SUGGESTIONS FOR A NEW CRITERION OF A POSITIVE WASSERMANN REACTION BASED ON AN ANALYSIS OF 2334 QUANTITATIVE TESTS[1].

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(With 3 Text-figures.)

CONTENTS.

I. INTRODUCTION.

The criterion of a positive result in the Wassermann test, which is commonly adopted, is that a given amount of complement, which along with a known negative serum and the "antigen," causes complete lysis of the test corpuscles, will with a positive serum in the same combination show no lysis or, at most, a faint trace, i.e. lysis not exceeding about 30 per cent. of the corpuscles. This may be termed the "all or nothing" criterion. Some workers adopt a fixed volume of guinea-pig's serum, e.g. in the original method of Wassermann and his co-workers, but it is to be remembered that the amount which these observers employed at first was subsequently reduced by half. Others (e.g. Boas, Fildes and McIntosh) employ a definite number of haemolytic doses of complement. The results thus obtained, although they afford useful indications, are by no means perfectly satisfactory. Thus Wassermann's one tube method neglects the following facts: (1) that the haemolytic power of different

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guinea-pigs' sera may vary considerably; (2) that there is no fixed relationship between deviability and dosage (see Table III); and, further (3) that since only one amount of complement is used, it is not possible to differentiate degrees of positiveness satisfactorily. With regard to the last-mentioned point, the range of lysis obtained with the mixture of patient's serum and antigen along with varying amounts of complement is not necessarily similar with different patients' sera (see Table I); also, owing to the comparatively large amount of complement employed by Wassermann, it is probable that a number of the less pronounced, but still significant, positive reactions are missed. This is a very serious defect when employing the reaction in the control of treatment or when cases are examined at an early or latent stage of the infection. Boas, in order to introduce a quantitative gradation, employs a series of varying amounts of patient's serum along with a definite number of haemolytic doses of complement, and this procedure was also adopted by Fildes and McIntosh; here again variations in the deviability of different specimens of complement are neglected. Latterly Fildes and McIntosh have used only a single amount of serum. All of the criteria employed above, therefore, are unsatisfactory in as much as they fail to allow for the varying sensitiveness of complement to the reagents, antigen and patient's serum, separately or together. Accordingly, it is necessary to discard the results when the control tests show that the complement is abnormal in its behaviour.

Browning and Mackenzie (1909) showed at a comparatively early period that when a given serum was tested with two different specimens of complement, all the other reagents being identical, the degree of positiveness as ascertained by the two series might show a marked difference. Recently Browning and Kennaway (1919) have reported the results obtained when the same specimen of syphilitic serum was examined for complement fixation in the Wassermann test on a number of different occasions and have found that: (1) the actual amounts of complement fixed, reckoned either by volumes or as haemolytic doses, varied greatly on the different occasions, and (2) the variations were quite irregular, and depended on factors which cannot, so far, be rendered constant. Thus it was not possible to fix upon any single amount of complement the behaviour of which, along with "antigen" and patient's serum, would determine whether that serum was positive or negative. In the modified original technique of Wassermann 0·05 c.cm. of complement was employed, under specified conditions, in all cases; sera showing no lysis with this standard amount were regarded as positive, and those showing complete lysis as negative. But the results with the positive serum obtained by Browning and Kennaway showed that on one day 0·04 c.cm. complement sufficed to produce commencing lysis, whereas on other occasions no less than three times this amount was required. Browning and Mackenzie (1911) adopted originally as the standard of a positive serum that along with the antigen it should require at least five doses of complement in addition to the sum of the amounts inhibited by the serum and the antigen separately,

in order to produce complete lysis of the test corpuscles. These workers also specified the limits of inhibitory effect on complement which each constituent might exercise by itself without disturbing the results of the test. A fixed amount of patient's serum and a series of varying doses of complement were accordingly employed. The method, however, appears to have been regarded as too complicated to commend itself generally and the number of tubes originally recommended (five or six) was such as to consume too much time in the performance of the test. It is obvious, however, that for a satisfactory method of performing the test it is necessary to institute a quantitative comparison between the complement-inhibiting property of the mixture of patient's serum and antigen on the one hand and a known negative serum along with antigen on the other. Accordingly, the object of the present investigation was to ascertain whether the study of the numerical results of a large number of Wassermann tests in which the actual amount of complement fixation was determined, would suggest any rule, more satisfactory than those already in use, by which the distinction between "negative" and "positive" sera could be defined. Clearly no system can wholly eliminate those reactions which must be reported as "suspicious," "doubtful," or "±," since the serum of every patient who has recently become infected, or who is passing into the state of latent infection either as the result of treatment or spontaneously, must at some period be in transition from the negative to the positive condition, or *vice versa*. But for purposes of clinical diagnosis it is well to determine how far the use of such terms may be restricted to a minimum.

II. METHOD OF ANALYSIS OF RECORDS.

With the above object in view, the quantitative records of over 2000 Wassermann reactions were expressed in graphic form, in the hope that the curve of distribution thus obtained would give some indication of the point at which the transition from negative to positive occurs. Three series of records were examined, comprising 2334 sera in all; Series I contained 540 sera, Series II, 533, and Series III, 1261. A fourth series of 508 sera is also utilised in the last section of this paper. The results in the first series were obtained during 55 days of routine testing, those in the second series during 37 days, and those in the third during 69 days. The first, third and fourth series comprise specimens of blood submitted for diagnosis from the wards and out-patient departments of a general hospital; a certain number of cases which have undergone anti-syphilitic treatment are, of course, present among these. We are indebted for Series II to the records of Dr H. Ferguson Watson; the cases were examined by him and were quite independent of the other series. Further, in Dr Watson's series there are certainly very few, if any, treated cases, as the specimens were taken from children and adults with a view to determining the prevalence of syphilis and not in order to diagnose the nature of diseased conditions.

