EXPERIMENTS WITH HUMAN AGGLUTINATING SERA.

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It is well known that the potency of human serum used for blood grouping frequently deteriorates with considerable rapidity. As a result, volunteers may be wrongly diagnosed as "universal donors," and time may be wasted in cross-matching the bloods of donor and recipient, and mistakes occur if this cross-matching is not carried out.

The object of the following experiments was to study methods of increasing the agglutinating power (or titre) of these sera, and to study what factors are of importance in maintaining this power as long as possible.

The agglutinating power of the serum is the highest dilution which is able to produce visible agglutination of the red cells. It is this agglutination of red cells by an incompatible or "typing" serum which causes the severe reaction in the patient when an incompatible donor is used.

The titre of the serum, however, varies according to the time allowed for agglutination to take place, the temperature, the method of reading the results (whether by naked eye, hand lens, or microscope), and the technique used. As the rate of deterioration of the serum can only be discovered by estimating the titre from time to time, it is obvious that some satisfactory and standard method of titre-estimation must be used in all the experiments.

Two methods were therefore tried out, which will be briefly described:—

(1) DILUTION TUBE METHOD.

The technique was based upon that used in the estimation of the heterophile antibodies in the blood serum of a patient suffering from glandular fever.

The serum was first inactivated for half an hour at 56°C. to destroy the complement and prevent hemolysis, and a series of increasing dilutions was made in Wassermann tubes. A 2 per cent suspension of washed red cells in saline was added to each tube, and the tubes were placed in the water bath at 37°C. for one hour and then stored in the cold room overnight. They were then inverted three times and the clumping of the red cells observed. The highest dilution of serum causing naked-eye agglutination of the red cells was taken as the titre (by this method).

(2) SLIDE METHOD.

Hollow ground glass slides were used and a series of doubling dilutions of the serum made on these in 1 per cent sodium citrate-saline solution.
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A 5 per cent suspension of whole blood in 1 per cent citrate-saline was prepared and an equal volume added to the diluted serum on each slide. The mixture was then stirred with a stiff wire immediately, after five minutes, and after twenty-five minutes at room temperature. The macroscopic titre, seen by the unaided eye, was read at five minutes, and the microscopic titre, seen under two-thirds magnification of the microscope was read at thirty minutes. The latter titre was invariably found to be four times as great as the former. In the earlier experiments, controls for false reactions (pseudo-agglutination, etc.) were put up but were later found to be unnecessary.

Comparison of Methods for Estimating the Titre.

By the dilution tube method, two sera gave titres of 256 and 512 respectively. By the slide method the microscopic titres of the same sera were 64 and 128. It was considered, therefore, that the slide method was sufficiently accurate to be employed in the further experiments to be undertaken, and it has, of course, the advantage of speed. The future titres expressed, therefore, are the microscopic titres by the slide method. Strong agglutination is expressed by the sign +, and weak agglutination by ±. It must be remembered, of course, that the agglutinability of red cells varies in different individuals, and therefore the sera used in the experiments (Group 3 or 4) were always tested against my own red cells (Group 2).

Raising the Titre.

It is obvious, of course, that the typing sera to be kept in stock should be of as high an initial titre as possible in order to remain efficacious for a longer period, but it is not always easy to procure a high titre serum—particularly a high titre of the rare Group 3 serum—in a limited military population. Some method, therefore, of artificially raising the agglutinating strength of serum is required. The following methods were used:

1) Physical Method.—A light yellow-brown serum with a titre of 64 was chosen; 10 cubic centimetres were placed in a narrow sterile test tube, frozen solid and thawed at room temperature four times. As a result of this the serum separated into sharply defined upper and lower layers, a nearly colourless upper two-thirds having a titre of 16 and a dark brown lower third having a titre of 128. The upper two-thirds were removed and discarded.

2) Biological Method.—This is based on the fact that red cells absorb specific agglutinins at room temperature and liberate them at 56°C. Groups 2 cells were added to a Group 3 serum having a titre of 32 and the mixture shaken occasionally for two hours at room temperature. The cells were then removed from the serum by centrifugation and washed in saline. They were afterwards added to a small quantity of saline, shaken, and heated to 56°C. for five minutes in a water bath, and shaken
thoroughly. The mixture was then rapidly centrifuged while hot in a preheated and well-padded centrifuge tube, a hot water centrifuge not being available. The supernatant saline was removed while hot, and had a titre of 128.

(3) Chemical Method.—This was carried out with a number of different sera. These were first diluted with double their volume of distilled water. The euglobulin, precipitated by adding 16 per cent ammonium sulphate crystals to the diluted sera, was removed by centrifugation or filtration and re-dissolved in 2 per cent salt solution, but did not agglutinate red cells. The pseudoglobulin precipitated by 28 per cent ammonium sulphate, removed by filtration in a water-saturated atmosphere, dried by expression between layers of filter paper, and redissolved in distilled water, gave a high titre of agglutination. The final filtrate containing the serum-albumin, etc., did not agglutinate red cells.

