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ULTRAFILTRATION OF PROTEINS THROUGH CELLOPHANE MEMBRANES OF ENHANCED PERMEABILITY

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SUMMARY

Proteins were ultrafiltered through cellophane membranes made permeable to large molecules by the swelling effect of zinc chloride. The time course of filtration follows a characteristic curve with an initial lag period, a sigmoid phase of rising protein output, a steady-state plateau at the level of the input concentration. The time scale of the filtration curve depends mainly on the molecular size of the protein. A mathematical expression has been derived on the basis of a diffusion model to describe the progressive changes of protein concentration in the ultrafiltrate.

INTRODUCTION

Dialysis and ultrafiltration offer a means of effecting molecular separations on the basis of differential migration through a barrier membrane. The removal of salts from high molecular weight solutes has been the main field of application of these methods[1]. An extension of the dialysis technique to achieve separations among large molecular species was made by CRAIG *et al.*[2,3] and this new technique was applied by them to the fractionation of polypeptides and proteins. Since simple dialysis is inherently a slow process, physical methods have been employed to accelerate protein transport through membranes; application of an electric field in electrodialysis[4,5], application of hydrostatic pressure in ultrafiltration[6–10]. Of special importance for protein fractionation was the development by MCBAIN AND STUEWER[11] of a chemical procedure, the swelling of cellophane in zinc chloride, to enlarge pore dimensions in a graded manner to permit passage of proteins according to molecular size[5,12,13].

Our present studies on the ultrafiltration of a number of purified proteins through zinc chloride-treated cellophane provide data on transmission rates in relation to molecular size. The time course of the filtration was followed with analytical measurements of protein output in the effluent solution. By assuming a diffusion mechanism to be at the basis of ultrafiltration we have arrived at a mathematical expression to describe the progressive changes in the protein concentration of the ultrafiltrate.

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EXPERIMENTAL

Filter holder

We have used the High Pressure Filter Holder (Millipore Filter Corp.) for 47-mm diameter filters, and the Laboratory Sterilizing Filter Holder (Millipore) for larger filters of 142 mm diameter. The filter disc is supported on a stainless steel screen assembly. The solution chamber is sealed against leakage by a rubber or teflon o-ring fitted into a channel in the upper plate. When agitation of the filtering solution is desired, several glass or plastic beads are suspended in the fluid and the filter holder is mounted on a reciprocating shaker. For our timed studies we have replaced the top plate of the commercial filter holder by one with a smaller solution chamber; 1.0 ml for the 47-mm filter, and 20.0 ml for the 142-mm filter (Fig. 1). The smaller chambers are provided with inlet and outlet ports to facilitate interchange of solutions.

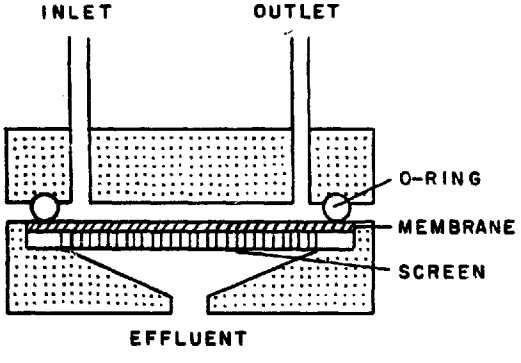

Fig. 1. Disc type ultrafilter.

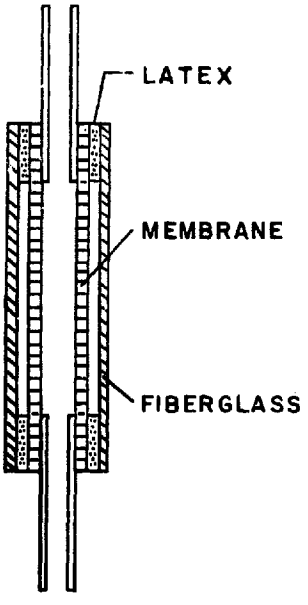

Fig. 2. Tubular ultrafilter.

The tubular type of filter membrane is attached to polypropylene or teflon tubing (1/8 in outside diameter) leading from a pump or from a pressurized reservoir (Fig. 2). Several grooves are impressed near the tip of the plastic tube to fashion a serrated hose nipple. The cellophane tube, in the vicinity of its attachment, is surrounded by a protective cuff of thin latex tubing (Penrose drainage tubing) and around this is applied a cotton or nylon ligature. Control valves are attached at both ends of the filter to aid in filling and wash-out operations. In order to limit the degree of inflation under pressure, the cellophane filter tube is surrounded by a tubular jacket of fiberglass cloth. Fiberglass sleeving is available commercially for electrical insulation in a variety of sizes, covering the useful range of 0.2 in to 0.4 in inflated diameter (Thermoflex 1200, L. F. Markel & Sons).

