sodium glycocholate with the same amount of an inactivated serum of a luetic patient. When the test tube containing the substances was left for twenty-four hours at room temperature, a manifest flocculation (precipitate) appeared in the fluid if the serum was luetic. Later, Klausner[5] claimed that the same results could be obtained by simply using 0.6 c.c. of distilled water and 0.2 c.c. of active luetic serum. Notwithstanding all the claims advanced on behalf of these various flocculation reactions, everyone had to agree that they were not to be compared with those obtained by the Wassermann reaction.

Soon after the communication of Porges and Meyer, Hermann and Perutz,[6] having observed that for complement-fixation Browning, Cruikshank and Mackenzie[7] had employed lecithin and cholesterin for their artificial antigens, tried to improve and so render more sensitive the solution of sodium glycocholate of Porges and Meyer by adding to it a certain amount of cholesterin. In fact they used two solutions. In Solution I they had two grams of sodium glycocholate, 0.4 grams of cholesterin and 100 c.c. of alcohol at 95 per cent. Solution II was a 2 per cent solution of sodium glycocholate, which had to be freshly prepared on each occasion at the moment of performing the test. The setting up of the test was very simple: to 0.4 c.c. of the active serum of the patient, 0.2 c.c. of Solution I, diluted 1:20, and 0.2 c.c. of Solution II were added. The test tube with its relative control was shaken, plugged with cotton wool and left at room temperature for 20 hours. After the lapse of that time positive syphilitic sera showed large floccules. According to the authors, out of 134 cases of syphilis 118 gave positive results; of 89 control cases only one gave a slight positive reaction.

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The great simplicity of technic, the economy of material in the test and the fact that it could be used by any general practitioner seemed to indicate that it would supplant the Wassermann reaction. But it was soon discovered that the newly proposed test was not as sensitive as the Wassermann reaction, and that a considerable number of sera reacting negatively with the Wassermann reaction gave positive results with the Hermann-Perutz.*

Other experimenters in the same field, e.g., Sachs and Georgi,[8] Sachs and Altmann,[9] Ternuchi and Toyoda,[10] Jacobsthal,[11] Bruck and Hidaka,[12] Hecht,[13] Meinicke,[14] etc., all tried to improve the Hermann-Perutz reaction, so attractive in its simplicity, and practically everyone proposed a method of his own.

Sachs and Georgi, following the idea of Michaelis[15] (who have found that organ extracts and serum of luetics in a given dilution gave a precipitate) prepared cholesterized organ extracts, such as are now used in the Wassermann reaction. They prepared their extracts from ox-heart muscle, freed from fat and minced finely. They added to it alcohol, in the proportion of 5 c.c. of alcohol to 1 gram of organ. The mixture is kept in the dark at room temperature in a glass-stoppered bottle. After five to six days the alcohol is filtered off in a well stoppered bottle and to the extract 1 per cent cholesterin alcohol solution is added until an optimal degree of solution is reached. As a rule they found that to 100 c.c. of raw alcoholic ox-heart extract 200 c.c. of alcohol and 13.5 c.c. of a 1 per cent alcoholic solution of cholesterin had to be added.

For carrying out the test 0.5 c.c. of this extract, diluted six times with 0.85 per cent NaCl solution, and 1 c.c. of patient's serum inactivated at 55° C. diluted 1:10 with 0.85 per cent NaCl solution, were put in a test tube, incubated at 37° C. for two hours and then taken out of the water-bath and left at room temperature for eighteen to twenty hours. After this time the results are read by means of the Kuhn-Woitke agglutinoscope.

It is understood that the extracts for this reaction as for all similar reactions based on the same principles, have to be titrated beforehand with a number of luetic and normal sera, and the proper dose has to be determined which will give with a certain positive serum a good flocculation and with a negative serum no sign of flocculation. When looked at in the agglutinoscope with a good magnifying lens, a strongly positive serum shows innumerable minute particles or flocculi in suspension in a clear fluid. If the observer has had no previous experience it is very difficult at first to differentiate a positive from a negative serum, and impossible altogether to judge of any borderline case. Furthermore, if the results are read in direct light even with the help of a good magnifying lens, no good readings will be obtained without long practice or the help of the agglutinoscope. The best way in such a case is to compare the serum in question with the control test tubes, which here as in other reactions are also indispensable.

*At the time, 1911, soon after the authors proposed this reaction, along with Dr. H. Hirschfeld of the Charitè Krankenhaus in Berlin, I tried the reaction on a number of sera, but the results were very unsatisfactory and as at the time I was engaged in other researches, I abstained from publishing the results.

Sachs and Georgi claimed that in 94.94 per cent of cases their reaction gave the same results as the Wassermann reaction. The Sachs-Georgi flocculation reaction has had a great vogue in Germany and even today in that country many workers claim to get with it results practically as reliable as those obtained by means of the Wassermann reaction. The object of the present paper is to compare the reliability of some of these "short-cut" methods with that of the Wassermann reaction.

