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Thin Film Dialysis Including Counter-Current Dialysis*,†

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MEMBRANE SEPARATIONS

There are many excellent reasons for a chemist to be interested in membrane separations, particularly if he is interested in the biochemistry of larger molecules. In living tissues, with their amazing ability to synthesize delicate, large polymers with unique structures, perform equally amazing separations of closely related solutes of complicated nature, maintain concentration gradients, and otherwise control a local environment, there seems always to be an intricate system of membranes. This has been realized for a long time and the literature dealing with biological membranes has become so voluminous as to be difficult to master. Nonetheless, it seems to fall far short of providing adequate explanations for all the phenomena observed.

It should be stated at the outset that no attempt will be made in this paper to deal with the membranes of living cells. Only the simplified case of inert, synthetic membranes will be considered. Even here, there are several approaches to the study that have only recently become possible.

One of these comes as a result of our increased knowledge of the precise structure, size, and conformation of certain pure solutes of larger size which can be isolated from living tissue. These include polypeptides, nucleotides, polysaccharides, and proteins. They can be used as models to study more precisely the permeation of various membranes by simple diffusive forces and the factors that

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either aid or hinder permeation. In addition, we now have a somewhat clearer concept of the nature of membranes and can possibly choose a simpler basis for study. It is granted at the outset that there is no such thing as an "ideal" membrane in the sense that it acts completely as either a mechanical sieve or a thin, solvent barrier. The problem of surface interaction is always present to some degree. These problems, however, can be reduced to second-order effects with the proper choice of a membrane for the class of solutes to be studied.

The approach we shall present has been worked out with commercial cellophane (Visking) casing. There are several reasons for this choice. Cellophane casing has long been the choice of biological laboratories for routine dialysis. Its reliability for this purpose is the by-product of the need for the strongest, thinnest semipermeable membrane possible for the meat packing industry. These properties also require the lack of fixed carboxyl groups and, thus, minimal ion exchange properties. A wide experience with cellulose as a support for chromatography indicates that many preparations are inert and have, in general, about the weakest adsorptive properties of any polymer with respect to substances of biological interest.

Experimentally, it would seem profitable to try to simulate as far as possible certain conditions found in tissues which depend on diffusion through membranes for their survival. For instance, laboratory dialysis as ordinarily performed is a slow process. Not so, however, in tissues. Certainly one of the important factors contributing to the difference is the relatively enormous membrane surface in contact with a solution of very limited depth. Thus, the solutes need diffuse only very short distances. There probably is no so-called Nernst layer in the sense of our experience in bulk dialysis, since the majority or all of the solution actually is in this layer. Moreover, on a microscopic level, the tissues, membrane as well as liquid film, are in constant oscillation or movement.

Irrespective of the relative importance of these concepts, we have devised a simple analytical dialysis cell which obviously falls far short of duplicating the marvellously favorable conditions found in tissues but, nonetheless, does give reliable results that are difficult to duplicate otherwise. We have called it a "thin film" dialysis cell [1].

A length of cellophane casing is pulled over a glass collar and held as shown in Fig. 1. It is tied off to form a sac of the desired length. A glass tube, whose diameter is nearly as large as the inflated casing diameter, is inserted. Its diameter is critical, so that with 0.5 ml of solution in the sac a thin film of solution will be spread reproducibly over the membrane when it is inserted as in Fig. 1b. The solution outside the membrane, which covers it and approximates a volume 10-fold that of the retentate, is held in a

