Gel immunofiltration

A new technique for the qualitative analysis of serum proteins

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SYNOPSIS The precipitin reaction has been used to locate serum proteins separated by thin-layer gel filtration on Sephadex G-200, thereby greatly increasing the sensitivity of the method. A pattern of lines reminiscent of immunoelectrophoresis is produced, but the proteins are separated in order of their molecular size and not in accordance with their electrical charge.

The introduction in 1961 (see Flodin, 1962) of Sephadex G-200, the most porous of the cross-linked dextran gels, was immediately followed by its application to the analysis of serum proteins: filtration of serum through a column of the gel was found to separate the serum proteins into three groups in accordance with their molecular volumes. The first group comprises the macroglobulins and lipoproteins, the second mainly γ G (7S γ) globulin and the third mainly albumin (Flodin and Killander, 1962).

In 1962 Determann introduced a thin-layer method of gel filtration analogous to thin-layer chromatography, which has been used by Johansson and Rymo (1964), Andrews (1964), and Morris (1964) for the analysis of serum proteins. An extension of this technique is now described, in which the separated proteins are detected by precipitation with antisera instead of by direct staining. It has resulted in an improvement in sensitivity comparable to that of immunoelectrophoresis over simple zone electrophoresis. Instead of two or three spots corresponding to the groups of proteins separated by the original column technique a whole range of individual proteins can be detected separated in order of their molecular volumes. Unlike the technique described by Carnegie and Pacheco (1964) it does not require transfer of the separated proteins into agar.

TECHNIQUE

The initial gel filtration stage is based on the technique of Johansson and Rymo (1964).

PREPARATION OF PLATES One gram of Sephadex G-200 superfine (Pharmacia, Uppsala) is left to swell for at least

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three days in 25 ml. of the following buffer solution, pH 7.2:—

Na ₂ HPO ₄	..	..	..	2.272 g.
KH ₂ PO ₄	..	..	..	0.544 g.
NaCl	..	..	..	11.7 g.
Sodium azide	..	..	..	1.0 g.
Water to	..	..	..	1 litre

The sodium chloride is added to increase the ionic strength, the sodium azide as a preservative.

Glass plates, 20 × 10 cm., are carefully cleaned by soaking in concentrated sodium carbonate solution followed by thorough washing in water as recommended by Determann.

A CAMAG applicator is used to spread a 0.5 mm. layer of gel on the plates. The sides of the plate are wiped free of gel.

GEL FILTRATION The apparatus is illustrated in Figure 1. It is a standard, horizontal strip, paper electrophoresis apparatus. A freshly spread plate is mounted horizontally on the inner walls of the buffer compartments on four small pieces of split rubber tubing. Whatman 3MM filter paper wicks slightly narrower than the width of the plate dip on each side into buffer compartments, one of which is filled right up and the other left almost empty. Consequently buffer solution flows slowly from the first compartment through the length of the thin layer of gel into

FIG. 1. Vertical section through apparatus.

the second compartment. The difference in level of buffer solution in the two compartments is adjusted to about 3.5 cm., the lid is put on to prevent evaporation, and buffer is allowed to flow through the gel overnight. Next morning the rate of flow is checked by applying about 5 μ l. of serum, containing a little bromophenol blue to stain the albumin, to a point on the plate about 2 cm. from the first paper wick. The difference in fluid level of the two buffer compartments is adjusted until the blue albumin spot moves at a rate between $\frac{1}{2}$ and 1 cm. per hour. The correct levels are then marked for future reference.

Sera for analysis are stained with bromophenol blue and 5 μ l. applied to a plate previously equilibrated by running buffer through overnight. Four samples 2 cm. apart can be analysed on one plate. The run is continued until the blue albumin spot has moved about 5 $\frac{1}{2}$ cm., a distance reached conveniently in about seven hours. During this time the macroglobulins will have moved about 9 $\frac{1}{2}$ cm.

PRECIPITIN REACTION The plate is removed and a pattern of troughs similar to that illustrated in Fig. 3 is made in the gel by placing it over a line diagram of the troughs and sucking them out with a Pasteur pipette attached to a suction pump, after arranging the stained albumin spots midway between the troughs and 2 cm. from one end. The pipette is steadied against a ruler held over the gel on supports placed on each side of the plate. The inner troughs cut parallel with the buffer flow are for the antisera: the outer rectangular trough, which is left empty and is cut 2 cm. from the outer antiserum troughs, was found to be essential for even diffusion of the antisera: even a narrow bridge of gel left inadvertently across this trough is enough to cause serious artefacts.

Appropriate antisera, bought, or raised locally as described elsewhere (Grant, 1964a), are then added to the central troughs using a 0.1 ml. micro-pipette with a curved tip such as the E-mil autaset. While emptying into the trough, the pipette is steadied and moved along against a ruler held over the plate as described above. It will be found that the serum is at once taken up by the gel, so it is advisable to use antiserum stained with bromophenol blue to confirm that it has been taken up evenly, on each side and throughout the length of the trough. The plate is then placed in a damp box at room temperature for 24 hours to allow the precipitin lines to develop.

