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Serum Protein Fractionation by Membrane Processes: Centrifugal Ultrafiltration, Osmosedimentation, and Multistage Ultrafiltration

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Abstract

Whole sera proteins may be fractionated according to their molecular weights by using three membrane techniques: centrifugal ultrafiltration, osmosedimentation, and multistage ultrafiltration. Ultrafiltration or dialysis cells were mounted in the swinging baskets of a centrifuge in all three cases, with the membranes aligned parallel to the centrifugation radius. As a result, solute accumulated over the membrane was convectively removed from its surface, thus preventing membrane polarization and fouling. During these experiments, smaller proteins migrated across the membrane, leaving behind the larger ones. Multistage filtration experiments were performed using cells fitted with three different membranes of successively narrower pores. Four different fractions were thus obtained and analyzed by gel permeation chromatography; separation factors as high as 2000 were obtained.

INTRODUCTION

The fractionation of protein solutes from a complex mixture is often necessary in the laboratory and in industrial processes.

Many techniques are currently used for this purpose; on a larger scale, fractional precipitation (*1*) (with ethanol, ammonium sulfate, etc.) and gel permeation chromatography are prevalent. Other techniques are available and used in the laboratory or plant: electrophoresis (*2*), electro-

decantation, adsorption and affinity chromatography, isoelectrofocusing, and ultracentrifugation (*3–5*).

Ultrafiltration seems to be ideally suited for protein–protein fractionation by size. Many pictorial descriptions of this technique show larger molecules being retained by the membrane while the smaller ones travel through the membrane pores. This simple and reasonable idea has met with little success in protein–protein fractionation.

Many authors have reported on their achievements and difficulties in fractionating proteins by ultrafiltration. DeFilippi and Goldsmith (*6*) mentioned the possibility of fractionating macrosolutes, but their examples refer to protein concentration, not fractionation. Van Oss and Bronson (*7*) showed that many protein–protein separations are feasible in a very small scale and in batch runs. However, they reported many problems. For example, albumin–globulin separation was obtained at higher but not at lower total protein concentrations. On the other hand, ethanol removal during serum albumin purification can be effectively done by ultrafiltration (*8*) as an alternative to gel filtration (*9*).

Accumulation of retained solute in the vicinity of a membrane during ultrafiltration is a major source of problems. This accumulation has been described by mathematical models (*10*, *11*), and it was experimentally determined (*12*, *13*) that solution layers as concentrated as ~60% were obtained during ultrafiltration of bovine serum albumin solutions.

Another important source of problems is the adsorptive interaction between membrane and protein solutes which causes membrane fouling (*14*) and is associated with membrane porosity reduction (*15*). Protein deposition kinetics on the membrane is relatively slow (6), causing flux decline in protein ultrafiltration. An attempt to alleviate these problems is the coupling of electrophoresis with ultrafiltration (*17*).

Mitra and Ng (*18*) recently reviewed the use of filtration and diafiltration in the plasma fractionation industry. From this work we conclude that membrane separation processes are very useful in concentration and microsolute removal steps during plasma fractionation. However, protein–protein separation according to protein sizes is not done by ultrafiltration. These authors conclude by stating that "... large-scale protein fractionation by ultrafiltration membranes has yet to fulfill its promise. Further improvements in membranes and hardware and in the management of fluid flow may help."

We have approached this problem by dealing with hardware and fluid flow management. In a recent work (*19*) we described a new type of device for centrifugal ultrafiltration in which the stagnant concentrated protein layer is effectively but gently removed from the vicinity of the membrane.

In this paper we describe the use of this device in whole sera fractionation. Results on serum fractionation by the osmosedimentation (*20, 21*) technique are also given.

EXPERIMENTAL

Ultrafiltration and osmosedimentation cells were built of acrylic, following Fig. 1(a). They contain two 17-mL compartments separated by a membrane supported on a perforated nickel sheet (1444 holes/cm^2, hole diameter 0.02 mm). Multistage ultrafiltration cells (Fig. 1b) have a similar construction except for two points: they contain four compartments, side by side, and the capacity of the first compartment is twice that of the others.

These cells fit the swinging buckets of a RC-3B Sorvall centrifuge; during a run the membranes are perpendicular to the centrifuge rotation axis. To start the osmosedimentation experiment, solution is placed in one cell compartment and solvent in the other. In the ultrafiltration experiments, solution is placed in the appropriate cell compartment.