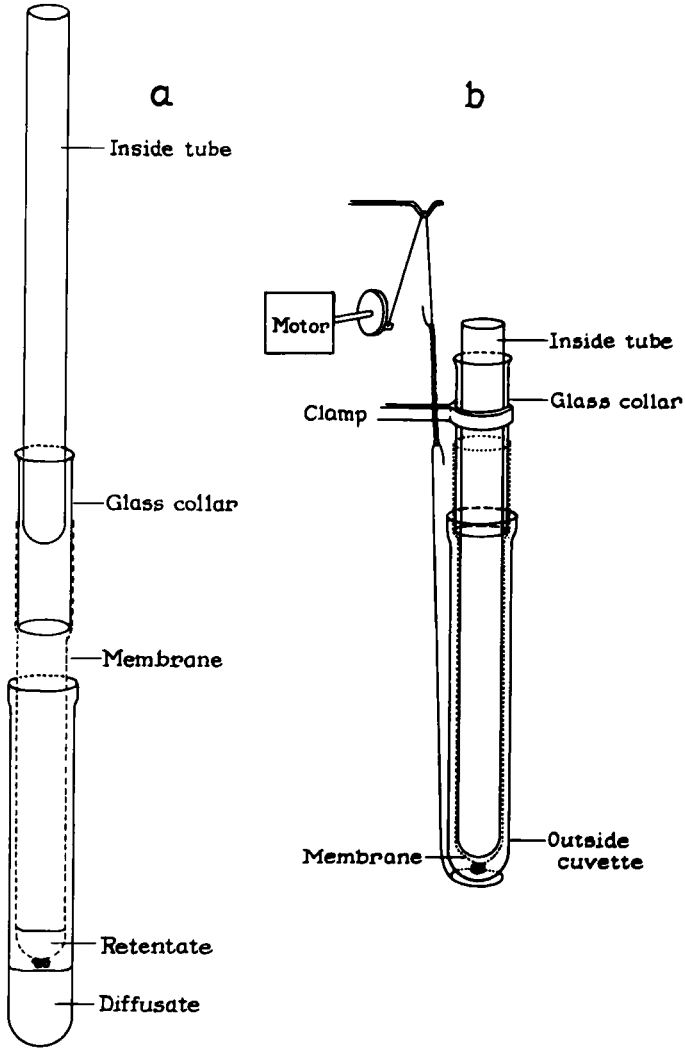


Fig. 1. Schematic drawing of the analytical thin film dialysis cell.

cuvet (see Fig. 1b). Stirring is accomplished by a piston-like movement of the outer cuvet; because of the flexibility of the membrane, the inside is also stirred. The retentate solution film against the membrane is of the order of 0.1 mm or less in depth.

In this arrangement the rate of transfer of the sample of solute placed inside to the solution outside is measured, keeping the outside concentration negligible by periodic removal and replacement of the outside solution until most of the solute has been transferred through the membrane. Thus, with a pure solute, first-order reaction rates should hold and do to an amazing degree with various solutes. The factors permitting adherence to the semilog plot, to be obtained as shown in Fig. 2A, have been worked out. Thus, relative diffusion rates can be expressed conveniently as half escape times. At 25°C the half escape time of NaCl or urea can be of the order of 0.5 min, and of lysozyme (molecular weight, 14, 000), 6 hr. A single membrane can be calibrated with various solutes of different known molecular size and used repeatedly with unknown solutes for long periods of time. Adsorption to the membrane can be easily recognized by recovery calculations. Any of the sensitive microestimations common in biochemistry can be applied when required.

When deviations from a straight-line escape occur, they can be diagnosed readily as due to a mixture (Fig. 2C) or a dissociating aggregate (Fig. 2B). Different conformational or slowly interconvertible forms are recognizable. A change in diffusional size or conformation brought about by addition of solutes such as urea, salt, detergents, etc., can be studied.

The real significance of such results seems to be a matter of whether or not they are true reflections of relative diffusional size and, if so, what is the smallest change in diffusional size that can be reliably detected. The first question is strongly supported in the affirmative by the many examples in our published work [1-6], none of which speaks against this view. The second can be estimated by the use of models and the choice of membranes.

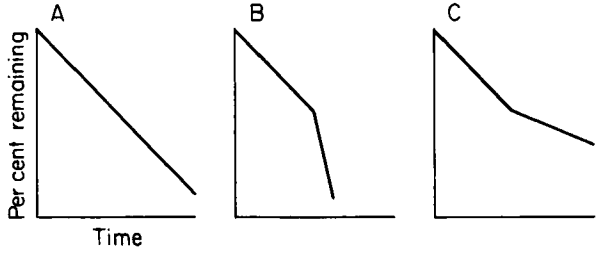


Fig. 2. Types of escape patterns given by (A) an ideal pure solute, (B) a dissociating solute concentration dependent, and (C) a mixture of two or more solutes of different diffusional size.

The highest selectivity is achieved for a particular solute when the membrane barely allows it to diffuse through the matrix [1]. There is not space here to develop the reason for this, but it is to be expected from ultrafiltration experiments and the Staverman coefficients [7] known in osmotic pressure studies. This means that the porosity of the membrane must be adjustable depending on the particular molecular size. We have been able to overcome this problem by simple procedures [1].

