Membrane Partition Chromatography: A Tool for Fractionation of Protein Mixtures

William F. Blatt

The availability of protein-retentive membranes with varying degrees of selectivity has permitted extension of ultrafiltrative techniques to the partition of simple as well as complex mixtures of macrosolute. However, attendant to all high flux ultrafiltration is the problem of concentration polarization, *i.e.*, accumulation of macrosolute at the membrane interface which, if left undisturbed, can act as a secondary membrane limiting flux as well as solute transport. Accordingly, management of this phenomenon is equally as important as the selection of the limiting membrane in applying the technique to fractionation. The evolution of ultrafiltrative procedures as well as recognition, theory, and control of macrosolute polarization are discussed initially; what follows are examples of practical systems for partition of binary and more complex mixtures using single cell and tandem systems.

With the introduction of membranes of graded porosity, ultrafiltration has been extended from its initial use as a rapid and efficient means of protein concentration (Blatt *et al.*, 1965; Pollak *et al.*, 1968) to further application in the larger field of macrosolute fractionation (Baker, 1969; Blatt *et al.*, 1967a; Lewis *et al.*, 1969; Zipilivan *et al.*, 1969). To function as efficient partition media, membranes should possess high degrees of selectivity (*i.e.*, well-structured pores in a narrow distribution range) and ultrafiltration rates should be of sufficient magnitude for reasonable separation times. Unfortunately, the membrane art to date is unable to produce membranes with ultra-sharp retentivity; and as we shall see later, the cut-off spectra are often even more diffuse under actual use. Typical views of the operational aspects of sharp *vs.* diffuse cut-off membranes are shown in Figure 1 (Michaels, 1968). In this figure the retention (the rejection coefficient) ($\sigma = 1 - C_f/C_b$ where C_f and C_b refer to solute concentrations in the filtrate and bulk solutions, respectively) is shown as a function of the molecular weight. Total membrane exclusion is 1, whereas 0 represents complete permeation of solute. It is recognized that the convenience of grading membranes on the basis of molecular weight cut-off is extremely arbitrary; retentivity based on molecular dimensions is far more applicable. With respect to the problems of membrane retentivity, nonuniform pore distribution is a major factor in diffusiveness. However, quite aside from this inherent difficulty, the separation of material varying in the geometry or in deformation during operating conditions can also preclude high orders of selectivity.

Applications Laboratory, Scientific Systems Division, Amicon Corp., Lexington, Mass. 02173

The examples cited above are associated with the membrane and/or solutes and, for the most part, are out of the operator's control. However, one of the greatest impediments to the use of membrane partition is concentration polarization, the accumulation of macrosolute at the membrane surface that both restricts flow and alters membrane selectivity. This factor is at least partially within the control of the operator, and the recent availability of equipment designed to minimize the consequences of this phenomenon resurrects the early promises of this method of separation. It is not our intent to go into the polarization problem in any depth. This has been dealt with adequately elsewhere (Blatt *et al.*, 1970). However, it would be impossible to discuss any separation techniques without some appreciation of this process.

CONCENTRATION POLARIZATION

Membrane polarization by macrosolute is, in its simplest terms, a concentration profile with solute content highest at the membrane surface and decaying to the bulk concentration at some distance from the membrane. Even in a well-mixed system this profile exists; if left undisturbed, the layer of concentrated solute at the membrane may consolidate into a secondary membrane completely obliterating the separatory function of the original ultrafilter.

Concentration polarization is multi-variable, both in cause and consequence. Solute concentration (a logarithmic relationship exists between ultrafiltrate flux and concentration), temperature (presumably operative through solution viscosity), as well as system geometry, notably contribute to this phenomenon. While most of the observations to date have related to solvent flux, they are equally applicable to solute transport.

An attempt to interrelate these factors is shown in Figure 2.

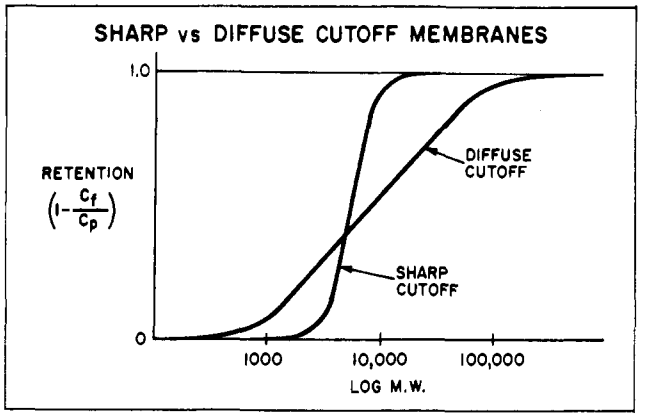

Figure 1. Solute retentivity in sharp and diffuse cut-off ultrafiltration membranes (Michaels, 1968). Reprinted with permission of: *Progress in Separation and Purification*, Vol. I, p 297, Wiley, New York, 1968

