Protein Solutions: Concentration by a Rapid Method

Abstract. *Protein solutions were concentrated, with no evidence of denaturation, by means of membranes formed from the complex interaction product of polyanions and polycations (Diaplex). The technique is considerably more rapid than conventional ultrafiltration with Visking tubing.*

To date, rapid methods for concentration of proteins without denaturation have not been available. While affording enrichment, freeze-thaw methods of concentration are subject to quantitative loss. Flash evaporation, even at reduced temperatures, should be used only for stable materials, while lyophilization is not feasible for extremely dilute solutions. Those procedures which utilize membranes with size-limiting pores (ultrafiltration) are by far the most selective and are least injurious to protein solutes. Saravis has recently described an apparatus for positive-pressure ultrafiltration which is relatively easy to manipulate (*1*). Although ultrafiltration utilizing Visking tubing is practical for handling protein solutions, the relatively slow rate, the significant changes in pore size induced by pressure in unsupported membranes (*2*), and losses due to adsorption on the tubing present drawbacks.

Recently, membranes fabricated from a novel class of materials have been evaluated as nonporous diffusive barriers for carrying out biochemical separations. These membranes are hydrous gels of the complex interaction product of polyanions and polycations and are marketed under the name "Diaplex" (*3*).

Water is transported across the membranes by a process of true molecular diffusion, whereas hydraulic pore flow is observed in conventional ultrafilters. It has been postulated (*4*) that the surprisingly high ultrafiltration rates observed for Diaplex are a consequence of "alignment-type diffusion" similar to that observed in self-diffusion of water. Similarly, the rejection of larger molecular entities is merely a manifestation of kinetic limitation of transport of these solute species in and through the filter. Hence, the Diaplex ultrafilter operates as a true semipermeable membrane.

An additional advantage of membranes which operate as barriers for diffusive transport is their increased resistance to "plugging," or decrease in flow rate with time. Diaplex membranes can become "plugged" only when interfacial, concentration polarization of macrosolute produces a surface scum, or when buildup of micro-ions at the membrane-solution interface results in reduction of the osmotic driving force across the membrane. Accordingly, it was necessary to utilize an ultrafiltration cell which minimized this concentration polarization, thus insuring a high filtration rate (*5*). A schematic diagram of the apparatus is shown in Fig. 1. The stainless-steel device can hold 175 ml of fluid. The apparatus is connected to a nitrogen tank with polypropylene tubing (*6*). The cell is provided with a bleeder valve for control of pressure. The magnetic stirring bar barely clears the membrane during filtration, and, with adequate stirring, concentration polarization of the solute is minimized. The Diaplex membrane is supported by a sintered steel disk. Teflon or rubber gaskets, or both, are used to insure an adequate seal; the apparatus is closed with spanner wrenches (no lubricants of any sort are used). Filtrate is collected from a side port; hence the cell can be mounted on a magnetic stirrer. No antibacterial agents were needed during the period (2 to 4 weeks) the membrane was left in the cell. The membrane must be kept wet at all times to prevent stress cracking, which would result from anisotropic drying.

To assess the efficacy of Diaplex ultrafiltration as a protein concentration technique, human serum, 2-percent stock solutions of human Cohn fractions III and IV-1, and 3-percent fraction V, were prepared and diluted with phosphate-saline, *p*H 7.4, to the concentrations shown in Table 1. Duplicate samples were prepared for each assay level, so that comparison could be made with results obtained by conventional

Table 1. Concentration of protein solutions by Diaplex (DUF) and conventional ultrafiltration (CUF).

Conc. (mg/ml)	Initial vol. (ml)	Filtration rate (10^{-2} ml min^{-1} cm^{-2})		Final vol. * (ml)		Protein recovery (%)	
		DUF †	CUF ‡	DUF	CUF	DUF	CUF
			Human serum				
1.01	300	6.9		9.0		90.5	
1.51	200	6.9		15.4		91.2	
2.42	250	6.4	0.22	10.5	9.0	95.9	87.6
3.03	100	5.9		13.0		92.4	
			Human serum, fraction III				
0.62	250	5.4	0.14	16.5	15.0	91.2	75.7
			Human serum, fraction IV-1				
0.57	250	5.4	0.16	12.5	15.0	83.5	77.4
			Human serum, fraction V				
1.01	250	10.9	0.22	10.5	15.0	90.5	91.9
			Phosphate-saline buffer				
0.15*M*	250	11.8		17.3			
			Distilled water				
	250	16.3	0.62	16.0			

* Final volume in all cases includes a chamber rinse. † Pressure used in the DUF cell was 7 atm, and the effective surface area was 20.2 cm^2. ‡ Rate given for CUF is based on a full casing (140 ml); when fluid level falls, the rate drops as a function of the exchange area. Pressure applied was 4.1 atm for all runs save water, where 6.8 atm was used. The area of exchangeable surface was 243 cm^2.

