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A SIMPLIFIED SIGMA TEST (PRELIMINARY NOTE)

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INTRODUCTION

THE Sigma Test of Dreyer and Ward ¹ has deservedly attracted considerable attention, especially in Denmark, Belgium, and Great Britain. Generally its attraction as a serum test lies in its greater sensitiveness than the Wassermann test and in providing a quantitative result in standard units. Since early in 1922 the writer has been engaged with workers in a number of Continental laboratories in a comparison, instituted by the Health Committee of the League of Nations, of the Wassermann test with the Sigma and other flocculation reactions, and as a result of an experience of over 3,000 tests has arrived at the conclusion that, for routine purposes in laboratories where large numbers of sera (100–200 per week) are dealt with, the Sigma test is too laborious and time-consuming even with the modifications of the original technique mentioned below, which were introduced from time to time by the authors or with their concurrence.

For details of the test the reader is referred to the original paper (*loc. cit.*) and to the Medical Research Council Special Report Series No. 78. For the sake of brevity it is proposed to indicate here, as an introduction to the description of a simplified technique, only those details (apart from the time occupied in reading) in which the test has proved laborious.

Incubation.—The original description required the first reading to be taken at the end of seven hours, but fortunately the authors of the test found that better results followed a prolongation of the first incubation period to twenty to twenty-two hours.

Serum Dilutions.—At first the test was conducted in nine tubes per specimen, but at a conference of workers engaged in the League of Nation's comparison which was held in Paris in November, 1922,² it was agreed, as a result of the experience of some thousands of tests, that

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the further investigation might be carried out with the first five tubes only. One of the chief effects of this modification consisted in halving the labour of shaking the tubes, no small item when it is remembered that each tube must be inverted on the thumb and shaken vigorously.

Measurement of Reagents.—Some months after the first publication of their technique, Dreyer and Ward introduced a mechanical syringe³ for the filling into tubes 2, 3, 4 and 5 of the β extract suspension, and this, while needing very careful preliminary standardisation, was found greatly to shorten the time consumed in putting up the test. Previously the β suspension was introduced with the dropping pipette (15 drops per tube = 60 drops for each five-tube test. The first of the five tubes, of course, received α suspension). The serum, saline, and α suspension of extract, had, however, still to be put into the tubes with the dropping pipette. This pipette can be arranged to deliver the *saline* automatically, being fed from a burette, while the racks containing the tubes are passed beneath it. It is connected to the burette by rubber tubing, the flow being most conveniently regulated by a screw clamp. Such an arrangement was approved by Professor Dreyer when the writer suggested it to him at Oxford in 1922.* But the same dropping pipette has to be used for each serum, and must be washed out and dried (best done with a suction pump) between each specimen. Much time is occupied thereby. A simpler method of measurement has been devised at the State Serum Institute, Copenhagen, consisting essentially in the use of pipette measurements instead of measurement by drops for serum, saline, and α suspension and the mechanical syringe for the β suspension. The advantages claimed are that the procedure "saves time and serum, as the total volume is smaller than in the dropping method." For the serum, a 0.2 c.c. pipette is used, and for the saline and α suspension a 1 c.c. pipette. The total quantity of fluid in each tube is arranged to be about half that with the dropping technique. The 0.2 c.c. pipette used for the sera is washed out, by means of a suction pump, with saline between sera and not dried, instead of with distilled water followed by acetone and drying in the flame, as laid down by Dreyer and Ward, for the dropping pipette.

* The use of this device for the extract suspensions is not permissible on account of the rubber connection.

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Parallel tests of the same sera carried out by the writer at Copenhagen using the routine method, and Dr. Mörch, of the State Serum Institute, using the pipette technique, showed that there was no appreciable difference in the results obtained.

A few experiments have also been made by the writer using the ordinary dropping technique to ascertain whether, in fact, different results would be obtained by the two methods of washing the dropping pipette between each serum. Thirty-nine syphilitic sera having their end-point within the range of the first five tubes were tested in parallel, the pipette being washed and dried by the routine method on the one hand, and on the other simply washed out with saline and not dried. In the first eighteen the saline wash was carried out with the suction pump; in the last twenty-one the pipette was washed out by drawing up saline and ejecting twice with the rubber test. The results with thirty-seven sera employing the routine dilutions were exactly the same, and in the remaining two the difference was negligible.