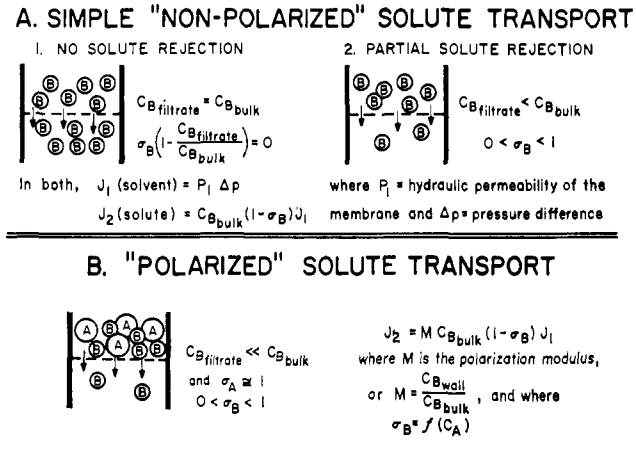

Figure 2. Solvent and solute transport in idealized nonpolarized and polarized ultrafiltration systems

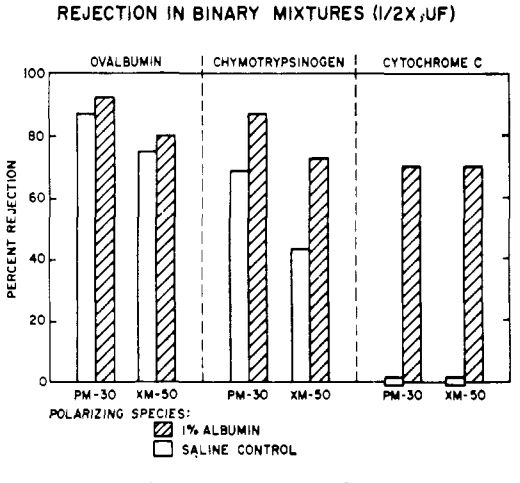

Figure 3. Rejection of membrane-permeable solutes in binary mixtures containing membrane-retentive macrosolute. Diaflo PM30 and XM50 membranes show single solute retentivity of 30,000 and 50,000 molecular weight, respectively

Membranes displaying retention for macromolecules only (of pore diameters >10 Å) appear to function as molecular sieves or screens. As seen at the top, solute transport in membranes showing no rejection for the species is a simple function of the solvent flow. With varying degrees of retention, the equations are modified to account for the fractional rejection. When a second highly-retentive species is

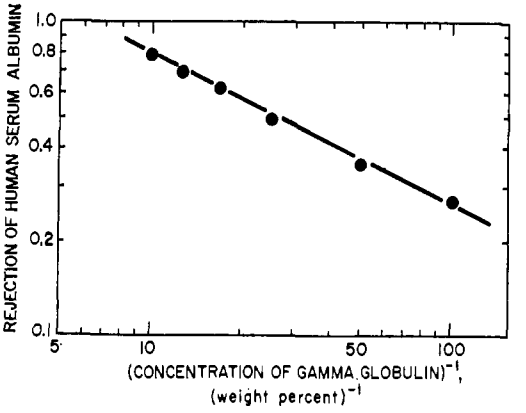

Figure 4. The rejection of human serum albumin by a Diaflo XM100 membrane as a function of varying gamma globulin content (stirred-cell system) (Blatt *et al.*, 1970). Reprinted with permission of: *Membrane Science and Technology*, p 47, Plenum, New York, 1970

introduced into the system, the rejection coefficient of B is elevated and the transport relationship must be further modified to account for the retention of B by the polarization boundary layer of component A at the membrane surface. Some examples that illustrate this observation follow.

Figure 3 denotes varying solute transport in different Diaflo membrane systems (anisotropic ultrafiltration membranes formed from synthetic polymers and distributed by the Amicon Corp., Lexington, Mass.) in the absence and then the presence of membrane-retained albumin. In all systems increased rejection of the permeable species was obtained on admixture with the retained species. Our studies thus far indicate that this inhibited transport is observed with macrosolutes to about 5000 molecular weight. Below that level little impedance is observed.