Fetal and adult bovine sera were obtained from Cultilab (Campinas). Other reagents were analytical grade. Gel-permeation chromatography was performed using LKB fittings and columns, a Buchler peristaltic pump, and a Micronal B 382 UV-VIS spectrophotometer fitted with a Thomas 0.25 mL flow cell. Column gels were Sephacryl S-300 and S-400, obtained from Pharmacia. Gel beds were 79 cm high, 1.6 cm diameter. Eluent was 0.1 *M* aqueous NaCl. Calibration proteins were obtained from SIGMA (cytochrome C, trypsin, BSA, bovine gamma-globulin, and beta-amilase), and Blue dextran was from Pharmacia. In figures containing many chromatograms, vertical displacement was used for clarity.

Membranes

Cellulose acetate membranes were cast following previous work from this laboratory (*22, 23*). Two casting solutions were used. A: 11% cellulose acetate (Carlo Erba), 23% twice-distilled water, 43% acetone, 23% acetic acid. B: 11% cellulose acetate, 23% water, 66% acetic acid. Retention and permeability of the membranes obtained using these solutions vary with the solvent evaporation time (prior to coagulation): 5 and 10 min were used.

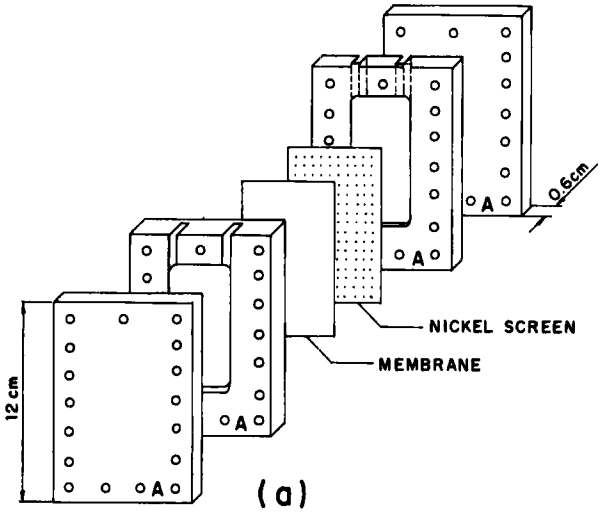


FIG. 1. (a) Centrifugal ultrafiltration cell. (b) Multistage ultrafiltration cell. A = acrylic sheet.

