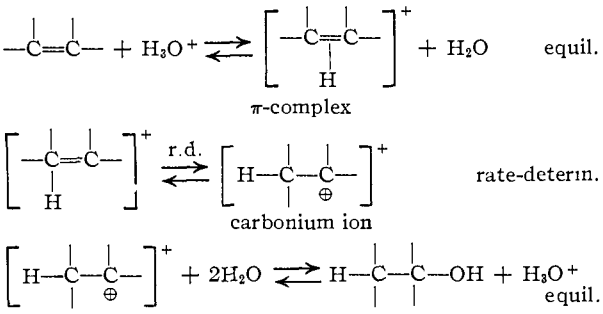
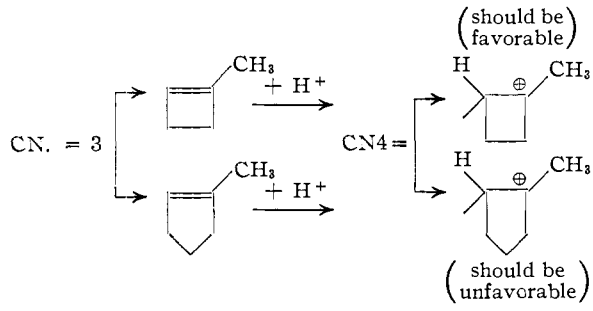


Application of the I-strain hypothesis to the relative rates of olefin hydration listed in Table V provides further evidence of importance concerning the structure of the transition state. If the positions occupied by the nuclei in the reaction transition state were those expected of a classical carbonium ion (in which the added H has formed a conventional C–H bond), then by arguments paralleling those used above, the rate of hydration of the cyclobutene should be favored and the cyclopentene retarded relative to the open chain olefins



However, as noted above, exactly the opposite order of rates is observed. We conclude therefore that the transition state does not possess the nuclear arrangement of a classical carbonium ion. On the other hand, the transition state is stabilized by those structural features which by electronic shifts (inductive or hyperconjugative electron-release) stabilize carbonium ions, as witnessed by the many powers of ten increase in rate in the series: ethylene << propylene << isobutylene.[21] The olefin hydration transition state may thus be ascribed the property of possessing carbonium ion character with respect to electronic but not with respect to molecular structure (nuclear arrangement). This property bears a striking similarity to the well-known Franck–Condon principle and, by analogy, leads to the proposal that the property results from more sluggish nuclear than electronic rearrangements in attaining the transition state.

It is a corollary of our conclusions that the transition state cannot be said (as frequently implied in discussions) to possess a fixed fractional character of the products and reactants of the rate-determining step, but this assessment, if it is to be generally useful, must be made with respect to both electronic and nuclear rearrangements.

Since the effects of structure on hydration rates shown in Table V are relatively small (compared to the effects on dehydration rates shown in Table VI), the π-complex appears to provide a suitable model for the molecular structure of the transition state. According to the structure proposed from the π-complex,[22] its formation from olefin should not require notable nuclear rearrangements.

(21) This is in contrast to the formation constants of the Ag^+ complexes of these olefins which show relatively little dependence on structure; *cf.* F. R. Hepner, K. N. Trueblood and H. J. Lucas, THIS JOURNAL, **74**, 1333 (1952).

(22) (a) M. J. S. Dewar, *J. Chem. Soc.*, 406 (1946); (b) C. C. Price, "Mechanisms of Reactions at the Carbon–Carbon Double Bond," Interscience Publishers, Inc., New York, N. Y., 1946, p. 46; (c) E. L. Purlee and R. W. Taft, Jr., THIS JOURNAL, **78**, 5807 (1956); (d) L. G. Cannell and R. W. Taft, Jr., *ibid.*, **78**, 5812 (1956).

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[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Dialysis Studies. II. Some Experiments Dealing with the Problem of Selectivity

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A convenient cellophane dialysis cell for quantitatively studying dialysis rates is described. A series of proteins of increasing molecular weight up to 45,000 have been studied. The rate of dialysis for each protein has been shown to fall in the order of increasing molecular weight but the membrane becomes more selective as the solute size approaches the membrane pore size. The method may offer a simple means for detecting a change in shape or size during certain types of transformation and for studying homogeneity with respect to size.

In the first paper[2] of this series an experimental approach for fractional dialysis with cellophane was described which permitted multiple fractional separations to be made in a manner entirely analogous to that accomplished with a few stages of countercurrent distribution.[3] Evidence of unanticipated selectivity was encountered at that time which was sufficient to make the procedure as it was described of practical use for the preliminary fractionation of complex mixtures of solutes differing widely in size. It seemed likely that the overall selectivity of the method could be improved either by enhancing the inherent selectivity at each stage or by some mechanical systematization so that many stages could be applied with a minimum of labor as was done in countercurrent distribution.

This latter is made difficult by the fact that for each unit in a stage the dilute dialyzate must be concentrated in some way to one tenth or less of its volume before being transferred to the inside of the next unit in the series. Before attempting to

(1) Fellow of the National Foundation for Infantile Paralysis.

(2) L. C. Craig and T. P. King, THIS JOURNAL, **77**, 6620 (1955).

(3) L. C. Craig, *J. Biol. Chem.*, **155**, 519 (1944).

design a physical set up for accomplishing the larger number of stages it seemed wise to make a more thorough study of some of the factors which could contribute to the inherent and unexplained selectivity possible in each membrane. The present paper will describe certain experiments along this line.

The results already published and other experiments soon showed that the inherent membrane selectivity was a function of many different factors such as the type of membrane, the solvent, the nature of the solute, the temperature, etc. The proper evaluation of each of these factors is made difficult by the fact that they are all more or less interdependent.

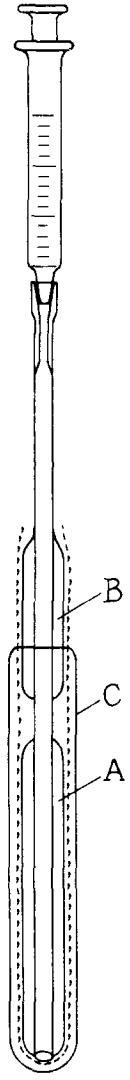

Fig. 1.—Dialysis cell.

