# OBSERVATIONS ON THE STANDARDISATION OF SCARLET FEVER REAGENTS.

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In 1927 we undertook certain work at the request of the Medical Research Council, as part of an international investigation into the standardisation of scarlet fever reagents promoted by the Health Organisation of the League of Nations. The laboratory work was carried out by the late Dr G. R. James, and his untimely death caused the suspension of the research. A great many observations, however, had been made. Since many of these have a general interest, and since up to the time of writing no completely satisfactory experimental method of assay has been devised, we have no hesitation in adding to the volume of evidence that is accumulating with regard to the methods at present in use.

## I. Comparative results of skin testing with undried and reconstructed dried toxins.

From a preliminary survey of the whole position it was considered that a more permanent basis for the comparison of results would be secured if reagents could be reduced to the dry form. This involved the elaboration of special methods for drying quantities of material containing no preservative under conditions ensuring complete sterility, and after this had been completed it was further necessary to estimate the specific activity of the final product. In this experiment a sample of a filtrate derived from a broth culture of a scarlet fever hemolytic streptococcus was provided by Dr O'Brien of the Wellcome Research Laboratories, and was a portion of the same batch of toxin referred to as Toxin *B* in Dr O'Brien's paper[1]. This was reduced to the dry condition, and reconstructed for ordinary testing by making up to its original volume with sterile distilled water. Dilutions suitable for skin testing were then prepared by adding the boric-borate diluting fluid introduced by Dr O'Brien and his co-workers, and the following groups of tests were performed:

(*a*) A preliminary comparison was carried out with 6 patients by the injection of 0·2 c.c. of a 1/1000 dilution of dried and reconstructed toxin, and a similar injection of a 1/1000 dilution of the undried preparation from which the dried reagent had been derived. A control of undried toxin inactivated by boiling for 1 hour was carried out at the same time. No reaction was obtained to the control injection in any of the cases, and to the test toxins 3 were

[1] Dr O'Brien's paper will appear in the *Journal of Hygiene*, Vol. xxix, No. 4.—Ed.

positive to both, and 2 negative to both, the positive reactions to both dried and undried reagents being exactly similar in size and intensity. In the remaining case while the undried toxin gave a doubtful result the dried gave a reaction 15 × 19 mm. There was thus no recognisable difference except such as might be ascribed to technique.

(*b*) The above experiment was repeated in 17 cases, the test toxin of each type being controlled by a similar injection of toxin inactivated by boiling as before. In this group of cases 11 persons reacted to both undried and reconstructed toxins, 6 were negative to both, and none showed reactions at the site of the control injections. Again there were no significant differences in size and intensity of the corresponding reactions.

(*c*) In this group 51 individuals were tested with 0·2 c.c. of three dilutions 1/2000, 1/4000 and 1/6000 of both undried and reconstructed toxins. No control of inactivated toxin was employed in this series and the results are classified in Table I.

Table I.

| | Undried | | | Reconstructed | | |
|---|---|---|---|---|---|---|
| Total | 1/2000 | 1/4000 | 1/6000 | 1/2000 | 1/4000 | 1/6000 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 |
| 24 | + | + | + | + | + | + |
| 5 | + | + | 0 | + | + | 0 |
| 3 | + | 0 | 0 | + | 0 | 0 |
| 1* | + | +(f.) | +(f.) | +. | 0 | 0 |
| 1 | + | + | 0 | + | + | + |
| 1 | + | + | + | + | + | 0 |
| 1 | + | + | 0 | + | + | +(f.) |
| 1 | + | + | 0 | + | 0 | 0 |
| 1 | 0 | 0 | 0 | +(f.) | 0 | 0 |

f. = faint reaction.

The intensity of the erythema of the corresponding reactions was estimated and all were carefully measured. No significant differences appeared. From these results it can be seen that the reactions of the two toxins were exactly similar in 45 cases. In five the difference could be attributed to technical error and favoured each toxin equally. In one anomalous case (*) the reaction favoured the undried.