A pressure head up to 60 lbs/in^2 is established by applying air pressure to the solution reservoir or by pumping fluid into the filter chamber with a chromatographic Minipump (Milton Roy Co.). The effluent flow rate runs from 5 to 300 ml/h depending upon the size and porosity of the membrane and upon the applied pressure.

Filter membranes

Filter discs are supplied by the Millipore Filter Corp. (MF, Type VF and VM) and by Carl Schleicher and Schuell Co. ("Ultraflex" Ultrafine Membrane Filters, Type UA). Experimental filters of cellophane were prepared in the laboratory by the McBAIN–STUEWER zinc chloride procedure. A flat strip cut from dialysis tubing (Visking, 2.25 in diameter, inflated) is suspended in zinc chloride solution, 65 % (w/w), for time periods of 0.5–1 h and the treated sheet is then transferred to rinse water. The membrane becomes gelatinous and friable in the chemical solution but regains toughness and elasticity after the water wash. When immersed in the zinc chloride the cellophane sheet should be kept free of folds and out of contact with solid structures. The strip is supported by a stainless steel film clip attached along a dry edge kept out of the chemical bath. Rinse solutions are passed through by pressure filtration; 0.1 N hydrochloric acid, 0.01 N acetic acid, buffer solution. Tubular filters are prepared from seamless cellophane dialysis tubing (Visking, 8/32 in diameter, inflated). A 10 in length is attached at the upper end by ligature to a glass tube. At the lower end, the cellophane tubing is closed with a short length of glass tubing the end of which has been drawn down to a thin sealed tip. The cellophane tubing is filled to within an inch of the upper glass tube with zinc chloride solution and is suspended in a glass cylinder containing the same chemical solution. Fluid levels are kept the same inside and outside the cellophane. At the conclusion of the treatment period the glass seal at the lower end is broken and the cellophane tube is withdrawn and rinsed. The filter is further washed by pressure filtration.

Proteins

Sources of supply were Worthington Chemical Co. for α-chymotrypsin (3 × crystallized); Pentex Inc. for β-lactoglobulin (3 × crystallized), human serum albumin (1 × crystallized), ovalbumin (5 × crystallized), and γ-globulin (Fraction II). The crystallized proteins had been dialyzed free of salt and were supplied as lyophilized preparations with an ash content of 3 % or less.

The proteins were dissolved in 0.01 N acetic acid in a concentration of 0.1 mg/ml to give a solution at pH 3.4 and of ionic strength $4.5–5 \cdot 10^{-4}$. Measurements of protein concentration were made with the LOWRY–FOLIN colorimetric method[14]. The ultrafiltrations were carried out at room temperature, but provision has been made to maintain the equipment at 0–4°.

RESULTS

The tubular type of ultrafilter permits of great flexibility in design. Both the cellophane dialysis tubing and the fiberglass sleeving are available in continuous lengths which can be cut to any size desired. The sleeving can be applied in a small diameter to prevent stretching of the inner filter tube, or in larger graduated diameters to provide different degrees of expansion. It has been technically inconvenient to handle the cellophane tubing in lengths greater than one foot in the zinc chloride bath. Sections of the chemically treated tubing can be connected with plastic couplings to form a filter of any desired length. Our previously reported studies[15] on oxytocin were carried out with tubular filters. The disc type filter is more conveniently manipulated in the zinc chloride solution. Since it is seated on a rigid stainless steel screen

the filter disc is not subjected to gross stretching so that the effects of chemical treatment can be isolated from those due to superimposed mechanical stretch. On the other hand, the disc filter does not permit of permeability enhancement by combining the effects of chemical treatment and physical extension. Commercial filter membranes are available only in the form of sheets and discs and this limits them to use in the disc type of filter holder.

