IMMUNOLOGICAL ANALYSIS OF ABNORMALITIES IN THE HUMAN SERUM PROTEINS BY A GEL-DIFFUSION METHOD

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Immunological methods allow of a more precise discrimination between similar proteins or high-molecular-weight carbohydrates than any chemical or physical method ; but they have in practice been little used in analysis. There are a number of reasons for this : only substances which are antigenic can be identified ; the major component of a mixture may provoke less antibody than a contaminant, or none at all ; there is no simple way of correlating a precip'tin reaction curve with the physical properties of the antigen molecule—shape, molecular weight, and so on ; quantitative estimation is a laborious and specialized technique ; furthermore there is no certainty that all the antibody measured has been brought down by the protein under investigation, rather than by contaminating antigen-antibody systems in the same optimal range.

The use of gel-diffusion methods such as those described below has altered this situation. It is now possible to identify a single line or "front" with near certainty as being due to a particular protein ; and, used with suitable precautions, this method of identification is far more specific and sensitive than any purely physico-chemical technique. It avoids the laboriousness and the uncertainties of immunological estimation, though it sacrifices its quantitative precision. However, quantitative estimates are possible, of a grade of accuracy usually sufficient for practical purposes.

The present study illustrates some clinical applications of the gel-diffusion method, with an approach to the problem of quantitative estimation. The method used is based on those of Ouchterlony (1948, 1949), Elek (1949), and Grabar (1954), in which the reagents diffuse towards one another from cups cut in agar in petri dishes.

Under these circumstances the reagents will meet at "optimal proportions" somewhere in the intervening space and a band of precipitate will occur there. If several antigen-antibody systems are present, several bands may develop, each corresponding with one antigen. If two differing preparations of antigen are placed in cups near one another, and both diffuse towards the antiserum, the lines due to proteins present in both will join at an angle, while if a particular protein is present in one but not in the other its characteristic line of precipitate will not be diverted (Fig. 1).

The methods using diffusion in test-tubes, such as those of Oudin (1946), Munoz and Becker (1950), and Oakley and Fulthorpe (1953), though giving well-marked band-patterns, do not allow of this side-to-side comparison of different preparations. Clearly with the petri dish method any convenient number of antigens can be arranged round the central antiserum cup, so that the lines due to a common antigen may join to form a polygon ; in practice, with fairly strong antisera, eight or 10 is the maximum giving lines of reasonable length.

A further refinement of technique is the combination of micro-electrophoresis (Grabar and Williams, 1953) with gel-diffusion. In this method the antigenic components are first separated by an electrophoretic run in agar, and then the antiserum is allowed to diffuse towards them from a trough cut at a suitable distance.

These techniques have as yet hardly been applied to the study of pathological sera. Clearly they are most readily adapted to showing qualitative or gross quantitative changes, their chief merit for this purpose being their very high specificity. Within their limits—that is, the power of discrimination of the antibody-forming cells of the animal species producing antibodies—there can be no sort of doubt about the identity of the fractions in which alterations occur. In the case of human sera, the antisera used in this work differentiate normally some dozen serum components, and other lines can be identified in
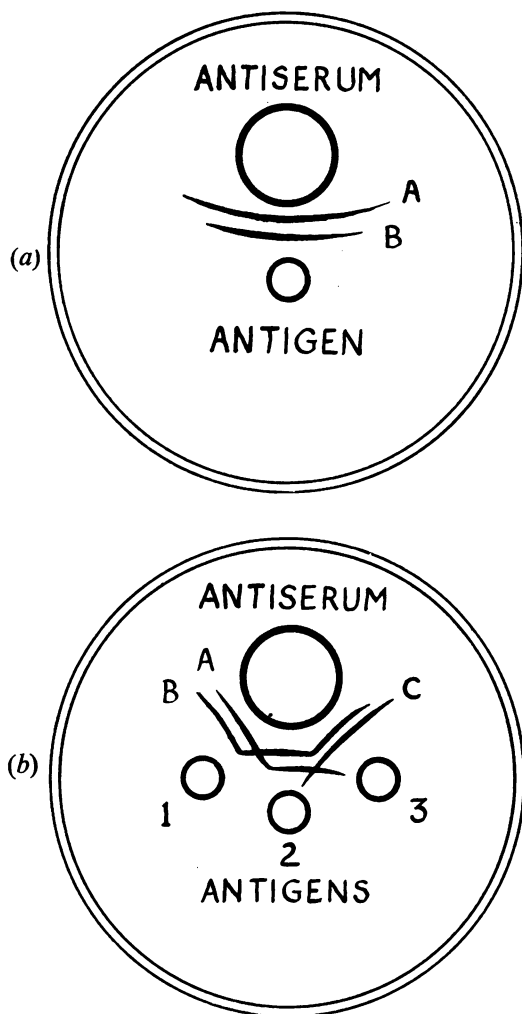
FIG. 1.—(a) Crude preparation of antigen (containing components A, B) diffuses towards antiserum where each pair meets as optimal proportions bands of precipitate form. (b) Three crude preparations of antigen diffuse towards antiserum—1 is seen to contain components A, B; 2 contains the same components but in different relative proportions since the lines join with those from 1; 3 contains one common component B and one fresh component C.

