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OF THE

SPECIAL COMMITTEE UPON THE STANDARDISATION OF PATHOLOGICAL METHODS.

No. 1.—THE WASSERMANN TEST.
(INTERIM REPORT.)

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Approved for publication by the Medical Research Committee,
1*st March*, 1918.

Medical Research Committee.

(National Health Insurance.)

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Sir Walter M. Fletcher, K.B.E., M.D., F.R.S. (*Secretary*).

In April, 1917, the Medical Research Committee invited the following special Committee to consider how far it may be practicable and desirable to obtain the standardisation of routine pathological methods, and to report to them. The special Committee were empowered to co-opt additional members for particular divisions of their subject.

Lieut.-Colonel J. G. Adami, M.D., F.R.S., C.A.M.C.

Major F. W. Andrewes, M.D., F.R.S., R.A.M.C., T.

Professor William Bulloch, M.D., F.R.S.

The subject of the Wassermann test was taken in the first place by the special Committee for their consideration. They invited the co-operation of Lieut.-Colonel L. W. Harrison, D.S.O., R.A.M.C., who by permission joined the Committee for this purpose.

REPORTS

OF THE

SPECIAL COMMITTEE UPON THE STANDARDISATION OF PATHOLOGICAL METHODS.

No. 1.—THE WASSERMANN TEST.

(Interim Report.)

13th November, 1917.

The Committee beg leave to present to the Medical Research Committee the following interim report:—

I. The Possibilities of Standardisation.

For the consideration of the technical methods employed in the Wassermann test and the possibilities of their standardisation, the Committee invited Lieut.-Colonel L. W. Harrison, D.S.O., R.A.M.C., to give them the benefit of his unrivalled experience and to serve as a member of the Committee. They are greatly indebted to him for the assistance he has given at all their meetings for the discussion of this subject and in the preparation of this Report.

The Committee have also to thank Colonel Sims Woodhead for attending at two of their meetings and for his aid in the consideration of the scientific and practical questions at issue. They further desire to express their thanks for the help given to them by Dr. Carl Browning, Staff Surgeon P. G. Fildes, R.N., and Major Bowman, Canadian A.M.C., each of whom has had very large recent experience in the use of his own particular variant upon the complete method of performing the Wassermann test. These pathologists were good enough to attend one or more meetings of the Committee in order to describe their own methods and to discuss them in relation to the subject in general. The Committee were further greatly indebted to Colonel Lyster, of the United States Army Medical Service, for his kindness in conveying to them information upon the general lines of action taken in the United States Army in regard to the performance of the Wassermann reaction.

In the opinion of the Committee there is no process of biochemical diagnosis that gives more trustworthy information or is liable to a smaller margin of error than the Wassermann test when it is performed with completeness and with proper skill and care.

At present the test is performed by different workers in different laboratories according to a great variety of methods, of which some depart from the test as originally described only in minor, though possibly important points of technique while others introduce fundamental modifications of undetermined or uncertain significance. Although it cannot be regarded as

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necessary for accurate diagnosis that all workers should perform the test with complete uniformity of technique, obvious advantages are to be gained by the standardisation of approved methods. In the present absence of standardisation, the value of the diagnoses provided by any given laboratory cannot be estimated either absolutely or in comparison with others obtained elsewhere. Diagnoses are commonly given at present without a declaration of the variety of the method used, and even if this declaration be made, it does not carry with it, in the absence of standardisation, any necessary implication that a particular course of technical manipulation has been followed. The resulting uncertainties must render, and, indeed, have rendered, valueless for scientific statistical purposes by far the greater part of the large volume of patient and laborious work by a multitude of workers from which information of great value might otherwise have been extracted.

The report of a pathologist that a given blood-serum has shown a positive or a negative Wassermann test has a greatly diminished value for statistical purposes unless the report is coupled with a statement of the particular method employed. It is well known that no statement of this kind is commonly provided. In earlier days, when relatively few pathologists performed the test, particular workers were known as practising particular varieties either of a general method or of detailed manipulation. But as workers have multiplied, it has become increasingly difficult to identify the method used for a given set of tests, and this difficulty has been gravely accentuated during the war by the accompanying multiplication of laboratories and the employment of many relatively inexperienced pathologists.

The Committee have considered two alternative courses. One, and the simpler, is to advocate a demand that every report upon Wassermann tests made officially for military or public health purposes shall bear upon it a statement of the special method employed. The second would be to lay down in precise terms the procedure of a standard method, setting limits upon minor variations of technique, and to recommend that this standardised method shall be made official and that all official tests shall be made by this method alone.

The disadvantages of the former course are that, in order to gain material for sound conclusions upon important questions such, for instance, as the frequency of syphilis in any given section of the population, Wassermann test reports, if given by various methods, would require the most careful analysis and sifting, and the margins of error in the different procedures employed would have to be estimated with great care before the figures based upon one series of tests could be fairly collated with those obtained by another. Direct scientific advantage would plainly be gained from this point of view if one standardised method received sole recognition and was alone employed for official returns. This adoption of an official method would not, of course, involve the exclusion of other methods so far as these might be employed for the more precise study of particular

cases for purposes of comparison and control; it would, however, be secured that official returns would be based upon a uniform known procedure common both in type and detail to all official workers in different places.

The Committee recognise, however, that in the present state of knowledge it is impossible to lay down with confidence the lines of optimum procedure for the performance of the test. We are still in ignorance of the exact physical basis of the reactions which are involved, and it is only by further laborious investigation of an exact quantitative kind into the various parts of the test that an approach can be made to full knowledge of the best conditions to lay down for a standardised test.

Quantitative investigations of this kind have already been undertaken in the Local Government Board laboratory under the general direction of Lieut.-Colonel Harrison, but it can hardly be hoped that these will bear fruit before the end of several months. The Medical Officer of the Local Government Board has kindly undertaken to communicate to the Committee the results of this work as it proceeds, and if necessary it will be supplemented by additional work undertaken elsewhere on behalf of the Medical Research Committee.

Therefore, while convinced that the Wassermann test when properly performed affords most reliable information, the Committee are of opinion that, for the protection of the individual, in all cases where the only evidence of syphilis is a positive Wassermann reaction, the serum should be retested before a diagnosis of syphilis is based on this sign alone.

In the meantime the Committee are strongly of opinion that no effort towards better standardisation of the test should be postponed. They desire to give cordial endorsement to the conclusion of the report of a Sub-Committee of the Pathological Section of the Royal Society of Medicine on the 21st December, 1914, which is quoted in the final report of the Commission upon Venereal Diseases. This Pathological Sub-Committee recognised that—

> "In the present state of our knowledge the Wassermann reaction is an empirical one. There is every reason for hoping that it may eventually be brought within the domain of exact physical chemistry and reduced to a simple chemical test. But it is the opinion of the Sub-Committee that, until this is so, the original Wassermann test, with its full controls, is the best adapted for general use, though it may be supplemented, for the control of treatment, by more sensitive short-cuts, that is, by those admittedly likely to yield a larger percentage of positive reactions."

The Committee would go further and recommend that in tests made for official returns only those methods shall be accepted which conform with the original Wassermann test as defined below and that the so-called "short-cut" methods are only to be accepted by way of voluntary supplement to the official tests.

The Committee are strongly of the opinion that it is greatly to the public interest that this recommendation made in 1914 should become effective as soon as possible, and they venture to urge that the Medical Research Committee should use their influence

with the authorities of the Admiralty, of the War Office and of the Local Government Board towards securing official action which shall make this recommendation effective, *as the minimum requirement*. It should be the universal official rule that for all Wassermann tests the findings given by the use of the original Wassermann test must be declared, with or without a supplementary report upon the results of other tests that may have been made in addition.

II. Definition of the Original Wassermann Test.

For the purposes of defining what is meant by the "original Wassermann test" the Committee desire to adopt the terms given by the Sub-Committee of the Royal Society of Medicine (Pathological Section) in the Report already mentioned. The Sub-Committee gave the following definition:—

> Since the method was first published by Wassermann, Neisser and Bruck, certain modifications of detail have been introduced and almost universally accepted. These especially concern the antigen employed, the original watery extract of syphilitic liver having been replaced by an alcoholic extract of syphilitic liver or of normal organs, with or without the addition of cholesterol or of purified phosphatids. With such details the Sub-Committee is not here concerned, though they hope to consider them fully in their final report. They would define the original test by its essential principles, as follows:—
>
> (1) The ingredients of the test (red corpuscles, "antigen," hæmolytic amboceptor, complement) are derived from *different* sources.
>
> (2) The serum to be tested is inactivated before use. An independent "hæmolytic system" is employed, consisting of a suspension of red corpuscles, an inactivated hæmolytic serum, and a fresh normal serum containing complement. The hæmolytic values of the anti-serum and complement are determined by a separate preliminary experiment.
>
> On general scientific grounds the Sub-Committee is unanimously of the opinion that, since the test is a quantitative reaction, the titre of the reagents ought, within practicable limits, to be accurately known.

With the opinions here expressed the present Committee are in full accord.

III. Details of Manipulation.

The Committee are not at present prepared to lay down an official method complete in all its technical details, because admittedly the various factors in the reaction, and the proportions in which the reagents afford the best results, have so far been selected only by individual experience and predilection. As already mentioned, the recommendation of a standardised *optimum* method must be deferred until a long series of standardising control experiments, as recommended three years ago by the Sub-Committee of the Pathological section of the Royal Society of Medicine, have been performed.

Nevertheless, the Committee desire to recommend strongly that even at this stage of knowledge there are some details in the

proper routine conduct of the tests which should receive official sanction and support. These will be embodied in their detailed recommendations given below.

IV. Standard Antigens and Standard Amboceptor.

It is evident that where there are various factors in a reaction, the smaller the number of these that are liable to variation the greater must become the likelihood of uniformity in results. Or otherwise, if admittedly the time is not ripe to recommend a standardised optimum method, everything is in favour of reducing the number of variables and of employing without further delay as many standard reagents as possible.

There are two essential reagents in the test which may be standardised, namely, the "antigen" and the haemolytic "amboceptor." The Committee therefore recommend that such steps be taken as are necessary to secure the provision of official standardised "antigens" and "amboceptor" and that these be issued with full particulars as to their respective titres and full instructions regarding the methods of preservation and use.

V. Laboratory Organisation.

The Committee after careful consideration have formed the opinion that the laboratories in which Wassermann tests are performed for the public services should be few rather than many. The concentration of this work in central laboratories at which large numbers of tests are performed as a matter of routine will secure gains both in economy and in efficiency. The performance of a large number of tests at the same time will obviously allow economy in staff and in material, because with proper organisation and drill an increase in the number of tests performed does not in any direct relation increase the time and trouble involved. It is suggested that on any one day the number of tests should be about 50.

The gain in efficiency by the concentration of tests at a few centres is two-fold. In the first place, the larger the number of tests performed under identical conditions, the larger is the number of strictly comparable results. In the second place, the pathologist in regular charge of the performance of tests as a matter of frequent routine and upon a large scale will be far more likely to produce trustworthy results, and to have a correct judgment in border-line cases, than one to whom a series of tests is only an occasional experience.

The Committee suggest for the guidance of administrative action that the central laboratories performing tests for any of the public services should be so chosen with reference to the expected needs of the service that not less than an average of 100 tests should be made weekly.

For the sake of rapidity in diagnosis it is most desirable that the test should be carried out not less than twice weekly.

VI. The Employment of Laboratory Assistants.

In some institutes laboratory assistants have been trained to perform Wassermann tests accurately as a matter of routine. Direct evidence, however, which has been before the Committee convinces them that the preparation for the tests, their performance, and the reports based upon them, should not be left to laboratory assistants, however skilled they may be in manipulation. While a carefully trained assistant may with proper supervision carry out most of the routine work involved in the test, the Committee are very strongly of opinion that the responsible pathologist, on every occasion when testing is to be done, should personally make the preliminary control observations. He should also inspect the reactions actually obtained, and be directly and personally responsible for the reports made. He should, of course, be responsible at the same time for the general supervision of the accuracy of any routine work done by his assistants.

The responsibility attaching to any imperfection or slovenliness of technique is so great, and the results of a false diagnosis may bear so heavily upon a patient and his family, that the Committee believe that the whole weight of responsibility for the reports made should be borne by a fully-qualified pathologist, and that in no circumstances should the reputation of the pathologist in charge be used as a "cover" for the work of an assistant, however long his experience or however great his manipulative skill.

The Committee would urge further that reports upon Wassermann tests should be accepted only from pathologists who can afford evidence of adequate training and experience in the performance of the tests and of the control observations which are necessary to it.

VII. The Methods of Collection and Recording.

In order to avoid confusion and incorrect reports, and for the preservation of exact statistics, the Committee think it highly desirable that standard methods should be employed for the description and transmission of each sample of blood to be tested, and for the entry of *data* related to it. They would make accordingly the following recommendations:—

(1) *Method of collection.*

Whenever possible the individual whose blood is to be tested should present himself at the laboratory or venereal clinic and the sample of blood be there obtained. When this is not possible and the specimen is obtained by a medical man outside and forwarded to the laboratory, it must be remembered that the minimum amount of blood required for conducting a Wassermann test is 1 c.cm. (15 ms.) and that it is preferable to have 2 c.cm. or more. Blood to this amount is most easily obtained from a vein, either by means of a syringe, or by a hollow (about 56 gauge) needle and a rubber tube; or it may be obtained by pricking the finger, thumb, great toe or lobe of the ear.