Curves are plotted for the ratio of protein concentration in the effluent ultrafiltrate, c_e, relative to the concentration in the input reservoir, c_i. This ratio varies as a function of time, or as a function of the volume of filtrate collected. The volume flow is kept constant by occasional adjustment of filtration pressure. The ultra-

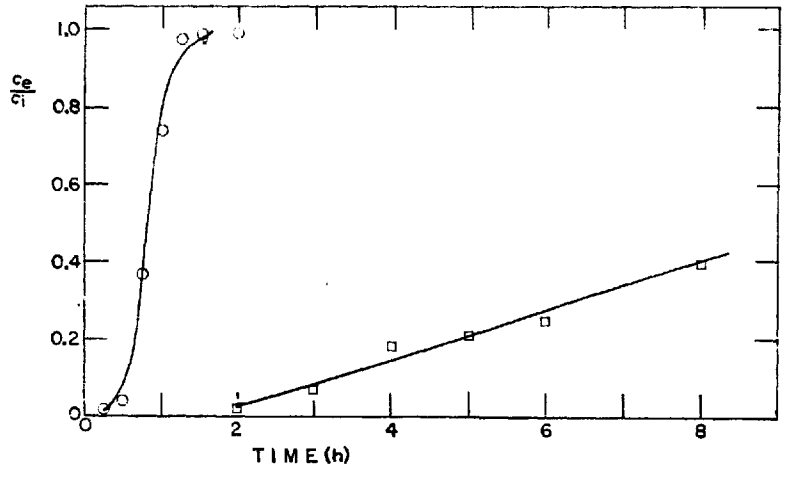

Fig. 3. Ultrafiltration through cellophane membrane given 30 min treatment in 65 % zinc chloride. O—O, α-chymotrypsin; □—□, human serum albumin. α-Chymotrypsin (mol. wt. 24500); D, $28 \cdot 10^{-10}$ cm²/sec; v_s, 0.2. Serum albumin (mol. wt. 69000); D, $2.8 \cdot 10^{-10}$ cm²/sec; v_s, 0. Membrane thickness (l), $5 \cdot 10^{-3}$ cm (original untreated dry membrane). Input protein concentration, 0.1 mg/ml; filtration pressure, 50 lbs/in²; flow rate, 5.7 ml/h; filter area, 12 cm²; protein solution, 0.01 N acetic acid (pH 3.4), $I = 5.10^{-4}$.

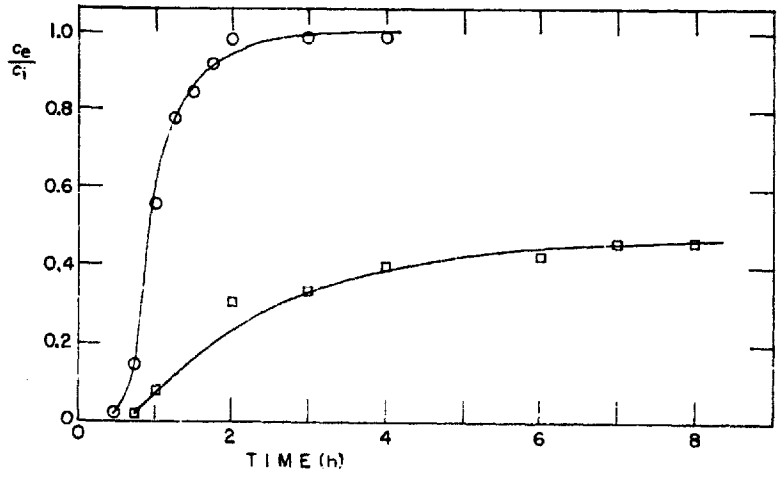

Fig. 4. Ultrafiltration through cellophane membrane given 45 min treatment in 65 % zinc chloride. O—O, β-lactoglobulin; □—□ ovalbumin. β-Lactoglobulin (mol. wt. 35000); D, $28 \cdot 10^{-10}$ cm²/sec; v_s, 0.2. Ovalbumin (mol. wt. 44000); D, $3.5 \cdot 10^{-10}$ cm²/sec; $v_s = 0$; l, $5 \cdot 10^{-3}$ cm. Input protein concentration, 0.1 mg/ml; filtration pressure, 50 lbs/in²; flow rate, 7.0 ml/h; filter area, 12 cm²; protein solution, 0.01 N acetic acid (pH 3.4), $I = 5 \cdot 10^{-4}$.

filtration curve shows a small lag period before measurable protein appears in the effluent. The curve has a sigmoid shape with an early inflexion point. The final plateau level approaches the value of the input concentration.

Three experiments are graphed (Figs. 3–5) to show the filtering characteristics of membranes with different degrees of augmented permeability. The membranes were treated in the same 65 % zinc chloride solution for graded time periods of 30, 45 and 60 min. In each experiment two proteins of differing molecular weight were filtered separately through the same membrane on successive days. The influence of molecular size is seen in the great divergence of the filtration curves. The smaller protein exhibits a shorter lag period and a more rapid rise toward the input level of concentration. As the permeability of the membrane is increased, one can differentiate between proteins of greater molecular dimensions.