## II. Comparison of the potency of two preparations of scarlet fever toxin in 119 scarlet fever patients.

In this work we have made comparative tests between the reconstructed toxin employed in Exp. I and a toxin kindly supplied by Dr McCoy of the Hygienic Laboratory at Washington. He informed us that the test dose of the Washington toxin was 0·1 c.c. of a 1/2000 dilution (solution *B*). For the purpose of the experiment the reconstructed toxin was used in two dilutions, viz. (i) at a dilution of 1/1000 in a dosage of 0·2 c.c. (solution *A*), and (ii) at a dilution of 1/2000 in a dosage of 0·1 c.c. (solution *C*). That is, the amount of toxin injected in series *A* is four times the amount in *C*. All toxin dilutions in this and other experiments were made in the boric-borate diluting fluid. These

three toxin solutions were tested on 119 scarlet fever patients at all stages of the disease, each patient receiving injections of each of the three toxins as described above. The results are summarised in Table II.

Table II.

| Day of disease | 1–5 | | 6–10 | | 11–15 | | 16–20 | | 21–28 | | 28+ | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reaction | + | − | + | − | + | − | + | − | + | − | + | − | + | − |
| Solution *A* | 12 | 2 | 8 | 6 | 11 | 5 | 11 | 4 | 8 | 5 | 27 | 20 | 77 | 42 |
| % positive | 86 | | 57 | | 69 | | 73 | | 61 | | 57 | | 65 | |
| Solution *B* | 8 | 6 | 4 | 10 | 5 | 11 | 7 | 8 | 2 | 11 | 9 | 38 | 35 | 84 |
| % positive | 57 | | 29 | | 31 | | 47 | | 15 | | 19 | | 29 | |
| Solution *C* | 7 | 7 | 4 | 10 | 7 | 9 | 8 | 7 | 4 | 9 | 21 | 26 | 51 | 68 |
| % positive | 50 | | 29 | | 44 | | 53 | | 31 | | 45 | | 43 | |

*A* = Reconstructed toxin. 1/1000. Dose 0·2 c.c.
*B* = Washington toxin. 1/2000. Dose 0·1 c.c.
*C* = Reconstructed toxin. 1/2000. Dose 0·1 c.c.

From the data set forth in Table II it will be noted that the reconstructed toxin even when used at the lower dilution is stronger than the Washington toxin when employed at the dilution shown. The difference in strength of solutions *A* and *C* is also reflected in the results in so far as the stronger solution gave 26 more positive reactions than the weaker solution. At the same time as the results were scrutinised the reactions were measured and their intensities noted. The comparative results of these show generally that *A* gave the largest and most intense reaction whilst *C* was next and *B* smallest. The reason that scarlet fever patients were selected for the comparative testing was to discover in addition to the relative potencies of the preparations what was the most satisfactory test dose for diagnostic purposes. One of us (Joe, 1925) in an early paper on the Dick test, when discussing the subject of standardisation, suggested that the behaviour of any given toxin towards the immunity process produced by the disease itself might be accepted as a criterion of the usefulness or otherwise of the reagent. Thus it was proposed that a satisfactory filtrate would be one which in suitable dilution would produce a positive skin reaction in 95 per cent. of cases of scarlet fever during the first 5 days of the disease, and would not produce a positive reaction in more than 30 per cent. of convalescent patients tested after the fourth week. Of the three solutions studied *A* comes nearest to these requirements. Only 14 patients were tested in the first 5 days of the disease, and of these 12 (86 per cent.) gave a positive reaction. On the other hand, of the 47 patients tested in the late convalescent stage, 59 per cent. still reacted positively to this dose and strength of toxin. When one-quarter of this amount was injected (solution *C*) only 50 per cent. of the early scarlet fever cases gave a positive reaction, and yet 45 per cent. of the 47 late convalescent cases still reacted positively to the weaker dilution of the same toxin. The inference from these results is that either the toxin is qualitatively unsuitable or the method of standardisation is inherently unsound. When it is realised, however, that for all practical purposes workers in this country have found the toxin under discussion a reliable indicator of immunity the former objection can