STAINING THE PRECIPITIN LINES By removing the soluble proteins, drying the gel containing the lines of precipitated protein and staining it, the pattern of precipitin lines formed in the soft and easily damaged thin layer of gel may be converted to a durable and permanent stained preparation as follows:—

The delicate layer of gel is first covered by a moistened cellulose acetate strip. In order to damp it evenly the strip is floated on a bath of water, then immersed for a minute, removed and blotted quickly. One end is placed on one end of the gel and then, to avoid trapping any bubbles of air, it is lowered very carefully and evenly over the whole pattern of lines. Two damped rectangles of 3MM Whatman filter paper are then placed gently in

succession onto the strip of cellulose acetate, and the whole is left in a moist box for a half to one hour. The filter papers but not the cellulose acetate are then removed, replaced by two further pieces of moist filter paper and the preparation left in the air until, viewed from below, all the blue dye can be seen to have been removed from the gel and cellulose acetate. If necessary, the filter papers are again renewed.

The filter papers are then removed, taking care not to disturb the cellulose acetate, and the plate is dried in an oven at 60°C. for a half to one hour.

One of the protein stains used for immunoelectrophoretic techniques (see Grant, 1964a) can then be used to stain the lines in general and a fat stain to stain the lipoproteins specifically. The amido black method of Heremans (1961) is very satisfactory for routine use:—

Amido Black 10B	..	..	0.5 g.
Ethanol	..	..	400 ml.
Acetic acid	..	..	100 ml.
Distilled water	..	..	500 ml.

The plate is placed in a bath of the stain. After five minutes the cellulose acetate strip may be removed. The plate is left staining for a further 20 minutes and then differentiated by two or three washes in the ethanol/acetic acid/water solvent mixture.

Sudan Black may be used to stain the lipoproteins. A fresh saturated solution of the dye in 60% ethanol is made by heating some in the solvent to boiling and filtering while hot through two Whatman number 44 filter papers (Swahn, 1953). The plate is placed in the dye upside down supported by match sticks for an hour. After differentiation in 50% alcohol it is redried in the oven at 60°C. for half an hour. It may be counterstained with azocarmine.

OTHER TECHNIQUES Details are described elsewhere: for analyses on columns of Sephadex G-200 see Grant (1964b); for immunoelectrophoretic analyses see Grant (1964a). For simple thin-layer gel filtration see Johansson and Rymo (1964); in this technique the proteins after separation are taken up into a strip of filter paper and stained therein.

RESULTS

The pattern of precipitin lines obtained on analysis of normal serum is shown diagrammatically in Fig. 2, where it is compared with the results obtained by the original staining technique. Examples of actual analyses of normal and pathological sera are shown in Figure 3. Specific antisera were used to identify the lines, as illustrated in Figure 4. As expected, precipitates were found to be formed by all those proteins known to be present in greatest concentration in serum. The following may be distinguished, listed in order of increasing mobility and therefore also in order of increasing molecular size:—

1 OROSOMUCOID This runs slightly more slowly than albumin. It is seen as a faint arc beginning

FIG. 2. Diagram of normal human serum pattern: (a) immunochemical technique; (b) simple staining.

FIG. 3. Analyses of normal and pathological human serum. Buffer flow left to right. Origins beyond illustration to the left. Note the outer rectangular empty trough, as well as the three inner ones containing antisera. The right hand and lower parts of the outer rectangular trough are outside the photograph.

FIG. 4. Analyses of normal and pathological human serum with specific antisera.

FIG. 5. Analysis of normal human serum on a column of standard grade Sephadex G-200. Immunoelectrophoretic analyses of fractions from the centres of peaks 1 'Macroglobulin fraction'; 2 'YG-globulin fraction'; 3 'Albumin fraction'.

before and then becoming covered by the albumin arc.

2 ALBUMIN Albumin forms the most intense precipitate. It is sometimes doubled with a sharper band near the antiserum trough and it extends as a hazy 'tail' far forward, usually to the beginning of the macroglobulin region.

3 SIDEROPHILIN Siderophilin moves slightly faster than albumin. The rearmost part of its arc is most clearly seen lying within the curve of the albumin arc.

4 γ G (7S γ)-GLOBULIN This forms a conspicuous arc at the centre. Its curved symmetry contrasts with the long precipitin line found on immunoelectrophoresis, and confirms that the family of proteins named γ G or 7S γ -globulin consists of molecules of similar size.

5. 6 These are two faint lines of similar mobility moving little if at all faster than 7S γ -globulin and not yet identified with specific antisera. One is probably haptoglobin, the other probably α -lipoprotein. The latter is stained by fat stains and is elongated extending some way forward, a finding consistent with its presence in both the macroglobin and γ G fractions separated by the column technique.

7 γ_{1A} -GLOBULIN γ_{1A} -globulin forms a third faint arc slightly further ahead.

8 α -MACROGLOBULIN α -Macroglobulin is one of the leading components always seen.

