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PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS

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SUMMARY

We have studied several variables affecting the migration and resolution of protein mixtures in preparative polyacrylamide gel electrophoresis. In the system we have used (Uniphor column, L.K.B.) the maximum sample load is 10 mg of protein per cm^2 per protein band.

When separating two closely adjacent bands, this value must sometimes be lowered to as little as 2 mg of protein per cm^2 per protein band. Of all the bed gel heights tested, the range 5-10 cm seems to be the most useful; shorter gels are convenient only when the components to be separated differ markedly in size or isoelectric points; longer gels have the disadvantage of increased electrophoretic times and consequent diffusion of the protein bands in the gel.

For most of the protein species usually found in biochemical analysis, gel strengths ranging from 4 to 8% acrylamide are most suitable. In particular, when dealing with a heterogeneous sample, a 4% gel is convenient as it allows all the protein species to enter the gel.

As for the elution buffer rate, we suggest the range 12-25 ml/h; slower rates will not remove the sample from the elution stopper efficiently, with the consequent risk of re-mixing the separated bands, while higher rates will bring about a marked dilution of the sample applied.

It is advisable to have in the gel and in the electrode chambers a buffer of low ionic strength (20-30 m*M*); this allows a high potential gradient to be applied across the gel, thus shortening considerably the electrophoretic run.

Sample recoveries in the system used are all greater than 90%.

INTRODUCTION

Since the introduction of analytical polyacrylamide gel electrophoresis (PAGE), numerous articles have appeared concerning devices for milligram-scale preparative PAGE (prep-PAGE)[1-25]. A few articles also deal with gram-scale preparative apparatus[26-27], but these apparatuses have so far not been made commercially available and are still under development. A common feature of many of them is that the

gel is cast as a hollow cylinder, with inner and outer cooling jackets, and that an elution chamber is built at the lower end of the gel bed, where a radial buffer flow sweeps the eluted bands (upwards or downwards) into a central capillary, connected with a monitor system and a fraction collector. Of the several models built, perhaps the two most common are that designed by JOVIN *et al.*[4], which has provided the basic design for "polyprep" apparatus (Buchler Instruments Inc., Fort Lee, N.J., U.S.A.), and that by GORDON AND LOUIS[17], now sold by Quickfit and Quartz Ltd. (Stone, Staffs., Great Britain).

Recently, however, a new device has been described by BERGRAHM[28,29]. This apparatus, called the Uniphor 7900, is commercially available from LKB Produkter AB (Bromma 1, Sweden). Despite its great versatility (the system can be used for molecular-sieve chromatography or electrophoresis in any type of gel bed as well as for electrophoresis in a sucrose density gradient, isotachophoresis, isoelectric focusing in both a density gradient and in gel, and finally for prep-PAGE), the apparatus does not seem to be very popular in the biochemical area, and it has even escaped the attention of one of the most recent and complete reviews in the field of PAGE[30].

We have also noticed very few publications on this subject[28,29,31]. In an attempt to characterize this system, we have made a study of many variables that affect the resolution in prep-PAGE and have tried to find optimal conditions with regard to gel bed height, gel strength, current and applied potential as well as elution buffer rate. We have also studied the recovery of the sample applied, using both low-molecular-weight and average-sized proteins.

MATERIALS AND METHODS

Acrylamide and N,N'-methylenebisacrylamide (Bis) were purchased from B.D.H. Ltd. (Poole, Great Britain). The former was recrystallized from chloroform[32], the latter from acetone[33]. A 30 % stock solution was made up and kept in a dark bottle at 4°. In the stock solution, the acrylamide–Bis ratio was 33.3:1 and was usually kept at this value in all the experiments. It has been customary in the literature[30] to call $\%T$ the total gel concentration (acrylamide + Bis) and $\%C$ the concentration of the crosslinking agent (expressed as a percentage of the acrylamide concentration). Because in our gels $\%C$ is always constant (it represents 3 % of the acrylamide concentration), we will not refer to it in the experimental section. We must emphasize, however, that when we refer to gel concentration, we only take into account the acrylamide percentage, and therefore our $\%T$ will be slightly less than the values reported by other workers.

Ovalbumin and cytochrome C were obtained from Sigma; bovine serum albumin (BSA), fraction V, from Miles; lysozyme was a gift from Società Prodotti Antibiotici (Milan) and ferritin (recrystallized five times) was obtained from Pentex. Haemoglobin was purified from human erythrocytes by the method of DRABKIN[34]. Immunoglobulins were commercially available from Istituto Sieroterapico Milanese. Coomassie brillant blue R 250 was purchased from Mann Research Laboratories and was routinely used because of its very high sensitivity (C.I. 42660)[35].