The best method of transmission of serum is in a small stout test tube, furnished with a rubber cork. If whole blood instead of serum be sent the tube should be filled as completely as possible to diminish the effects of shaking.

(2) *Labelling.*

It should not be permissible to write the name or number on a glass capsule or tube by means of a glass pencil, because, in handling, the writing may easily be rubbed off and become illegible. Each specimen should bear a firmly attached label, with the name or number and the date written upon it in *ink*.

(3) *Necessary data to accompany each sample.*

It must be borne in mind that the pathological observer has duties beyond the mere reporting to the practitioner or Medical Officer whether the sample gives a positive or negative reaction. It is only by his work in the laboratory that adequate *data* can be collected with regard to the various groups of cases and their reactions, and statistical information of scientific value compiled.

It is recommended that the minimum *data* to accompany each sample required by the Local Government Board, as given here, be demanded also *mutatis mutandis* in the case of the Navy and of the Army.

Form used by Local Government Board.

Particulars to be Supplied with each Specimen sent to the Laboratory.

Identification letter or number of patient_____

Age_____ Sex_____

County or County Borough in which the patient resides_____

Name of clinic, if any_____

Character and source of specimen sent_____

_____

Date and hour of its collection_____

Situation and character of lesion_____

Length of time the disease has existed_____

Has patient received any form of treatment?_____If so, state particulars as to:—

(*a*) Internal administration.
(*b*) Inunction.
(*c*) Application to local lesion.
(*d*) Duration of above treatment.
(*e*) Whether now discontinued.
(*f*) If so, since what date.

Has a previous specimen from this patient been sent to this or any other laboratory?_____

If so, give particulars and date_____

Special Remarks, including a statement of the symptoms pointing to a diagnosis of venereal disease.

Doctor's Name _____

Address _____

NOTE 1.—The identification letter or number should be kept by the doctor for verification when the pathologist's report is received, and for use in correspondence, or for any subsequent examination.

NOTE 2.—It is important to fill in accurately the information required under the various headings, and to supplement this under "Special Remarks."

(4) *Laboratory Records.*

It is important that a full record in the form of a book or of a card index be kept at the laboratory in which on receipt of a sample all the *data* indicated upon its covering label shall be entered, together with the results of the examination as obtained later.

(5) *Form of Report.*

The Committee think it advisable that a common form of report should be used for transmission to the Medical Officer or Practitioner who has forwarded the sample. It should contain at least the following information:—

Identification letter or number of patient_____

Date of receipt of specimen_____

Condition of specimen_____

Nature of test performed_____

Result _____

_____

_____

Date of Report _____

(6) *Form for quarterly return.*

Each laboratory conducting official Wassermann and other tests for venereal disease should forward to the proper authority a return of the results obtained at least once a quarter. In the opinion of the Committee this would allow a steady supervision of laboratory results, and of the accuracy of laboratory routine, at periods not too far apart to permit serious defects to remain long unnoticed.

It is recommended that such returns should be made in the following form :—

WASSERMANN TESTS OF BLOOD SERUM.

Period : from to . Method employed : .

Stage of Syphilis indicated by clinical Report. (A short note should be added in cases of doubt as to the stage.)	Number of Tests.	Number Positive.	Number Negative.	Number Doubtful.
Primary				
Secondary :—				
(*a*) untreated				
(*b*) treated				
Tertiary				
Latent :—				
(*a*) untreated				
(*b*) treated				
Congenital :—				
(*a*) under age of Puberty, with symptoms.				
(*b*) under age of Puberty, without symptoms.				
(*c*) over age of Puberty, with symptoms.				
(*d*) over age of Puberty, without symptoms.				
Non-syphilitic Diseases, as detailed below :—				
(*a*)				
(*b*)				
(*c*)				
(*d*)				
(*e*)				
WASSERMANN TESTS OF CEREBRO-SPINAL FLUID.*				
I. Without clinical symptoms of disease of central nervous system, but with constitutional symptoms, indicating :—				
(*a*) Primary syphilis				
(*b*) Secondary syphilis untreated ...				
(*c*) Do. treated ...				
(*d*) Latent syphilis				
(*e*) Tertiary				
(*f*) Congenital				
II. With symptoms of disease of central nervous system. Variety of disease indicated by clinical report :—				
(*a*) Meningeal and arterial affections apart from Tabes and General Paresis				
(*b*) Tabes dorsalis				
(*c*) General Paresis				
III. Non-syphilitic affections, as detailed below :—				
(*a*)				
(*b*)				
(*c*)				

* The report should be supplemented by examinations of the cerebro-spinal fluid for other abnormal features, such as Lymphocytosis, excess of globulin, &c.

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(4) Officially standardised "antigens" and "amboceptor" should be prepared and issued with full particulars as to their respective titres, and full instructions regarding methods for their preservation and use.

(5) That where the clinical history and clinical manifestations are doubtful, an individual giving upon one examination a positive Wassermann reaction should invariably be retested before a diagnosis of syphilis is made.

(6) In rendering quarterly and other statistical returns it should be clearly stated by the pathologist which method or methods have been employed.

The Committee make detailed recommendations as to the methods of collection and transmission of blood samples, on labelling and of supplying the data to accompany each sample. They further point out the importance of keeping full laboratory records and of the use of common forms for individual reports and for the statistical returns which should be rendered at least once a quarter. Suggestions are made as to the best form to be employed for each purpose. These recommendations will be found in Section VII. of the text.

J. G. Adami. William Bulloch.
F. W. Andrewes. L. W. Harrison.

APPENDIX.

Selected Methods of Performing the Full Original Wassermann Test.

The Committee are indebted to the authors of the following descriptions, for permission to print them here.

I.—Method used at the Rochester Row Military Hospital.

Amended extract from an article by Lt.-Col. L. W. Harrison, D.S.O., R.A.M.C., in "A System of Syphilis," by D'Arcy Power and J. Keogh Murphy, 2nd edition, Vol. III. With the permission of the Managers of "The Oxford Medical Publications."

Preparation of the Reagents.—The requirements for the Wassermann test are as follows: (1) serum to be tested, (2) a serum containing complement, (3) an extract of some suitable solid organ, and (4) a suspension of sensitised cells, the hæmolytic system which is required to test for free complement after (1), (2), and (3) have been incubated together. In addition to these, test-tubes, an incubator regulated to 37°C., an inactivating water-bath, pipettes, watch-glasses and a supply of fresh, sterile 0·85 per cent. salt solution are also required. If the reagents are to be measured out by Donald's dropping method, suitable droppers, made as described at the end of this article must also be provided.

Patient's Serum.—After the clear serum has been separated from the blood its complement must be removed by heating it at 55°C. It is usually recommended to heat for half an hour, but I think that this is longer than is necessary. The serum loses some of its reacting power at a temperature of 55°C., and this loss increases with the time during which the serum is heated. As I have found that ten minutes in a water bath at 55°C. is sufficiently long to inactivate 0·5 c.c. (the quantity I prepare for the test) of serum, I do not heat beyond this time.

Another reason advanced for heating for half an hour is that unheated serum is itself anti-complementary. Heated serum is also anti-complementary to a certain degree, and it is necessary to make provision for this property in calculating the proportion of the respective reagents. As far as my experience goes, sera vary no more in anti-complementary power after ten than after thirty minutes' heating, so that heating for ten minutes does as much as heating for a longer time, viz., inactivates the natural complement, and lowers the anti-complementary power sufficiently for working purposes; it has the advantage that it does not remove so much of the specific reacting substance. Browning and Mackenzie have pointed out that sera which have stood for some days develop increased anti-complementary power, although they may have been heated previously. On this account the tested sera should be heated on the day of the test, whether they have been previously heated or not.

Complement is contained in practically every fresh serum, but some of these contain other substances which are disturbing to the test, and others do not contain much complement, so that for the original test it is the common practice to use guinea-pig serum. The blood can be obtained from the ear if required for only a few tests, or it may be removed by aspiration from the heart, but the simplest plan is to cut the animal's throat over a wide glass funnel leading into a suitable receptacle. The blood may be whipped at once and centrifugalized, or allowed to clot and the serum to separate.

Browning and Mackenzie do not recommend guinea-pig serum to be used till it is eighteen hours old, as they have found younger serum unduly sensitive in deviation. I prefer to use it when four to eight hours old from the clot, or any time the same day after whipping and centrifugalizing the blood. In its original state, complement deteriorates and becomes useless in a few days at room temperature or in the ice-chest, and this is a great disadvantage of the original test. To overcome it, various devices have been suggested from time to time.

Complement keeps well when frozen hard, and for the past six years or so I have found the following a convenient method of keeping it frozen; the serum is pipetted off into small capsules, such as are used for vaccine, and these, in turn, are placed in a test-tube which is capped and immersed in a freezing mixture within a vacuum flask, such as a thermos, which is kept in the ice-chest. In a good flask it will keep frozen hard for two or three days, but should be examined daily, and the freezing mixture renewed as required. The serum should be kept frozen quite hard, or it will deteriorate.

Extract ("Antigen").—There can be no doubt that the quality of the extract determines that of the results obtained, and until a uniform source and method of preparing this ingredient has become established, the results obtained by different workers must differ in a certain proportion of cases. It is not so much in frankly negative and strongly positive sera that this happens, as in an important class of what may be described as border-zone sera. These are provided by cases of very early syphilis, latent, and well-treated cases, in which the reaction to the most delicate methods is not strong. A number of instances have been recorded where specimens of blood sera have been divided and sent to different laboratories for test. In one laboratory positive results have been obtained with sera which gave negative reactions in another, and this has led in some quarters to distrust of the test. More important than this, it is almost impossible under present conditions to compare the results of treatment obtained by different workers. One has only to test a batch of sera with

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from the firms responsible for their manufacture. They agree with McIntosh and Fildes regarding the uniformity of the heart cholesterin extracts, since three such behaved identically in their hands. Curiously, however, the extract prepared by Walker and Swift seems to have been less anti-complementary than that of McIntosh and Fildes, since in the first case not less than 1·2 c.c. of a 1 in 6 dilution was required to deviate 2 minimum hæmolytic doses of complement when acting alone, and the second required only 0·8 c.c. of a 1 in 8 dilution to deviate 2½ m.h.d. of complement, the quantity of test cells being the same. Possibly the difference may be accounted for by the different methods of preparing the crude heart extract employed by each. Experience of extract prepared according to the method of McIntosh and Fildes has shown me that its anti-complementary power is as stated by them. I have also found it better than an extract prepared according to the directions of Sachs, and now use it as routine.

The Hæmolytic System.—This is a suspension of red blood-cells sensitized by the addition of the heated serum of an animal which has been immunized against those cells. For the original test it is not very material what variety of blood-cells is used. Most workers use sheep's, which are probably most convenient to obtain. Browning and Mackenzie use ox cells, and Noguchi claims advantages from the use of human cells. Whatever variety of cells is chosen it is necessary to immunize some animal with them, and the most convenient is the rabbit. For the sake of convenience we will describe the technique when sheep's cells are used.

The blood can be drawn from the jugular vein, but is usually obtained at the slaughter-house. It may either be received into a 1 per cent. solution of citrate of soda in physiological salt solution or into a bottle containing a number of glass beads. The bottle, &c., should be sterile, and it is best to collect the blood after the sheep has bled for some time, as the first blood to flow must wash down many micro-organisms from the wool. If beads are used, as soon as the bottle is half-full it is stoppered and vigorously shaken so as to whip the blood in it. It is best to keep the blood in the bottle until required for the test or for immunizing purposes. As a rule, it keeps good in the cold for three or four days. For use it is necessary to remove the serum from the blood. The blood is placed in a centrifuge tube, with an equal quantity of 0·85 per cent. salt solution, and spun till the cells are all deposited. The clear, supernatant fluid is removed and replaced with salt solution, and this operation is repeated not less than five times. After the last washing the supernatant salt solution is removed and the deposit suspended in salt solution so as to make of it a 6 or 10 per cent. suspension. The strength of the suspension which is used in the test is 3 or 5 per cent., according to the practice of the individual worker, and this is obtained by mixing the 6, or 10, per cent. solution with an equal quantity of a suitable dilution of hæmolytic amboceptor. I, personally, prefer a 3 per cent. suspension.

For titration of hæmolytic amboceptor (see later) the strength of the suspension is made 3 (or 5) per cent. at once.

