THE ESTIMATION OF THE INDIVIDUAL HUMAN SERUM PROTEINS BY AN IMMUNOLOGICAL METHOD

BY

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Now that something is beginning to be known about the functions of the individual serum proteins there is need of a simple and satisfactory way of estimating their concentrations in normal and pathological conditions. It is difficult to do this by physico-chemical methods since serum is a mixture of so many different components. Methods such as electrophoresis distinguish about five main groups, while a more delicate method like the "immunoelctrophoresis" of Grabar and Williams (1953) can demonstrate numerous further components both in the \( \alpha \) and \( \beta \)-globulin groups. Complete purification of one such component by ordinary chemical means is hardly possible for routine estimation, but advantage can be taken either of chemical groupings which may be sufficiently specific to be estimated individually, for example, porphyrin or copper, or of the immunological specificity of antibodies, as in the Grabar-Williams method mentioned above. Antibodies are of course extremely precise in their discrimination, but it is hard to make immunological estimations both quantitative and simple. Measurement by the micro-Kjeldahl technique of precipitated antibody-nitrogen is extremely sensitive and accurate when properly carried out, but the antigen must be genuinely pure, and the method is slow and laborious. New gel-diffusion methods discussed by Augustin (1955) show promise, but are all somewhat difficult for routine use and, moreover, demand initially a fairly precise idea of the protein concentration to be measured. By the simplified serological methods described in this paper it is possible to decide within 24 hours whether the concentration of a given protein in the serum under test is within normal limits, and, if it is deficient, to give an estimate of its concentration to within about 25% each way. The only difficulty of this type of estimation is that of producing the antiserum in the first place, since it is desirable to use a purified or semi-purified preparation for immunization. Although antisera produced by immunization with even apparently pure preparations of proteins nearly always show the presence of antibodies against contaminants, it is nevertheless usually possible when one of the gel-diffusion methods is used, by which a given "line" can be correlated with the presence of a particular protein, to demonstrate with certainty a single antibody component reacting with the protein under investigation.

Methods and Materials

Antisera.—The following are required:

**Gamma Globulin.**—The antiserum against the \( \gamma \)-globulin was raised by the injection of purified \( \gamma \)-globulin (Lister Institute, kindly supplied by Dr. W. d’A. Maycock) into crossbred Copenhagen rabbits. The course of immunization used, which was successful in producing a strong antiserum, began with intradermal injections of 0.1 ml. of concentrated \( \gamma \)-globulin (10%). These were continued until a well-marked Arthus reaction was induced. When this reaction was at its height a course of thrice-weekly intravenous injections with alum-precipitated \( \gamma \)-globulin (protein concentration 0.15%) was given (Kabat and Mayer, 1948). This course was continued intermittently for four months, with occasional intradermal injections to test the occurrence of Arthus reactions. The first successful bleed was taken about 18 weeks after the beginning of the course; after this the animals were rested for three months. A further course of injections of the concentrated material was then given, first intraperitoneally (0.1 ml.) and then intravenously (0.2–0.5 ml.). Estimation of the precipitable antibody in this serum showed that it contained something of the order of 5 mg./ml. in all animals. Fig. 1a shows the immunoelectrophoretic picture given by the reaction of this antiserum with normal human serum. It can be seen that the antibody is almost exclusively directed against the gamma component, and other lines will not interfere with the reading except in special cases (see below).
Coeruloplasmin. — The serum against coeruloplasmin was raised against a preparation supplied through the courtesy of Professor Schultz of Marburg, to whom my thanks are due. This was precipitated with alum according to the previously mentioned technique and injected intravenously into four rabbits. Only one animal responded satisfactorily to the first course, but this supplied an antiserum which was quite sufficient for a number of quantitative estimations, although it did not contain a large amount of precipitable antibody; a second course produced a rather better serum (Fig. 1b).

Siderophilin (β-Metal-carrying Globulin).—This antiserum was also raised against preparations kindly supplied through the courtesy of Professor Schultz of Marburg. The animals were first given two subcutaneous injections of an emulsion of the antigen (5%) prepared according to Freund’s adjuvant method (Kabat and Mayer, 1948). This was followed by a short intravenous course with an alum-precipitated antigen prepared in a similar manner to that previously described. A satisfactory serum was raised by these means in a matter of six weeks (Fig. 1c).

The lines formed by this antiserum were not quite as precise as those formed with the two previous ones, possibly owing to the fact that it was prepared as the result of so short a course of immunization.

Estimation

These methods were devised initially for the routine estimation of sera from cases suspected of hypogammaglobulinaemia, and for the control of the treatment of such cases with γ-globulin.

The critical level of γ-globulin in the serum required for "normal" resistance to infection is 0.15 to 0.20 g. % (Janeway et al., 1953), a value below the level for satisfactory estimation by electrophoresis. A single method was therefore required to find out both whether the level of γ-globulin in the patient’s serum differed significantly from the normal level of 0.8 to 1.2 % (less in an infant), and also to detect and estimate it in a deficient serum down to very low concentrations.

