Antistreptolysin-O: Its Interaction with Streptolysin-O, Its Titration and a Comparison of Some Standard Preparations

H. GOODER, Ph.D., F.R.I.C. 1

The quantitative determination of antistreptolysin-O in the serum of a patient is frequently used as an aid in the presumptive diagnosis of a recent streptococcal infection. Difficulties have been experienced in obtaining consistently reproducible results, when large numbers of sera are tested, with accurate titration methods simple enough to be used routinely in a serological laboratory.

An investigation of the streptolysin-antistreptolysin reaction has revealed that the time of interaction at 37°C routinely in use in this test is very critical. Results are presented suggesting that this critical effect is due to the instability of the toxin, which is possibly attacked by the active proteinase present in streptolysin preparations. It was possible to develop a modified method using twofold dilutions of serum and a 50% haemolysis endpoint under conditions where proteinase has little effect on the toxin. This method has proved satisfactory in routine laboratory use with large numbers of sera.

A quantitative comparison of various standard antistreptolysin preparations available throughout the world revealed a reasonable constancy of absolute value when compared with the original Todd standard. Differences in their neutralization reactions with streptolysin-O were, however, apparent.

INTRODUCTION

The appearance of antibody to streptolysin-O (antistreptolysin-O) in the serum of a patient, or an increase in antistreptolysin-O titre, ordinarily reflects a recent streptococcal infection. Accordingly, the test for antistreptolysin-O is frequently carried out as an aid in the differential diagnosis of those disorders believed to be the sequelae of group-A streptococcal infections—notably, rheumatic fever. Ideally, in order to demonstrate the rise in antistreptolysin-O titre the initial serum sample should be obtained at the time of the streptococcal infection (e.g., sore throat). This is clearly impracticable in many cases, so that acute-phase sera only are available for diagnosis. Thus the antistreptococcal antibody test differs from the antibody tests employed in the retrospective diagnosis of many infectious diseases, where it is possible to detect a rise in titre by a comparison of acute-phase and convalescent sera. Consequently, the absolute value of the antistreptolysin-O titre becomes of diagnostic importance, and an accurate titration method simple enough to be used routinely in a serological laboratory is required.

Liao (1951) described a 50% end-point method which appeared to possess the required accuracy and yet avoid the complicated dilution schemes which are a disturbing feature of many methods (Hodge & Swift, 1933; Ipsen, 1944; Rantz & Randall, 1945; Johnson, 1955; Kusama, 1958). In 1955 Liao's method was put into routine use in the Streptococcus and Staphylococcus Reference Laboratory, Public Health Laboratory Service, Colindale, London. Trouble was encountered in obtaining consistently reproducible results. For this reason we investigated the reaction between streptolysin-O and its antibody and ultimately developed a modified method which has been in satisfactory routine use for approximately three years.

This paper describes our investigations of the fundamental reaction involved in the test, and the modified method adopted. This method was used to effect a comparison of various standard anti-streptolysin preparations with the original Todd standard (Todd, 1932).

MATERIALS

**Medium**

The medium used throughout was a Hartley broth with 0.3% (w/v) added maltose.

**Strain**

The streptolysin was prepared from a group-A, type 3, *Streptococcus* (National Collection of Type Cultures (NCTC) No. 9994). This strain was received originally from the Statens Seruminstitut, Copenhagen (through the courtesy of Dr E. Kjems).

**Streptococcal haemolysin**

For the preparation of the haemolysin, 10 ml of a young (6-hour) culture of the *Streptococcus* were inoculated into 2 litres of Hartley maltose broth (previously checked for sterility) at room temperature and the inoculated broth was then placed in the 37°C incubator for 15-16 hours. At the end of this period, a sample was removed and centrifuged, and the supernatant was tested for haemolytic activity by the method given below. If the supernatant contained more than 64 minimal haemolytic doses (MHD) per ml then the bulk of the culture was checked for purity and the pH adjusted to 7.0. Cysteine hydrochloride (4 g) was added and the culture was clarified by centrifugation at 2500 r.p.m. for 20 minutes. The supernatant was filtered through a Seitz pad and bottled in 20-ml amounts in screw-capped bottles which were stored at 4°C. No preservative was added.

