ISOELECTRIC FRACTIONATION OF PROTEINS ON POLYACRYLAMIDE GELS

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The introduction of isoelectric fractionation of proteins by Svensson [1] and the development of suitable carrier ampholytes by Vesterberg and Svensson [2] represents one of the greatest advances in preparative electrophoresis. In this type of electrophoresis a stable pH gradient is generated and amphoteric molecules migrate into a pH region at which they are isoelectric and will concentrate as a narrow zone. The width of the zone depends premarily on the ratio of electrophoretic concentration to diffusional dilution for the particular molecule. The method has an extraordinary high resolving power, capable of separating substances with isoelectric points differing by only 0.02 units, is extremely reproducible and allows quantitative recovery of products.

In this paper the method is adapted to gel electrophoresis. The replacement of the sucrose density gradient by a gel medium renders this method suitable for small scale analytical separation and the greater stabilising qualities of the gel might be expected to give enhanced resolving power. Of the gel stabilising media available, polyacrylamide was chosen since it has negligible electro-osmotic flow, a property essential for maintaining a stable pH gradient within the gel.

Glass tubes 3-6 mm diameter and 15-20 cm long are supported vertically, stoppered at the bottom end and filled to within 5 cm of the open end with a solution containing acrylamide (8%), *N,N'*-methylene bisacrylamide (0.24%), Ampholine * (1%) of the desired pH range and riboflavin (0.0005%). The solution is polymerised by exposure to daylight or artificial light for a period varying from 15 min to 2 hr depending on the intensity of illumination [3].

After removing the stopper the lower end of the tube is placed in a small electrode vessel 1 cm diameter, containing a platinum electrode (cathode) and filled with 0.5-1.0 ml tetramethyl ethylene diamine solution (1%). The sample dissolved in 10-20 μl Ampholine solution (1%) is layered on top of the gel, followed by 0.5-1.0 ml Ampholine solution (1%). A platinum electrode (anode) is dipped into this solution and a potential gradient of 4-6 v/cm is applied and increased to 15 v/cm after 2-4 hr.

For multiple analysis, a number of glass tubes can be sealed with Araldite (Ciba Ltd.) into a perspex frame (fig. 1) incorporating a narrow trough as the anode compartment. The frame is held vertically in a perspex support with a base containing a narrow cathode compartment. This apparatus has proved highly satisfactory for the analysis of myoglobins and abnormal haemoglobins using LKB Ampholine pH range 7-10 (fig. 1). The gels are readily removed from the tubes and the isoelectric point of the protein can be obtained by measuring the pH of the zone with a spear type miniature glass electrode.

The detection of colourless protein zones has proved difficult since the Ampholine reacted with all protein stains tested, giving an intense background colour. It is first necessary to fix the protein zone by precipitation with aqueous trichloroacetic acid (10%), followed by repeated washings with the same reagent to remove the electrolyte. The gel is then stained with Coomassie Brilliant Blue [4] (0.01% in trichloroacetic acid solution) and finally washed with aqueous ethanol (40% v/v) until a clear background is obtained.

* Obtainable in various ranges pH 3-10 from LKB Instruments, Ltd., 232 Addington Rd., South Croydon, Surrey, CR2 8YD.

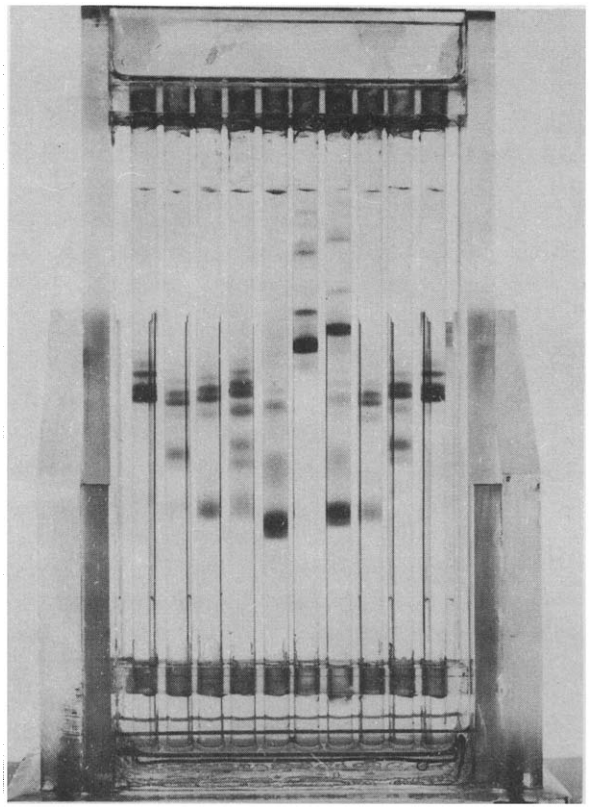

Fig. 1. Isoelectric fractionation of carbonmonoxy-haemoglobins and carbonmonoxy-myoglobins in polyacrylamide gel containing Ampholine pH 7-10. Left to right, 1 and 10 normal adult Hb, 2 and 9 Hb A+S, 3 and 8 Hb A+C, 4 Mixture of Hb A+S and Hb A+C, 5 Whale myoglobin, 6 Horse myoglobin, 7 Mixture of Whale and Horse myoglobins.

In addition to improved resolution, the method described in this paper offers several advantages over conventional zone electrophoresis. The application of the starting zone is not critical and may be made as a wide irregular zone of dilute protein solution. In homogeneity studies, blocks of gel cut from previous electrophoresis experiments may be used as starting zones. With the conditions described above cooling of the gel is unnecessary. During the course of the electrophoresis the current decreases and the potential may be correspondingly increased, reaching a maximum of 40-50 v/cm. At this potential the proteins concentrate to form very narrow zones which remain almost stationary, except for a slow migration towards the cathode, due to drifting of the pH gradient.

It is of interest to comment on the bands obtained by isoelectric fractionation of myoglobins and haemoglobins in polyacrylamide gel (fig. 1). The myoglobins, which were commercial samples, are known to be heterogeneous containing several minor components that are concentrated to narrow zones in the isoelectric fractionation and are therefore more readily detected. The main zones when cut from the gel and re-run give single bands and behave as homogeneous components.

It will be observed that the haemoglobins give bands that appear as doublets; haemoglobin A_2 which is barely visible in the photograph also shows double banding. For example, a sample of normal haemoglobin gives 4 bands close together, 2 major and 2 minor, well separated from 2 faint haemoglobin A_2 bands. When the 2 major bands are cut from the gel and re-run in separate gels they each form 2 zones, suggesting a reversible equilibrium.

It is possible that the additional bands are caused by complexing with the electrolyte (Ampholine) but this is unlikely as shown by the following experiment. A series of gels was run where the amount of haemoglobin applied was varied, but the electrolyte concentration remained constant. Quantitative evaluation of the distribution of zones was obtained using a Unicam SP 800 spectrophotometer which has been adapted as a tube scanning densitometer. By taking advantage of the absorption at various wavelengths, and the scale expansion facilities on the SP 800 it was possible to scan 5 gels with a 250 fold range of haemoglobin concentration. All the gels gave essentially the same distribution of bands, showing an independence of haemoglobin concentration.

This aspect of the problem is being investigated further.

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References

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