The records in Series I and II were dealt with in the following manner. The amount of complement required to produce just complete lysis with the negative control serum in the presence of antigen is reckoned as 100. The amounts of complement required to produce just complete lysis with each of the other sera tested upon the same occasion can then be expressed as percentages of the amount required by the negative control serum. In this way a quantitative comparison of the character of the different sera is obtained. Further, if the point of initial lysis, as well as that of complete lysis, can be observed, the amount of complement required to complete the haemolytic process when once begun can be expressed upon the same system of percentages. This latter amount is remarkably variable even in groups of sera which require the same amount of complement to produce complete lysis; some will show a short, others a long range of partial lysis. An example is given in Table I below; in this, the range with one serum (*N*) is about twice as great as it is with the other (*A*). This feature is obviously a subject for study, although its significance is by no means clear.

Table I.

Example of difference in range of lysis with different positive sera tested with the same complement. Antigen, liver-lecithin + cholesterol.

Date: 15. x. 12.

					Serum control	
Complement c.c.	0·024	0·035	0·05	0·07	0·01	0·02
Serum (*A*)	None	None	Trace	Complete	Almost complete	Complete
Serum (*N*)	Faint trace	Trace	Almost complete	Just complete	Almost complete	Complete

One point of great practical importance immediately arises out of this fact, however, viz. the unsatisfactory character of any method which employs a single amount of complement, e.g. Wassermann's original procedure. In examining a series of quantitative tests it is easy to pick out sera which along with antigen give marked or complete lysis in a mixture containing 0·05 c.c. complement and which, therefore, would be returned as negative; but a quantitative examination shows that they give little or no lysis with 0·03 c.c. complement, whereas the negative control is complete with 0·01 c.c.

The application of the method of calculation described above may be illustrated by the following examples (Table II). The amount of complement required to produce just complete lysis with the negative control serum (0·015 c.c.) is taken as 100; the amounts in the other three tubes (0·03, 0·045, and 0·06 c.c.) are therefore represented by 200, 300, and 400. With serum *A*, lysis begins at some point below 100, and extends to 200. With serum *B*, lysis begins near 200 and extends to 400. With serum *C* lysis begins near 300 and extends beyond 400, the whole range not being observed. These results may be represented graphically as in Fig. 1. The arrows indicate that the complete range of lysis could not be observed within the series of tubes employed.

Table II.

Tube	(1)	(2)	(3)	(4)
Amounts of complement in c.c.	0·015	0·03	0·045	0·06
Doses	2	4	6	8
Percentage	100	200	300	400
Negative control serum . .	[1]Just complete lysis	Complete lysis	—	—
Serum *A*	Very marked lysis	[1]Just complete lysis	Complete lysis	
Serum *B*	No lysis	Trace of lysis	Distinct lysis	[1]Just complete lysis
Serum *C*	No lysis	No lysis	Trace of lysis	Distinct lysis

[1] I.e. the fluid is nearly but not perfectly clear, and a trace of red corpuscles, amounting to about two per cent. of the total quantity originally added, is visible at the bottom of the tube on standing.

Haemolytic dose of guinea-pig's complement for 0·5 c.c. 3 per cent. suspension of ox or sheep corpuscles sensitised with at least 5 doses of immune body = 0·0075 c.c.

The great advantage of this method of representing the results is the fact that the behaviour of the known negative serum constitutes its basis[1]. This must always be the chief guide in diagnosis, since it serves as a control upon the variations which occur both in the deviability of complement (in the direction especially of over-deviability)[2] and in the complement-absorbing power of different samples of antigen. However, when any large series of

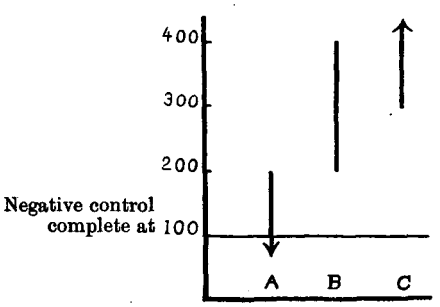

Fig. 1.

results is dealt with in this way the following difficulties, omitted purposely from the examples given above, are encountered. (1) The negative control may show quite complete lysis in the first tube; the base-line (100) cannot then be exactly ascertained, as the amount of complement required to produce just complete lysis is unknown. The results obtained on such days are, therefore, not perfectly suitable for presentation by this method. (2) In a large proportion of cases, the points of initial and of just complete lysis cannot be observed; they will either lie outside the series of tubes, as in sera *A* and *C*

[1] Considerable importance attaches to the choice of a negative control serum. This point is discussed in detail on p. 93.

[2] The use of a known weak positive control serum is chiefly of value for controlling the deviability of the complement in the direction of under-deviability (see p. 104).

