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DISPLACEMENT ELECTROPHORESIS IN GEL AS A TECHNIQUE FOR SEPARATING PROTEINS ON A PREPARATIVE SCALE

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SUMMARY

A preparative displacement electrophoresis apparatus has been developed for the fractionation of proteins and other macromolecules. The separation is carried out down a vertical annular gel which is supported by a cylindrical glass cooler. The gel is protected from evaporation by a heavy inert insulating liquid with the terminating electrolyte floating on it. Very high potential drops and instabilities in the terminating electrolyte are avoided by continuously lowering the level of the inert liquid during the course of the separation.

Fluorescent mobility markers have been synthesized, which make it possible to follow the progress of the separation and to divide the gel into fractions, once the separation is complete.

INTRODUCTION

Electrophoresis in gels has been an outstandingly successful technique for the analytical separation of proteins, especially when conducted in two dimensions. No correspondingly high-resolution preparative technique has been available. The major reason for this is that workers have attempted to handle larger amounts by increasing the thickness of the medium, when temperature differences across the thickness of the medium introduce difficulties. These temperature differences not only cause changes in the rate of migration, because of increased mobility with increasing temperature, but also changes in the ionization constants of both of the substances whose separation is desired, and of the buffers. Electroendosmosis can seldom be completely avoided. It gives rise to changes in the volume of the gels, which, when the gels are confined in a tube, make good separations impossible. If these difficulties are minimized by using a low potential gradient, and a long tube for separation, then diffusion prevents clear cut separations.

The zone electrophoretic method is difficult to use as a preparative method. The zones will only move without broadening, other than by diffusion, if the solutions

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are so dilute that the conductivity of the substances to be separated is negligible in comparison with that of the buffer. This means that the apparatus has to be very large, and even if the layers are kept thin to avoid difficulties due to heating, the separation is exceedingly slow. Good detection of the volume occupied by a given substance becomes very difficult for those substances present in only small amounts. These may well be the substances in which one is most interested.

Analytical isoelectric focusing in gels and displacement electrophoresis both show the advantages of a method in which the concentration of the separated substances can be kept high. For instance, many proteins can be made visible simply by precipitation with trichloroacetic acid. It is of interest to note that immunoelectrophoresis, which works well as a detector for zone electrophoresis, itself supplies the concentrative step as well as introducing separation in another dimension.

Preparative isoelectric focusing in sucrose density gradients is limited by thermal difficulties and by precipitation. There is also a time limitation, caused by migration of the carrier ampholyte from the end of the gradient to the electrodes. Until recently, membrane isoelectric focusing using simple buffers[1] was seriously limited by the paucity of usable membranes. These can now be tailored to a particular purpose. Such an apparatus is especially useful for the separation of large amounts of materials. The limits of resolution have not yet been established.

When displacement electrophoresis has been run to a steady state, every substance lies within a narrow range of ionic strengths. The concentrations are limited by the properties of protein solutions. Though dilute ionically, they may have concentrations of several per cent by weight, and may considerably change the viscosity, dielectric constant and thermal conductivity of the solution, and hence alter both the mobility and charge of the proteins. Several hundred milligrams can be handled in an annular gel 20 mm in diameter and 1.2 mm thick with a zone length of several centimetres. The effective cooling means that high potential gradients are possible and that resolution is very sharp. The sharpness of the zones exceeds the accuracy with which they can be cut out.

If a good detector is available, a substance present in a substantial amount will show a zone of constant properties and all but the ends of this zone will consist of a single, or at most a few, substances. This centre portion may be excised and the material remaining can be run a second time, this time at a higher dilution or on a smaller diameter apparatus. It will then occupy a length comparable to the original length during the first run, and zones occupying only a small proportion of the whole length the first time, will now be so much longer that they can be more easily cut out.

Such fractions can be re-run under different conditions of pH or dielectric constant, or in the presence of complexing substances. Substances with almost identical mobilities during the first runs can then be expected to separate. A small amount of interzonal material will be left, which is as complicated as the original sample, but which could be included in any subsequent separation. We believe that the essential resolution by this method is better than by any other general method except of isoelectric focusing.

Conditions for good separation of proteins by displacement electrophoresis

The method of displacement electrophoresis (also called by others isotachophoresis) has been used very successfully for small molecules by Everaerts *et al.*[2], and

