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TECHNICAL REPORT NO. 68-40

THE APPLICATION OF SELECTIVE ULTRAFILTRATION TO PROTEIN CHEMISTRY

by

William F. Blatt, PH. D.

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UNITED STATES ARMY
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NATICK, MASSACHSETTS

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THE APPLICATION OF SELECTIVE ULTRAFILTRATION TO PROTEIN CHEMISTRY

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FOREWORD

Characterization of the regulatory factors involved in man's adaptation to his environment must be preceded by the rapid and non-denaturant separation of these moieties from various body tissues and fluids. Accordingly, part of our laboratory effort has been devoted to the development of new procedural techniques which involve minimal changes to these compounds during isolation.

This report represents a summary of membrane ultrafiltration methods which were developed to fulfill the aforementioned criteria, as well as their application to the general field of protein chemistry.

MILTON MAGER
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Biochemistry and Pharmacology Laboratory

APPROVED:

JAMES E. HANSEN, Col., MC
Commander & Scientific-Technical Director

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ABSTRACT

Preformed ultrafiltration membranes, with molecular weight exclusion limits varying from 1,000-100,000, are uniquely suited for preparative methods in protein chemistry. The applications include non-denaturing protein concentration, rapid dialysis, micro solute binding, limited purification, and fractionation of complex mixtures. In the latter procedures, selective ultrafiltration obviates the use of cumbersome electrophoretic methods, or initial column chromatography. Increases in the separation rate, as well as the total quantity of material fractionated, can be obtained by increasing the exchangeable surface of the filter.

THE APPLICATION OF SELECTIVE ULTRAFILTRATION TO PROTEIN CHEMISTRY

INTRODUCTION

The availability of high flux, preformed ultrafiltration membranes, serving as diffusive barriers to the transport of macromolecular species, makes them uniquely suited for applications in protein chemistry where rapid non-denaturing techniques are desirable. Although a number of membranes have been examined at this laboratory, the most promising results to date have been obtained using Diaflo ultrafilters*. Hydrous gels, representing the complex interaction products of polyanions and polycations (1), are used to synthesize membranes of compact structure with high solute retention. In this system, water is transported through the membrane by molecular diffusion rather than the hydraulic pore flow observed with conventional ultrafilters. A diagrammatic representation of the differences in the diffusive transport of micro ions through Diaflo membranes, contrasted with passage in pore-limited ultrafilters (regenerated cellulose or Visking tubing) is shown in Fig. 1. The pore clogging, evident in the more conventional ultrafilters (Fig. 1, right), is obviated with the gel structures. When the membrane structure was expanded to attain higher orders of selectivity, it was necessary to resort to pored structures as a partitioning device.

To date, filters with molecular weight exclusion limits of 1000-100,000 have been prepared. Some of their potential applications, as a function of the retentive capacity, are illustrated in Fig. 2. In order of increasing exclusion, the uses cited range from techniques for rapid dialysis with compacted structure membranes through methods for the fractionation of complex protein mixtures employing ultrafilters of more expanded composition. The order of presentation, varied to permit a more logical development of the topics under consideration, is as follows: (1) protein concentration, (2) dialysis, (3) micro solute binding, (4) proteolysis, (5) limited fractionation, (6) fractionation of complex mixtures, and (7) immunochemical applications. Although a number of these topics have been previously presented in detail (2-5), their unique contribution to preparative methods of protein chemistry warranted integration into a single report.

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*Amicon Corporation, Cambridge, Mass.

PROTEIN CONCENTRATION

With high flux membranes, ultrafiltration is a rapid and essentially non-denaturant method for the concentration of solutions containing labile macromolecules. The cross section of the stainless steel ultrafiltration cell used in initial studies with the Diaflo membranes is shown in Fig. 3. (This unit has since been replaced by an entirely plastic apparatus[+]). With the earlier apparatus, an ultrafilter graded at a retention value of 1000 was evaluated and the system contrasted with the more conventional method (as exemplified by the NIL Saravis ultrafilter[†]) which uses Visking tubing as the ultrafiltration membrane. Table 1 compares various protein preparations, in phosphate buffered saline, pH 7.4, as concentrated by the two considered ultrafiltration modes (2). Since the areas of the filters are different, the ultrafiltration rates have been normalized to unit surfaces of exchange. It is apparent from the data that the Diaflo membrane ultrafiltration procedure is extremely rapid relative to Visking tubing (up to 50-fold faster) and that high recoveries may be expected. The actual filtration rates of the various fractions relative to buffer and distilled water are shown in Fig. 4. On inspection, it appears that the type of protein rather than the absolute concentration (given in Table 1) is the prime variable in determining the effective filtration rate.

The electrophoretic distribution of three protein solutions, prior to and following concentration by both procedures, was determined on analysis of the electropherograms obtained using cellulose acetate as the supportive medium (see Table 2). With either method, essentially uniform results were obtained with respect to the starting material, and, consequently, denaturant changes capable of resolution by this technique were assumed to be minimal. Additional evidence for non-denaturation is shown in Fig. 5 wherein immunoelectrophoresis patterns of human serum aliquots prior to dilution and after ultrafiltration with Diaflo membranes or Visking tubing are contrasted. The concentrates appear to be identical to the starting material. Although the membrane used in these studies was initially assigned a retentive limit of 1000, it should be noted that the exclusion values given for all membranes are quite arbitrary. While molecular weight serves as convenient measure, better indices of membrane exclusion would be molecular dimensions and/or configuration.

For the rapid concentration of solutions containing macrosolutes with molecular weights in excess of 1000, it is suggested that the ultrafilter with an exclusion limit of 10,000 be used.

[+] Ibid

[†] National Instrument Laboratories, Bethesda, Md.

A METHOD OF RAPID DIALYSIS

The extremely rapid rate of micro ion exchange permits us to use these membranes for dialysis by either the discontinuous technique of repetitive dilution and concentration (diafiltration) or a continuous method as afforded by the specialized assembly shown in Fig. 6. In this latter procedure, a dialysate reservoir and valve assembly have been placed above the standard ultrafiltration cell. With the fluid connecting valve closed, both chambers are pressurized, then the air line to the lower assembly is closed and the fluid valve opened. Thereby, a relatively constant volume is maintained in the ultrafiltration cell. Figure 7 illustrates the technique wherein salt was removed from fixed volumes of solution as well as the reverse process; i.e., the introduction of salt into the solution. In these and subsequent studies, the effluent from the side port of the cell was passed through a conductivity meter[‡] which served to monitor the salt content. In Fig. 8 are depicted the results where solutions of differing molarity were dialyzed in forward and reverse manner. The introduction of protein (see Fig. 9), while reducing the ultrafiltration rate, does not change the effective volume of reservoir solution necessary for effective dialysis. Using this system, it is apparent that the volume of solution necessary for dialysis is independent of the macro or micro ion content of the retentate. The approximate volumes necessary for effective exchange can be calculated from the equations given below (6):

$$(1)\ Q = v \times \ln \frac{\text{coupled fraction}}{\text{residual fraction}}$$

where Q = volume of dialysate

v = volume of sample being dialyzed

(2) at 95% completion and using a 10 ml sample,

$$Q = 29.5 \text{ ml}$$

This technique greatly differs from conventional dialysis in that in the latter, the concentration of the exchangeable ion determines the effective dialysis volume and subsequently the time necessary for removal. With the membrane method, 95% dialysis can be achieved using approximately three times the retentate volume, and with ultrafilters chosen appropriate to the size of the solute to be retained, dialysis can be accomplished in less than 1 hour. It should be noted that this analysis presumes that the micro ion to be eliminated is not bound to any of the protein constituents, nor is there any appreciable micro solute rejection by the membrane. When this rejec-

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[‡] Radiometer Corp., Copenhagen, Denmark

tion does occur, a considerably larger volume of dialysate is necessary. Accordingly, the exact conditions for microsolute removal should be delineated for the specific system under study.

MICRO SOLUTE BINDING

The use of an apparatus which permits constant volume to be maintained during continuous flow of an exchange solution enables one to measure the interaction of binding species as they pass through solutions of membrane-retained macro solute (6). To illustrate this we have studied the binding of two small molecules, dimethyl orange (M.W., 327) and ionic calcium to human serum albumin. At the outset, it was necessary to establish the diffusivity of the micro solute through the protein limiting membrane. Figure 10 illustrates the comparative diffusivity of sodium chloride, calcium chloride and methyl orange through the 10,000 retention membrane as contrasted with the passage of NaCl through a standard paper filter. The sodium and calcium exchange was monitored by on-line determination of the conductivity of the ultrafiltrate while methyl orange in the ultrafiltrate was indicated by its absorbance at 425 mµ. Free diffusivity of the calcium ions occurred, but methyl orange was retained to a large degree by the membrane. It appeared that this was due to interaction of the dye with the membrane. Nevertheless, binding profiles can be obtained in that the same degree of membrane interaction will occur in protein-free and protein-containing solutions, and these will in effect cancel each other out. Salt solutions, approximately 0.45M, were effective in clearing the dye from the membranes. Figure 11 illustrates a methyl orange-albumin binding study. In the absence of protein, the curve shown at the extreme left was obtained. With increasing albumin content, there is a progressive shift to the right. For rapidly evaluating total binding, the diafiltration of micro solute is continued until equilibration (the absorbance of the final eluate approximates that of the initial solution). Noting any changes in the volume of the protein retentate solution and its absorbance relative to the initial dye solution, we may calculate the molar binding ratio. The data and necessary calculations are shown in Table 3. The same method was used for the determination of calcium binding to albumin. Figure 12 illustrates the study where solutions of approximately 5 x 10^{-3}M calcium chloride were diafiltered through progressively increasing concentrations of albumin. The calculation of the molar binding ratios under varying conditions is shown in Table 4.

In summary, membrane ultrafiltration provides a rapid method for the determination of binding profiles and calculation of molar interaction ratios. Whereas the more conventional methods of equilibration, typified by equilibrium dialysis, are tedious and require 48-72 hours for effective completion, this procedure requires no more than a few hours.

SEPARATION AND ANALYSIS OF THE PRODUCTS OF PROTEOLYSIS

The results of the prior studies suggested that membranes which selectively retain macromolecules during ultrafiltration can be used to separate low molecular weight constituents from either native protein mixtures or from unreacted substrate following enzymatic degradation. Accordingly, a system was devised for the rapid and continuous ultrafiltration of proteolytic digestion products as they were formed through a size-selective membrane (3). The commercially-available ultrafiltration cell was reduced in size and altered to permit continuous on-line ultrafiltration without the necessity for a separate reservoir assembly. This modification is shown in the upper portion of Fig. 13. The substituted center portion permitted reduction of the internal volume to approximately 16 ml. The top-supported stirring assembly was replaced by a teflon-coated magnetic stirrer supported by a teflon collar force-fitted in that portion directly above the membrane. The lower figure displays the assembled unit on a magnetic stirrer with appropriate valve connections for sample introduction and buffer flow as well as ultrafiltrate egress to a flow-through UV analyzer. This last system provides for continuous spectral evaluation of the ultrafiltrate during proteolysis. An expanded diagrammatic view of the ultrafiltration cell is shown in Fig. 14. For these studies, a 10,000 membrane was used as the partitioning membrane. The cell was filled with the substrate solution and buffer circulated through the system at a constant rate until equilibrium was achieved; i.e., a stable baseline representing the absence of UV absorbing material in the eluate. Following this, the pump was stopped, the enzyme solution directly introduced through the sampling valve into the cell, and the buffer flow then restored. In these studies, the substrate consisted of a 1% solution of bovine milk whey in phosphate buffered saline, pH 7.4. Prior to digestion, the solution was diafiltered through a 10,000 retention membrane to remove any lower molecular weight moieties. After the cell was filled with this solution and equilibrated, 1 ml of a 1% solution of alpha chymotrypsin was introduced into the sampling valve. The absorbance of the ultrafiltrate was monitored at 225, 260 and 280 mμ.

Figure 15 shows the UV absorption of the ultrafiltrate (flow rate, 25 ml/hr) produced during 12 hours of digestion, as well as the Sephadex G-100 chromatographic evaluation of each of the constituents in the system. Reduction in the proportion of larger molecular weight components (substrate; fractions B and C) is noted in the material retained by the membrane; lower weight moieties formed during proteolysis are denoted by appearance of fractions D and E in the ultrafiltrate. A further evaluation of the digested material was made by taking time-collected aliquots of the ultrafiltrate (Fig. 15, left, Fractions 1-4) and chromatographing these on Sephadex G-25 (see Fig. 16).

Other possible applications of this technique are in the recovery of the biologically active peptides formed during the proteolysis of certain plasma proteins as well as the selective removal of the products of digestion from degraded substrate after interaction with a variety of enzymes.

LIMITED FRACTIONATION

With the availability of lower retentive capacity membranes, i.e., those with more expanded structure, preparative techniques become feasible. Accordingly, an experimental membrane with a molecular weight retention value graded at 35-45,000 was examined as a partitioning device for the removal of larger molecular weight contaminants from smaller sized species (4). For evaluation, we chose crystalline bovine alpha lactalbumin (M.W., 14,500) a material physically able to diffuse through the membrane and containing a small quantity of albumin as a contaminant. The modified flow-through cell was unavailable for these studies. Consequently, a standard 65 ml ultrafiltration cell was employed using repetitive dilution and concentration. Aliquots of a 2% solution were diluted to 50 ml with pH 7.6, TRIS buffered saline and ultrafiltered to a concentrate volume of 5 ml, rediluted and the ultrafiltration repeated (final retentate referred to as Concentrate A). The two ultrafiltrates were combined, then concentrated on a 1000 retention filter (Concentrate B). The final filtrate (< 1000) was reduced by flash evaporation. The protein content of all fractions was determined by biuret analysis. A summary of the separation scheme, recovery values, as well as chromatographic evaluation of each fraction are shown in Fig. 17. From the recovery values shown to the right of the figure, it is apparent that a substantial portion of the alpha-lactalbumin diffused through the membrane with only 23% remaining in the membrane retentate. Accordingly, 69% of the biuret positive material with molecular limits of 1,000-35,000 was obtained in Concentrate B. No more than 8% of the total biuret positive material appeared in the final filtrate. The central portion of the figure denotes the qualitative evaluation of the various membrane-separated fractions following chromatography on Sephadex G-100. The appearance of the UV absorption pattern of Concentrate B attests to the removal of albumin (Fraction B) as well as to the uniformity of the major component (Fraction C). In order to test the ability of this membrane to remove more substantial quantities of albumin, an equivalent amount of impure albumin was added to grossly contaminate the crystalline alpha lactalbumin sample. The extent of purification by membrane partition is shown in Fig. 18. Additional evidence substantiating the selective removal of albumin from either crystalline or admixed fractions was obtained by immunochemical evaluation of the various fractions using the titration immunodiffusion procedure described by

Saravis (7). With this technique, two-fold serial dilutions of antigen were reacted with horse anti-albumin antisera, using preformed microporous cellulose acetate as a supporting medium. The precipitin bands obtained are shown in Fig. 19. The lack of interaction in Concentrate B would indicate the relative absence of albumin in this fraction.

The availability of membranes with higher orders of selectivity permitted us to extend our studies into the fractionation of more complex protein mixtures (described below).