hardly be sustained. With regard to the other objection, we are now aware of the very wide range of individual response to the infective process in scarlet fever, and there remains the difficulty in deciding the actual time of its onset before the efflorescence of the rash. These difficulties could be overcome to a certain extent by reducing the five-day limit, during which a very high percentage of positive reactors would be expected, to the first 2 days of the disease, but since relatively few cases of scarlet fever are seen in hospital at this early stage the disadvantages of this method, even if the conception be well founded, are immediately apparent.

### III. Comparison of potency of two preparations of scarlet fever toxin by testing with serial dilutions.

In another series of experiments the reconstructed and Washington toxins were compared in a different way, an attempt being made to determine whether an end point might be demonstrated with each toxin which would indicate its potency in relation to the other, and whether there was any significant grading in the size of the reactions with variations in dose. The total number of cases tested was 26, similar dilutions of the two toxins as shown in Table III being injected into the same individual, the reconstructed toxin in one arm, and the Washington in the other.

The table demonstrated again that the reconstructed toxin is more potent than the Washington. In this series of cases no definite difference could be made out in 15, but in 7 cases there was a definite difference in favour of the reconstructed toxin. In 3 cases the difference is hardly sufficient to be regarded as definite, while in 1 case the difference is definitely in favour of the Washington toxin. In view of the general trend of the results we are obliged to regard this exception as due to faulty technique, though the most scrupulous care was taken at the time of injection to prevent this. To a similar defect we must ascribe the one or two instances in which a negative result appears in a series in which lower dilutions still give measurable reactions. As in all this work each series of reactions was graded according to intensity, but no material difference could be observed at the different dose levels. The table brings out other points of general interest. For example, it will be noted that so far as a juvenile hospital population is concerned, a definite scale of immunity may be recognised and separation into sharply defined Dick positive and Dick negative reactors cannot easily be made. Also 4 cases showed a reasonably good reaction to the injection of as small an amount of toxin as 0·2 c.c. of a 1/12,000 dilution, and 3 others not tested at a lower dilution showed a similar reaction to the injection of a 1/10,000 dilution. In this connection and with regard to the stability of the toxin in high dilution, we may mention that 1/6000 dilutions of the reconstructed and Washington toxins have been stored in the cold room (4° C.) for 2 months and have been found to produce good reactions in susceptible individuals when further diluted to 1/12,000 and injected in 0·2 c.c. quantities.