THE SACHS-GEORGI REACTION AS COMPARED WITH THE WASSERMANN REACTION

Having prepared a series of three different extracts according to the method given by the authors, and having titrated each of them in different concentrations (keeping the quantity of extract constant, I added alcohol and 1 per cent alcoholic solution of cholesterin in different amounts), I obtained extracts of the following concentrations:

	I	*II*	*III*	*IV*	*V*	*VI*
Raw Alcoholic Extract, in c.c.	1	1	1	1	1	1
Alcohol, in c.c.:	–	1	2	–	1	2
1% Solution of Cholesterin, in c.c.:	0.1	0.1	0.1	0.15	0.15	0.15

In a series of small test tubes each extract was diluted six times, and of this suspension 0.5 c.c. was used. The sera, the one positive and the other negative, were diluted 1:10. In each small test tube there was now 0.5 c.c. of each extract diluted 1:6, and 1 c.c. of the serum diluted 1:10. Each extract had a control tube containing 0.5 c.c. of extract at the same dilution as used for the main test and 1 c.c. of 0.9 per cent NaCl solution. Another control tube contained 1 c.c. of serum diluted 1:10 and 5 c.c. of 0.9 per cent NaCl solution without extract.

Having carried out the same operation for each extract, I put the test tubes in the water-bath at 37° C. for two hours, then took them out and left them at room temperature for two hours. Thereupon the results were read with the agglutinoscope. That dose of the extract was chosen which gave an optimum flocculation with a positive serum, while the test tubes containing the negative serum and the controls, remained absolutely clear, the uniform milky appearance of the emulsion being readily distinguishable from flocculation.

The dose of the extract having been thus determined, I passed to the setting up of the main test, the extreme simplicity of which is no doubt the main advantage of this reaction. For the main test I used two rows of test tubes, the front row contained 1 c.c. of the serum to be examined, diluted 1:10, and 0.5 c.c. of the extract, diluted 1:6. The test tubes in the second row contained 1 c.c. of the same serum as the front row, diluted 1:10, to which, moreover, to make up the same volume as was contained in each of the front row test tubes, 0.5 c.c. of 9 per cent NaCl solution, instead of 0.5 c.c. of extract, was added. The rack containing all the test tubes was now put in the water-bath at 37° C. and left there for two hours, after which time it was taken out and left at room temperature for twenty hours. The reading has to be done either with an agglutinoscope or with an improvised appa-

ratus consisting of a good sized square wooden box, blackened all over and containing an efficient electric lamp. In the front portion of the box a narrow slit, less than half a centimeter wide is left, so that the rays of light are made to pass through it and strike the fluid contained in the test tube on a less than 0.5 cm. space. In this way any slight degree of flocculation in the fluid will be observed. The reading takes place in a dark room or may be carried out in ordinary light under a piece of black cloth. As mentioned before, the use of a hand lens is indispensable.

The two test tubes, one of the front row and one of the second row, are held slightly slanting against the slit in the box. If the serum comes from a luetic patient, the test tube of the first row will show a mass of very fine whitish granules or flocculi, like ground glass, suspended in a clear fluid, while the test tube of the second row containing the serum without extract will show a clear limpid fluid. The reading, as stated, is very hard at times, especially with sera on the borderline, which as usual are exactly those we want to know how to classify.

I have tested more than a hundred sera with this reaction and must venture to say that the reaction is not as sensitive as the Wassermann reaction. The Sachs-Georgi has given me 49 per cent negative results, 43 per cent positive and 8 per cent doubtful. The same sera tested by the Wassermann reaction gave me 28 per cent negative, 68 per cent positive, and 4 per cent doubtful; so that 25 cases with a relatively strong positive Wassermann reaction and with a definite history of syphilis were left undetected by the Sachs-Georgi reaction. The want of correspondence revealed in these results constitutes a serious verdict against the Sachs-Georgi reaction. One fact of interest noted was that the sera giving anticomplementary results with the Wassermann gave also exactly the same result with the Sachs-Georgi, illustrating the fact already noted by Gengou[16] that precipitates will remove complement from a mixture.

THE Σ REACTION OF DREYER AND WARD AS COMPARED WITH THE WASSERMANN REACTION

Quite recently Dreyer and Ward[17] proposed an improvement upon the Sachs-Georgi flocculation reaction, which they recommend not only as a qualitative but also as a quantitative test. They claim that the Σ reaction has given, in their hands, as good results as the Wassermann reaction, that it may be easily standardized and the results expressed in standard units, and that consequently the different methods of treatment may readily be compared one with another.

They work with extracts prepared according to the method of Bordet and Ruelens,[18] which extracts are said to be more stable and to give more reliable results.