special cases by the use of fractionated or " pathological " sera (see below). Grabar and Williams (1953) quote at least 15–17 substances as being demonstrable. This very simple method for distinguishing so many different proteins can be used for analysis of pathological sera in large numbers, and may both throw light on the function of individual proteins and prove a more refined aid in diagnosis than paper electrophoresis alone.

Useful results have been obtained in the study of a- and hypo-gammaglobulinaemia, and promising ones in nephrosis, multiple myeloma, etc. ; some of these are described below.

Technique

Antisera.—These were raised in rabbits either by the use of alum-precipitated antigens given intravenously (Kabat and Mayer, 1948) or by subcutaneous injections of emulsions made according to the method of Freund and McDermott (1942). All sera are preserved with 0.02% sodium azide. The antigens used should in general be as crude as possible, that is, they should contain all the proteins which will later need identification ; special investigations will of course call for partially purified antigens. Satisfactory antisera will contain of the order of 0.25 mg. precipitable nitrogen per ml. Individual rabbits are exceedingly diverse in the pattern of antibodies which they produce to a multiple antigen, even if the total weight of antibody varies little, and there is some advantage in not bulking antisera but in keeping them separate.

Medium.—For protein antigens, agar is the most convenient medium. In this work New Zealand agar (Davis) has been used throughout at 0.5–0.6%, made up in phosphate buffer saline (pH 7.4) containing sodium azide (0.02%) ; this is filtered and poured in specially selected flat-bottomed petri dishes to a depth of 3 mm., care being taken to keep the dishes perfectly level. (Agar may be unsuitable for systems containing carbohydrate antigens, for obvious reasons.)

Setting up the Reaction.—For satisfactory results it is necessary to keep the arrangement of the cups strictly uniform : the exact dimensions will depend upon the strength of the antiserum. With antisera of the antibody content quoted above, a distance of 10 to 12 mm. between the edges of the antiserum and antigen cups, and about the same distance between the individual antigens, is sufficient for clear differentiation of the lines. With these distances the results can be read at 18 hours ; if even a slightly greater distance is allowed the time is sometimes considerably increased. Such dimensions allow a symmetrical arrangement of six to eight cups for antigens surrounding the antiserum cup.

It is convenient to cut the cups by means of corkborers ; their size and distances apart can thus be varied at will, which is impossible if a template is used. It is not necessary to seal the bases of the cups. The nearer the reagents are together, the easier it is to get visible lines with weak or dilute antisera. At 4 mm. one drop of a strong antiserum diluted 1/32 gives a clear line (Fig. 2). The limiting dilution of antiserum is that which just gives a positive ring (interfacial) test.

Antigen Concentration.—For clear-cut lines it is necessary that the total amount of antigen in each cup should be approximately in " equivalent proportions " to the total amount of antiserum, but a fairly wide variation is permissible (Fig. 3). If there are two

antigen-antibody systems present whose optimal ratios are very widely different, it may only be possible to bring one of them out satisfactorily. It is desirable in starting on any new batch of antiserum to put up a
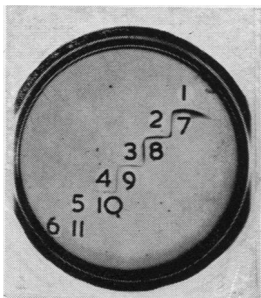

