MICROCHROMATOGRAPHY OF PROTEINS ON WEDGE-COMPRESSED NITROCELLULOSE MEMBRANES

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(Received February 14th, 1967)

In another paper[1] we described in detail a simple procedure for compressing nitrocellulose membrane filters into a wedge thus forming a continuous pore size gradient along the membranes. Examples were presented of the chromatographic behaviour and separation of phenolsulphophthalein and a high-molecular denatured protein on membranes compressed at different angles. It was evident from those first experiments that the proposed principle of filter chromatography on compressed membranes really operated. The present communication refers to further results achieved by this technique during the chromatography of very small amounts of proteins.

EXPERIMENTAL

VUFS membranes (VCHZ Synthesia, Uhříněves, Czechoslovakia; the membranes are now distributed by "Chemapol", Prague, under the name "Synpore 8"), 2 × 4 cm, impregnated with 2 % Tween 60 as described before[2,3], were used. A 0.1 *M* TRIS–HCl + 1 *M* NaCl buffer, pH 7.9, was used in all gel filtration and chromatographic experiments. Ovalbumin, human γ-globulin, dog and bovine serum, two protein fractions isolated by gel filtration on agar pearls[4] from the plasma expander Resorba[5], a Czechoslovak modified bovine serum MBS[6] and phenolsulphophthalein were chromatographed. Amido black 10 B, Ponceau S and nigrosine were used for the detection of the proteins[1,7].

Gel filtration of Resorba was done on a 84 × 1.4 cm column of 4 % pearl condensed agar as described before[5]. The isolated fractions were concentrated twice by dialysis against 25 % crude dextran in the TRIS buffer. The compression of the membranes was done between polished steel blocks, the values *a* and *b* (*cf.* ref. 1) on the pressing device were constant (8 mm and 15 mm respectively), the controlled pressures *P* were in the range 15–100 atm, the supports *c* were 0.08 mm and 0.16 mm respectively. Horizontal chromatography was performed as usual[8], 1–3 % samples were applied in amounts of the order 10^{-5}–10^{-4} ml. For photographic documentation the chromatograms were made transparent with paraffin oil.

RESULTS AND DISCUSSION

Fractionation of the blood expander Resorba (Fig. 1) led to the isolation of two

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main peaks[9], A (mol. wt. of the order 10^6) and B (mol. wt. of the order 10^5), which were very suitable for further chromatographic experiments.

It was assumed[1] that in the course of chromatographing macromolecules on wedge-compressed membranes from the larger pores towards the smaller ones the larger particles would be intercepted at a certain distance d from the line of maximum compression h_2. Fig. 2 shows that this phenomenon actually takes place. In one experiment, the samples were applied onto starting points at different distances from h_2. After 7–8 h when the indicator dye had passed to the other end of the strip the protein spots gathered at a line (I) or showed a tendency to approach a line (see also II, III) perpendicular to the direction of pore size gradient, regardless of the position of the starting points. Beyond this line the pores were evidently too small and therefore unpenetrable to the macromolecules under study. This can be seen very well with the sample nearest to h_2 which could not even diffuse from the start. The degree of penetration towards h_2 depended on the pressure and the angle of compression (*cf.* Fig. 2, II, III) as has already been shown before[1], and on the molecular size (*cf.* Fig. 2, I, II) as was expected. A sufficient "overflowing" of the buffer, as indicated by the indicator dye, is assumed here. Otherwise, in shorter chromatographic runs (about 4 h), where the larger particles were only retarded but not fully immobilized, the time factor also played an important role. To get comparable results in those shorter runs, it was necessary to stop the chromatography at the moment when the indicator dye reached the same distance behind the line h_2 on all chromatograms. Experiments were made under such conditions to obtain information on the role of the pressures used to deform the membranes. The results led to the following conclusions (Fig. 3):

Pressures higher than 25 atm led to constant results as far as the distances d from h_2 are concerned. However, the running time (over 8 h) was too long to be practical when higher pressures up to 100 atm, had been used, so that 25 atm was found to be most convenient in our experiments when the parameter $c = 0.08$ mm. For $c = 0.16$ mm, $P = 20$ atm was sufficient. When lower pressures were applied (the