**Buffer solution**

The buffer solution used throughout was made as follows: 1 g of Armour's plasma albumin, fraction V, was dissolved in 500 ml of physiological saline (0.145 M NaCl); 270 ml of 0.2 M NaH₂PO₄, 2H₂O and 126 ml of 0.2 M Na₂HPO₄, 12H₂O were added and the volume was adjusted to 1000 ml with distilled water.

**Red cell suspension**

Rabbit blood was collected from the ear vein into about half its volume of Alsever's solution (Kabat & Meyer, 1948). This red cell suspension could be stored at 4°C if necessary for 3 to 4 days. When required, the cells were washed twice with buffer solution and an approximate 5% suspension was prepared for standardization. The 5% suspension was adjusted (by concentration or by the addition of buffer) so that when 1 ml of the red cells was mixed with 11 ml of distilled water and allowed to stand for 5 minutes the resulting haemoglobin solution gave an optical density reading of 0.46-0.50 at a wave-length of 520 mµ.

**Standard antistreptolysin globulins**

Two different standard globulin preparations were used. The primary standard was a solution of horse globulins containing 20 000 antistreptolysin units per ml. This was received from Dr L. F. Hewitt, Medical Research Council Serum Laboratories, Carshalton, Surrey, England; it is the original Todd standard (Todd, 1932) and has been used extensively in the United Kingdom. The batch used throughout was No. 7, dated 19 September 1944. This standard was diluted with buffer solution to contain 20 units per ml for a working solution and was stored frozen.

Standard human antistreptolysin globulin (Col.) was prepared from a pool of high-titred patients' sera by the ammonium sulfate fractionation scheme of Kendall (1937). The preparation was sterilized by filtration through a sintered-glass filter (bacteriological grade) and stored at 4°C. The solution was standardized by the method described below, using the horse antistreptolysin globulin as standard.

Standard antistreptolysin human globulins of nominal value 10 units per ml were received from the Statens Seruminstitut, Copenhagen, Denmark (SSI), and also from Behringwerke, Serumvertrieb, Marburg/Lahn, West Germany (Mar.). Standard antistreptolysin globulin was purchased from Difco Laboratories, Detroit, Mich., USA (Diff.), nominal value 3.30 units per ml, and standard antistreptolysin rabbit globulins were received from the Institut Pasteur, Paris, France (Past.), nominal value 60 units per ml.

**Reducing agent**

Thioglycollic acid was used to reduce the lysin. An approximately 0.2 M solution was freshly prepared each day as follows: 0.1 ml of the acid was diluted with 2 ml of buffer, 2 drops of BDH (British Drug Houses) universal indicator were added and the solution was neutralized with 10 N sodium
hydroxide solution. The volume was adjusted to 5.0 ml with buffer.

METHODS

Estimation of the degree of haemolysis

One millilitre of the standardized red cell suspension was diluted with 11 ml of distilled water. This corresponded to the final haemoglobin solution obtained when complete lysis of the red cells had occurred owing to the streptolysin-O in the test system. From this haemoglobin solution others were prepared corresponding to the range 5% to 95% haemolysis in steps of 5%. The haemoglobin content of these solutions was then estimated colorimetrically in a spectrophotometer at a wavelength of 520 mμ, using either 10 mm × 10 mm cuvettes or matched 125 mm × 10 mm test-tubes. A graph was constructed of the relation between the optical density values of these solutions at 520 mμ and the percentage haemolysis. This is shown in Fig. 1, which is the best straight line for the average of three separate determinations. This graph was used to estimate the percentage haemolysis. It was checked at frequent intervals.

<table>
<thead>
<tr>
<th>FIG. 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>RELATION OF OPTICAL DENSITY AT 520 mμ AND PERCENTAGE HAEMOLYSIS IN TEST</td>
</tr>
</tbody>
</table>

Estimation of the haemolytic activity of streptolysin-O (MHD50)

The haemolytic dose used was that described by Herbert (1941); it is defined as the amount of toxin haemolyzing 50% of the red cell suspension in 30 minutes at 37°C and is referred to as the MHD50. The test system consisted of 1.0 ml of buffer to which was added 0.5 ml of buffer containing various amounts of streptolysin-O. The tubes were shaken, 0.5 ml of the red cell suspension was added and the tubes were then incubated for 30 minutes at 37°C (water-bath), being shaken after 15 minutes. The tubes were centrifuged at 2500 r.p.m. for 5 minutes and 1.0 ml of the supernatant was removed. This was diluted with 2 ml of distilled water and the haemoglobin content of the resulting solution was estimated as previously described. Duplicate tubes were always employed and the results averaged. From the values between 15% and 85% haemolysis the MHD50 was calculated, using von Krogh's equation (von Krogh, 1916).