In spite of such interdependence it was obvious from the data published[2] and from other preliminary data that the major factor governing selectivity, at least with cellophane membranes, and similar types of solutes, was primarily the size of the solute. Moreover, the effect of size appeared much greater than would be expected on the basis of difference in rates of diffusion in free solution. Apparently the membrane not only prevents mixing due to convection but in addition exerts a selective action of its own in inhibiting the diffusion of the larger molecules through it to a greater degree than the smaller ones. If this is true then it might be expected that relatively higher selectivity with a given membrane would be found for those molecules which due to their size are barely able to pass through the membrane.

Rather than search for a membrane which would slowly pass only small solutes or try to decrease the permeability of one already available it seemed more profitable first to study the passage of larger molecules through the commercial membranes already available to everyone.

In order to pursue this line of attack it seemed advisable first to consider some arrangement for shortening the time of passage of the more sluggish solutes through the membrane both in the interest of saving time and in minimizing the chance of transformation due to bacterial growth. The desired arrangement should not alter the inherent permeability of the membrane in any way such as stretching of the membrane by hydrostatic pressure. This point will be discussed later.

As might be expected, the net total rate of passage of solute caused solely by a concentration gradient of that solute across a membrane is directly proportional to the effective area of the membrane. When the rate is considered in terms of per cent. of the total solute placed in the membrane at the beginning, as would always be the case in fractionation studies, it is apparent that in order to accomplish the above stated objective, the smallest volume possible of solution inside the sac should be in contact with the largest area of membrane while only the solvent with essentially no solute in it should be present on the other side of the membrane.[4]

These requirements were easily met in the very simple apparatus shown schematically in Fig. 1. The data given in the present paper were all obtained with a dialysis cell of this type.

Rather early in the development of the method of countercurrent distribution,[5] it was realized that the simple calculations of the binomial theorem could be applied only when the partition isotherms of the solutes were essentially linear, *i.e.*, the partition ratios for a given system did not shift with a change in solute concentration. This ideality proved to be best also from the standpoint of overall separation although non-linear isotherms often permitted better resolution of one component at the expense of the purity of the others.

Since with countercurrent dialysis the rate of escape replaces the partition ratio in C. C. D., it would seem that here again the same mathematics can be applied only when the rate of escape on a percentage basis does not change with solute concentration. This means that the rate of decrease of the amount of solute remaining on the high concentration side of the membrane should follow first order reaction kinetics, and if the solute escapes through the membrane against essentially zero solute concentration, a plot of the logarithm of the per cent. solute remaining against time should give a straight line. In countercurrent dialysis such a straight line behavior is analogous to a linear partition isotherm in C. C. D. Departure from linearity in the dialysis process would be expected to have an effect similar to that already so familiar in C. C. D.

Experimental

The simple apparatus shown in Fig. 1 is constructed easily by an amateur glass blower. A Pyrex glass tube of outside diameter slightly smaller than the diameter of the extended cellophane tubing is chosen and a 6 mm. tube sealed inside it is shown with the lower end of the smaller tube left open. The smaller tube passes through a ring seal at the upper end of the larger tube, A, and at distance of about one cm. from this ring seal into a second section, B, of the larger tubing. This second section of the larger tubing is approximately 0.5 mm. larger in diameter than the lower section, A, and approximates 4 cm. in length. After passing through the upper ring seal of the upper section, the 6 mm. tube is joined to the appropriate part of a standard Becton–Dickinson adapter so that a 2-ml. syringe can be fitted to it.

The dialysis casing to be used is soaked for a few moments in water and one end is tied off with a single knot exerting care not to stretch in any way the part of the membrane above the knot which will later serve as the dialysis membrane. The knot can be tightened adequately by holding the initial loosely formed knot with two fingers while it is pulled tight with the short piece extending below the knot. The extension is then cut off with scissors. The tubing is cut to form a sac of such length that most of section B will be covered when it is pulled over A. This can be done by slowly rotating the wet glass part while the wet membrane

(4) R. E. Stauffer in Weissberger, "Technique of Organic Chemistry," Vol. III, 2nd edition, Interscience Publishers, New York, N. Y., 1956, p. 79.

(5) L. C. Craig, C. Golumbic, H. Mighton and E. Titus, *J. Biol. Chem.*, **161**, 321 (1945).

is gently pulled over it. That part of the membrane covering A is kept immersed in water but that over B is permitted to dry. The latter almost immediately contracts in air sufficiently to fix the membrane in place.

Even though kept wet the membrane around A contracts slightly and a small allowance must be made for this in fixing the membrane to B. Likewise, allowance for this contraction must be made in the initial choice of the tube to give the correct outside diameter of A.

C is the part of the cell to contain the solvent into which the solute dialyzes. It is of such size that a volume of solvent 10–20 times that initially used inside the sac will provide a level as high as the upper part of A. The rim of C is heated above the melting temperature and permitted to collapse sufficiently so that A and B will barely pass through. This contracted opening later rests against B and serves to center the upper part of A in the cell C. When in use the unit is supported by a clamp gripping it just below the ground joint but C rests on the table. The lower end of A can then be centered in C by a small shift of the latter on the table. A small spout is made in the rim of C to facilitate pouring.

Before the solution to be dialyzed is inserted, a small hole is punched with a needle through the membrane at a point well above the top of A and just below the bottom of B. This is to avoid pressure when the solution is forced into the sac. The membrane is washed several times with portions of the solvent both inside and out. For the inside wash, the solvent is added through the ground joint. Depression of the plunger of the syringe attached to the top forces the liquid out around the annular space inside the membrane. The solution can be moved into the inside tube again by raising the plunger and then withdrawn by a pipet or a second syringe.

A second small syringe fitted to a plastic tube is most convenient for placing and withdrawing solutions from the lower end of the inside tube. The flexible plastic tube can be of polyethylene or better yet of "Kel F." It is attached to a standard Becton–Dickinson adapter with the end drawn out to a size slightly larger than the inside diameter of the plastic tube. If the tubing is forced over the prepared tip while it is kept hot in a steam jet it will remain firmly fixed in place. The other end of the glass adapter carries the ground joint for attachment to the syringe.