Further attesting to reduced transport even at extremely low concentrations of retained solute is the data of Figure 4. Employing a high cut-off membrane, varying amounts of 7S

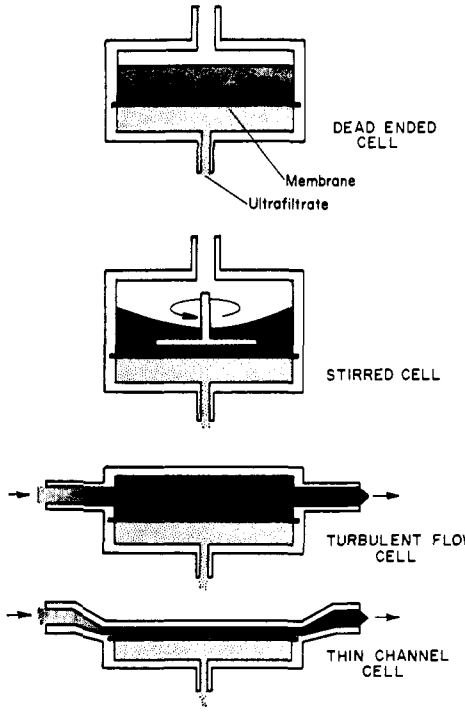

Figure 5. A comparative view of basic ultrafiltration systems

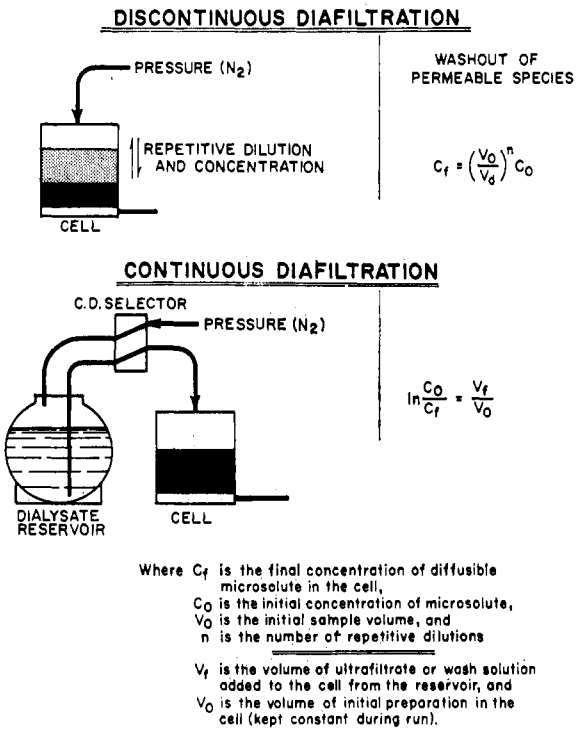

Figure 6. Washout of membrane permeable species by the diafiltration technique in both continuous and discontinuous modes

globulin sharply decreased the net transport of albumin. The reason for the relationship

$$\sigma_{alb} = 2.4\sqrt{\text{Conc. } \gamma\text{-globulin}}$$

is not known; similar relationships, with varying exponential values, appear to exist in other systems we have examined.

POLARIZATION CONTROL

Clearly then, membrane selection is but a part of any partition regimen. Selection of systems to optimize membrane performance is probably more important. Ultrafiltration systems generally fall into the categories shown in Figure 5.

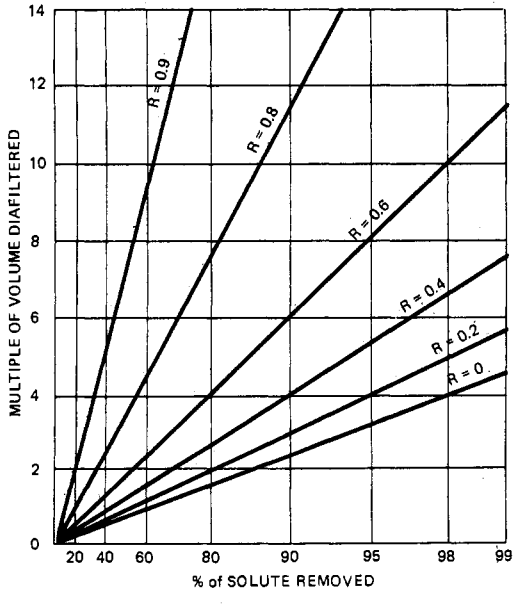

Figure 7. Removal of membrane-permeable species with varying rejection coefficients by the continuous diafiltration technique