By this method a serum had its titre raised from 8 + to 64 ±. It was found, however, that all the sera so treated gave a somewhat “cloudy” effect which interfered with the macroscopic and microscopic readings. Further experiments showed that if ammonium sulphate crystals (acid) or sodium chloride crystals (B.P., alkaline) were added to a serum, the titre of this was lowered, although insufficient chemical was added to produce any precipitation in the serum. It was evident that the resulting hypertonicity of the serum rendered it unsuitable, and the same “cloudy” effect as above was noted in every case.

The solutions of pseudoglobulin in distilled water was therefore dialysed in collodion bags suspended in water in order to get rid of the ammonium sulphate. Dialysis was continued for six days at 15° to 18° C. By this method a solution was obtained giving a fourfold increase of titre over the original serum, and much less “clouding.”

**Comparison of Methods for Increasing the Titre.**

The method of concentration recommended is that of freezing and thawing, as less working time is required and there is less risk of contamination.

**Effect of Temperature on Sera.**

Sera were kept in the incubator at 37° C., at room temperature (17° to 20° C.), and in the ice box (10° to 14° C.). All were protected from light.

A serum with a titre of 16 was so kept for four months. At the end of this time the titre had dropped to 0 at 37° C., but was still 16 at room temperature and in the ice box. A second serum was chosen with a titre of 64. In nine months at room temperature the agglutinating power had dropped from 64 + to 64 ±, but in four months at 37° C. it had fallen to 0. The titre of a third serum fell from 32 ± to 16 ± in four months at room temperature and to 0 at 37° C. in the same period. As will be seen later the rate of deterioration of the serum titre depends
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upon the reaction of the glassware as well as upon the temperature. It is evident that the problem is chiefly one concerning the preservation of sera at high temperatures, such as would occur on active service in tropical countries.

Effect of Mineral Acids and Glycerine on Sera.

The following experiments were carried out at 37° C. Preliminary experiments showed that the keeping power of a serum varied in an irregular manner when samples of it were kept in tubes of different kinds of glass. It was thought that this might be due to varying alkalinity of the glass, and investigation showed that the more alkaline samples of serum (high pH) had the lowest titres. From this it seemed likely that the addition of an acid substance to the serum would improve its keeping power. As glycerine (acid, pH below 6.8) was experimented with at the same time, it will be necessary to consider both glycerine and other acid substances together.

The first essential was of course to standardize some method of measuring the reaction of the sera by estimating the pH value. This was carried out as follows:

A flask of distilled water containing 10 per cent of 0.01 per cent phenol red solution as indicator was adjusted by the addition of caustic soda solution until it was exactly neutral in reaction, the pH remaining stable at 7.0 when tested daily. Three cubic centimetres of this fluid were placed in a "cordite" glass tube and 4 drops of serum were added from a size "56" dropper. The tube was shaken and the pH read colorimetrically by comparison with the standard tubes.

Experiment 1.—The following experiment was then carried out to discover the effect of acid substances and glycerine on the keeping power of a serum. The latter was kept in narrow sealed tubes of the same make and glass, which were prepared for use by boiling in alkali soap, rinsing, soaking in 1 per cent hydrochloric acid for twenty hours, rinsing in distilled water (acid, pH below 6.8), and sterilizing by dry heat. The serum was obtained from blood taken into a plain sterile test tube (not new).

<table>
<thead>
<tr>
<th>Sample of serum</th>
<th>Initial Controls</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
<th>Sample 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance added</td>
<td>—</td>
<td>—</td>
<td>1½% sulphuric acid</td>
<td>Acid sodium phosphate</td>
<td>Glycerine</td>
<td>Sodium phosphate</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>Amount of substance added</td>
<td>—</td>
<td>—</td>
<td>10%</td>
<td>Trace</td>
<td>About 60%</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Titre of serum</td>
<td>128+ Both 8.4 and 8.0</td>
<td>32+</td>
<td>16+</td>
<td>32+</td>
<td>0</td>
<td>0</td>
<td>16+</td>
</tr>
<tr>
<td>pH of serum</td>
<td>7.6</td>
<td>8.0 and 8.1</td>
<td>7.7</td>
<td>6.8</td>
<td>8.0</td>
<td>Above 8.4</td>
<td>Above 8.4</td>
</tr>
</tbody>
</table>
Two samples of the same serum, untreated, were kept as "controls" under the same conditions as the tubes containing serum and the added substance. The nature and amount of the latter are shown in the table on page 34, which also indicates the titre and pH at the end of the experiment with the various samples of this serum. The second column of the table headed "Initial Serum" gives the titre and pH of the serum at the commencement of the experiment. The duration of the experiment was ten weeks at 37°C.