Wet cellophane is a rather rigid gel which is deformable. It shows a degree of elasticity but, if stretched beyond a certain limit, will remain deformed. Schematically, the average pore shape would be modified as in Fig. 3b, and the membrane should become less

Fig. 3. Hypothetical change of pore shapes caused by stretching the membrane in one direction.

porous for those solutes barely able to pass scheme a. On the other hand, if stretched equally in two 90° directions, the membrane would become more porous. That this is the case is shown in Fig. 4. Even more interesting is the fact that the measured distance of stretching can be correlated with actual known molecular diameters, as shown in Fig. 5. Here, a membrane stretched the calculated amount will pass chymotrypsinogen (molecular weight, 25, 000) at essentially the same rate as ribonuclease (molecular weight, 13, 600) in the unstretched membrane. Clearly, cellophane is acting as a mechanical sieve.

Now we can treat more effectively the question of selectivity by the use of models. For this purpose, there is available a series of rigid cyclic polysaccharides, the Schardinger dextrans, whose size and shape have been well documented by X-ray studies [4]. They are doughnut-shaped molecules of similar thickness but differing in width. Cycloheptaamylose is about 11% wider than cyclohexaamylose, and cyclooctaamylose is also about 11% wider than cycloheptaamylose. A membrane adjusted to provide a half escape time of 6 hr for cyclohexaamylose will give 12 hr for cycloheptaamylose (Fig. 6). We can estimate from this that we could detect differences in diffusional diameters of the order of 2-3%. Other studies with rigid molecules support this estimate.

If the foregoing views regarding the basis of selectivity are correct, a still higher order should be obtainable by still more precise adjustment of pore size and slower rates of diffusion per unit area

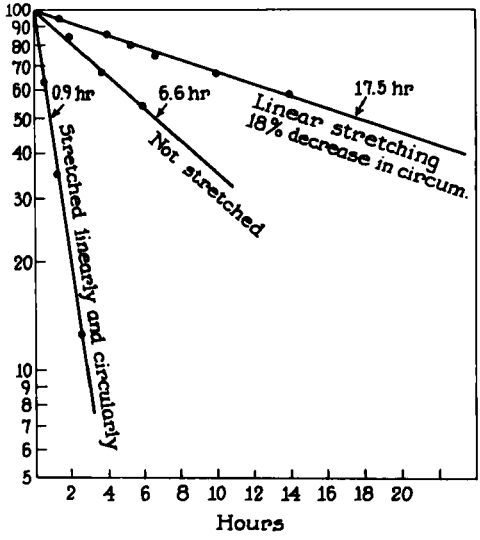


Fig. 4. Effect of the different types of stretching on escape rates.

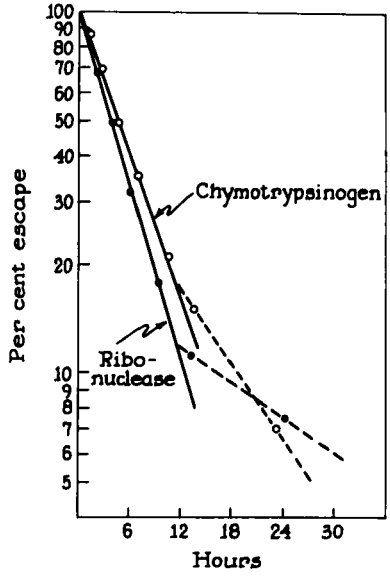


Fig. 5. Comparison of the escape rate of ribonuclease in an unstretched membrane with that of chymotrypsinogen in a membrane stretched the calculated amount.

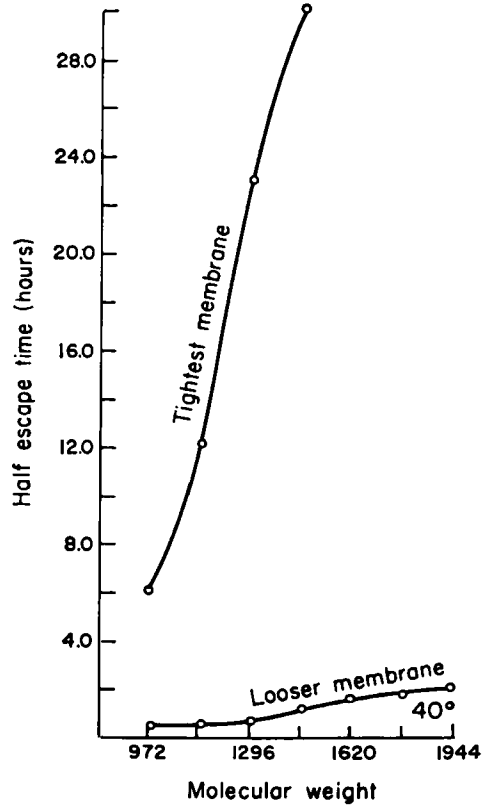


Fig. 6. Relative escape rates of Schardinger dextrins in a membrane adjusted to a selective pore size as compared with a more porous membrane.