After withdrawing all the wash solution from the inside, the solution to be dialyzed is inserted and gently forced out into the annular space with the syringe. The solution must be of such volume that when the upper level in the annular space inside the membrane reaches the top of A none of it will remain in the glass tube inside A but all will be essentially in intimate contact with the membrane. In the case of the 20/32 and 18/32 membranes the volume inside was 0.6 ml. In these cells the outside diameter of A was 14.8 mm.

Since the annular space between the glass and the membrane only provides for a thin film of solution it is important that the distribution be uniform. This can be ensured by gentle manipulation of the membrane with freshly washed finger tips and by drawing the solution back and forth into the glass tube inside A with the plunger of the syringe.

After satisfactory adjustment the unit is hung in the solvent in C to a height so that the levels of liquid inside the membrane and outside are equal. The time is then recorded.

After an arbitrary interval the outside solution is changed and the first solution set aside for analysis. At each change of outside solution the solution inside the membrane is drawn back into the glass tube by moving the plunger of the syringe and redistributed over the membrane.

At the end of the dialysis experiment each change of solution is analyzed as well as the solution remaining inside the sac so that the rate of escape can be calculated on the basis of total recovery of the solute. The recovery may be slightly greater or less than the material taken, but too great a divergence serves to indicate analytical errors.

At the beginning of a dialysis run when the concentration of solute is highest inside the sac, water will tend to flow into the sac due to osmotic pressure. This dilution will become progressively less as the solute moves out. The effect of the dilution is included in the escape rates indicated by the plot of the decrease of solute against time. No attempt to correct for this effect has been made. The increase in volume inside the sac is usually not more than 20 to 50% but with certain proteins may be much greater. When the latter is the case the escape rate is seldom linear and may indicate strong association.

The cellophane tubing used thus far has steadily given off a small amount of solute absorbing in the range of 280 mμ in spite of the preliminary washing treatment. This absorption has amounted to about 0.010 to 0.015 density unit in the 1-cm. cell at the beginning. That it was not due to an analytical error was confirmed in a 10-cm. cell but has not been of sufficient amount to give a weighable residue in the weight analysis procedure when 1.0 ml. was evaporated.

The bacitracin used in this study was the A fraction obtained by countercurrent distribution. The salmine came from the Nordisk insulin laboratories in Denmark and the "protamine" from GBI. Cytochrome C from horse heart was obtained from Sigma. Subtilin was obtained from the Western Regional laboratories. Ribonuclease and lysozyme were obtained from Armour. The pituitary lactogenic hormone was a sample considered pure by C. C. D. and obtained from Dr. R. D. Cole. We take this opportunity to thank him and Dr. C. H. Li for it. The insulin was a fraction obtained by C. C. D. of a sample of Boots beef insulin. The other proteins were obtained from Worthington.

Results and Discussion

If a single pure solute behaves ideally in that it does not associate to form larger molecules, or be adsorbed appreciably by the membrane, it should be possible to set up experimental conditions for dialysis so that the net rate of passage of solute per unit area of membrane would be proportional only to the concentration gradient across the membrane.[4] That this has been achieved can be shown best experimentally by frequent replacement of dialyzate with fresh solvent. A plot of the logarithm of the per cent. of the original solute remaining in the sac against time then should give a straight line.

Actual experiments with a number of pure solutes in distilled water or dilute acid in the dialysis cell described above have given experimental results which adhered to a straight line to a remarkable degree over a wide concentration range. The solutes studied have included glucose, sucrose, leucine, tryptophan, aspartic acid, bacitracin, subtilin and others. Curves of escape rates for aspartic acid and for bacitracin already have been published.[6] A representative experiment is that made with a good preparation of the polypeptide subtilin and shown in Fig. 2. The result for bacitracin with the same membrane is also shown. As mentioned in the introduction this represents "ideal" behavior as far as fractionation is concerned. However, not all preparations studied were found to give straight lines and it was soon concluded that deviation from a straight line could arise from causes other than impurity as discussed later.

The membranes most used in the studies reported in this paper were Visking 20/32 dialysis tubing selected for dialysis work and 18/32 Visking seamless cellulose tubing. In a cell of the type described the former was found to pass solutes dissolved in 0.01 *N* acetic acid even up to the size of ovalbumin, mol. wt. 45,000, at an appreciable rate. Table I gives the 50% escape time[2] for a number of readily available proteins differing in molecular weight from 5743 to 45,000, plus a few smaller solutes for comparison. The 20/32 membrane is not the most suited for the smaller solutes as ex-

(6) L. C. Craig and T. P. King, THIS JOURNAL, **78**, 4171 (1956).

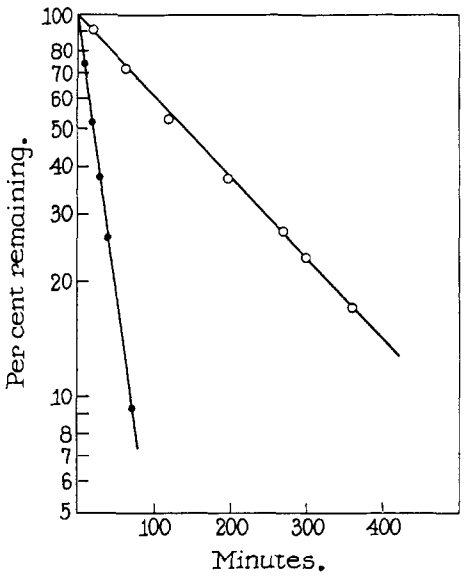

Fig. 2.—Escape curves through 18/32 cellophane in 0.01 *N* acetic acid: ●, bacitracin; ○, subtilin.

plained below. The cells used in gathering these data provided approximately 45 sq. cm. of dialyzing surface with a volume of solution inside the membrane of 0.6 ml. at the start of the dialysis. Each dialysis experiment required from 5 to 20 mg. of solute. A temperature of 25° was used throughout.