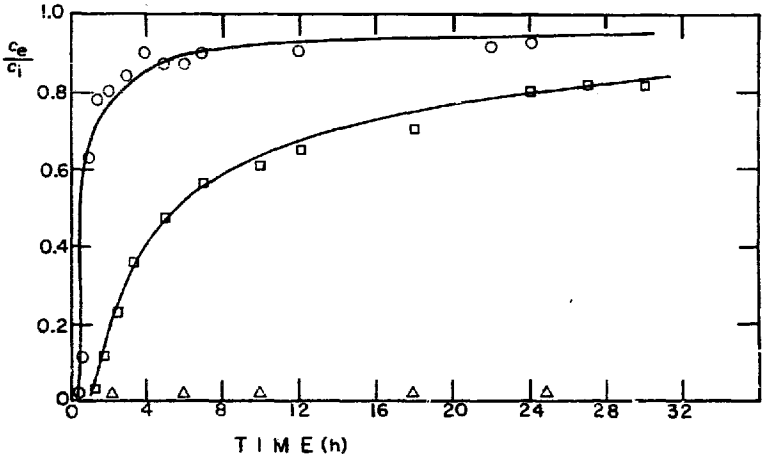

Fig. 5. Ultrafiltration through cellophane membrane given 60 min treatment in 65 % zinc chloride. O—O, ovalbumin; □—□, human serum albumin; △—△, γ-globulin. Ovalbumin (mol. wt. 44000); D, $14 \cdot 10^{-10}$ cm²/sec; v_s, 0.2. Serum albumin (mol. wt. 69000); D, $7 \cdot 10^{-10}$ cm²/sec; v_s, 0. γ-globulin (mol. wt. 160000); l, $5 \cdot 10^{-3}$ cm. Input protein concentration, 0.1 mg/ml; filtration pressure, 50 lbs/in²; flow rate, 8.4 ml/h; filter area, 12 cm²; protein solution, 0.01 N acetic acid (pH 3.4), $I = 5 \cdot 10^{-4}$.

THEORETICAL

In ultrafiltration the hydrostatic pressure head sets up a flow of water, J_w, across the membrane. A constant flux of solute, J_1, into the input surface of the membrane is established.

$$J_1 = c_1 J_w, \tag{1}$$

The fluid stream within the membrane exerts a frictional drag on the solute molecules tending to drive them through the membrane. Passage of the particles is impeded by frictional interaction with the structural matrix of the membrane[16]. Multiple impacts between the solute molecules and the macromolecular structures within the membrane impart random displacements to the solute particles that might be considered analagous to Brownian motion. This model suggests that the transport of solute through the membrane may be subject to the mathematical laws of diffusion.

Ultrafiltration as a diffusion process

The basic differential equation governing linear diffusion is

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} \tag{2}$$

This relationship is often referred to as FICK's second law. Here c is the concentration of solute in a plane located at distance x from the origin, D is the diffusion coefficient, t is time. The solution to this equation satisfying the conditions that seem applicable to ultrafiltrative flow is given by CARSLAW AND JAEGER[17],

$$c = \frac{J_0 x}{D} - \frac{8 J_0 l}{D\pi^2} \sum_{n=0}^{\infty} \frac{(-1)^n}{(2n+1)^2} \exp\left(-\frac{D(2n+1)^2\pi^2 t}{4l^2}\right) \sin\frac{(2n+1)\pi x}{2l} \tag{3}$$

J_0 is the numerical value of the solute flux, J_1, where $J_1 = -J_0$. This is a case of linear diffusion in a solid bounded by a pair of parallel planes, $x = 0$ and $x = l$. The region, $0 < x < l$, is initially at zero concentration. There is a constant flux, J_1, into the region at $x = l$. The effluent surface, $x = 0$, is kept virtually at zero concentration.