To obtain the hæmolytic amboceptor a rabbit is injected repeatedly with washed sheep cells. It is best to immunize a number of rabbits at the same time, since some may die, and in any case rabbits vary very considerably in their response to the injections—some rapidly producing a potent serum, and others, the reverse. The injections may be made intravenously (into the marginal vein of the ear), intraperitoneally, or subcutaneously. For intravenous injections it is not advisable to give more than 2 c.c. at a time, and if the suspension is too strong (*e.g.*, 50 per cent.) the rabbit may die suddenly about the fourth or fifth injection. I have obtained excellent hæmolytic amboceptor by giving six intravenous injections each of 2 c.c. of a 5 per cent. suspension at weekly intervals. As a rule intraperitoneal injections should be stronger and in increasing doses, 2, 5, 7, 10 and 15 c.c. of a 50 per cent. suspension at weekly intervals, though Boas obtains powerful amboceptor by giving intraperitoneal injections of 1, 2 and 4 c.c. at intervals of 3–4 days and bleeding three days after the last injection. Subcutaneous injections are apt to result in abscesses, but, these avoided, a potent serum can be obtained in this way.

After a few weeks the rabbit's serum should be titrated for hæmolytic

amboceptor in the manner to be described, and when sufficiently potent (usually about ten days after the sixth injection) the rabbit is bled to death. The blood can be collected in various ways, of which the most convenient is the following.

The carotid is exposed and stripped down as far as possible after ligaturing it in two places above; the end is then turned into a sterile test-tube and snipped half-way across with a pair of fine, sharp-pointed scissors, the cut end of the artery being held in position within the test-tube by forceps.

The blood is whipped and centrifugalized, or set on one side for twenty-four hours before the serum is removed, but when it has clotted the clot should be separated from the sides of the tube, or flask, in which it is contained, so as to allow it to shrink properly.

It is convenient to bottle the clear serum in 1 c.c. vaccine ampoules. Every care should be taken to prevent contamination.

After bottling, the complement should be removed by heating the serum for half an hour at 55°C. The serum will maintain its potency for many months, but should be kept in the ice-chest.

Standardization of Reagents.—It must be remembered that extract alone and serum alone, as well as normal serum in combination with extract, will deviate a certain amount of complement. The distinguishing characteristic of syphilitic serum is that, in combination with extract, it will deviate more complement than any of these.

Another point to remember is that some syphilitic sera, especially those of well-treated cases, will not deviate much more complement than will normal sera under the same conditions. It follows from this that the proportions which the respective ingredients bear to one another must be such that, on the one hand, no non-syphilitic serum will react positively nor, on the other, an undue proportion of syphilitic sera give a negative reaction. On this account, after fixing arbitrarily the amount of the patient's or control serum to be used in the test, it is necessary to estimate quantitatively the strength of each of the other reagents, and from the information thus obtained to fix the amounts of these to be used.

In describing my own technique, I propose to make a departure from the usual practice of expressing values in cubic centimetres and fractions thereof, by speaking of volumes in varying dilutions.

Thus, if in the usual nomenclature the minimum hæmolytic dose of complement for 1 c.c. of sensitized cells were found to be 0·02 c.c., I should describe it as 1 volume of a 1 in 50 dilution for 1 volume of sensitized cells, or, briefly, as 1 in 50. Since it is the proportions which matter, and not arbitrary quantities, this amounts to the same thing. The size of the volume does not matter, within limits.

In the practice of most workers it is either 0·5 c.c or 1·0 c.c., but in my own practice, chiefly for the sake of economy, I have fixed the volume at about 0·12 c.c. A fairly large number of capillary pipettes are graduated with mercury to measure this amount, and are used chiefly for the measurement of undiluted sera. For dilution of the latter with saline, it is convenient to have a few chambered pipettes graduated to measure four volumes. (For details as to the technique of making capillary pipettes, the reader is advised to consult "Technique of the teat and capillary glass tube" by Sir A. E. Wright.)

Using such small quantities of reagents, the various tests can be carried out in test-tubes measuring 5 × 1 centimetre. Instead of the usual type of air incubator, it is very convenient to have a water-bath fitted with trays into which the test-tubes are slipped.

Titration of Hæmolytic Amboceptor.—To ascertain the minimum amount of rabbit's anti-sheep-cell serum which is sufficient to hæmolyze completely a volume of sheep cells with the help of an excess of complement, a number of test-tubes are set out in a row, and into each is placed 1 volume of a 3 per cent. suspension of the washed sheep-cells deposit. A series of dilutions of the anti-sheep serum is prepared, *e.g.*, 1 in 125, 250, 500, 750, 1,000, 1,500, 2,000, and from each of these dilutions in turn a volume is taken and added to the sheep cells in one of the tubes, so that the row of tubes now contains a series of equal quantities of sheep cells in contact with varying amounts of hæmolytic amboceptor. After half an hour one

volume of a 1 in 10 dilution of fresh guinea-pig serum (containing complement) is added to each of the tubes. A volume of saline is placed in each tube to make the bulk of fluid equal to that in the test itself. It is important in all titrations which bear on the Wassermann test that the bulk of fluid in each tube should be the same as it is in the test itself, since concentration affects the titre. In my own technique four volumes are used in the test proper—one each of serum, complement, extract and cells. All the tubes are placed in the incubator at 37° C. The tubes are shaken about every ten minutes, and the reading is taken at the end of an hour. The tube is sought which contains the smallest amount of amboceptor, and in which there is complete hæmolysis. The dilution of amboceptor serum in this tube represents the minimum hæmolytic dose (M.H.D.) of the titrated amboceptor. An amboceptor of lower titre than 1 in 1,000 should not be used, since a low-titre amboceptor serum has to be added in such large amounts to the cells that agglutination of the latter is apt to occur. Another objection to the use of low-titre amboceptor is that, other things being equal, the titre of complement against cells sensitized with such an amboceptor is lower than when the cells are sensitized with a powerful amboceptor. This affects the test, since, as Browning points out, hæmolytic activity and deviability of a complement do not run *pari passu*. An excess of complement is used since one M.H.D. of amboceptor will not effect complete hæmolysis when working with one M.H.D. of complement, and, within limits, the larger the amount of amboceptor with which the cells are sensitized, the smaller the M.H.D. of complement. This is mentioned here as it affects the question of the amount of amboceptor to use in sensitizing the cells for the titration of complement and for the test itself.

For sensitizing the cells some workers use 2½, others 4 M.H.D., and still others larger quantities of amboceptor. Whatever quantity is to be used in the test proper must be used in titrating the complement. Since in the test proper the cells come into contact with a small but variable amount of hæmolytic amboceptor which is present in the human serum, I have always considered it better to sensitize the cells beforehand with a large amount of amboceptor, so that in the test proper the M.H.D. of the complement as ascertained by previous titration will not be affected by the additional amboceptor in the tested sera. For this reason I sensitize the sheep cells with not less than 5 M.H.D. of hæmolytic amboceptor.

To make up the sensitized cells for titration of complement and for the test, assuming that 20 c.c. of sensitized cells are required, and that the M.H.D. of amboceptor is 1 in 1,500, 10 c.c. of a 1 in 150 dilution of the amboceptor is prepared and added to an equal quantity of a 6 per cent. suspension of sheep cells, making 20 c.c. of a 3 per cent. suspension of cells in a 1 in 300 dilution of amboceptor. A sufficient quantity of sensitized cells should be prepared to suffice for all preliminary titrations and for all the tests carried out on any one day. There is a possibility of error if one batch is sensitized for titrations and another for the tests. It is well to allow the cells to remain in contact with the amboceptor for half an hour before using them.

Amboceptor is very stable, and need not be titrated more often than once in about three months.

Titration of Complement—Complement must be titrated each time the test is performed, to ascertain the amount of it to be used.

The titration is done in a similar manner to that employed with amboceptor. A series of tubes is laid out and into each is placed one volume of a varying dilution of guinea-pig serum, *e.g.*, 1 in 10, 20, 30, 40, 60, 80 and 100. To each is then added one volume of sensitized cells prepared as above, and two volumes of salt solution, to make the bulk the same as in the test proper. The mixtures are incubated for half an hour and at the end of this time the tubes are examined. As in the titration of amboceptor, the tube containing the smallest amount of complement and in which the hæmolysis is complete is sought, and the amount of complement is noted as the M.H.D. of the complement.

I now use, as a minimum, 3 M.H.D. of complement. For instance, if lysis is complete in the 1 in 60 tube, the amount used in the test is one volume of 1 in 20. Besides this, minimum, amount of complement I set up the same serum and extract with larger amounts of complement, as

will be mentioned in more detail when discussing the quantitative estimation of the reaction.

In addition to determining the M.H.D. of the complement, in order to guard against the employment of a complement which is hypersensitive to deviation, Browning and Mackenzie recommend that the number of M.H.D. complement deviated by the amount of extract and of serum, respectively, to be used in the test should be ascertained. I have usually followed this practice, but not to the extent of testing the auto-deviating power of every one of the tested sera. A convenient plan of carrying it out is the following: Two additional rows of tubes are set out; into each tube of one row is placed a volume of the extract, diluted as it will be used in the test, and into each of the other row a volume of any of the sera to be tested, in the same dilution as will be employed in the test. Into each tube of each row is placed a volume of the guinea-pig's serum in the same varying dilution as in the titration for M.H.D., and one volume of saline. The mixtures are incubated for half an hour, and to each tube is then added one volume of sensitized cells. After incubation for half an hour longer, the tubes are read. To take a common example of such a reading, when the M.H.D. of complement is 1 in 60, the highest dilution of complement in the extract row which gives complete lysis is 1 in 30, showing that the extract by itself has absorbed one M.H.D. of complement. In the serum row, using 1 in 5 serum, it is common under these circumstances to find that the tube containing 1 in 40 complement is the last to show complete lysis, showing that the serum alone has absorbed rather less than one M.H.D. of complement. It is not practicable in a large batch of tests to estimate the deviating power of every one of the tested sera, and, in any case, in the test itself the serum is put up with the minimum amount of complement used in the test, so that any abnormal deviating power which may be displayed by a serum acting alone is guarded against.

The *extract* may vary in its deviating power according to the manner in which it is diluted. It was pointed out originally by Sachs and Rondoni that if the dilution is done slowly the result is a more turbid emulsion, with greater deviating power, than when the dilution is effected suddenly. McIntosh and Fildes find, however, that in the case of heart-cholesterin extract the deviating power is rather greater when the extract is quickly diluted. They recommend that the extract be floated on the necessary amount of saline, and the mixture quickly shaken, or, more recently, to put the extract in a large tube, and add saline suddenly. Probably the observation of Sachs and Rondoni applies more particularly to liver extracts. When working with the latter I have usually diluted slowly, according to the method recommended by Browning and Mackenzie. The extract is floated on the top of the necessary amount of saline, just as urine is floated on nitric acid; the tube is set to one side for a time, and a white ring forms at the junction of the two fluids. The tube is then rolled between the hands, at first slowly till the salt solution has diffused into the extract lying above, and then more quickly. Finally, when the two fluids are almost mixed, the tube is inverted, and the result is a turbid emulsion. With heart-cholesterin extract I have diluted quickly according to the original plan of McIntosh and Fildes.

Before titrating the extract it is advisable to ascertain that it is not unduly hæmolytic. To do this a volume of a 1 in 5 dilution of the extract should be incubated with a volume of the suspension of cells and two volumes of saline for an hour. If no hæmolysis is apparent at the end of this time the extract is so far satisfactory.

The strength of the extract to be used in the test is determined in many ways. Many workers use arbitrarily an amount which is equivalent to one volume of a 1 in 5 dilution. McIntosh and Fildes consider that in the case of heart-cholesterin extract made according to their directions, the titre is so constant that it is not necessary to determine it for each fresh supply of extract. In a system where the amount of tested serum is 0·1 c.c., the complement, 2½ M.H.D., and the quantity of cells eventually added, 0·5 c.c., they use 0·5 c.c. of a 1 in 15 dilution of a heart extract, 3, cholesterin solution, 2, mixture. This is one quarter the amount which when incubated alone with 2½ M.H.D. complement is just sufficient to inhibit hæmolysis. Walker and Swift using a heart extract containing

0·4 per cent. cholesterin in a system where the serum is 0·1 c.c., the complement, 2 M.H.D., and the amount of cells eventually added, 0·5 c.c., recommend 0·5 c.c. of a 1 in 10 dilution, or one quarter the amount which by itself is able to inhibit lysis. I have found the strength recommended by McIntosh and Fildes satisfactory when using their heart-cholesterin extract, but for the sake of safety, prefer to titrate every extract according to the following method, which is perhaps rather troublesome, but gives a good idea of the value of the extract. As the titration need be carried out only at the outset, and occasionally afterwards, the extra trouble is not of any great moment.