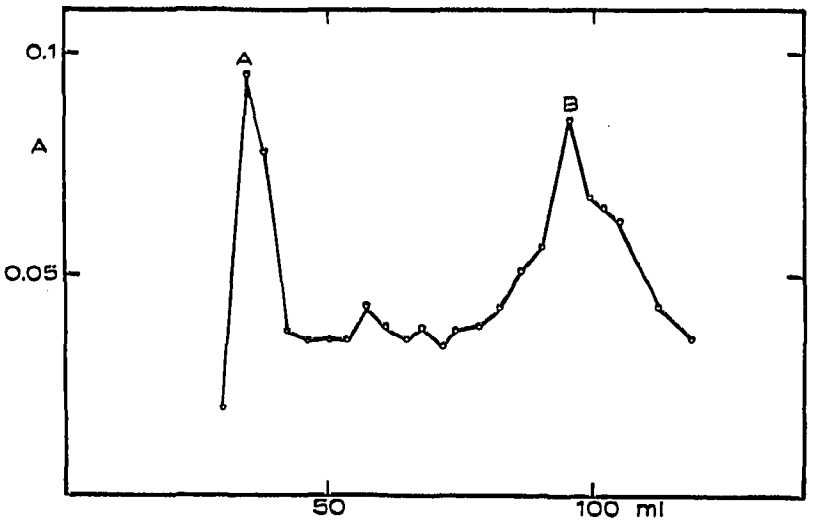

Fig. 1. Gel filtration of the plasma expander Resorba[5] on pearl condensed agar. Buffer: 0.1 *M* TRIS–HCl + 1 *M* NaCl, pH 7.9; 4% agar pearls; column: 84 × 1.4 cm. Estimation of proteins was made turbidimetrically. Fractions A and B were used for further experiments.

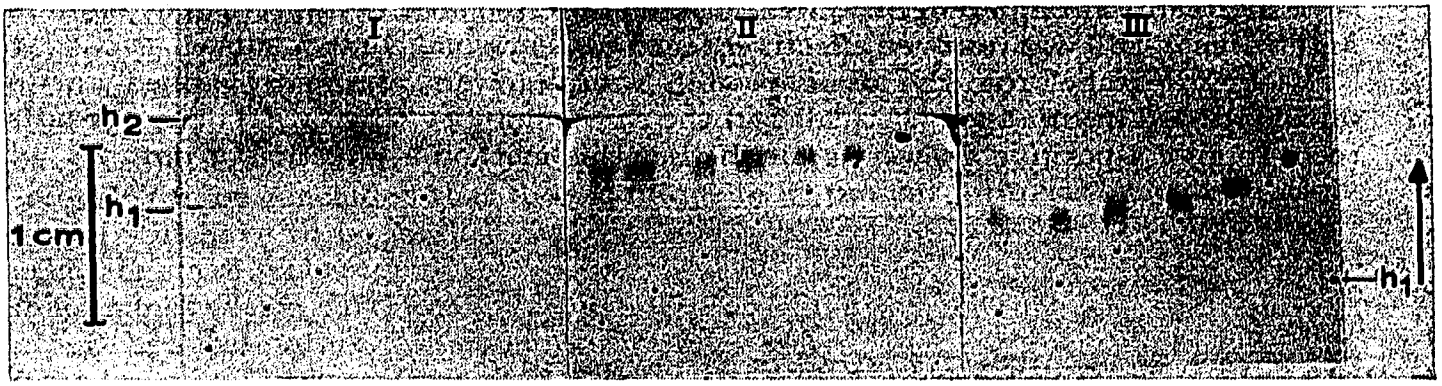

Fig. 2. Chromatography of modified proteins on wedge-compressed VUFS membranes impregnated with Tween 60. h_1 = Line of minimal compression; h_2 = line of maximal compression. Strip I: fraction B of Resorba. Strip II: fraction A of Resorba; both strips were compressed under the same conditions at the pressing device[1]: P = 20 atm, a = 8 mm, b = 15 mm, c = 0.16 mm. Strip III: fraction A of Resorba; the strip was compressed at P = 25 atm, a = 8 mm, b = 15 mm, c = 0.08 mm. Starts are indicated by dots. Buffer (see Fig. 1). Chromatographed for 4.5 h.

other conditions remaining constant) the membranes were compressed insufficiently so that fraction B often reached or even passed through the line h_2 (Fig. 3).