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above, or they will, so to speak, fall between two tubes. A number of tubes impracticable in routine work would be required in order to obtain results ideally suitable for statistical purposes. Suppose for instance, that lysis is well advanced, but by no means complete, in one tube of the series, and is quite complete in the next; where is the point of just complete lysis? The point in question can be expressed as a percentage only by a process of guessing which cannot be dignified by the term "interpolation." If all sera exhibited the same range of lysis (i.e. required the same amount of complement in order to cause the same increment of lysis, e.g. from distinct to very marked), and differed only in the region of the whole scale in which this range lay, it would be possible to interpolate in a satisfactory manner; but, as was pointed out above (see p. 90), this extension of partial lysis is a most variable character. In practice one must judge as best one can, from the rate of advance of lysis seen in the preceding tubes, whereabout the point of just complete lysis would lie. An alternative method which would eliminate guessing, would be to take as "just complete" always the first tube showing "quite complete" lysis; but owing to the necessarily large intervals (generally 100 per cent.) between the amounts of complement employed in successive tubes, this would seem to involve a greater error than does guessing, at any rate in cases where lysis is far advanced in the preceding tube. (3) As one cannot in practice measure and record all the different degrees of partial lysis which occur, the results fall inevitably into groups which do not give a strictly accurate representation of the facts. These degrees of partial lysis are, of course, infinite in number, and could be estimated with accuracy only by the examination of the contents of each tube in a haemoglobinometer, which is not practicable. Accordingly, in recording the results of Wassermann reactions we employ only eight terms to denote the stages of lysis; the quantitative significance of these terms is discussed below (p. 95). The grouping which results from this necessary limitation in the number of descriptive terms is evident in the general form of the curves shown in Figs. 2 and 3. However, this source of error tends to produce a series of flat segments or steps in the distribution curve, and one must, therefore, attach the more significance to any portions of it which show a gradient. Thus, it is evident that the records of Wassermann reactions cannot yield material which is thoroughly suitable for statistical purposes unless (1) a very long series of tubes be used, and (2) the degrees of lysis be estimated by some accurate method. Since it is impracticable to realise these conditions in routine work, one must either abandon examination of the abundant material which is at hand or make what use of it one can in its relatively defective state. In view of the importance of the question involved and of the fact that we are probably in possession of the fullest data available on the subject, we have judged it advisable to make a comprehensive examination.

III. THE NEGATIVE CONTROL SERUM.

The quantitative method of performing the Wassermann reaction proved clearly at an early stage that all negative sera are not alike in their behaviour. Thus in Figs. 2 and 3, a number of sera are represented at the left-hand side of the graph as lying below the horizontal line at 100; that is to say, they are more negative than the negative control and the majority of the negative sera. They amount to 54 out of the 760 sera in the two figures, and constitute 7 per cent. of the whole number, or 11 per cent. of the negatives (i.e. those showing complete lysis at or below 150). On the other hand, some non-syphilitic sera, including those from certain adults who are apparently in good health, will tend to fix more complement in the presence of antigen than the great majority of other negative sera do. To these sera we have applied the term of *border-line negatives*. They are not by themselves specially anti-complementary. But with a hyper-sensitive complement such sera will yield an apparently positive reaction, while in the same series many other sera will react definitely negative; this is illustrated in Table III. The experiments

Table III.

Behaviour of different non-syphilitic sera tested simultaneously with two complements, illustrating an extreme degree of over-deviability in Complement II. Antigen, liver-lecithin + cholesterol.

Complement, c.c.	0·015	0·025	0·04	0·06	0·09	Serum control 0·015
Complement I (M.H.D. ·005 c.c.)						
Negative control serum *A*	—	Very marked	Complete	—	—	Complete
Negative control serum *B*	—	Complete	—	—	—	Complete
Emulsion control	Complete					
Complement II (M.H.D. ·005 c.c.)						
Negative control serum *A*	—	None	None	Very marked	Complete	Just complete
Negative control serum *B*	—	Faint trace	Trace	Very marked	Complete	Complete
Patient's serum *C*	—	Complete	—	—	—	Complete
Emulsion control	Complete					

recorded in this table were carried out upon the same day with two samples of complement (I and II) each obtained from the pooled blood of two guinea-pigs and treated in the same manner before use; all the other reagents employed were the same throughout the series. While both complements showed a normal behaviour in the serum and antigen controls, and had the same M.H.D. (0·005 c.c.), Complement II showed extreme over-deviability in the presence of sera *A* and *B* together with antigen, while another serum (*C*) gave a typical negative result. The negative character of sera *A* and *B* is known from many years' experience, yet with Complement II they require no less than eighteen M.H.D. to give complete lysis. Complement II represents

an excessive degree of over-deviability, which in our experience is extremely rare; but the results illustrate what is, in a less pronounced form, very common.

We have, therefore, attached great importance to the use of such a negative serum and have for several years past constantly employed as the negative control a serum of this kind derived from one individual. Its normal behaviour thus becomes known, and any unsuitability in the other reagents is the more readily detected, and if not too great, allowed for. A stock sufficient for several months can be kept frozen in amounts each suitable for a single day's work, whereby repeated thawing is avoided[1]. In this laboratory we have now used the serum of one person upon 355 occasions. This serum constitutes the negative control in Series III (heart-cholesterol antigen) and IV and on six of the 55 occasions in Series I; in Series II for which the serum in question was not available, the negative control differed from day to day and was not selected on the above principle. The results in Series II indicate that the conclusions arrived at in this paper regarding the criterion of a positive reaction hold generally and are not dependent on the use of any particular negative control serum. Thus when the use of a single serum, such as that described above, is impracticable we would suggest that *the negative control should consist of a pooled specimen of at least twelve negative sera derived from cases which have not recently received anti-syphilitic treatment and in which there is no question of early syphilis before a positive reaction has developed.*

IV. TECHNIQUE EMPLOYED IN THE TESTS.

All the Wassermann tests of which the records were utilised in Series I and II were carried out by the method of Browning, Cruickshank and Mackenzie. Two antigens were employed, namely a solution of ox-liver "lecithin" and the same with the addition of 1·2 per cent. of cholesterol; one volume of the alcoholic solution was mixed slowly with seven volumes of saline, and each tube received 0·3 c.c. of this emulsion together with 0·025 c.c. of patient's serum. Only those results obtained by the use of the latter antigen are dealt with here, as the process of drawing any conclusion is very much complicated if two figures have to be considered in the case of each serum. In Series III, the antigen was a rapidly mixed 1 in 30 dilution of a mixture of 3 volumes of alcoholic extract of human heart and 2 volumes of a 1 per cent. solution of cholesterol, i.e. it corresponds to the antigen employed in the method of Fildes and McIntosh. Each tube received 0·5 c.c. of emulsion and 0·05 c.c. of patient's serum. The haemolytic system consisted of 0·5 c.c. 3 per cent. suspension of washed ox (or occasionally sheep) blood sensitised with at least 5 doses of immune body from the rabbit[2].