Analytical PAGE was carried out with a gel slab on a tray of dimensions

9 × 15 × 0.6 cm with a glass bottom. After moulding the gel, the cover with the slot former was removed, the sample was applied and the gel tray was sandwiched between two cooling jackets, both having glass surfaces. In this way, we could reproduce in the analytical apparatus the same conditions as were used in the preparative apparatus. For staining, the gel slab was allowed to soak for 2 h at 50° in a solution of 0.05% Coomassie blue in 25% isopropanol and 10% acetic acid. De-staining was carried out by leaching out the dye in 7% acetic acid at 50–60°.

For prep-PAGE, the Uniphor 7900 column was connected with a Uvicord II flow analyzer (LKB 8300), which was connected with a recorder (LKB 6520). The fractions were collected with an LKB 7000 Ultrorac fraction collector. The elution buffer was pumped through the lower coupling by a peristaltic pump (LKB Varioperpex 12000).

RESULTS

Choice of the correct gel bed height

In preliminary experiments, we tried to separate a mixture of 15 mg of BSA and 30 mg of ferritin in a gel bed of 6 cm, containing 4% acrylamide. This sample is very heterogeneous, because it contains polymeric forms of albumin[36] and ferritin[32]. In fact, in such a system, the two monomers and the various aggregates would contaminate each other to a considerable extent, as demonstrated by the analytical PAGE of isolated fractions.

Much better resolution was obtained when a mixture of 35 mg of BSA and 45 mg of ferritin was separated in a gel bed of 10 cm. Fig. 1 shows the actual elution profile of the various bands; the unmarked peak represents UV-absorbing material given off from the gel; the other peaks are, in order, albumin monomer, albumin dimer and ferritin monomer, dimer and trimer. In this instance the ferritin monomer has been completely separated from albumin and ferritin dimers. The shaded areas represent pooled fractions concentrated in dialysis bags against Sephadex G-100,

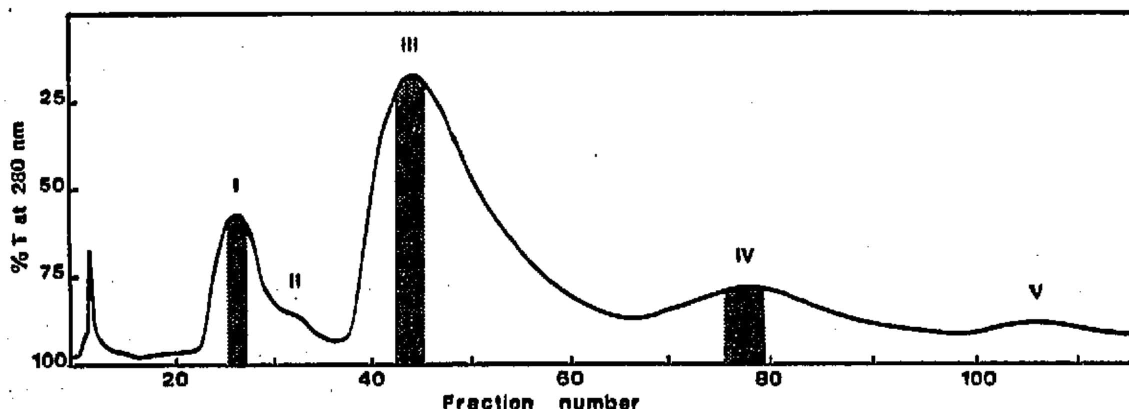


Fig. 1. Elution profile from a mixture of 35 mg of BSA and 45 mg of ferritin (in 3.0 ml of 2.5 m*M* Tris–glycine buffer of pH 8.7) applied to a 4% acrylamide gel, 10 cm high. Recorder chart speed, 2 cm/h; elution buffer rate, 26 ml/h; electrophoretic conditions, 700 V and 18 mA; fraction volume, 3.9 ml each tube. Unmarked peak: UV-absorbing material from the gel. The peaks are in the following order: I = albumin monomer; II = albumin dimer; III = ferritin monomer; IV = ferritin dimer; V = ferritin trimer.

dialyzed against 2.5 mM Tris-glycine buffer of pH 8.7 and fractionated in analytical PAGE.