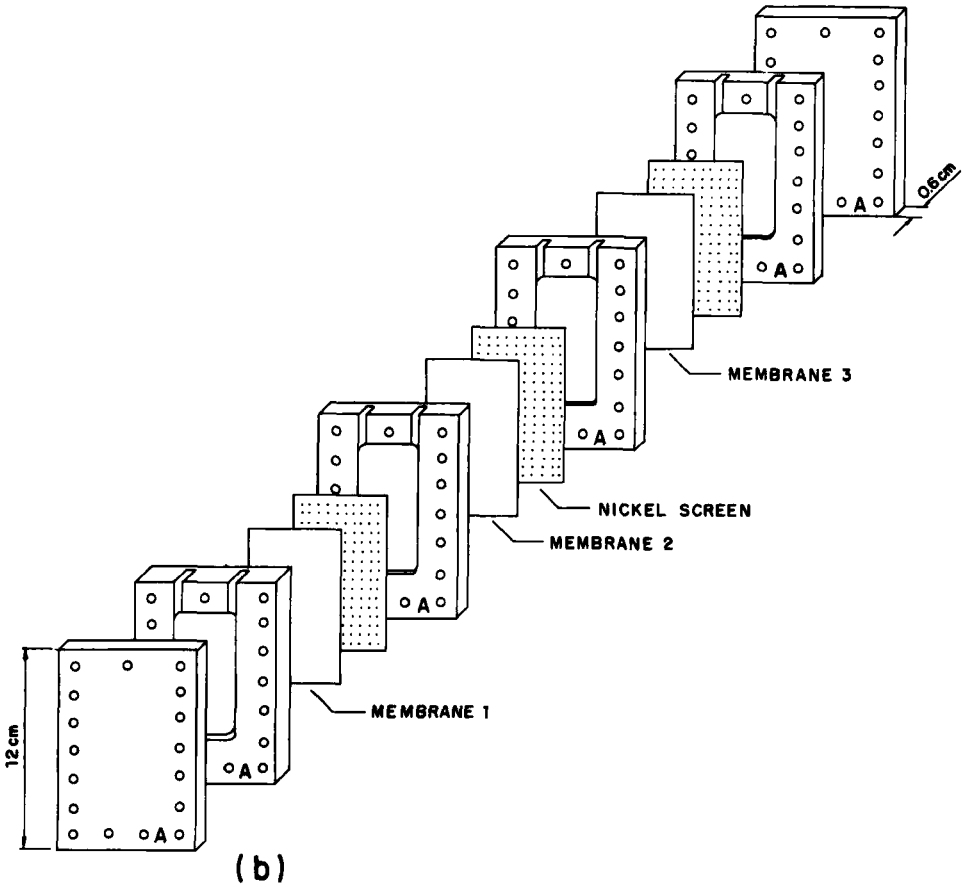
RESULTS

Centrifugal Ultrafiltration of Fetal Bovine Serum

Fetal bovine serum (Lot 1033-1) was diluted (3:10) with 0.1 *M* NaCl. 17.0 mL of this solution was centrifuged for 2 h at 3.000 rpm, 4°C, within a filtration cell such as that depicted in Fig. 1(a). After this time, 5.0 mL filtrate was obtained; the feed compartment was completed with 0.1 *M* NaCl aqueous solution and the cell was centrifuged again for the same time and conditions. An additional 5.1 mL filtrate was collected. The solution retained in the feed compartment was withdrawn and divided into six fractions from cell top to bottom.

Chromatograms of the feed solution, the two filtrates, and of the upper and lower fractions of the retentate are given in Fig. 2. From this figure we observe that the filtrates contain considerable concentrations of the low-MW components of serum but very little of the high-MW components. Moreover, the upper and lower retentate fractions show some differences: the top fraction is richer in the low-MW components than the lower fraction. Note that the lower fraction of the retentate shows a considerable increase in overall concentration over the upper fraction.

From these results we conclude that fetal bovine serum proteins can be



concentrated and fractionated at the same time by centrifugal ultrafiltration. Various types of cuts with different compositions can be obtained.

Osmocentrifugation of Fetal Bovine Serum

Fetal bovine serum (1033-2) was diluted in aqueous NaCl, as described in the previous section, and osmocentrifuged for 2 h, 3000 rpm, 4°C, in a cell such as that described in Fig. 1(a). Samples were taken from both cell compartments: one from the solvent compartment (after drawing and