FRACTIONATION OF COMPLEX MIXTURES BY MEMBRANE PARTITION

For this application, it was necessary to evaluate the complete array of available membranes: 100,000; 60,000; 40,000; 10,000; and 1,000 (5). The retentive capacity was determined using the modified flow-through cell (identical to that described for proteolysis evaluation). Non-pulsatile buffer flows were established through cells containing each of the membranes using sufficient ultrafiltrate passed through the UV monitoring system to achieve baseline stability. Following this, 1 ml of 1% protein solutions in phosphate-buffered saline, pH 7.4, were injected through the sampling valves and the UV absorption of the ultrafiltrate contrasted with recordings of flow patterns through cells in which the membranes were omitted. The amount of protein passed was determined from the differential peak areas of the ultrafistrates relative to the areas of non-membrane systems. Retention was calculated by difference. An illustration of this method is shown in Fig. 20 wherein serum albumin was evaluated. When a filter graded at 100,000 was used, no retention was observed, whereas, with a 60,000 filter, considerable albumin was retained. The holdback capabilities of the available ultrafilters for a spectrum of proteins is shown in Table 5. Insofar as many of the commercial preparations contain impurities that could partially invalidate the results, we selected protein standards that are used for the determination of molecular weight by gel diffusion chromatography. The heavy line serves to bracket the major exclusion area (greater than 60% retention). The data reinforced an earlier observation that exclusion limits, based upon other model polymers, are only arbitrary and that true characterization of retention entails considerably more than the gross molecular weight. In addition, the limited diffusivity of essentially uniform proteins, e.g., albumin and hemoglobin through the 60,000 filter, may be due to the following: a portion of the protein can be affixed to the surface or entrapped within the membrane matrix, thus altering its retentive capacity.

On the basis of the observed exclusion limits, an assembly was devised consisting of three of the ultrafiltration units, placed in series, each containing a progressively lower exclusion filter (60,000:10,000:1,000). The apparatus is shown in Fig. 21. In the initial evaluation, approximately 80 mg of an equal part mixture of albumin, cytochrome c, cyanocobalamin and phenylalanine in phosphate buffered saline, pH 7.4, was placed in the upper cell and a constant buffer flow of 10-15 ml/hr. established through the system. The separations obtained, as characterized by chromatographic evaluation of each of the membrane-retained fractions, is shown in Fig. 22. A transition of high to lower molecular weight species was accomplished by the graded retention filters. Moreover, these separations were in accord with what had been predicted on the basis of the single evaluation schema shown in Table 5. It would appear then, that the diffusivity of a given protein through these membranes was not altered by using a protein mixture.

In another series, aldolase, ovalbumin, cytochrome c, and cyanocobalamin were separated in a similar unit. The results are shown in Fig. 23. Once again, the progressive change in distribution as evaluated by gel chromatography substantiates the findings of the earlier study. In additional studies, we examined different mixtures in systems containing the filter graded at 100,000 but with less success. The larger molecular weight materials, e.g., gamma globulin and apoferritin, show a tendency to plug the filter when used for protracted periods, and consequently interfere with the passage of material normally diffusible through these membranes. If charge sites could be included within the structure of the membrane, selective rejection of gamma globulin with admission of species of different charge and lesser size might be effected. Additionally, with tailoring of these membranes to other exclusion limits, tandem systems can be devised for the separation of proteins more similar in molecular weight than those used in this evaluation.

IMMUNOCHEMICAL APPLICATIONS USING MEMBRANE PARTITION

This final application represents a synthesis of the preceding areas of study, where the processes of peptide separation, limited purification and fractionation have been integrated to provide a system suitable for immunochemical studies. This aspect is preliminary and this report will, for the most part, provide a conceptual approach rather than a definitive statement of results. In this study, changes in the antigenicity of a substrate following enzymatic degradation were noted. The lower molecular weight of most proleolytic enzymes, relative to the substrate used, necessitated coupling of the enzyme to an insoluble matrix so that it would not diffuse through the 60,000 membrane used in these studies. Accordingly, the enzyme was interacted with polyacrilamide gel (8). During evaluation, the cold-stored enzyme-gel suspension is placed in the top cell of the tandem assembly and sufficient antigenic substrate is introduced through the sampling valve prior to establishing buffer flow through the system. A diagrammatic illustration showing enzyme-gel, substrate, and degradation products in the various filter compartments is shown in Fig. 24.

Following digestion, aliquots can be removed from each of the filter units and evaluated. Immunoelectrophoresis as well as double diffusion in agar are applicable in systems yielding precipitin reactions. In those cases in which haptenic fragments are formed (presumably less than 10,000) a titration immunodiffusion procedure (vide supra) can be employed. The test solution is admixed with the antisera, reacted against native substrate, and the results contrasted with the reaction of saline-diluted antisera. Where haptenes exist, reduction in the extent of interaction can be noted and appropriate quantitation made.

SUMMARY

Specific applications of selective ultrafiltration to pertinent problems in protein chemistry have been presented. An advantage of this system, apart from the use of these membranes in removing ionic or low molecular weight species, is its ability to resolve complex mixtures into size-graded classes without the use of precipitating reagents, cumbersome electrophoretic methods, or initial column chromatography. Increase in the separation rate and the total quantity of material fractionated can be obtained by increasing the exchangeable surface of the filter. In that limited fractionation rather than total resolution is achieved, membrane partition chromatography appears to be an apt title for this preparative method.

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APPENDIX

Figures 1-24

Tables I-V

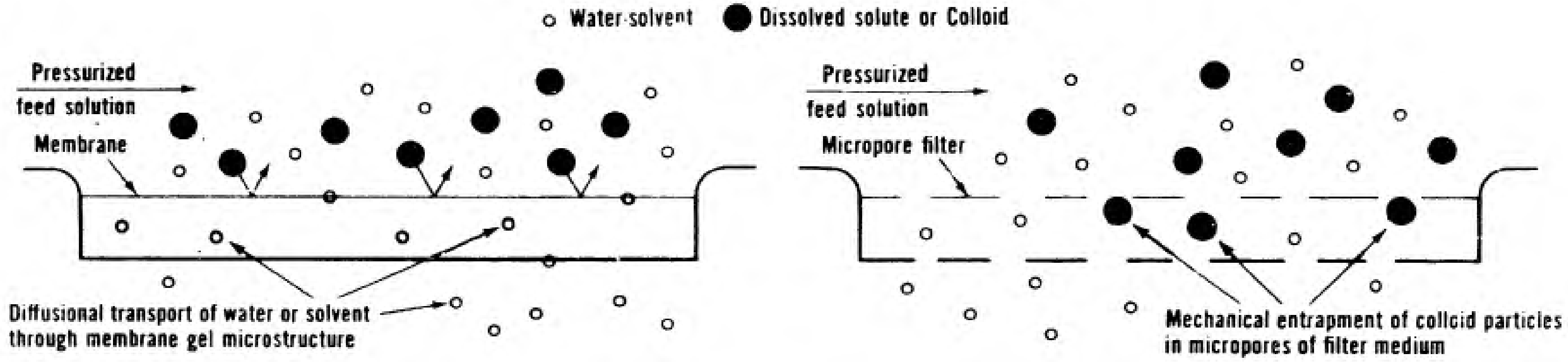


Fig. 1. Diffusive transport of microsolute in Diaflo membranes (left) contrasted with transport in conventional ultrafilters (right) as used in positive ultrafiltration.

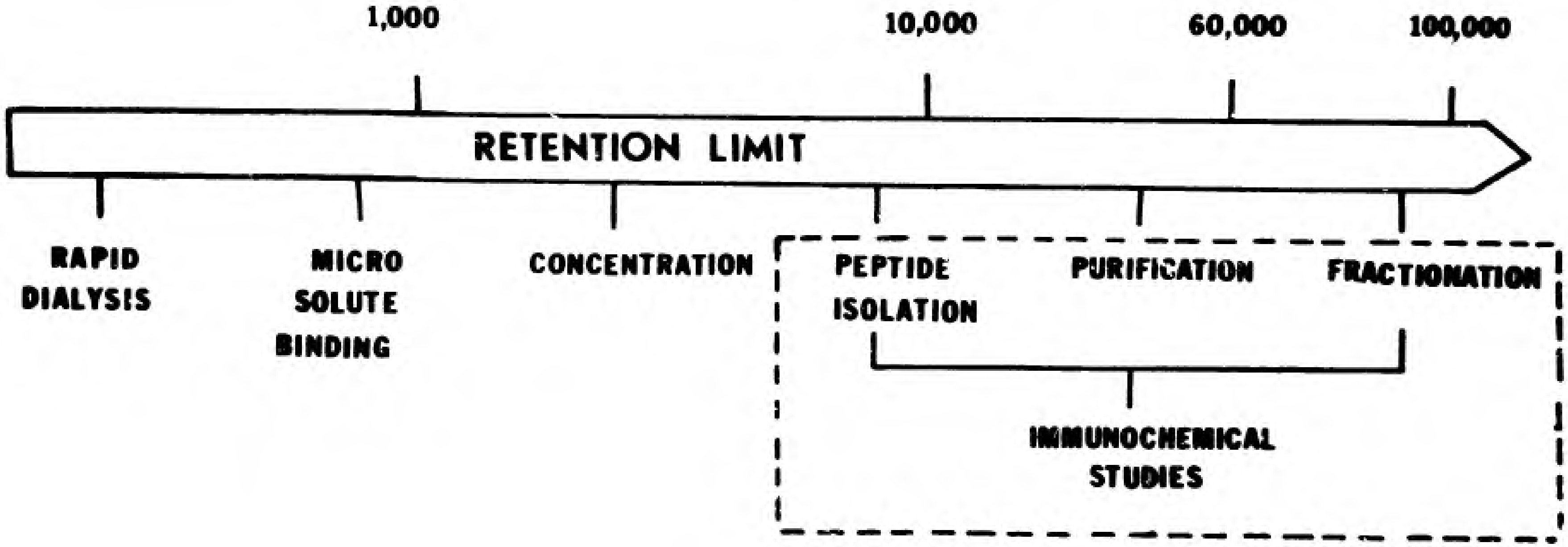


Fig. 2. The application of size-selective membranes to
Molecular weight retention limits are indicated
application are shown above.

ein chemistry.
the areas of

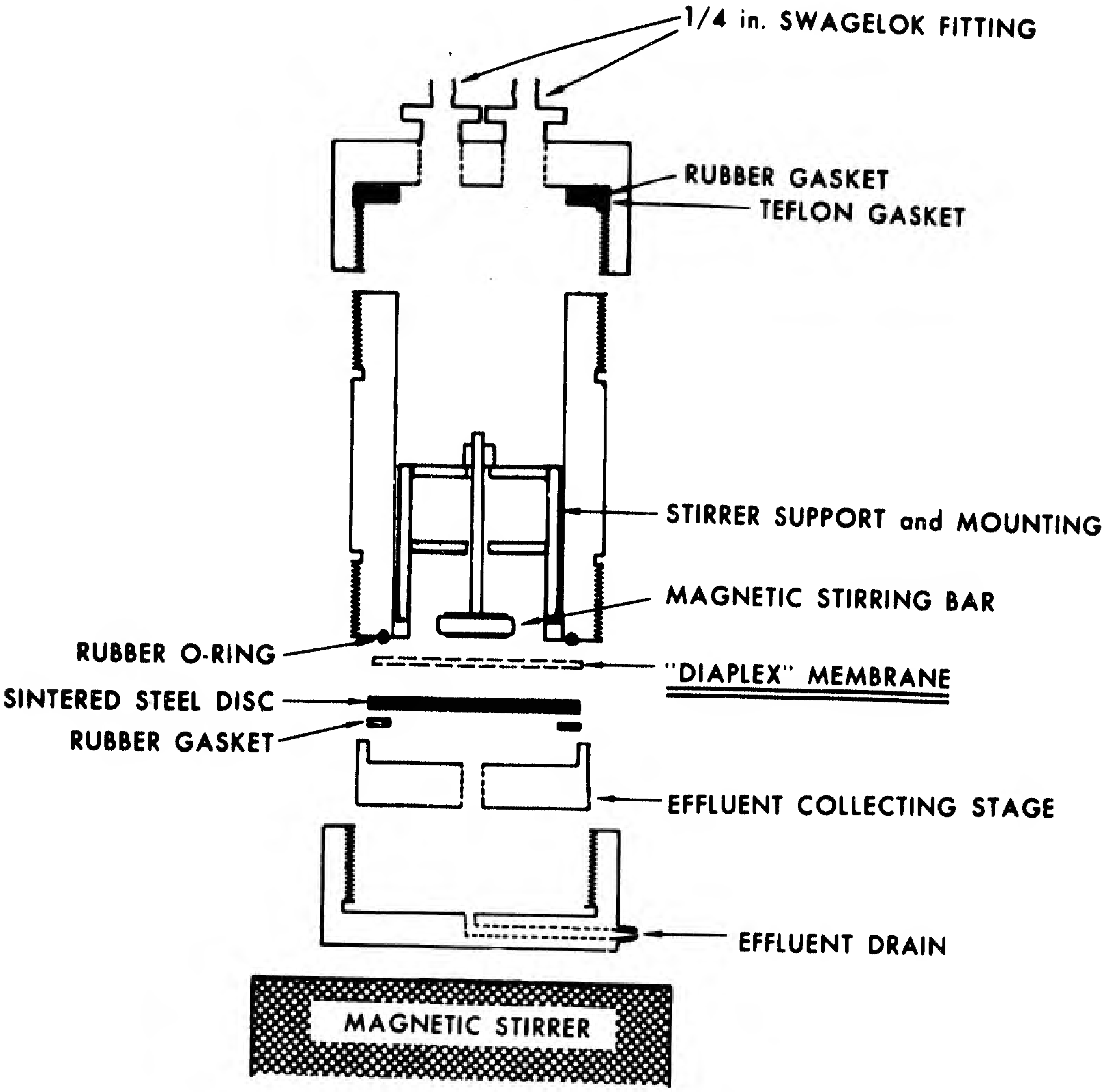


Fig. 3. Vertical cross section of a positive pressure membrane ultrafiltration unit. (From reference 2).