Table 2. Distribution, in electrophoresis, of starting material and ultrafiltration concentrates. The values listed represent the percentage distribution of each of the electrophoretic components as determined by densitometric evaluation of electrophoretograms stained with Ponceau S.

Electrophoresis fraction	Human serum			Fraction III			Fraction IV-1		
	Starting material	DUF	CUF	Starting material	DUF	CUF	Starting material	DUF	CUF
Albumin	66	64	69				18	18	20
Alpha-1-globulin	3	3	3	11	10	10	82	82	80
Alpha-2-globulin	8	8	8	26	30	28			
Beta-globulin	10	12	7	63	60	62			
Gamma globulin	13	13	12						

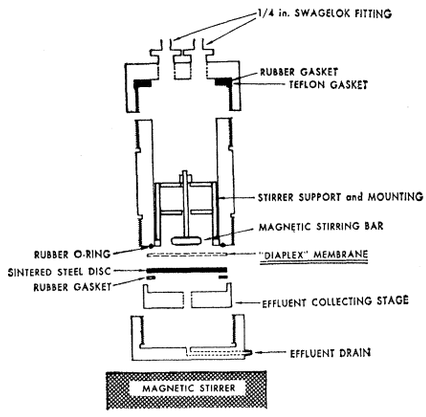

Fig. 1. Vertical cross section of the Diaplex membrane filtration apparatus.

ultrafiltration with the Saravis apparatus. In both cases, small buffer rinses were employed at the conclusion of a run to wash residual protein from the chambers. In the Diaplex study, a pressure drop of 6.8 atm (100 lb/in.^{2}) was imposed across the membrane. For conventional ultrafiltration procedures with protein solutions 4.1 atm was used, while 6.8 atm was used to establish the water filtration rate. Whereas conventional ultrafiltration was carried at 4°C, the more rapid Diaplex method permitted concentration at room temperature. No attempt was made during these studies to evaluate the effects of temperature on the filtration rate.

The results of the study are summarized in Table 1. Protein levels were determined by the biuret technique (*7*) and confirmed by micro-Kjeldahl analysis. Rates obtained with Diaplex considerably exceeded those obtained with conventional dialysis tubing. Flux rates normalized for pressure drop are approximately 18-fold higher for Diaplex ultrafiltration than for cellophane ultrafiltration. The average recovery of protein was higher with Diaplex. The rate characteristics of the Diaplex ultrafiltration method are shown in Fig. 2. The same filter was used for all studies, and the series with phosphate-saline and distilled water were run after the studies of protein had been completed. The average salt content of the Diaplex concentrates, as reflected by the conductivity, was found to be 22 millimhos, somewhat higher than the average value of 15 millimhos found for both filtrates and starting solutions. This difference may reflect some rejection of the microsolute by the membrane. Initially, the rates appear to be linear, but decrease is noted near the end of each run, probably due to concentration polarization of the solute. As both Fig. 2 and Table 1 indicate, the type of protein rather than the absolute concentration appears to exert a greater rate-limiting effect on the flux rate. To evaluate any denaturation that might result from these techniques, the starting material and the concentrates were examined by electrophoresis and immunoelectrophoresis on cellulose acetate (*8*). The electrophoretic profiles shown in Table 2 confirm the lack of marked changes in either technique, whereas the representative immunoelectrophoretograms of human serum shown in Fig. 3 substantiate the absence of significant alterations demonstrable by this assay.

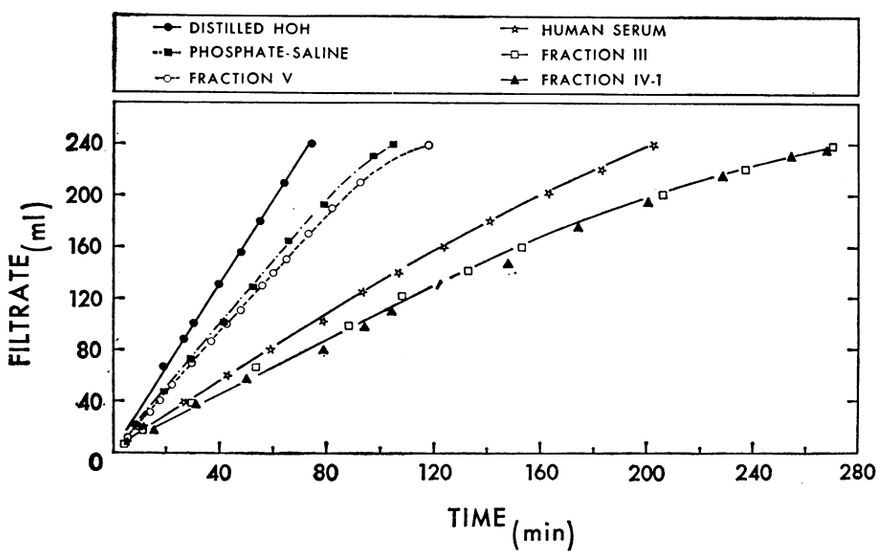

Fig. 2. Filtration rates of various solutions through the same Diaplex membrane. Filtration order: fraction V, fraction IV-1, human serum, fraction III, phosphate-saline buffer, and distilled water. Pressure used was 6.8 atm and the effective surface area was 20.2 cm^2.