TABLE I

COMPARATIVE ESCAPE RATES OF A SERIES OF POLYPEPTIDES AND PROTEINS

Solute	Mol. wt.	50% escape time, min. 0.1 *N* HAc 20/32	0.01 *N* HAc 20/32	0.01 *N* HAc 18/32
Tryptophan	204	.....	4	6
Bacitracin	1,422	.....	15	21
Salmine	2000–8000	.....	40	150
Subtilin	3,300	.....	54	138
Insulin	5,733	1.5 hr.	..	Will not pass
Cytochrome C	12,000	.....	60	
Ribonuclease	13,600	3.5 hr.	2 hr.	
Lysozyme	14,000	3.5 hr.	2.3 hr.	
Trypsin	20,000	.....	4 hr.	
Trypsinogen	20,000	.....	6.5 hr.	
Chymotrypsin	24,500	.....	5 hr.	
Chymotrypsinogen	25,000	12 hr.	9 hr.	
Pit. lact. hormone	26,000	.....	13 hr.	
Gliadin	27,000	.....	29 hr.	
Ovomucoid	28,000	.....	35 hr.	
Pepsin	35,500	.....	80 hr.	
Ovalbumin	45,000	.....	More slowly than pepsin	

About half the solutes listed in Table I showed more or less of a tendency to deviate from straight line behavior—an indication of impurity, association or unexplained membrane effect of some kind. In these cases analysis of the escape rate by several different analytical methods as discussed below did not clearly reveal size inhomogeneity for the ones listed. However, the results from a number of preparations studied were thrown out on this basis and not listed.

In spite of the deviation it is worth comparing the 50% escape times with the latest accepted molecular weight values somewhat arbitrarily selected from the more recent literature. The values for most of the molecular weights of the proteins appear to be subject to a certain amount of revision (except for insulin and ribonuclease) and should not be considered as too exact. Certainly the 50% escape times should not be interpreted too strictly at this time. In spite of such uncertainties the correlation between molecular weight and escape rate is very striking. Only cytochrome C (granting its molecular weight range to be correct) would appear to be appreciably out of line. It is a strongly basic protein with a high isoelectric point (10.65) and contains many lysine residues. Earlier work[2] had indicated that strongly basic peptides with a high proportion of basic amino acids such as the polymyxins would show a higher escape rate in acetic acid solution than would otherwise be expected from their molecular size. The result given here could indicate that cytochrome C is a more tightly coiled molecule than has been suspected and thus of smaller molecular size than its molecular weight would indicate.

The proteins in Table I include ones with widely differing isoelectric points ranging from pepsin to lysozyme and cytochrome C. Yet they fall in the proper order according to size which indicates that charge does not play a very significant role in determining escape rate under the conditions chosen. It seems justifiable to conclude that molecular size is by far the most important factor in determining escape rate in a given cell, solution and membrane.

Tryptophan is one sixth the size of bacitracin but its 50% escape time approximates one fourth that of the latter. Ribonuclease is a little larger than one half the size of chymotrypsinogen yet its 50% escape time is also of the order of one fourth that of chymotrypsinogen.[7] The selectivity of the membrane obviously increases as the molecular size approaches the limit passed by the membrane. This is confirmed by comparison of the escape rates of bacitracin and subtilin in the 20/32 and 18/32 membranes.

It would now appear on the basis of these data, those in the previous paper, and other data still being accumulated in this Laboratory, that the relative molecular size of solutes up to molecular weights of 45,000 probably can be approximated by very simple, controlled dialysis experiments with considerable reliability. The reliability of the size derived for an unknown would of course depend on the assumption that the substance is reasonably globular in shape as are all proteins thus far studied. The deviation from spherical shape of the proteins listed in Table I as indicated by their published frictional ratios[8] is not sufficient to upset the relationship of molecular weight to 50% escape time.

Although considerable caution should be taken in interpreting the meaning of an escape rate until wider experience has been gained, there would appear to be no difficulty as far as the duplication of an experiment is concerned. The escape rates given in Table I for several of the proteins were checked by different workers at different times on more than one preparation and with membranes

(7) The value reported in (6) for chymotrypsinogen is a misprint.

(8) J. T. Edsall in "The Proteins," Neurath and Bailey, Vol. IB, Academic Press, Inc., New York, N. Y., 1953, p. 634.

prepared from rolls of cellophane purchased at different times. Apparently, within a given size the membrane is a remarkably uniform product as far as this type of test is concerned. The 50% escape times for check runs at different times have agreed to well within 10%.

It is of interest that trypsin gives a considerably smaller 50% escape time than trypsinogen. Although not shown to be significantly smaller by direct molecular weight determination heretofore, it is known that the activation of trypsinogen to trypsin involves a type of proteolytic splitting,[9] with loss of at least a hexapeptide.

Similarly chymotrypsin gives a significantly shorter 50% escape time than chymotrypsinogen. Again the former is formed from the latter by proteolysis[9,10] with loss of a peptide moiety.

In both pairs the enzyme is formed from the zymogen by loss of probably something less than 10% of its original weight. The difference in escape rate noted must therefore arise from a significant change in shape. That the transition from zymogen to active enzyme does involve a change in shape has been indicated strongly by the studies of Neurath, Rupley and Dreyer.[11] They found that the changes in optical rotation and viscosity produced by reversible urea denaturation were such as to indicate the active enzyme to be more tightly coiled or folded than the zymogen but capable of being unfolded to the same degree by urea.

Further information on the changes involved in the activation of trypsinogen and chymotrypsinogen is given by Neurath and Dreyer[12] who consider the molecular weights to be 23,800 and 23,000, respectively.

The foregoing and Table I have been presented to enable some rough over-all correlations to be drawn. Obviously, it is of equal importance to consider the finer details of the individual behavior of each substance or preparation.

For instance, a commercial protamine sample did not give a straight line. Its escape curve was characterized by two distinct breaks in the curve as shown in Fig. 3, curve 1. Since it has already been shown[6] by a curve of the escape rate of an artificial mixture of aspartic acid and bacitracin that a rather sharply breaking curve will be given by a mixture of two solutes provided their individual escape rates are sufficiently different, it is only natural to deduce from curve 1 that this sample of protamine is a mixture with at least three or more different molecular sizes present.

Further experience with the method, however, showed that such a conclusion could not be drawn unless the diffusion behavior of each pure solute with the same membrane under identical conditions was known already. Indeed, a few pure solutes have been found not to give a straight line with the 20/32 membrane. Thus tyrosine repeatedly gave a curve indicating two components. Histidine and threonine similarly did not give a straight line. Obviously a greater tendency to deviate from ideality is to be expected with these solutes, perhaps, due to some form of association between like molecules or with the membrane surface. These are the amino acids with substituents which would most likely permit some form of hydrogen bonding.