In Fig. 2 are shown the electrophoretic patterns of these fractions and respective controls, revealed either by specific iron stain[37] (lower gel) or by Coomassie blue stain (upper gel). The areas corresponding to each slot were cut lengthwise,

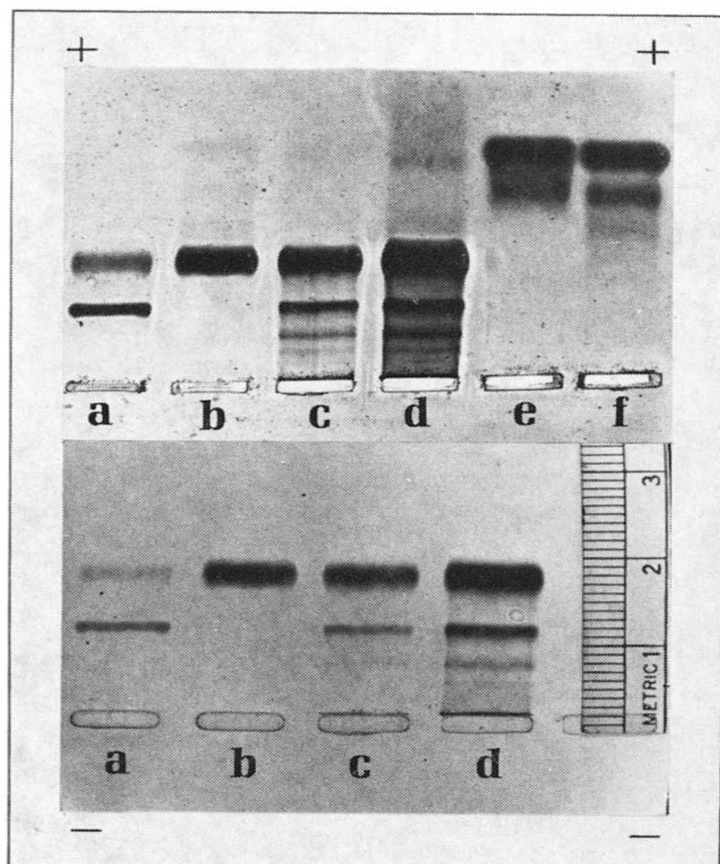


Fig. 2. Analytical PAGE of the shaded peaks of Fig. 1. A gel slab containing 4% acrylamide and 25 mM Tris-glycine of pH 8.7 was run for 3 h at 400 V and 25 mA. Approximately 40 μg of protein in 50 μl were applied to each pocket. All the samples had been dialyzed overnight against the same buffer, 2.5 mM Tris-glycine of pH 8.7. The gel was first stained for iron (below) and subsequently for proteins with Coomassie blue (above). The slots contain (a) ferritin dimer (peak IV of Fig. 1); (b) ferritin monomer (peak III of Fig. 1); (c) and (d) control, unfractionated ferritin in increasing amounts; (e) BSA monomer (peak I in Fig. 1); (f) BSA control, unfractionated.

placed in a cuvette and scanned in a Gilford spectrophotometer, fitted with a scanning device, at 555 nm. Fig. 3 shows the corresponding electropherograms from which the relative abundance of each band could be calculated. Thus an unfractionated sample of ferritin was found to be composed of 55% monomer, 23% dimer, 16% trimer, 5% tetramer and 1% pentamer (Fig. 3d). It is of interest to note that, as 40 μg of sample were applied to the gel, the pentamer band represents 0.4 μg of total protein detected in this system; this value is very close to the lower limit of sensitivity for Coomassie blue (0.2 μg) found by FAZEKAS DE ST. GROTH *et al.*[35] in cellulose acetate strips. In comparison with the ferritin control, the monomer eluted from the Uniphor column is completely free from albumins and ferritin