[1] The temperature was maintained at $-15°$ C. or lower. It is possible that temperatures more closely approaching 0° C. may be less suitable owing to the occurrence of autolytic changes. This point requires further investigation.

[2] For further details of the methods see Browning and Watson (1919), *Venereal Diseases*. London.

V. METHOD OF RECORDING DEGREES OF LYSIS.

The various degrees of lysis which occur in the series of tubes employed in the test have been described in the records by an arbitrary series of terms, namely: *faint trace*, *trace*, *distinct*, *marked*, *very marked*, *almost complete*, *just complete*, *complete*. In order to ascertain the range of lysis over which the observers concerned would apply each of these descriptive terms, a number of experiments with mixtures of known proportions of lysed and unlysed corpuscles were made in the following manner: 3 c.c. of washed red corpuscles are mixed with 7 c.c. of normal saline; 5 c.c. of this suspension are made up to 50 c.c. with normal saline to form a 3 per cent. suspension of corpuscles as used in the Wassermann test. The remaining 5 c.c. of the mixture are added to 41 c.c. of distilled water; when lysis is complete, 4·2 c.c. of 10 per cent. sodium chloride are added, giving a strength of 0·85 per cent. salt in the whole amount; the lysed solution will then not produce any further lysis when mixed with the suspension of corpuscles. Mixtures are then made as indicated in Table IV; the last column shows the manner in which the terms in question were found to be employed.

Table IV.

0·4 *c.c. normal saline is placed in each tube to represent the volume of the other constituents present in a Wassermann test.*

Tube	Unlysed suspension c.c.	Lysed solution c.c.	Percentage of corpuscles lysed	Terms used to record degrees of lysis
1	0·4	0·1	20	Faint trace
2	0·35	0·15	30	
3	0·3	0·2	40	Trace
4	0·25	0·25	50	
5	0·2	0·3	60	Distinct
6	0·15	0·35	70	
7	0·1	0·4	80	Marked
8	0·05	0·45	90	Very marked
9	0·025	0·475	95	Almost complete
10	0·02	0·48	96	
11	0·01	0·49	98	Just complete
12	0·005	0·495	99	
13	0·0	0·5	100	Complete

It is evident that the system given in this table is by no means ideal for statistical purposes. Firstly, from a quarter to a half of the corpuscles require to be lysed before the appearances which one would describe as "faint trace" or "trace" are produced; the point of commencing lysis cannot therefore be determined with much accuracy. Secondly, as it is much easier to estimate the smaller than the larger amounts of corpuscles, the later stages of lysis are over-represented; three of the seven percentages lie at or above 90. However, these defects seem unavoidable by any method which is practicable in testing large numbers of sera; and further, for the purpose of the present

investigation, the point of complete, or rather just complete, lysis is by far the most important, hence defective observation of the early stages of lysis is of no great significance.

VI. DISCUSSION OF RESULTS.

The range of lysis observed with each serum was expressed graphically in the manner described above (p. 91); thus the amount of complement required to produce complete lysis with the negative control serum was reckoned as 100, and the amount required with each of the other sera to produce the whole range of lysis observed was expressed upon the same scale. The result is shown in Figs. 2 and 3. (1) The sera are plotted from left to right in the order of magnitude of the amounts of complement which they require in order to give complete lysis, that is, in the order of height reached by the vertical lines. (2) When lysis is complete in the first tube, the serum in question is represented by a dot only, placed at the level corresponding to the amount of complement in this tube. (3) A horizontal line is drawn across each figure at the level 100, which represents complete lysis with the negative control serum. Dots falling upon this line are not represented, but the length of this line between 50 and 100 is of course proportional to the number of these dots; there would be 199 dots on this line in Fig. 2, and 91 in Fig. 3. (4) A "faint trace" or "trace" of lysis has unavoidably to be taken as indicating the actual starting point of the process, though this is of course not accurate (see Table IV). (5) When the degree of lysis in the first tube is more than a trace, but is still incomplete, the line representing the serum bears an arrow directed downwards, to indicate that no point of initial lysis could be observed. (6) Sera with which lysis is still incomplete in the last tube, that is, with the largest amount of complement employed, are excluded from the graph, since it is the position of the point of complete lysis which is the basis of classification. The latter sera are all undoubtedly positive, and their removal does not affect the consideration of the doubtful zone. The negative control sera of each of the 92 days' tests are also excluded. By this elimination, the number in Series I is reduced from 540 to 408 and in Series II from 533 to 352.

In considering the results presented in the graphs Figs. 2 and 3, the sera were divided into groups, each increase of 50 per cent. in the amount of complement required to produce complete lysis constituting a group. Thus Group I shows complete lysis with not more than 50 per cent. of the amount of complement giving complete lysis with the negative control serum; Group II shows complete lysis with 51 to 100 per cent. of this amount, and so on up to Group X which extends from 451 to 500 per cent. The limits of each group are marked upon the horizontal line at the top of each figure. The length of each group was then measured, and these lengths calculated as percentages of the total length of the series; the distribution of the sera was thus ascertained, and is shown in numerical form in Table V.