However, proteins having molecular weights less than about 100,000 (*e.g.* ovalbumin, serum albumin), passed the line h_2 even when pressures up to 100 atm had been used. This indicated that there was a certain limit in the sizes of particles which could penetrate the "highly compressed" membranes. For VUFS membranes and globular proteins this limit seemed to be at a mol. wt. of about 100,000.

To confirm the applicability of this technique to the fractionation of macromolecules according to their size, a model experiment was made using ovalbumin, γ-globulin, fraction "B" and indian ink. Whereas indian ink did not penetrate even the uncompressed membrane VUFS, and remained entirely on the start, the other substances differed very distinctly in their chromatographic behaviour (Fig. 4, I). Thus ovalbumin penetrated "freely" through the line h_2, fraction "B" and γ-globulin

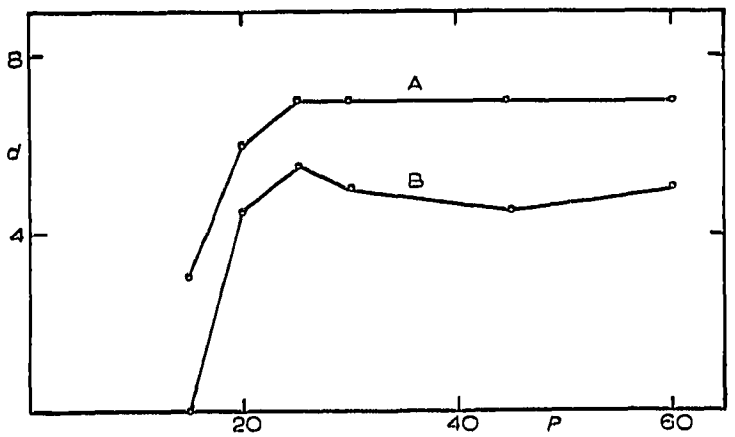

Fig. 3. Migration of protein samples on membranes compressed at different pressures. d = Distance (in mm) of the centre of the spot from h_2; P = pressure (in atmospheres) applied to deform the VUFS membrane; other conditions (*cf.* ref. 1): a = 8 mm, b = 15 mm, c = 0.08 mm. Fractions A and B of Resorba (*cf.* Fig. 1) were applied. Phenolsulphophthalein migrated 2 mm over line h_2. Chromatography lasted 4 h.

were intercepted at different distances in the compressed area. A mixture of ovalbumin and fraction "B" was resolved effectively into both original components.

In a parallel run the same protein samples were chromatographed on an uncompressed membrane, where all had the same mobility (Fig. 4, II). These results seem to give a positive answer as to whether this method is effective or not.

