POLYACRYLAMIDE-ISOELECTRIC-FOCUSING

A NEW TECHNIQUE FOR THE ELECTROPHORESIS OF PROTEINS

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In this new technique, proteins migrate through a pH gradient to equilibrium positions within a slab of large-pore polyacrylamide gel. The pH gradient is established, and maintained, by carrier ampholytes ('Ampholines', L.K.B. Instruments Ltd.) under the influence of the applied electric field. The technique offers the advantages (ease of sample application and high resolving-power dependent only upon protein charge) of existing procedures utilising the 'isoelectric-focusing' principle (Hoch and Barr, 1955; Tuttle, 1956; Svensson, 1962; Vesterberg and Svensson, 1966) and, in addition, employs simple apparatus, conserves expensive carrier-ampholytes, is very resistant to convective mixing, and permits the simultaneous separation of several mixtures. The gels may be stained for protein, subjected to densitometric analysis and dried for storage. Examples have been described of the use of the technique for the resolution of complex protein mixtures, the separation of isoenzymes, the determination of the isoelectric points of proteins and for preparative purposes.

Since a preliminary account of this work was presented (Leaback and Rutter, 1968), a note has appeared concerning a similar procedure conducted in polyacrylamide rods (Dale and Latner, 1968).

Methods

The apparatus devised for this work (Fig. 1) comprises a perspex lid bearing terminals (T) connected to horizontal carbon or platinum electrodes (E),

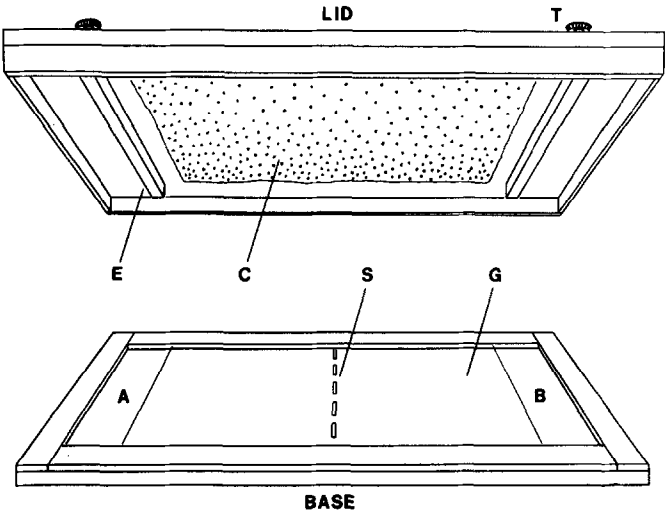

Fig. 1.

and a glass base to carry the gel (G) and the electrode wells (A, B). The only other major requirement is for a power supply of 0-300 Volts D.C. at 0-3mA. The base consists of a glass plate to which four strips of glass are bonded (with 'Araldite' epoxy-resin) to form a water-tight well 18x8x0.2 $cm.^3$ A perspex strip 8x2x0.2 $cm.^3$ is placed at A and another at B before the well is filled with polymerisation mixture (either 30ml. of solution containing 0.85g. acrylamide, 50mg. NN'-methylene-bis-acrylamide, 25µl. NNN'N'-tetramethyl-ethylene-diamine and 20mg. ammonium persulphate or, for photopolymerisation, 1.43g. acrylamide, 75mg. NN'-methylene-bis-acrylamide, 0.04mg. riboflavin and 0.3-0.6ml. of the appropriate 40% Ampholine solution) and is overlaid by a rigid perspex sheet bearing moulds for sample-slots (five slots 10x1x1$mm.^3$ are convenient). After setting, persulphate-polymerized gels are washed with 2-3 changes of water before the appropriate 24% Ampholine solution (0.5-1.0ml.) is spread upon the gel surface and allowed to stand at room temperature for several hours before use.

Electrode wells A and B are filled with aqueous solutions of phosphoric acid (0.1M) and diamino-ethane (0.15M) respectively, the samples are added to

slots S and, with the lid in position, a potential difference is applied to the electrodes such as to keep the current at or below 2mA. A moist pad (C) prevents excessive evaporation from the gel surface. At field strengths of 20-25V./cm. homogeneous proteins give sharp bands at room temperature whereas ampholytes of low molecular weight (e.g. methyl red, haematoporphyrin) require field strengths over 70V./cm. for bands of comparable sharpness. When equilibrium is attained, the gel (and glass base) is immersed in aqueous trichloroacetic acid (5% w/v).

Since carrier ampholytes complex with protein dyes, the gels were washed several times in the trichloroacetic solution before staining (30min. with 0.05% Naphthalene Black in methanol;glycerol;water;acetic acid;50;50;20;1 by vol.), and subsequent destaining in aqueous acetic (7%v/v). For storage or densitometric analysis the fixed gels were washed several times with 7% (v/v) aqueous acetic acid, spread upon cellulose tri-acetate sheets and air-dried.