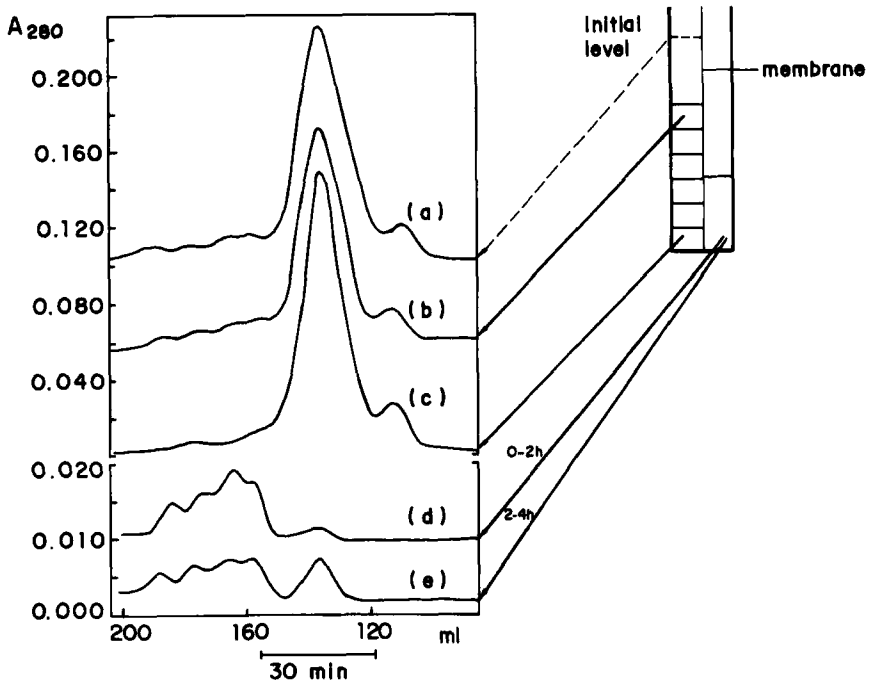


FIG. 2. Gel permeation chromatograms (S-400 Sephacryl) of filtrate and retentate of fetal bovine serum. Membrane cast from Solution A, 10 min evaporation. Centrifugal ultrafiltration conditions: 4 h at 3000 rpm, 4°C. Samples: (a) 1.0 mL serum (diluted 3:10 with water, made to 0.1 *M* NaCl); (b) 1.0 mL retentate upper fraction; (c) 0.5 mL retentate lower fraction; (d) 1.0 mL filtrate, withdrawn after the first 2 hours; (e) 1.0 mL filtrate, withdrawn after another 2 hours.

mixing its contents) and two from the solution feed compartment (top and bottom).

Data in Fig. 3 show that: 1) low-MW components migrate to the solvent compartment; 2) the material in the feed compartment is depleted of low-MW components; 3) the solution taken from the cell bottom is more concentrated than the feed solution.

Centrifugal Ultrafiltration of Bovine Serum

Bovine serum was fractionated by centrifugal ultrafiltration by using two different membranes of Types A and B. Experiments were analogous

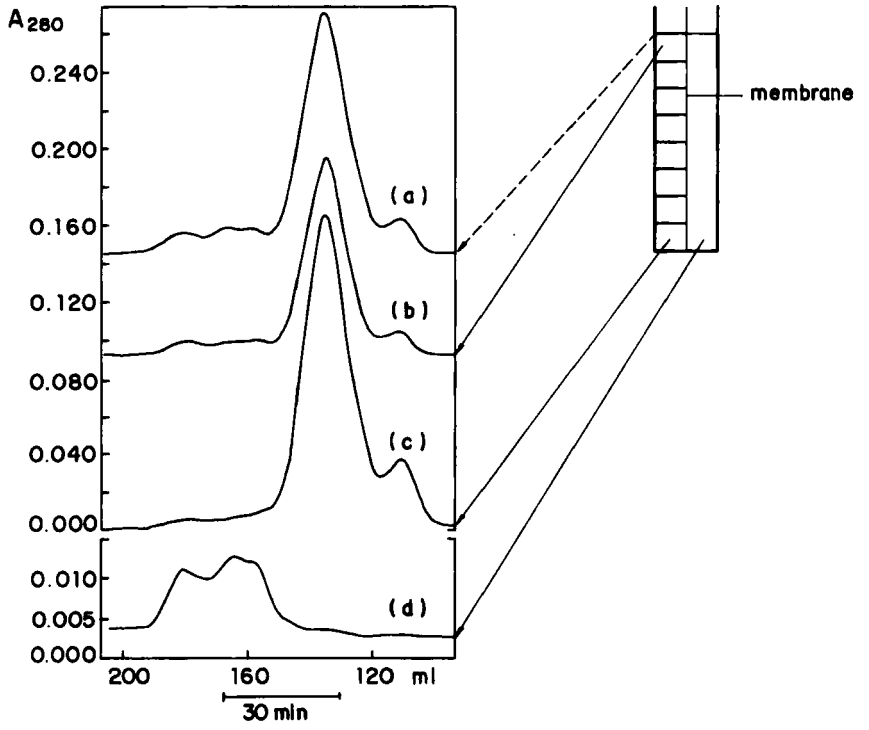


FIG. 3. Gel permeation chromatograms (S-400 Sephacryl) of filtrate and retentate of fetal bovine serum. Membrane as in Fig. 2. Osmosedimentation conditions: 2 h at 3000 rpm, 4°C. Samples: (a) 1.0 mL serum (as in Fig. 1); (b) 1.0 mL retentate upper fraction; (c) 0.7 mL retentate lower fraction; (d) 1.5 mL solution collected from the compartment initially filled with solvent.

to those described for fetal bovine serum in this work. The results given in Figs. 4 and 5 show that different degrees of fractionation are possible by using membranes of different characteristics.