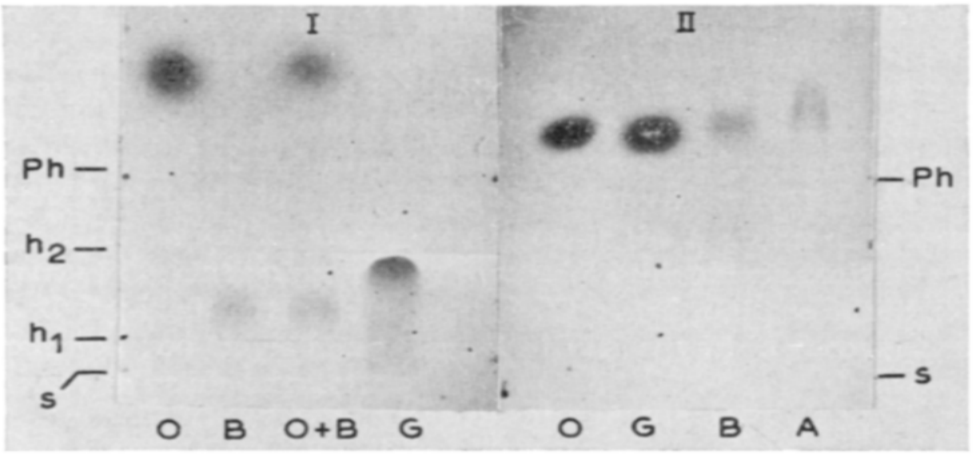

Fig. 4. Chromatography of various protein samples on VUFS membranes impregnated with Tween 60. s = Start; Ph = position of the phenolsulphophthalein (centre of the spot). Strip I: O = ovalbumin; B = fraction B of Resorba; O + B = mixture of both samples; G = γ-globulin. The strip was compressed at P = 25 atm, a = 8 mm, b = 15 mm, c = 0.16 mm. Chromatography lasted 4.5 h. Strip II: O = ovalbumin; G = γ-globulin; B = fraction B of Resorba; A = fraction A of Resorba. The VUFS membrane was not compressed. Chromatography lasted 1 h.

In other experiments, more complex mixtures of macromolecules were chromatographed to investigate the possibilities of the microchromatographic technique on wedge-compressed membranes. As is shown in Fig. 5, certain characteristic separations were achieved, suitable for some special although rather informative qualitative microestimations. Thus a characteristic difference can be seen between the chromatogram of native serum with a well separated albumin fraction and the chromatogram of the modified serum of a plasma expander MBS (Fig. 5). This change is somewhat similar to the changes of the electrophoretic patterns[10] but here some information is

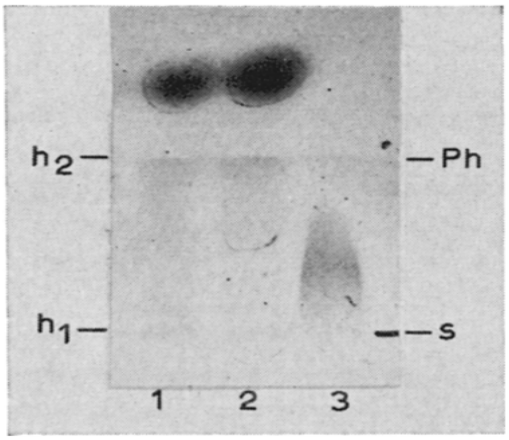

Fig. 5. Chromatography of native and modified sera. 1 = Dog serum; 2 = bovine serum; 3 = MBS. Membrane VUFS, impregnated with Tween 60, compressed at P = 25 atm, a = 8 mm, b = 15 mm, c = 0.08 mm. Buffer TRIS–HCl, pH 7.9. Chromatography for 6 h.

also given indicating the increase of particle size. However, no significant fractionation of Resorba or MBS was achieved comparable to gel filtration (*cf.* Fig. 1 and ref. 4) although separate fractions A and B had different mobilities (*cf.* Fig. 2). It is possible that a partial "stopping" of the pores by particles having very similar particle sizes causes interference here.

It can be concluded that the technique in its present form has some limitations which still have to be studied and eventually overcome, in order to achieve more accurate small scale separations of complex systems. For this purpose other types of membranes with a narrower distribution of pore sizes, as well as membranes of another chemical nature, are under investigation now. The results will be published later.

ACKNOWLEDGEMENT

The author is grateful to Mrs. ZD. NOVOTNÁ for technical assistance.

SUMMARY

Small scale chromatography of various protein samples was done on wedge-compressed nitrocellulose membrane filters impregnated with Tween 60. Evidence is presented that particles of different sizes can be separated according to the principle of filtration chromatography during migration along the continuous pore size gradient from the greater pores towards the smaller ones. Some important factors influencing the separation were examined.

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