## Table III.

| Case no. of patient | Washington 1/1000 | 1/2000 | 1/4000 | 1/6000 | 1/8000 | 1/10,000 | 1/12,000 | Reconstructed 1/1000 | 1/2000 | 1/4000 | 1/6000 | 1/8000 | 1/10,000 | 1/12,000 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **No difference:** | | | | | | | | | | | | | | |
| 1, 11 | II | II | II | II | — | — | II | II | II | II | II | — | — | II |
| 2 | II | III | 0 | 0 | — | — | 0 | II | III | 0 | 0 | — | — | 0 |
| 3 | II | II | II | II | — | — | II | I | II | II | II | — | — | II |
| 4 | 0 | 0 | 0 | 0 | — | — | 0 | 0 | 0 | 0 | 0 | — | — | 0 |
| 5, 6, 10 | II | II | 0 | 0 | — | — | 0 | II | II | 0 | 0 | — | — | 0 |
| 8 | II | II | II v.f. | II v.f. | — | — | II v.f. | II | II | II f. | II f. | — | — | II f. |
| 14, 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | I | II | II | II | II | II | II | I | II | II | II | II | II | II |
| 20 | II | II | II | 0 | — | — | II | II | II | ± | ± | ± | — | II |
| 22 | II | ± | 0 | 0 | 0 | 0 | — | II | ± | 0 | 0 | 0 | 0 | — |
| 26 | II | II | II | II | II | II | — | II | II | II | II | II | II | — |
| **Definite difference in favour of reconstructed:** | | | | | | | | | | | | | | |
| 7 | 0 | 0 | 0 | 0 | — | — | 0 | II | III | 0 | 0 | — | — | 0 |
| 9 | II | II | II f. | II f. | — | — | 0 | II | II | II f. | II f. | — | — | II f. |
| 12 | II | III | III | ± | 0 | 0 | 0 | II | II | II | III | III | III | III |
| 13 | II | II | ± | III f. | 0 | 0 | 0 | II | II | II | II | II f. | 0 | 0 |
| 21 | II | II | II | III | III | 0 | 0 | II | II | II | II | II | II | — |
| 24 | II | II | II | 0 | 0 | 0 | — | II | II | II | II | II | II | — |
| 25 | II | II | II | 0 | 0 | 0 | — | II | II | II | II | 0 | 0 | — |
| **Doubtful difference:** | | | | | | | | | | | | | | |
| 15 | II | II | II | ± | II f. | 0 | 0 | II | II | II | 0 | II f. | II f. | 0 |
| 18 | II | II | III | 0 | ± | ± | ± | II | II | III | 0 | III f. | III f. | 0 |
| 23 | II | ± | 0 | 0 | 0 | 0 | — | II | II | 0 | 0 | 0 | 0 | — |
| **Favours Washington:** | | | | | | | | | | | | | | |
| 19 | II | II | III | III | 0 | 0 | 0 | II | II | ± | 0 | 0 | 0 | 0 |

I = reactions whose diameter was 30 mm. or over.
II = ,, ,, between 10 and 30 mm.
III = ,, ,, less than 10 mm.
0 = negative reaction.
— = test not performed with this dilution.
± = indefinite reaction not capable of being measured.
f. = faint reaction.
v.f. = very faint reaction.

## IV. Standardisation of scarlet fever antitoxin by skin neutralisation methods.

Three separate groups of 34, 27 and 30 individuals respectively were examined as to their suitability for neutralisation experiments by injections of 0·2 c.c. of 1/1000 dilution of reconstructed toxin and diluted horse serum. It has been suggested that only those subjects giving a reaction of not less than 30 mm. diameter should be chosen for skin neutralisation tests, but since only 8 cases in the total number tested fulfilled these conditions we enlarged our numbers by including all those who gave a reaction of 20 mm. or more. In this way we were able to select 10, 8 and 12 individuals respectively from the first mentioned groups, and these of course were also non-reactors to the injection of the highest amount of horse serum about to be employed in the experiment. The mixtures to be injected were prepared in the laboratory and consisted of equal volumes of the reconstructed toxin at a dilution of 1/500 and various dilutions of antitoxin. Injections of 0·2 c.c. were made $1\frac{1}{2}$ to 2 hours after the mixtures had been made and readings were taken and measured at the end of 24 and 48 hours. The results of the three neutralisation experiments are combined in Table IV.

Table IV.

| Dose of toxin | Dose of antitoxin | No. of cases tested | No. of cases in which: Complete reduction occurred — 24 hr. | | No. of cases in which: Complete reduction occurred — 48 hr. | | No. of cases in which: Complete reduction, or reduction to 10 × 10 mm. or less occurred — 24 hr. | | No. of cases in which: Complete reduction, or reduction to 10 × 10 mm. or less occurred — 48 hr. | |
|---|---|---|---|---|---|---|---|---|---|---|
| 0·1 c.c. of 1/500 | 0·1 c.c. of 1/250 | 17 | 14 | 82% | 11 | 65% | 16 | 94% | 14 | 82% |
| 0·1 c.c. of 1/500 | 0·1 c.c. of 1/500 | 25 | 19 | 76% | 17 | 68% | 22 | 88% | 19 | 76% |
| 0·1 c.c. of 1/500 | 0·1 c.c. of 1/1000 | 28 | 21 | 75% | 17 | 61% | 24 | 85% | 21 | 75% |
| 0·1 c.c. of 1/500 | 0·1 c.c. of 1/2000 | 28 | 13 | 46% | 6 | 21% | 20 | 71% | 11 | 39% |
| 0·1 c.c. of 1/500 | 0·1 c.c. of 1/4000 | 13 | 5 | 38% | 2 | 15% | 7 | 54% | 2 | 15% |
| 0·1 c.c. of 1/500 | 0·1 c.c. of 1/6000 | 5 | 1 | 20% | 0 | 0 | 1 | 20% | 0 | 0 |