If a reversible association complex or dimers, trimers, etc., were formed, dilution would favor the monomeric form and it would be expected that the rate of escape would increase with dilution. This should lead to a deviation from a straight line as shown in the hypothetical case of the curve of Fig. 4. However, a striking deviation of this type has thus far not been observed and it seems most likely that the type noted in actual practice with the small solutes could be caused by a membrane effect of some sort. That it was not a concentration effect was established by beginning the dialysis with different amounts of solute. There is the unlikely possibility that those solutes with strong associating tendencies form dimers which pass through the membrane more rapidly than their monomers due to a different degree of adsorption on the membrane surface.

A further suggestion that the deviation stems from a particular membrane was obtained from the finding that the amino acids mentioned above gave a straight line when Visking 18/32 seamless cellulose tubing was substituted for the 20/32 membrane. The former gives a slower rate of escape and in fact will not permit any of the proteins thus far studied including insulin to pass in appreciable amount. It could be that the time interval was too short in this experimental set up for the small solutes to behave ideally in the 20/32 membrane.

Irrespective of the basic cause of such deviations it is not difficult to differentiate a divergence due to this phenomenon from that due to the presence of a mixture of molecular sizes. Thus the solute which has passed through until the time of the first break can be recovered and run again. If the original comprised a mixture of sizes, the escape rate of the re-run would then more nearly approximate a straight line, or at least show a faster escape rate than the original. Such a re-run of the first 20% of the sample of protamine gave curve 3 in Fig. 3.

In evaluating whether or not a mixture of sizes is present in a preparation it is helpful to analyze the different portions of the dialyzate with several analytical techniques. These could include weight analysis,[13] optical density, ninhydrin,[14] Folin–Ciocalteau,[15] acid or alkali titration, biological assay, etc.

If a single molecular size is present in the sample, the same curve will be obtained regardless of the analytical method used for determination of the escape curve. When it is possible to use direct weight analysis[13] this is the most reliable from the

(9) J. S. Fruton and M. J. Mycek, *Ann. Rev. Biochem.*, **25**, 63 (1956).

(10) B. Meedom, *Acta Chem. Scand.*, **10**, 881 (1956).

(11) H. Neurath, J. A. Rupley and Wm. J. Dreyer, *Arch. Biochem. Biophys.*, **65**, 243 (1956).

(12) H. Neurath and Wm. J. Dreyer, *Dis. Faraday Soc.*, **20**, 32 (1955).

(13) L. Craig and D. Craig, in Weissberger, "Technique of Organic Chemistry," Vol. III, Part I, 2nd Ed., Interscience Publishers, New York, N. Y., 1956, p. 321.

(14) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

(15) O. H. Lowry, N. J. Rosebrough, L. A. Farr and R. J. Randall, *ibid.*, **193**, 265 (1951).

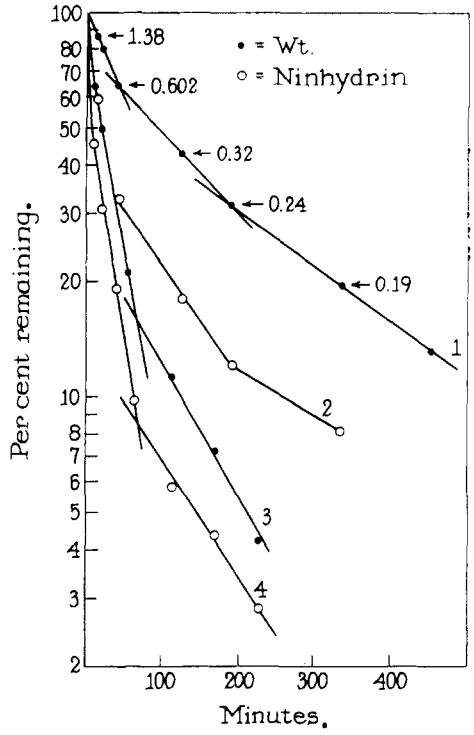

Fig. 3.—Escape curves of "protamine" through 18/32 cellophane in 0.01 *N* acetic acid: 1, weight curve of original; 2, ninhydrin curve of original; 3, weight curve of re-run; 4, ninhydrin curve of re-run.

standpoint of interpretation since it gives the per cent. escape rate based on total solute. The contrast of this with that based on one of the other selective methods then may be very enlightening as can be seen from curves 2 and 4 of Fig. 3 which are based on ninhydrin. As would be expected, the weight curve and ninhydrin curve more nearly agree on the re-run. Since the total solids in each dialyzate fraction is known, the change of ninhydrin color yield per mg. along the curve can be determined. The numbers given above curve 1 of Fig. 3 refer to leucine microequivalents of ninhydrin per mg. for the corresponding dialyzate. It is evident from these data that there is a steady partial resolution of molecular size since the smaller molecular sizes would be expected to give higher ninhydrin values per mg. of solute. It is also evident that further fractionation will be required.

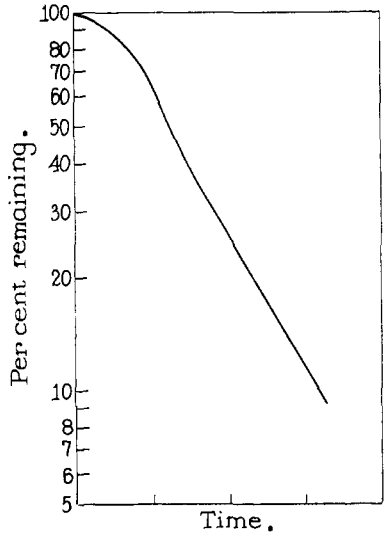

Fig. 4.—Hypothetical escape curve of solute which shows association-dissociation.

Figure 5 gives the comparative weight curves for the protamine mixture in the 20/32 and 18/32 membrane. The latter gives a much more distinct break in the curve indicating greater selectivity. It also shows a second break not revealed by the first. This again confirms the deduction from Table I that the selectivity becomes greater as the solutes approach the limit of size which will pass through the membrane.