of membrane. Thinner membranes, thinner solution films, and larger membrane areas could offset the slower rate of diffusion. We have not as yet devised a practical way to do this experimentally, although the conditions are certainly met in living tissue. Another factor concerns the distribution of effective pore size. The narrower the range of pore size, the more selective the membrane should be. Through the kindness of Dr. W. F. Underwood of the Visking Company, we have tested different cellophane preparations and have found considerable differences in selectivities even at critical escape rates. We have ascribed the findings to differences in the distribution of pore sizes and suggest the approach as a possible way to compare cellophanes.

Thus far, we have dealt only with the rates of diffusion of solutes through cellophane under the simplest possible static conditions and with the highest possible concentration gradient. Is it possible to devise a continuously flowing arrangement so that the countercurrent principle can be added to the advantage of the thin-film arrangement? Other workers have published designs [8-10], and we also have become interested [11] in the problem. Our most recent design is shown in Fig. 7.

In this scheme the membrane is held in the annular space between two glass tubes, one inside the other. The tubes are 90 cm long with an annular space of approximately 0.3 mm. Retentate solution is pumped through a capillary inside the inner glass tube, emerging at the bottom inside the membrane. It rises in a thin film and is removed by a pump and capillary tube at the top with minimal holdup. The outer diffusate stream flows downward in a

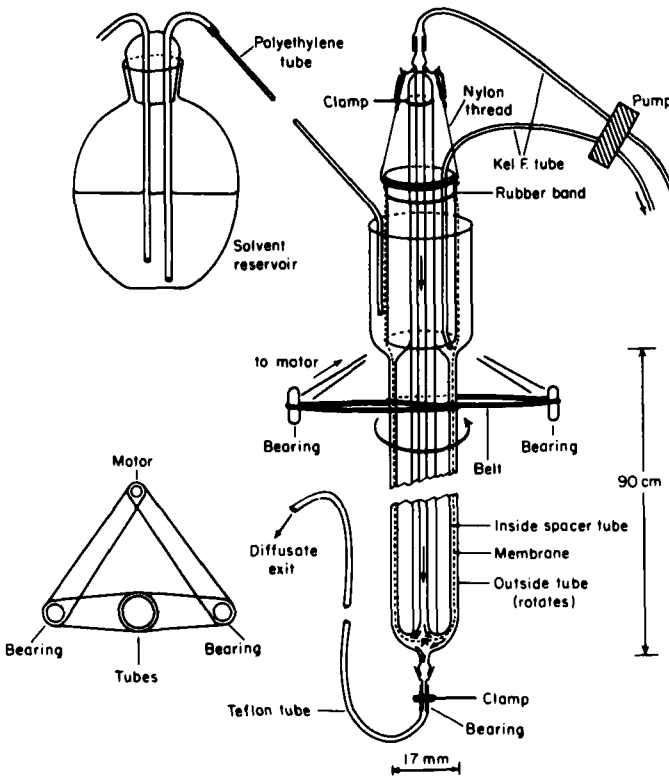


Fig. 7. Schematic drawing of a thin film countercurrent dialyzer.

thin film outside the membrane. Channeling is prevented by continual rotation of the outer tube. Both retentate and diffusate streams can be collected in a fraction collector. The membrane area approximates 500 cm^2; the volume inside the membrane during operation approximates 3 ml and that outside, 6-10 ml.

When a pulse of 1 ml of a 1.0 M solution of sodium chloride containing dextran blue was passed through at a retentate stream rate of 0.5 ml/min against a diffusate stream rate of 1.5 ml, analysis of the two streams gave the result shown in Fig. 8. The salt was essentially all removed from the retentate stream, leaving a coherent band of the nondialyzable dye. The apparatus described was the result of many experimental attempts to achieve the narrowest and most coherent bands possible in such an experiment.

The next logical question is the completeness of removal of the salt on a single pass. We have investigated this question with tritiated water, which diffuses through the membrane at a rate comparable to sodium chloride. Here, it was found that a pulse containing 10^8 counts/min (scintillation) introduced in the entering retentate stream would be cleared to the extent of background in the emerging retentate stream.