From these it is interesting to note that in some cases dilutions of serum are able to inhibit the toxin reaction for 24 but not for 48 hours, and this inability increases roughly with the progressive dilution of the antitoxin. Taking the 24 hours' reading as an index it would with two exceptions have been possible to put these mixtures in a descending order of their antitoxin content, whether the complete reduction had been regarded as neutralisation or whether the less stringent condition of reduction to 10 × 10 mm. or less had been imposed. The two exceptions are in the mixtures containing 1/500 and 1/1000 dilutions of serum. On the other hand if only the 48 hours' reading had been considered, and complete reduction taken as the sign of neutralisa-

tion, a similar difficulty would have arisen in estimating the antitoxin values of the 1/250, 1/500 and 1/1000 dilutions. If all the data are considered, it does seem possible to give an approximate estimate of antitoxin content if a sufficient number of cases are tested, and provided great care is exercised in choosing suitable reactors. The importance of the latter consideration has already been indicated in Table III, where we have seen how widely the state of immunity or susceptibility may vary. Our inclusion of the cases giving a reaction of 20 mm. and over has not apparently defeated the object of this experiment, but as the next experiment indicates, it might be well to limit neutralisation tests to those individuals giving a reaction of not less than 30 mm. in diameter, even though this may tend to restrict greatly the number of individuals available for test purposes.

In another experiment (Table V) an attempt was made by means of neutralisation tests to estimate the potency of an American antitoxin in terms of the antitoxin employed in the above experiment. The reconstructed toxin was employed in a dilution of 1/300, 0·1 c.c. being injected with 0·1 c.c. of various dilutions of each antitoxin. The mixtures were injected $1\frac{1}{2}$ to 2 hours after being made. Readings were carried out after 24 and 48 hours, measured and recorded. All reactors chosen for this comparison had been tested, 48 hours previously, with 0·2 c.c. of 1/600 reconstructed toxin, and none gave a reaction to the lowest dilution of each antitoxin employed. The actual protocols of this experiment are put on record in Table V as they illustrate the difficulties experienced in the practical application of this method. We had reason to believe, and for the purposes of this test it was assumed, that our antitoxin was ten times stronger than the American. The results of the test suggest that this assumption was not entirely correct, and that the American antitoxin was stronger than we anticipated. As a matter of fact the experiment must be regarded as unsatisfactory as in order to limit the number of injections in each individual we only compared two dilutions of each antitoxin in any single case. It was hoped, however, by multiplying the number of cases that we should find a sufficient number of reactors giving at one or another dose level an end point with each antitoxin, which would afford a basis of comparison. Owing to the fine grading of the immunity scale previously referred to, similar end points did not occur and the results suggest that it was unwise to include in the series such relatively insensitive reactors, indicated by the preliminary test, as cases 1, 4, 5, 7 and 8. The inference is that we should have obtained more definite information by carrying out a large series of comparative tests in a few of the most sensitive individuals, and even in these the possibility of variation must be fairly wide.