It is at once evident upon inspection of Figs. 2 and 3 that no very abrupt

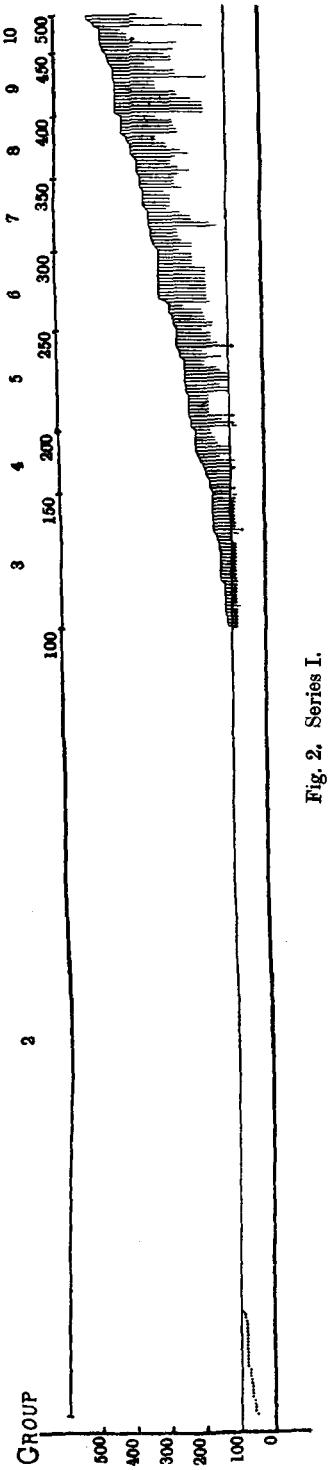

Fig. 2. Series I.

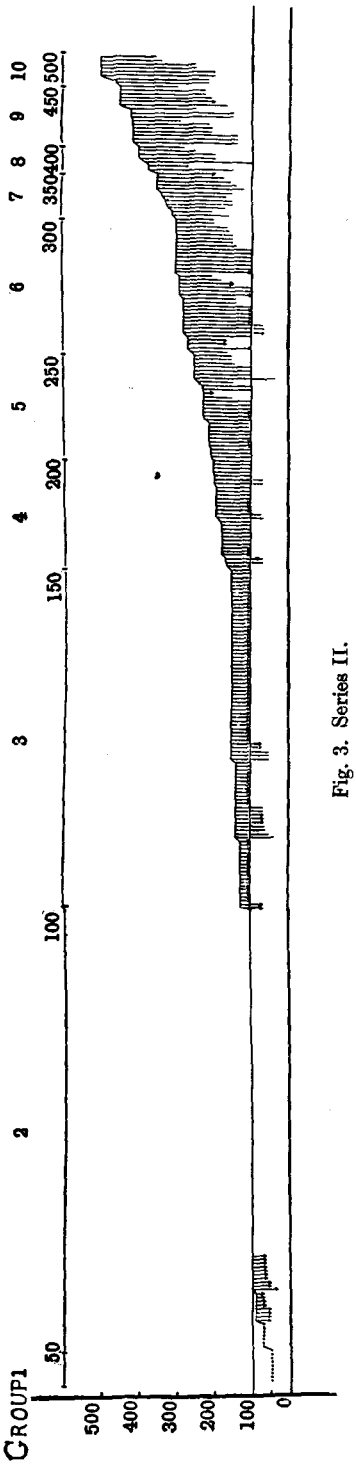

Fig. 3. Series II.

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Table V.

Numerical results from all sera represented in Figs. 2 and 3.

Amount of complement required to produce complete lysis with negative control serum = 100		Series I			Series II			Series I and II combined		
		Total	per cent.	Total of per-centages	Total	per cent.	Total of per-centages	Total	per cent.	Total of per-centages
Group	Lysis complete									
I	at or below 50	0	—	—	9	2·5	—	9	1·2	
II	51–100	229	56·1	—	116	32·8	35·3	345	45·4	46·6
III	101–150	40	9·8	65·9	91	25·9	61·2	131	17·2	63·8
IV	151–200	18	4·4	70·3	29	8·2	69·4	47	6·2	70·0
V	201–250	29	7·2	77·5	28	8·0	77·4	57	7·5	77·5
VI	251–300	23	5·6	83·1	36	10·3	87·7	59	7·8	85·3
VII	301–350	21	5·1	88·2	12	3·4	91·1	33	4·3	89·6
VIII	351–400	19	4·7	92·9	7	2·0	93·1	26	3·4	93·0
IX	401–450	18	4·4	97·3	16	4·6	97·7	34	4·5	97·5
X	451–500	11	2·7	100·0	8	2·3	100·0	19	2·5	100·0
	Total ...	408			352			760		

change or discontinuity occurs. Hence the distinction between positive and negative sera is not capable of being very sharply defined. This is what would be expected by anyone who has experience of the Wassermann test when carried out by a quantitative method. But in both graphs a definite steepening of the gradient appears at the beginning of Group IV (151 to 200); in Table V this is represented by a fall in the percentage distribution, namely, from 9·8 to 4·4 in Series I, from 25·9 to 8·2 in Series II, and from 17·2 to 6·2 in the combined series. If the strongest positives, the omission of which from the graphs was mentioned above (p. 96), were included the fall in the percentage would be from 8·2 to 3·7 in Series I, and from 18·5 to 6·1 in Series II. The evidence, therefore, points to this region as the one in which is to be found the change in character of the sera from negative to positive. In the light of this result, we would propose that the following system should be applied in diagnosis:

(1) Sera which allow of complete lysis with not more than 150 per cent. of the amount of complement giving complete lysis with the negative control serum are negative (Groups I, II, and III).