by other investigators using a long thin tube containing a more or less viscous liquid. However, attempts to separate proteins in the same type of apparatus have met with little success. The difficulties arise because of adsorption on the walls of the tube, because the ratio of surface to volume is very large, and because the protein solution can differ considerably in density, from the solution at the end of a zone, thus making the zone front unstable. Hjertén[3], by rotating a horizontal tube about its axis, has lessened this latter difficulty but at the expense of considerable complication of the apparatus. The use of a gel instead of a viscous liquid is impractical in a narrow tube. If the gel is very weak and dilute, electroendosmosis causes enough pressure to crack the gel, which results in the simultaneous presence of gel and synerezed liquid in which the proteins run irregularly. If, on the other hand, the gel is strong enough to prevent syneresis, bubbles containing only water vapour are formed, which result in excessive heating below the bubbles. If a thin slab of gel is used uncovered, it can shrink as the result of electroendosmosis, without necessarily causing trouble. However, if the potential gradient is high enough to give good resolution, evaporation from the wet surface eventually leads to dry, non-conducting regions, and before complete dryness occurs the resistance becomes so high that excessive heat is generated and the protein is denatured. If evaporation is prevented by placing a plastic or glass cover on top of the gel, the gel synereses at the points of contact with the cover, and liquid flows in an irregular pattern and causes distorted zones. As the cooling is always better at the edges than in the middle of a thin slab, the temperature difference leads to different speeds of movement of the fronts and also causes different rates of electroendosmosis. Syneresis then occurs where there is swelling, and the synerezed liquid flows to the place where the gel is shrinking. If the lid extends beyond the sides of the gel, syneresed liquid may well run outside the gel area into the space between the lid and the side supports. Another compelling reason for using a thin layer of gel with one exposed surface, rather than a completely enclosed tube or slab, is that it is possible to choose good conditions for polymerization of the gel, and then to replace the solutes present in the gel with a lead electrolyte of chosen composition and strength. This can be achieved simply by washing with a solution of the desired composition.

In a completely enclosed gel the electrolytes can be exchanged for others by electrical migration, but the ionic strength is not under independent control. In general, it is very difficult to work with very low ionic strengths in totally enclosed gels.

We have avoided the irregularity of temperature at right-angles to the current by using a vertical glass tube surrounded by a thin annulus of gel, and we have avoided evaporation by immersing the gel, where it is not making contact with the rest of the electrical circuit, in a chemically inert water-immiscible insulating liquid, whose density is sufficiently different from that of water to cause the meniscus to be well defined. Only *o*-dichlorobenzene was found to be inert enough and dense enough for this purpose. Other chlorinated solvents were found to hydrolyse sufficiently to make the gel acidic. Under these conditions we have found a surprisingly complete absence of syneresis, and have had no difficulty in obtaining very regular zones.

It is desirable to have the zones migrating downwards, so that the hotter displacer is above the separating proteins. Convection in the insulating liquid carries the heat generated in the displacer, upwards away from the proteins. This can be clearly seen as an upward moving Schlieren pattern in the insulating liquid.

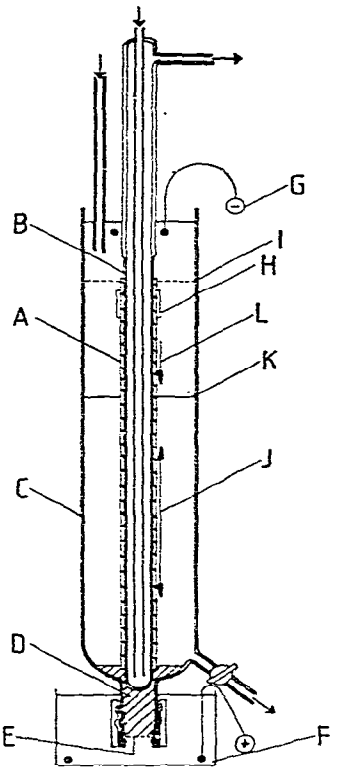

Fig. 1. Diagram of apparatus. A = cross-linked polyacrylamide gel; B = cylindrical glass tube supporting the gel; C = glass container; D = agarose plug containing lead electrolyte; E = electrically conducting membrane supported by screw-cap and O-rings; F = bottom electrode compartment with electrode; G = top electrode; H = paper impregnated with sample at start of separation; I = displacer/*o*-dichlorobenzene interface at start of separation; J = extent of protein band at the end of the separation; K = position of displacer *o*-dichlorobenzene interface at the end of the separation; L = direction of migration of protein.

If separation has to be made between two proteins of closely similar mobility, then the zones have to be run for many times their combined length, to obtain separation, and hence there is a big potential drop over the region of the gel which has become occupied by the displacing electrolyte (terminating electrolyte). We have found that lengths of more than a few centimetres of displacing electrolyte are unstable, probably because the upper part becomes excessively hot with irregular current distribution and that ultimately the protein zone is also affected. It is, however, easy to reduce the level of the inert liquid, replacing it with a solution of displacing electrolyte. The current can then be carried by a large area of electrolyte with a correspondingly low resistance. This results in much less heat being produced, and little change in potential is required to maintain the current at a constant value. Indeed a constant voltage power pack can be used, and the rate of addition of electrolyte and of removal of inert liquid can be adjusted to maintain a constant current. The conductivity of the lead zone can usually be made so high that its length is unimportant.

It is essential for good separation that the interface between the dichloro-

benzene and the terminating electrolyte moves evenly down the annular gel as the *o*-dichlorobenzene level falls. In order to achieve this, it is necessary to use an outer tube of rather large diameter, since although the gel is preferentially wetted by the aqueous terminating solution, the glass outer tube is preferentially wetted in some places by the aqueous layer and in other places by the organic layer. This results in an uneven rate of fall of the interfacial meniscus around the circumference of the outer tube. If the distance between the wall of the outer tube and the surface of the gel is too small, the irregular distribution of the surface tension forces causes the meniscus at the gel surface to fall unevenly as well.