FIG. 2.—Limiting concentration of antibody. The cups 1–6 are filled with human serum albumin, decreasing by serial fourfold dilutions from 1% to 1/1024%, the cups 7–11 with antiserum at 1/1, 1/4, 1/16, 1/64, 1/256 (2 drops of each): the distance from antigen to antiserum is 4 mm. The limiting concentration of antibody under these circumstances is seen to be 2 drops of 1/64, assuming that the reagents are at approximately optimal proportions.

plate containing serial dilutions of standard antigen against a standard volume. With most of the systems used in this work, 4–24 drops of antiserum, 1–4 drops of antigen (human serum, undiluted) at 11 mm. gives a satisfactory general test of a pathological specimen. When the nature of the change is known the proportions can be altered, absorbed sera used, etc., to bring it out clearly and if possible quantitatively. Fig. 5 shows an example where the use of absorbed sera, and lesser distances, brings out with a very high degree of sensitivity the effects of therapy in a case of hypogammaglobulinaemia.

Identification of the Lines.—With human serum this is most conveniently done by means of fractions eluted from electrophoretic paper strips. The albumin and the γ-globulin can be easily identified with certainty

in this way. The β-globulins form a " family " containing four members at least ; this fraction is always contaminated with α_2-globulin, which with our standard (rabbit) antiserum has always appeared to be a single entity, though Grabar and Williams (1953) using horse antisera find several. The α_1-globulin has not been studied in this investigation. These identifications can be checked by means of a modification of the agar-electrophoresis method of Grabar and Williams (1953) as described below.

Quantitative Estimations.—The amount of antigen in a sample influences both the distance away that its line develops, and the density of the line. The distance is not at all sensitive to small alterations ; in one system it was found that $2 \times$ changes in concentration had the effect of shifting the line one millimetre. However, it is often sufficient to know the order of concentration, particularly where one is investigating the deficiency of a protein—a research to which the method, because of its high sensitivity, is particularly applicable. Thus a side-by-side comparison of the pathological serum with normal serum at a known dilution or with a known concentration of γ-globulin (Fig. 4) gives a useful working estimate of the amount of γ-globulin present. More precise methods of estimation are described below. In the rare cases in which there is an absolute deficiency of a protein the method can be pushed to the limit of its sensitivity, and is far more sensitive than any other at present available (Fig. 6).

Recording and Preservation of Completed Reactions.—The photographs reproduced here were obtained by placing the petri dish on the horizontal lens of a Leitz projector and photographing the image on the screen ; this is only practicable, however, with this type of projector. Attempts to photograph the reactions on a black background by obliquely transmitted light were only moderately successful.

It is quite easy to make the preparation permanent by fixing with formalin, washing thoroughly, removing
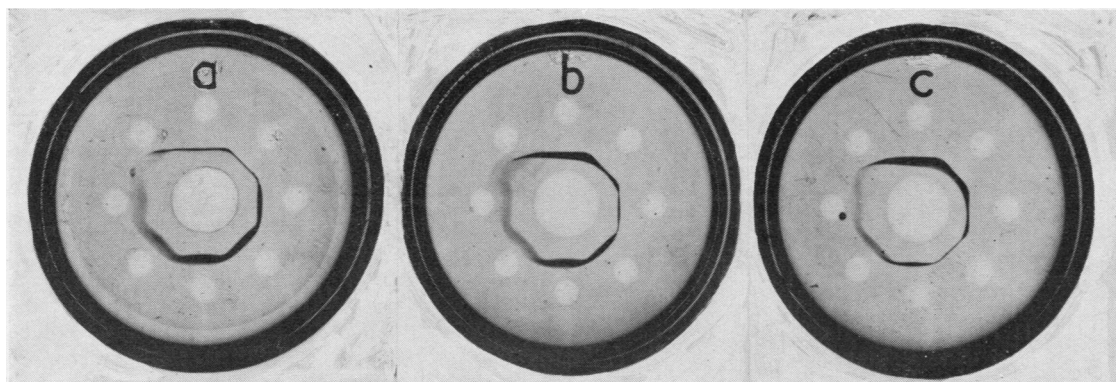