From this experiment it will be seen that the "control" sera became more alkaline on keeping, as shown by the rise of the pH value, and that the titre fell considerably. The addition of acid or acid salt produced a lower pH and a higher titre, while the two alkaline sodium salts produced a higher pH and a lower titre. Glycerine (acid) gave a higher titre, but glycerine made alkaline with a few drops of caustic soda solution was not so satisfactory.

Experiment 2.—A second experiment was undertaken with a different serum to confirm the above results. As alkali is presumably given off by the glass, the glass tubes (new) and the bottles (not new, having been used many times previously for media) used in this experiment were prepared as in the previous experiment, but the acid used was raised to 2 per cent. HCl for two days.

The serum was obtained from blood taken into an agar-lined test tube. (In this experiment only three drops of serum were used in the estimation of the pH.) The serum was divided into two portions which were treated as follows:

(a) The first portion was kept at 37°C for five weeks in sealed glass tubes. Three "controls" of the same serum; untreated, were kept in the same manner as the treated specimens. The following table, arranged as before, shows the titre and pH of the various specimens of serum at the end of the experiment.

<table>
<thead>
<tr>
<th>Sample of serum</th>
<th>Initial serum</th>
<th>Controls 1, 2 and 3</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
<th>Sample 6</th>
<th>Sample 7</th>
<th>Sample 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance added</td>
<td>-</td>
<td>-</td>
<td>10% sulphuric acid</td>
<td>10% sulphuric acid</td>
<td>N/10 sulphuric acid</td>
<td>N/10 sulphuric acid</td>
<td>N/10 hydrochloric acid</td>
<td>Acid sodium phosphate</td>
<td>Glycerine</td>
<td>Glycerine</td>
</tr>
<tr>
<td>Amount of substance added</td>
<td>-</td>
<td>-</td>
<td>10%</td>
<td>20%</td>
<td>10%</td>
<td>20%</td>
<td>20%</td>
<td>Trace</td>
<td>About 60%</td>
<td>About 80%</td>
</tr>
<tr>
<td>Titre of serum</td>
<td>32</td>
<td>All 0</td>
<td>8</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>pH of serum</td>
<td>7-8</td>
<td>All above 8.4</td>
<td>8-0</td>
<td>Below 6-8</td>
<td>Above 8-4</td>
<td>8-4</td>
<td>7-7</td>
<td>6-8</td>
<td>8-3</td>
<td>8-2</td>
</tr>
</tbody>
</table>
(b) The second portion of serum was kept at 37°C for seven and a half weeks in the screw-capped bottles. Two "controls" of the same serum, untreated, were kept under the same conditions. The following table shows the titre and pH as before at the end of the experiment.

<table>
<thead>
<tr>
<th>Sample of serum</th>
<th>Initial serum</th>
<th>Controls 1 and 2</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
<th>Sample 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance added</td>
<td>-</td>
<td>-</td>
<td>1% sulphuric acid</td>
<td>N sulphuric acid</td>
<td>Acid sodium phosphate</td>
<td>Glycerine</td>
<td>Glycerine</td>
<td>Glycerine</td>
</tr>
<tr>
<td>Amount of substance added</td>
<td>-</td>
<td>-</td>
<td>10%</td>
<td>10%</td>
<td>Trace</td>
<td>About 33%</td>
<td>About 60%</td>
<td>About 80%</td>
</tr>
<tr>
<td>Titre of serum...</td>
<td>32+</td>
<td>Both 2 ± 4</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>2 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>pH of serum ...</td>
<td>7.8</td>
<td>Both 8.2</td>
<td>7.4</td>
<td>8.1</td>
<td>Below 6.8</td>
<td>8.1</td>
<td>8.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

The two controls gave titres of 8 + and 8 ± when tested at the end of five and a half weeks. At this period the other bottles did not show any improvement over these figures.

Experiment 3.—A final test was carried out with a third serum on the same lines as the two previous experiments. In this case more energetic measures were taken to neutralize the glassware. This was boiled in 6 per cent sulphuric acid and potassium bichromate solution for three hours, instead of alkali-soap, soaked in distilled water (acid, pH below 6.8) overnight, and sterilized by dry heat. Four of these tubes were then tested by keeping in them for a week at room temperature some of the neutral fluid (pH 7.0) used in estimating the pH. At the end of the week this fluid was still neutral or only very slightly alkaline (pH 7.0 to 7.1).