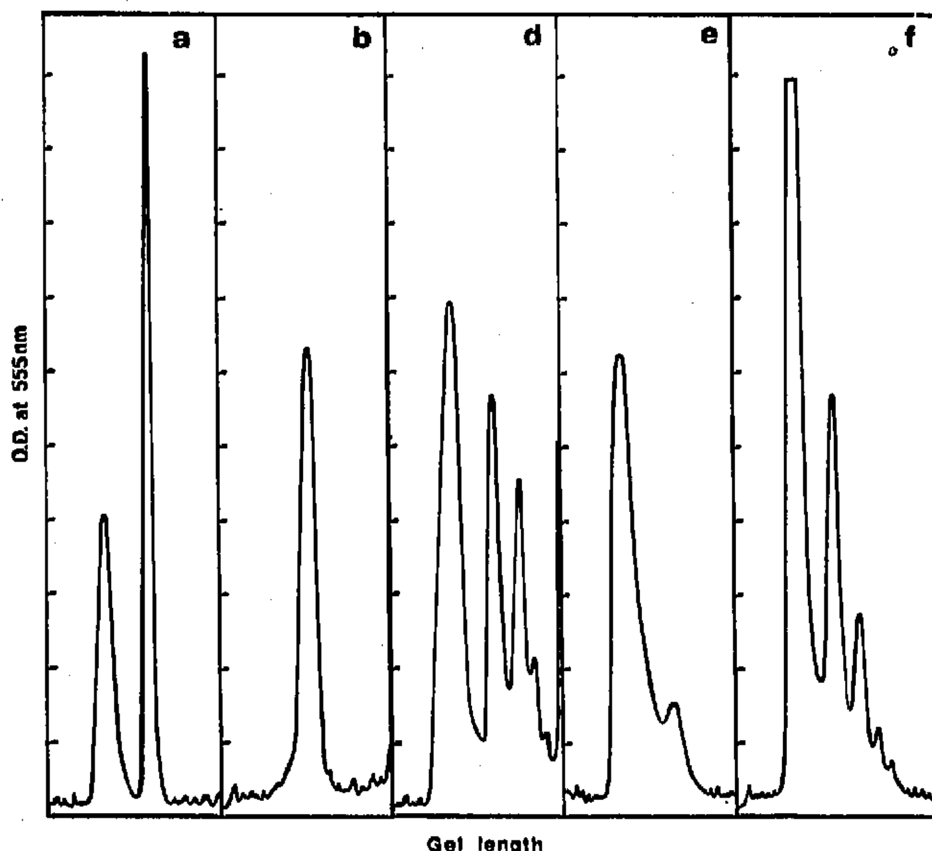


Fig. 3. Electropherogram of the bands fractionated by analytical PAGE in Fig. 2 (upper gel). The area corresponding to each slot was cut lengthwise and the gel placed in a cuvette of a Gilford spectrophotometer fitted with a scanning device. Wavelength, 555 nm. Lettering as in Fig. 2. Integration of the peaks was performed by triangulation. The right-hand end of each section of the graph represents the cathodic region of the gel.

aggregates (Fig. 3b). The dimer, however, is much enriched (72 % dimer compared with 28 % monomer) but not completely pure (Fig. 3a). This can also be seen in the skewed elution profile of the ferritin monomer peak. This asymmetry does not seem to be due to the high protein load (as it is apparent also when loading smaller amounts), but it could be correlated to a heterogeneity of this material, as revealed by isoelectric focusing in horse spleen ferritin[38] and HeLa ferritin[39]. The analysis of the albumin peak showed no contamination by the ferritin, but there was still partial overlapping with the dimer (91 % monomer and 9 % dimer) (Fig. 3e). As a comparison, unfractionated BSA is composed of 69 % monomer, 20 % dimer, 9 % trimer, 2.5 % tetramer and 0.6 % pentamer (Fig. 3f). The correct choice of gel bed is also correlated to the complexity of the mixture to be separated: in this instance, where at least five different components had to be separated, we had to use a 4 % acrylamide gel and a relatively high bed. Had the protein sample been less heterogeneous, such as in the fractionation of albumin polymers alone, a simple 5.5 % acrylamide gel, 6 cm high, would have completely resolved the aggregates (Fig. 4). The mixture albumin-ferritin is also a good example of a separation that can be achieved only by varying either gel strength, bed height or both: the components cannot be separated simply by using a different pH or ionic strength

of the buffer, as these proteins have very similar isoelectric points. In fact, even ferritin aggregates move as single bands in cellulose acetate electrophoresis[40].