Washed 'persulphate' gels were used throughout the results presented here to obviate the possibility of protein interactions with components of the polymerisation mixture (cf. Mitchell, 1967) and since difficulties were experienced in obtaining reproducible large-pore gels (3%) by the photopolymerisation procedure.

Endosmosis in gels prepared as above was low (1-2cm./16hr.). In contrast, experiments with strips of paper, methylated paper (cf. Tiselius and Flodin, 1954) or cellulose acetate indicated that the endosmosis on these materials precludes the establishment of adequately stable pH gradients.

Results

The technique has been applied to the separation of the common haemoglobins A, C, F and S. These proteins, as the met-, carbonmonoxy-, or oxy- forms (Figs. 2a, 2c and 2d respectively) separate as sharp, well-resolved bands after 3-6 hr. electrophoresis in a 6-8pH gradient. The discreteness of the separation of oxyhaemoglobins A and F in particular indicates that the

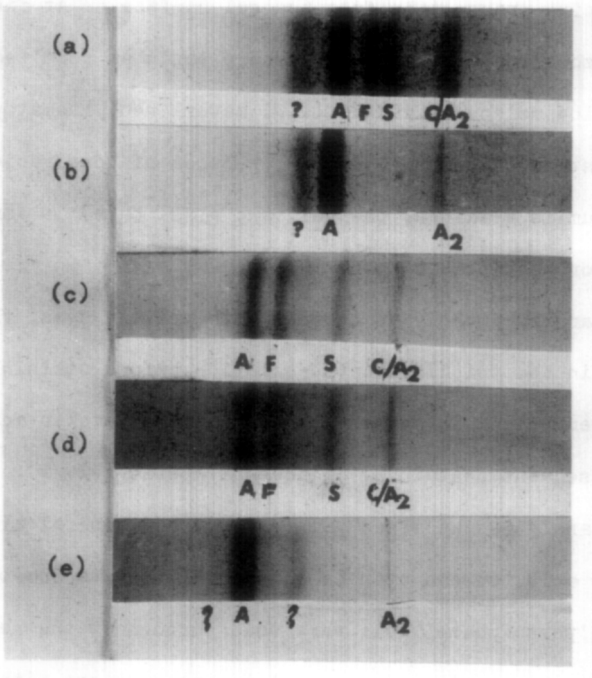

Fig. 2. The portions of gels shown here correspond approximately to pH 6.5-8.0 (left to right) with mixtures of met- (a and b), carbonmonoxy- (c), and oxy- (d and e), forms of the haemoglobins indicated above. Bands marked ? were of unknown species.

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technique has advantages over cellulose acetate (Briere, Golias and Batsakis, 1965) or polyacrylamide electrophoresis (Dowding and Tarnoky, 1967). By using carbonmonoxy-haemoglobins A and C as marker proteins of known characteristics (cf. Svensson, 1962), the iso-electric points of carbonmonoxy-haemoglobins F and S have been estimated from gel measurements to be 7.07 and 7.23 respectively.

The use of the technique to follow the chemical modification of a protein was investigated by labelling bovine serum albumin according to the method of Rinderknecht (1962); the introduction of the fluorescein moiety

brought about a marked acid-shift in the principal protein band to the point F (Fig. 3(b)). The location of erythrocyte acid phosphatases after spraying gels with 4-methylumbelliferyl phosphate and exposure to ammonia (Fig. 3(d)) gave multiple fluorescent bands (A) which appeared to be sharper than those described for a starch-gel procedure (Hopkinson, Spencer & Harris, 1964).

Zones have been assigned for some important components of a typical human serum (Fig. 3(k)) using authentic purified proteins (Hoechst Pharmaceuticals Ltd.) as marker substances (e.g. haptoglobins, Fig. 3i, transferrins, Fig. 3j); more detailed assignments will follow the application of specific localisation techniques.

Since protein bands sharpen as equilibrium is reached, very minor components of protein mixtures can often be detected. Thus, a few percent of haemoglobin A_2 can readily be demonstrated in the presence of excess haemoglobin A (Fig. 2(e)) and minor components in crystalline samples of ovalbumin (Sigma Chemical Co.), bovine serum albumin (Armour Pharmaceutical Co.) and bovine pancreatic ribonuclease (British Drug Houses) have been detected (Figs. 3(a), 3(b) and 3(c) respectively).

Advantage may be taken of the ease of sample application in a procedure for re-running protein bands. Thus, 1cm. squares were excised from a gel containing a series of bands (Fig. 3(e)) obtained from a human immunoglobulin G (homogeneous to immuno-electrophoresis); the squares were placed in $1cm^2$ holes in another gel and the proteins re-run to give the patterns shown in Figs. 3(f), 3(g) and 3(h). It was concluded that the banding in Fig. 3(e) represented true molecular heterogeneity and not, for example, the complexing of an homogeneous protein with various Ampholine species.