The Copenhagen pipette technique shortly indicated above undoubtedly reduces the time taken up by the test, and it was felt that if now one of the chief features which still rendered it very laborious and time-consuming, namely, the necessity for shaking each individual tube, could be removed, the practicability of the Sigma test would be much increased. In the technique now to be described it is believed that this object has been attained without undue interference with the value of the test or the expression of the results in standard units.

Mixing the tube contents by means of bubbles of air was tried, but although this is practicable when ordinary round-bottomed test tubes are used, it was found to be unreliable where the narrow pointed agglutination tube is concerned. In the technique about to be described, the tube contents are mixed by simple shaking of the racks.

TECHNIQUE

The chief features are summarised as follows :—

(1) Economy of time. One hundred tests can be prepared for incubation in less than three hours.

(It may here be remarked that the preparation of the extract suspensions, which is the same for the proposed

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as for the original technique, requires the supervision of the pathologist, but need not occupy him throughout. The writer has adopted the procedure of personally mixing the required amount of extract and cholesterin solution ; of measuring out each 1 c.c. of the mixture which is to be diluted ; of starting the syphonage and of checking the quantity of suspension in the cylindrical measure when each dilution is completed, leaving it to the laboratory attendant to check the dilution times with a stop-watch and to prepare the cylindrical measure for further use.)

(2) No dropping pipette is used. For each serum a fresh 1 c.c. pipette is taken ; 1 c.c. and 5 c.c. pipettes are used for saline and a 1 c.c. pipette for the α suspension.

(3) No mechanical syringe is required for the β suspension. This is delivered with a 5 c.c. pipette, though the use of a mechanical syringe as employed in the routine technique is, of course, optional.

(4) No pointed agglutination tubes are used. These are replaced by round-bottomed tubes, easier to clean than Dreyer's tubes and of wider bore (see below). The relation of the time occupied by the laboratory attendant in washing 100 of the rounded tubes, and 100 Dreyer tubes, was found to be 2 : 3.

(5) No shaking of the individual tubes is necessary, as with the narrower pointed agglutination tubes. The tubes are shaken *in situ* in the racks.

A. APPARATUS

(1) Baths, racks, and constant water-level apparatus, as in the original technique, except that as regards the racks the five holes between each two dilution-tube holes must be slightly enlarged to accommodate the larger-sized tubes employed.

(2) Tubes. These are dwarf test tubes, 6 to 6.5 cm. in length and 8 to 8.3 mm. in internal diameter. It is useful to have the mouths flanged like the Dreyer tubes. Before the tubes are first taken into use, 1 c.c. of water should be measured into each, and only those tubes should be employed in which the fluid reaches the same level.*

The size of tube was arrived at as the result of a long series of experiments. Having determined what was

* The tubes were accurately made for me by Messrs. R. B. Turner & Co., 9 and 10, Eagle Street, Southampton Row, W.C. 1.

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to be the final volume of fluid in each tube, it was necessary so to fix the internal diameter that while the degree of flocculation would not materially differ from that in the routine agglutination tubes, the column of fluid should be sufficiently high to obviate any difficulty in the arrangement of the immersion level. At the same time the bore of the tubes had to be of such size as would enable the contents to mix readily by simple shaking of the tubes *in situ* in the rack. As a practical point, it has been found that a degree of accuracy in the height of the column is attained by the use of the wider tube, with rounded end, which is in excess of that attained with the Dreyer tubes. This is due to the permitted variation of 0.7 mm. in the internal diameter of the Dreyer tubes (5.5 to 6.2 mm.). This variation may itself account for differences in level of about 8 mm., which differences may be further exaggerated (or on the other hand diminished) by differences in the content of the conical ends.

There is no difficulty in arranging the immersion level in the proposed technique so that the column of fluid is one-half to two-thirds below water-level. If it is desired not to interfere with the existing water-level, the ordinary Sigma rack can be raised with accuracy to the required level by standing each end on a heap of glass slides held together by a rubber band.

- (3) Floating syphon as in the original technique.
- (4) Graduated pipettes : 1 and 5 c.c.

B. METHOD OF SETTING UP THE TEST

Table I. gives details for ten serum dilutions. It is proposed, however, that in a routine test only the first five tubes should be employed. These require a total of 1.6 c.c. serum, as compared with about 1.45 c.c. in the original technique. Place six tubes in the stand. The left-hand tube is the dilution tube.

Saline.—With a 5 c.c. pipette measure 0.9 c.c. into the dilution tube. With a 1 c.c. pipette measure 0.2 c.c. into tube 3, 0.3 c.c. into tube 4, 0.8 c.c. into the α extract control tube, and 0.4 c.c. into the β extract control tube.

