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Note

Protein titration curves using modified cellulose acetate membranes

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Righetti et al. [1] first introduced titration curves in polyacrylamide gel slabs for the study of proteins and their genetic variants. We have modified the technique for use with cellulose acetate membranes and have thereby made more economical use of the carrier ampholyte. An essential requirement of such a modification is the formation of a suitable pH gradient and its subsequent stability during electrophoresis in the second dimension. Methylated cellulose acetate as described by Ambler and Walker [2] provides a suitable alternative to polyacrylamide and has been used in this work to investigate haemoglobin variants.

MATERIALS AND METHODS

Membrane preparation

Sepraphore III cellulose acetate membranes (Gelman, Hawksley Ltd., Northampton, Great Britain) were methylated according to the method of Ambler and Walker [2]. The membranes (12.5 × 14.5 cm) were taken through increasing concentrations of methanol until they were equilibrated with absolute methanol, after which they were transferred to methylating reagent, 4% boron trifluoride in methanol, and incubated in this solution for 45 min at 46°C. The treated membranes were rinsed in three washes of methanol and stored in methanol. They were drained of excess solvent before being placed in ampholyte solution: 7.5% Ampholine (LKB) pH range 3.5—10, glycerol 6%, 6 m*M* each of aspartic acid (Asp), glutamic acid (Glu), lysine (Lys) and arginine (Arg). An 8-ml volume of solution was sufficient for two 12.5 × 14.5 cm membranes. Equilibration was allowed to take place for at least 8 h, occasionally agitating the membranes.

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Isoelectric focusing and electrophoresis

A membrane was removed from the ampholyte solution, held vertically for 10–20 sec to drain and placed on a 12.5 × 26 cm LKB glass plate. Care was taken not to allow air bubbles to enter between the membrane and the plate. A 14.5-cm length of electrode wick, soaked in 0.3 *M* ethanolamine, was placed at the cathode and another, soaked in 0.2 *M* citric acid, was placed at the anode. To establish the pH gradient isoelectric focusing (IEF) was carried out in the first dimension using the LKB Multiphore 2117 apparatus with a 2103 power supply and maintaining a constant 3.5-W power at 3–6°C for ca. 1.75 h. The voltage across the membrane rose from an initial ca. 350 V to ca. 700 V on completion of focusing, with the degree of wetness of the membrane and electrode wicks causing some variation. A marker haemolysate (2 μl) was applied close to the anode and another close to the cathode at one edge of the membrane; the movement of the two markers into one set of focused zones signalled the completion of IEF.

The electrode wicks and 1.5 × 14.5 cm strips of membrane were then cut from the anodal and cathodal edges and discarded. A vertical section 2 × 9.5 cm containing the focused marker was also removed, leaving a membrane measuring 9.5 × 12.5 cm. This was lifted in one quick movement from the glass plate, moved through a 90° angle and carefully positioned, again in one movement, on to a second glass plate. New electrode wicks, 9.5 cm in length and soaked in the same solutions as for IEF, were applied to the membrane. Approximately 2 μl of the haemoglobin solution (25 g/l) under investigation were applied across the membrane in the direction of the pH gradient. Electrophoresis in the second dimension was carried out at a constant 300 V for 30 min at 3–6° C. The pH gradient existing at the end of this procedure was determined by cutting 5 × 10 mm sections across the membrane and eluting the carrier ampholytes into 200 μl of 10 m*M* potassium chloride and measuring the pH of the resulting solutions.

Fixing and staining were carried out simultaneously for 15 min in the following solution: Coomassie Brilliant Blue R 250 2.5 g, methanol 455 ml, deionised water 455 ml, glacial acetic acid 90 ml. Destaining was carried out in a solution containing methanol 500 ml, deionised water 500 ml, glacial acetic acid 100 ml, until the background was clear. The membrane was then transferred to 0.83 *M* acetic acid; it could then be photographed in reflected light without drying.

RESULTS AND DISCUSSION

Fig. 1 shows the pH measurements taken across the membrane after electrophoresis. The gradient is not linear and shallows out in the pH range 7–8.5. This has the effect of enhancing separation of proteins with isoelectric points (p*I*) in that range. Ampholyte mixtures can be altered to create optimum separations over different pH ranges. Haemoglobin A (p*I* 7.0) and C (p*I* 7.4) form two well separated curves as shown in Fig. 2. Other faint titration curves can be seen and represent other proteins present in the haemolysate; the more pronounced of these lines is found halfway between the two haemoglobin curves and is probably the hybrid molecule described by Righetti's group.

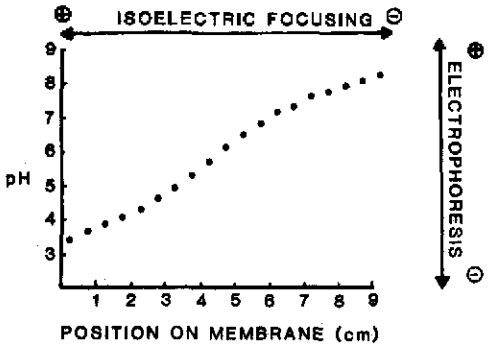

Fig. 1. pH gradient established in Sepraphore III membranes after isoelectric focusing followed by electrophoresis.

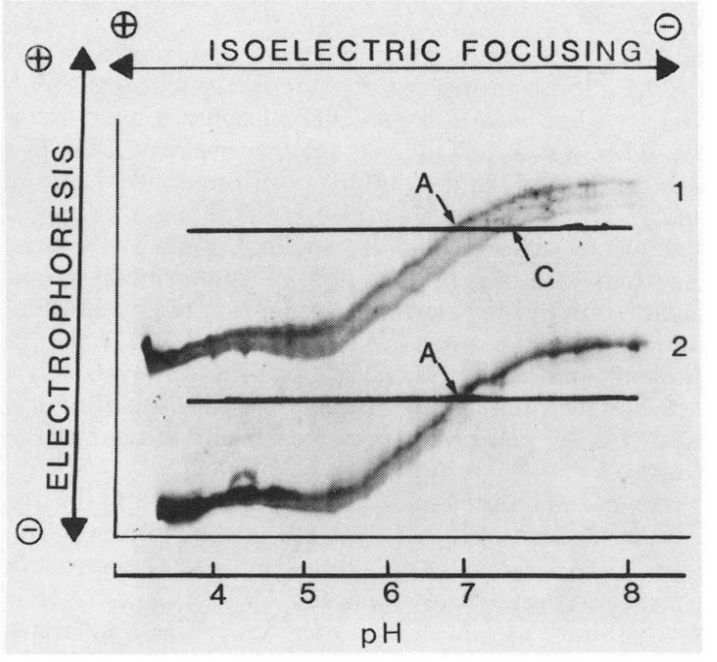

Fig. 2. Titration curves of haemoglobin A + C (1) and haemoglobin A (2) on Sepraphore III membranes. The arrows indicate the p*I* values as points of intersection with the application lines.

Instability of both haemoglobin species is demonstrated by the blurring of the curve in the acid regions of the membrane.

The proposed support medium has a number of advantages over polyacrylamide gel. It is non-toxic, easier to handle and prepare and, once methylated, membranes can be stored in methanol for up to four weeks. Staining and destaining times are short. Smaller quantities of protein can be analysed and the problems associated with molecular sieving are reduced; the medium should therefore be more suitable when large protein molecules are under investigation. Two membranes take up half the amount of carrier ampholyte used by

the original method [1] and slightly less than the reduced LKB modification [3], with the added saving that two haemolysates instead of one can be run on a single membrane.

REFERENCES

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3 LKB Application Note 319, 1981.