FIG. 3.—Optimal proportions. Three different amounts of antiserum (*a*, 16 drops, *b*, 8 drops, *c*, 4 drops) against eight serial dilutions of antigen (human serum albumin: 4 drops of 1%–1/128% arranged clockwise starting at the top). The optimum (sharpest line) is clearly 1/2 : 16 drops, 1/4 : 8 drops, 1/8 : 4 drops.
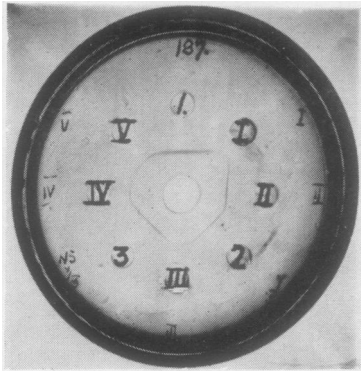
FIG 4.—A- and hypo-gammaglobulinaemia.   (1) Normal human serum 1 1.   (2) γ-globulin 0·3%.   (3) Normal human serum 3 10. I, Patient G. P. (Grant and Wallace): "total a-gammaglobulinaemia." F., 23 yr. II, Patient P., F., 47 yr. III, Patient N., F., 33 yr. IV, Patient W. F., M., 3 yr. V, Patient H., F., 38 yr. The deficiency of γ-globulin in the samples of serum from patients II–V can be seen by the faintness of the line of precipitate and the decrease in its distance from the (antigen) source. The line from normal serum 1 1 is in " antigen excess " to the amount of antiserum present. There is no sign of the presence of any antigen in Serum I.

FIG. 6.—A-gammaglobulinaemia. These two reactions demonstrate a more rigid test on the serum of Case 1 for traces of γ-globulin. (a) 1, Normal serum 1 100. 2, Serum, Case 1. 3, Serum, Case 2. 4, Serum, Case 3. While Cases 2 and 3 show more γ-globulin than normal serum at 1 100 (their lines are further from the antigen source), there is still no sign of a junction with any line from the serum of Case 1 (the other lines visible are due to minor globulins). (b) 1, 2, normal serum at 1/1,000; 3, normal serum at 1/10,000; 4, serum, Case 1. Compared with (a) the antiserum has been diluted 1/10; distance is 2 mm. There is still no sign of any deviation of the γ-line, i.e., the amount of γ-globulin present in Case 1 serum is (if any) less than 1 1,000 of the normal, but, since normal serum at 1 10,000 is too weak to react at all, there may be the equivalent of this amount at least present.

the agar slab, and drying it on a lantern slide. When completely dry the fainter lines disappear: the plates may either be stored dry and moistened for subsequent re-examination, or covered before they are quite dry with several coats of " betacryl 122X " (I.C.I. Dyestuffs), a plastic soluble in xylene. The final coat of plastic might be oiled or varnished to render it impermeable to water.

Such preparations, if they are not too thick, give better results in a projector than a photographic lantern slide.
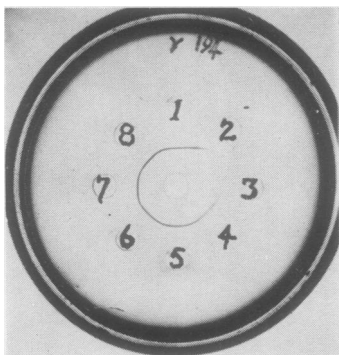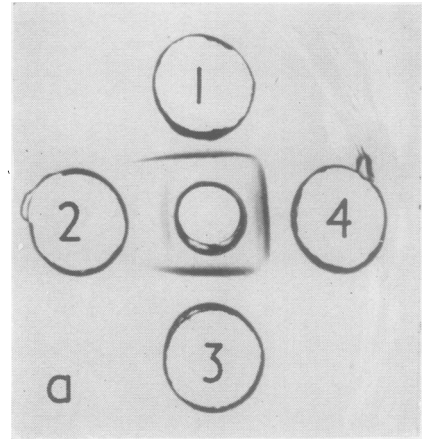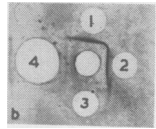

FIG. 5.—Effect of therapy on a case of hypogammaglobulinaemia. Case 4 (a boy of 3) had less than 0·05% gammaglobulin initially. He was given 0·5 g. of gammaglobulin weekly for four months, during which time the serum γ-globulin rose to about 0·25%. This figure illustrates this response to therapy. (1) γ-globulin (of the batch administered) 0·3%; (2) patient's serum before start of treatment; (3) one week; (4) three weeks; (5) five weeks; (6) nine weeks; (7) 13 weeks; (8) normal serum ½. The antiserum has been " adsorbed " (with the serum of Case 1) and reacts only with γ-globulin.