This result suggested a possible use of this dialyzer in tritium exchange studies with proteins, since the residence time in the column approximates 6 min. We found it ideal for this purpose

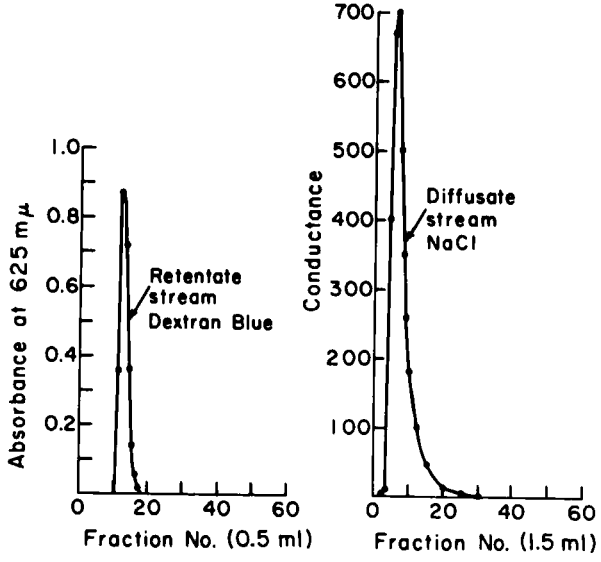


Fig. 8. Performance data for thin film countercurrent dialyzer.

and are using it this way. Thus, a 2-mg sample of growth hormone, provided by Dr. J. M. Dellacha of Buenos Aires, was allowed to equilibrate in 0.5 ml of tritiated water and was then passed through the dialyzer to remove quickly the unbound tritium. Growth hormone (molecular weight, 21, 000) would not diffuse through the membrane. Analysis of the retentate stream by absorbance gave the results in Fig. 9 in two separate experiments with slightly different buffers, one of which causes the hormone to aggregate. Analysis gave a strikingly superimposable result which showed little back-exchange of the bound tritium during the 6-min dialysis time in the column. The number of slowly exchangeable tritiums is easily calculable.

Relatively slow tritium-proton exchange rates carried out under suitable conditions presumably reflect the binding through hydrogen bonding of one part of a chain to the other and thus are part of the forces that determine conformation, or alternatively, they could be part of the forces that help to bind two or more molecules together to form aggregates. These forces and ways of studying

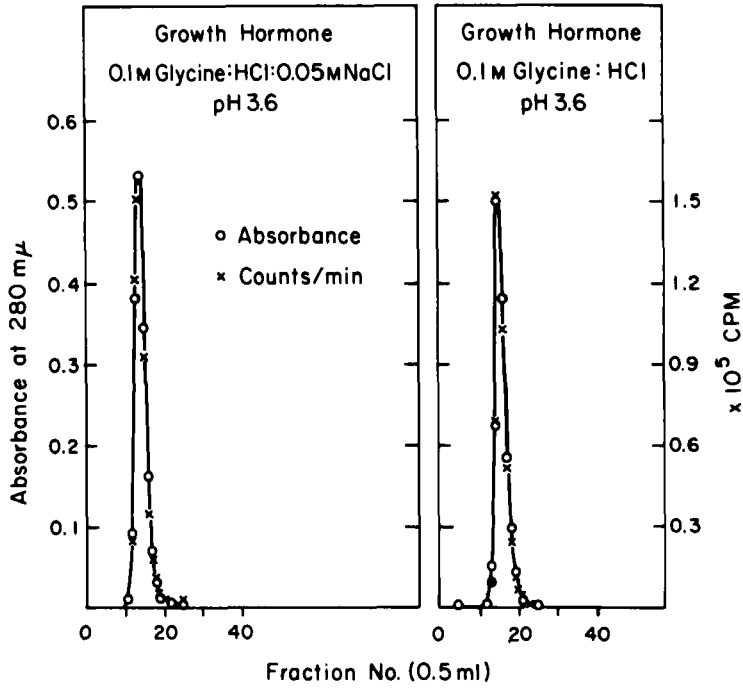


Fig. 9. Use of the countercurrent dialyzer in a tritium exchange study of growth hormone.

them are currently of tremendous interest in biochemistry. Curiously enough, it is also of interest that cellophane itself is a porous gel by virtue of the fact that certain short sections of the cellulose chain are tightly held by secondary forces to complimentary parts of other chains. Other adjoining sections are held apart by the solvent action of the water and thus form "pores."