## CONCLUSIONS.

1. A scarlet fever streptococcal toxin after drying and reconstruction retained its full specific activity.

2. The relative potencies of different toxins, or different dilutions of the

Table V.

| Case no. of patient | Original test with 1/600 recons. | Antitoxin *A*: 1/200 A.T. + 1/300 t. | | Antitoxin *A*: 1/400 A.T. + 1/300 t. | | Antitoxin *A*: 1/800 A.T. + 1/300 t. | | Antitoxin *A*: 1/1600 A.T. + 1/300 t. | | Antitoxin *B*: 1/20 A.T. + 1/300 t. | | Antitoxin *B*: 1/40 A.T. + 1/300 t. | | Antitoxin *B*: 1/80 A.T. + 1/300 t. | | Antitoxin *B*: 1/160 A.T. + 1/300 t. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 | 48 | 24 | 48 | 24 | 48 | 24 | 48 | 24 | 48 | 24 | 48 | 24 | 48 | 24 | 48 |
| 1 | 15/21 | 0 | 0 | 0 | 0 | — | — | — | — | 0 | 0 | 0 | 8/6 | — | — | — | — |
| 2 | 22/38 | 0 | 0 | 0 | 0 | — | — | — | — | 0 | 0 | 0 | 0 | — | — | — | — |
| 3 | 22/32 | 15/15 | 20/26 | 14/10 | 20/21 | — | — | — | — | 0 | 5/6 | 11/10 | 18/23 | — | — | — | — |
| 4 | 15/13 | 0 | 5/4 | 0 | 9/6 | — | — | — | — | 0 | 0 | 0 | 0 | — | — | — | — |
| 5 | 18/25 | 0 | 0 | 0 | 0 | — | — | — | — | 11/19 | 0 | 0 | 0 | — | — | — | — |
| 6 | 25/34 | — | — | 20/24 | 33/28 | 28/23 | 40/47 | — | — | — | — | 12/10 | 12/9 | 22/17 | 27/27 | — | — |
| 7 | 17/22 | — | — | 0 | 0 | 0 | 0 | — | — | — | — | 0 | 0 | 0 | 0 | — | — |
| 8 | 16/20 | — | — | 0 | 0 | 0 | 0 | — | — | — | — | 0 | 0 | 0 | 0 | — | — |
| 9 | 22/25 | — | — | 11/14 | 17/15 | 13/14 | 20/20 | — | — | — | — | 10/14 | 0 | 13/12 | 13/10 | — | — |
| 10 | 31/34 | — | — | 0 | 12/12 | 11/14 | 12/13 | — | — | — | — | 0 | 0 | 0 | 7/5 | — | — |
| 11 | 15/23 | — | — | — | — | 0 | 0 | 0 | 0 | — | — | — | — | 16/14 | 0 | 14/13 | 0 |
| 12 | 15/19 | — | — | — | — | 0 | 0 | 15/19 | 0 | — | — | — | — | 17/25 | 0 | 15/20 | 0 |
| 13 | 20/26 | — | — | — | — | 0 | 0 | 0 | 14/10 | — | — | — | — | 0 | 6/5 | 0 | 9/7 |
| 14 | 20/30 | — | — | — | — | 12/20 | 13/20 | 15/14 | 20/20 | — | — | — | — | 17/20 | 19/10 | 16/20 | 18/21 |

Antitoxin *A* is antitoxin employed in Table IV.
Antitoxin *B* is an American antitoxin.

same toxin, can be ascertained by comparative tests if a sufficient number of individuals are available, but it is impossible by this method to give a numerical value to one toxin in terms of another. The method previously suggested of estimating a suitable test dose of scarlet fever toxin by noting the results in early and late cases of scarlet fever presents great practical difficulties.

3. Skin testing with serial dilutions of different toxins indicates the relative potencies of these toxins and reveals that the scale of immunity is very gradual. The end point between positive and negative reactors is not sharply defined.

4. The titration of scarlet fever antitoxin by means of skin neutralisation tests presents many difficulties. Markedly sensitive reactors are required and mixtures made with a wide range of dilutions must be employed. Skin tests should be scrutinised at 24 and 48 hours and the results of similar titrations in any two individuals would probably disclose wide differences.

(*MS. received for publication* 24. VIII. 1929.—Ed.)