In this work we used an accurately ground cylindrical glass tube with an external diameter of 20 mm as the support for the annular gel, which has a thickness of 1.2 mm and a length of 320 mm. The gel is immersed in the cooling liquid which is contained in an external glass tube 70 mm in diameter. Fig. 1 shows the general arrangement of the apparatus.

Fluorescent mobility markers for the displacement electrophoresis of proteins

When a mixture of proteins is subjected to displacement electrophoresis it arranges itself into separate zones of graduated mobility as it progresses along the apparatus, with the most mobile in front and the least mobile behind. If only two proteins are considered, and the mobility difference is sufficiently large, then they will separate themselves from each other with a more or less sharp boundary between them. Before the separation is complete there will be three zones; a zone of separated fast component, followed by a zone containing a mixture of both components, followed by a zone of separated slow component. If the separation is not carried on for long enough then this situation will obtain at the end of the experiment, the length of time required for complete separation of a given pair of substances being an inverse function of the ratio of the mobilities and of the sum of the time taken for the two substances to pass a given point. If the mobility differences are small and the zones short, the electrical forces sorting the molecules are comparable in strength with diffusional forces, and practically no separation will occur[4]. A slight partial separation will occur at each end of the mixed zone, which will be of constant composition over most of the rest of its length. More complex mixtures can form mixed zones containing more than two components. With a complex mixture of proteins it is to be expected that it will be impossible, under any one set of conditions, to separate all the components completely, although it ought to be possible to devise conditions which separate a particular pair of components. The fluorescent mobility markers we have devised are intended to provide information about the course of such a separation and to aid the establishment of such optimal conditions, as well as aiding the proper cutting up of the gel, on completion of the separation.

Imagine a mixture of fluorescent molecules having a vast range of mobilities, and fluorescent enough that their presence can be detected at concentrations which are very small compared with the concentration of the proteins, so that they do not add significantly to the total mass of the mixture, and therefore have a negligible effect upon its separation.

If a mixture of two easily separated proteins contains a very small amount of mixture of fluorescent markers, whose mobilities vary continuously or almost continuously from that of the leading electrolyte to that of the displacing electrolyte, then

as the separation proceeds, the markers most different in mobility from the mean mobility of the mixture of proteins will form fluorescent edges to the mixed protein band. Later, as the proteins themselves begin to separate, two more fluorescent bands form, one at the junction between the slower moving protein and the mixed zone and the other at the junction between the mixed zone and the faster moving protein. When the separation has reached a steady state these two fluorescent bands coalesce.

With a complicated mixture fluorescent bands will form, at first, at all emerging junctions. When the separation is completed each front between two zones will have the fluorescent marker with mobility intermediate between that of the proteins concentrated in it. In the case of incomplete separation the fuzziness or sharpness of the bands gives an indication of which zones are pure and which are still mixed. It is possible, but not yet proved, that the distribution of residual fluorescence between the fluorescent delineating zones will show whether the particular zone has any variation in mobility along its length. An attempt has been made at preparative displacement electrophoresis by mixing the sample with a large amount of the ampholyte buffer used for isoelectric focusing[7]. The proteins will then separate in an order closely similar to the isoelectric order, but the separation will be very slow and the zones will have no sharp fronts, mixed zones with the ampholytes always occurring.

We synthesized the fluorescent markers by reacting LKB carrier ampholytes, which are mixtures of polyaminopolycarboxylic acids, (other carrier ampholytes containing sulphonic acids and phosphonic acids can also be used), with either fluorescein isothiocyanate or with Rhodamine B200 sulphonyl chloride:

$$Fl\text{–}\vec{N}C{=}\tilde{S} + NH_2\text{–}\tilde{R} \rightarrow Fl\text{–}NH\text{–}\underset{\underset{S}{\|}}{C}\text{–}NHR$$

or

$$Rd\text{–}SO_2Cl + NH_2R \rightarrow Rd\text{–}SO_2\text{–}NHR$$

In each instance the reaction is accompanied by the loss of an ionizable amino group, so that at a given pH it is more negatively charged or less positively charged that the parent molecule. However, the mobility can be adjusted by reaction with an amino epoxide:

$$R_2N\text{–}CH_2\text{–}\underset{\diagdown\ \diagup}{\overset{O}{CH}}\text{——}CH_2 + NH_2\text{–Marker} \rightarrow R_2N\text{–}\overset{OH}{\underset{|}{CH_2}}\text{–}CH_2\text{–}NH\text{–Marker, etc.}$$

Other amine-containing reagents could also be used, and the mobilities could be altered in the opposite sense, by reaction with acidic reagents such as glycidic acid, chloroacetic acid or chlorosulphonic acid, although we have not yet done this. All of these modifications have the advantage of greatly increasing the numbers of different species of different mobility in the mixture. After reaction, mobility markers with a particular range of mobility can be separated out, if desired, by displacement electrophoresis between leading electrolyte and displacing electrolyte of chosen mobility.