The method described here depends upon the use of the Elek (1948)–Ouchterlony (1948) principle, whereby...
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antigen and antibody diffuse towards one another in agar plates to form "lines" of precipitate at optimal proportions. The character of such lines is very much more sensitive to changes in the concentration of antibody than of antigen, which makes the straightforward comparison of the lines formed by a standard and by an unknown antigen of little use for purposes of estimation. In the method described here, therefore, the lines formed by the unknown reacting with several different concentrations of antisera are compared line by line with those formed by several standard preparations of different concentration.

In preliminary tests, a range of dilutions of antisera is found, which, when reacting in agar with a range of antigen dilutions, will give satisfactory lines, neither too strong nor too faint. If now six dilutions of antisera within this range are arranged, in cups cut in agar, around a central cup containing, for example, \(\gamma\)-globulin in (appropriate dilution), a hexagon of precipitate will be formed and the shape and appearance of such a hexagon will be different for different concentrations of the antigen in the central cup. If appropriate volumes or dilutions of the serum in which the \(\gamma\)-globulin is to be estimated are similarly made to form hexagonal precipitates with the same dilutions of antisera, these can be compared line by line with the hexagons produced by the various \(\gamma\)-globulin standards. A standard hexagon which matches the appearance of a test sample most exactly will give the concentration of \(\gamma\)-globulin in that dilution of the unknown serum; with experience intermediate values can be suggested with reasonable accuracy. Three or four times the standard volume of the unknown serum can be used undiluted and the test thereby made extremely sensitive, though in this case care must be taken not to be misled by lines due to other antibodies, particularly those often developed against trace components of human serum. A further advantage is that grossly haemolysed and even infected samples of serum can still give reliable results, though these should of course always be checked on a satisfactory sample later.

Results

Table I gives details of the volumes of the various reagents used in the estimations, and Figs. 3, 4, and 5 show examples of actual tests.

\(\gamma\)-Globulin.—From Table II it can be seen that concentrations of \(\gamma\)-globulin can be estimated from 1.2% (that is to say a high normal level)

![Diagram](image)

**Fig. 2.—Diagrammatic arrangement of antisera (A to F), standards (S1 to S4) and unknowns (T1 and T2): for quantities used in the actual estimations see Table I. The reactions are done in flat plates containing phosphate-saline-agar (KH₂PO₄, 2.75%; KOH 0.8%; NaCl, 1.7%; New Zealand agar 0.7%; pH 7.4) poured to a depth of approximately 2.5 mm. The cups are cut with a No. 2 cork-borer (or larger if required for increased volumes of unknown sera). A convenient template for cutting the cups can be made from "chartwell" isometric graph paper (4801). In reading it should be noted that the lines of each hexagon run in reverse order to those of the adjacent ones.**

<table>
<thead>
<tr>
<th>Estimations of Sera</th>
<th>(\gamma)-Globulin</th>
<th>Coeruloplasmin</th>
<th>Siderophilin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cup T1: Un-unknown</td>
<td>4 drops or 1/8 drop</td>
<td>3 drops</td>
<td>1 drop 1:25 or 1:20</td>
</tr>
<tr>
<td>Serum</td>
<td>1 drop or 1/64 drop</td>
<td>1 drop</td>
<td>1 drop 1:10</td>
</tr>
<tr>
<td>Cup S1: Standard</td>
<td>1 drop 1:50</td>
<td>2 drops</td>
<td>Normal serum*</td>
</tr>
<tr>
<td>(\gamma)-globulin</td>
<td></td>
<td></td>
<td>1 drop 1:2/5</td>
</tr>
<tr>
<td>Serum</td>
<td>1 drop 1:400</td>
<td></td>
<td>Normal serum*</td>
</tr>
<tr>
<td>(A) Antiserum</td>
<td>1 drop 1:100</td>
<td></td>
<td>1 drop 1:5</td>
</tr>
<tr>
<td>(B)</td>
<td>1 drop 1:200</td>
<td></td>
<td>1 drop 1:1</td>
</tr>
<tr>
<td>(C)</td>
<td>1 drop 1:400</td>
<td></td>
<td>1 drop 1:4</td>
</tr>
<tr>
<td>(D)</td>
<td>1 drop 1:100</td>
<td></td>
<td>1 drop 1:4</td>
</tr>
<tr>
<td>(E)</td>
<td>1 drop 1:100</td>
<td></td>
<td>1 drop 1:4</td>
</tr>
<tr>
<td>(F)</td>
<td>1 drop 1:100</td>
<td></td>
<td>1 drop 1:4</td>
</tr>
</tbody>
</table>

Distance apart of centres of cups . . . . . . 0-9 cm.

* Or appropriate amounts of the purified protein.