Determination of the test dose of streptolysin-O against 1 unit of antistreptolysin-O (LH dose)

The capacity of the streptolysin-O to combine with its homologous antibody was determined by the 50% end-point method described by Liao (1951). The test dose (LH) is the amount of streptolysin-O which in the presence of 1 unit of antistreptolysin haemolyses 50% of the red cell suspension in a stated length of time at 37°C.

The conditions used in most experiments were as follows: To 1 ml of buffer containing 1 unit of antistreptolysin-O were added varying amounts of toxin in 0.5 ml of buffer. The tubes were incubated at 37°C (water-bath) for 15 minutes, after which 0.5 ml of the red cell suspension was added. Incubation was continued for a further 75 minutes (the tubes being shaken after 15 minutes) and then tubes showing haemolysis in the region of 50% were removed and centrifuged. The percentage haemolysis and 50% end-point were determined. Liberation of haemoglobin was essentially linear throughout the 75 minutes, except for a short initial lag period.

Table 1 shows a typical result for the determination of the test dose of streptolysin-O. The end-

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td>DETERMINATION OF THE TEST DOSE OF STREPTOLYSIN-O (LH)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Streptolysin reciprocal dilution</th>
<th>Percentage haemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>75</td>
</tr>
<tr>
<td>7.2</td>
<td>68</td>
</tr>
<tr>
<td>7.4</td>
<td>57</td>
</tr>
<tr>
<td>7.6</td>
<td>47</td>
</tr>
<tr>
<td>8.0</td>
<td>21</td>
</tr>
<tr>
<td>8.5</td>
<td>12</td>
</tr>
</tbody>
</table>
point of the titration shows that 0.5 ml of a dilution of
the lysin of 1:7.5 gives 50% haemolysis of the red
cells in the presence of 1 unit of antistreptolysin-O.

To demonstrate the variability of the end-point
each lysin was retitrated at the appropriate 50%
dilution, using duplicate dilutions with 5 samples
from each.

One series gave values for the 5 samples of 50%,
44%, 49%, 42% and 45% haemolysis (mean,
46%) and the duplicate series values of 57%, 52%,
55%, 47% and 44% haemolysis (mean, 51%).

Once standardized, the streptolysin preparation
has been found to store well at 4°C, showing only
minor variations, of the order of 5%, over periods
as long as 3 months. On only one occasion has a
large sudden drop in titre been encountered. This
occurred in all the bottles of a particular lysin
preparation and was not due to a change in activity
of the antistreptolysin. No explanation can be
offered for this occurrence.

**Determination of the antistreptolysin-O titre of
human sera**

The antistreptolysin-O titre of a serum was
determined by the method of Liao (1951). Dilutions
of the serum (inactivated at 56°C for 30 minutes)
were prepared in 1.0 ml of buffer. One LH dose of
streptolysin was added to each tube and the tubes
were then treated as described under the determina-
tion of the LH dose. The serum dilutions employed
were an initial dilution of 1:50 (0.1 ml to 4.9 ml of
buffer), followed by serial doubling dilutions of 1 ml
to a final dilution of 1:800.

**Determination of the neutralizing ability of the
standard antistreptolysin preparations**

The neutralization studies were performed with
a constant streptolysin concentration and varying
antibody concentrations. This method was chosen
because of the instability of the activated streptolysin
preparations; it allowed the chosen toxin solution
to be added almost simultaneously to all tubes in an
experiment. The activation of the toxin, the com-
bination of the toxin with the antiserum and the
determination of the residual haemolytic activity
were performed as described in the modified routine
test.

**Streptococcal proteinase**

Proteinase activity was determined by the milk
thioglycollate test of Elliott (1945).

**RESULTS**

**Streptolysin preparations**

The streptolysin preparations were sterile culture
filtrates of a group-A type 3 *Streptococcus*, activated
by sodium thioglycollate. They contained varying
proportions of active and inactive haemolysins, both
of which combined immunologically with antibody,
as previously shown by Hodge & Swift (1933). For
use in the antistreptolysin test it was considered that
a preparation with a high MHDs0/LH ratio would
be the most sensitive indicator.