Multistage Centrifugal Ultrafiltration

Bovine serum was also fractionated in a 4-compartment cell fitted with three different membranes and prepared by using Solution B (5 and 10 min drying) and Solution A (10 min drying). In this case the effluent of the first membrane is filtered through the second and third membranes.

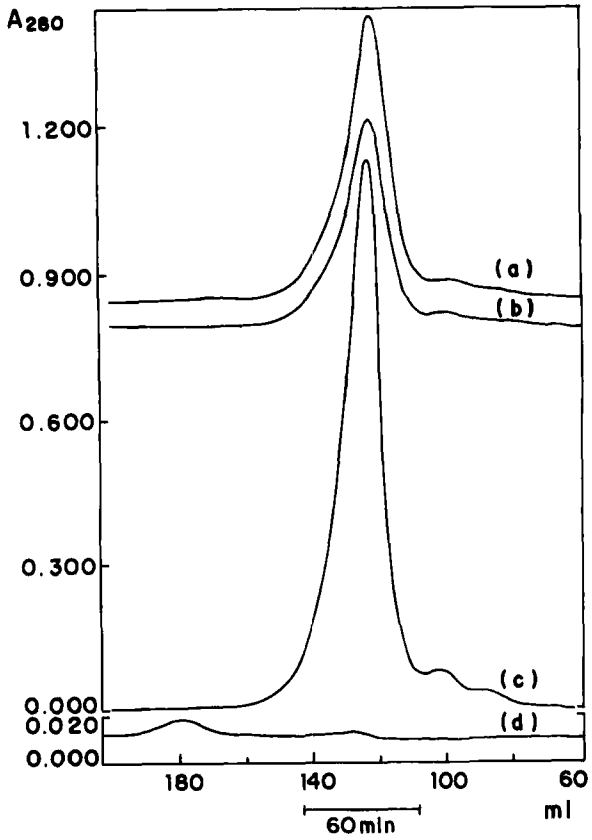


FIG. 4. Gel permeation chromatograms (S-400 Sephacryl) of filtrates and retentates of bovine serum. Membrane as in Fig. 2. Centrifugal ultrafiltration conditions: 3000 rpm, 4°C, 2 h. Serum diluted as in Fig. 1. Membrane type used: A. Samples: (a) 0.5 mL feed; (b) 0.35 mL retentate upper fraction; (c) 0.25 mL retentate lower fraction; (d) 1.0 mL filtrate.

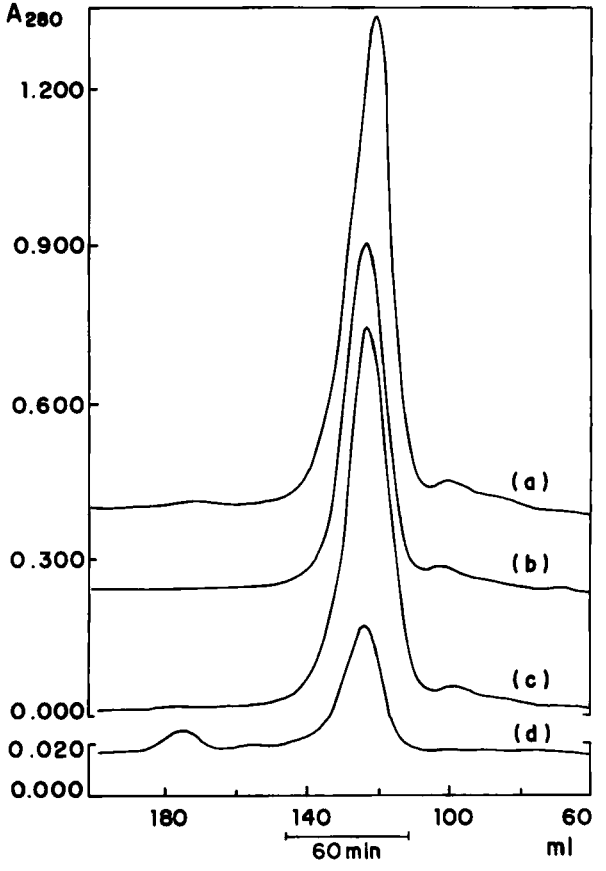


FIG. 5. The same as in Fig. 4 but using a membrane cast from Solution B. Samples: (a) 0.75 mL feed; (b) 0.75 mL retentate upper fraction; (c) 0.25 mL retentate lower fraction; (d) 1.0 mL filtrate.