Serum.—This is inactivated as in the routine method. Draw up 1 c.c. pure serum into a 1 c.c. pipette. Deliver 0.8 c.c. into tube 1. Now draw up into the same pipette a further 0.6 c.c. pure serum, so that it contains 0.8 c.c.

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Deliver half this amount (0.4 c.c.) into tube 2 ; 0.2 c.c. into tube 3 ; 0.1 c.c. into tube 4 ; and the final 0.1 c.c. into the dilution tube, the dilution of serum in which is now 1/10. Mix the contents of the dilution tube by drawing up into the pipette and blowing out twice, and then draw up 0.4 c.c. and deliver into tube 5, afterwards discarding the dilution tube. A separate 1 c.c. pipette is used for each serum. While the serum dilutions are being made, the preparation of the extract suspensions may be in progress. These are made according to the original technique.³

Extract.—With a 1 c.c. pipette measure 0.2 c.c. α suspension into tube 1 and into the α control tube. With a 5 c.c. pipette measure 0.6 c.c. β suspension into tubes 2, 3, 4 and 5, and into the β control tube.

Shaking.—Shake the rack and place it in the water-bath. The tubes should be shaken in a rigid rack like that used in the ordinary technique, and the holes should not be greatly larger than the tubes. If these conditions are observed, it will be found that the tube contents mix quite readily.

TABLE I.

Number of Tube.	Serum, in c.c.	Saline, in c.c.	Extract, in c.c.	Total dilution in which serum acts.
1	0.8 of 1/1	0.0	0.2 α suspension	1/1.25
2	0.4 „	0.0	0.6 β „	1/2.5
3	0.2 „	0.2	0.6 β „	1/5
4	0.1 „	0.3	0.6 β „	1/10
5	0.4 of 1/10	0.0	0.6 β „	1/25
6	0.2 „	0.2	0.6 β „	1/50
7	0.1 „	0.3	0.6 β „	1/100
8	0.4 of 1/100	0.0	0.6 β „	1/250
9	0.2 „	0.2	0.6 β „	1/500
10	0.1 „	0.3	0.6 β „	1/1,000

α Extract control = Saline 0.8 c.c. + α Extract suspension 0.2 c.c.

β Extract control = Saline 0.4 c.c. + β Extract suspension 0.6 c.c.

The quantities in the above Table, for details of which I am indebted to I. R. Mörch, of the State Serum Institute, Copenhagen, are double those used in the pipette method already referred to, employed at that Institute.

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RESULTS

Parallel tests were carried out on fifty-six occasions, totalling 332 sera. The method adopted was to test only such syphilitic sera whose end-point fell within the first five tubes. These were discovered in the course of the routine work of the laboratory, and they were then put aside and subsequently re-tested by the routine and the proposed method under strictly parallel conditions. The investigation was spread over a period of twelve months. The average number of sera tested at one time was six.

The 332 sera tested comprised :—

Cases of syphilis	.	.	.	.	286
Controls	.	.	.	.	46

Out of the 286 cases of syphilis :—

- (i.) 62 were positive * by both methods in Tubes 1 or 2.
- (ii.) 201 were positive by both methods in Tubes 3, 4, or 5.
- (iii.) 23 were negative by both methods, but showed some degree of flocculation in Tubes 1 or 2.

The controls comprised twenty-one cases of gonorrhœa, twenty-three without venereal disease, and two cases of soft chancre. All were negative by both methods.

(i.) Of the sixty-two cases which were positive by both methods in tubes 1 or 2, fifty-one were positive at twenty hours and eleven at forty hours. Of the fifty-one which were positive by both methods at twenty to twenty-two hours, thirty-two gave identical results, the average of the thirty-two readings being 1.8. Out of the nineteen which differed, sixteen gave lower and three gave higher units by the modification than by the routine method. But the differences were small. The greatest difference was 0.41, in No. 209, a case of treated syphilis, which at twenty hours was S in tube 1 (= 1.3 units) by the routine method, and tr⁺ in tube 1 (= 0.89 unit) by the modified method ; in forty to forty-four hours the reading was 1.3 by the routine, and 1.1 by the modification. The average difference for the nineteen cases was 0.27 unit.

Of the eleven readings which were positive by both

* By "positive" is meant more than 1.0 units in a known case in twenty to twenty-two or forty to forty-four hours, and more than 1.4 units in an unknown case in twenty to twenty-two hours.