The properties of some of the streptolysin prepare-
ations are shown in Table 2. Considerable variation
will be noted. In preparation 4 a sudden drop in
both the MHDs0 and the LH values was noted after
3 months' storage at 4°C, but the ratio remained
constant. The work described in this paper has been
performed with streptolysin preparations with a
MHDs0/LH ratio greater than 20.

**Table 2**

**Properties of streptolysin-O preparations**

<table>
<thead>
<tr>
<th>Streptolysin preparation</th>
<th>MHDs0 per ml</th>
<th>LH dose per ml</th>
<th>Ratio MHDs0/LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90</td>
<td>3.1</td>
<td>29.0</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>5.2</td>
<td>9.2</td>
</tr>
<tr>
<td>3</td>
<td>150</td>
<td>15.0</td>
<td>10.0</td>
</tr>
<tr>
<td>4A (20.5.57)</td>
<td>550</td>
<td>14.0</td>
<td>39.3</td>
</tr>
<tr>
<td>4B (12.8.57)</td>
<td>450</td>
<td>11.0</td>
<td>40.9</td>
</tr>
<tr>
<td>Difco</td>
<td>48</td>
<td>3.3</td>
<td>14.5</td>
</tr>
</tbody>
</table>

**Reaction of streptolysin-O and antistreptolysin-O**

Most methods for the determination of anti-
streptolysin-O titres suggest a combination time of
15 minutes at 37°C for the reaction of antigen and
antibody. No evidence seems to have been produced
to support the choice of this figure, which appears in
the original paper of Todd (1932). We therefore
investigated the variation of percentage haemolysis
given under standard conditions by mixtures of
1 LH dose of streptolysin-O and 1 unit of anti-
streptolysin-O when they were allowed to combine
for various times at 37°C. Fig. 2 shows the results
obtained with three different lysin preparations.

It appeared that the time allowed for the com-
bination of antigen and antibody at 37°C was a
such antiserum concentration of the neutralizing effect proteinase, but this was complicated by the fact that such antiserum (and also normal rabbit serum) had a neutralizing effect on streptolysin-O. This effect was found to reside in the globulin fraction. The results indicated, however, that the excess free toxin in these mixed systems was more stable than the toxin alone. The stability at 37°C of toxin activated by sodium thioglycollate (0.04 M) was also compared with that of toxin mixed with sodium acetate (0.04 M) as a control system. Five minutes before adding red blood cells to each mixture, to test for the amount of toxin remaining active, sodium acetate (final concentration, 0.04 M) was added to the previously activated sample and, correspondingly, sodium thioglycollate (final concentration, 0.04 M) to the as yet unactivated toxin. Separate experiments had previously shown that 5 minutes at 37°C was sufficient to produce maximum activation of streptolysin-O. As a control, completely unactivated samples of toxin were used, diluted as necessary throughout with sodium acetate.

The results of these tests (illustrated in Table 3) showed that unactivated enzyme is more stable than activated enzyme. This again could be interpreted as indicating that the loss of toxin was due to proteinase, which would be active only under the reducing conditions found in activated toxin.

Elliott (1945) reported that proteinase had little activity at low temperatures. This suggested that dilute streptolysin-O would have correspondingly greater stability at low temperatures, and this was found to be so (Fig. 3).

If the reaction of streptolysin-O and antistreptolysin-O were carried out at 4°C, then the residual toxin should have greater stability and this might remove the major source of the variation that had been found in the routine test. A study was therefore made of the combination of streptolysin-O and antistreptolysin-O at 4°C. Appropriate dilutions

**Stability of streptolysin-O**

Herbert (1941) showed that dilute streptolysin-O (3 MHD₅₀ per ml) was unstable at 37°C but was relatively stable at 2°C. This finding was confirmed. Herbert suggested that the toxin underwent surface denaturation. An alternative explanation is that under the reducing conditions present in the test the streptococcal proteinase is activated and brings about a loss in potency of the streptolysin-O. Proteinase activity was demonstrable in all our streptolysin preparations, usually after preliminary concentration of the protein present by ammonium sulfate precipitation. An attempt was made to stabilize the streptolysin preparations by incorporating antiserum prepared against crystalline proteinase, but this was complicated by the fact that such antiserum (and also normal rabbit serum) had a neutralizing effect on streptolysin-O. This effect was