(2) Sera which do not allow of complete lysis with less than 200 per cent. of the amount of complement required by the negative control serum are positive (Group V and subsequent groups).

(3) Sera falling between these two classes (from 150 to 200 per cent.) must be regarded as "doubtful" or "suspicious," i.e. Group IV.

It is to be noted also that the majority of those sera which fall into the next higher (200–250) and subsequent groups, give no lysis with 100 per cent. of complement, i.e. with the amount which causes complete lysis of the negative control. Accordingly, they would be reckoned as positive on the older one tube, "all or nothing," criterion and thus far the two methods of estimating a positive serum appear identical in their results. But, although

the sera of the 150–200 group show in many cases only commencing lysis at 100, the estimation of the amount of complement required to produce just *complete* lysis indicates that these sera are only very little removed from negative sera. This fact appears clearly when the sera of the 150–200 group are compared with those falling into the higher groups. Hence the latter test affords much more precise quantitative information as to the reacting power of the serum than does the one tube, "all or nothing," criterion. On the other hand, certain sera which are quite definitely positive, since they require 250 per cent. or more of complement to give complete lysis, may show a very extensive range, with commencing lysis below 100 per cent. (see Fig. 3); the positive nature of such sera would appear questionable on the "all or nothing" basis.

It may be asked why the doubtful group should be arbitrarily limited to the 150 to 200 per cent. zone. In reply to this it may be said that the character of a serum which requires for complete lysis double the amount of complement required by the negative control, is definitely abnormal and, with the known exceptions, such abnormality indicates syphilis. But, as a further measure of precaution in the reckoning, those sera may be returned as *weak positives* which require for complete lysis not less than 200 nor more than 250 per cent. of complement. The rest, which number 87 per cent. of all those requiring 200 per cent. of complement or more for complete lysis, are frank *diagnostic positives* (see footnote, p. 103).

Confirmatory evidence of the value of the above criterion has been obtained from two sources, viz. (1) the examination of a large series of sera with heart-cholesterol antigen, the results of which have been computed by a somewhat different method (see Section VII (*a*)), and (2) a comparison of the results arrived at by the method used in Series I with the original diagnoses returned according to Browning and Mackenzie's five dose criterion. (See Section VII (*b*).)

VII. CONFIRMATORY EVIDENCE.

(*a*) Series III.

As the method of calculation applied in Series I and II to over one thousand sera is somewhat laborious, an attempt was made to apply to a still larger number a method which, while consuming much less time, would extend the information as to the general distinction between positive and negative sera. Records of 1193 patients' sera were employed, together with the corresponding 67 tests of a negative control serum derived from the same person as in the greater part of Series I. The human heart-cholesterol antigen was employed as described in Section IV. The percentages of lysis (see Table IV) shown in the three tubes containing a given serum were entered upon a card, and the cards arranged in the order of magnitude of these percentages. The results are summarised in Table VI.

The following points are exhibited by the table: (1) the negative control

serum showed 90 per cent. or more of lysis in the first tube on 66 out of 67 days; probably its appearance in Group IV on the remaining occasion was due to some error of technique. (2) With more than half the sera (57·6 per cent. comprising Groups I and II) the range of lysis in the first two tubes is identical with that shown by the negative control serum on the same or other occasions. Such sera cannot be regarded as other than negative and it is justifiable to conclude that they give complete lysis with at most 150 per cent. of the

Table VI.

Results of the test of 1193 *sera with heart-cholesterol antigen.*

The amount of complement in the first tube varied on different occasions from 1·3 to 3 M.H.D.; the second and third tubes always contained respectively 2 and 3 times as much complement as the first tube.

	Percentage lysis in			Patients' sera			Patients known to have been treated		Number of times that negative control serum fell in group
	1st tube	2nd tube	3rd tube	Number of sera	percentage	Total of percentages	No.	percentage of group	
Relative amounts of complement ...	1	2	3						
Group I	100	—	—	557	46·4	—	101	14·6	51
II	90–98	100	—	134	11·2	57·6			15
III	25–80	100	—	56	4·7	65·1 (7·5)	16	28·6	0
IV	—	90–98	100	34	2·8		10	29·4	1
V	—	0–80	100	21	1·75	66·8			
	—	—	90–98	28	2·3	69·1 (34·3)	18	13·2	
	—	—	25–80	87	7·25	76·3			
VI	—	—	0	276	23·0	100	33	11·9	
Total...	—	—	—	1193	—	—	178	—	67

= 14·8 per cent. of 1193

complement which causes complete lysis of the negative control. (3) The next two groups (III and IV) taken together show in the second tube the same range of lysis as is given by the negative control serum in the first. Since the ratio of the amounts of complement in the first, second, and third tubes was in all cases as 1 : 2 : 3 these sera (III and IV) taken all together require therefore twice as much complement as do the negative controls, also taken all together, to allow of a given degree of lysis. To compare in detail Groups III and IV with the negative control is a more difficult matter; for instance, the opinion to be formed of sera in Group III will depend upon whether, on the day in question, the negative control falls in Group I or Group II. The most satisfactory comparison is that between a serum in Group IV and the negative control in Group II; here one can state definitely that the respective amounts of complement required to produce 90 to 98 per cent. of lysis are as 1 : 2. A negative control in Group I is a less satisfactory basis for comparison, since one cannot tell whether or not the amount of complement present is in excess of that required to produce complete lysis.