Choice of correct gel strength

Fig. 5 shows the elution profile obtained from a mixture of 25 mg of ovalbumin, 25 mg of BSA and 40 mg of haemoglobin applied to a gel column containing 6% acrylamide and 10 cm high. The only peak almost completely resolved

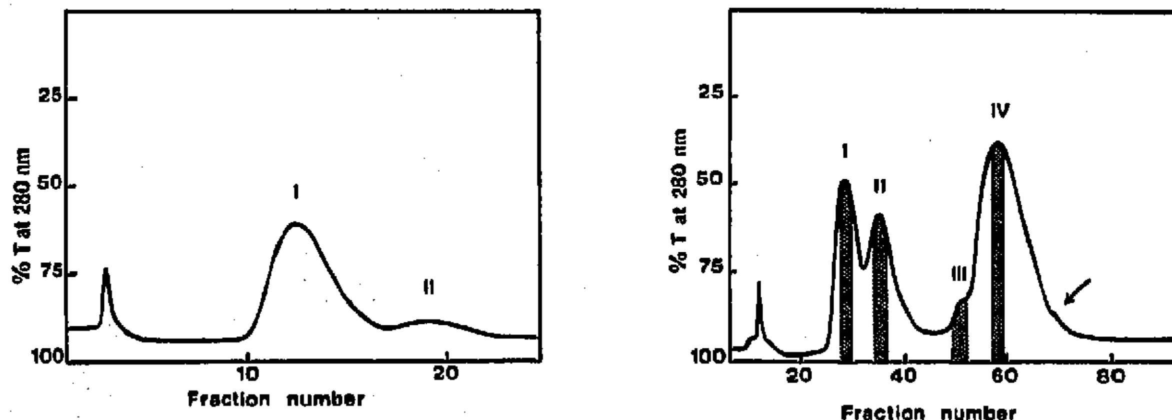


Fig. 4. Elution profile from 20 mg of BSA (in 2 ml of a 2.5 m*M* Tris–glycine buffer of pH 8.7) applied to a 5.5% acrylamide gel, 6 cm high. Recorder chart speed, 3 cm/h; elution buffer rate, 20 ml/h; electrophoretic conditions, 400 V and 25 mA; fraction volume, 5 ml each tube. Peak I, albumin monomer; peak II, albumin dimer.

Fig. 5. Elution profile from a mixture of 25 mg of ovalbumin, 25 mg of BSA and 40 mg of haemoglobin (in 3 ml of 2.5 m*M* Tris–glycine buffer of pH 8.7) applied to a 6% acrylamide gel, 10 cm high. Recorder chart speed, 1 cm/h; elution buffer rate, 20 ml/h; electrophoretic conditions, 600 V and 18 mA; fraction volume, 3.5 ml each tube. The peaks are as follows: I = ovalbumin; II = BSA; III = BSA dimer; IV = haemoglobin. The arrow indicates haemoglobin A_2 ($\alpha_2 \delta_2$).

is the haemoglobin band. In fact, when the shaded areas were analyzed by analytical PAGE (Fig. 6), this peak showed only 5–8% impurity due to the albumin dimer. Ovalbumin and BSA monomer are only partially resolved. When the Coomassie blue-stained regions of Fig. 6 were analyzed in the Gilford gel scanner at 555 nm, they appeared to be approximately 63% pure, the remaining 37% protein impurity being due mostly to reciprocal contamination among the two protein species. It can be seen that the haemoglobin peak is not symmetrical. Even though this protein, when isolated from human erythrocytes, is 95% pure, it must be borne in mind that it still contains several haemoglobin species: A_{IA}, A_{IB}, A_{IC}, A($\alpha_2 \beta_2$) (which is the most abundant, being 90% of the total haemoglobins) and A_2 ($\alpha_2\delta_2$)[41]. Of these, only the last has an isoelectric point (7.4) that is significantly different from that of haemoglobin A (6.95). All the other species have very close p*I*s. In fact, when the haemoglobin peak was analyzed by gel electrofocusing by the technique of RIGHETTI AND DRYSDALE[42], it showed all these components with the exception of $\alpha_2\delta_2$, which would begin to separate as a small peak on the tail of the main haemoglobin band (indicated by an arrow in Fig. 5).