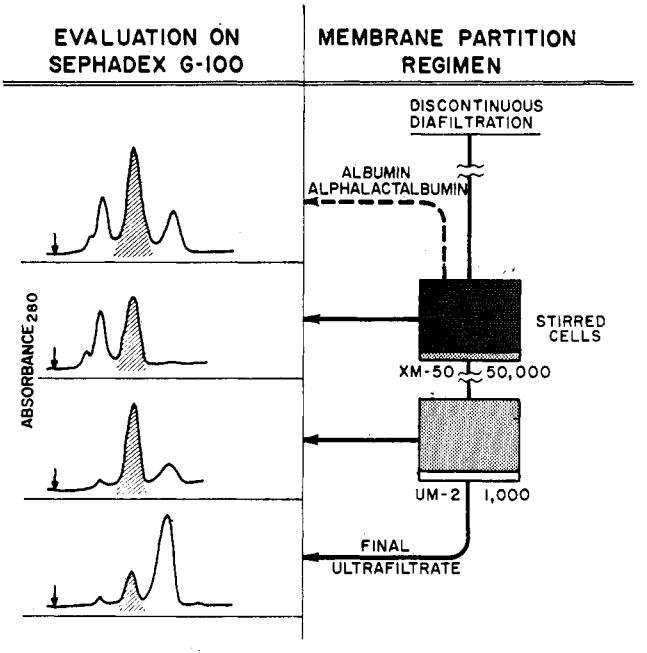

Figure 8. The partial purification of alphalactalbumin admixed with human serum albumin by membrane partition. Redrawn from data of Blatt *et al.*, 1967b

Insofar as they make no attempt at polarization control, dead-ended systems are useless for other than concentration of very dilute solutions of macrosolute. Stirring at or near the membrane surface affords a measure of turbulence that decreases the polarization boundary layer; but both turbulent flow and laminar flow in thin channels (<50 mil) at high velocity across the membrane surface affords the greatest reduction in concentration polarization, thus maximizing flux and solute transport.

Solute Extraction: The Diafiltration Technique. Generally, the simple filtration of a solution of mixed solute, for other than microsolute or salt removal, is not sufficient for adequate partition. Furthermore, as we have shown, the rejection coefficients are elevated in mixed systems to values whereby only fractional solute extraction is observed in the filtrate. Accordingly, a diafiltrative, or washout technique, must be employed whereby fresh solvent is added to the bulk solution (Blatt *et al.*, 1968a). Figure 6 illustrates two ways for accomplishing this. The first, or discontinuous method, simply involves repetitive dilution and concentration; the

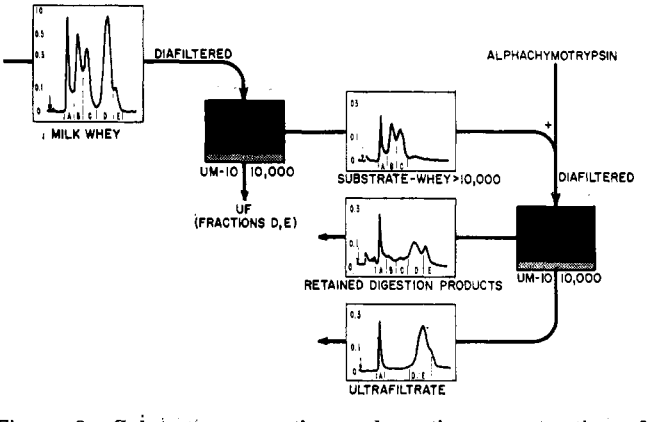

Figure 9. Substrate preparation and continuous extraction of proteolytic products, as formed, from bovine milk whey admixed with alphachymotrypsin. Redrawn from data of Blatt *et al.*, 1968b

Table I. A Comparison of Membrane-Permeable Solute Extraction in Stirred-Cell and Thin-Channel Systems (5× Diafiltration)

Solute separation Solutes	Membrane	Nominal cut-off value[a]	Total mg in ultrafiltrate Thin-channel	 Stirred-cell	Ratio[b] (TC/SC)
1% mixed globulin	XM300	300,000	93	25	3.7
0.9% bovine serum solids	XM300	300,000	223	122	1.8
1% Albumin–Cytochrome C	PM30	30,000	118	38	3.1
1% 7S globulin-alphachymotrypsin	XM50	50,000	123	33	3.7
2.5% milk whey[c]	PM30	30,000	815	662	1.2

Total diafiltration time Solutes	Time in minutes Thin-channel	 Stirred-cell	Ratio (TC/SC)
Serum	109	340	0.32
Whey[c]	97	377	0.26

[a] Nominal cut-off established by single solute evaluation. [b] TC and SC refer to thin-channel and stirred-cell, respectively. [c] A 10× diafiltration was used for the whey solutions.

second, a continuous system employing a reservoir and valve, permits fixed volume solvent replacement (the solvent replacement volume in the dual pressurized system matches the ultrafiltration rate). The increased efficiency of the second procedure can be readily gleaned from the mathematical expressions for washout. In the first case, removal of 99% of a nonrejected solute from a 5-ml sample would require 90 ml of filtrate; in continuous diafiltration, 23 ml of throughput would accomplish the same reduction. At comparable filtration rates, the time reduction is obvious as well as the lessened requirement for secondary concentration of the extracted material. For the more usual situations in solute partition ($\sigma_B > 0$), Figure 7 is a convenient method of ascertaining the necessary throughputs for the extraction of solutes displaying varying degrees of rejection in the system.