The extract of Bordet and Ruelens is prepared so that to 100 grams of calf's heart-muscle, freed from fat and minced very fine, 125 c.c. of 95 per cent alcohol is added and the mixture left at room temperature for seven

days. The mixture, kept in a well stoppered glass bottle, is occasionally shaken. After a week the alcohol is poured off and the organ residue is dried in the incubator at 37° C. for twenty-four hours. When it is well dried 200 c.c. of pure acetone is added and the mixture kept in a well stoppered glass bottle in the incubator at 20° C. for seven days. The acetone is then filtered off and the residue is again treated with another 100 c.c. of acetone and kept at 20° C. for one day. The acetone is again filtered off and the residue is dried in the incubator at 20° C. for two hours, whereupon 200 c.c. of 95 per cent alcohol is added to the residue. This mixture is put in a glass-stoppered bottle in the incubator at 20° C. for ten days and then filtered through filter paper. The filtrate, which contains the acetone-insoluble, alcohol-soluble substances of the heart-muscle, is kept in the dark at room temperature in a well stoppered glass bottle and is ready for use.

Besides the extract, a 1 per cent alcoholic solution of cholesterin is prepared and kept under the same conditions.

From the extract they prepare two heart-cholesterin saline suspensions, which they call α and β suspensions. For the preparation of α suspension they pipette into a clean, dry test tube 5 c.c. of the extract and 0.25 c.c. of the cholesterin solution and mix them. From this, with a clean pipette, 1 c.c. of the heart-cholesterin extract is carefully pipetted into a 100 c.c. measuring cylinder and the fluid is left running out of the pipette when this has touched the bottom of the cylinder, without letting the extract come in contact with the wall of the cylinder. Then 10.7 c.c. of a 0.9 per cent NaCl solution is dropped in (dropping distance 36 cm.) and the NaCl solution dropped at a constant rate by a special mercury pressure dropping apparatus described by the authors and which is very easily constructed. Afterwards, without shaking, the resulting suspension is gently mixed by inverting the cylinder several times. (This slow method of preparing the suspensions is the same as is used in the Sachs-Georgi and also in the Wassermann reaction). This represents α suspension. Suspension β is prepared in a like manner, only instead of 10.7 c.c., 34 c.c. of NaCl solution is added.

Two dilutions of the patient's serum are now prepared, one 1:10, the other 1:20.

I have tested the Dreyer and Ward reaction only qualitatively.

In a specially constructed brass rack nine agglutination test tubes (6 in. long by 0.6 in. internal diameter) with conical end were placed. In the first five I put a different number of drops of the serum diluted 1:10; in test tube I, 20 drops; in II, 10 drops; in III, 5 drops; in IV, 2 drops and in V, 1 drop. In the remaining four test tubes I put as many drops of the same serum but diluted 1:20, viz.: in test tube VI, 10 drops; in VII, 5 drops; in VIII, 2 drops and in IX, 1 drop. Into test tube I, I put 6 drops of α suspension, into the remaining eight test tubes 15 drops of β suspension. NaCl solution is now added, drop by drop, to each test tube until each, with the exception of the first which already contains 26 drops, contains 25 drops altogether.

The dilutions in this way vary from 1/1.25 to 1/462. A control tube containing 20 drops of NaCl solution and 6 drops of α suspension, and another control tube containing 10 drops of NaCl solution and 15 drops of β suspension, are placed in the same rack. After each test tube has been well shaken, the rack containing the test tubes is put in a water-bath set at 37° C. and left there for seven hours. After this long incubation the rack is taken out and left at room temperature for 10 minutes, whereupon the results are read in the same way as mentioned before in the case of the Sachs-Georgi. All the various precautions advised by the authors (for details see their paper) were carefully observed. Having examined each serum in the dilution of 1:10 and 1:20, and having found that positive results are very rarely obtained with dilutions of 1:20 (even then they are inessential for merely qualitative tests), I carried on the research solely with the first 5 test tube dilutions. The Σ reaction was tried on more than 150 sera and the results were in each case compared with those given by the Wassermann reaction. The percentage obtained is the following: 34 per cent negative, 58 per cent positive, 8 per cent doubtful. As already stated in an earlier part of this paper with the Wassermann reaction carried out on the same sera I obtained 28 per cent negative, 68 per cent positive, 4 per cent doubtful. Ten sera which gave a positive Wassermann reaction *responded negatively with the Σ reaction*. When now the three reactions, the Wassermann, the Sachs-Georgi and the Dreyer-Ward are compared, it is found that the first is the most sensitive of the three and the second quite unreliable, while in view of its simplicity and the ease with which it may be standardized the Dreyer-Ward reaction, granted an increase in its sensitivity and improvement in the matter of reading the results, might in future very well stand alongside the Wassermann reaction.

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