<table>
<thead>
<tr>
<th>Table II</th>
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**CONCENTRATIONS OF \(\gamma\)-GLOBULIN \(\mu\)GAMMA-PROTEIN TO VOLUMES OF UNKNOWN SERA**

<table>
<thead>
<tr>
<th>Sera (Vol.)</th>
<th>20</th>
<th>10</th>
<th>5</th>
<th>2-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4) (T1)</td>
<td>a</td>
<td>5.0</td>
<td>2.5</td>
<td>1.25</td>
</tr>
<tr>
<td>(1) (T2)</td>
<td>20</td>
<td>10</td>
<td>5</td>
<td>2-5</td>
</tr>
<tr>
<td>(8) (T1)</td>
<td>160</td>
<td>80</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>(164) (T2)</td>
<td>1,280</td>
<td>640</td>
<td>320</td>
<td>160</td>
</tr>
</tbody>
</table>

Gamma-globulin content when given volume of unknown serum gives a hexagon matching one of the concentrations of standard \(\gamma\)-globulin.
Estimation of $\gamma$-globulin: the TI cup (4 vol. serum) shows marked "antigen excess"; the T2 cup (1 vol.) is suitable for reading, and is intermediate between S2 and S3 but rather nearer the latter. The $\gamma$-globulin in the unknown serum may therefore be estimated at 7 mg. per 100 ml. (0.007%) and confirms the presence of gross hypogammaglobulinaemia. (Reaction stained with amidoschwartz and printed direct.)

Coeruloplasmin.—Coeruloplasmin is the copper-containing protein of plasma described and isolated by Holmberg and Laurell (1948). Its deficiency is associated with the condition known as Wilson's disease (hepato-lenticular degeneration); its normal concentration is of the order of 35 mg. per 100 ml. serum. It can be estimated with good accuracy in serum by copper analysis, but this method demands very meticulous technique, in particular as regards the exclusion of contaminating copper while taking the blood. The serological method, on the other hand, which

![Figure 3](http://jcp.bmj.com/)

**Fig. 3.**—Estimation of $\gamma$-globulin: the T1 cup (4 vol. serum) shows marked "antigen excess"; the T2 cup (1 vol.) is suitable for reading, and is intermediate between S2 and S3 but rather nearer the latter. The $\gamma$-globulin in the unknown serum may therefore be estimated at 7 mg. per 100 ml. (0.007%) and confirms the presence of gross hypogammaglobulinaemia. (Reaction stained with amidoschwartz and printed direct.)

![Figure 4](http://jcp.bmj.com/)

**Fig. 4.**—Estimation of coeruloplasmin: the T1 cup contains 1 vol. of the test serum, the T2 cup 1 vol. of this serum at 1:4. For other quantities see Table I. T1 is read as slightly stronger than S1 and T2 as intermediate between S2 and S3, i.e., 1:4 vol. of test serum is equivalent to between 1:1 vol. of normal serum: coeruloplasmin is present at between 200% and 400% of normal. (The test serum came from a thyrotoxic patient; its copper content was 308 $\mu$g., which is about three times the normal level.)

![Figure 5](http://jcp.bmj.com/)

**Fig. 5.**—Estimation of siderophilin: the T1 cup contained 1 vol. of the test serum at 1:5, the T2 cup 1 vol. of the serum at 1:20. For other quantities see Table I. T1 is read as slightly weaker than S1 and T2 as weaker than S3 but stronger than S4, i.e., 1:20 vol. serum is equivalent to between 1:10 and 1:10 vol. of normal serum, nearer the latter: siderophilin is about 175% of normal. (Same test serum as in Fig. 4.)
of course measures the protein rather than the metal, though much less precise, supplies a useful rapid method for, e.g., family surveys of the relatives of cases of Wilson's disease.

Siderophilin (β-Metal-carrying Globulin).—This serum protein is responsible for the transport of ionizable iron in the blood stream; its normal concentration is of the order of 250 mg. per 100 ml. serum. Its immunological and immunoelectrophoretic behaviour have been described by Grabar and Burtin (1955). As with coeruloplasmin, siderophilin can be measured by its metal content when saturated with iron; however, measurement of the protein per se by the serological method (Fig. 5) gives an independent estimate of the iron-carrying capacity of the plasma and in this and other ways may be of value.

The general method described here can clearly be applied to any protein whose line in an agar-diffusion test can be recognized with certainty and distinct from contaminants. Moreover, its specificity combined with extreme sensitivity allow of its application to other materials than serum. Its use in the analysis of proteins in small amounts in urine is under investigation.

Summary

A general method is described for the rapid serological estimation of individual proteins in whole human sera with an accuracy of about 50%. This method may be made to cover a very wide range of concentrations. Results are given of estimations of γ-globulin, coeruloplasmin, and siderophilin (β-metal-carrying globulin).

The method involves the comparison in agar-diffusion tests of the pattern of precipitation of the unknown serum, against different concentrations of antiserum, with the patterns formed by standard preparations of the antigen to be estimated, or by normal serum.

References