STIRRED-CELL *vs.* THIN-CHANNEL OPERATION. Using this diafiltrative technique, the heightened efficiency of solute extraction in thin-channel (tc) *vs.* stirred-cell systems becomes evident (Table I). For the most part, nearly four-fold increases in solute permeation were obtained with low velocity tc systems. The exceptions were serum and whey. As we shall see later, the macrosolute content of serum is such that polarization control for solute extraction is marginal for low velocity tc systems. With respect to whey, nearly 50% of whey is comprised of solutes below 5000 molecular weight; and, as we have noted, their transport is not inhibited by membrane polarization. Moreover, with respect to filtrate flux, the superiority of tc operation is clearly obvious (lower half of Table I). Whenever possible, the overall effect of increased solvent flux with an augmented solute transport obtained with thin-channel systems strongly recommends their use.

OPERATIONAL VARIABLES. Use of thin-channel equipment alone does not maximize fractionation efficiency performance. In general, low operating pressures, reduced macrosolute content, elevation in temperature (where possible), and maximum shear (commensurate with lability of the species toward shear) (Charm and Lai, 1970) will do much to optimize separations.

MACROSOLUTE SEPARATIONS

Purification by Extraction. A solution of crystalline alpha-lactalbumin was admixed with a human serum albumin preparation stabilized with amino acids and then a membrane procedure was devised for its isolation. Separation was accomplished by discontinuous diafiltration [this study repre-

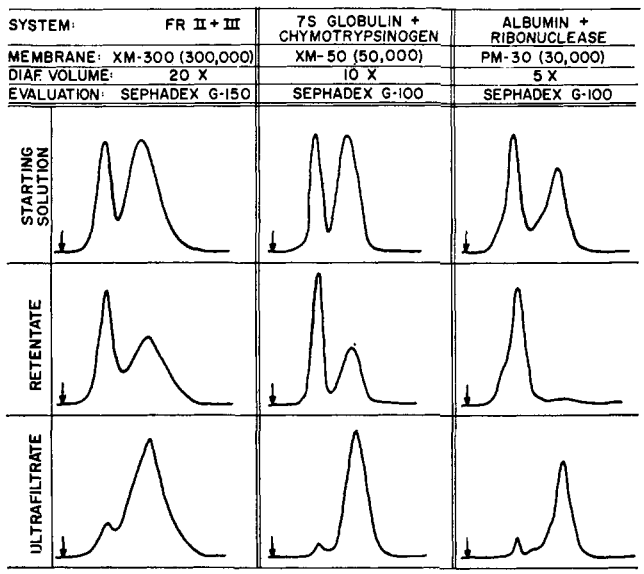

Figure 10. Thin-channel membrane partition of binary mixtures at differing diafiltration volumes

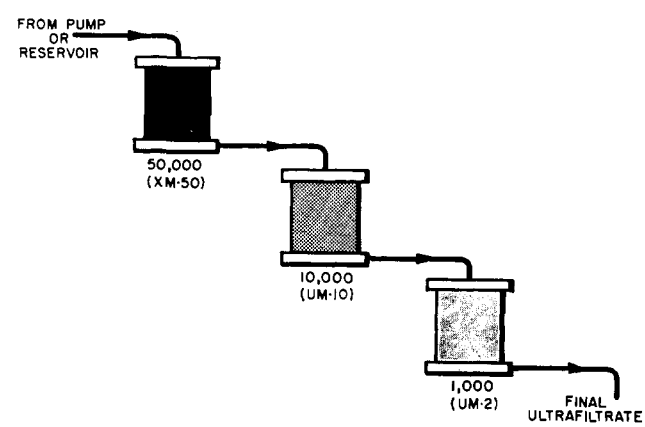

Figure 11. Schematic representation of a tandem system for the partition of mixed solutes into size-graded classes based on membrane retentivity of the components. Redrawn from Blatt *et al.*, 1967a