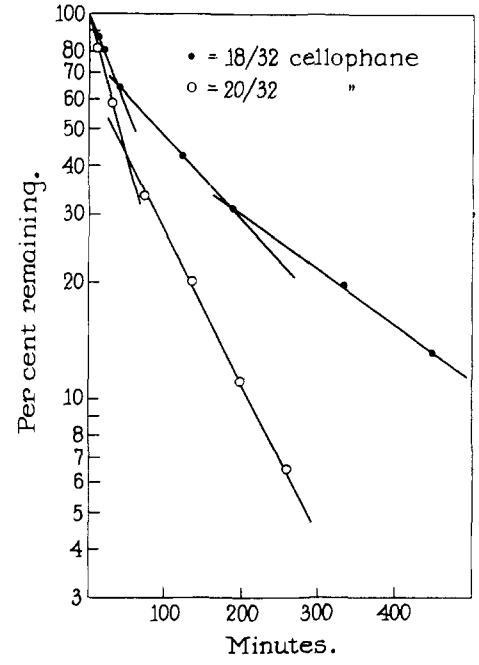

Fig. 5.—Escape curves of "protamine" through 20/32 and 18/32 cellophane in 0.01 *N* acetic acid.

The results in the case of insulin are interesting since more is known about the properties and structure of it than for any other protein. It is an unusually stable protein. These considerations make it a good model for basic studies.

A sample of beef insulin purified by extensive fractionation by countercurrent distribution[16] gave the lower curve in Fig. 6. The experiment was made with 12 mg. of insulin in 0.1 *N* acetic acid. Straight line adherence was observed until 85% of the solute had passed through the membrane. It should be pointed out that a result of this type is on a logarithm scale which has the effect of magnifying the relative importance of the last part of the solute to escape. Actually almost 99% of the insulin had passed through the membrane by 21 hours time.

This result contrasted sharply to that shown in the upper curve of Fig. 6, which was obtained with

(16) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **74**, 3083 (1952).

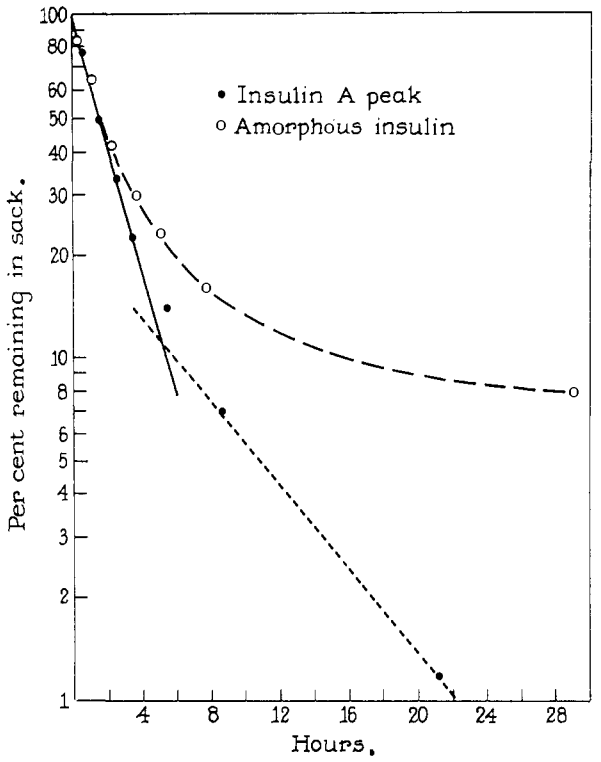

Fig. 6.—Escape curves of insulin through 20/32 cellophane in 0.1 N acetic acid.

an amorphous commercial sample. The latter was known by countercurrent distribution to be of the order of 70% pure.

Insulin is known to associate in the neutral pH range to a size with six or more sub-molecules in a particle. It also can form fibrils at acid pH ranges.[17] This tendency suggests that the deviation noted even in the lower curve could be due to associated molecules which do not return rapidly to the monomeric form.

Irrespective of the truth of this speculation it was found experimentally that a sample of insulin brought into solution in water with a minimum of dilute ammonia would not pass through the 20/32 membrane in appreciable amount after several hours in the standard cell when distilled water was used outside the membrane. During this time the original pH of the insulin solution had dropped from about 8 to 6. Ribonuclease (mol. wt. 13,600) readily passed under these same conditions.

In the first paper[2] of this series it was noted that the solvent had considerable influence on the rate of passage of smaller molecules through cellophane. Distilled water nearly always permitted the fastest rate. This appears to be even more true for the larger protein type molecules. All salt solutions thus far studied irrespective of the salt used have had a definite retarding effect as compared to distilled water when they were present in ionic strength greater than 0.01. The effect increased with increasing salt concentration to a certain salt concentration above which there was little effect.

(17) D. F. Waugh, in "Internal Secretions of the Pancreas," Ciba Foundation Symposia, Vol. IX, Little, Brown & Co., Boston, Mass., 1956, p. 122.

The pH also was found to have an effect but somewhat complicated by interrelationship with the salt effect. The data in Table I show that with several proteins studied 0.01 N acetic acid permitted a faster escape rate than 0.1 N acetic acid. The former permitted a rate not significantly different from distilled water.

With the larger molecules part of the experimental work has involved a search for conditions which would permit the most rapid dialysis without loss of selectivity. The use of distilled water or 0.01 N acetic acid as a solvent and with the 20/32 membrane has been uniformly the best thus far found. Fortunately, these conditions also have been those providing the nearest adherence to straight line escape rates. A straight line adherence until approximately 84% of the ribonuclease had escaped was noted, Fig. 7, if no salt was present in the solvent. Even somewhat better adherence was found when the solvent was distilled water alone. Here, although the main part of the curve coincided with that noted with 0.01 N acetic acid, a break in the curve was not observed until 93% of the solute had passed the membrane as shown by the dashed curve.

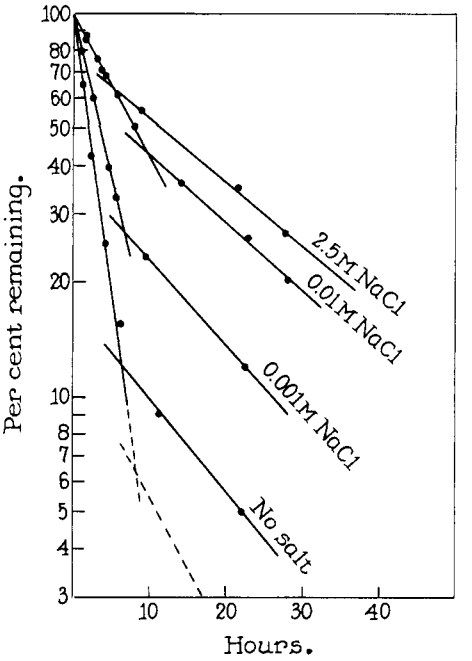

Fig. 7.—Escape curves of ribonuclease through 20/32 cellophane in 0.01 N acetic acid at various salt concentrations and in distilled water- - -.