Four rows of test tubes are set out, and may be marked A, B, C, and D, respectively. Into each tube of row A is placed one volume of a 1 in 5 dilution of the serum of a normal person. Into each tube of row B a similar amount of a non-syphilitic which is known to fix complement more than the average normal serum. Amongst others, the sera of many patients suffering from psoriasis have this property, and unless the test is so graded that such sera cannot give a positive reaction, errors in diagnosis are apt to occur. Into each tube of row C the same amount of serum which is known to give a strong Wassermann reaction (generally that of a florid secondary syphilitic). Lastly, into each tube of row D is placed the serum of a patient who gives a feeble Wassermann reaction. A series of dilutions of the extract to be tested is prepared, *e.g.*, 1 in 5, 7·5, 10, 15, 20, 40, 60, 90, and so on to 200. Into the first tube of each row is put one volume of the 1 in 5 dilution of extract; into the second, one volume of the 1 in 7·5; into the third, one volume of 1 in 10, and so on. In every tube is placed a volume containing 3 M.H.D. of complement. After incubating for half an hour at room temperature and the same time at 37° C., a volume of sensitized cells is added to each tube, and the incubation continued for another half-hour. The tubes are then examined, and the results vary with the quality of the extract. With a good extract, such as a heart-cholesterin extract made according to McIntosh and Fildes, all the tubes containing normal serum (row A) will show complete hæmolysis, or, at most, there will be some fixation in the tubes containing one volume of 1 in 5 extract and slight inhibition in the tube containing 1 in 7·5 extract. In row B there may be slight evidence of complement fixation in the tube containing 1 in 10 extract, but none in the 1 in 15 tube. In the strongly syphilitic serum tubes (row C) lysis may be complete only in the tube containing 1 in 200. In the weakly syphilitic lysis may be partial in the tubes containing 1 in 15 or 20, and complete in those containing higher dilutions of extract. A poor extract may fail to deviate with a strongly syphilitic serum when in a dilution of 1 in 10, and yet show partial deviation with a normal serum when in a dilution of 1 in 5.

The strength of extract to be used in the tests is provisionally fixed at half the amount which just fails to deviate 3 M.H.D. of complement when in contact with a normal serum. This amount is then incubated alone with a number of varying dilutions of complement, just as was described, in the titration of complement to ascertain how many M.H.D. of complement it will deviate. If it deviates only one M.H.D. or less, the amount is so far satisfactory.

Finally, before taking it into use the new extract is tested alongside an accredited extract with 20 non-syphilitic and 20 syphilitic sera. The former should include as many sera of psoriasis cases as possible.

The Test.—A normal and a known positive serum should be tested with the sera under examination. It is a distinct advantage to estimate the strength of the reaction. Here again the practice of different workers varies. Some consider that the strength of the reaction varies inversely with the amount of tested serum which will deviate a constant amount of complement with a constant amount of extract, and use varying quantities of tested serum. Generally this appears to be true, florid cases of syphilis reacting with smaller amounts of tested serum than well-treated cases; but sometimes a given amount of serum will give a negative and half this a positive reaction. Others vary the amount of extract, believing that the strength of the reaction varies inversely with the amount of extract which produces a positive reaction. This is certainly a useful method

with modifications. In over 7,000 Stern tests I have never found a serum react with the smaller and yet fail with the larger amount of extract, and in treated cases the reaction with the smaller amount of extract is the first to die out. In the original test, carried out for routine purposes, I have always estimated the strength of the reaction by varying the amount of complement, on the principle that the stronger the reaction the more complement will be deviated. The results by this method, judging by over 10,000 tests, have coincided very closely with clinical symptoms and the effect of treatment

Assuming that a constant amount of tested serum and of extract respectively is to be used and that to estimate the strength of the reaction two different amounts of complement are to be used, a rack containing four parallel rows of holes is taken, and along the back row are set out the sera to be tested.

The front three rows are filled with empty test-tubes, so that there are three tubes in front of each serum. Each serum is taken in turn, diluted to 1 in 5 with 0·85 per cent. salt solution, and one volume of this dilution is placed in each of the three tubes. A convenient method of carrying this out is as follows:—A row of clean watch-glasses is laid out in front of the rack and into each is measured four volumes of saline, with the appropriate pipette. Each serum is taken in turn, a volume of it placed in the four volumes of saline in one of the watch-glasses, and, after mixing, a volume of the 1 in 5 dilution so made is measured into each of the appropriate test tubes.

When every serum has been dealt with in this way, into every tube of the two front rows is put one volume of the diluted extract, and, to make the bulk equal in all tubes, a volume of saline is placed in each of the third row tubes.

The complement is next added. Into each tube of the front and third rows is put a volume containing 3 M.H.D. of complement (*e.g.*, if the M.H.D. of the complement is 1 in 60, a volume of 1 in 20 is placed in each of these tubes). Into every tube of the second row is put a volume containing 5 M.H.D. of complement.

It will be seen from this arrangement that the third row differs from all the others in containing no extract. It is a control to show that the amount of serum in each test cannot by itself deviate the smallest amount of complement used in the test.

The tubes are shaken, allowed to stand at room temperature for half an hour, and then placed in the water-bath incubator at 37°C. for half an hour. Formerly it was the practice to place the tubes in the incubator at once, but a difficulty was raised when Jacobsthal pointed out that in some cases binding of complement in the Wassermann test took place better at 0°C. than at 37°C. Guggenheimer then found that in some cases it was better at 37°C. than at 0°C., and Thomsen and Boas that in no case did binding occur better at 0°C. than at room temperature, but that in the majority of cases the reaction took place better at room temperature (or at 0°C.) than at 37°C. In a series of 207 sera which I tested at the same time by both these methods the results practically confirmed these conclusions. Of the total number tested, 35 were positive after preliminary incubation in the water-bath, as well as after standing at room temperature, and 158 were negative. Of the remaining 14 sera, 7 gave a positive reaction only after incubation at 37°C. and 7 only after standing at room temperature. The practical solution of the difficulty is that suggested by Boas, to incubate first at room temperature and then at 37°C.

At the end of this period, to each tube is added a volume of sensitized cells, and incubation allowed to proceed. The tubes are shaken every five or ten minutes and if the normal control serum is placed last it is sufficient to take the reading when this shows complete hæmolysis. As a rule, the reading can be taken in half an hour.

The following are the rules to be observed in reading the results: (1) Failure of the normal serum test to show complete hæmolysis in all its tubes entails rejection of the whole batch of tests. (2) Failure of any tube in the third row (control) to show complete lysis entails rejection

on the test of the serum of which it is a control. These conditions being satisfied, (3) failure of hæmolysis in each of the two front tubes (containing extract) devoted to the testing of any serum may be marked as strongly positive (+ +). No hæmolysis in the front, but lysis in the second row tube, not so strongly positive (+). Incomplete lysis in a front row tube may be marked as doubtful (± or ±), and complete in a front tube is returned as negative.

If the test is for diagnosis purposes, it is a good plan to re-test the serum if the reaction is simply +, to eliminate any possibility of an error in technique; this reaction, however, is good enough for a diagnosis. Doubtful reactions, ± and still less so ±, should not be considered diagnostic. Such reactions are, however, generally given by very early or by well-treated cases of syphilis, and an early opportunity should be taken of testing the serum again, no treatment being administered in the meantime unless this is designed to provoke a definite reaction.

When the results are read so soon as recommended above some difficulty may be experienced in deciding whether or no there is lysis in certain tubes since the cells have not sunk to the bottom. With experience, partial lysis can be detected though the mixture is still turbid, but in case of doubt the tubes in question can be spun in the centrifugal machine.

Wassermann Test of the Cerebro-Spinal Fluid.

It was formerly the custom to test the cerebro-spinal fluid in exactly the same quantity as the blood-serum. Hauptmann and Hössli, however, showed the advantage and safety of using relatively larger quantities of fluid than of serum, and this practice is now followed by practically all workers. The result has been to increase the percentage of positive reactions found in tabes, and whereas formerly it was said that in ordinary syphilis of the central nervous system the Wassermann reaction of the cerebro-spinal fluid, as a rule, was negative, it now appears that quite a large proportion of patients in the secondary stage give a positive reaction, especially in syphilitic affections of the cranial nerves and other syphilitic lesions of the central nervous system, apart from tabes and general paralysis.

As the cerebro-spinal fluid contains no complement and has practically no anti-complementary action, it is unnecessary and even undesirable, to heat it.

I have usually carried out the test as follows:—

Into a series of tubes are put varying quantities of the fluid thus:—

2 volumes of undiluted fluid.
1 volume of undiluted fluid.
1 volume of a 1 in 2·5 dilution.
1 volume of a 1 in 5 dilution.

Into each of these is put a volume of extract and of complement, respectively, in the same dilutions as in the serum test. Into another tube (control) are put two volumes of undiluted fluid, and one of complement. The rest of the procedure is exactly as in the serum test, and the strength of the reaction determined by the number of tubes, excluding the control, in which a positive reaction occurs. As a general rule the cerebro-spinal fluids of cases of general paralysis give the strongest reactions, and then those of tabetics, while the fluids of other cases of syphilis of the central nervous system give reactions only in the tubes containing the larger quantities, undiluted, and 1 in 2·5 dilutions.

Measurement of Reagents by Donald's Dropping Method.

The Wassermann test involves the addition of equal quantities of the same reagents, such as saline, complement, extract, and sensitized cells, to numbers of test-tubes, and the monotony and eye-strain involved when a large number of tests have to be carried out with pipettes can become very trying. Another disadvantage of the hand pipette method of measurement is that, unless it is carefully delivered, the full amount of the measured fluid does not reach the test-tube, some of it remaining behind in the pipette.

The disadvantages of the ordinary pipette method can be overcome by the use of dropping pipettes, as devised by Donald. These depend on the principle that, at constant temperature and pressure and at a constant delivery rate which does not exceed one drop per second, the size of drop of any given fluid which is delivered by a vertically held nozzle is constant and depends on the circumference of the delivery nozzle at its outlet.

For fuller details of this method of measurement, the reader is referred to the papers mentioned below.* It is proposed here to deal only with its application to the method of conducting the Wassermann test which has been described above.

I have carried out a large number of Wassermann tests by the dropping method in parallel with the hand pipette, and the two series have agreed so consistently as to results that the dropping method has been adopted as routine at Rochester Row for all occasions when more than twenty tests have to be carried out.

In the test as described above the volume of neat human serum is one-fifth that of the diluted extract, diluted complement, and sensitized cells, respectively. For practical purposes the same nozzle delivers complement, saline and sensitized cells in drops which are of equal size, but to obtain the same-sized drop of extract it is necessary to have a larger nozzle. Droppers of three different sizes are required—one for the human serum, one for the diluted extract, and one for the saline, &c. The appropriate sizes can be obtained by the use of a Starrett's Morse drill and wire gauge, No. 186 (L. S. Starrett Co., 36, Upper Thames Street, London, E.C.), for the smaller sizes, and a Rawco gauge, Columbus pattern, for the larger.

The sizes of the nozzles used at Rochester Row were found by a process of trial and error, and are now made as follows: For saline, complement, and sensitized cells, a piece of glass tubing is cut off squarely at the point where its outside diameter is 0·76 c.m. on the Rawco gauge. At about two inches from this point the tubing is softened in the flame and drawn out to a short, wide capillary, where it is cut off. This results in a short nozzle with its upper end throttled. For extract, a nozzle is cut in the same manner so as to gauge 0·9 c.m. For human serum, a piece of glass tubing is softened in the flame and drawn out into a capillary, the sides of which are as nearly cylindrical as possible. This is passed through the No. 56 hole in the Starrett gauge until lightly gripped by the latter and is cut off with a sharp, glass-cutting knife as closely to the upper surface of the gauge as possible. (For the details of making such a knife, the reader is referred to "Technique of the teat and capillary glass tube," mentioned above.) The tube is just nicked, then withdrawn from the gauge, and broken across at the nick. The cut end must be square, as in the case of the larger pipettes. The gauges used for these purposes must be kept as free from grease as possible.

Each of the nozzles made as described above must be carefully tested, as follows: The dropping end of the nozzle is freed from grease by dipping it in an alcoholic solution of caustic potash and thoroughly rinsing under the tap. It is then set up as shown in figure I, being attached to the delivery end of a 1 c.c. pipette, graduated in 0·01 c.c., by a short length of rubber tubing. The pipette is, in turn, passed into a larger tube, one end of which has been closed in the flame, and the other fitted with a short length of rubber tubing. The latter fits the sides of the graduated pipette so as to make a moderately air-tight joint, which, however, allows the outer tube to slide over the pipette (a little glycerine smeared over the sides of the latter acts well as a lubricant). The combination is set up vertically in a burette stand, taking especial care to have the dropping nozzle vertical.