Chromatograms of the feed solution, retentate, and the successive filtrates are given in Fig. 6.

Pertinent data relative to this experiment are presented in Table 1 which gives the mass balance and separation factors of this experiment.

Effect of Working Conditions on Separation Factors

To evaluate the effect of centrifugation speed on the protein/protein separation factors obtained by centrifugal ultrafiltration, 1:1 mixtures of bovine gamma-globulin and serum albumin were fed to ultrafiltration cells and spun for 0.5 h. The filtrate was removed and the cells were spun for another 0.5 h, after which a new filtrate was collected. Chromatograms of the filtrates are given in Fig. 7 together with the separation factors relative to the two main peaks (Table 2).

We observe that the separation factors depend on the centrifugation speed and that they increase with time in both runs.

DISCUSSION

Lower and higher MW proteins can be effectively separated by the three techniques used in this work. Multistage ultrafiltration results, summed up in Table 1, are quite interesting: they show that 26% of Component A can be obtained in a single experiment in 1 h, contami-

TABLE 1
Solute Recovery and Protein-Protein Separation Factors (SF) in Bovine Serum Multistage Ultrafiltration

Sample	Volume (cm^3)	% of solute component[a] A	B	C	D	SF_A^B	SF_B^C	SF_C^D
Feed	30.0	100	100	100	100	—	—	—
Retentate	12.8	~60	95.5	97.5	102	0.6	1.0	1.0
1st filtrate	4.8	11.4	0.6	0.5	0.4	19	1.2	1.3
2nd filtrate	6.2	12.8	0.1	—	—	130	>100	—
3rd filtrate	6.2	13.3	—	—	—	>2000	—	—

[a]Chromatogram peaks are labeled as in Fig. 6.

$$SF_j^i = (C_j/C_i)_{\substack{\text{filtrate,} \\ \text{retentate}}} / (C_j/C_i)_{\text{feed}}$$