Illustrative Cases

Hypogammaglobulinaemia.—Cases of this syndrome are being more frequently found with the increasing use of paper electrophoresis in clinical laboratories.   Fig. 4 shows a routine test on the serum of five cases, in four of which γ-globulin is shown to be present though reduced in amount, while in one (Case 1) it appears to be absent entirely.   This case has been described by Grant and Wallace (1954) (to whom my thanks are due for the supply of samples): at the highest antiserum dilution practicable no suggestion of a γ-globulin line was found.

Patients with hypogammaglobulinaemia usually present as recurrent pneumonia, bronchiectasis, mastoiditis, etc., the deficiency of γ-globulin being correlated with a deficiency in antibody defences.   Of the cases from which serum samples have been examined here, four (Cases 1, 2, 3, 5) were in adult females, and one in a boy of 3 (Case 4).   In all except Case 1 there is some indication from the history that the condition was not present at birth ; in the child the first evidence of deficiency appeared at the age of 1, in Cases 2, 3, and 5 during the 20s or late 'teens.   None of the cases had any signs of deficiency of the

lymphoid system, as far as could be ascertained by clinical examination and blood counts. A number of cases of this syndrome have recently been described in Europe and the U.S.A. (Bruton, 1952; Young and Wolfson, 1954; Janeway, Apt, and Gitlin, 1953), some with and some without changes in lymphoid tissue.

Treatment is directed towards replacement of the absent antibodies, either with plasma, or preferably, because of the lesser risk of jaundice, with purified γ-globulin. These materials usually contain a sufficient titre of antibodies against the commoner infections to afford a reasonable measure of protection. Janeway *et al.* (1953) state that a blood level of 0.1 to 0.15% γ-globulin is necessary to obtain therapeutic results. This is about the limit of sensitivity for paper electrophoresis, and quantitative results are hardly possible. Fig. 5 shows the effect of therapy and Fig. 7 an attempt at quantitative immunological estimation: in the original plate at least there was
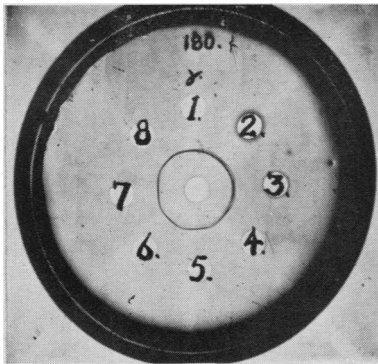

FIG. 7.—Quantitative estimation of γ-globulin. "Reference" (1) γ-globulin, (2), (3), (4), (5), (6), (7), (8) volumes of serum from Case 4 on 14/3 which if matched with (1) give equivalent concentrations of 0·1, 0·15, 0·2, 0·25, 0·3, 0·35, 0·4 (approx.). The three reactions, 2, 3, 4, on the right are definitely in "antigen excess" (the first interferes with the precision of part of the "reference" line): careful comparison of the characteristics of the lines on the original plate showed that (6) gave the best match to the density and sharpness of (1). It has not been possible to bring this out convincingly in the photograph.

no doubt, from comparison of the density and diffuseness of the lines, that 0.25 to 0.30% γ-globulin was equivalent to the concentration in the serum under test. This sample was obtained from the child of 4 (Case 4), who was being treated with 0.5 g. of γ-globulin intramuscularly once a week; the sample was taken just before an injection, and, since it represented presumably a minimum concentration, the treatment had evidently satisfied the criterion of Janeway *et al.* quoted above.

Comparison of Serum and Urinary Proteins in Nephrotic Syndrome.—Another clinical condition in which the specificity of the immunological method is of value is in the examination of the urine in proteinuria. Fig. 8 shows the result of a parallel examination of urine and serum in two
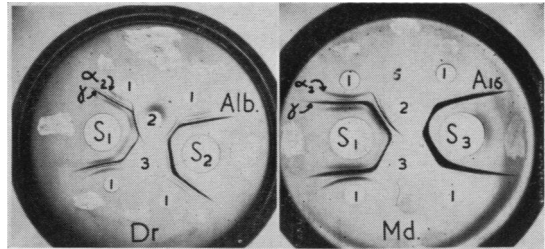