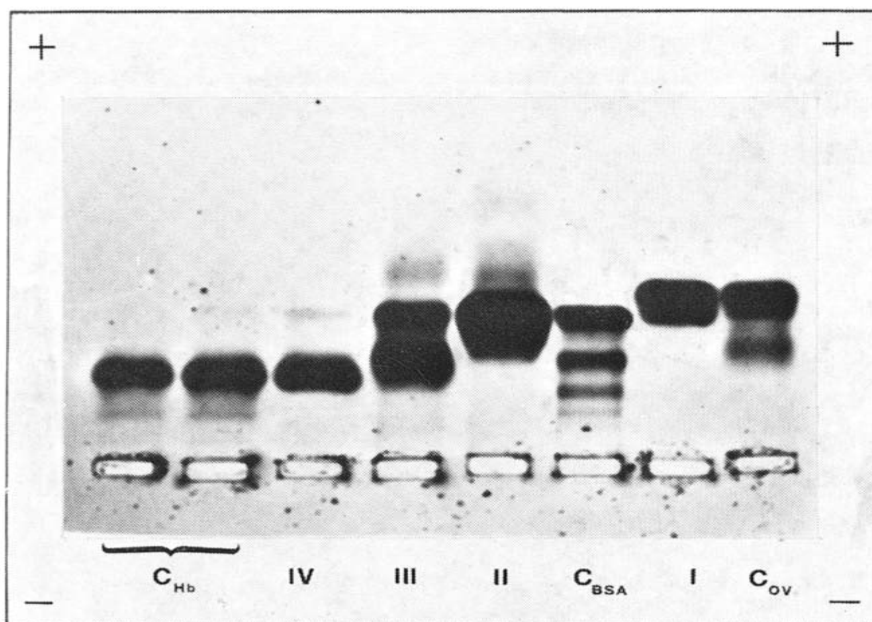


Fig. 6. Analytical PAGE of the shaded areas from Fig. 5 and controls. C_{OV} = unfractionated ovalbumin; C_{BSA} = BSA control; C_{Hb} = control, unfractionated haemoglobin in increasing amounts from left to right. The numbers represent the same peaks as in Fig. 5. The run was performed in a gel slab containing 4% acrylamide and 25 mM Tris–glycine buffer of pH 8.7. The electrophoresis was run for 3 h at 400 V and 25 mA. Approximately 40 μg of protein in 50 μl were applied for each sample. The shaded fractions from Fig. 5 were concentrated and then dialyzed against 2.5 mM Tris–glycine buffer of pH 8.7 before analytical PAGE. The gel was stained with Coomassie blue.

Much better resolution was obtained when the same protein mixture was run in a gel of the same bed height, but containing 7.5 % acrylamide. This time ovalbumin and BSA monomer and dimer were almost completely separated from each other (Fig. 7). Therefore, by adjusting the gel strength to the size of the proteins to be separated, one can often achieve a separation even better than that obtained by varying the gel bed length. As a general rule, when dealing with a very heterogeneous sample, the size and molecular weight of the component of interest will dictate the correct acrylamide concentration to be used in the gel matrix. Therefore, when

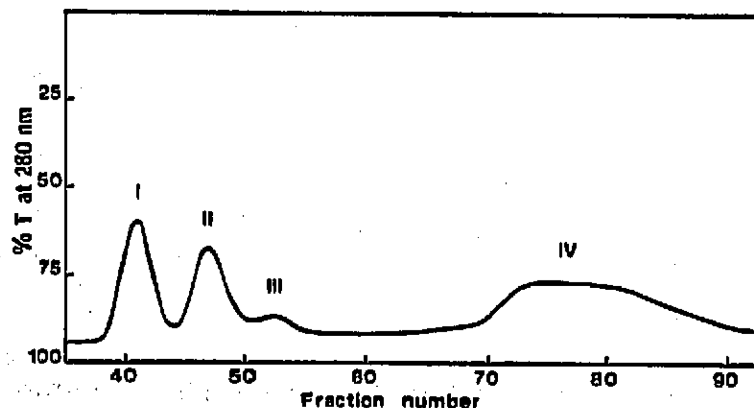
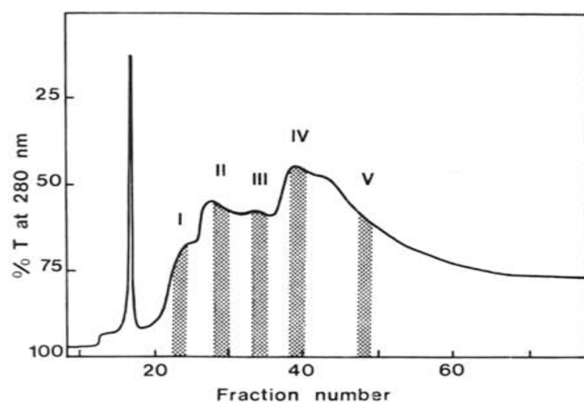


Fig. 7. Elution profile from the same protein mixture as in Fig. 5 applied to a 7.5% acrylamide gel, 10 cm high. The conditions were the same as in Fig. 5.