To measure the drops, the outer tube is first pushed down over the graduated pipette as far as the place where the latter is gripped by the burette stand. A vessel containing the particular fluid which the dropper is designed to deliver is lifted up so that the delivery nozzle dips below

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* Donald R. Proceeds of the Royal Society B., vol. lxxxvi., 1913, pp. 198–202. Lancet, June 29, 1912, p. 1752. Lancet, December 4, 1915, vol. II., p. 1243. Lancet, September 2, 1916, vol. II., p. 423.

the surface of the liquid, and nozzle and graduated pipette are filled to a convenient mark near the top by sliding the outer tube upwards. The vessel is removed and, by manipulation of the outer tube, the face of the fluid at the mouth of the delivery nozzle is made quite flat, *i.e.*, with no meniscus projecting either inwards or outwards. A note is made of the level of the fluid in the pipette and then, by spirally twisting

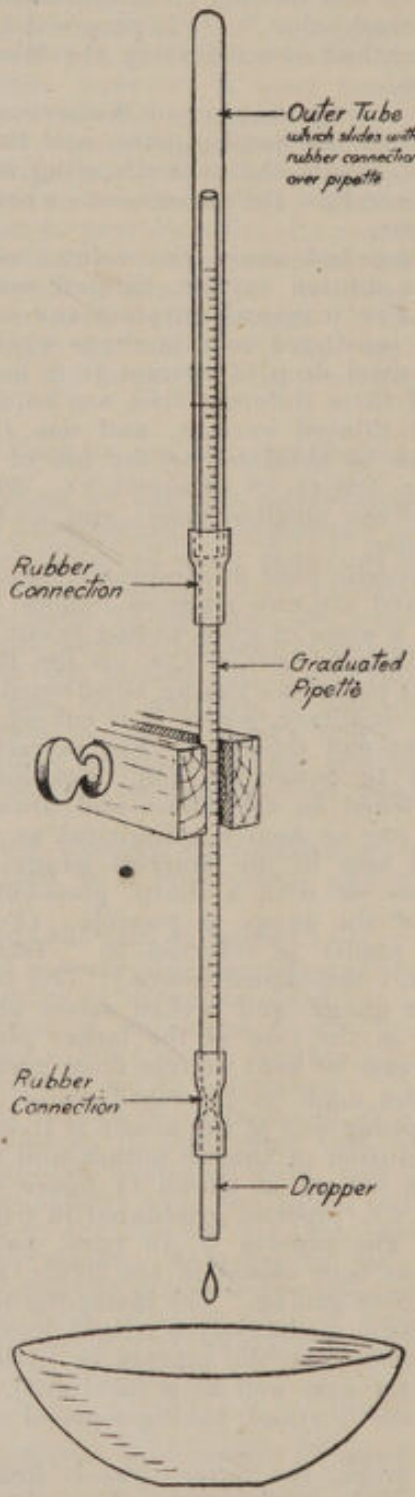

FIG. 1.—Apparatus for standardizing the dropper.

the outer tube and rubber connection very slowly and steadily in a downward direction, the fluid is forced out drop by drop. The drops are counted, and when the level of the fluid is near to the bottom of the graduated pipette the delivery is stopped. The outer tube is then manipulated so as to make the face of the fluid at the mouth of the delivery

nozzle quite flat, and the reading again taken. The difference between the first and second reading, divided by the number of drops which have fallen, gives the size of each drop. The measurement should be repeated several times to insure accuracy, and successive series of drops of the same fluid should also be weighed against one another.

At a certain temperature, droppers gauged as above delivered as follows:—

Gauge 0·76 c.m., 6 drops = 0·66 c.c. saline. Gauge 0·9 c.m., 6 drops = 0·66 c.c. extract diluted 1 in 15. Starrett Morse, No. 56, 30 drops = 0·66 c.c. heated human serum.

The large droppers mentioned above for saline and extract are rather sensitive to vibration and require also a separate gauge. Drs. Scott and Griffith working in the L.G.B. laboratory with droppers gauged in the Starrett Morse plate found that—

5 drops of human serum dropped by a capillary of 56 gauge ... ... = 2 drops of saline dropped by a tube of Nr. 30 gauge ... = 2 drops of extract diluted 1 in 15 dropped by a tube of Nr. 18 gauge.

Droppers of 18 and 30 gauge can be employed, therefore, in place of the larger nozzles mentioned above, using two drops from the former in place of one from the latter. They have the advantage of being less sensitive to vibration than the larger droppers.

Technique of the Test itself.

The tubes are laid out in racks, as described for the pipette method, with the serum specimens in the back row. The human serum capillary dropper is set up as shown in Fig. 2, exactly vertical. The rack is lifted up to the dropper until the latter dips into the first serum to be tested.

FIG. 2.—Dropper for Serum.

A small quantity of serum is drawn into the pipette, by manipulating the teat, and the rack lowered until clear of the pipette. Then a drop of serum is slowly expressed in turn into each of the three tubes in front of the serum tube, the balance returned to the serum tube, and the rack replaced on the bench. The pipette is cleaned by filling and emptying two or three times with saline, and the operation is then repeated with the succeeding sera. For each of the remaining ingredients it is advisable to have a separate dropper. These are set up as shown in Fig. 3, which

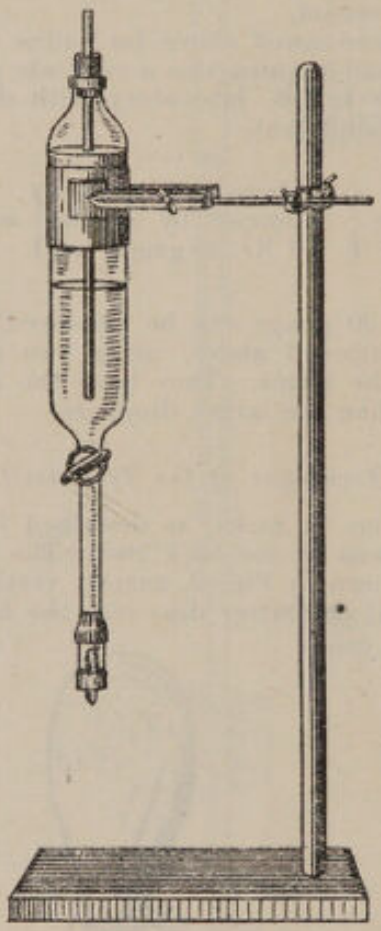

FIG. 3.—Arrangement of dropper for Saline, diluted Complement, etc. (after Donald).

illustrates a separator funnel fitted with the appropriate dropping nozzle and with a Mariotte's tube. The latter serves the double purpose of keeping the pressure constant, whatever the height of fluid in the funnel, and of stirring up the fluid (by the rising of bubbles from the lower end of the Mariotte's tube).

Saline is next added to each tube in the front row. By turning the tap on the funnel, the dropping is started, and when the delivery rate is steadily just about one drop per second the rack is passed along under the dropper so that each tube is dosed in turn.

Assuming that the saline drop is five times the serum drop, one drop of saline is allowed to fall into each tube of the two front rows, and two drops into each of the back row tubes (which will receive no extract). If the saline drop is 5/2 of the serum drop, as described above, naturally, two drops are placed in each tube of the two front rows, and four in each third row tube.

Complement of the lower strength is then dropped, on the same principle, into each of the front and third row tubes, and complement of the higher strength into each of the middle row tubes. The two strengths of complement can be dropped in turn by the same nozzle.

Extract of appropriate strength is dropped into each tube of the front two rows, and, after suitable incubation, sensitized cells are added to each tube by a dropper of the same size as that used for saline.

It will be seen from the above that the hard work connected with the dropping method is in the beginning, when the appropriate nozzles are being found. After this it is almost automatic, and accuracy is independent of fatigue. In this it has a great advantage over the hand pipette method. A worker may be quite accurate at first with the latter, but after the measurement of some hundreds of volumes of various fiuids in one day, accuracy is apt to suffer.

In using the drop method some precautions are necessary. (1) The nozzles should not be allowed to become greasy. A good plan is to keep them in saline between tests, and before use to dip them in alcoholic solution of potash, which is removed by rinsing under the tap. (2) The set of nozzles should be tested at intervals to see that they are still delivering drops of the correct relative sizes. (3) The dropper should always be vertical when in use. (4) Drops should be delivered as nearly as possible at uniform rate. This is fairly easy to arrange with the separator funnel, but demands some little skill with the teat. Donald recommends that the fundus of the teat be squeezed firmly between the ulnar edge of the thumb tip and the radial of the index finger. Then by rolling the thumb towards the fundus of the teat the pipette is filled, while rolling towards the latter empties it steadily. This is better than manipulating the teat delicately between finger and thumb.

I have found the method of conducting the Wassermann test, which is described above, entirely satisfactory in over 10,000 tests. Its great advantage is that it is economical in reagents and incubator space, and this is a consideration when large numbers of tests have to be carried out in one day, and when a class has to be supplied with materials for practice.

II.—Method used by C. H. BROWNING, M.D., at the Bland-Sutton Institute, Middlesex Hospital (based on that of Browning, Cruickshank, and Mackenzie, modified by Browning and Kennaway).

A fixed amount of each patient's serum plus a fixed amount of "antigen" is tested along with varying amounts of complement; the amounts of complement are conveniently measured in hæmolytic doses, but it must be emphasised that *no invariable relationship exists between the hæmolytic power of a complement and its capacity for being fixed in the presence of syphilitic serum + "antigen."*

(In spite of statements to the contrary, it is definitely proved that similar variations in complement are found when using both ox's and sheep's blood + IB (from the rabbit), provided that the tests are arranged so as to enable differences of this kind to be detected when present.)

The antigens consist of alcoholic ox-liver "lecithin" solution alone and plus cholesterin; one part of each solution being emulsified with 7 parts of saline so as to yield maximum turbidity.

Different batches of antigen are standardised by using varying strengths of lecithin and cholesterin till concentrations are found which, when repeatedly tested with standard positive and negative sera, give practically the same quantitative results, qua amount of complement fixed, as the specimen of antigen already in use.

Preliminary to each day's tests, the hæmolytic dose of complement (guinea-pig's serum which has been kept for about 18 hours after withdrawal of the blood) is determined as in the Complement Control (*see* B. below), except that the mixture of complement and saline is not incubated prior to the addition of the sensitized red corpuscles. (Immune body, as a rule, need not be titrated on each occasion; insufficiency of immune body for the particular specimen of red corpuscles in use is generally indicated by great "tailing off" of lysis when the preliminary estimation of the hæmolytic dose of complement is made.)

A. *Series of 7 Tubes for Serum of each Patient.*

Each tube receives 0·025 c.c. patient's serum (heated at 56°C. for ½ hour).

Lecithin	0·3	0·3		
Complement	0·015	0·03		
Lecithin-Cholesterin	...	0·3	0·3	0·3
Complement	...	0·03	0·045	0·06
Serum Control—				
Saline	0·3	0·3		
Complement	0·01	0·02		

B. *Controls for Whole Series of Patients' Sera.*

(1) *Antigen Control*—				
3 tubes each of—				
(*a*) Lecithin	0·3	0·3	0·3	
(*b*) Lecithin-Cholesterin	0·3	0·3	0·3	
Complement to (*a*) and to (*b*).	0·015	0·03	0·045	
(2) *Complement Control*—				
Saline	0·3	0·3	0·3	0·3
Complement (1 in 4 dilution)	0·01	0·02	0·03	0·04

All the amounts mentioned are fractions of a c.c.

For measuring amounts up to 0·1 *c.c. a graduated pipette with a total volume of* 0·1 *c.c. is employed; for larger quantities a* 1·0 *c.c. pipette is used. The pipettes are operated preferably by means of a mouth-piece. This is considered to be the procedure which combines an optimum degree of rapidity and accuracy for measuring quantities of the order here employed.*

NOTE.—*The above amounts of complement apply as a rule when the hæmolytic dose for* 0·5 *c.c. RCs + IB is* 0·0075 *c.c.*

The mixtures are incubated for 1½ hours at 37°C., *then* 0·5 c.c. ox blood suspension (3 per cent. of washed sediment) sensitised ad maximum (5 doses IB or more) a quarter of an hour previously, is added; incubate 1 hour at 37°C. Read results best after the tubes have stood for several hours further at room-temperature to sediment.

Attention is directed to the following points:—

(1) *A series of increasing amounts of complement is used.*

This is judged necessary because it has been found as the result of many years' routine tests with longer series of tubes than those above noted, that no single amount of complement, measured either by volume or by hæmolytic doses, will serve as a criterion for { positive, owing to slight or no lysis occurring with it: / negative, owing to complete lysis occurring with it.

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*** No further grading of the results can be recommended.

When the same control sera are kept frozen and used in successive tests, then on comparing the results obtained with these controls one can decide that on a given day the complement is little deviable or over deviable. It is also possible to preserve complement for long periods—weeks or months—by keeping it frozen; thus a pooled specimen may be used in repeated tests.

When the complement is *over deviable* weak positive results should be regarded as not highly significant.

When the complement is *little deviable* weak positives are in danger of being classed as negative.

> Careful observations have shown that no advantage is gained by using varying amounts of patient's serum in the above method; with a large amount of patient's serum the most noteworthy effect is the inhibitory effect of the latter by itself on complement as seen in the serum control.

Strong emphasis is laid on the necessity for using a *series* of amounts of complement with the serum of each patient, since it is this procedure which both permits the variability of complement to be allowed for and also affords the means of instituting a thoroughly satisfactory quantitative comparison between different patients' sera, so as to enable the complement-fixing property of a positive serum in the presence of "antigen" to be distinguished from that of a negative one.*

The record, as kept for routine purposes, of the results of an actual series of tests is appended by way of illustration.