In the column procedure of Svensson (1962) the sucrose concentration gradient employed is only partly successful in opposing mixing due to sample overloading, to convection and to turbulence while drawing off the fractions. The technique in polyacrylamide gel is very resistant to

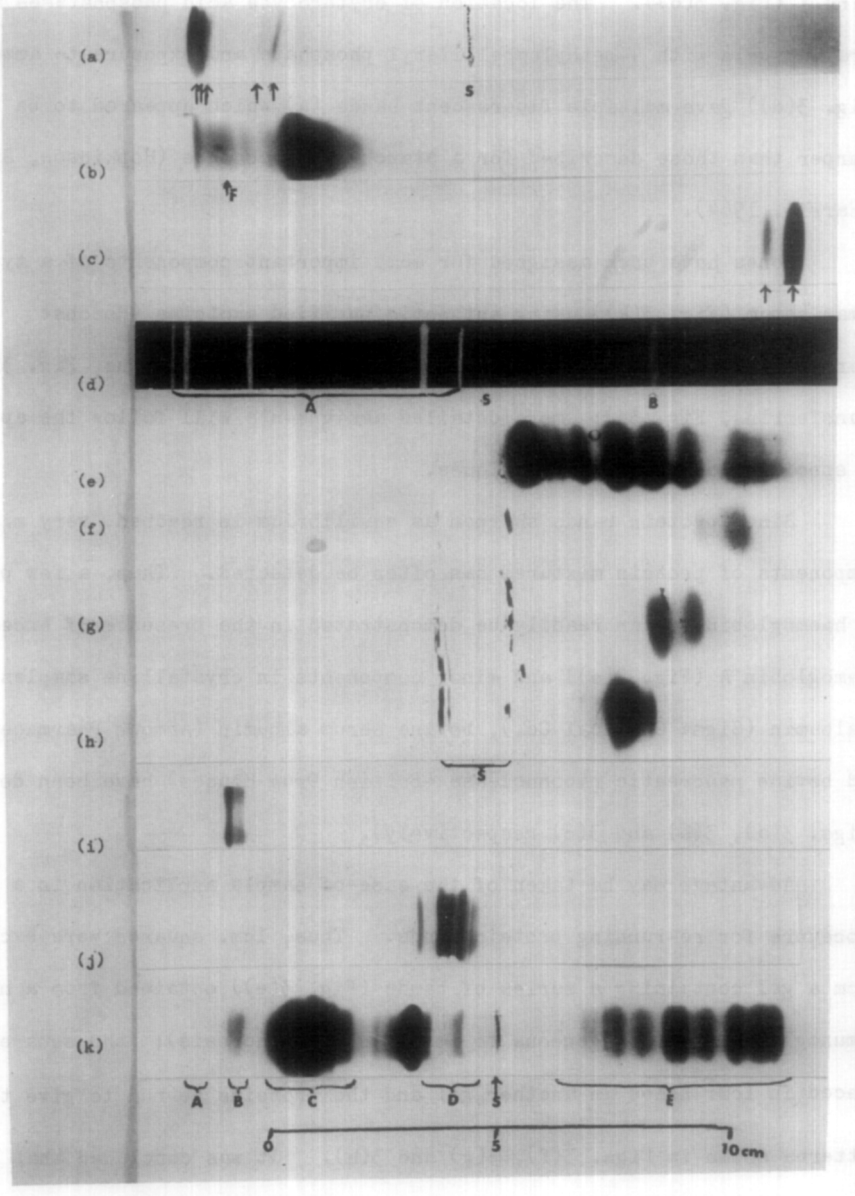

Fig. 3. The portions of gels shown here correspond approximately to pH3-10 (left to right) with (a) ovalbumin, (b) bovine serum albumin, (c) ribonuclease, (d) drawing of erythrocyte phosphatase activities (A) with oxyhaemoglobin A indicated at position B, (e) human γ-globulins, (f), (g) and (h) fractions of (e), (i) human haptoglobins, (j) human transferrins and (k) a 'normal' human serum with prealbumin (A), haptoglobin (B), albumin (C), transferrin (D) and γ-globulin (E) zones. Sample slots are marked S.

convective mixing and to sample overloading. Furthermore, the high resolving-power of the method can be preserved by excising appropriate portions of the gels (colourless protein bands being located with respect to coloured marker ampholytes). Another technique has been used successfully to avoid the necessity of extracting gel segments for preparative purposes. Thus, the sample is applied to a 70x2x3mm^3 slot (S) and, after sharpening, bands are carried by endosmosis into another such slot (slightly towards the cathode end of the initial equilibrium position) where the protein is removed and, if necessary, replaced by carrier ampholyte and the process repeated. As much as 30mg. of a haemoglobin A/A_2 mixture have been applied to a single gel and separated as electrophoretically-pure components by this procedure.

The technique offers potential for a wide range of routine and research applications.

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