FIG. 8.—Nephrotic syndrome: serum and urine proteins. The serum and urine of two cases (Dr, Md) of nephrotic syndrome analysed with the assistance of three antisera: S_1 reacting with γ-globulin, α_2-globulin and some others (but not albumin), the others (S_2, S_3) reacting mainly with albumin. (1) Normal serum 1 vol. (2) Patient's serum 1 vol. (3) Patient's urine 2 vol. In both plates α_2-globulin appears to be absent from the urine: γ-globulin is present in both, and albumin is in very high concentration in the urines and deficient in the sera.

cases of the nephrotic syndrome. The urinary threshold of excretion closely parallels the molecular weights, α_2, and to a lesser extent γ, being retained in the serum, and albumin appearing in high concentration in the urine.

This figure also demonstrates the antigenic identity of the urinary and the serum proteins. As far at least as the rabbit antibody-producing system can discriminate, the process of excretion does not appear to have had any deleterious effect upon the protein molecules.

Serum Protein Abnormalities in Multiple Myelomatosis.—Fig. 9 shows the results obtained with myeloma sera and the antisera raised against them. Fig. 9a shows the results with a "γ-myeloma" with a typical electrophoretic picture : here the increased γ is evident in both the homologous and the heterologous (anti-normal-serum) reactions, i.e., the increase is due to excess of serologically normal γ-globulin, though whether the γ_1- or γ_2-fractions are involved is not shown by this method. In the β-myeloma the reaction with anti-normal-serum shows no marked deviation from the normal pattern, though the proportions of some of the components are changed, but the homologous reaction shows the presence of a strong antigenic component, apparently absent from normal serum, though further analysis suggests that the antibody produced cross-reacts with one of the normal β-globulins.

Finally Fig. 10 shows the reactions of a serum which contains a globulin of an unusual kind. This substance was precipitated on dilution of the serum
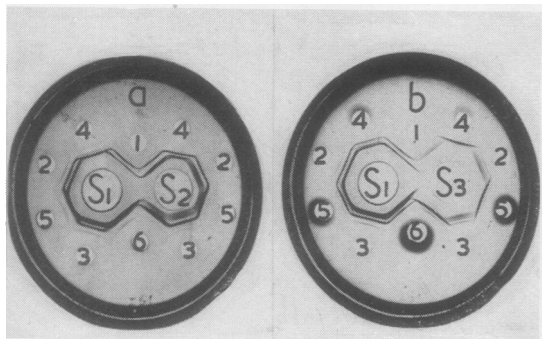
FIG. 9.—Multiple myeloma. (a) " Gamma myeloma " (Case Ou).
1, 2, 3 = Normal serum, 4, 5, 6 = myeloma (Ou) serum, at $\frac{1}{2}$, 1,
and 2 drops respectively. S_1 = Antiserum raised against normal
human serum, reacting mainly with γ-globulin (outer strong line)
and albumin. S_2 = Antiserum raised against serum of myeloma
patient (Ou): reactions similar. (b) Beta myeloma (Case Pk).
Arrangement as in (a), but the serum used is from Case Pk and
the antiserum S_3 was raised against this. The reactions of serum
Pk with " anti-normal-serum " are much the same as those of
serum Ou in A, but reaction with the homologous S_3 reveals
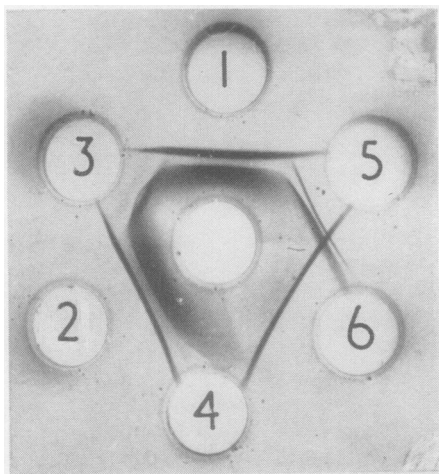the presence of an apparently " specific " component.