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methods at forty to forty-four hours, ten were identical (1·1), and one—No. 217—gave 1·6 by routine and 1·4 by the modification.

Thus, with the exception of Case 209, which was positive by routine and negative by the proposed method at twenty hours, though positive by both at forty hours, no serum out of the sixty-two which reacted positively in tubes 1 or 2 only (*i.e.*, a weak positive) with the routine, failed to react positively also, either at twenty or forty hours by the modified method, and indeed it is seen that differences in readings, when they occurred, were, for practical purposes, negligible.

(ii.) Of the 201 readings which were positive in tubes 3, 4 or 5 by both methods at twenty to twenty-two hours, fourteen gave identical readings. Out of the fourteen readings two were 13·4 and 17·2 respectively. Of the remaining twelve the highest was 6·2, and the lowest 2·6, the average being 3·5.

Out of the 187 readings which differed, 169 gave higher, and eighteen gave lower readings by the routine than by the proposed method. But the differences generally were small. They were greatest in nine cases, as follows (Table II).*

TABLE II.

No.	Units obtained by Routine Method.	Units obtained by Proposed Method.	Difference in Units.
83	9·6	6·0	3·6
107	7·4	11·4	4·0
130	14·0	18·0	4·0
153	18·1	25·0	6·9
227	19·2	15·2	4·0
255	21·6	28·8	7·1
288	18·6	14·4	4·2
315	19·0	14·5	4·5
321	30·0	20·4	9·6

* But, as has been shown^{4, 5}, different workers, testing the same specimen of serum by the same technique, may obtain widely different readings. And it will be seen, in a report to be published by the Medical Research Council, that variation in unit readings to the extent here seen may be found when the observer retests the same specimen of serum after an interval. It is concluded that the expression of the results in units, whilst certainly useful, does not lend itself to a too rigid interpretation for clinical purposes.

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Out of the remaining 178 cases, the average difference in reading was 0.98 unit. In thirty cases out of the 178 the difference was 0.1 unit, and in sixty-eight further cases out of the 178 the difference was less than one unit.

(iii.) Out of the twenty-three cases of syphilis which were negative by both methods, but in which there was some slight flocculation at twenty or forty hours, the readings were identical in eleven at twenty and forty hours, and differed in the remaining twelve, the only marked difference, however, being in one case, which at twenty to twenty-two hours was tr^- in tube 1 by routine and $tr^?$ by the proposed method, and at forty to forty-four hours was S^+ in tube 1 by routine, and tr by the proposed method.

It is seen that, generally, lower unit readings were obtained with the proposed than with the routine method. Whilst the dilution in which the serum acts is the same in both methods in tubes 1 and 2, it is slightly less by the proposed method from tube 3 onwards, thus:—

Tube	1	2	3	4	5	
	1/1.25	1/2.5	1/5.2	1/13.1	1/26.4	Routine method.
	1/1.25	1/2.5	1/5	1/10	1/25	Proposed method.

The slightly higher concentration in which the serum acts from tube 3 onwards in the proposed method appears to compensate, but not completely, for the slightly lessened flocculation in these tubes due to their wider bore.

In tubes 1 and 2, in which the concentration of the serum is the same in both methods, the weaker flocculation due to the wider bore of tube is, however, apparent only with sera reacting weakly in these tubes, and then only, as has been shown, in some cases and to a negligible extent.

Fine degrees of flocculation are slightly more difficult to read than in the routine method, but the difficulty is easily overcome by practice. Thus when the flocculated contents of a tube showing, say, tr^- by the proposed method are transferred to a routine agglutination tube, the flocculation becomes easier to see on account of the more slender column of fluid. This difference tends to become exaggerated with cloudy sera. Generally one has the impression that individual flocculi tend to be rather less regular in size than in the routine tubes.

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CONCLUSION

The simplified Sigma technique here described has yielded, in a series of parallel tests, results which seem to compare favourably in point of accuracy with the ordinary technique whilst effecting a considerable economy of time.

REFERENCES

- (1) *Lancet*, i., 956, 1921.
- (2) League of Nations, Reports of Serological Investigations presented to the Second International Conference on the Standardisation of Sera and Serological Tests held at the Pasteur Institute in Paris, in November, 1922.
- (3) Medical Research Council, Special Report Series, No. 78.
- (4) *Lancet*, p. 58, July 12th, 1924.
- (5) League of Nations, Investigations on the Sero-Diagnosis of Syphilis—Report of the Technical Laboratory Conference held at Copenhagen, November 19th to December 3rd, 1923.