However, this is not usually necessary, since if the crude mixture is added to the protein, then those markers which are too mobile will travel zone electrophoretically in the leading electrolyte, and those markers which are too slow or which have the wrong charge will migrate zone electrophoretically in the displacing electrolyte, either in the same direction as the protein or in the opposite direction. In any event they will travel separately from the protein zone and will not interfere with the separation or hinder its observation.

EXPERIMENTAL

Method of operation of preparative apparatus

The annular gel is cast on to the glass cooling tube using a split mould, preferably made from an oxygen-impermeable substance. If the mould is not oxygen impermeable it must be previously degassed to obtain complete polymerization. The mould is removed and the gel washed overnight with 2 l of leading electrolyte. The sample solution, up to 0.4 ml, but more usually 0.2 ml, in volume, containing fluorescent markers if desired, is applied to a Whatman No. 1 filter-paper strip 20 mm wide with a length corresponding exactly to the circumference of the gel, about 75 mm. The paper with the sample on it is wrapped carefully round the circumference of the gel, about 7.5 mm below its top, and tied on with rayon thread.

The bottom of the wide tube is sealed with Visking dialysis membrane, which is then overlaid by a pool of molten agarose containing lead electrolyte. The glass cooler is then clamped vertically with the bottom end of the annular gel dipping into the pool of agarose. The agarose is allowed to congeal, a process which can be accelerated by circulating cold water through the glass cooler. An electrical connection with the bottom electrode is made by dipping the Visking-covered end of the outer tube into the bottom electrode chamber.

When the agarose has congealed, the wide tube is filled with *o*-dichlorobenzene, until the top of the sample paper is 5 mm below the surface of the *o*-dichlorobenzene. Then displacing electrolyte is poured gently on top of the *o*-dichlorobenzene to a depth of about 100 mm. The top electrode is immersed in this solution and a potential difference of 500 V or less is applied until all of the sample has migrated into the gel and is a few millimetres below the paper. When anionic proteins are being separated, the cathode is at the top and the anode at the bottom. For cationic proteins the polarity is reversed. Then *o*-dichlorobenzene is run out of the apparatus until its interface with the terminating electrolyte is below the paper. The potential is then increased to give the desired running current and *o*-dichlorobenzene is run out of the apparatus during the separation, at such a rate as to keep its interface with the terminating electrolyte 5–10 mm above the top of the protein zone. At the same time enough terminating electrolyte is added to the top of the apparatus to keep the electrode immersed. When the protein is near the agarose the inner tube is pulled out of the wide tube and mounted in a lathe. Then, while the tube is rotating the protein zone is divided into rings with a fine inclined needle using the fluorescent markers as a guide. One cut is then made parallel to the axis and each ring is lifted away from the surface of the supporting tube by wrapping a narrow strip of filter-paper round it to which the gel adheres, so that it can be peeled away from the glass and from the adjacent rings with the help of a scalpel blade if necessary.

The rings of gel are pressed through a stainless-steel gauze of 50 mesh. The broken gel is packed into a 5-mm column and the protein eluted from it with a buffer solution.

For the purpose of analysis by analytical isoelectric focusing in gel, small pieces of preparative gel can be laid on the surface of the analytical plate. It is not necessary to extract the protein into free solution before analysis. A two-dimensional separation can be made by taking a strip cut longitudinally from the tube and laying this at right-angles to the direction of the current in the isoelectric focusing step. The fluorescent markers are of no value in the isoelectric focusing step.

Materials used in preparative electrophoresis

Gel. The gel was cross-linked polyacrylamide, with N,N'-methylenebisacrylamide as the cross-linking agent. In the usual nomenclature the total gel concentration was 3.4% (w/v) and the cross-linking agent concentration was 2.9% (w/w). The gel was made up in trishydroxymethylaminomethane–phosphate buffer solution (pH 8.1) containing 0.1 *M* trishydroxymethylaminomethane and brought to pH 8.1 with phosphoric acid. The solution also contained N,N,N',N'-tetramethyl-1,2-diaminoethane (0.002 *M*) as accelerator, and the polymerization catalyst was ammonium persulphate (0.14%, w/v).

Electrolyte concentrations for the separation of anions at pH 8.1. The leading electrolyte was trishydroxymethylaminomethane brought to pH 8.1 with phosphoric acid. The trishydroxymethylaminomethane concentration was varied in the range 0.1–0.005 *M*. The terminating electrolyte was 0.115 *M* 6-aminohexanoic acid and 0.037 *M* trishydroxymethylaminomethane.

Electrolyte concentrations for the separation of cations at pH 8.1. The leading electrolyte was sodium veronal buffer (pH 8.1) (0.0025 *M*). The terminating electrolyte was 4-aminobutyric acid (0.1 *M*).

Samples. Bovine serum was dialysed against water. The euglobulin precipitate which formed was removed by centrifugation and the material freeze-dried.

Crude haemaglobin was prepared by the lysis of horse red blood cells. The crude lysate was centrifuged to remove cell ghosts, dialysed against water and then freeze-dried.

For experiments with the positively charged fluorescent markers an aqueous butanol extract of pig gut was used.