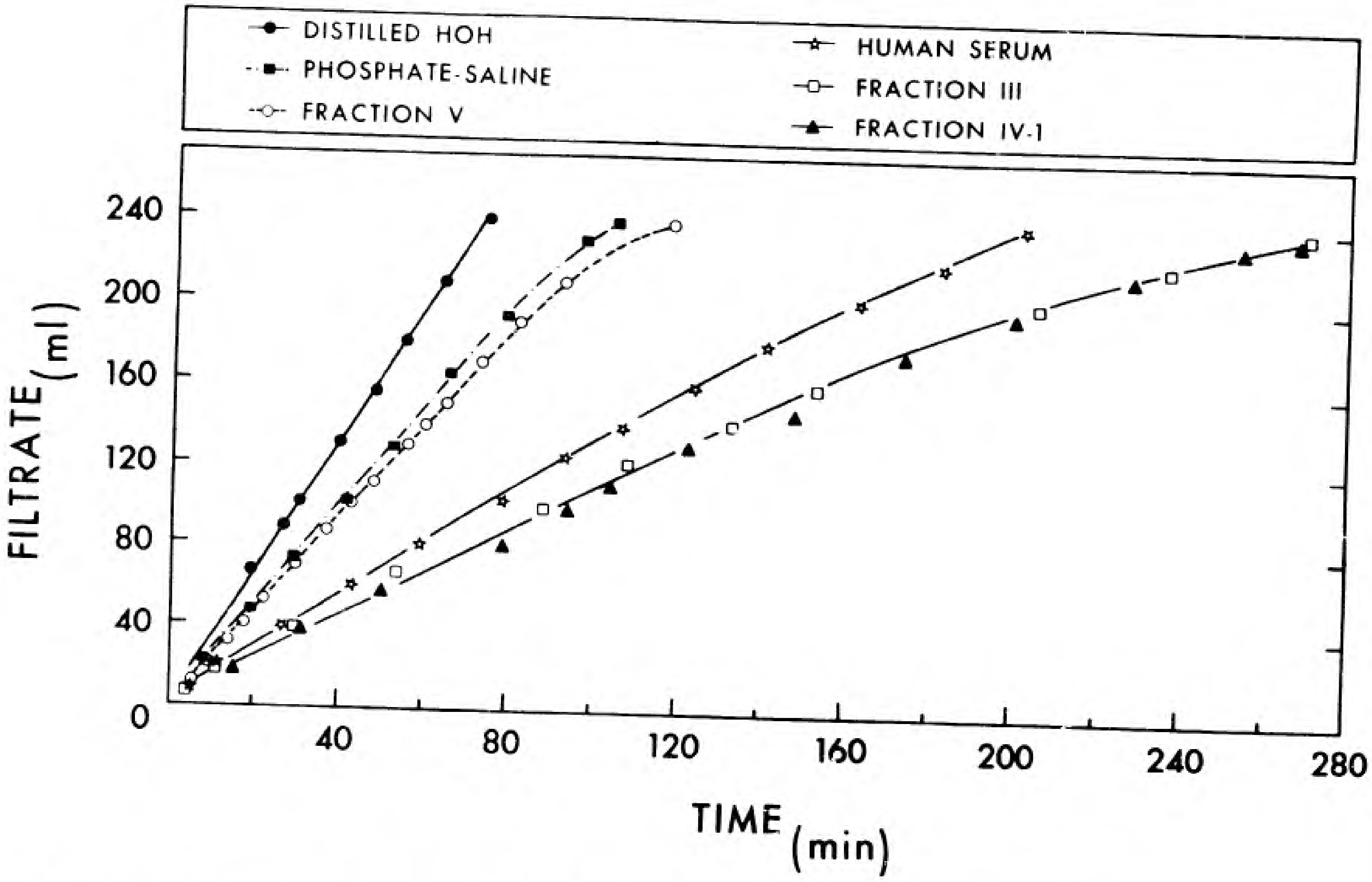


Fig. 4. Filtration rates of various solutions through the same retention membrane (1,000). Filtration order: fraction V, fraction IV-1, human serum, fraction III, phosphate-saline buffer and distilled water. Pressure used was 6.8 atm and the effective surface area was 20.2 cm^2. (From reference 2).

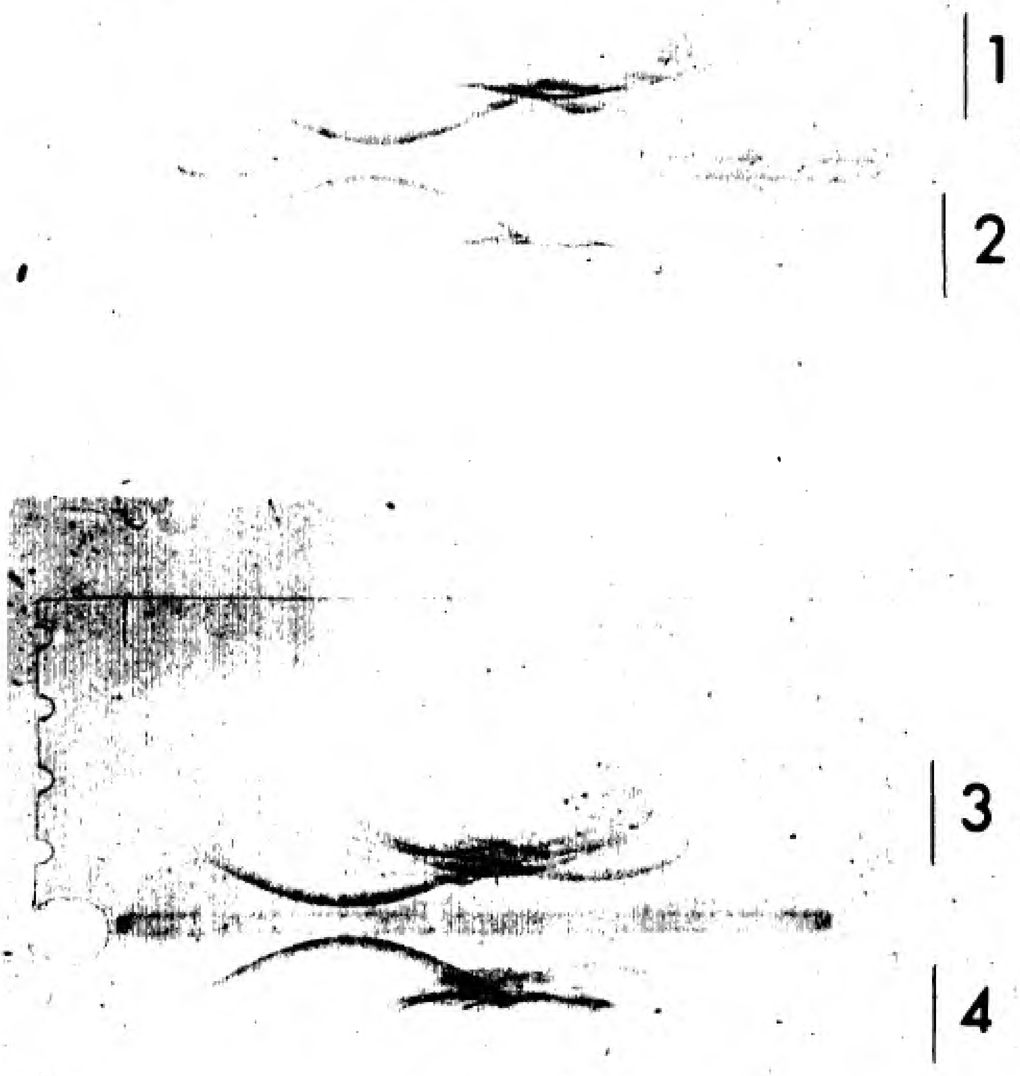


Fig. 5. Immunoelectrophoresis of human serum before and after concentration by ultrafiltration: 1, membrane concentrate (Diaflo); 2, starting material; 3, conventional concentrate (Visking); 4, starting material. (From reference 2).

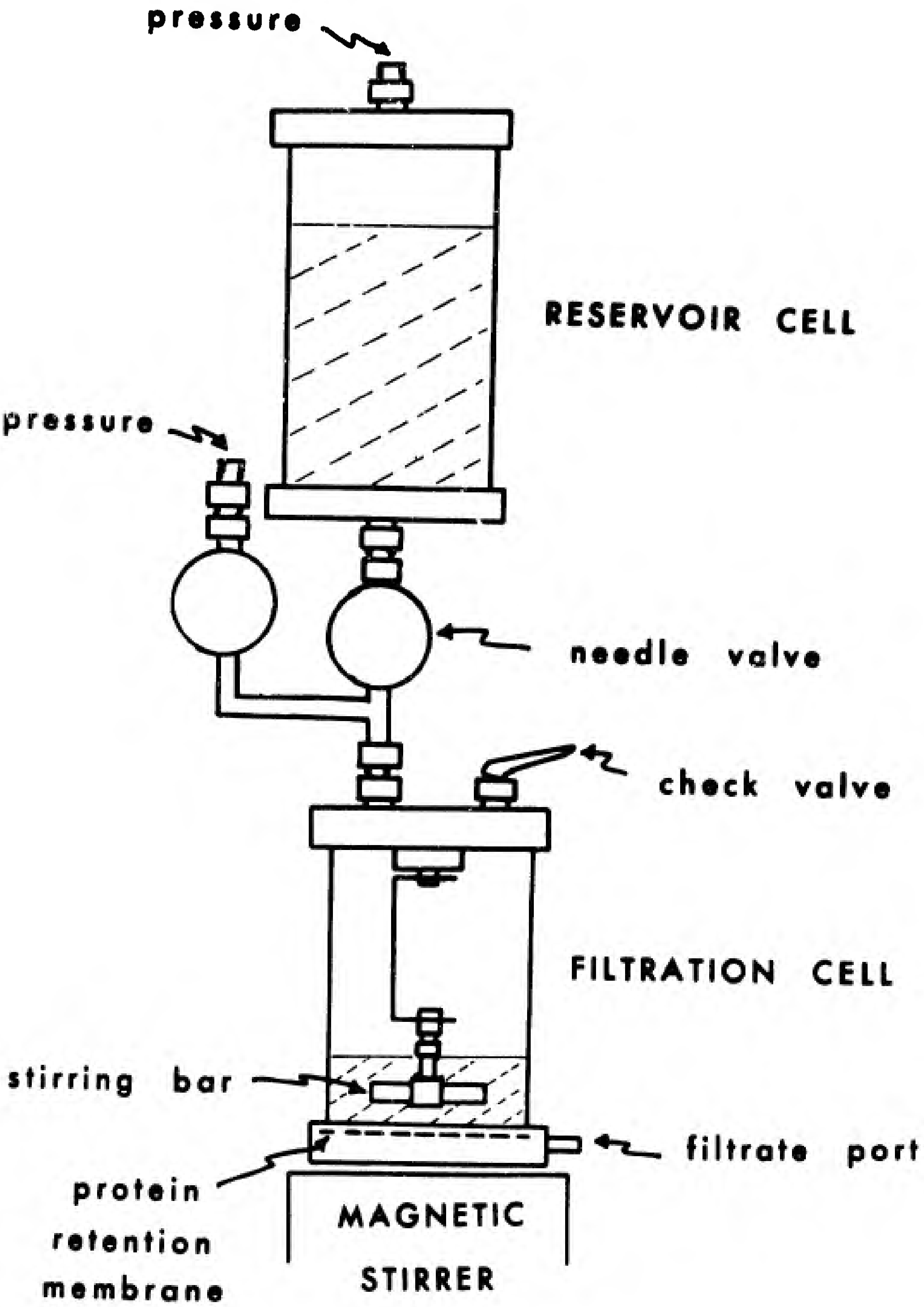


Fig. 6. Diafiltration assembly for rapid dialysis and microsolute binding evaluation. Lower filtration cell formulated of plexiglass with a teflon-coated internal stirrer assembly. Upper reservoir of similar construction. Swage-lok fittings, needle valves and 1/8" stainless steel tubing comprise the connecting assembly.

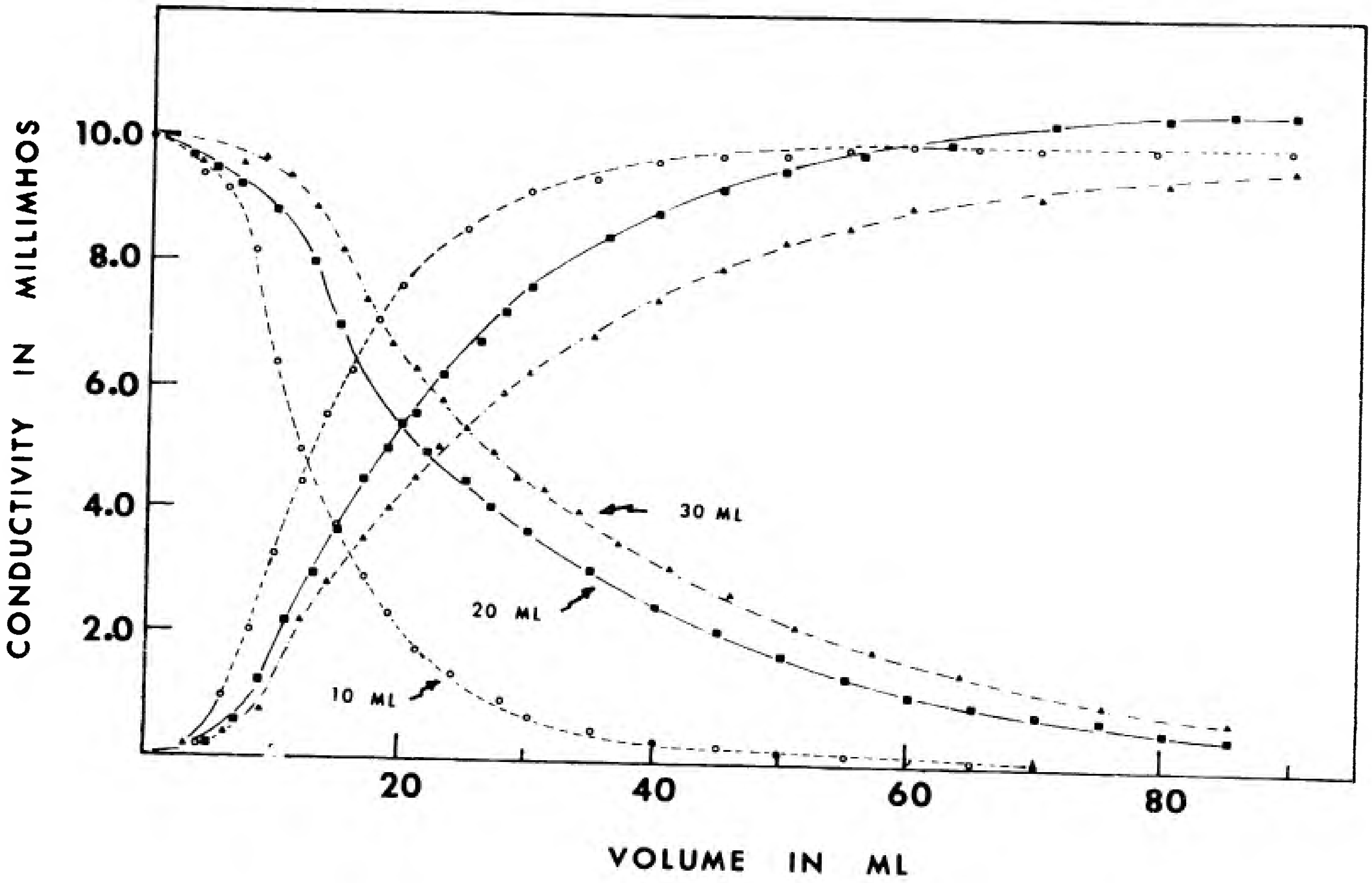


Fig. 7. The continuous fixed-volume filtration (diafiltration) of distilled water and 0.15M NaCl solutions against NaCl and distilled water, respectively.