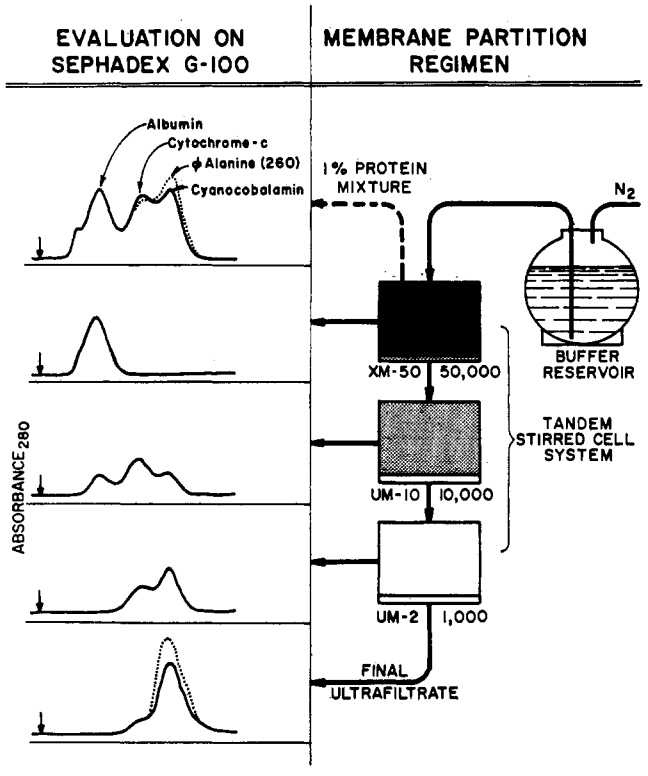

Figure 12. The fractionation by membrane partition chromatography of a mixed protein solution in a three-cell (stirred) tandem system. Redrawn from Blatt *et al.*, 1967a

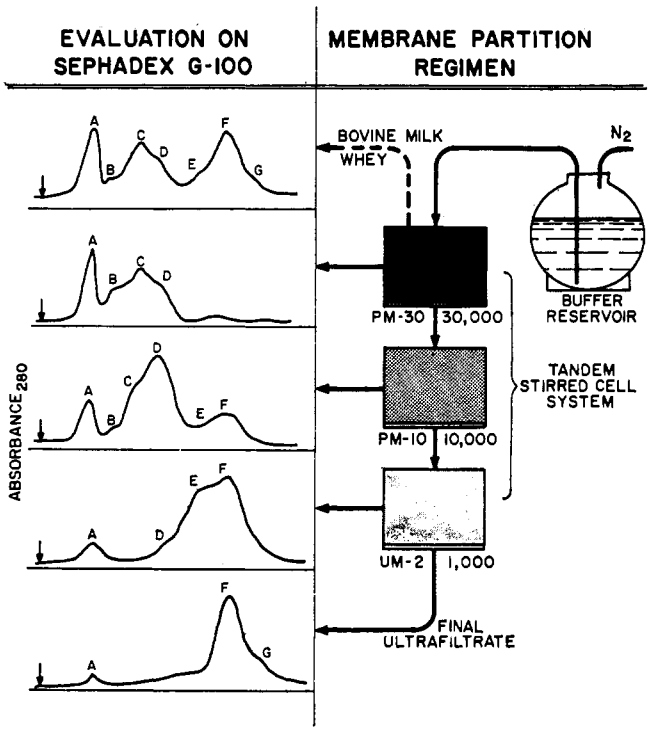

Figure 13. The fractionation by membrane partition chromatography of defatted bovine milk whey in a three-cell (stirred) tandem system

sents work done prior to our continuous methods (Blatt *et al.*, 1967b)] using the membrane regimen shown at the right of Figure 8. Evaluation of starting material and membrane-separated components by Sephadex chromatography (shown at the left) yielded the indicated degree of enrichment. Were the diafiltration to be extended, presumably more of the lower molecular weight species would have been removed. With respect to overall yield, approximately 70% of the initial alphalactalbumin content was recovered in the enriched fraction.

In quite a contrasting application, the digestion products of an enzyme–substrate mixture retained in a membrane compartment were isolated (Figure 9) (Blatt *et al.*, 1968b). In a multi-stage diafiltration system, the higher molecular weight components of milk whey were initially separated from lower molecular weight species by diafiltration using a 10,000 molecular weight retentive filter. This starting substrate was then admixed with alphachymotrypsin, and under continuous diafiltration, the digestion products were washed out as they were formed. The chromatographic evaluations indicate the breakdown of substrate in the retentate as well as the appearance of the lower molecular weight species in the ultrafiltrate.