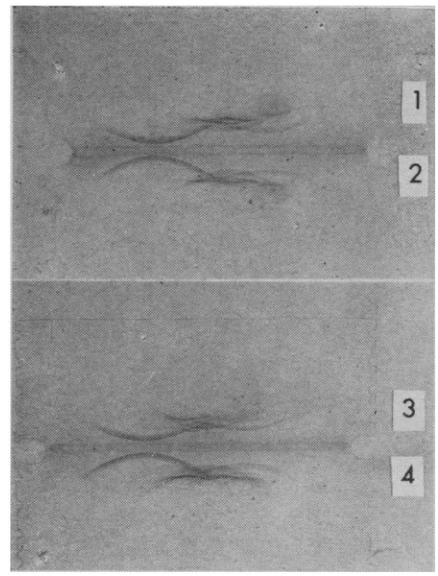

Fig. 3. Immunoelectrophoresis of human serum before and after concentration by ultrafiltration: *1*, Diaplex concentrate; *2*, starting material; *3*, conventional ultrafiltrate; *4*, starting material.

The Diaplex membranes can be made in forms tailored to the specific ultrafiltration task. Where high flux rate is a requisite, anisotropic membranes composed of a dense, permselective skin supported by a spongy, highly stable, expanded phase are used. The skin (upstream surface) properties can be formulated to produce ultrafilters capable of screening solutes of varying molecular weights; membranes are available which will effectively reject microsolutes as well as macromolecular solutes. As one might expect, membranes which are suitable for screening microsolutes are inherently less permeable to water than the macromolecular ultrafilters. The anisotropic membranes we used were devised to retain microsolutes of molecular weight greater than 500.

Our method for concentrating proteins from biological fluids seems to be reliable, safe, and extremely rapid.

WILLIAM F. BLATT
MICHAEL P. FEINBERG
HAROLD B. HOPFENBERG
U.S. Army Natick Laboratories, Natick, Massachusetts
CALVIN A. SARAVIS
Protein Foundation, Jamaica Plain, Massachusetts

References and Notes

1. Marketed by National Instrument Laboratories, Bethesda, Md., as the N.I.L.–Saravis ultrafiltration apparatus. The unit used in these studies was 2.5 cm (inside diameter) by 30 cm; it held 140 ml of fluid. The total exchange area was 243 cm^2.
2. L. Craig and W. Konigsberg, *J. Phys. Chem.* **65**, 166 (1961).
3. These membranes were developed by and are available from the Amicon Corp., Cambridge, Mass.
4. A. Michaels, *Ind. Eng. Chem.*, in press.
5. The filtration unit was built to specifications supplied by Amicon.
6. Polypropylene tubing and fittings (Imperial Eastman) were obtained from A. E. Borden Co., Boston, Mass.
7. J. Reinhold, in *Standard Methods of Clinical Chemistry*, M. Reiner, Ed. (Academic Press, New York, 1953), vol. 1, p. 88.
8. M. Feinberg, L. Mann, Jr., W. Blatt, *Am. J. Clin. Pathol.*, in press.

28 June 1965

Hemolysin of Mycoplasma pneumoniae: Tentative Identification as a Peroxide

Abstract. Mycoplasma pneumoniae *produces a soluble hemolysin active against guinea pig erythrocytes. This hemolysin appears to be a peroxide, since catalase or peroxidase inhibits its activity. The action of catalase and peroxidase is specific, since heating the enzymes abolishes their effect on the hemolysin. In addition, 3-amino-1,2,4-triazole, a potent inhibitor of catalase, reverses the inhibitory effect of the enzyme. The hemolysin of* M. laidlawii *is also a peroxide. The hemolysins of* M. pneumoniae *and* M. laidlawii *seem unique for microbial organisms since the bacterial hemolysins which have been described have been protein or lipid in nature.*

Colonies of *Mycoplasma pneumoniae* and *M. laidlawii* produce a soluble hemolysin active against guinea pig and sheep erythrocytes (*1*). When an agar culture containing mycoplasma colonies is overlaid with agar containing red blood cells, each colony releases sufficient hemolysin to produce a circular area of complete or almost complete red cell lysis. We call these areas hemolytic plaques. Plaques also develop when horse, human, or rabbit erythrocytes are used, but with these cells hemolysis is often incomplete. We have speculated that the antibodies to red cells, which develop in some cases of primary atypical pneumonia (cold agglutinins) may be related to the action of the *M. pneumoniae* hemolysin (*2*).