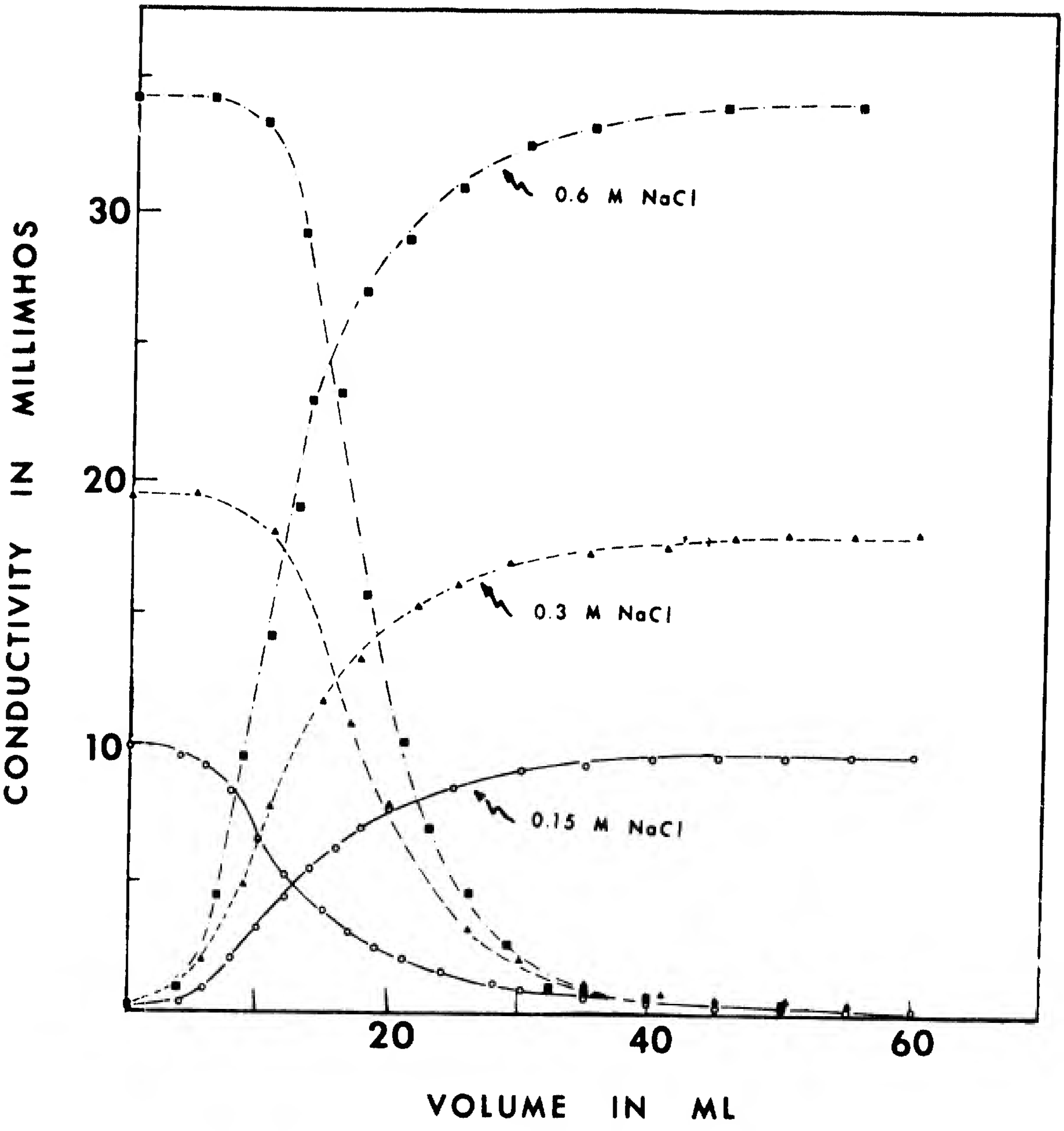


Fig. 8. The diafiltration of distilled water and varying concentrations of NaCl against varying salt and distilled water, respectively. Volume in the filtration cell was maintained at 10 ml.

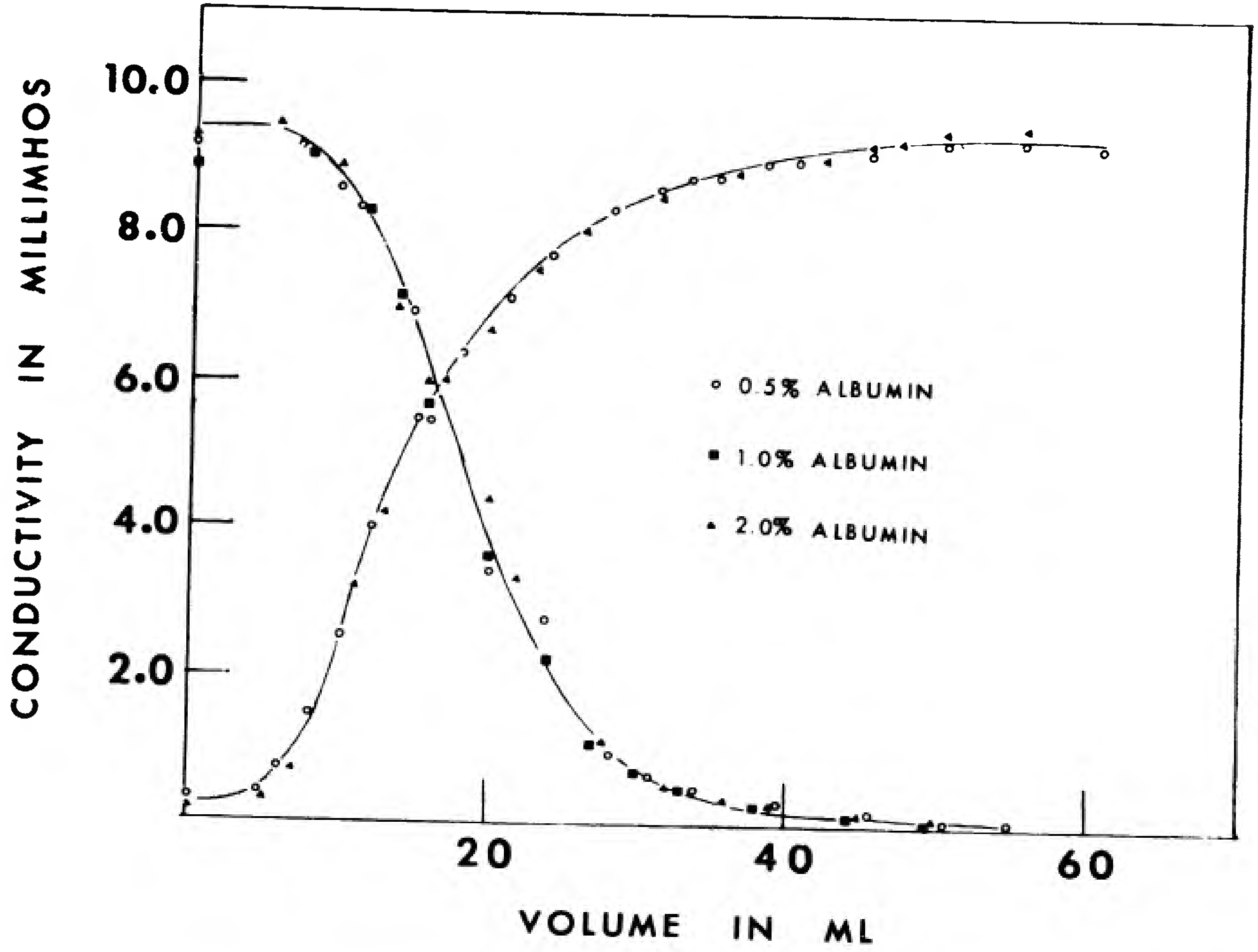


Fig. 9. The diafiltration of aqueous and saline solutions of albumin against saline and distilled water, respectively. Volume in the filtration cell was maintained at 10 ml.

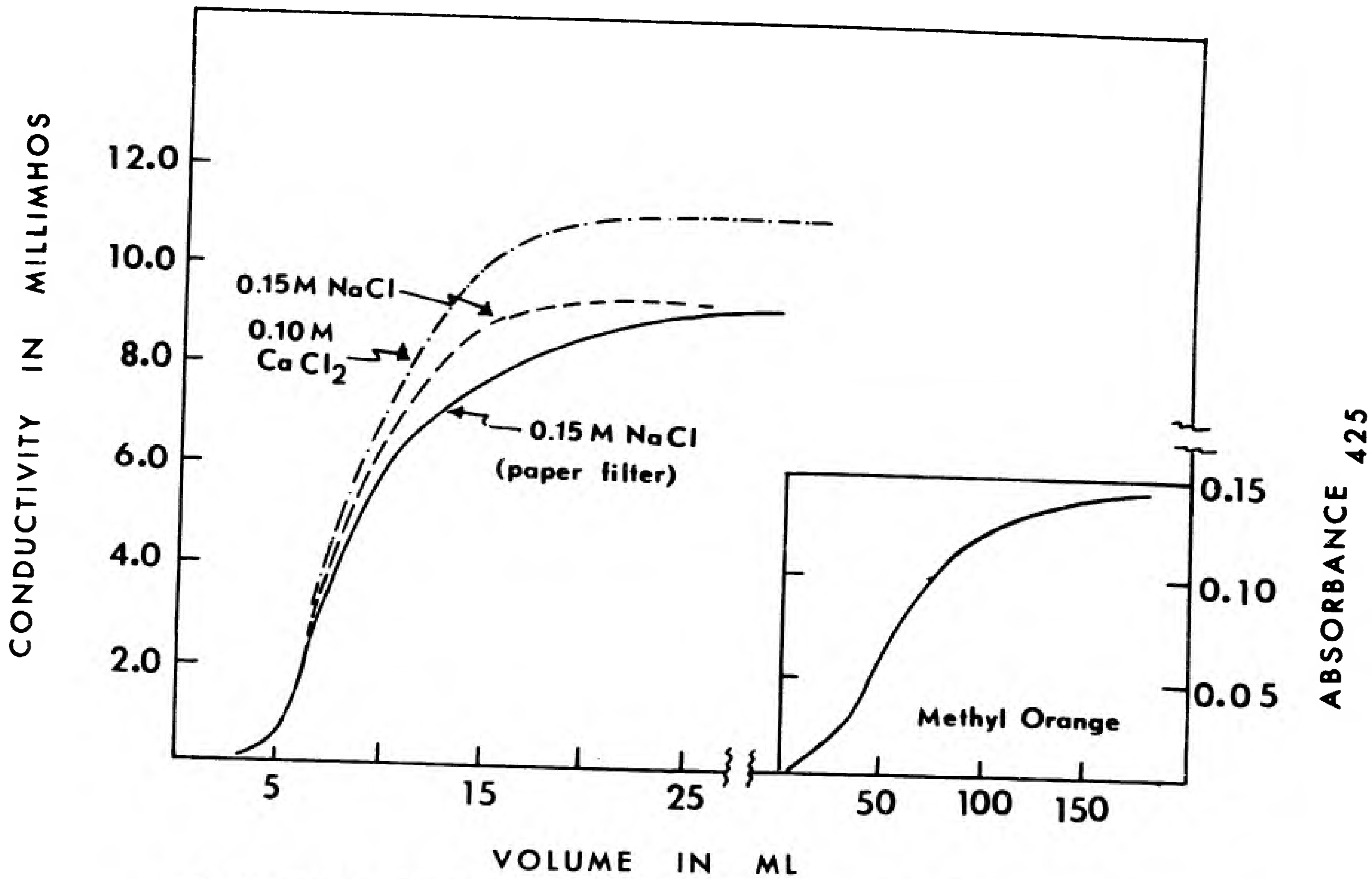


Fig. 10. The diffusivity of NaCl, $CaCl_2$ and methyl orange, through Diaflo membranes (retention; 10,000). The salt passage as evaluated by change in conductivity of the filtrate is shown by the broken lines, the heavy line denotes the passage of NaCl through a paper filter. (Exchangeable membrane surface area, 36.3 cm^2). The diffusivity of methyl orange (insert) was monitored by changes in the absorbance of the filtrate solution. Another assembly was used with an exchangeable membrane surface of 11.3 cm^2.

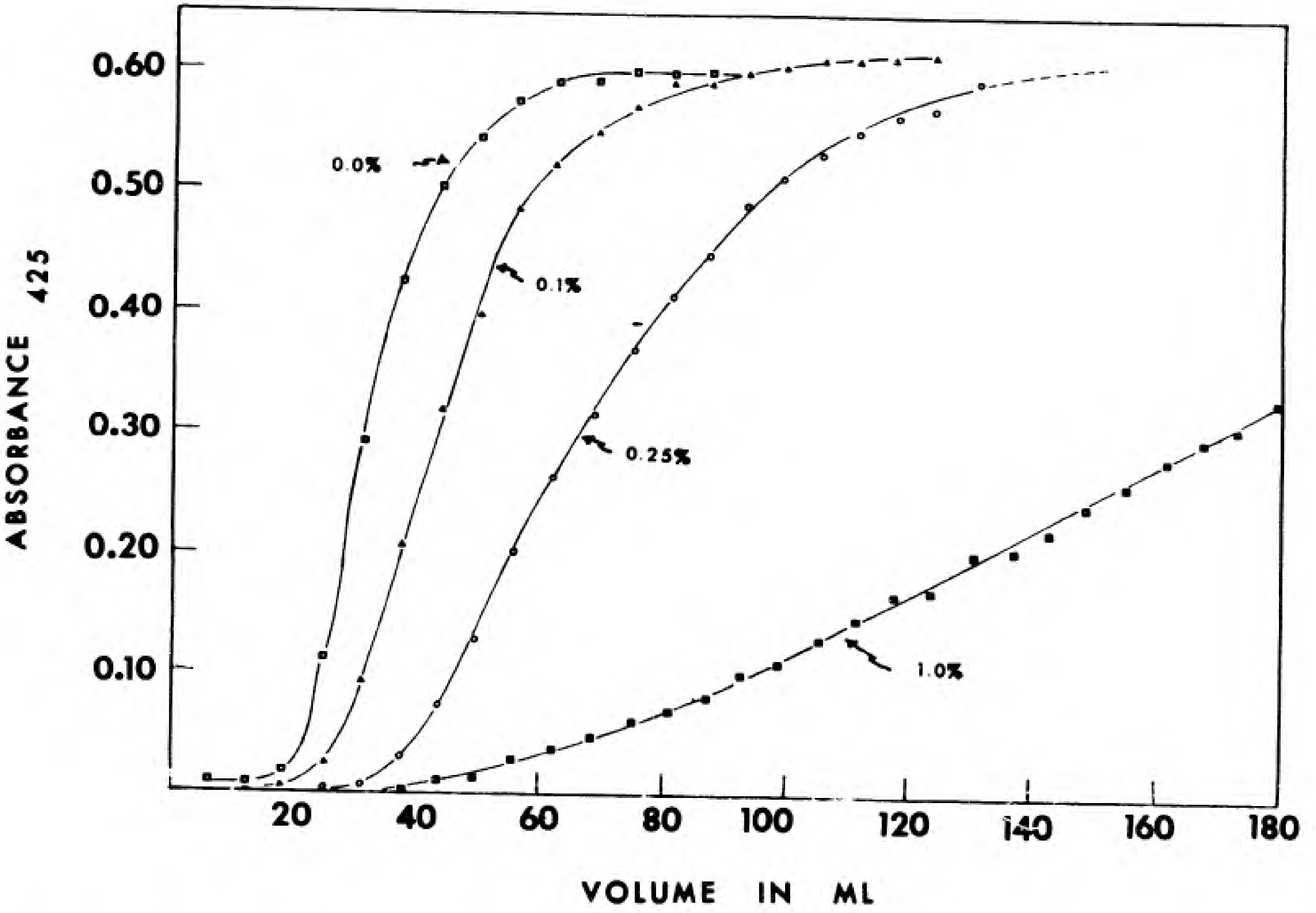


Fig. 11. The diafiltration of human serum albumin in phosphate buffered saline, pH 7.4, against 2.45×10^{-5}M methyl orange in the same solvent. Volume in the filtration cell, 10 ml; temperature, 22 ± 2C. The figures in % denote the albumin content.