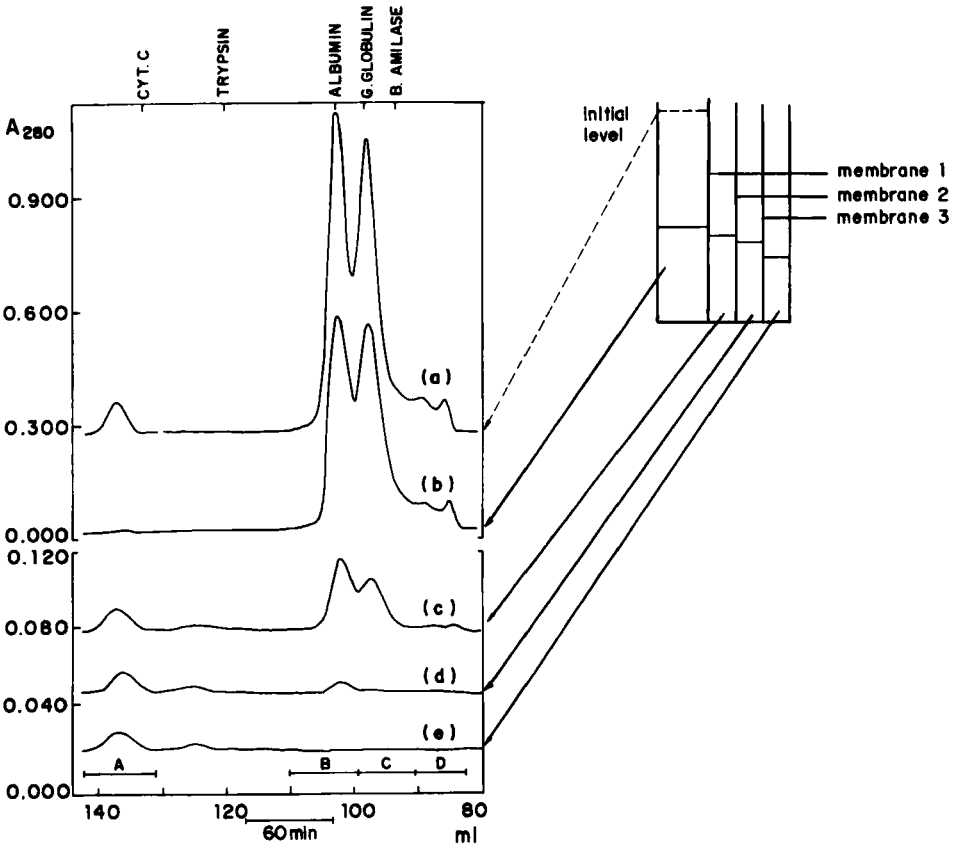


FIG. 6. Gel permeation chromatograms (S-300 Sephacryl) of bovine serum fractions, obtained by multistage ultrafiltration. Running conditions: 1 h, 3000 rpm, 4°C. Serum diluted as in Fig. 1. Samples: (a) 0.75 mL feed solution; (b) 0.5 mL retentate; (c) 1.0 mL of 1st filtrate; (d) 1.0 mL of 2nd filtrate; (e) 1.0 mL of 3rd filtrate. Membranes used: 1st and 2nd, Solution B, 5 and 10 min evaporation; 3rd, Solution A, 10 min evaporation.

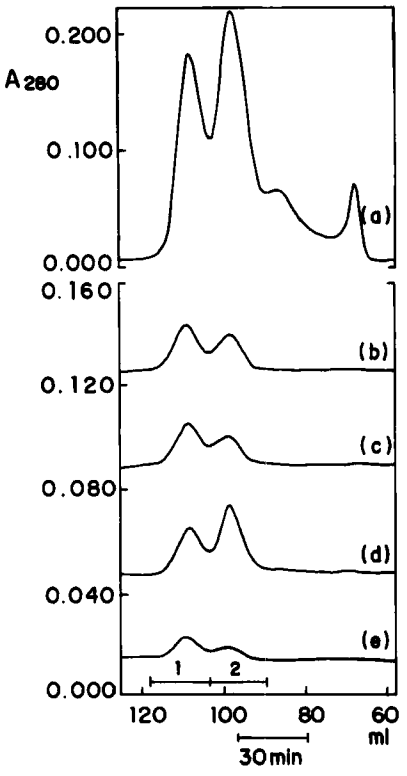


FIG. 7. Effect of centrifugation speed and time on albumin-gamma-globulin separation. (a) Chromatogram of a feed solution, 1:1 (w/w) albumin:gamma-globulin ratio, 0.5% total concentration; sample size, 1 ml. (b) Filtrate obtained after 0.5 h, 1000 rpm. (c) Filtrate after 1 h, 1000 rpm. (d) Filtrate after 0.5 h, 3000 rpm. (e) Filtrate after 1 h, 3000 rpm. Membrane as in Fig. 5.

nated with less than 0.1% of the amount of Component B found in the feed, and undetected amounts of Components C and D.

Useful separation factors have also been obtained for albumin-gamma-globulin fractionation which is a difficult separation.

We believe that further progress in protein-protein separation by porous membranes will depend on the availability of membranes containing well-defined pores in the relevant pore size ranges. McGregor (*24*) recently showed that statements regarding membrane "cuts," including those made by many commercial suppliers, have very little meaning. This matter has been irrelevant until now because the actual

TABLE 2
Albumin-Gamma-Globulin Separation Factors as a Function of Centrifugation Speed and Time

Running conditions (speed/time)	SF_1^2 [a]
1000 rpm/0.5 h	1.7
1000 rpm/1.0 h	2.0
3000 rpm/0.5 h	1.1
3000 rpm/1.0 h	2.4

[a] 1 = albumin, 2 = gamma-globulin.

selectivity of the membranes was much affected by the techniques in which they were used and by the extent of polarization and fouling.

In centrifugal ultrafiltration and osmocentrifugation experiments, lower MW components movement through the membrane is unimpaired by retained solutes. In these cases, separation could benefit from membranes having sharp cuts, i.e., a paucidisperse pore size distribution.

Last, but not least, we should stress that centrifugal ultrafiltration is the simplest conceivable route to multistage ultrafiltration: fairly high pressures (in our case, up to 10 bar) are reached in a self-regulated device; in these cells, pressure within each compartment increases with its load. A simple Plexiglas box within a centrifuge basket handles a job which would otherwise require many cells, pumps, recirculation loops, etc. Moreover, the feasibility of scaling up is clear, and work in this direction is currently in progress in this laboratory.

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