9 γ -MACROGLOBULIN γ -Macroglobulin has a similar mobility. Although clearly seen in cases of macroglobulinaemia (see Figs. 3 and 4), in normal serum it usually has too low a concentration to form a visible precipitate.

10 β -LIPOPROTEIN β -Lipoprotein has the same mobility and is easily identified with fat stains.

It is probable that other components in lesser concentration could be detected if specific antisera and larger amounts of sample were used: this has not yet been investigated.

DISCUSSION

The main technical requirements are first the clear separation of the protein components, and then a sensitive method for their detection. It is of interest to contrast the separation achieved by the thin-layer technique with that by the column technique. In Fig. 5 are shown the results of some preliminary experiments in which normal serum was analysed on a column of standard grade Sephadex G-200; the fractions from the centres of each peak were re-concentrated and analysed further by immunoelectrophoresis. It will be seen that separation of some proteins is incomplete; in the case of albumin, which can be seen even in the macroglobulin fraction, this could be partly due to polymerization. In the light

of these results the degree of separation obtained by the thin-layer technique is unexpectedly good, and it is not at all surprising that an albumin 'tail' can be seen extending so far forwards. Analyses of highly viscous solutions, such as concentrated sera, are unsatisfactory by the thin-layer technique just as on the column.

The requirements for good separation are:—

1 That diffusion of the protein solute molecules between the solvent outside and inside the gel particles should reach equilibrium at a given point in the thin layer, before being swept on to the next. This means first that the rate of solvent flow should be adjusted to be as slow as is possible without allowing too much diffusion at right angles to the direction of buffer flow. Fortunately, when locating a protein by the precipitin reaction such sideways diffusion is not so damaging as with simple staining. Secondly, the gel particles used should be very small so that the large surface area allows equilibrium within and without them to be rapidly attained. The new 'superfine' grade of Sephadex G-200 with particles between 40 μ and 10 μ in diameter has been designed to do this.

2 That interaction between proteins be prevented; it may be minimized by using a buffer solution of high ionic strength.

3 That conditions during analyses be standardized; consequently a freshly spread plate is used, and to prevent buffer tracking sideways, the plate is supported from below, so that nothing touches its edges, and these are wiped free of extraneous gel. Evaporation is prevented by enclosure in an air-tight box: buffer solution is allowed to flow overnight so that equilibrium is reached before applying the protein solution, and the buffer compartments are large enough for their fluid levels not to change appreciably during a run.

In order to select the correct rate of flow a coloured marker protein is required, and a method of varying the rate. Johansson and Rymo (1964) use fluorescein conjugated proteins as markers; it seems simpler merely to add a little bromophenol blue to the test serum; this produces a clearly stained albumin spot. Previous authors have adjusted the rate of buffer flow by varying the slope of the plate and most of our analyses have been done by this method using a specially made perspex box with a screw adjustment for slope. The rate of flow, however, depends on the difference in heights of the buffer baths as well as on the angle of slope of the plate. It is therefore also possible to hold the plate horizontal and adjust the rate entirely by varying the heights of the two buffer levels.

For the development of satisfactory precipitin lines in gel diffusion analyses the reagents must be

able to diffuse within a stationary body of solvent. In the continuous gels ordinarily used, such as 1% agar, the solvent is held stationary in the gel, but in the slurry required for gel filtration, which consists of gel particles lying in free buffer, there is nothing to prevent this buffer flowing in any direction. This means that the area of the thin layer in which the precipitin reaction is to be carried out must be carefully isolated from any outside influence.

Ten serum proteins were detected by this technique but it is reasonable to suppose that improvements in sensitivity will be made in the future.

The method has many possible applications as a technique complementary to immunoelectrophoresis in the qualitative analysis of soluble protein mixtures, especially as a preliminary test before more complex procedures. In the many problems concerned with the permeability of living membranes a technique is required which separates protein molecules in accordance with their size rather than their charge. Previously information on these lines required fractionation on a column of Sephadex G-200 followed by further analysis of the individual fractions, for example, by immunoelectrophoresis. The thin-layer technique described above appears to separate the proteins as efficiently and has the great advantages of speed and of only requiring 5 μ l. of serum.

The technique may well be useful in the investigation of tubular proteinuria and massive proteinuria, where the sizes of the protein molecules excreted are of diagnostic importance. Other applications are likely to arise when studying proteins of similar electrophoretic mobility but different molecular size. The most obvious example is the separation of

γ -macroglobulin from γ G or 7S γ -globulin in the investigation of antibodies and in the differential diagnosis of macroglobulinaemia from myelomatosis (see Grant, 1964b).

As pointed out by Andrews (1964), although mobility on gel filtration depends primarily on molecular size, the technique may be used to estimate the molecular weight of an unknown protein; there is an approximately linear relation between the rate of migration and the logarithm of the molecular weight.

Another possible application is the study of the polymeric forms of enzymes and other proteins.

The use of thin layers of superfine Sephadex G-75 and G-100 as well as G-200 to analyse urine proteins is being investigated.

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