Irrespective of whether or not this is the correct concept or adequate in all respects, semipermeable membranes offer one of the most informative ways of studying the binding of a small solute in solution to a much larger one. The use of dialysis in equilibrium studies of binding is well known. The static thin film dialysis cell described above is excellent for this purpose [12, 13] because of the rapid equilibration time provided. The continuous countercurrent device is also interesting in this connection and is capable of greater versatility. An example can be taken from certain polypeptide-nucleotide studies under way in our laboratory.

Actinomycin is an extremely toxic polypeptide antibiotic of molecular weight approximately 1200 and apparently owes its toxicity to the fact that it strongly binds to a deoxyguanosine site [14] in deoxyribonucleic acids. It binds also to the nucleotide, deoxyguanosine-5′-phosphate. A calibrated membrane of such porosity that it shows a 50% escape time for actinomycin in the thin film dialysis cell of 1 hr will rapidly pass the nucleotide. But when the nucleotide and actinomycin are placed together with the nucleotide in slight excess molar proportions, the 50% escape time of the actinomycin is 2 hr. Other nucleotides do not have this effect.

This binding effect is revealed by the countercurrent thin film dialyzer, as shown in Fig. 10. A pulse containing actinomycin alone run through the dialyzer shows that most of the polypeptide passes into the diffusate stream. But when deoxyguanosine-5′-phosphate is in the solvent solution, a considerably smaller proportion passes through the membrane.

Solutes that show strong tendencies to aggregate are interesting special cases of binding. The antibiotic polypeptides called tyrocidines are especially interesting models for this type of study. We have established their structure [15] to be that shown in Fig. 11. They are cyclic decapeptides, which apparently stack to form aggregates containing 50 or more monomers, as shown by the ultracentrifuge, depending on the solvent environment.

In the thin film static dialysis cell, a calibrated membrane which passes a molecule of 1200 with a half life of 15 min will allow tyrocidine to pass only slowly. Addition of dimethyl formamide speeds up the rate of dialysis. Breaking the polypeptide ring structure [15] eliminates the aggregation, and the expected 15-min dialysis rate of the monomer is observed.

In the thin film countercurrent dialyzer the results shown in Fig. 12 were observed when a 0.5-ml pulse containing 2 mg of pep-

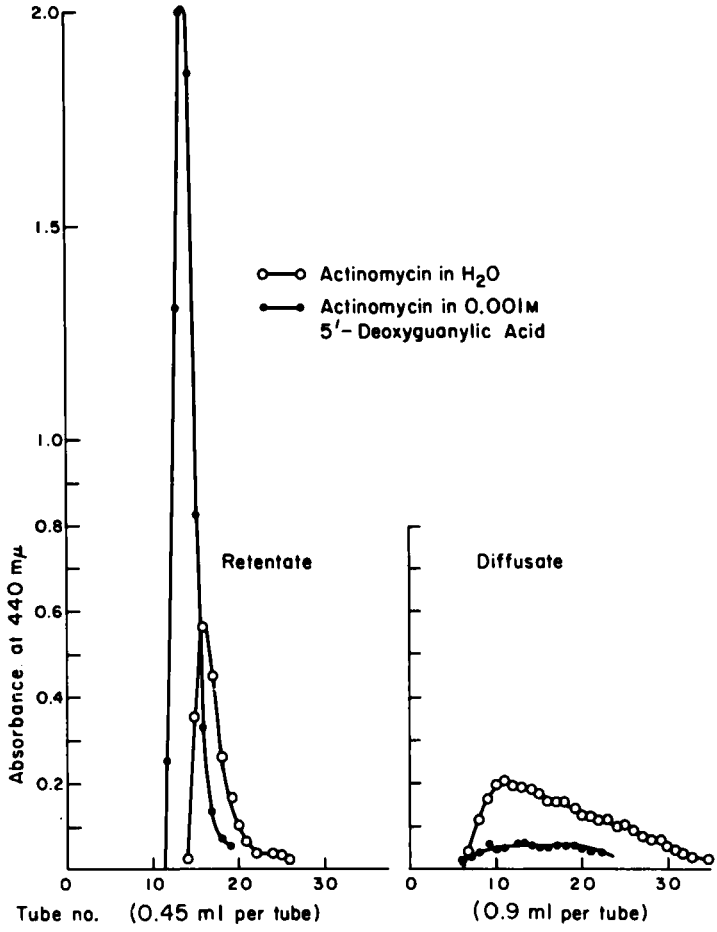
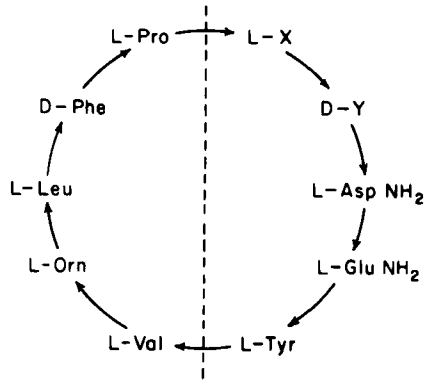


Fig. 10. Use of the countercurrent dialyzer in the study of the binding of 5′-deoxyguanylic acid to actinomycin.