Analytical isoelectric focusing. This was performed using an LKB 2117 Multiphor system according to the manufacturer's instructions[5]. Using a pH 3.5–9.5 gradient, gel samples from the preparative electrophoresis were laid directly on to the surface of the gel. Haemaglobin bands were immobilized by soaking in 10% trichloroacetic acid solution. Serum proteins were immobilized and made visible using acidic bromophenol blue by the method of Awdeh[6].

Synthesis of fluorescent mobility markers which are anionic at around pH 8

LKB carrier ampholyte (pH range 9–11; concentration 20%, w/v) (0.1 ml) was mixed with water (0.7 ml) and with 0.2 *M* carbonate–hydrogen carbonate buffer (pH 9.2) (0.2 ml), and to this was added fluorescein isothiocyanate (2 mg) in dioxane (0.25 ml). The mixture was left at room temperature overnight and extracted twice with 1 ml of chloroform. The aqueous layer was then warmed under a water pump

vacuum to remove trace amounts of chloroform and used without further treatment. A 2.0-μl volume of the mixture was ample to produce clearly visible fluorescent markings.

Synthesis of fluorescent markers which are cationic at pH 8

A 1-ml volume of anionic markers produced as described above was treated with 5 μl of 2,3-epoxypropyldiethylamine, and the mixture was allowed to react overnight. Next day it was treated with another 5 μl of 2,3-epoxypropyldiethylamine and again allowed to react overnight. The product, which was slightly less fluorescent than the anionic markers, was used without further purification, 10 μl being used per separation.

RESULTS AND DISCUSSION

Electrophoresis of crude haemoglobin using the anionic buffer system

More than 100 mg of crude haemoglobin can successfully be loaded on to the gel. It was found that a leading electrolyte concentration of 0.1 *M* in trishydroxymethylaminomethane was too high. The protein band was unstable with curved leading and trailing edges, so that the zone length was not constant around the whole circumference of the annulus. This presumably results from the high protein concentration affecting the electrical properties of the zone. At leading electrolyte concentrations of 0.01 *M* or less, the protein zone became stable and symmetrical. Within the haemoglobin zone bands of different intensity began to appear. It was not possible to decide by inspection whether the banding was caused by a complete separation of the haemoglobin species or merely by a partial separation.

When fluorescent mobility markers were added to the sample only the front and rear of the haemoglobin zone were delineated by fluorescence; no fluorescent boundaries could be seen within the zone. The extent of the separation of the various haemaglobin species within the coloured zone was studied by cutting a 2-mm wide strip along the protein zone, parallel to the axis of the annulus, so that the protein distribution along the length of the strip was representative of that along the zone as a whole.

The strip was laid on the surface of an analytical isoelectric focusing plate, with its axis at right-angles to the field. A sample of the crude mixture was placed alongside it for comparison. The electropherogram obtained is shown in Fig. 2. The separation on the left-hand side (A) is that of the 2-mm wide sample strip, whose anodic end was on the left and cathodic end on the right. The right-hand separation is that of the crude mixture (B), which contains four major coloured bands. However, the mixture is more complex than this, as it contains many uncoloured proteins, which can only be revealed by staining. It can be seen from the left-hand sample that there is a considerable, although not complete, separation of the major haemoglobin species along the length of the sample strip, and hence along the length of the haemoglobin zone in the preparative separation. No attempt was made to optimize the conditions for this separation and it may be that under other conditions a more perfect separation could be obtained. Nevertheless, the partial separation obtained is sufficient to allow a significant purification of the major bands. Also, and possibly more important, it would permit the isolation of minor components in greatly enriched form