To obtain the flux of solute, J_e, at the output face, $x = 0$, we make use of the relationship expressed in FICK's first law,

$$J_e = -D\left(\frac{\partial c}{\partial x}\right)_{x=0} \tag{4}$$

Differentiating Eqn. 3 with respect to x and evaluating the expression at $x = 0$ we obtain

$$\left(\frac{\partial c}{\partial x}\right)_{x=0} = \frac{J_0}{D}\left[1 - \frac{4}{\pi}\sum_{n=0}^{\infty}\frac{(-1)^n}{2n+1}\exp\left(-\frac{D(2n+1)^2\pi^2 t}{4l^2}\right)\right] \tag{5}$$

We introduce the Relationships 1 and 4 into Eqn. 5 and make use of the fact that the concentration of solute in the effluent fluid, c_e, is given by

$$c_e = \frac{J_e}{J_w} \tag{6}$$

We arrive at the equation,

$$\frac{c_e}{c_1} = 1 - \frac{4}{\pi}\sum_{n=0}^{\infty}\frac{(-1)^n}{2n+1}\exp\left(-\frac{D(2n+1)^2\pi^2 t}{4l^2}\right) \tag{7}$$

Eqn. 7 gives the time course of the build up of solute concentration in the effluent solution. The quantity D can be referred to as the ultrafiltration–diffusion coefficient. Fig. 6 shows a plot of Eqn. 7 in the generalized time units of $4l^2/D\pi^2$. For comparison we have inserted curves for solutes having ultrafiltration-diffusion coefficients larger and smaller then the original value, D.

Ultrafiltration as a combined process of diffusion and convection

In their treatment of electrokinetic phenomena in membrane transport, SCHLÖGL AND SCHÖDEL[18] called attention to a convection term, cv, that should be appended to the NERNST–PLANCK flux equation,

$$J = -D\frac{dc}{dx} + Dc\frac{F}{RT}\frac{d\psi}{dx} + cv \tag{8}$$

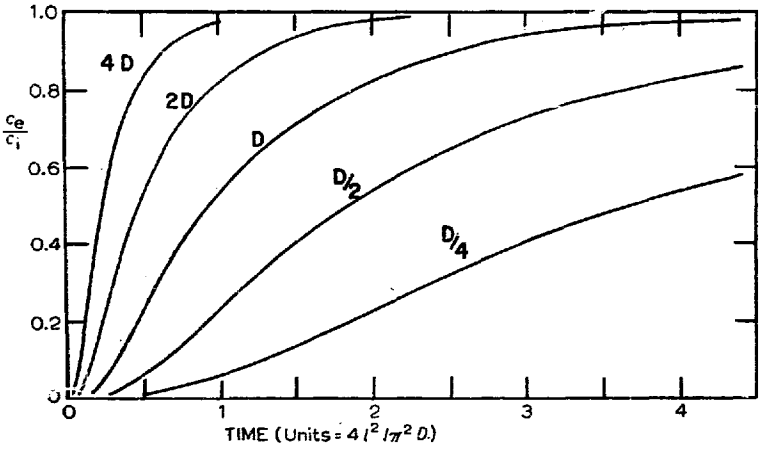

Fig. 6. Theoretical ultrafiltration curve. The curve D is a plot of Eqn. 7. Other curves are for solutes having a D value greater and less than D.

In the transport of small ions, v was taken to be the bulk velocity of fluid in the membrane. In the case of a large molecular solute with a retarded velocity we will substitute the convection velocity of the solute, v_s. In the absence of an applied electric field, ψ, and assuming a relatively uncharged membrane, the electric transport term vanishes since $d\psi/dx = 0$. This leaves only the diffusion and convection terms,

$$J = -D\frac{dc}{dx} + cv_s \tag{9}$$

If solute were transported by convection alone a moving front would be established traversing the membrane with velocity, v_s. In this velocity term we will measure distance in fractional parts of the membrane thickness and time in the units, $4l^2/D\pi^2$. The distance, l_1, remaining ahead of the solute front at time, t, is given by the following expression, where l is the total thickness of the membrane,

$$l_1 = l\,(1 - v_s t) \tag{10}$$

We can regard the diffusion process to be operating across this distance, l_1, which is progressively being reduced in magnitude as the front advances. The diffusion equation (Eqn. 7) is to be applied to a continuously thinning membrane. Graphically this situation may be represented as a progressive contraction of the time scale expressed in the time unit, $4l^2/D\pi^2$. The time, t_1, to which we shift an ordinate point corresponding to an original time coordinate, t, is then expressed by the following relationship,

$$t_1 = \int_0^t \frac{l_1^2}{l^2}\,dt = \int_0^t (1 - v_s t)^2\,dt$$

or

$$\frac{t_1}{t} = 1 - v_s t + \frac{1}{3}v_s^2 t^2 \tag{11}$$

In Fig. 7 we have shown how the original diffusion curve is altered by applying a series of differing ultrafiltration-convection coefficients, v_s.