The effect of various concentrations of sodium chloride in the 0.01 N acetic acid can be seen from Fig. 7. The salt effect is more pronounced up to 0.01 molar after which there is less increase in effect.

When the effect of pH was studied a marked influence other than that due to the salt effect of the buffer was not noted until a rather high pH was reached. The escape rate for 0.01 N ammonia was much less than for 0.01 N acetic acid.

The effect of urea also was studied. With insulin in 0.01 N acetic acid and six molar with re-

spect to urea, the 50% escape time was increased from 1.5 to 5 hr. Straight line adherence was noted until about 70% of the solute had escaped but the remainder appeared to dialyze very slowly.

Similarly ribonuclease dialyzed very slowly through the 20/32 membrane in 6 molar urea, 0.01 *N* acetic acid. Here straight line behavior was not found, perhaps an indication of progressive denaturation or transformation of some sort.

Since conditions favoring denaturation appeared always to result in a much slower escape rate, it seemed likely that the deviations from straight line behavior even in 0.01 *N* acetic acid might be due to a tendency toward denaturation. However, before this could be considered the question of size inhomogeneity in the samples used should be considered.

With ribonuclease this question was studied in the following way. A standard run was made, but the escape rate was determined by weight determination, ninhydrin, optical density and quantitative enzymatic activity.[18] With the latter a reproducibility of the values of ±10% was found in several experiments. All of these gave a curve superimposable within the experimental error, but showing the same break in the curve as noted in the next to the lowest curve of Fig. 7. The first third of the solute to dialyze and the last third to dialyze were then separately recovered. Comparative dialysis experiments were made on each.

The first fraction gave an escape curve similar to the original, but the break did not occur until several per cent. more had escaped. On the other hand the last fraction showed a break a few per cent. earlier in the dialysis than did the original. Both were equally active enzymatically. They also were compared by ion-exchange chromatography[19] and found to be very similar, but two small bands present in the original and the first fraction were not present in the last fraction. Neither this small difference nor the dialysis difference noted on re-dialysis seemed sufficient to explain fully the break obtained with the original material.

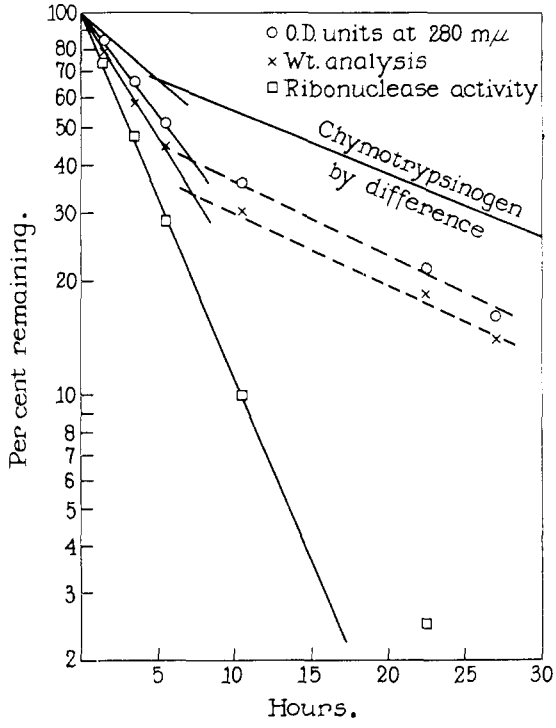

Fig. 8.—Dialysis of an artificial mixture of equal parts of ribonuclease and chymotrypsinogen through 20/32 cellophane in 0.01 *N* acetic acid.

(18) M. Kunitz, *J. Biol. Chem.*, **164**, 563 (1946).

Chymotrypsinogen gave a result very similar but with the break occurring earlier. It also did not give as marked a shift on re-dialysis as was expected. The results thus far appear to suggest that the behavior may be caused in part by impurity, and in part by a tendency to denature. On recovery a part of that which had shown a slower escape rate due to its being in the denatured state could shift back into the undenatured form and thus confuse the quantitative aspects of the experiment somewhat.

As stated in the introduction it is the purpose of this paper to report some of the basic studies fundamental to the use of a stepwise countercurrent dialysis process. Perhaps this can be emphasized best by the experience with an artificial mixture of ribonuclease and chymotrypsinogen. Although the larger has less than twice the molecular weight of the smaller the data shown in Fig. 8 would indicate that they could be separated quite well in only a few dialysis stages.[2] It so happens that these proteins are separated easily by other methods and Fig. 8 is of value only for an example.

Our studies thus far have shown that selectivity can be improved by choice of a membrane which will pass the solutes of interest only slowly under favorable conditions. For certain mixtures the selectivity can be improved by doubling the membrane.[6] It can be varied by choice of the solvent[2] and by substitution of the solutes, for instance, as by preparation of DNP derivatives of amino acids or peptides.[2]

A given roll of cellophane can be made considerably more porous by placing it under hydrostatic pressure. Thus 20/32 Visking will withstand a hydrostatic pressure of 200 mm. of Hg, but will stretch from a diameter of 15 to 24 mm. After release of the pressure the diameter will return to 21 mm. A cell made from this membrane will now pass larger proteins than the unstretched membrane. A brief treatment with sodium hydroxide also makes the membrane more porous.[4]

On the other hand acetylation of the membrane appears to make it less porous. A preliminary experiment with the 20/32 membrane has given a membrane which would not pass subtilin, but would permit bacitracin to pass slowly (50% escape time = 2.8 hr.). It readily passed tryptophan (50% escape time = 11 min.). Acetylation of 18/32 Visking gave an even less porous membrane which would scarcely permit bacitracin to pass although it gave a 50% escape time of 15 minutes for tryptophan. These findings with modified membranes are again in line with the thesis that size is the primary factor governing an escape rate for a given membrane and solution.