FIG. 10.—Abnormal globulin. 1, Normal serum. 2, Serum of patient
Ed. 3, Fraction from serum of patient Ed. 4, Fraction similarly
extracted from normal serum. 5, Fraction eluted from electro-
phoretic strip of serum Ed. 6, Serum of Case 1 (a-gammaglobu-
linaemia): the antiserum is against normal human serum. The
reaction is put up at relative " antigen excess " for most serum
components. The reactions with 2 and 3 do not show the
presence of the abnormal protein owing to interference by the
γ-globulin reaction (the fluffy innermost line). The well-marked
outer line is due to α_2-globulin, removed by fractionation from
3, 4, and 5. The sharp outermost line opposite 5 is due to an
abnormal protein, which has here been concentrated relative to
γ-globulin and therefore brought out clearly. Note that it is
" deviated " by normal serum (1), though not by the a-gamma-
globulinaemia serum (6), hence it must be present in normal
serum (though at too low a concentration for its faint line to
appear on the photograph), a conclusion which is inevitable since
antibodies to it have been provoked by the normal human serum
used as the original antigen. But its metabolism is apparently
linked in some way with γ-globulin, in spite of complete lack of
serological cross-reaction, unless its absence from the a-gamma-
globulinaemia serum is purely coincidental.

with three parts of water, and had haemagglutinating
properties, although it did not appear to be an anti-
body in the strict sense. The patient suffered from
haemolytic anaemia, and the " haemagglutinin " was
assumed to have something to do with this. The
interesting point brought out is that this very unusual
substance is evidently present in normal serum, in
very low concentration, and is there strongly anti-
genic ; not only does the antiserum raised against
normal serum contain an appreciable amount of anti-
body against it, but it can actually be observed as a
hitherto unidentified faint line in the normal serum
pattern, very near the antigen source, i.e., in low
concentration, but definitely joining with the line pro-
duced by the purified substance from the pathological
serum.

**Combination of Immunological Analysis with
Electrophoresis.**—The method described by Grabar
and Williams (1953), in which antigen is electro-
phoretically separated in agar before the reaction with
antiserum, is the one of choice in certain cases, but
it suffers from some limitations for routine use ;
though technically fairly simple there are more things
which can go wrong than in the petri-dish method,
not more than two antigens can be directly compared,
and those not side by side, it takes some 24 hours
longer, and needs rather more antiserum.

The technique employed here is not quite identical
with that of the above authors, and will be described
in a later publication. Essentially, however, an
electrophoretic run is carried out in a 3 mm. layer of
barbiturate buffer agar spread on a glass plate. Elec-
trodes of platinum wire are contained in separate com-
partments : a filter paper bridge connects each of
these compartments with a reservoir of buffer, and
another connects this reservoir with one side of the
agar on the plate ; using this method we have experi-
enced no difficulties with pH changes, etc. One drop
of the serum to be examined, and of a control normal
serum (both containing a little bromphenol blue) are
added to cups cut in the agar ; a current (4–8 mA) is
passed, sufficient to move the albumin (as marked by
the blue dye) about 4 cm. in 18 hr. Under these condi-
tions the γ-globulin moves backwards with the endos-
motic flow, β-globulins remain near the origin but are
clearly separated, and the α_2- and α_1- are intermediate.
After stopping the flow of current a trough is cut
parallel with the direction of movement, 8–12 mm.
from the original cup, and this is filled with antiserum.
Arcs of precipitate develop centred on the point of
maximal concentration of the antigen concerned
(Fig. 11).

For testing a pathological serum it is convenient
to cut two cups 25 mm. away from one another,
one for the test serum and one for normal serum ;
at the conclusion of the run the trough can then be
cut between them, and one can reasonably though
not certainly conclude that arcs which are opposite
one another represent identical components.

This is of particular value in the examination of
the various sorts of γ-globulin. In agreement with
Grabar we have found that these are not serologically