Tyrocidine	Residue X	Residue Y
A	Phe	Phe
B	Try	Phe
C	Try	Try

Fig. 11. Amino acid sequence of the tyrocidines.

tide was injected in the retentate stream. Only the retentate effluent is represented in the figure. When the dialysis solution was a 0.001 M solution of sodium chloride, only the sharp narrow band of a nondialyzable solute was observed. When, however, the dialysis solution was 10% aqueous dimethylformamide (DMF), no such band was observed because the peptide passed through the membrane and emerged in the diffusate stream. Addition of salt sufficient to make the 10% DMF solution 0.001 M gave the lower curve with some loss to the diffusate stream. When the salt concentration was increased to 0.01 M, the intermediate curve resulted. Clearly, the presence of salt promotes aggregation, whereas DMF promotes disaggregation. We have concluded [15] that the aggregation is a result of hydrophobic bonding strengthened by a particular fixed conformation.

When the tyrocidine is dissolved in the 0.001 M NaCl solution and injected as a pulse into the apparatus previously operating with the solution containing 0.01 M NaCl and 10% DMF, the intermediate curve would be expected if the dissociation should take place immediately. Adjustment of the DMF and NaCl equilibrium would be very rapid. However, the effluent curve found coincided almost exactly with the dashed pattern except for a slight broadening on the right-hand base. Dissociation, therefore, does not occur to a large extent in the 6-min residence time in the column: Time dependency for dissociation was also observed by R. Williams of this laboratory

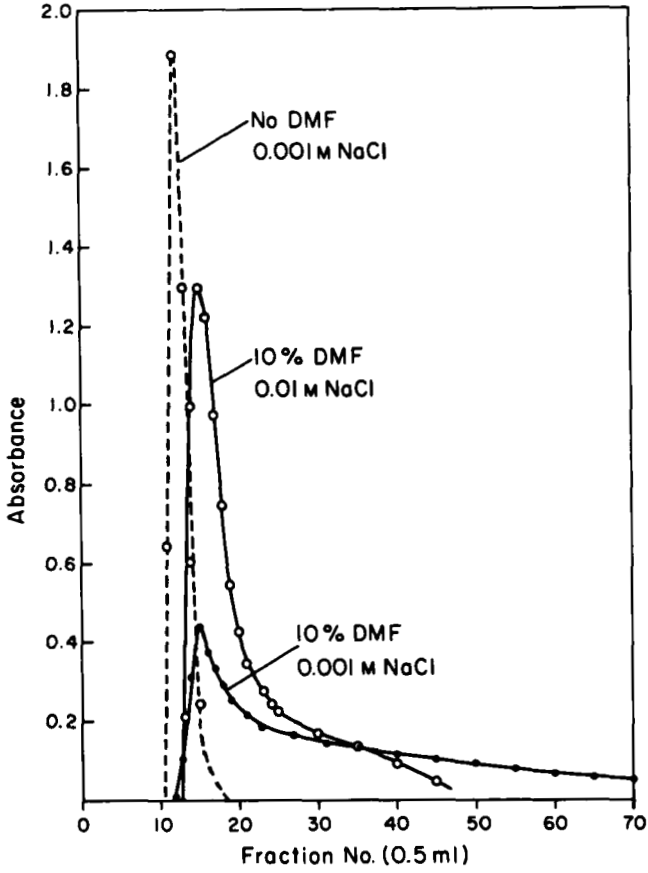


Fig. 12. Effect of sodium chloride and demethylformamide on the aggregation of tyrocidine B.

in ultracentrifuge studies. These results suggest that a conformation shift of some sort is involved in the association-dissociation phenomenon.