(19) C. H. W. Hirs, S. Moore and W. H. Stein, *ibid.*, **200**, 493 (1953).

A closer study of modified membranes must be left for a later communication. It is apparent, however, from the data given in this paper that much greater over-all selectivity for separations can be achieved by use of a membrane of the optimum pore size. When mixtures containing molecules of widely differing size are to be separated a series of membranes with different porosities will be needed.

From our data at present with cellophane membranes and 0.01 *N* acetic acid five standard sizes are easily available as follows. (1) A stretched 20/32 membrane which readily passes native ovalbumin. (2) The 20/32 Visking cellophane which readily passes native proteins up to ovalbumin. (3) The 18/32 Visking cellophane which will not pass proteins as large as insulin. (4) Acetylated 20/32 Visking which will not pass subtilin but will pass bacitracin. (5) Acetylated 18/32 Visking which will scarcely pass bacitracin. A study of the optimum way to integrate the use of these different sizes with the countercurrent approach is in progress.

The porosity of the membrane does not correlate with the thickness as can be seen from the data in Table II, which gives five sizes of cellophane studied with insulin. The thickness given is that found with a micrometer on the dry membrane before use.

TABLE II

COMPARISON OF THICKNESS, SIZE AND POROSITY OF SEVERAL SIZES OF VISKING TUBING

Size	Thickness, mm.	Result with insulin
8/32	0.064	Slowly passes
18/32	.0175	Does not pass
20/32	.020	Rapidly passes
23/32	.0275	Does not pass
27/32	.025	Passes less rapidly than with 20/32

NEW YORK, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE]

The Methylation of Deoxyuridine[1]

BY ELIZABETH A. PHEAR AND DAVID M. GREENBERG

RECEIVED JANUARY 15, 1957

The methylation of deoxyuridine, with formaldehyde as the methyl group precursor, has been demonstrated with cell-free preparations from various tissues. The most active of the extensively studied tissues was the thymus gland. Employing a Dowex-1-Cl^--treated, dialyzed rat thymus preparation, the primary product of the reaction appeared to be thymidylic acid, which was then partially converted to thymidine. Tetrahydrofolic acid, reduced phosphopyridine nucleotide, adenosine triphosphate and Mg^{++} were required as cofactors for the reaction.

Friedkin and Roberts[2] showed that deoxyuridine (UDR)[3] was incorporated into DNA-thymine and free TDR by suspensions of chick embryo and by rabbit and chick bone marrow. They concluded that the TDR was formed by a folic acid-dependent methylation. Reichard[4] also showed that deoxyuridine was incorporated into DNA-thymine. The observations of Friedkin and Wood[5] suggested that CDR also may be incorporated into DNA-thymine by the same or a similar reaction.

This paper describes studies of the methylation of UDR with $H_2C^{14}O$ in a cell-free preparation of rat thymus gland, and a survey of the distribution of this enzyme system in various other tissues.

Experimental

Materials.—Thymus glands from 1 month old Long-Evans rats were used for most of the work. In the determination of the distribution of the enzyme in various tissues, spleen and bone marrow from 1 month old rabbits, and marrow from pork, lamb and veal bones,[6] and thymus glands from steer, lamb and pig[7] were tested.

The sources of the following chemicals were: DPNH from Sigma Co., ATP from Nutritional Biochemical Co., UDR and TDR from California Foundation for Biochemical Research, folic acid from Mann, CF was a gift of the Lederle Laboratories, and the $H_2C^{14}O$ was purchased from the Bio-Rad Co., Berkeley. THF was prepared by the reduction of folic acid by the method of O'Dell, *et al.*[8]

Enzyme Methods.—Cell-free tissue preparations were obtained by homogenizing the minced tissue with 9 volumes of 0.05 *M* veronal buffer at *p*H 7.6 in a Potter–Elvehjem glass homogenizer, followed by centrifugation for 10 minutes at 20,000 × *g*. The thymus preparations were then batch-treated with Dowex-1-Cl^-,[9] centrifuged at 59,000 × *g*, and dialyzed overnight against veronal buffer.

The incubations were run for 2 hr. in 25-ml. beakers at 37° under nitrogen in a Dubnoff shaking metabolic incubator. Each vessel contained 2 ml. of enzyme (5–20 mg. protein) and substrates and cofactors at the following levels: $H_2C^{14}O$, 5 μmoles (98,400 c.p.m. per μmole); UDR, 10 μmoles; carrier TDR, 1.2 μmoles; ATP, 10 μmoles; DPNH, 2 μmoles; $MgSO_4$, 10 μmoles; THF 500 μg. The total volume was 2.7 ml. and when any of the above constituents was omitted the volume was made up with buffer; 0.05 *M* veronal buffer at *p*H 7.6 was used throughout unless otherwise indicated. Carrier thymidylic acid was added after incubation, since it inhibited the reaction, but the addition

(1) Aided by research grants from the National Cancer Institute (CY-2915 and 3175), National Institutes of Health, U. S. Public Health Service and the Cancer Research Funds of the University of California.

(2) M. Friedkin and D. Roberts, *Federation Proc.*, **14**, 215 (1955); *J. Biol. Chem.*, **220**, 653 (1956).

(3) Other abbreviations employed: TDR, free thymidine; TA, "thymidylic acid"—see Analytical Methods section; TTC, total acid-soluble compounds yielding thymine on hydrolysis; CDR, deoxycytidine; CF, citrovorum factor, leucovorin; ATP, adenosine triphosphate; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; TCA, trichloroacetic acid; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; THF, tetrahydrofolic acid.

(4) P. Reichard, *Acta Chem. Scand.*, **9**, 1275 (1955).

(5) M. Friedkin and H. Wood, IV, *J. Biol. Chem.*, **220**, 639 (1956).

(6) Kindly supplied by Swift and Co., San Francisco.

(7) These were a gift from James Allen and Sons, 3rd Street, San Francisco.

(8) B. L. O'Dell, J. M. Vandenbelt, E. S. Bloom and J. J. Pfiffner, THIS JOURNAL, **69**, 250 (1947).

(9) B. E. Wright and T. C. Stadtman, *J. Biol. Chem.*, **219**, 863 (1956).