The hemolysin of *M. pneumoniae* is extremely labile, because hemolysis does not occur when a mixture of red cells and agar is added to solid medium on which mycoplasma colonies had been grown and then removed. Omission of yeast extract from the mycoplasma growth medium, or incubation under reduced oxygen tension inhibits hemolysin production (*3*). That hemolysin passes through a viscose dialysis membrane indicates its low molecular weight and probable nonprotein nature.

We studied the nature of the hemolysin by incorporating specific enzymes in the erythrocyte-agar overlay and determined which enzymes inhibited lysis. Plastic dishes containing 5 to 6 ml of mycoplasma agar medium (*4*) were inoculated with a broth suspension of *M. pneumoniae*. The inoculum produced approximately 100 to 300 colonies after 7 to 10 days of aerobic incubation. In the first experiment, the addition of 12,000 units of catalase (*5*) into the erythrocyte-agar overlay completely suppressed the development of hemolytic plaques (Fig. 1). Plaque production could be prevented with 400, but not with 40, catalase units. The addition of boiled catalase did not affect plaque production. These observations indicated that peroxide was involved in the hemolytic reaction.

The incorporation of catalase directly into the overlay did not reduce the viability of the mycoplasma colonies. The contents of *M. pneumoniae* agar cultures overlaid with red cell-agar containing added catalase or boiled catalase were ground into a broth suspension (20 percent) which was then titrated for viable organisms. Decimal dilutions of the suspensions were inoculated onto mycoplasma agar medium. Cultures overlaid with either catalase or boiled catalase contained 2×10^9 colony-forming units.

Peroxide could produce hemolysis either by direct action on the red cells, or indirectly by reacting with constituents of the erythrocyte-agar medium to release hemolysin or induce its production. To determine whether the hemolysin itself was a peroxide, various enzyme preparations were enclosed in a double-dialysis membrane placed over the colonies. The hemolysin could diffuse through a dialysis membrane and still lyse guinea pig erythrocytes placed over the membrane (*3*). Viscose tubing (Visking brand, 44-mm flat width, 0.0254 mm thickness) was cut to fit the agar surface and moistened with Alsever's solution. Approximately 0.15 ml of various enzyme solutions, including catalase (30,000 unit/ml) and trypsin (25 mg/ml) were inserted between the layers of the dialysis membrane. To prevent bacterial contamination, 150 units of penicillin was added to each enzyme preparation. The solution was dispersed throughout the dialysis "bag" by exerting finger pressure. The dialysis bag was placed over the colony-containing agar surface, and the plates were then overlaid with 3 ml of a guinea pig erythrocyte and agar mixture. The catalase, acting as a barrier between the colonies on the agar surface and the erythrocyte in the agar overlay, completely suppressed hemolysis (Fig. 2). However, plaques of hemolyzed cells were observed in areas not covered by the membrane, particularly at the periphery of the plate. When trypsin or boiled catalase was placed in the dialysis bag, hemolytic plaques developed throughout the overlay. These findings indicate that the *M. pneumoniae* hemolysin is a peroxide.

The specificity of the catalase effect

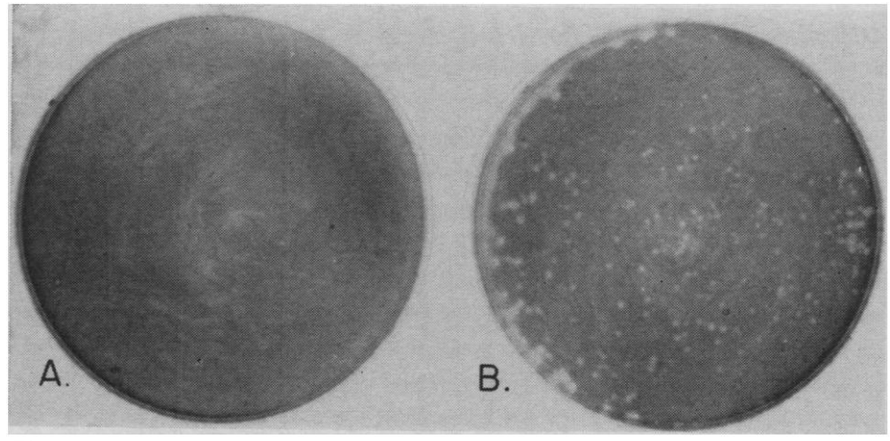

Fig. 1. Effect of catalase on development of *M. pneumoniae* hemolytic plaques. (*A*) 12,000 units of catalase added to guinea pig erythrocyte–agar overlay. (*B*) Catalase, boiled for 30 minutes, added to overlay.