The theoretical ultrafiltration curve reproduces the major features of the experimental curves. The fit, however, is not yet precise enough to justify a definitive

assignment of diffusion and convection coefficients. We prefer, at this time, to treat the experimental curves on an empirical basis since most of the significant information can be obtained directly from the graphic plots of the observed data. Approximate values for the ultrafiltration-diffusion coefficients have been inserted in the figure legends to show their order of magnitude.

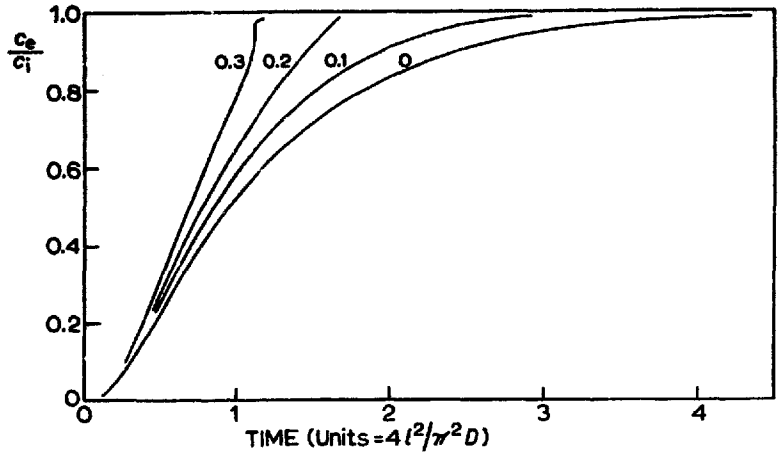

Fig. 7. Modification of ultrafiltration curve by convection term. The curves correspond to different values of the ultafiltration-convection coefficient.

DISCUSSION

It is evident from these experiments and from our previous work with the pituitary peptides that one can adjust the pore size of a cellophane membrane to discriminate with good resolution among polypeptides and proteins ranging in molecular weight from 1000 to 100000. In the case of ovalbumin (mol. wt. 44000) and human serum albumin (mol. wt. 69000) there was, at 5 h of filtration, a ratio of 3:1 in the cumulative output of the smaller protein compared to serum albumin. Differential filtration is evident only during the transient state of the ultrafiltration process. When the steady state is reached the concentration in the effluent approximates the input concentration and there is no longer a differentiation according to molecular size. BARRER[19] has called attention to this characteristic of a diffusion controlled process "in the transient state of flow separation factors may be very great indeed. The faster moving component may reach the outgoing surface of the membrane long before the slower moving component".

Our treatment of ultrafiltration invokes a constant flow of solute into the membrane on the high pressure side. The concentration at this surface rises progressively until a steady state is attained. In the steady state the flow of solute away from the input surface into the membrane is equal to the flux entering this surface from the exterior. Once the equilibrium concentration is attained there should be no continued accumulation of solute at the interface. Experimentally, two measures are taken to promote this behavior, the membrane is made suitably permeable relative to the size of the filtered protein and the protein is maintained in high dilution on the input side. Most of the previous work with ultrafiltration has utilized the phenomenon of molecular sieving at the surface of the membrane rather than the diffusion mechanism. A theory was proposed by FERRY[20] to account for molecular separation of the type

dependent upon sieve action at surface pores. Many of the unfavorable aspects of ultrafiltration seem to be associated with excessive protein deposition resulting from this sieving effect. Precipitation, film formation, blocking of pores are among these difficulties. The measures that favor the diffusional over the sieving process seem to be effective in reducing the surface accumulation problems.

The ultrafiltration equation was derived on the basis of a model system operating through a diffusion mechanism. The salient features of the protein build-up in the filtrate are reproduced by the theoretical curve. The curve is defined primarily by the ultrafiltration diffusion coefficient and is subject to secondary modification by the ultrafiltration-convection term. The ultrafiltration-diffusion coefficient is not directly related to the coefficient of free diffusion in water. It is a complicated quantity determined by the interaction of the streaming protein molecules with the structural matrix of the membrane. The size and conformation of the protein, its flexibility, degree of hydration, frictional interaction with water are some of the parameters that contribute to the make-up of the ultrafiltration coefficient. In view of the sensitivity of the filtration curve to dimensional properties of the solute, the ultrafiltration technique should become a means of discriminating between different proteins for purposes of characterization and fractionation.

ACKNOWLEDGEMENT

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