It is noteworthy that of the cases in Groups III and IV the percentage which had undergone anti-syphilitic treatment, is twice as great as that

present in the whole series (roughly 28 per cent. and 14 per cent. respectively). The high proportion of treated cases in these groups is due to two factors: (1) in the course of cure, the serum of a person who has given a positive reaction must pass back through this doubtful zone, and (2) many persons are now treated before their serum has developed the full positive reaction; this will occur especially in clinics where the examination for spirochaetes is systematically carried out.

The general results derived from Series III are as follows:

(1) A large number of sera (57·6 per cent.) require for complete lysis not more than 150 per cent. of the complement which causes complete lysis with the negative control.

(2) A comparatively small number (7·5 per cent.) require from 150 to 200 per cent. of this amount. It is noteworthy that this group contains a greater proportion (double) of cases known to have been treated than any other.

(3) The remainder (34 per cent.) require more than 200 per cent. of complement. Two-thirds of these shows no lysis with 300 per cent. of the complement which lyses the negative control completely.

These results are therefore confirmatory of those given in the preceding section in the following points: (1) the frequency distribution of the sera shows an abrupt fall in the region corresponding roughly to Group IV of Series I and II, namely, where the amount of complement required to produce complete lysis reaches 150 to 200 per cent. of that required by the negative controls collectively. (2) This region, which it is proposed to regard as that of "suspicious" sera, shows a very high proportion of treated cases; and it is certain that such cases must always yield a large number of reactions which are doubtful on any system of diagnosis.

(*b*) Comparison of the proposed method of diagnosis with that previously used.

When the sera included in Series I were submitted to the Wassermann test, now several years ago, the diagnoses were then made on the system stated on p. 88 above, under which a positive serum must require at least five doses of complement, in addition to the amounts inhibited by the serum and the antigen separately, to allow of complete lysis of the test corpuscles. One can now compare these original diagnoses with those suggested by the subsequent examination of the results which forms the subject of the present paper. This comparison is presented in Table VII and it will be seen that there is a close agreement between the diagnoses arrived at on the earlier and the later systems[1].

The new data brought forward in this paper have suggested that (1) a serum in order to be counted as negative must allow complete lysis with

[1] Many of the diagnoses in Series II are not now available, so that the comparison in that Series cannot be made.

not more than 150 per cent. of the amount of complement required by the negative control; (2) a positive serum must require not less than 200 per cent. of this amount, and (3) sera in the intervening zone must be regarded as doubtful. Now it is seen in the table of original diagnoses that: (1) no

Table VII.

Comparison of the proposed method of diagnosis with that previously used.

Amounts of complement giving complete lysis (100 gives complete lysis with negative control)	Original diagnoses Series I		
	−	?	+
50–100	229	0	0
101–125	12	1	0
126–140	11	3	0
141–150	9	4	0
151–175	0	5	1
176–200	0	4	8
201–225	0	3	9
226–250 251–275 276–300 301–325 326–500	0	0	186

negatives occur above 150; (2) the doubtful cases, though extending below 150 and above 200, attain their maximum between these two points; (3) there is only one positive below 175; (4) above 225, all were diagnosed as positive.

The original "5 dose rule" was arrived at by the combined clinical and laboratory examination of a large number of cases; hence the fact that it gives diagnoses in general agreement with those which would be made on the new system put forward here provides valuable support for the latter. Discrepancies in the case of sera in the doubtful zone are bound to occur when such results, obtained on different days with complements of varying deviability, are pooled. Thus, nine sera were diagnosed as positive which would be considered doubtful by the new rule; on the other hand, three sera originally returned as doubtful would now be called positive. The discrepancies, therefore, amount to only 2·5 per cent. and do not involve any transference from the negative to the positive category or *vice versa*. In the face of this difficulty, the agreement between the two systems seems to be quite as close as can be expected. Of course, the delimitation of the "doubtful" region must always be a difficult and wholly arbitrary matter.

VIII. THE APPLICATION OF THE RESULTS IN DIAGNOSIS.

In practice a compromise has to be made between (1) the large number of tubes which is needed to show adequately the range of lysis with each serum, and (2) the small number of tubes which is practicable in routine testing on a large scale. Latterly we have used three tubes (exclusive of the serum control) containing amounts of complement in the ratio of 1 : 2 : 3;

these amounts being such as to give 2, 4, and 6 M.H.D. in accordance with the preliminary estimation of the dose (see note I at the end of this paper). With this arrangement the great majority of sera will fall into two classes, namely, (*A*) negatives, showing in the first tube the same, or nearly the same, degree of lysis as does the negative control in this tube; and (*B*) positives, showing in the second tube either nearly the same degree of lysis as does the negative control in the first (weak positives), or less than this (diagnostic positives)[1]; i.e. the positive sera deviate not less than twice as much complement as does the negative control. For the diagnosis of the weakest positives, it is very desirable that the negative control should not show complete lysis in the first tube, so that an exact comparison can be made. It is, of course, the sera intermediate between the classes (*A*) and (*B*) above which cause difficulty. In the series of tubes described above there is no provision for exact observation of the "doubtful" class which requires from 150 to 200 per cent. of the amount of complement required by the negative control; on this account it seems that 2, 3, and 4 doses of complement would be preferable to the 2, 4, and 6 doses used in the work which led subsequently to these conclusions. It would certainly be advisable to use the 2, 3, 4 dose range when retesting suspicious sera. When 2, 4 and 6 doses are used, and one requires to pick out the 150 per cent. group of sera, one has to assume

Table VIII.

Scheme of diagnosis of negative, suspicious and weak positive sera.