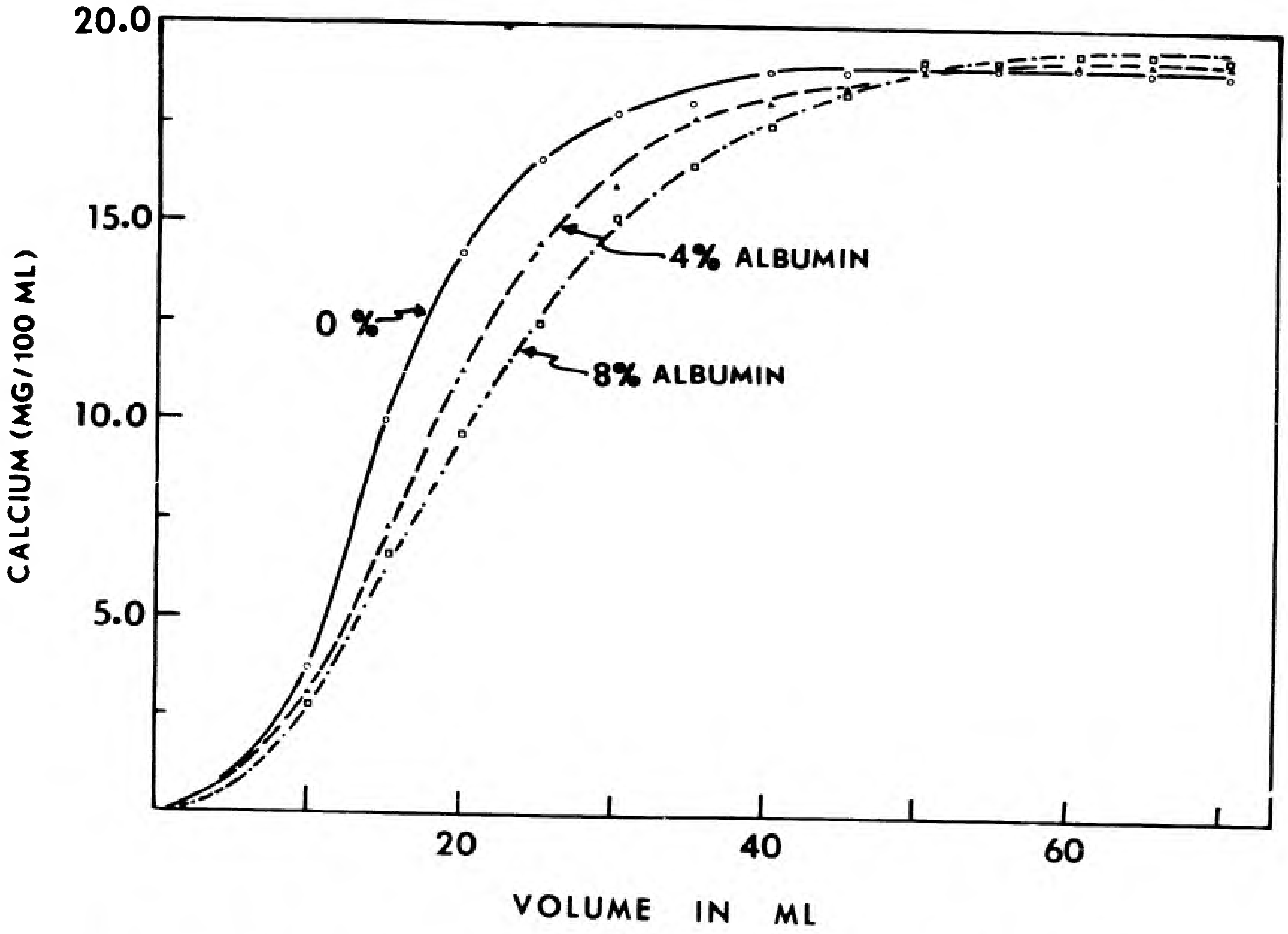


Fig. 12. The diafiltration of human serum albumin in TRIS-HCl, pH 7.4, against 5×10^{-3}M $CaCl_2$ in the same solvent. Volume in the filtration cell, 10 ml, temperature 22 ± 2C. The figures in % denote the albumin content.

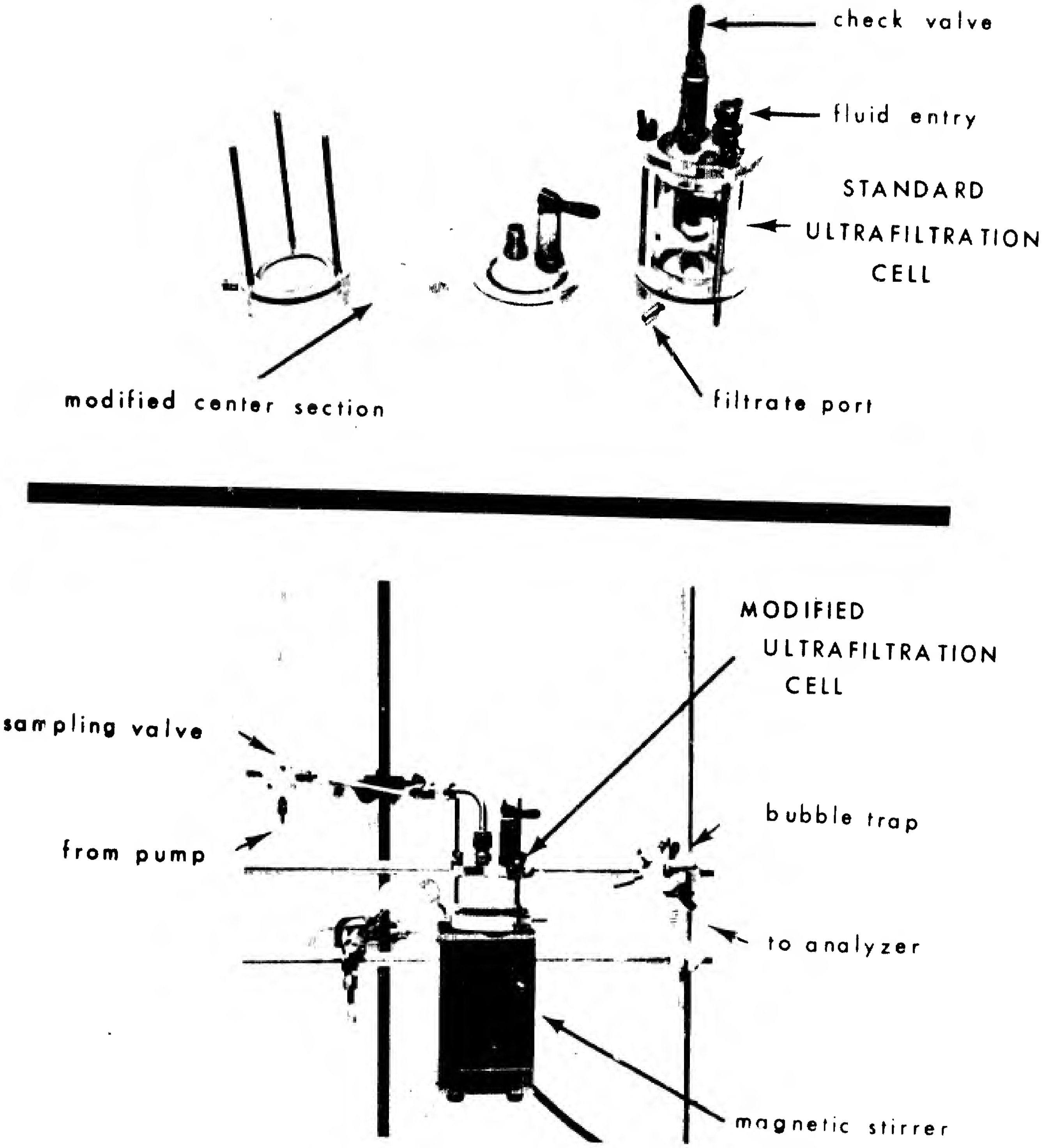


Fig. 13. Upper: Modified center section of a flow-through ultrafiltration cell contrasted with the standard assembled unit. Lower: Complete assembly with sampling valve and bubble trap. (From reference 3).

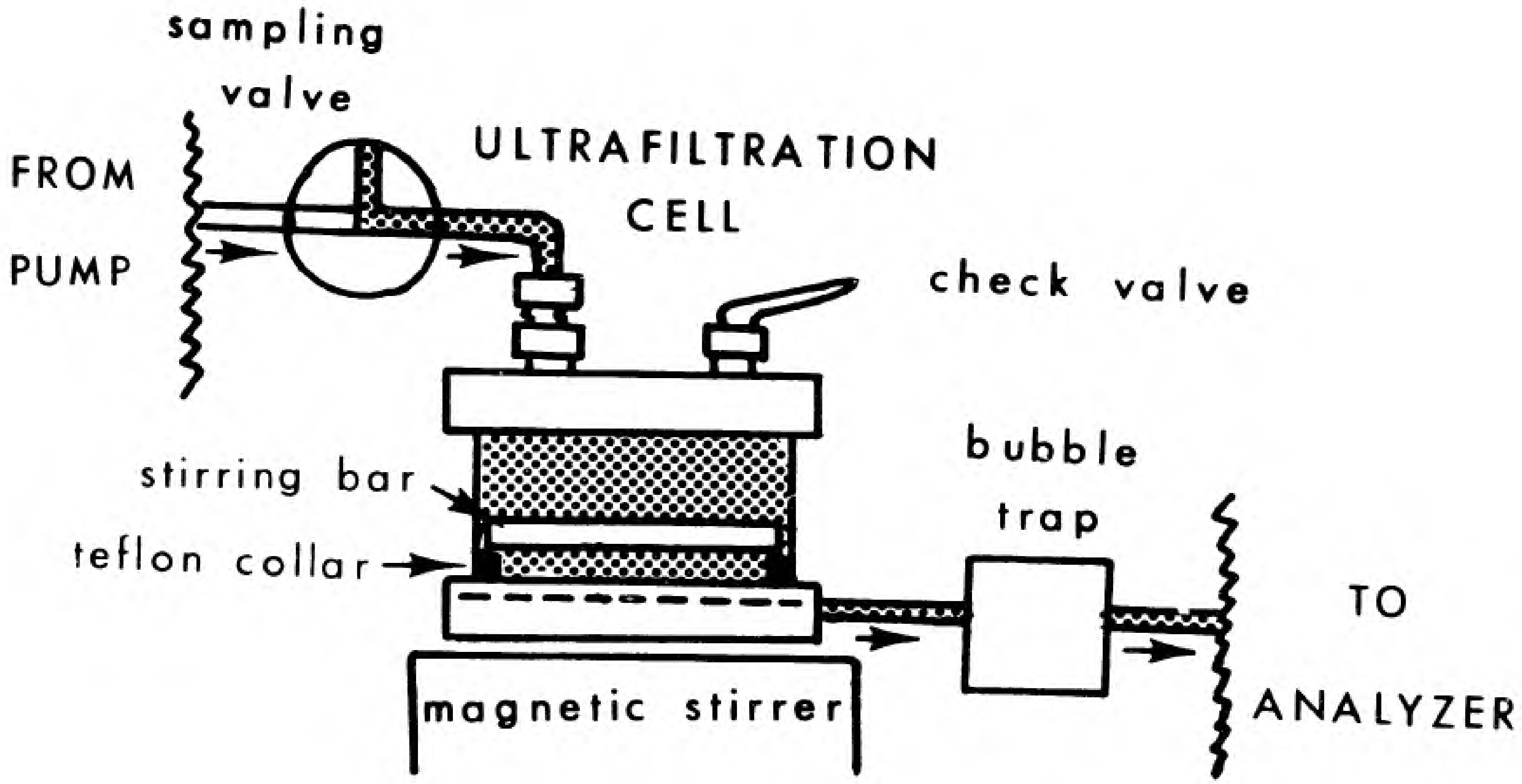


Fig. 14. Constant flow ultrafiltration cell (16.5 ml capacity) with buffer and sample introduction valve, ultrafiltrate egress port, bubble trap and entry to the UV analytical system. The dashed line above the stirrer denotes the ultrafiltration membrane. (From reference 5).

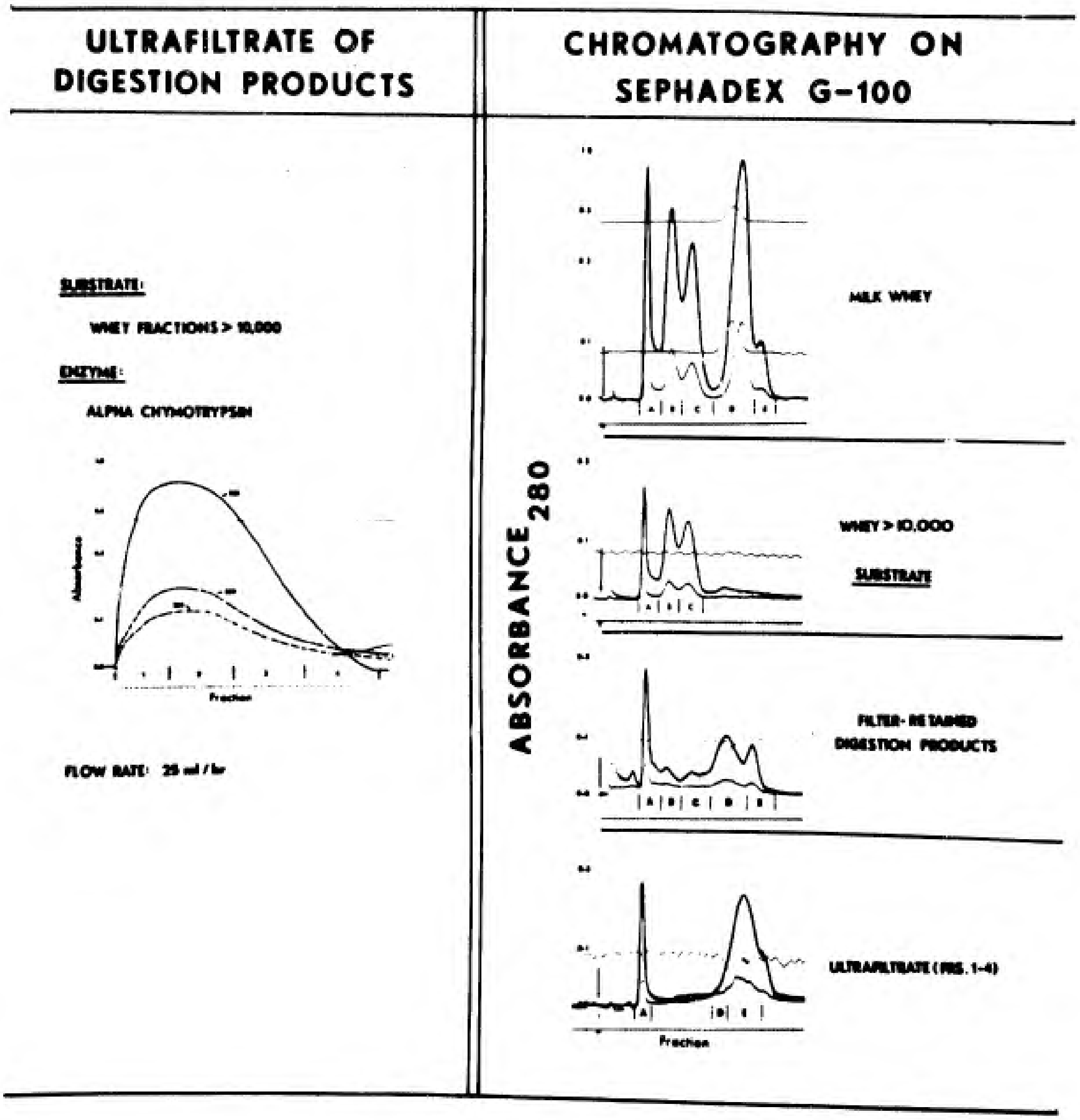


Fig. 15. Left: Absorbance (225, 260 and 280 mμ) of the ultrafiltrate obtained during the alpha chymotrypsin digestion of the higher molecular weight components (>10,000) of bovine milk whey. Right: Gel chromatography of (a) bovine milk whey, (b) the fraction used as substrate, (c) the filter retained digestate, and (d) the ultrafiltrate of this latter step. Phosphate-buffered saline, pH 7.4, was used for elutions on the 1.5 x 50 cm columns of Sephadex G-100, at a flow rate of 25 ml/hr. (From reference 3).