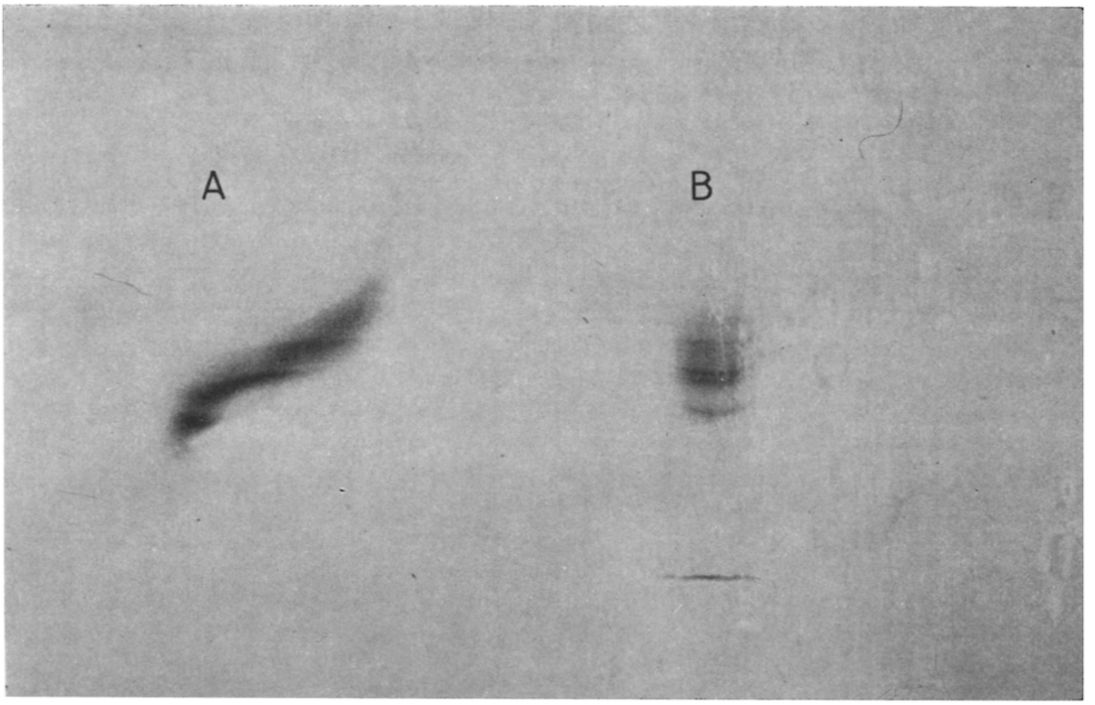

Fig. 2. Displacement electrophoresis of 50 mg of haemoglobin. (A) Analytical isoelectric focusing of the protein contained in a 2-mm wide strip cut through the protein zone of the preparative separation parallel to the axis of the annulus. The anodic end of the strip is to the left, the cathodic to the right. (B) Analytical isoelectric focusing of a sample of the crude haemoglobin. The conditions of the preparative separation were: sample, 50 mg of haemoglobin in 0.2 ml water; leading electrolyte, Tris–phosphate (pH 8.1) containing 0.0076 *M* Tris; loading conditions, 400 V, 4 nA; time required to load, 25 min; running conditions, 10–12 mA, 1200 V; running time, 40 min; distance run along gel, 21 cm; zone length, 1.6 cm.

such as the lower mobility minor components seen on the right-hand side of the separation, which are hardly detectable in the electropherogram of the crude mixture.

Fig. 3 gives another illustration of these effects. In this instance the haemoglobin zone was divided arbitrarily into four equal length fractions (A, B, C and D), and a further fraction (E) of the same length was taken from the clear material immediately behind the haemoglobin zone. The first four fractions represent partial but useful purifications of the four major haemoglobin bands. The fourth fraction also contains a considerable amount of lower mobility material. The fifth fraction (E) consists entirely of these lower mobility components.

If the gel is broken into small particles by forcing it through a small-mesh sieve, and the particles are packed into a small glass column, then the haemoglobin can be recovered virtually quantitatively by elution with a few column volumes of water or of appropriate buffer solution.

Electrophoresis of dialysed bovine serum in the anionic electrolyte system using the fluorescent markers which are anionic at pH 8.1