		Percentage of lysis			
	Complement M.H.D.	Tube 1	2	3	Diagnosis
		Ratio 1	: 2	: 3	
Type I	Known negative	100	—	—	(Control)
	Patients' sera	60–70	100	—	Negative (border line)
		50 or less	100	—	Suspicious
		20–80	95–98	100	Weak positive (lowest limit)
Type II	Known negative	90	100	—	(Control)
	Patients' sera	40 or more	100	—	Negative (border line)
		Less than 40	More than 90	100	Suspicious
		Less than 40	Less than 90	100	Weak positive (lowest limit)

Note 1. The range of lysis given for the negative control is the same as that observed on 66 occasions in Series III (Table VI).

2. Very abrupt increase of lysis (e.g. faint trace in one tube and complete in the next) is suggestive of possible errors in technique, as is likewise a very gradual increase (e.g. very marked, almost complete, just complete, in the three tubes) and it is well to retest such sera, though some will be found to act constantly in this way.

[1] It is to be understood that there are two criteria of positive: firstly the absolute positive which is required for diagnosis in an unknown case, and to which one would swear in a court of law, and secondly the weak positive or suspicious reaction, which is as good as positive in a case of known treated syphilis or in one which had reacted positive prior to treatment; the latter reaction might be termed the *therapeutic* positive in contra-distinction to the former, the *diagnostic* positive.

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that a serum which requires 150 per cent., or 3/2 of the complement required by the negative control, should theoretically show 2/3 or 66 per cent. lysis (i.e. distinct) with the amount which gives complete lysis with the negative control. A general scheme of diagnosis suggested by these considerations is given in Table VIII (p. 103). It is, of course, impossible to state any such method in a form which can be applied mechanically in all cases. In proportion as the negative control serum deviates more or less complement on any particular day, so must the classification of patients' sera be shifted in one or the other direction; the table is intended to provide a basis for judging of the results in this way.

Use of a positive control.

In addition to the negative control, a positive control serum should, of course, always be included in each series; the latter should be comparatively weak, giving complete lysis with the highest amount of complement used. When the same positive control is employed on repeated occasions one obtains a check upon the deviability of the complement; thus if the positive control on a given day requires less complement than usual to give complete lysis, one knows that a less deviable complement has been used and therefore that more significance attaches to suspicious or weakly positive results and *vice versa*.

The serum control.

The action of each patient's serum on complement when salt solution is substituted for the "antigen" should never be omitted, since occasionally human sera are met with which are abnormally inhibitory toward complement (see Kennaway and Wright). Any serum which, along with two doses of complement, gives less than almost complete lysis, is abnormal in this respect and should be retested, since, of course, such inhibitory action will vitiate the result.

SUMMARY.

Examination of the records of a large number of quantitative Wassermann tests has suggested a new and simple criterion of a positive reaction. This is based upon a comparison of the amounts of complement giving complete lysis with any given serum, and with the negative control, respectively. Reasons are given for regarding this criterion as more satisfactory than that in common use. Details are given of the methods employed for the selection of a negative control serum, the estimation of degrees of lysis, and the provision of a suitable range of amounts of complement.

NOTE I.

The Amounts of Complement used in Wassermann Tests.

The consideration of the results of Wassermann tests is facilitated if the different amounts of complement used bear a simple ratio to one another and it is preferable that these amounts should contain whole numbers of haemolytic doses; the latter arrangement is especially to be recommended if the results are to be used for statistical purposes. Unfortunately the haemolytic dose as estimated in the course of the actual test of the sera (complement control) is not always the same as that found in the preliminary test of the complement, in the light of which the amounts to be used in the actual test are selected; hence adherence to any system of dosage sometimes breaks down in practice. However, the following scheme has been found useful (Table IX).

Table IX.

Number of Haemolytic Doses to be used (for three tubes of antigen plus patient's serum).

Amounts of complement giving number of doses required	Haemolytic dose (for 0·5 c.c. of 3 per cent. red corpuscle suspension sensitised with 5 doses of immune body)			
	0·0025 c.c.	0·005 c.c.	0·0075 c.c.	0·01 c.c.
·01 c.c.	4 doses	2 doses		
·015 „	—	—	2 doses	
·02 „	8 „	4 „	—	2 doses
·03 „	12 „	6 „	4 „	
·04 „	—	—	—	4 „
·045 „	—	—	6 „	
·06 „	—	—	—	6 „

NOTE II.

Proportions of Positive and Negative Sera.

It is of interest to note the proportions of negative and positive sera observed in ordinary hospital practice, as shown in Table X. In Series I and IV the liver-lecithin-cholesterol antigen was used and in Series III heart

Table X.

Results (per cent.)	Liver-lecithin-cholesterol antigen			Heart-cholesterol antigen
	Series I (485 sera)	Series IV [1] (508 sera)	Series I and IV combined (993 sera)	Series III (1193 sera)
Negative	53·8	53·6	53·8	59·6
Suspicious	4·1	7·0	5·6	2·7
Weak positive ...	2·6	2·9	2·8	2·3
Positive	39·5	36·5	37·8	35·4
	100·0	100·0	100·0	100·0

[1] The analysis of Series IV in the manner described in this paper was not carried out owing to the number of occasions on which the point of just complete lysis with the negative control serum could not be observed.

extract plus cholesterol. The strongest positives of Series I, which were omitted from Fig. 2 (see p. 96), are of course included here.

The heart-cholesterol antigen appears to give the slightly smaller number of suspicious results, but the number of weak positives is the same with both antigens. An unqualified return of "negative" or "positive" was made in 92–95 per cent. of the cases.

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