The samples of this serum were kept for seven weeks at 37°C, and then the pH and titre of these (including three "controls") were measured. The samples containing 7 per cent of 1 per cent sulphuric acid, and 14 per cent of N/10 sulphuric acid, both gave titres of 32, as against 16 in two of the controls and 8 in the third control; and pH values of 7.8 and 8.0 respectively against 8.2 to 8.3 in the controls. The initial serum, from an agar-lined tube, had a pH value of 7.7 and a titre of 64. Presumably a definite amount of alkali was given off from the glass in seven weeks at 37°C, and the beneficial effect of mineral acid was again evident.

In the course of these experiments the following observations were made:

(a) Phenol Red.—The addition of 14 per cent of 0.01 per cent phenol red solution as indicator to a serum was not found to have any deteriorating effect, and the further addition of sulphuric acid to the mixture improved the keeping power of the serum as compared with untreated serum controls. This fact might be used, sufficient acid being added to produce a standard colour in the serum.
(b) Reaction of Serum from Clots.—As the reaction of serum is evidently of importance in regard to its keeping power, the following observation may be of importance in standardizing any method of obtaining serum. All the serum was removed from a blood-clot in an agar-lined tube after twenty-four hours and gave a pH value of 7.8. After four days the serum further exuded gave a pH of 7.4. Another serum, stored in a plain glass tube over a small sediment of red cells gave a pH which fell from 7.5 to 7.2 in three days. Neutral fluid stored in control tubes of the same batch showed no alteration of pH.

(c) Effect of Salts on Sera.—The addition of 6 per cent ammonium sulphate to a serum had no effect. About 10 to 12 per cent ammonium sulphate crystals dissolved in a serum had no effect upon its microscopic titre (16) but reduced the macroscopic titre from 4 to 2. In another case approximately 14 per cent ammonium sulphate caused no trace of turbidity in a serum but reduced the (microscopic) titre from 8 to 4. A fourth serum diluted with equal volumes of either normal, half saturated or saturated sodium chloride solution gave titres of 8, 4 and 4 ±. Although, therefore, the protein was not precipitated from the sera by any of the above hypertonic solutions, the effect was to lower the titre and cause a "cloudy" type of agglutination. These facts indicate the importance of dialysing the pseudoglobulin solution.

(d) Experiments with Dried Sera.—These experiments were not completed owing to change of station, but the following results may be of some interest. Two sera were frozen in the refrigerator and then evaporated to a dry powder in vacuo at -10°C. Samples of each were immediately redissolved in sufficient distilled water to restore the original volume, and in both cases the titre had fallen to one-half. Neither of the dried sera when subsequently stored at room temperature or in the ice chest showed any advantage over liquid "control" sera similarly kept; a saturated solution in distilled water gave the same titre as the "controls" and of course a smaller volume. Dried sera kept at 37°C. would not yield a satisfactory solution. It must be noted, however, that these dried sera were not stored in vacuo.

(e) Other Experiments.—Other methods were tried in which the oxygen dissolved in liquid serum was as far as possible removed or absorbed. This was achieved by (1) addition of glucose; (2) exhausting the air by suction pump for twenty minutes (trial tubes when broken under water showed an almost complete vacuum); (3) removal of oxygen by pyrogallate; (4) a combination of glucose and either of the other two methods. None of these methods improved the keeping power of the sera.

Discussion.

In all the foregoing and other experiments it was shown that the sera became more alkaline when stored at 37°C. or at room temperature, in spite of the various efforts made to use neutral glassware. Associated with this
was a marked fall of titre. When mineral acid or acid salt was added to the serum, this was less alkaline or slightly acid (pH lower) and the titre of the serum was definitely higher. The serum also kept much better, and became less alkaline in the old bottles than in the new glass tubes. It was found that excess of acid or acid salt producing a pH well below 6.8 was inimical to the maintenance of a high titre in the serum, and that alkalies producing a pH above 8.4 had the same effect. Glycerine was not found to be wholly satisfactory as a preserving agent. Although 60 to 80 per cent of glycerine added to sera improved their keeping qualities, in no case were the titres obtained so high as when 1 per cent sulphuric acid was used, and a more serious objection was that the type of agglutination was “cloudy” and therefore more difficult to read. In the case of acids and traces of acid salt, however, the agglutination was always quite distinct. Smaller amounts of glycerine had neither good nor bad effect on the sera.

CONCLUSIONS.

(1) A method of raising the titre of human agglutinating serum is described.

(2) The importance of the reaction of agglutinating serum in regard to its keeping quality is also shown, together with the improvement obtained by the use of mineral acid.

I should like to express my thanks to Brevet Colonel L. T. Poole, D.S.O., M.C., K.H.P., R.A.M.C., for his advice and the interest he has taken in these experiments. I am, however, responsible for the results.