TC Partition of Binary Mixtures. The studies depicted in Figure 10 illustrate a number of variables attendant to polarization even in thin-channel systems. Using an open membrane (300,000 cut-off) with the more polarizing high molecular weight species, *i.e.*, the globulins of Fr. II and III, a 20-fold diafiltration produced a highly enriched 7S content in the ultrafiltrate. However, the throughput was insufficient to completely remove the lower molecular weight species from the membrane-retained fraction. With a tighter membrane far better resolution was obtained with only a ten-volume diafiltration of admixed 7S globulin and chymotrypsinogen. In a system of lower molecular weight solutes and employing a tighter membrane (albumin and ribonuclease with a PM30 membrane), good separation was obtained with a five-volume exchange. Comparable studies, using stirred cells, yielded filtrates of similar composition; however, at the same diafiltration volumes, the retentates showed a greater content of retained membrane-permeable species as a result of the reduced solute flux in these systems. Obviously the membrane still displayed the primary solute discriminatory function. What was altered was solvent and solute flux. These observations will be borne out later on more complex mixtures.

TANDEM SEPARATION SYSTEMS

Figure 11 illustrates the physical design of tandem systems. Initially we used four-stage stirred-cell units; later we employed three-stage thin-channel apparatus. In operation the solution to be fractionated is placed in the uppermost compartment of the train equipped with the most open filter. Below are buffer-filled compartments with progressively lower cut-off membranes. Solvent from a reservoir is either pumped or gas-driven through the system until the desired throughput is reached. Insofar as the cells are completely fluid-filled and the final ultrafiltrate is at atmospheric pressure, we generally assume a step pressure drop throughout the system. Flow, therefore, is defined by what can be obtained at the system pressure of the tightest membrane. Sephadex chromatography was used to evaluate the distribution of the membrane-separated components.

Four-Stage Stirred-Cell Separations. Figure 12 shows the resulting separation patterns when a 1% mixture of the components indicated on the figure was separated in the membrane regimen depicted to the right of the figure. The fraction shown near the top of the uppermost figure, albumin, and the lower pattern, phenylalanine and cyanocobalamin, were obtained with little admixture. However, the intermediate fractions were not clearly separated.

Even more illustrative of this partial separation is the de-

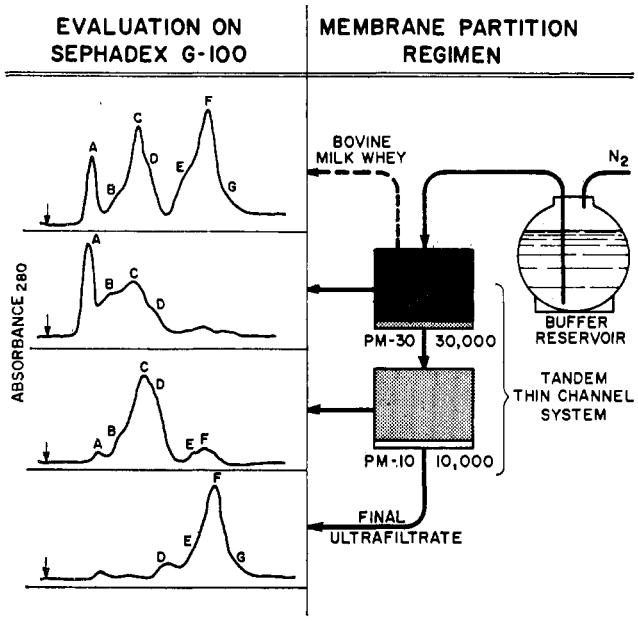

Figure 14. The fractionation by membrane partition chromatography of defatted milk whey in a two-cell (thin-channel) tandem system

tailed data obtained using defatted milk whey in a similar stirred-cell regimen. The regimen and chromatographic patterns are shown in Figure 13. Distributive content of each of the fractions, as well as molecular weight estimates, were obtained from these chromatographic results. From the upper portion of Table II it is obvious that while the yield of the lower molecular weight fractions (<2000) is in excellent agreement with the theoretical content, attesting to nonimpeded passage of lower molecular weight species, retarded permeation of the intermediate fractions was observed. The chromatographic patterns of Figure 13 substantiate the admixture of these intermediates.