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* For further details, *see* Browning and Mackenzie's "Recent Methods in the Diagnosis and Treatment of Syphilis" (London: Constable, 1912).

Antigen, 6th Product. Complement, frozen pooled sera of three guinea pigs.
Date, July 2nd, 1917.

Doses of complement		2	4	6	8	Serum 1·3	Controls 2·6	Result
Amounts		·015	·03	·045	·06	·01	·02	
Negative Control Serum (A known "borderline" Specimen.)	L	jc	c			c	c	
	L–C	jc	c	c				
Positive Control Serum (A rather strong positive).	L	ftr	jc			c	c	
	L–C		o	ftr	tr			
Antigen Control ...	L	c	c	c				
	L–C	c	c	c				
H. McD.	L	ftr	m			c	c	+
	L–C		o	o	ftr			
T.B. and eleven other sera identical.	L	c	c			c	c	—
	L–C		c	c	c			
M.F.	L	o	ftr			c	c	+
	L–C		o	o	o			
L.M.	L	d	jc			c	c	+
	L–C		o	ftr	c			
J.D.	L	ac	c			c	c	+
	L–C		o	d	c			
E.S.	L	tr	jc			c	c	weak +
	L–C		tr	c	c			
G.J.	L	o	vm			c	c	+
	L–C		o	o	ftr			
W.W.	L	tr	jc			c	c	+
	L–C		o	ftr	ftr			
A.B.	L	o	d			c	c	+
	L–C		o	o	o			
D.	L	m	c			jc	c	weak +
	L–C		ftr	c	c			
Complement Control: serum diluted 1 in 4 ...		·01	·02	·03	·04			Dose 0·0075
		tr	ac	c	c			

L = Lecithin. L–C = Lecithin-Cholesterin.
Grades of Hæmolysis—c = complete. jc = just complete.
ac = almost complete. vm = very marked. m = marked. d = distinct.
tr = trace. ftr = faint trace

III.—Method of Staff-Surgeon P. G. FILDES, R.N.V.R., and JAMES McINTOSH, M.D., used at the Royal Naval Hospital, Haslar, and at the London Hospital.

(Based on description of the method published in *The Lancet*, October 28th, 1916.)

The following is the technique of the Wassermann reaction as carried out by us at the present time in ordinary diagnostic work. The present form of the test is an improvement upon that described by us in *Brain*, 1913, XXXVI., 193, but this earlier paper may be consulted for the more general discussion of many matters of technique.

The chief characteristic of our method is the use of a particular antigen composed of tissue extract mixed with cholesterin. Cholesterin was first used as an antigen by Fleischmann but apparently abandoned, and credit is due to Browning, Cruickshank, and Mackenzie for demonstrating first that cholesterin is capable of much increasing the specific action of another antigen. Browning and his co-workers amalgamated the cholesterin with "lecithin" extracted by themselves, but Sachs (1911) suggested the addition of small amounts of cholesterin to the organ extracts already in use.

In 1912 we published an extensive comparative test which we had carried out between various antigens, including Browning's and our own which we had founded upon the suggestions of Sachs. These tests showed that the formula worked out and suggested by us was more satisfactory than those hitherto in use and fulfilled the requirements of a "standard" antigen which would give comparable results in the hands of different workers. During the past five years this antigen has been extensively adopted, and is probably more widely used now than any other.

An improvement embodied in the present technique is the performance of the "antigen control" before the tests proper are carried out instead of at the time of the tests. This "control" is elaborated into a quantitative estimation of the amount of complement absorbed by the antigen, and the amount of complement actually used in the test is based on this estimate.

1. *Collection of the serum to be tested.*—About 0·5 to 1·0 c.c. of blood is required. This is very easily collected from the finger or thumb. The patient swings his right arm rapidly in order to drive the blood into the finger tips; a piece of fairly thin drainage tubing is then twisted round the thumb and two or three punctures are made with a glass "pricker" or surgical needle just proximally to the nail and towards the ulnar side. The blood is collected in a Wright's capsule of suitable size. Fuse the straight end of the capsule. Attach a gummed label inscribed with the number of the case and enter up the particulars in the book under the same number. Put away the capsule into an efficient ice chest until required.[1]

At this point it may be strongly emphasised that it is of no value to spend time upon an elaborate Wassermann technique if the organisation permits of any possibility of mixing one serum with another. Grease pencil should never be used for writing numbers on glass. Every action involved in collecting the sample and "booking" it must be uninterrupted and rigidly according to routine.

2. *Materials required for the test.*—

(*a*) Copper racks to hold 24 test-tubes, as sold by Messrs. Baird and Tatlock, 14, Cross-street, Hatton-garden, E.C., for this purpose.

(*b*) Test tubes, 4 × $\frac{5}{8}$, washed and finally rinsed in distilled water and dried.

(*c*) 1 c.c. and 10 c.c. graduated pipettes, specially long, graduated to tip (Baird and Tatlock).

(*d*) Washed sheep's corpuscles. Collect the blood from a freshly killed animal into a stoppered bottle containing a few pieces of glass rod and iron wire. Fill only half full. Shake continuously for sufficiently long to

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[1] If the ice chest is inefficient or if the sera have been sent by post or badly collected, it is better not to keep the capsules for several days but to centrifuge them at once and keep the serum separate from the corpuscles.

defibrinate it. The blood will keep in the ice chest for three or four days. When required, fill the glass centrifuge buckets with the blood and drive down the corpuscles. Remove serum and add saline; shake and centrifuge. This constitutes the first washing. There must be three washings in all. The first may be carried out the day before the corpuscles are required. After the third washing remove the saline without shaking up the corpuscles. At each washing there must be from 8 to 10 times as much saline as corpuscles.

(*e*) Guinea-pig complement. On the day of the test kill a guinea-pig by cutting its throat and collect the blood in a saucer. The animal is first stunned *slightly* by knocking its head upon the edge of the table. Pour the blood from the saucer into a small conical glass. "Whip" to defibrinate with a piece of cotton-wool upon the end of an iron wire. Centrifuge and pipette off the serum.

(*f*) Antigen. This must be kept separate in two bottles A and B. Preparation of heart extract (A.) Obtain a fresh human heart. With a pair of scissors cut off the muscular portions of the ventricles and elsewhere, but do not take fat. Mince these pieces and weigh them. Place in a mortar with a little sand and grind them up with absolute alcohol, using 9 c.c. of alcohol to 1 grm. of heart. Transfer the whole to a well-fitting glass-stoppered bottle, and shake occasionally for one and a half hours. Filter into another *well-fitting* glass-stoppered bottle through paper and preserve in ice chest. A deposit will be found to form and this may filtered off again. Cholesterin solution (B). Take 1 grm. of pure cholesterin, such as Kahlbaum's, and place in a *well-fitting* glass-stoppered bottle. Add 100 c.c. absolute alcohol and put in the stopper tightly. *Shake and heat in water bath* until the cholesterin is dissolved.

(*g*) Amboceptor. This will usually be purchased, although most sera upon the market are somewhat feeble in action.

3. *Standardisation of the amboceptor.*—To be performed with two different guinea-pigs to ensure average complement action. The standardisation should be repeated after several months.

a. Materials. 1. Saline solution. 2. Tube containing 0·5 c.c. guinea-pig's serum and 0·5 c.c. saline. 3. Tube containing amboceptor diluted 1 in 1,000 thus: Tube *a* = ambo. 0·1 c.c. (*exact*) + saline 9·9 c.c. (shake); tube *b* = 1 c.c. from tube *a* + 9·0 c.c. saline (shake)—this is 1 in 1,000. 4. Tube containing 1 c.c. of deposited washed corpuscles + 19 c.c. saline (shake). 5. Rack with 9 test-tubes 4 × §.

b. Method. Fill reagents into the tubes as indicated.

No. of test-tube ...	1	2	3	4	5	6	7	8	9	Pipette to be used.
1. Saline	0·8	0·7	0·6	0·5	0·4	0·3	0·2	0·1	0	10 c.c.
2. Ambo. (tube 3*b*)	0·1	0·2	0·3	0·4	0·5	0·6	0·7	0·8	0·9	1 c.c.
3. Complement (tube 2) ...	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	1 c.c.
4. Blood (tube 4) ...	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	10 c.c.

Shake. Incubate for one hour at 37° C. and then read off the minimal hæmolytic dose—*i.e.*, the first tube which is absolutely clear. If the m.h.d. does not fall between 0·0001-0·0009 c.c. the amboceptor should be rejected. A weak amboceptor sometimes gives obscured readings in the test proper.

4. *Daily Routine.*—(1) Wash or complete washing of the blood corpuscles.
(2) Kill the guinea-pig and prepare the serum.
(3) Make up fresh saline solution.
(4) Standardise the complement without antigen and in the presence of antigen. (All subsequent measurements under 1 c.c. are made with the 1 c.c. pipette; up to 10 c.c. with the 10 c.c. pipette; above 10 c.c. with a 50 c.c. measure.) Materials: (*a*) Four tubes in the rack front row; four

tubes in the rack back row. (*b*) Complement diluted 1 in 24 (0·1 c.c. complement + 2·3 c.c. saline). (*c*) Complement 2½ times stronger (0·2 c.c. complement + 1·7 c.c. saline). (*d*) 5 per cent. corpuscle suspension containing amboceptor This is made up as follows: The quantity required is 0·5 c.c. for every tube in the test + 10 c.c. to allow for the amount used in the standardisation of the complement and for emergencies. Two tubes are required for each case. Thus if 60 bloods are to be tested 60 + 10 = 70 c.c. of suspension are required. The quantities will be as follows: (γ) corpuscles (centrifuged deposit) 3·5 c.c.; (β) amboceptor, 4 m.h.d. per tube—*i.e.*, if m.h.d. = 0·0005 c.c., the amount per tube will be 0·002 c.c., and for 140 tubes 0·28 c.c.; ( ) saline 66·22 c.c. Every half c.c. of this mixture will contain 0·002 of amboceptor, the correct quantity per tube. Make up in the order of the Greek letters and shake after each addition. (*e*) Dilute antigen, made up as follows:—Rinse out and clean a 1 c.c. pipette with spirit. Take 0·3 c.c. of heart extract and 0·2 c.c. of cholesterin and place in a dry test-tube. Run in 7 c.c. of saline and shake.

Method: Fill in the 8 tubes in the rack as indicated.

Front Row for Estimating m.h.d. of Complement.

	1	2	3	4
1. Saline	0·85	0·8	0·75	0·7
2. Complement (tube *b*)	0·15	0·2	0·25	0·3
3. Corpuscles + amboceptor (tube *d*) ...	0·5	0·5	0·5	0·5

Back Row for estimating m.h.d. of Complement in the presence of Antigen.

1. Saline	0·35	0·3	0·25	0·2
2. Complement (tube *c*)	0·15	0·2	0·25	0·3
3. Antigen (tube *e*)	0·5	0·5	0·5	0·5

Shake. Put rack into a water bath at 37° C. for 10 minutes to see whether the front-row tubes are laking properly. Tube No. 4 should be laked by this time. If not completely clear very soon after, another guinea-pig must be taken as the complement is too weak. If laking is satisfactory transfer to the incubator until the hour is complete. Read the m.h.d. of the complement in the front row and put 0·5 c.c. of corpuscles (tube *d*) into each of the back row tubes and heat in the water bath for 10 minutes. If the tube immediately behind that showing the m.h.d. in the front row is *completely* laked continue as in following table:—

If m.h.d. is tube 1 use 0·5 c.c. of a 1 in 33 dilution of complement for each tube.
" 2 " 1 in 24 " " "
" 3 " 1 in 19 " " "
" 4 " 1 in 16 " " "

If the tube immediately behind is not completely laked and the next one to the right is, this latter will indicate the amount of complement to use, but if this also is not clear the complement is unsatisfactory and that of another guinea-pig must be taken.

5. *Preparation of serum for testing.*—While the complement is undergoing standardisation, or at any other time on the day of the test, the sera should be distributed into the tubes. If the serum has not been pipetted off already the numbered capsules are centrifuged and a series of test-tubes are set out in the racks, each pair numbered to correspond to the capsules. The numbering of the tubes must be indelible, and is best carried out by Donald's method. 1. Warm the tube in the flame. 2. Inscribe a bold figure on it with ordinary blue-black ink. 3. Burn in the ink by heating in the flame to a point short of the fusing temperature. From each capsule transfer 0·1 c.c. of clear serum to each of the corresponding tubes. This is conveniently done with Donald's dropping pipettes, thus:—1. Pull out a piece of glass tubing into two pipettes. 2. Pass the pipette through a particular hole in a wire gauge plate, when it engages cut it off flush with the plate. The actual hole to

be used is found once and for all by experiment. A pipette made in No. 53 Stubbs will deliver 0·1 c.c. of serum in 4 drops. Always hold the pipette vertically and wash with water between each serum. When the sera are all filled in make the tubes into a bundle in some safe and convenient manner and suspend them in a water-bath standing at 55°–56° C. for 30 minutes. Then replace them in the racks in sequence. When testing cerebro-spinal fluid use twice as much and do not heat it. If sera are removed from the capsules for storage in the ice chest, they may be inactivated in the pipettes in which they have been stored and distributed from these pipettes by the method of Donald.