With selectivities with respect to diffusional size of the order discussed above, thin film dialysis should be of considerable interest in the study of the effect of various factors, such as temperature, pH, ionic strength, urea, etc., on the conformation of many large molecules: polypeptides, proteins, nucleotides, etc. That this is true is supported by the considerable data we have published [1-3, 16] but cannot present here because of lack of time. In general, rigid molecules used as models, which are tightly cross-linked, show

little change in diffusional size by changes in environment. Those having no covalent cross-links, however, show large shifts when the solvent environment is changed. The secondary forces involved are similar to those that cause association phenomena and are complicated and subtle in nature. For instance, tRNA (molecular weight, 27, 000) will diffuse through a suitably porous membrane in dilute $MgCl_2$ but not at all in water [17]. ACTH will diffuse through a suitable membrane 15 times as fast in 0. 01 N acetic acid as in 0. 15 M ammonium acetate [16], as shown in Fig. 13a. Urea expands the molecular conformation and slows the rate of dialysis. On the other hand, where there is aggregation, as in glucagon, it accelerates

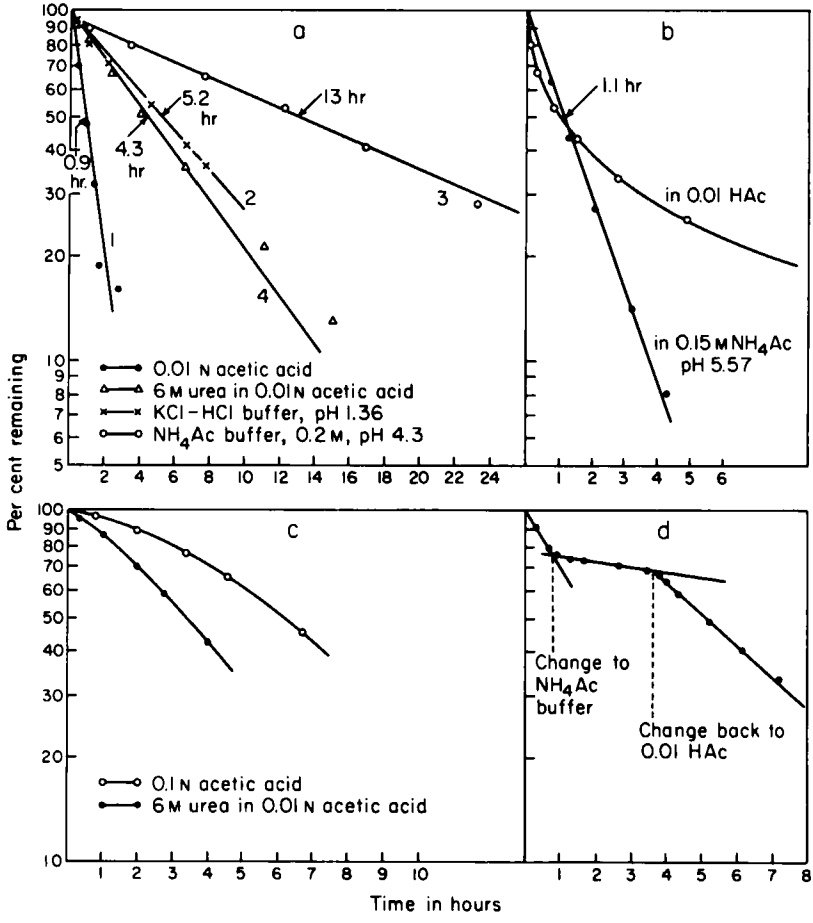


Fig. 13. Escape patterns showing the effect of various solvent components on the conformations of various polypeptides.

the rate of dialysis, as shown in Fig. 13c. Ammonium acetate markedly slows the rate of dialysis, as shown in Fig. 13d. Here, after a time, the original 0.01 N acetic acid diffusate solution was changed to ammonium acetate buffer. Almost at once, dialysis was markedly retarded. It quickly increased on changing the diffusate solution back to the 0.01 N acetic acid.

Salt increases the diffusional size of conformationally labile peptides with alternate positive and negative charges scattered along the chain. It has the opposite effect when the charged groups are only negative, as in the oxidized A chain of insulin, Fig. 13b. This effect shows up strikingly in the transfer ribonucleic acids, where the strongly charged groups are phosphates.

Obviously, a review of the extensive data we have accumulated, both published and unpublished, cannot be presented here. This paper is an attempt to present the general experimental approach we are developing and some of the reasoning on which it is based. The results thus far have convinced us that simple dialysis can be much more widely used as a versatile tool in biochemistry—for separations, for proof of purity, for determination of diffusional size, and for studying conformational stability, aggregation, and molecular interactions. In pursuing such objectives, much can be learned about the nature of semipermeable membranes.

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