analyzing a commercial sample of immunoglobulins, we choose a 4% acrylamide with a 10 cm bed height. The elution pattern of this material (Fig. 8) shows at least five peaks, whose shaded areas were analyzed by analytical PAGE. As seen in Fig. 9, while the control, unfractionated sample, shows an almost continuous spectrum of bands, covering the entire length of the gel from the application point (which indicates a very complex mixture of proteins with several p*I*s and molecular weights), the Uniphor peaks can be seen to correspond to clearer and less heterogeneous regions of the starting material. Here the separation was complicated by the fact that the commercial sample was probably mixed with high-molecular-weight material, almost uncharged, which would slowly pack on the gel surface and form a gelatinous layer. This phenomenon causes severe disturbances in prep-PAGE because the gel pores become mechanically clogged. This effect caused the pronounced tailing and very high base line toward the end of the elution profile in Fig. 8.



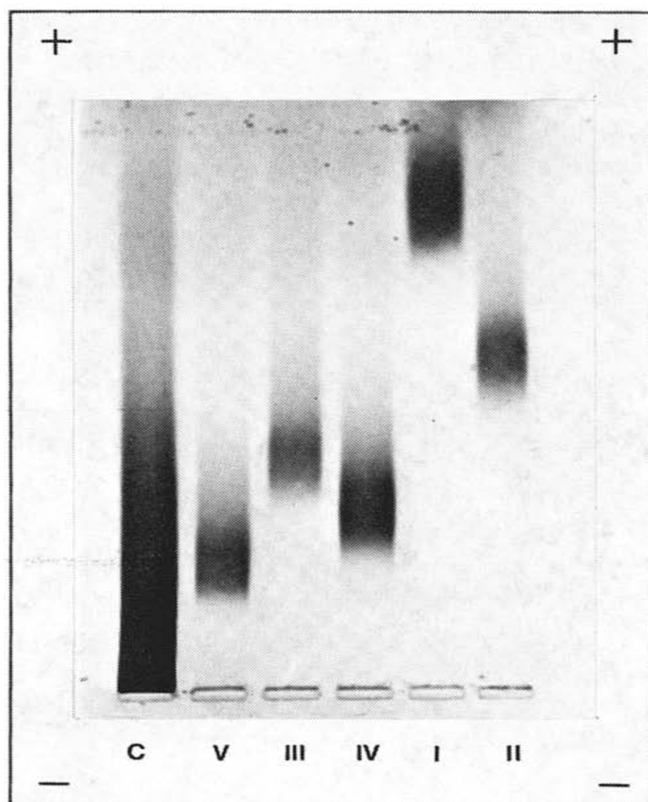


Fig. 8. Elution profile from 150 mg of commercial immunoglobulins (dissolved in 3 ml of 2.5 m*M* Tris–glycine of pH 9.5) applied to a 4% acrylamide gel, 10 cm high. Electrophoretic conditions, 800 V and 18 mA; elution buffer rate, 18 ml/h; recorder chart speed, 1 cm/h; fraction volume, 3.9 ml each tube.

Fig. 9. Analytical PAGE of the shaded areas from Fig. 8 and control. C = commercial unfractionated immunoglobulins. Lettering of fractions as in Fig. 8. All the other conditions were the same as in Fig. 6.

Recovery studies

Owing to the peculiar design of the elution stopper in the Uniphor column, we felt that it was important to check the recovery of the samples applied, to ascertain whether or not there were any losses in the electrode compartment. For

this purpose, we analyzed a series of protein standards, ranging from low to higher molecular weights. As seen in Table I, for all but cytochrome C the recovery was greater than 90 % of the amount applied. The low recovery for cytochrome C, however, was not due to its crossing the dialysis membrane in the elution stopper and consequent loss in the lower electrode chamber, but rather to smeared, reddish protein material that was not eluted from the gel bed (probably partially denatured or adsorbed cytochrome C). When the gel was homogenized and the coloured protein was extracted and added to the eluted band, the recovery was 95 % of the applied material. It is important to note that, because both cytochrome C and lysozyme are below their p*I*s in phosphate buffer at pH 6.0, the experiments with these two markers were carried out with the cathode in the lower compartment.