Three-Stage Thin-Channel Systems. Using similar solutions (defatted whey) in a tandem thin-channel system (two cells), we obtained the results shown in Figure 14 and the summary data in the lower portion of Table II. A heightened degree of component resolution was obtained; and upon

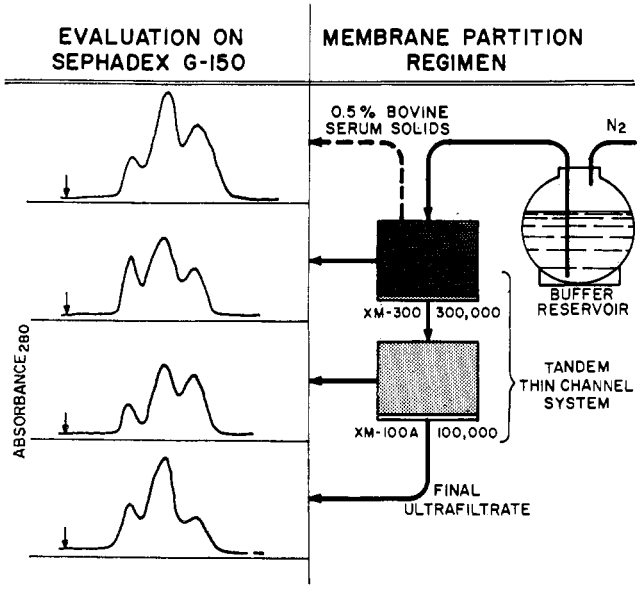

Figure 15. The fractionation by membrane partition chromatography of diluted bovine serum in a two-cell (thin-channel) tandem system

Table II. Comparison of Actual and Theoretical Yields for Whey Separations in Tandem Membrane Systems

Nominal membrane compartment molecular weight range[a]	Actual protein yield %	Theoretical yield %
Stirred-Cell System		
>30,000	50.3	17.6
10,000–30,000	2.4	38.1
2,000–10,000	5.7	4.1
<2,000	41.5	40.1
Thin-Channel System		
>30,000	24.7	13.2
10,000–30,000	32.8	41.5
<10,000	42.5	45.2

[a] Based on known Diaflo membrane retention characteristics for single solutes in solution.

examination of the theoretical *vs.* actual membrane yields, considerably better agreement was noted.

In these systems we believe the following to be true. In the presence of highly retained macrosolute, the rejection coefficients of permeable species are elevated. The lessened degree of polarization control in stirred-cell systems does not permit adequate separation; however, with tc operation, retentivity of permeating species is not as great and separations can be obtained in the diafiltration volumes employed. These are examples where no effective polarization control can be obtained even in thin-channel operation. The regimen and evaluation of a 0.5% bovine serum solids solution is shown in Figure 15. In spite of low velocity tc operation, the rejection coefficients induced by the layer of retained macrosolute for the membrane-permeable species are too high to allow fractionation; and, as a consequence, little discrimination is noted in each of the membrane compartments.

In summary then, the following must be considered in membrane fractionation. System selection must be such that polarization is minimized; membranes must be selected that are retentive to desired species so that discrimination can be made between these and species that have had their rejection coefficients elevated due to polarization. With respect to operating conditions, generally low pressure and high shear (where possible) are recommended.

In its current state, membrane partition can serve as an adjunct to more refined separation techniques. What remains is the evolution of sharp membranes together with advanced equipment providing that degree of polarization control which will make the technique sufficient unto itself.

LITERATURE CITED

Baker, R. W., *J. Appl. Polym. Sci.* **13,** 369 (1969).
Blatt, W. F., Feinberg, M. P., Hopfenberg, H. P., Saravis, C. A., *Science* **150,** 224 (1965).
Blatt, W. F., Hudson, B. G., Robinson, S. M., Zipilivan, E. M., *Nature (London)* **216,** 511 (1967a).
Blatt, W. F., Robinson, S. M., Robbins, F. M., Saravis, C. A., *Anal. Biochem.* **18,** 91 (1967b).
Blatt, W. F., Robinson, S. M., Bixler, H. J., *Anal. Biochem.* **26,** 151 (1968a).
Blatt, W. F., Hudson, B. G., Robinson, S. M., Zipilivan, E. M., *Anal. Biochem.* **22,** 161 (1968b).
Blatt, W. F., Dravid, A., Michaels, A. S., Nelsen, L. M., "Membrane Science and Technology," p 47, Flinn, J. E., Ed., Plenum, New York, N.Y., 1970.
Charm, S. E., Lai, C. J., *Biotechnol. Bioeng.* in press (1970).
Lewis, U. J., Cheever, E. V., Seavey, B. K., *Endocrinology* **84**(2), 325 (1969).
Michaels, A. S., "Progress in Separation and Purification," Vol. I, p. 297, Perry, E. S., Ed., Wiley, New York, N.Y., 1968.
Pollak, V. E., Gaizutis, M., Rezaian, J., *J. Lab. Clin. Med.* **71**(2), 338 (1968).
Zipilivan, E. M., Hudson, B. G., Blatt, W. F., *Anal. Biochem.* **30,** 91 (1969).

Received for review September 29, 1970. Accepted December 3, 1970. Presented at the Division of Agricultural and Food Chemistry, 160th Meeting, ACS, Chicago, Ill., September 1970.