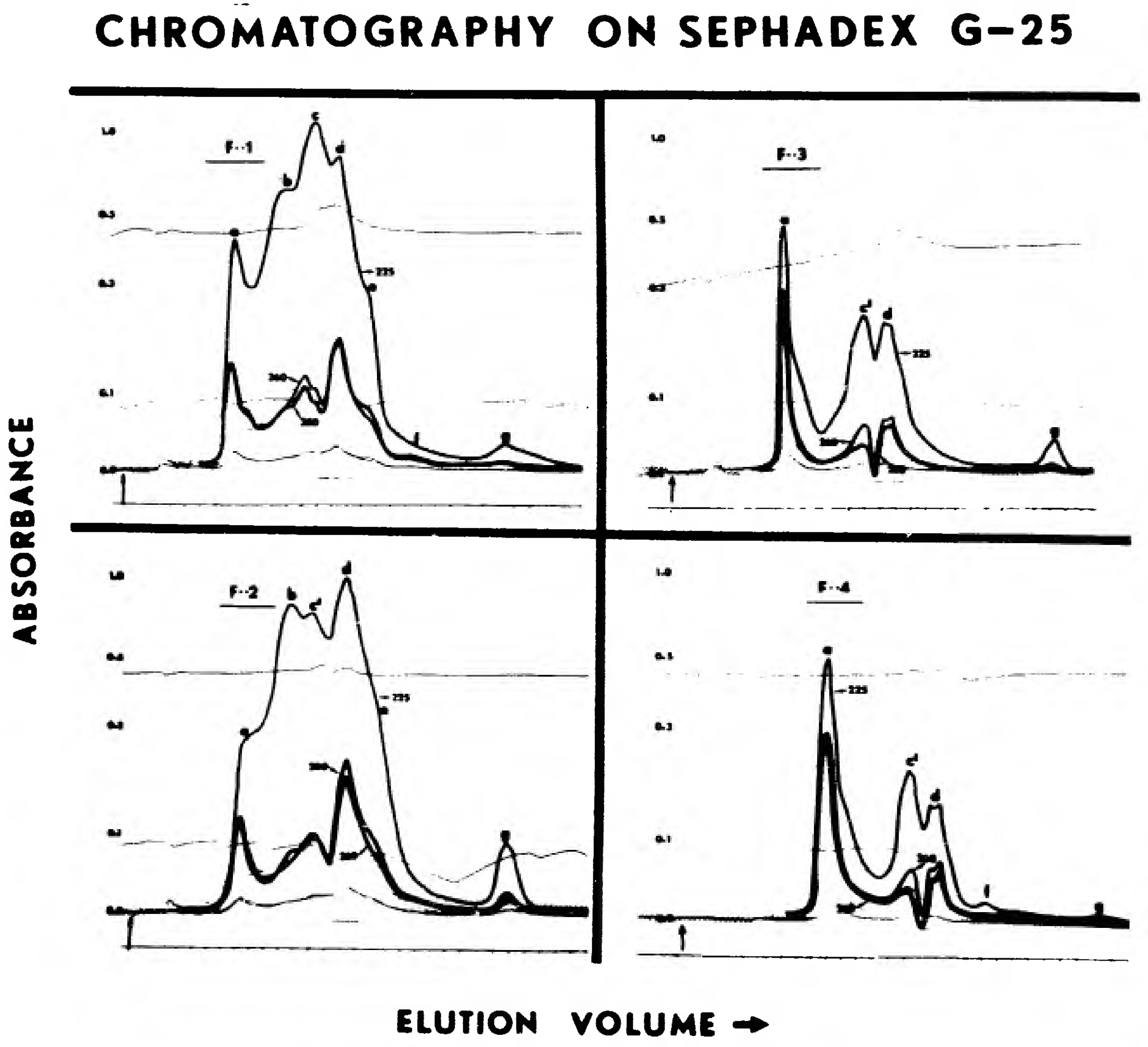


Fig. 16. Gel chromatography of the subfractions of the ultrafiltrate obtained during proteolysis of modified milk whey on 0.5 x 50 cm columns of Sephadex G-25, using phosphate-buffered saline as the eluting fluid at a flow rate of 25 ml/hr. (From reference 3).

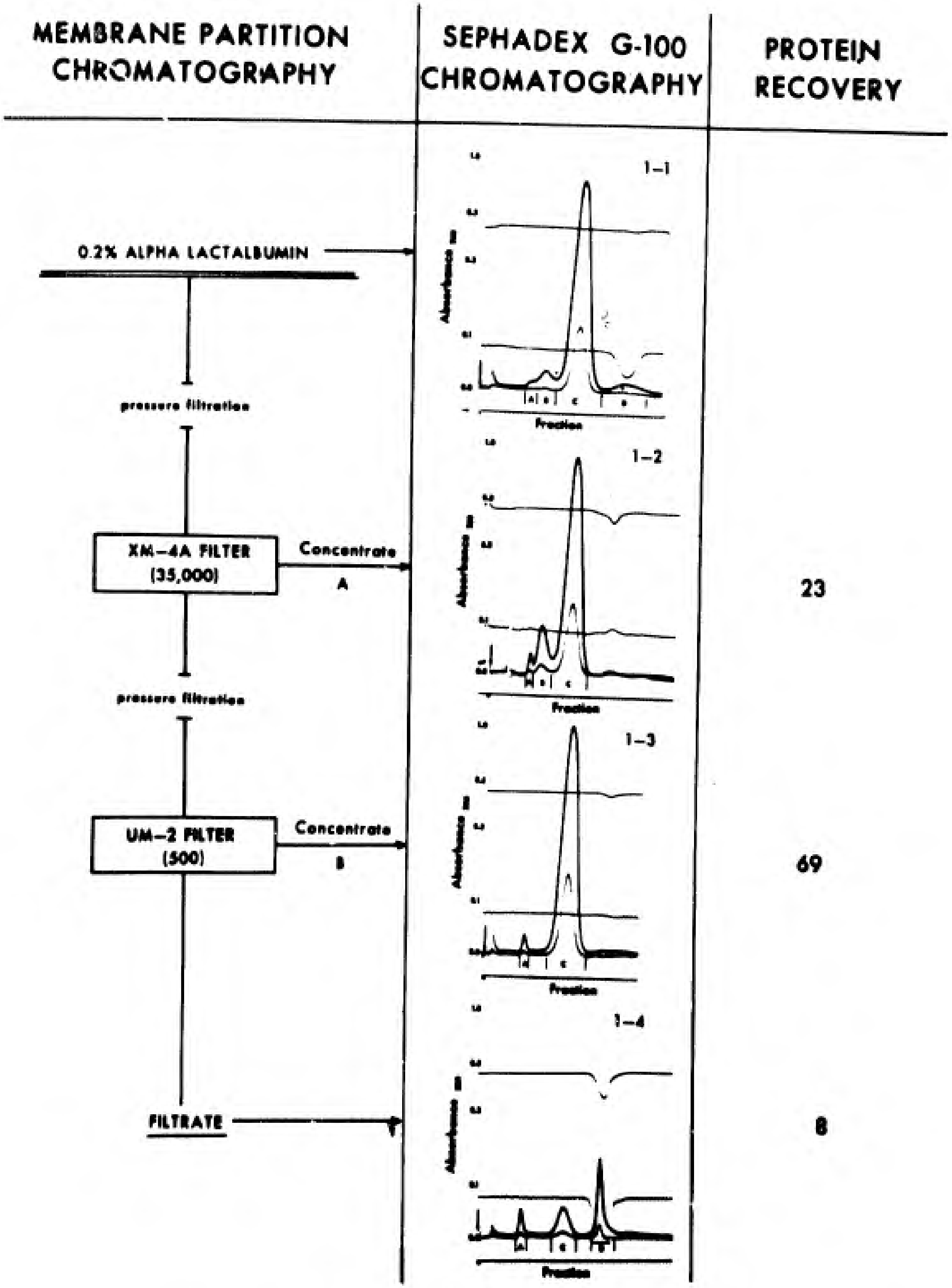
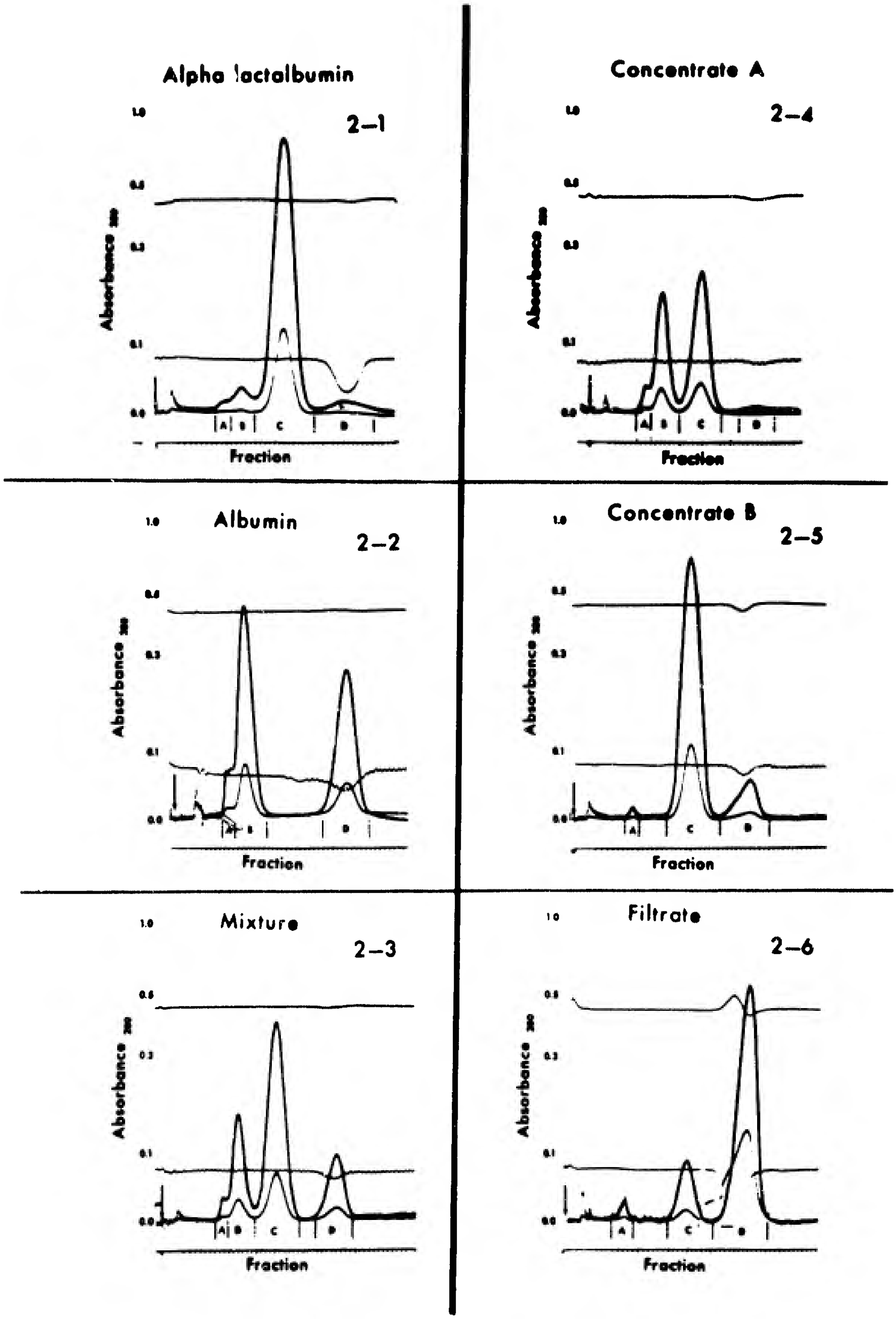


Fig. 17. Separation, chromatographic evaluation and yields of bovine alpha lactalbumin. Left: separation scheme (membrane partition chromatography). Center: Gel diffusion chromatography of the membrane-prepared fractions on 1.5 x 50 cm columns of Sephadex G-100. Right: Distribution in percent of the total recovery of protein for the indicated fractions. (From reference 4).



Alpha lactalbumin 2–1

Concentrate A 2–4

Albumin 2–2

Concentrate B 2–5

Mixture 2–3

Filtrate 2–6

Fig. 18. Gel chromatography on 1.5 x 50 cm columns of Sephadex G-100. Identification of the concentrates and filtrate can be made from Fig. 17. (From reference 4).