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**TABLE 3**

<table>
<thead>
<tr>
<th>Time of incubation at 37°C (minutes)</th>
<th>Incubated in the activated state</th>
<th>Incubated in the inactivated state and then activated for testing</th>
<th>Incubated and tested without activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>55</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>31</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>28</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>10</td>
<td>36</td>
<td>0</td>
</tr>
</tbody>
</table>
of activated toxin (0.5 ml) were mixed with 1 unit of antistreptolysin-O (1 ml) and left at 4°C; at intervals tubes were removed, 0.5 ml of red cell suspension was added and the haemolysis recorded after incubation at 37°C for 30 minutes. The results obtained with two different toxin preparations are given in Fig. 4. The interaction of toxin and antibody at 4°C appears to be completed in 1 1/2-2 hours and the residual toxin is apparently fairly stable for the next 2-3 hours. This type of result was found to be general with toxin preparations and antistreptolysin-O, so our routine test was modified to use a combination time of 2 hours at 4°C in place of the original 15 minutes at 37°C. Advantage was also taken of this change in the routine test to substitute 30 minutes at 37°C, in place of 75 minutes as used previously, as the time of incubation when testing for residual toxin. The results of a comparative test using the old and new methods (Fig. 5) showed that there was no significant disagreement between the methods.

Reproducibility of the modified test

The 50% end-point method using the 2-hour combination time has been in use at Colindale for approximately three years. Standard tubes, which are incorporated in the test each week, include as primary standard the original Todd horse globulin solution and subsidiary standards of human globulin. These globulins appear to have the same neutralization curves.

From the results obtained each week on these standard sera we have accumulated a large number...
of values for the same serum which give a measure of the reproducibility of the test from week to week and also of duplicate samples of the same serum in the same test (Table 4). It will be seen that the errors involved in week-to-week testing are no greater than those found between duplicate sera in the same test.

The magnitude of the standard deviation varies with the magnitude of the estimated titre and appears to be about ± 10% of the titre. Similar results were obtained by Liao (1951).

**Standard antistreptolysin-O**

For a test in which a single determination of the titre has diagnostic value, a reliable reference standard is essential. Previous investigations have made use of standard sera from a wide variety of sources. The initial standard was undoubtedly that of Todd (1932). This was a pepsin-refined horse globulin preparation containing 20 000 units per ml. The globulins had originally been prepared for therapeutic use and were issued as a standard serum because of demand and convenience (L. F. Hewitt, personal communication). For laboratory use such a standard serum is far too concentrated. Other centres and also some commercial houses have prepared and issued their own standards, usually with reference to the Todd globulin preparation.

No standard technique for effecting this comparison seems, however, to have been suggested. Ipsen (1944) recommended that a human globulin preparation should be adopted as standard. He was under the impression that the Todd standard was a solution of rabbit globulins and he showed that such globulins had a quantitatively different neutralization curve with streptolysin-O when compared with human globulins.

A second batch of the Todd globulin preparation was prepared in 1944 and reasonable quantities of this are still available. It appeared that a comparison of this standard preparation and the standard sera frequently quoted in the literature would be useful and instructive, since the Todd standard is the only common link with much of the earlier work in the field. Accordingly a comparison was made of five antistreptolysin preparations with regard to both their absolute content of neutralizing antibody and the type of neutralization curve they show with streptolysin-O. Each experiment was complete in itself since Ipsen (1941) had demonstrated the impossibility of obtaining exactly similar rabbit cells for haemolysis studies from different bleedings. The determinations were made in duplicate and the results averaged. Owing to the small amount of some of the sera, it was possible to carry out the comparison only three times. The Todd standard was used as a primary standard for the standardization of the toxin.

**TABLE 4**

Reproducibility of the value of the standard globulin preparation in use in the weekly routine test

<table>
<thead>
<tr>
<th></th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean squares</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between pairs</td>
<td>11 964</td>
<td>19</td>
<td>629.7</td>
</tr>
<tr>
<td>Within pairs</td>
<td>13 269</td>
<td>20</td>
<td>663.5</td>
</tr>
</tbody>
</table>

![Fig. 5](image-url)
Table 5 records the results of a typical experiment showing the resulting haemolysis after mixing one LH₉₀ unit of streptolysin-O with various amounts of the different antisera. The antisera had been diluted in buffer to a final concentration of approximately 2 units per ml.