6. *Preparation of the reagents for use.*—(a) When the complement is standardised make up a sufficient quantity of the correct dilution to allow 0·5 c.c. for each tube and a little over. (*b*) Also make up diluted antigen as shown in the table. Mix in the manner already described for antigen, allowing 0·5 c.c. per tube for half the total number of tubes.

No. of tubes.		C.c.		Heart.		Cholesterin.		Saline.
30	=	15	=	0·6	+	0·4	+	14·0
40	=	20	=	0·8	+	0·53	+	18·6
50	=	25	=	1·0	+	0·67	+	23·3
60	=	30	=	1·2	+	0·8	+	28·0
70	=	35	=	1·4	+	0·93	+	32·6
80	=	40	=	1·6	+	1·06	+	37·3
90	=	45	=	1·8	+	1·2	+	42 0
100	=	50	=	2·0	+	1·34	+	46·6
110	=	55	=	2·2	+	1·47	+	51·3
120	=	60	=	2·4	+	1·6	+	56·0
130	=	65	=	2·6	+	1·73	+	60·6
140	=	70	=	2·8	+	1·86	+	65·3
150	=	75	=	3·0	+	2·0	+	70·0

7. *The test proper.*—Mix the diluted antigen with an equal quantity of diluted complement, and then measure 1 c.c. of the mixture into the left hand of each pair of tubes to which the serum has been already added. Shake. It is to be understood, of course, that the mixture is to be distributed as soon as it is mixed, and is not to be allowed to stand. If the number of cases to be tested is large, it will be necessary to mix antigen and complement in smaller batches in order to avoid loss of complement before the test begins.

Mix the rest of the diluted complement with an equal quantity of saline, and measure 1·0 c.c. into the right hand tube of each pair. Shake. Incubate for one hour in the air incubator. Add 0·5 c.c. of the corpuscle-amboceptor mixture to each tube. Shake. Place the racks in the water-bath and read results when some of the tubes are quite laked while others are quite opaque—*i.e.*, in about 15 minutes.

All the right-hand tubes should be laked completely, but as it requires considerable skill and practice to read the results at this stage it is advisable to allow the racks to stand at room temperature for two or three hours (or in the ice chest overnight) to deposit the corpuscles partially.

8. *Reading results.*—In reading the results it must be remembered that as individuals becoming infected with syphilis pass from negative to positive, and after successful treatment pass from positive to negative, varying degrees of inhibition may be met with from time to time.

If the supernatant fluid is colourless or only slightly tinged the result is to be considered "positive." Such reactions are diagnostic. Tubes showing inhibitions of lesser degrees than the above can be judged only when all the facts of the case, including treatment, are known. In the case of a known syphilitic patient these results should be returned as "slightly positive" or "negative, trace," according to the degree of inhibition. Otherwise they are to be returned as negative. Complete hæmolysis is a "negative" reaction.

Note.

Owing to the necessity for economy, the test has been carried out with half quantities for the past 5 months. Half-inch test tubes are used, with racks to fit. All the preliminary tests remain as above, but in the test proper everything is halved.

IV.—Method Number Four.

Requirements in apparatus, etc.

Stoppered glass, amber-coloured bottles	250 c.c. ...	6
	100 c.c. ...	6
	50 c.c ...	6
Glass graduated cylinders	25 c.c. ...	6
	50 c.c. ...	6
	250 c.c. ...	6
	1,000 c.c. ...	6
Erlenmeyer flasks	1,000 c.c. ...	6
	500 c.c. ...	12
	250 c.c. ...	12
Glass pipettes graduated to tip	10 c.c. in 0·1 c.c. ...	24
	5 c.c. in 0·05 c.c. ...	24
	1 c.c. in 0·01 c.c. ...	24
Wright's teat pipettes	0·5 c.c. ...	12
	0·25 c.c. ...	24
	0·1 c.c. ...	60

(These should be standardised to deliver the exact quantity of distilled water, at the normal rate of working, as weighed on a chemical balance.)

Test-tubes	12″ × 1″ ...	36
	6″ × ¾″ ...	4 gross
	3″ × ½″ ...	12 gross
Rubber teats		36
Electrically or water-driven centrifuge, four-bucket		1
Refrigerator		1
Dry air steriliser		1
Koch's steam ditto		1
Autoclave		1

Water-bath, thermo-regulated to 37° C., fitted with removable trays, with the front row of holes of each tray numbered in series, thus—first tray, 1–12; second tray, 13–24; and so on. Each tray to take 48 tubes in four rows of 12 each ... 1

Water-bath, thermo-regulated to 56° C., similar to above, but to hold two trays, each tray numbered in series as to every hole, thus—number 1 tray, 1–48; number 2 tray, 49–96 1

The glass-ware used for the test should invariably be scrupulously clean. The following procedure is recommended to insure perfect cleanliness:—

Immediately after use the test-tubes are placed in a large bowl of water, and the pipettes, after thorough rinsing, in a tall cylinder of water, and allowed to remain there for several hours. Reagents should never be allowed to dry on glass-ware. Test-tubes are then brushed out in running water, placed in a solution of washing-soda, and brushed again. Pipettes are rinsed out thoroughly in water, followed by soda solution. The glass-ware is then washed out in a weak solution of hydrochloric acid, thoroughly rinsed in tap-water until free from acid, and placed in distilled water for some hours. On removal, tubes, etc., are drained by placing them inverted in wire baskets, and finally dried by placing them in the hot-air steriliser for 30 minutes at 150° C.

Chemicals.

Sodium chloride.
Sodium carbonate.
Alcohol absolute.
Acid hydrochloric.

Special Reagents.

Complement.—The best complement for the Wassermann test is contained in guinea-pig serum. The animals should be well-fed and not pregnant. The blood may be obtained by cutting the animal's throat over a glass funnel; from the ear, if only a small quantity is required; or by heart puncture.

The blood, however obtained, should be placed in the incubator or water-bath at 37° C., and the serum removed from it as it separates. Finally, the serum should be centrifugalised until perfectly free from cells.

The complement-containing serum should be used from blood drawn on the day of the test, and its hæmolytic activity and other properties invariably determined by titration (as shown below) as a preliminary to every day's tests.

"Antigen" or Extract.—Of the various extracts which have been recommended at one time or another, that prepared from heart and reinforced with cholesterol, as recommended by McIntosh and Fildes, will be found simple to prepare and as satisfactory as any in use at the present moment. It may be prepared as follows:—Having obtained a fresh heart, cut off some pieces of the left ventricle, avoiding fat, mince in a machine, and weigh out 10 grammes of the mince. Place in a mortar with some clean sand, and grind up with 90 c.c. of absolute alcohol. Place the whole in a well-stoppered bottle, and shake at frequent intervals during the next few hours. Allow to stand over-night, and filter into a stoppered, amber-coloured, glass bottle through filter-paper which has been washed through with ether and allowed to dry. The filtrate should be allowed to stand for a week before being used. During this time a deposit settles to the bottom of the bottle; in drawing off extract for the tests the deposit should be avoided.

Extract will keep in good condition in the ice chest for six months.

Solution of Cholesterol.—Weigh out one gramme of pure cholesterol and place in a well-stoppered bottle with 100 c.c. absolute alcohol. Shake well, warming in a water-bath at 37° C. until the cholesterol has dissolved.

Immediately before the test three parts of the extract are mixed with two parts of cholesterol solution, and a 1 in 15 dilution of the mixture is made in 0·85 per cent. saline solution in the following manner:—

The necessary amount of heart-extract-cholesterol mixture is placed in a large test-tube, and the saline added suddenly to it.

Suspension of Washed Sheep's Cells.—A number of large glass beads are put into a stoppered bottle of litre size, and the whole sterilised in an autoclave, or a Koch's steamer. The sterile bottle is taken to the slaughter-house and half filled with sheep's blood, after the sheep has bled for a few minutes. The stopper is replaced, and the bottle vigorously shaken for at least five minutes. Until required for use the blood should be kept in the refrigerator, and fresh blood should be obtained every three days. Instead of being whipped, the blood may be received into an equal quantity of a 2 per cent. solution of citrate of soda in 0·85 per cent. salt solution. To prepare the suspension of washed sheep's cells, pour about 5 c.c. of the whipped blood into each of two 15 c.c. centrifuge tubes and fill up to the top with sterile, 0·85 salt solution. Spin until the cells are all deposited. Remove the supernatant fluid, replace with saline, and shake. Repeat the operation four times, using a clean pipette each time. Finally, remove the supernatant saline and make a 6 per cent. suspension of the deposit in saline.

Anti-sheep Cell Amboceptor may be prepared as described on p. 16, or obtained from a central laboratory. Whether prepared locally or obtained elsewhere, it should invariably be titrated before being taken into use, and at intervals of about three months afterwards. An amboceptor of lower titre than 1 in 1,000 should not be used. The method of titration is described below.

Serum to be Tested.—The blood (at least 1 c.c.) may be obtained from the patient by pricking the thumb or lobe of the ear and pipetting off the blood with a capillary pipette into a small test-tube; or by veni-puncture as follows:—Sterilise a needle of about 56 gauge in alcohol and flame off the alcohol. Sterilise a test-tube by steaming, and make sure afterwards that no water is left in the tube. Fasten a rubber band round the upper arm to make the distal veins prominent, and paint the selected site with iodine. Fix the skin below the site of the puncture by grasping the fore-arm, and, keeping it almost parallel with the line of the vein, with the bevel looking upwards, push the needle into the vein. Receive the blood into the test-tube. A convenient device for the purpose is that illustrated in Fig. 4, p. 42. The blood is put on one side for a few hours until the serum has separated. The clear serum is prepared for the test by heating it for half-an-hour at 55° C., as will be shown below.

It is of the highest importance that, as soon as the specimen of blood has been obtained, it should be labelled with an adhesive paper label on which the patent's identification particulars are written in ink. This should be done before the specimen leaves the hand. The particulars as written on the label must correspond with those entered in the record book; the latter should again be verified before going on to another patient.

In the laboratory, as a preliminary to the day's tests, it is necessary to pipette off the required amount of serum into a small test-tube and inactivate it by heating. This operation may easily be a source of error from the confusion of one patient's serum with another's, and, as such an error may lead to a patient's serum being returned as positive when negative, and vice versa, it it most important that the sera be laid out on such a system as will prevent any possibility of confusion. The following system is recommended as least likely to lead to error:—

(1) Draw a red line in the record book above the particulars of the first patient whose serum has to be tested on the day in question, and another red line below the particulars of the last patient.

(2) Write just below the top red line the number of the syphilitic serum chosen for the syphilitic control, and just above the bottom red line that of the serum chosen for the normal control.

(3) Having centrifugalised those specimens which require it in order to obtain clear serum, lay out the specimens in a rack as shown in Fig. 5, in the order in which they appear in the record book, commencing with the syphilitic control and ending with the normal.

(4) Number the identification particulars in the book and on each specimen serially in red ink, thus—syphilitic control in book and on specimen, "1"; first patient's serum, "2"; and so on to the normal control. Compare all particulars on specimens and in the book to make quite sure that they correspond.

(5) Place in the hole provided for it in front of each specimen a small test-tube, 3 in. by ½ in., numbered to correspond with the red ink number on the specimen in front of which it stands.

(6) Commencing with Nr. 1, pipette or pour from the original specimen about 0·5 c.c. clear serum into the empty test-tube standing in front of it and bearing the corresponding number. As each specimen of serum is placed in the small test-tube, put the latter into that hole of the inactivating bath-rack the number of which corresponds to the number on the test-tube. Remove each original specimen from the rack and put it out of the way as soon as it has been disposed of in the above manner.

(7) Place the loaded rack in the inactivating water-bath and heat at 55° C. for half-an-hour.

(8) Remove the rack from the water-bath and lay out the sera as shown below under test-proper.

Titration of Reagents.

Hæmolytic Immune Serum or Amboceptor.—This should be titrated in order to determine the minimum amount of it which is required to produce complete hæmolysis of the standard amount of cells used in the test, when in the presence of excess of complement. The titration should be carried out on receipt of every fresh supply of amboceptor and at intervals of about three months afterwards.

Prepare a series of dilutions of the amboceptor serum as follows:—With a Wright's pipette measure 0·1 c.c. of the serum into a test-tube, and add to it 9·9 c.c. saline, making thus a dilution of 1 in 100. Of this dilution, take 0·5 c.c., and add to it 4·5 c.c. saline, producing a 1 in 1,000 dilution.