Even fairly large amounts of colourless protein mixtures, such as bovine

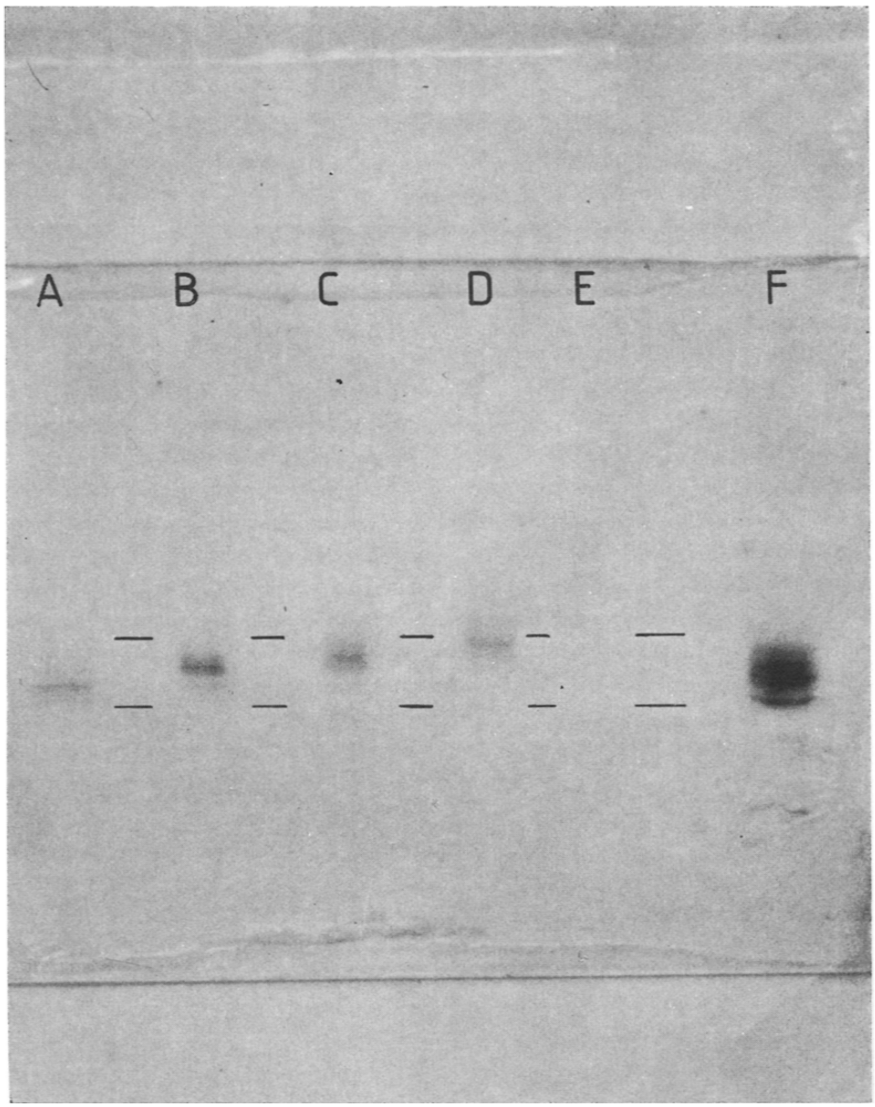

Fig. 3. Displacement electrophoresis of 50 mg of haemaglobin. Analytical isoelectric focusing of fractions A–E and of crude haemoglobin, F. The fractions were cut arbitrarily, the coloured protein zone was cut into four equal length fractions, A–D, taken from the anodic to the cathodic end. Fraction E was of equal length cut from the colourless region immediately behind the coloured protein zone. Conditions of electrophoresis as in Fig. 2.

serum, are invisible on the column during displacement electrophoresis. However, using the fluorescent mobility markers which are anionic at pH 8, it is possible to gain some information about the progress of such a separation and also, at the end of electrophoresis, to decide where to divide the gel in order to obtain the most useful separation. Fig. 4 is a photograph of such a separation. During the running of this gel the fluorescent bands at the top of the picture were evident soon after the beginning of the separation whereas the fluorescent band at the bottom only appeared later.

The darker region between the bands was originally quite fluorescent, but this fluorescence disappeared as the separation progressed. Fractions were taken from an identical gel according to the divisions shown in Fig. 4. These fractions were analysed by analytical isoelectric focusing and a photograph of the separation is shown in Fig. 5. The letters in Fig. 5 correspond to the fractions indicated in Fig. 4.

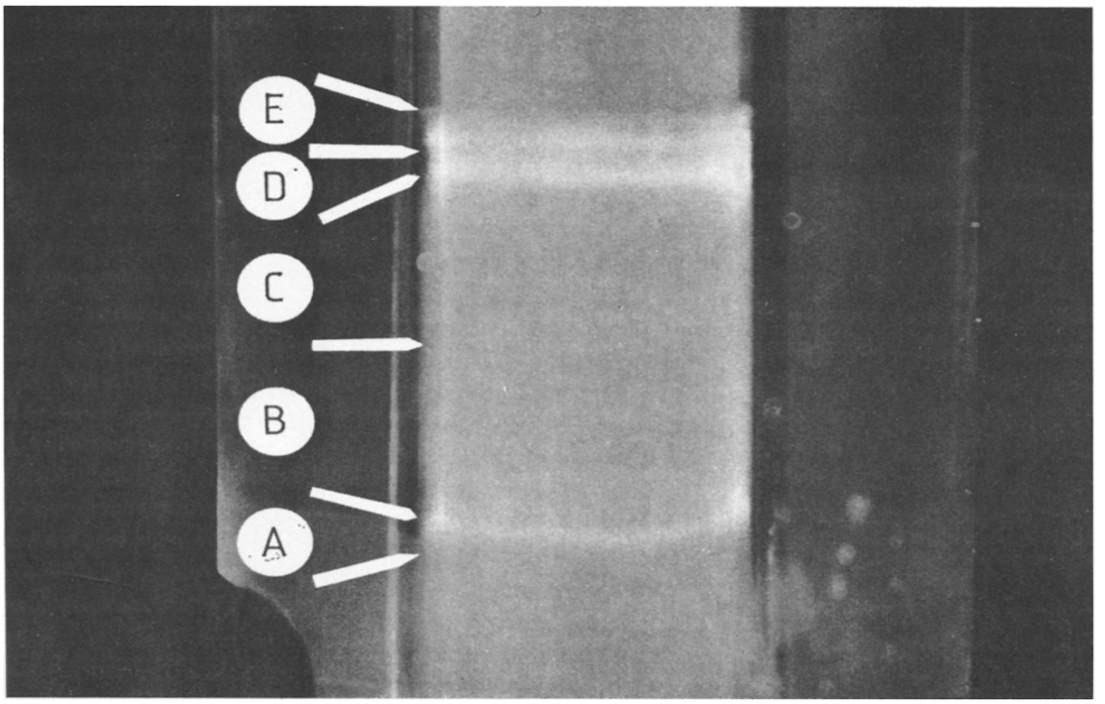

Fig. 4. Displacement electrophoresis of dialysed bovine serum proteins; 75 mg made visible by means of fluorescent markers. Electrophoresis conditions: leading electrolyte, Tris–phosphate buffer solution (pH 8.1); Tris concentration, 0.0076 *M*; sample, 75 mg of protein in 0.2 ml of water; loading conditions, 400 V, 3.8 mA; loading time, 35 min; running conditions, 12–14 mA, 1200 V; running time, 30 min; distance run along gel, 230 cm; length of zone, 2.8 cm. The fractions into which a replicate gel was divided are indicated. The analytical isoelectric focusing patterns of these fractions are shown in Fig. 5.