To interpret the type of result shown in Table 5, Liao (1951) applied von Krogh's equation (von Krogh, 1916) to the streptolysin-antistreptolysin system. The von Krogh equation was derived as an adsorption formula, but owes its characteristics largely to the inhomogeneity of red cells with respect to their susceptibility to lysis by, in this case, streptolysin-O. Alternatively, the interpretation of this inhomogeneity can be made by applying the method of probit analysis as suggested by Ipsen (1941). In order to produce a straight-line relationship the haemolysis values had to be restricted with both methods to those lying between 15% and 85%.

Fig. 6 shows the figures from a similar experiment plotted according to von Krogh's equation:

\[ X = k \left( \frac{Y}{100-Y} \right)^{1/n} \]

where \( X \) is the amount of antistreptolysin, \( Y \) is the percentage haemolysis, \( 1/n \) is the slope of the line when \( \log X \) is plotted against \( \log \left( \frac{Y}{100-Y} \right) \), and \( k \) is a constant that is equal to \( X \) at the 50% end-point.

From such graphs for each experiment the 50% end-points \( \left( \frac{Y}{100-Y} \right) = 1 \) in terms of millilitres of antistreptolysin were obtained for each serum. These values were multiplied by the initial dilution.

### Table 5

**Residual Percentage Haemolysis Given by Mixtures of Antisera (Antistreptolysin Units per ml) and 1 LH Dose of Streptolysin-O**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Percentage Haemolysis after Mixture with Following Amounts of Antiserum:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Past.</td>
<td>91</td>
</tr>
<tr>
<td>Mar.</td>
<td>93</td>
</tr>
<tr>
<td>SSI</td>
<td>89</td>
</tr>
<tr>
<td>Dif.</td>
<td>89</td>
</tr>
<tr>
<td>Col.</td>
<td>88</td>
</tr>
<tr>
<td>Todd</td>
<td>85</td>
</tr>
</tbody>
</table>

### Fig. 6

**Estimation of LH₉₀ of Different Antistreptolysin Preparations (Log Scale)**

![Graph showing estimation of LH₉₀ of different antistreptolysin preparations](image-url)
that had been used for each particular serum and the results transformed into 50% units per ml of antistreptolysin-O. In order to allow comparison between experiments, the value for the Todd serum was adjusted where necessary to 1.0 unit per ml and the other values in that experiment were multiplied by the same factor. Table 6 gives the values obtained by this means for the antistreptolysin titre of these sera in terms of units per ml. The nominal value is that of the appropriate issuing body.

Within the error of the techniques used, the nominal values appear reasonably consistent with the Todd standard. Equally important with the absolute value are the characteristics of a neutralizing antiserum which is to be used as a standard for human clinical values. That differences exist in the manner in which the standard globulin preparations neutralize the streptolysin are apparent from Fig. 6, in which the slope of the line given by von Krogh's equation could be used as an index of similarity of the neutralization curves. Accordingly, the slopes were determined and are given in Table 7.

**DISCUSSION**

The reasons for attempting to use a 50% haemolysis end-point method in preference to the usual end-point of the tube with *no trace* of haemolysis were adequately stated by Liao (1951). Ipsen (1944) had previously made a similar recommendation, but his method proved too elaborate for routine use. The test described here covers the clinically important range of values without requiring a preliminary testing of the sera to find an approximate value or the careful pipetting of very small quantities.

**TABLE 6**

**UNITS OF ANTISTREPTOLYSIN-O IN THE ANTISERA TESTED**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Antiserum tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.6</td>
</tr>
<tr>
<td>2</td>
<td>5.8</td>
</tr>
<tr>
<td>3</td>
<td>6.9</td>
</tr>
<tr>
<td>Mean value</td>
<td>5.7</td>
</tr>
<tr>
<td>Nominal value</td>
<td>6.0</td>
</tr>
</tbody>
</table>

*The Todd globulin solution taken as standard and adjusted to 1.0 unit per ml where necessary; other values adjusted appropriately.*

The test has been easily adapted to the use of semi-automatic pipettes for the dispensing of the diluent buffer and the red cell suspension. The initial 1 : 50 dilution on each serum is prepared by means of a Seligson automatic burette (Seligson, 1957) and the subsequent serial 1-ml dilutions are carried out with a 1-ml syringe pipette.