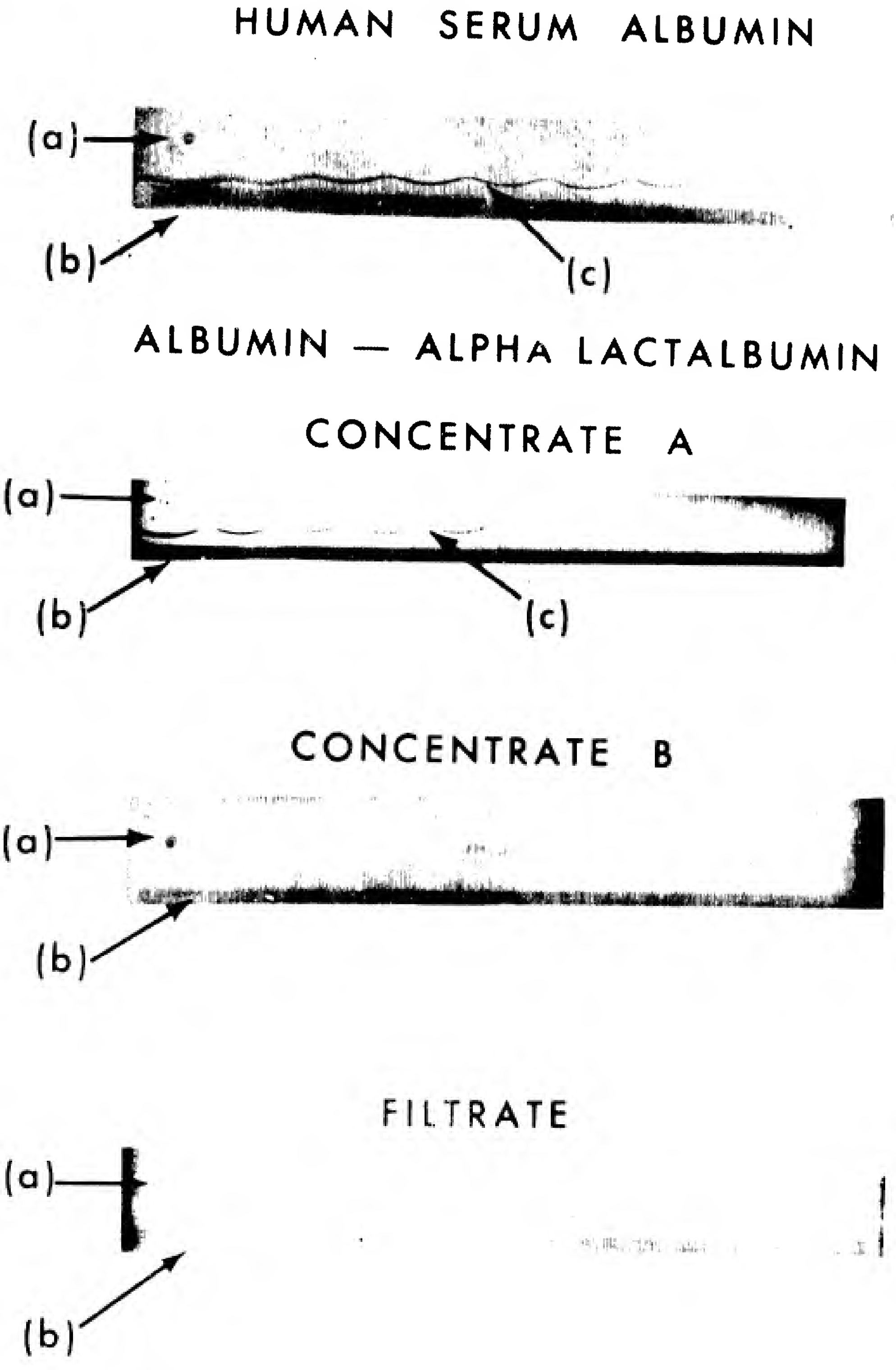


Fig. 19. Titration immunodiffusion on cellulose acetate (albumin and bovine alpha lactalbumin admixed fractions): (a) antigen well, (b) antisera trough, (c) immunoprecipitin lines indicating reaction. (From reference 4).

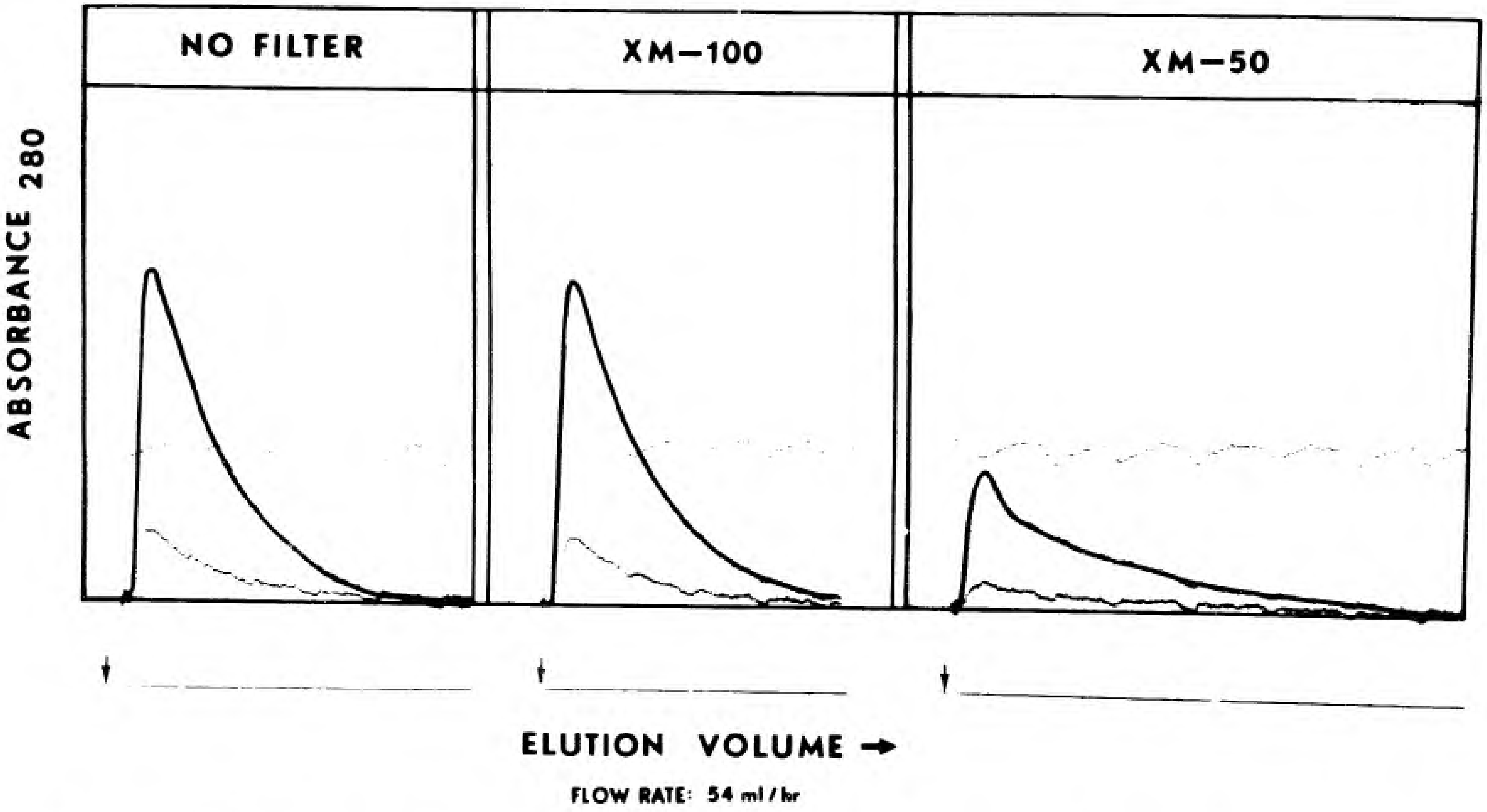


Fig. 20. The absorbance (logarithmic) at 280 mµ of the ultrafiltrate of bovine serum albumin through filters graded at 100,000 and 60,000 exclusion, respectively, contrasted with a non-filter system. Flow rate 54 ml/hr; total protein, approximately 10 mg. (From reference 5).

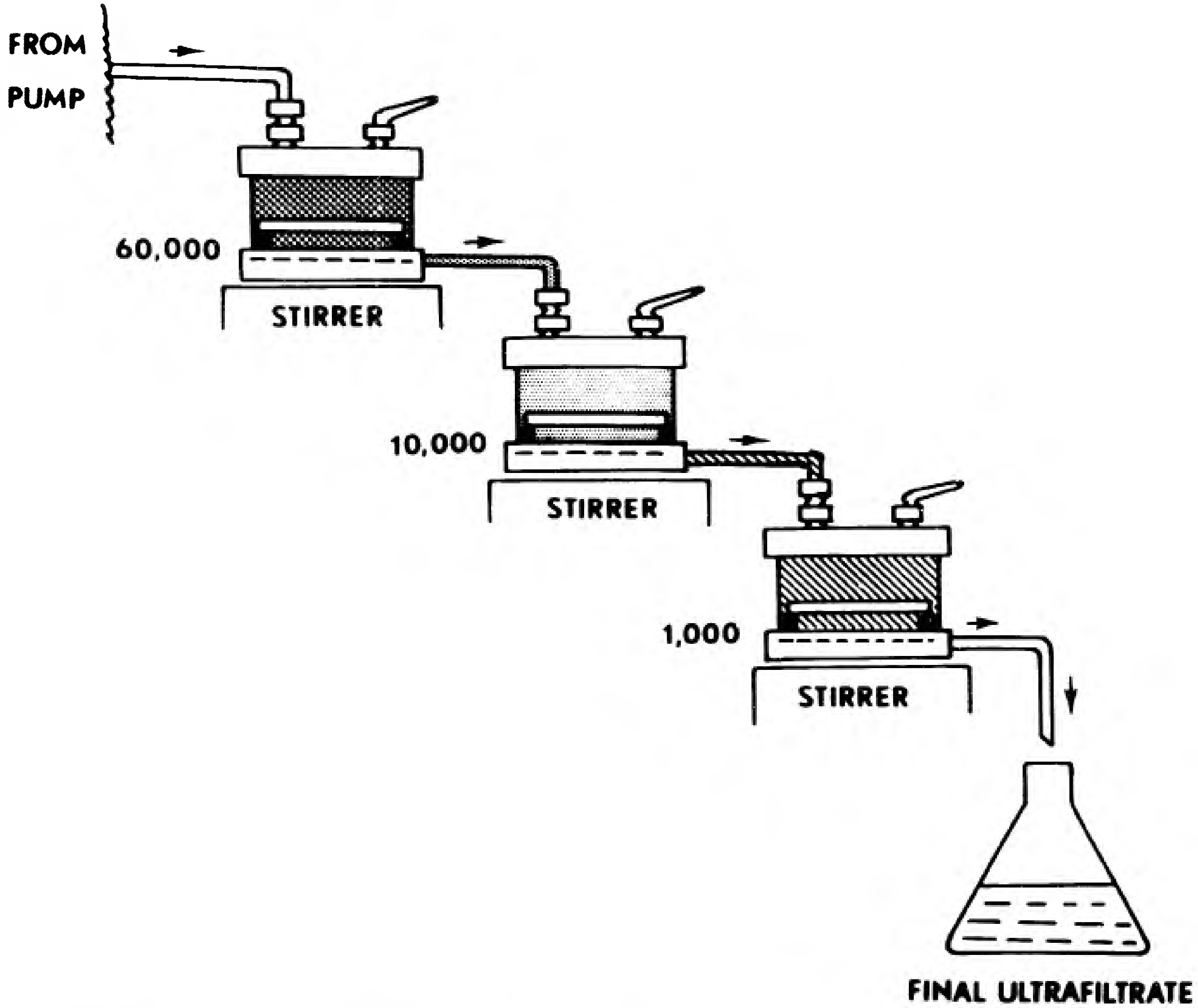


Fig. 21. A three-chamber tandem assembly with membranes graded at 60,000, 10,000 and 1,000, respectively. Each cell capacity, 16.5 ml.

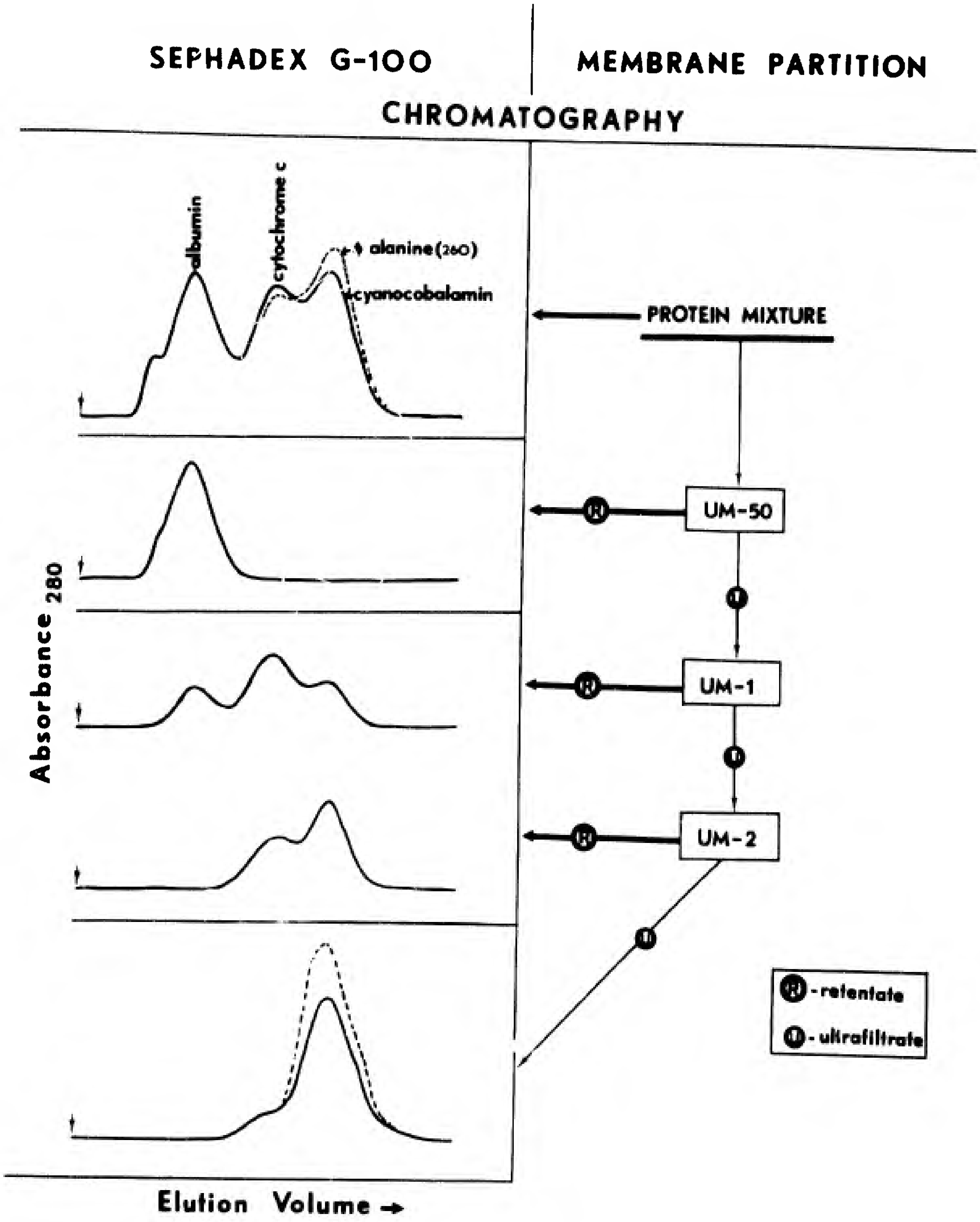


Fig. 22. The membrane partition scheme and the Sephadex G-100 separation patterns of the following protein mixture: albumin, cytochrome c, cyanocobalamin and phenylalanine. Flow rate, 10-15 ml/hr; temperature, 4C. (From reference 5).