Gamma₂ globulin

Gamma₁ globulin

Beta globulins

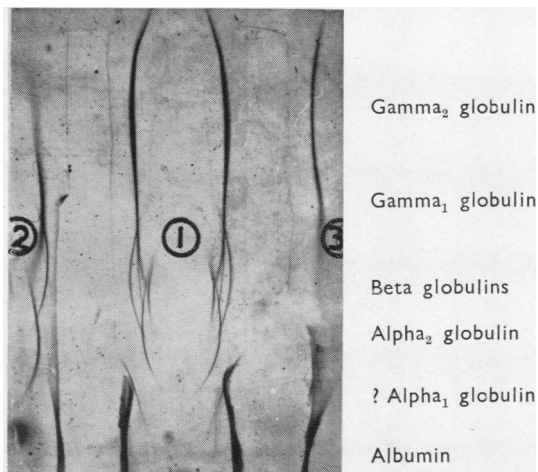Alpha₂ globulin

? Alpha₁ globulin

Albumin

FIG. 11.—Immuno-electrophoretic method. 1, Normal serum. 2, Serum Ha., an undiagnosed case with hypoproteinaemia. 3, Serum Ta., a case of disseminated lupus erythematosus. The fractions are labelled on the right, at approximately the level of maximum concentration, i.e., the centre of the area formed by the precipitate.' There is a striking deficiency of γ_2-globulin in 2, and a less marked but still evident deficiency of γ_1-globulin in 3. The a- and β-reactions in 3 do not appear, as the sample of serum was old and these fractions are apt to deteriorate on keeping at 4° C. The albumin and a_1-reactions are unsatisfactory in this plate, for technical reasons.

differentiable; the gamma does not, however, form a true arc of precipitate with antiserum but an elongated and sometimes humped line. It is reasonable to equate these " humps " with the γ_1 and γ_2 globulins (or γ and " β_2 " globulins), whose relationship is discussed by Oncley (1953) : and the shape of the line can give some evidence as to their relative proportions. Routine analysis of a large number of sera from conditions showing abnormal values for " total γ-globulin," so as to differentiate between these fractions, should supply valuable data as to their pathogenesis. Fig. 11 shows an example of the application of the method to such an analysis.

Discussion

Once an antiserum has been raised and titrated, the immunological method of analysis has some advantage over more usual methods such as paper electrophoresis, though it is not likely to replace the latter for routine use. Its chief disadvantage is its lack of quantitative precision; and although

as described above it is possible to adapt it to give a quantitative answer within 10 to 20% this necessitates the expenditure of some thought and possibly repeated tests, a more time-consuming process than a quantitative paper electrophoretic run, by a method such as that described by Hardwicke (1954). On the other hand, there are three great advantages which recommend it as a method for investigating more thoroughly cases shown to be abnormal ; its very high sensitivity, valuable in cases of deficiency of a serum protein, its great discriminative power, demonstrating as it can more than 15 different fractions in normal serum as against the five to seven fractions of conventional electrophoretic techniques, and the fact that it depends upon biological or at least biochemical properties of a protein rather than upon the purely physical properties responsible for both the electrophoretic and the ultracentrifugal pattern. In general, immunological analysis by the gel-diffusion method provides a valuable supplementary form of analysis, but cannot displace the usual electrophoretic techniques.

Summary

Methods are described for the laboratory analysis of human serum proteins by immunological and immuno-electrophoretic techniques, carried out in agar. Results in cases of a- and hypo-gammaglobulinaemia, the nephrotic syndrome, myelomatosis, etc., are described. Adaptations leading to semi-quantitative results are suggested.

REFERENCES

Bruton, O. C. (1952). *Pediatrics*, **9**, 722.
Elek, S. D. (1949). *Brit. J. exp. Path.*, **30**, 484.
Freund, J., and McDermott, K. (1942). *Proc. Soc. exp. Biol., N.Y.*, **49**, 548.
Grabar, P. (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 65.
—— and Williams, C. A. (1953). *Biochim. biophys. Acta*, **10**, 193.
Grant, G. H., and Wallace, W. D. (1954). *Lancet*, **2**, 671.
Hardwicke, J. (1954). *Biochem. J.*, **57**, 166.
Janeway, C. A., Apt. L., and Gitlin, D. (1953). *Trans. Ass. Amer. Phys.*, **66**, 200.
Kabat, E. A., and Mayer, M. M. (1948). *Experimental Immunochemistry*, pp. 543 and 544. Thomas, Springfield, Illinois.
Munoz, J., and Becker, E. L. (1950). *J, Immunol.*, **65**, 47.
Oakley, C. L., and Fulthorpe, A. J. (1953). *J. Path. Bact.*, **65**, 49.
Oncley, J. L. (1953). In *Blood Cells and Plasma Proteins*, ed. Tullis, J. L., pp. 180–184. Academic Press, New York.
Ouchterlony, O. (1948). *Acta path. microbiol scand.*, **25**, 186.
—— (1949). *Ibid.*, **26**, 507.
Oudin, J. (1946). *C.R. Acad. Sci., Paris*, **222**, 115.
Young, I. I., and Wolfson, W. Q. (1954). *Clin. Res. Proc.*, **2**, 101.