TABLE I

PROTEIN RECOVERIES FROM THE UNIPHOR COLUMN

All the samples were applied to a 6 cm high gel bed. BSA and ovalbumin were run in gels containing 5% acrylamide and 25 m*M* Tris–glycine buffer of pH 8.7. Electrophoretic conditions: 600 V and 18 mA. Lysozyme and cytochrome C were run in gels containing 6% acrylamide and 10 mM phosphate buffer of pH 6.0 (anode uppermost). Electrophoretic conditions: 400 V and 30 mA. These experiments were also performed in 10 cm high gel beds, with essentially the same recoveries. The lower recovery for cytochrome C is not due to loss of this protein in the elution stopper, but to part of the sample being denatured and forming a smear in the gel column.

Sample	*M.W. (daltons)*	*Amount applied (mg)*	*Amount eluted (mg)*	*Recovery (%)*
BSA	69000	20	18.44	97
Ovalbumin	43000	31.5	29.0	91
Lysozyme	14400	31.45	30.85	97
Cytochrome C	12300	32.50	26.0	80

DISCUSSION

There are several variables that must be controlled to optimize a preparative run in the PAGE system. As a general rule, it is always advisable to have a buffer of low ionic strength in the gel as well as in the electrode compartment; this allows a high potential difference to be created across the gel, with relatively low current, thus increasing the sample migration rate and minimizing diffusion phenomena due to longer runs. For a typical run, a 20 m*M* Tris–glycine buffer of pH 8.7 was found to be the most satisfactory; it allows potentials up to 800 V to be used, with a corresponding current of 15 mA. When using phosphate buffer, it is better to decrease the molarity even further (5 or 10 m*M*, compared with 20 m*M* for the Tris system).

As the Uniphor column is built to accomodate a solid gel cylinder, with cooling only on the outside, whereas in several other systems the cooling is also applied in the core of the gel by polymerizing a hollow gel cylinder, it is important not to apply power of more than 10–12 W to the column. At higher powers, the band profile becomes concave, as the protein at the centre of the gel, where the heat

is not rapidly dissipated, moves faster than the protein near the cooling jacket, and there is a risk of the separated zones mixing again during elution.

There is still controversy in the field of PAGE with regard to the use of "continuous" and "discontinuous" buffer systems. Actually, as pointed out by HJERTÉN *et al.*[43], a continuous system gives the same sharpening as the discontinuous one described by ORNSTEIN[44] and DAVIS[45] provided that the sample is applied in a buffer having a lower conductivity than the electrode and gel buffer. Therefore we routinely apply our material to the gel column in a buffer having one-tenth of the ionic strength of the electrode buffer; the conductivity jump thus created gives very sharp starting zones even when the sample is applied in a volume of 3–4 ml. When using this method, however, one must introduce the sample into the gel by applying a low potential, so as to avoid thermal convection in the sample zone, which might counterbalance the conductivity jump effect. As for the optimal gel bed height, we have found the range 5–10 cm to be satisfactory; it is not convenient to use gels longer than 10–12 cm, as the longer electrophoresis time will bring about a greater diffusion of the sample bands and will also adversely affect enzymes that are not very stable.

It is also very important to choose the correct protein load. In the Uniphor system, the gel has a surface area of approximately 5 cm^2, which allows loading up to a maximum of 50 mg per protein species. This upper limit has to be lowered significantly when closely related proteins, having very similar p*I*s or molecular weights or both, are to be separated. Because of this and because gram-scale preparative PAGE apparatus is not generally available, it is advisable, in a scheme for the purification of a protein, to carry out prep-PAGE as one of the last steps, when the amount of starting material has been considerably reduced.

Several workers have suggested very long pre-running times to free the gel from persulphate and UV-absorbing material. We usually do not pre-run the gel for more than 1 h, and we found that our gel columns give off very little impurity absorbing at 280 nm. This is due to the fact that we use high purity, recrystallized acrylamide and Bis. Reagent purification is very important in PAGE, as it gives gels with higher degree of polymerization and more reproducible pore size[30]. Particulate material in the sample to be applied should also be carefully avoided. Any insoluble component should be centrifuged and discarded, otherwise it will clog the gel surface and give the undesirable effects mentioned in the results section.

Finally, there is an over-all strategy to be used before carrying out pre-PAGE. It is advisable to perform first an analytical gel electrofocusing step so as to obtain an idea of the range of the p*I*s of the protein components of the mixture to be analyzed[42]. This allows optimization of the buffer pH for the protein of interest. It is also important to carry out analytical PAGE at various gel strengths, together with SDS-electrophoresis[46–48]; this will give an indication of the range of molecular weights of the proteins in the sample, and will permit optimization of the gel strength for the protein to be purified.

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