When only small numbers of sera (up to 20) were to be examined, Liao's original method proved adequate, but with larger numbers discrepancies were found. Duplicate sera placed first and last in a series were always found to have apparently increased in titre. This appears to be due to the unstable nature of the streptolysin under the conditions of the test and we suggest that it is being digested by the active proteinase present. When conditions were chosen so as to minimize the effect of proteinase, the streptolysin activity was maintained. We have therefore adopted 2 hours at 4°C as our standard procedure for the combination of streptolysin and antistreptolysin. A shortened incubation period of 30 minutes at 37°C when testing for the residual activity was also introduced with no loss of accuracy or sensitivity.

In a recent paper, Kusama, Ohashi, Shimazaki & Fukumi (1958) also noticed that the streptolysin-antistreptolysin reaction did not appear to go to completion under the conditions advocated by Todd. These authors recommend overcoming this difficulty by increasing the amount of streptolysin used in each tube to 80 haemolytic units with a combination time of 1 hour—a recommendation that appears to us rather wasteful and surprising. They also confirm the original suggestion of Gillen & Feldman (1954) regarding the advantageous effects of incorporating plasma albumin in all diluting solutions when working with streptolysin-O.
The method described here is flexible enough to be used for special investigations in a restricted range of values simply by changing the initial dilution. For example, a series of hypogammaglobulinaemia patients undergoing frequent passive antibody injections have been followed in the range 10 to 80 units simply by adopting a 1 : 10 initial dilution.

The routine accuracy appears to be good. The weekly values obtained for our standard human globulin solution suggest a standard deviation of ± 10% of the titre which is in keeping with the amount of error found by Liao (1951). Our values were obtained during the course of normal routine testing of clinical material over a 6-month period. Since many investigations have shown that 85-90% of the normal population have titres below 200 units, it is felt that the test described here could be an aid in the presumptive diagnosis of a previous streptococcal infection when only a single serum specimen is available.

The different standard antistreptolysin preparations which we were able to study showed a reasonable constancy in absolute value. This suggests that the values of antistreptolysin-O in normal subjects reported in various surveys employing these standard preparations are broadly comparable. Considerable variation in the slopes of the neutralization curves of streptolysin-O was noted, however, and in tests employing quantitative determinations of the haemolytic power of lysin-antibody mixtures this might lead to important differences. This variation may in part be related to the animal species from which the globulin preparation is obtained, since the greatest variation was observed between a rabbit globulin standard and the other standards, which were horse and human globulin solutions. A similar difference was found by Ipsen (1944). However, Kusama et al. (op. cit.) have recently reported considerable variation among globulins obtained from a single species, either horse or man. Such a variation would suggest that it is immaterial which animal species is chosen to furnish a preparation for use as an international standard. The standard globulin preparation which has recently been established as the International Standard for Antistreptolysin-O is prepared from human globulins (Spaun, Bentzon, Larsen & Hewitt, 1959). Comparison of the International Standard and the Todd horse globulin standard has revealed no obvious dissimilarities. This suggests to us that the Todd horse globulin preparation, which is freely available, would be quite suitable for use as a working standard.

RÉSUMÉ

Il est fréquent que l’on détermine la teneur d’un sérum en antistreptolysine O, pour étayer le diagnostic de streptocoque récent. Des difficultés ont surgi lorsqu’il s’est agi de titrer l’antistreptolyse dans une série de sérums, au moyen d’une méthode simple, applicable couramment dans les laboratoires d’analyses sérologiques.

L’auteur a cherché les causes de l’inconstance des résultats et a découvert que l’incubation à 37° C du mélange toxine-antitoxine en vue de la neutralisation de la toxine, diminuait la teneur en toxine résiduelle, qui doit être titrée. La toxine perdait progressivement, au cours de 30 minutes, une bonne part de son activité, attaquée probablement par une protéinase que l’on sait exister dans les préparations d’antistreptolysine. En revanche, lorsque le mélange est maintenu à 4° C pendant 2 heures, la réaction atteint un équilibre, et la toxine non neutralisée reste stable pendant une heure au moins.

La méthode de titrage de la toxine a été modifiée en conséquence. On utilise des séries de sérums dilués à raison 2, tamponnés, et un point final 50% est calculé d’après la quantité d’hémoglobine libérée des érythrocytes de lapin par la streptolysine O.

En outre, l’auteur a comparé la préparation étalon d’antistreptolysine de Todd avec les étalons utilisés dans diverses parties du monde. Si les valeurs absolues sont assez semblables, de notables différences sont apparues dans les réactions de neutralisation.

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