Fraction A was taken from just ahead of the fastest running fluorescent band, and can be seen to contain a concentrate of the faster moving proteins, together with a little material which is identical with one of the zones found in fractions B and C. The material between the first fluorescent band and the second was divided by area into two equal fractions, fractions B and C. These can be seen, in Fig. 5, to be identical. They consist of two protein bands and represent albumin. Crystalline bovine serum albumin also migrates as two major and several minor bands under these conditions of isoelectric focusing. It is worth noting that these albumin fractions, although containing two species, are very nearly free from minor impurities. As the analytical isoelectric focusing plate was overloaded with protein, this is a sensitive test for trace amounts of other proteins. That the two albumin components are at least partially

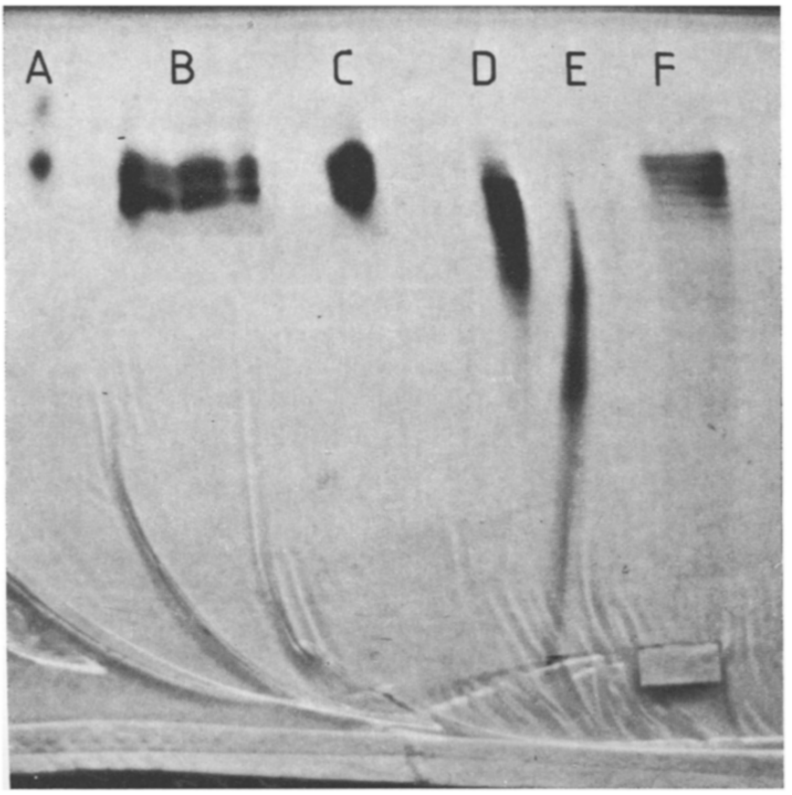

Fig. 5. Displacement electrophoresis of 75 mg of dialysed bovine serum proteins. Analytical isoelectric focusing of fractions A–E (see Fig. 4) into which the gel was cut using the fluorescent markers for guidance; sample F is the original mixture.

separated is indicated by the presence of only the more mobile of these subcomponents in fraction A of Fig. 5. The area between the second and third major fluorescent bands was divided by area into two equal fractions, fractions D and E of Figs. 4 and 5. These fractions can be seen to be different in composition although there is a little overlap. The reason why the zones containing these proteins (β- and γ-globulins) are so short in the preparative separation is not only that albumin is the most abundant protein in serum but also that it is one of the most acidic, so that at the pH of the separation it has a much higher negative charge density than do the less mobile components. Hence, for an equivalent ionic strength, the concentration of this protein is much smaller than that of the less mobile proteins. As the ionic strengths of all of the protein zones must be similar, the proteins in the later zones will be much more concentrated. More favourable conditions for the separation of these less mobile proteins from each other can probably be found by raising the pH, when they will have a higher negative charge, or by lowering the pH to such an extent that they are positively charged.

Although this particular example was run with only 75 mg of protein, very similar results were obtained with 200 mg of protein using a higher leading electrolyte concentration of 0.01 *M* when the total length of the protein zone was 100 mm. An

analogous separation to that illustrated in Fig. 5 was obtained. A different batch of fluorescent markers was used in this experiment and more fluorescent zones were visible during the separation than are visible in Fig. 4. Obviously the preparation of the markers must be carefully standardized.

The use of fluorescent markers in the pH 8.1 buffer system for cationic substances

The anionic markers contain no positively charged fluorescent species, as tested by displacement electrophoresis in the system for cationic proteins at pH 8.1. However, after treatment with 2,3-epoxypropyldiethylamine the same test showed the presence of ample cationic fluorescent material. Its usefulness as a mobility marker was tested using a butanol extract of pig gut, which contained a significant amount of highly basic polypeptides. It was possible to delineate the polypeptide zone and to detect some structure within it, using these cationic markers.

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