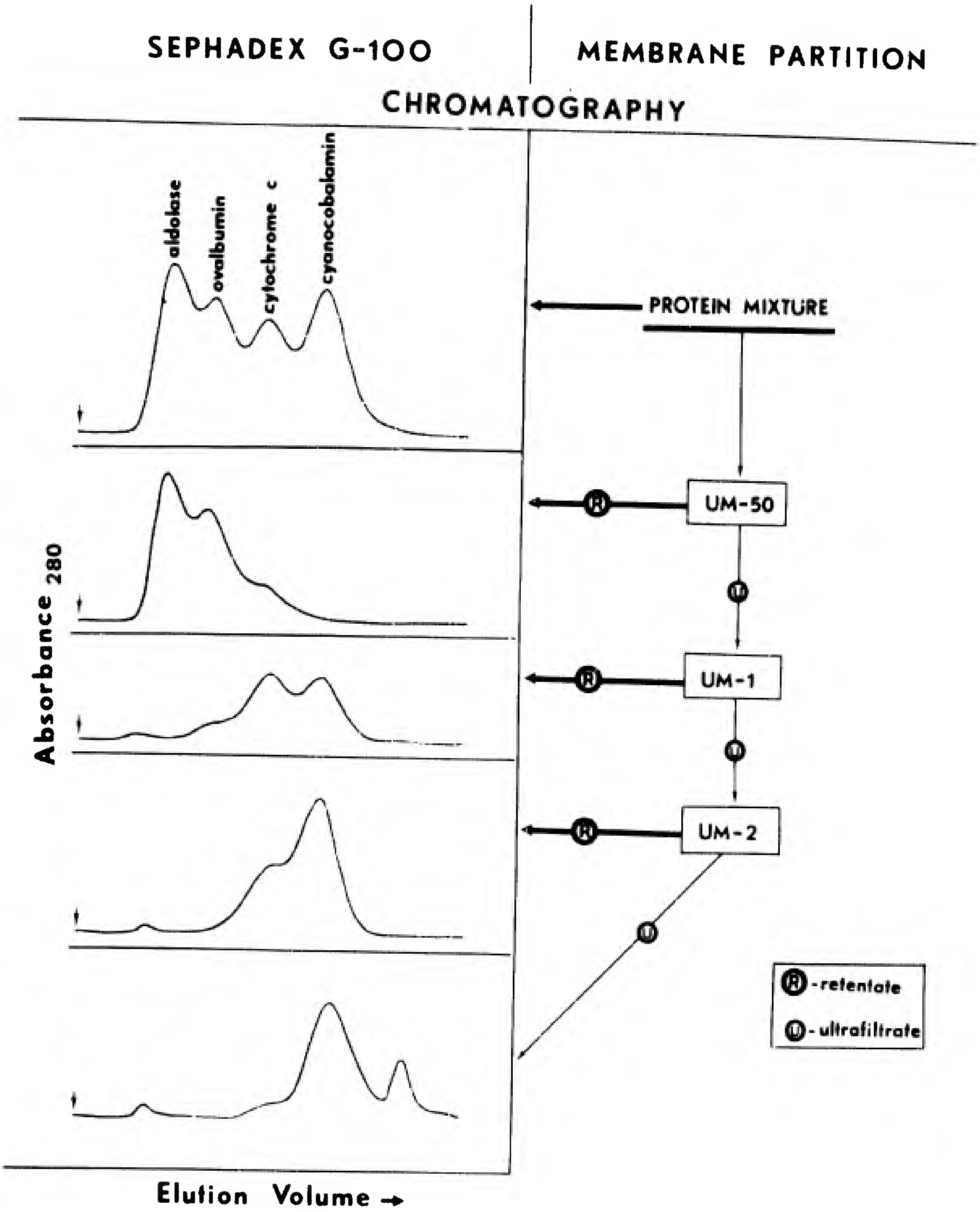


Fig. 23. The membrane separation scheme and the Sephadex G-100 separation patterns of the following protein mixture: aldolase, ovalbumin, cytochrome c, and cyanocobalamin. Flow rate, 10-15 ml/hr; temperature, 4C. (From reference 5).

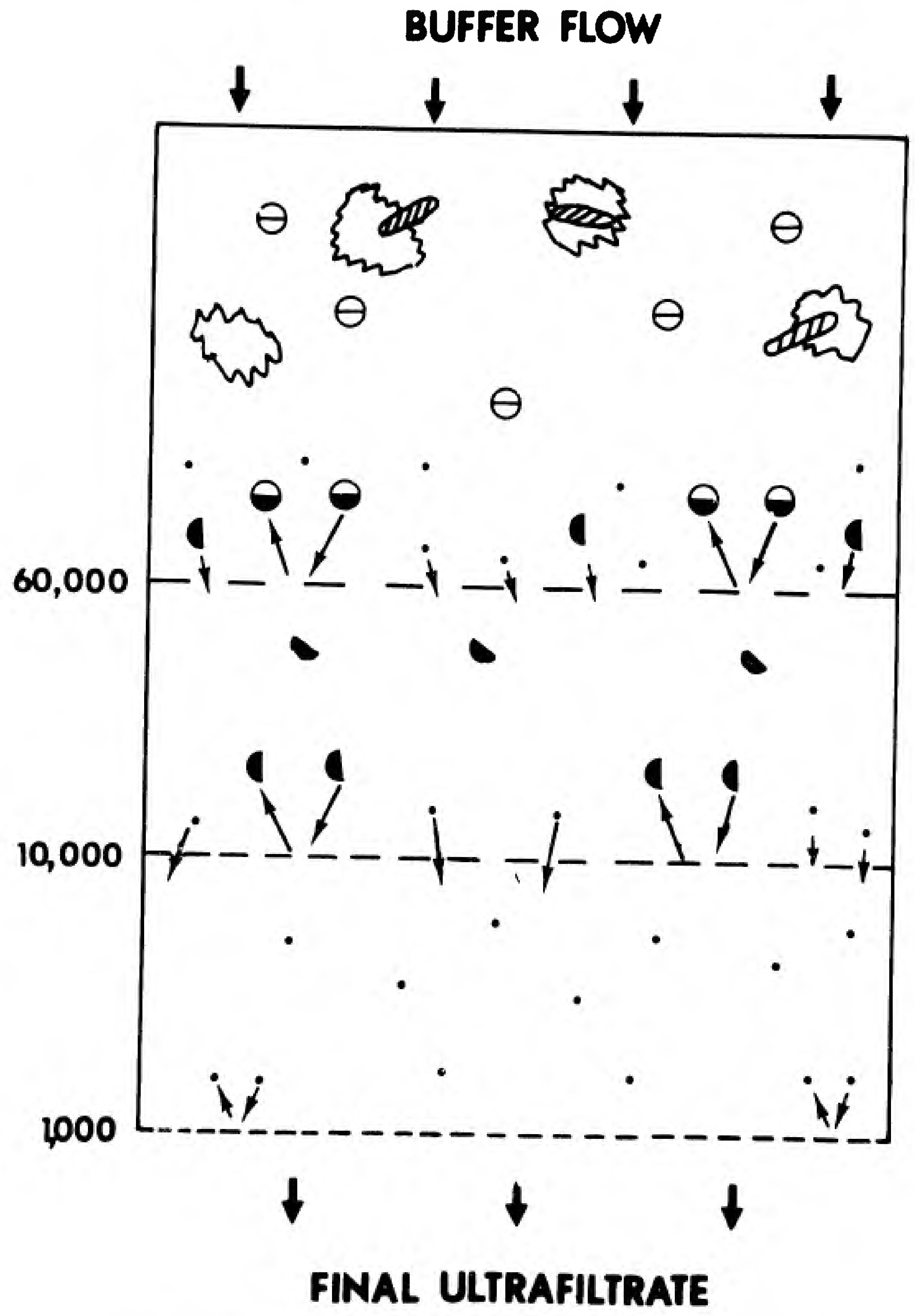


Fig. 24. The digestion of antigenic substrate by polyacrilamide-coupled enzyme in a tandem membrane assembly. Free gel particles, enzyme totally entrapped and active enzyme gel, as well as native substrate (⊖), enzyme-modified substrate (◓), and proteolysis products (◗, •), are shown in the upper portion. The diffusion of the digestion products through the size-limiting membranes is shown by the appropriate symbols in the lower chambers.

TABLE 1. Concentration of protein solutions by Diaplex and conventional ultrafiltration (2).

Material	Concn. (mg/ml)	Initial Volume (ml)	Filtration Rate (10^{-2}ml min^{-1}cm^{-2})		Final Volume[3] (ml)		Protein Recovery (%)	
			DUF[1]	CUF[2]	DUF	CUF	DUF	CUF
Hu 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	---
Hu Fr. III	0.62	250	5.4	0.14	16.5	15.0	91.2	75.7
Hu Fr. IV-1	0.57	250	5.4	0.16	12.5	15.0	83.5	77.4
Hu Fr. V	1.01	250	10.9	0.22	10.5	15.0	90.5	91.9
Phosphate-saline	0.15M	250	11.8	---	17.3	---	---	---
Distilled HoH	---	250	16.3	0.62	16.0	---	---	---

[1] Pressure used in the Diaflo ultrafiltration cell (DUF) was 100 psi and the effective surface area was 20.2 cm^2

[2] Rate given for conventional ultrafiltration (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 60 psi for all runs save water where 100 psi was used. The area of exchangeable surface was 243 cm^2.

[3] Final volume in all cases includes a chamber rinse.

TABLE II. The electrophoretic distribution of starting material and ultrafiltration concentrates (2).

Electrophoretic	Hu Serum			Fr. III			Fr. IV-1		
Fraction	Pre	DUF[1]	CUF[2]	Pre	DUF	CUF	Pre	DUF	CUF
Albumin	66	64	69	--	--	--	18	18	20
Alpha-1-glob	3	3	3	11	10	10	82	82	80
Alpha-2-glob	8	8	8	26	30	28	--	--	--
Beta-glob	10	12	7	63	60	62	--	--	--
Gamma-glob	13	13	12	--	--	--	--	--	--

[1] Diaflo ultrafiltration

[2] Conventional ultrafiltration (Visking tubing)

TABLE III. The binding of methyl orange to human serum albumin as determined by membrane ultrafiltration.

Initial Concentration (x 10^{-5}M)		Dye Concentration (x 10^{-5}M)		Final Cell	Total Bound	Molar[1]	
Protein C_p	Dye C_o	Cell C_T	Filtrate C_F	Volume V_T	Dye $V_T\ (C_T - C_F)$	Binding Ratio	
0	0.50	0.55	0.52	7.7	0.23[2]	---	
0	0.49	0.52	0.44	6.0	0.48[2]	---	
0.725	0.49	1.47	0.49	6.8	6.66	0.92	>1.09
0.725	0.49	1.68	0.49	7.6	9.04	1.25	
1.450	0.49	2.84	0.50	8.0	18.72	1.29	>1.17
1.450	0.49	2.62	0.46	7.0	15.12	1.04	
2.175	0.49	3.13	0.45	8.8	23.58	1.08	
0.725	2.83	5.66	2.77	6.8	19.65	2.71	>2.75
0.725	2.87	5.96	2.83	6.4	20.16	2.78	

[1] Initial volumes of 10 ml (V_o) were placed in the filtration cell. Calculation of the molar binding ratio was from the following:

$$M.B.R. = \frac{V_T(C_T - C_F)}{V_o\ (C_p)}$$

[2] Insofar as no protein was in the filtration cell, the values reported as bound dye represent the microsolute rejection by the membrane.

TABLE IV. The binding of calcium to human serum albumin as determined by membrane ultrafiltration.

Initial Concentration ($\times 10^{-3}$M)		Ca Concentration ($\times 10^{-3}$M)		Final Cell Volume	Total Ca Bound	Molar Binding Ratio[1]	
C_p	C_o	Cell C_T	Filtrate C_F	C_T	$V_T(C_T-C_F)$		
0	4.74	4.43	4.74	7.8	---	---	
0	4.92	4.67	4.82	11.6	---	---	
0.29	4.53	5.53	4.55	8.6	8.43	2.91	>3.22
0.29	5.00	6.25	5.03	8.4	10.25	3.53	
0.58	4.98	6.65	4.75	10.8	20.52	3.54	>3.65
0.58	4.83	6.65	4.97	13.0	21.84	3.76	
1.16	5.00	7.75	4.92	10.0	28.30	2.44	>3.04
1.16	4.95	9.01	4.78	10.0	42.30	3.64	
0.29	19.13	21.25	19.08	11.0	23.87	8.23	>8.15
0.29	19.13	20.48	19.18	18.0	23.40	8.07	
0.29	95.00	98.15	96.13	10.2	20.60	7.10	>6.35
0.29	95.60	95.25	94.00	13.0	16.25	5.60	

---

[1] Initial volume (V_o) of 10 ml within the diafiltration cell. Calculation as shown in Table 3.

TABLE V. Protein retentive capacity of size-selective membranes (5).

Compound	Mol. Wt.	% Retention				
		XM-100 (100,000)[1]	XM-50 (60,000)	XM4-A (35,000)	UM-1 (10,000)	UM-2 (1000)
Apo-Ferritin	480,000	100[2]	100	---	---	---
Gamma Globulins	160,000	100[2]	100	---	---	---
Aldolase	142,000	0	85	100	100	100
Albumin	67,000	0	68	100	100	100
Hemoglobin	64,500	0	62[2]	70	100	100
Ovalbumin	45,000	0	77	98	100	100
Chymotrypsinogen A	25,000	0	0	0	90	100
Alpha-Chymotrypsin	24,500	0	0	0	95	100
Trypsin	20,000	0	0	0	95	100
Myoglobin	17,800	0	0	0	88	100
Cytochrome c	12,400	0	0	0	85	100
Cyanocobalamin	1,355	0	0	0	0	0[3]
Phenylalanine	165	0	0	0	0	0

[1] Values in parenthesis represent arbitrary cutoff limits ascribed to the various membranes.

[2] Membrane plugging was observed with these compounds as evidenced by a considerable increase in pressure during ultrafiltration. This was not observed in the non-porous membranes (35,000 and below).

[3] With the other compounds, diffusivity, where present, was effected at a rate comparable to the clearance observed in a non-filter system. However, with cyanocobalamin, a slow escape was noted, and given sufficient time, complete passage through the membrane was obtained.

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13. ABSTRACT

(U) Preformed ultrafiltration membranes, with molecular weight exclusion limits varying from 1,000-100,000, are uniquely suited for preparative methods in protein chemistry. The applications include non-denaturing protein concentration, rapid dialysis, micro solute binding, limited purification, and fractionation of complex mixtures. In the latter procedures, selective ultrafiltration obviates the use of cumbersome electrophoretic methods, or initial column chromatography. Increases in the separation rate, as well as the total quantity of material fractionated, can be obtained by increasing the exchangeable surface of the filter. (U)

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