Successive dilutions are then made as follows:—

0·25	c.c.	of	1	in	1,000	and	0·25	c.c.	saline	= 1 in 2,000
,,	,,	,,	,,	,,	,,		0·5	c.c.	,,	= 1 in 3,000
,,	,,	,,	,,	,,	,,		0·75	c.c.	,,	= 1 in 4,000

and so on to—

0·25	c.c.	of	1	in	1,000	and	2·25	c.c.	,,	= 1 in 10,000

Of each of these dilutions take 0·25 c.c., place in a small test-tube, and add 0·5 c.c. of a 1 in 4 dilution of the 6 per cent. cell suspension prepared as shown above; the dose of cells per tube is thus 0·25 c.c. of a 3 per cent. suspension. Add to each tube 0·25 c.c. of a 1 in 10 dilution of the guinea-pig serum. Incubate in the water-bath at 37° C. for one hour, and find the tube containing the greatest dilution of amboceptor in which there is complete lysis of the cells. This dilution expresses the minimum hæmolytic dose (M.H.D.) of the amboceptor in question. No amboceptor should be used the M.H.D. of which is less than 1 in 1,000.

Preparation of Sensitised Cells.—As a general rule it will be found that cells sensitised with 5 M.H.D. give complement titres which are as high as can be obtained. The number of doses of a given amboceptor required to produce highest possible complement titres may be determined more closely by repeating the titration of complement, as shown below, against cells sensitised with varying amounts of the amboceptor in question, but for ordinary purposes it serves to sensitise with 5 M.H.D. as follows:—Prepare a dilution of the amboceptor serum which is ten times as strong as that representing the M.H.D., and add it quickly to an equal volume of a 6 per cent. suspension of cells. Thus, say the M.H.D. is 1 in 5,000 and 30 c.c. sensitised cells are required for the day's tests, prepare 15 c.c. of a 1 in 500 dilution of the amboceptor and pour it into 15 c.c. of the 6 per cent. suspension of cells, which thus becomes a 3 per cent. suspension in a 1 in 1,000 dilution of amboceptor.

Complement Titration, Alone and in the Presence of "Antigen."—This should be carried out as a preliminary to each day's tests. Prepare a 1 in 5 dilution of the guinea-pig's serum, and from this make successive dilutions as follows:—

0·25	c.c.	of	a	1	in	5	dilution	and	0·75	c.c. saline = 1 in 20
,,	,,	,,	,,	,,	,,	,,			1·0	c.c. ,, = 1 in 25
,,	,,	,,	,,	,,	,,	,,			1·25	c.c. ,, = 1 in 30
,,	,,	,,	,,	,,	,,	,,			1·50	c.c. ,, = 1 in 35
,,	,,	,,	,,	,,	,,	,,			1·75	c.c. ,, = 1 in 40
,,	,,	,,	,,	,,	,,	,,			2·25	c.c. ,, = 1 in 50
,,	,,	,,	,,	,,	,,	,,			2·75	c.c. ,, = 1 in 60
,,	,,	,,	,,	,,	,,	,,			3·75	c.c. ,, = 1 in 80

Lay out 24 small tubes in a rack, in three rows of eight each. Pipette 0·25 c.c. of the 1 in 80 dilution of complement into the first tube of each row; pipette the same quantity of the 1 in 60 dilution into the second tube of each row; and so on to the 1 in 20 dilution.

Place 0·5 c.c. saline into each tube of the first row and 0·25 c.c. into each of the second row. Place 0·25 c.c. of the 1 in 15 dilution of heart-extract-cholesterol mixture in each tube of the second row. After allowing them to stand for half-an-hour at room temperature put the tubes of the first and second rows into the water-bath at 37° C., and after another half-hour add to each tube 0·25 c.c. of the 3 per cent. suspension of sensitised cells. Thus the total volume of fluid in each tube is 1·0 c.c., as in the test proper. Shake at once and every 10 minutes afterwards. At the end of half an hour read the result. The tube in the front row, which shows complete hæmolysis and contains the highest dilution of complement, represents the minimum hæmolytic dose of complement. The tube in the second (antigen + complement) row containing complement twice as strong as the above should also show complete hæmolysis. Thus, if the last tube in the front row which showed complete hæmolysis were that containing 1 in 80 complement, this would represent for the purpose of the test the hæmolytic dose of the complement; then in the second row hæmolysis should be complete in the tube containing 1 in 40 complement. If not, the complement should be rejected. Complement, the titre of which is less than 1 in 30, should also be rejected.

The third row is reserved for incubation with the test proper, and serves to detect any deterioration of the complement which may have occurred during the time it was standing before addition of the sensitised cells in the test proper.

The Test Proper.

Remove from the water-bath as many trays as will be required for the test, commencing with that numbered 1-12, and so on serially. Set out the inactivated sera in the back row of each rack, in serial order—Nr. 1, the control syphilitic serum, being opposite Nr. 1 hole, and so on to the last number, the control normal serum. Fill the remaining holes of each rack with empty test tubes so that three tubes stand in front of each specimen of inactivated serum. Take Nr. 1 serum and measure 0·25 c.c. of it into a small test-tube (not in this rack). Add to it 1 c.c. saline. Mix and measure 0·25 c.c. of the 1 in 5 dilution so obtained into each of the three tubes standing in front of Nr. 1 serum. Repeat the procedure with Nr. 2 and the remainder of the sera in turn.

Make a dilution of the complement which is three times as strong as that representing the minimum hæmolytic dose. Thus, if the M.H.D. of the complement on the day in question were 1 in 60, a dilution of 1 in 20 would be made here. Add 0·25 c.c. of this dilution to each tube of the front and third rows. Similarly, make a dilution of the complement which is five times as strong as that representing the minimum hæmolytic dose. Add 0·25 c.c. of this dilution to each tube of the second row.

Add 0·25 c.c. of the 1 in 15 dilution of antigen, prepared as already described, to each tube of the first and second rows, and 0·25 c.c. saline to each tube of the third row.

Also add 0·5 c.c. saline to each tube of the complement titration row which was held in reserve when this operation was carried out.

After allowing all the tubes to stand at room temperature for half an hour, place the racks in the water-bath at 37° C. After half an hour add to each tube of the first three rows and of the complement titration reserve row 0·25 c.c. sensitised cells. Shake at once, and again after 15 minutes. Watch the third row (serum control) and mark for re-test, if necessary, any sera where hæmolysis lags definitely behind the others in this row. Watch the last set of three tubes (normal serum control) for hæmolysis. Ten minutes after hæmolysis is complete in the front row tube of this set, remove the tubes from the warm bath and place them in a water-bath containing ice. Read the results in an hour or so, when the cells will have settled somewhat, and the condition of the supernatant fluid can be gauged more easily. With experience, it will be found easy to read results 10 minutes after the serum control tubes show complete lysis.

Reading of Results.

(1) Where any third row tube has lagged behind the others in showing complete lysis the test of the serum of which that tube is the control should be rejected and the test repeated with another specimen, unless there is hæmolysis of the cells in the front row tube, indicating that the serum is giving a negative reaction.

(2) If the front row tube of the Nr. 1 set (syphilitic serum control) shows any hæmolysis, or the corresponding tube of the last set (normal serum control) fails to show complete hæmolysis, the whole set of tests should be rejected. This is a very rare occurrence, and investigation would usually disclose an error in technique. The tests must be repeated with another complement, and it is advisable then to introduce three or four additional syphilitic and normal controls.

(3) The following notation is suggested for recording the different grades of reaction:—

(*a*) Complete inhibition (no tinge of hæmoglobin in the fluid around or above the cells) in the front and second row tubes = ++

(*b*) Complete inhibition in the front row tube, some opacity in the second row tube, but also some hæmolysis = +±

(*c*) Complete inhibition in the front row tube but complete hæmolysis in the second row tube = +

(*d*) Slight hæmolysis in the front row tube = ±

(*e*) Considerable hæmolysis in the front row tube = $\underline{\pm}$

As a matter of precaution it is advisable to re-test sera which give merely + where the case has not been diagnosed as syphilis previously.

Interpretation of Results for Purposes of Diagnosis, etc.

Where the case has not previously been diagnosed as syphilis (by finding of spirochætes or previous Wassermann test), no weaker reaction than + should be accepted for diagnostic purposes.

Where, in such cases, the reaction is ± or $\underline{\pm}$, it should be regarded with suspicion, and steps taken to repeat the test on another specimen taken at an early date, preferably after a provocative injection of "606" or "914."

In cases where the patient has already been diagnosed as syphilitic, and the test is required as a guide to the administration of further treatment ± or $\underline{\pm}$ should be regarded as an indication that such further treatment is necessary.

Elaboration of the above Test for the Purpose of Indicating more closely the Strength of the Reaction.

Three courses are available:—

(1) To test the serum against diminishing quantities of extract, keeping the amounts of the other ingredients the same.

(2) To use diminishing amounts of the serum, keeping the amounts of the other ingredients the same.

(3) To test the serum against increasing quantities of complement such as 3, 5, 10, and 15 doses.

In the case of (1) and (2) the strength of the reaction would naturally vary inversely with the amount of the varied ingredient required to deviate three minimum hæmolytic doses of complement. In the case of (3) the strength of the reaction would be determined by the number of doses of complement deviated. Of these methods, (2) and (3) are recommended.

FIG. 4.

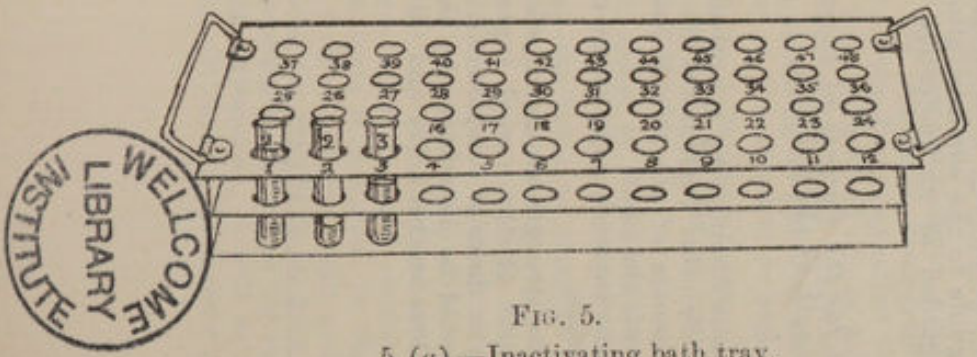

FIG. 5.

5 (*a*).—Inactivating bath tray.

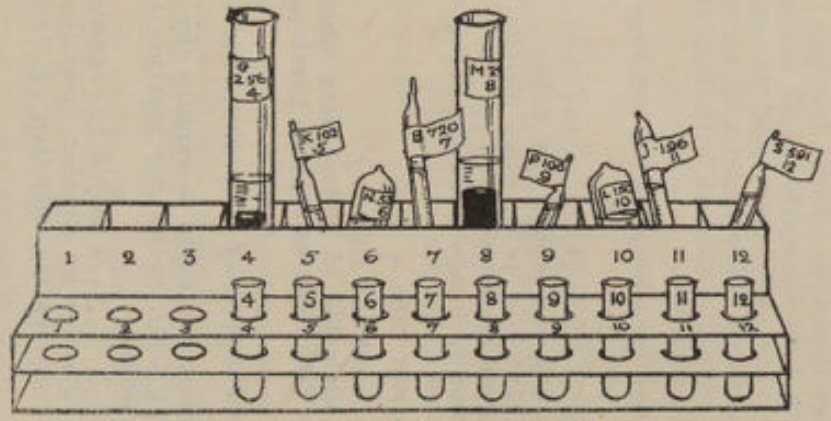

FIG. 5.

5 (*b*).—Tray for arrangement of specimens in serial order. Letter and upper number are identification particulars. Lower number is serial number in day's test.

5 (*c*).—Specimens put away.

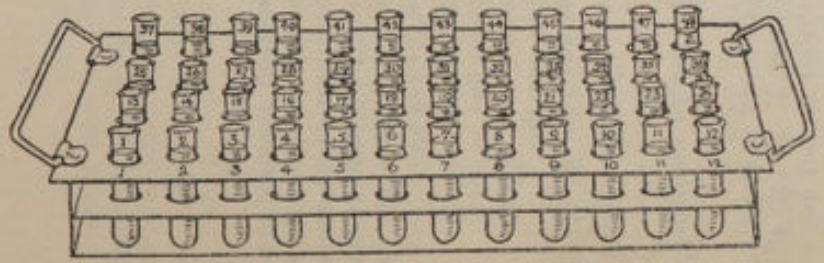

FIG. 6.

Inactivating rack filled.

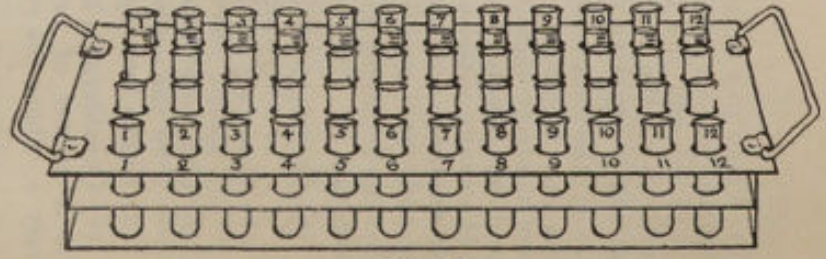

FIG. 7.

Arrangement for test proper. Sera in back row. Three empty tubes in front of each serum.

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