Dialysis Studies. VI. Experiments with Amino Acids*

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A study has been made of the membrane diffusion behavior of amino acids with the thin-film dialysis technique. Membranes of low porosity and high selectivity were made by acetylating cellophane. The results demonstrated conclusively that the charge on the solute has very little effect on the rate of diffusion and that this type of membrane carries very little if any fixed charge. The rate of diffusion at a given temperature therefore is a true measure of the effective diffusional volume except where adsorption is found. Data are presented to show that reversible adsorption can accelerate the rate of diffusion through the membrane.

Amino acids play such a central role in biochemistry that they now have been studied from almost every conceivable standpoint. Their behavior in free diffusion has naturally not been overlooked (Longsworth, 1957). Diffusion under conditions imposing selective restrictions based on effective molecular volume and shape, however, can be quite different from free diffusion and should be a subject worthy of close study. The environment of living tissue in which membranes, gels, and interfaces abound could offer conditions greatly enhancing the relatively small differences noted in free diffusion.

Diffusion through a dialysis membrane is one form of restricted diffusion which can be studied in a very simple and reproducible way (Craig and King, 1962). The present investigation was undertaken with this in mind and at the same time in order to throw light on certain questions connected with the thin-film dialysis technique itself.

One of these concerned the question of the effect of fixed charges on the membrane. Cellophane, the membrane used exclusively in these studies thus far, might be expected to carry a few carboxyl groups and therefore to behave as a weak cation-exchange membrane. If so, the rate of diffusion through the membrane could then be a function of the charge carried by the solute molecule as well as the effective volume and shape. The amino acids are interesting in this connection because the charge is relatively large in relation to their size as compared to peptides and proteins. They are therefore particularly interesting models for studying the effect of charge.

MATERIALS, METHODS, AND DATA

The amino acids used were analytically and chromatographically pure preparations purchased from several different supply houses. The cellophane tubing was of the 18/32 and 23/32 sizes purchased from the Visking Co., Chicago, Ill., during the year 1961.

Dialysis cells with membranes of the required low porosity were made from the tubing as received by linear stretching and subsequent acetylation in pyridine with acetic anhydride (25%) as previously described (Craig and Konigsberg, 1961). A time of heating in the acetylation mixture of 7 hours at 65° gave reproducible membranes with the 18/32 size which would scarcely allow amino acids to pass at 25°. With the 23/32 tubing, an acetylation time of 2 hours gave a more porous membrane. At 40° the half-escape time was of the order of several hours for the less porous membrane.

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The cells and procedure were of the type previously described (Craig and Konigsberg, 1961). The volume of solution inside the membrane was 0.4–0.5 ml for cells affording approximately 50 cm^2 of dialyzing surface. Analysis of the diffusate was made by the ninhydrin procedure of Moore and Stein (1954), by weight analysis (Craig, 1960), or by optical density in the Beckman ultraviolet spectrophotometer in the case of the aromatic amino acids. In a number of instances the ninhydrin analyses were checked by weight analysis. All the samples tested gave straight-line escape plots. The weight of the sample used for a run varied between 1 and 10 mg. No concentration effect was noted in this range. The total material accounted for in the diffusate plus that in the retentate where a significant amount remained, agreed to within a few per cent of the weight taken.

The data in Table I were obtained with two membranes. Nearly every value given was checked in a separate experiment with the same membrane. One value which was not checked was the 7.2-hour value for leucine in the third column. Unfortunately, this particular membrane developed a pinhole before the value could be rechecked. Exact matching of porosities or sufficient matching to use in a series of this type is sometimes troublesome. However, it is almost certain that this value is too high. Comparative studies with another membrane did not reveal a significant difference between leucine and isoleucine. The relative differences between other amino acids in this table were supported by studies with membranes of slightly different porosities.

A rather limited study was made with one membrane prepared in the same way but by chance with significantly lower porosity. These data obtained also at 40° are given in Table II.

A series of experiments was made in which the potential across a membrane was measured when different concentrations of an ionized solute were placed on each side of the membranes. The solutes studied were sodium chloride, lysine hydrochloride, and monosodium glutamate. The solutions on each side of the membrane were connected to saturated calomel half-cells by means of Ostwald bridges containing the solutions in contact with the membrane. The potential between the two calomel half-cells was measured on the millivolt scale of a Radiometer *p*H meter.

In the data given in Table III, three different membranes were used. No. 1 was the most porous. It was made from 18/32 untreated Visking cellulose casing. No. 2 was a tighter acetylated membrane (No. 2 of Table I). No. 3 was still tighter and almost prevented the diffusion of amino acids as shown in Table I (membrane No. 1 of this table).

TABLE I

HALF-ESCAPE TIME OF AMINO ACIDS IN HOURS AT 40°

Amino Acid	In Water		In 0.01 HAc
	Membrane No. 1[a]	Membrane No. 2[b]	Membrane No. 1[a]
Tryptophan	3.2	0.35	2.0
Tyrosine	—	—	2.8
Phenylalanine	4.4	0.36	2.8
Leucine	5.8	0.5	7.2
Isoleucine	5.7	0.5	5.5
Valine	5.3	0.47	4.5
Alanine	2.9	0.43	2.7
Glycine	2.5	0.27	2.1
Threonine	3.9	0.43	—
Serine	3.2	0.42	3.5
Proline	3.3	0.37	—
Glutamic acid	7.0	0.45	5.2
Aspartic acid	5.0	0.55	4.7
Lysine	8.7	0.43	6.0
Histidine	9.3	0.42	7.0
Ornithine	5.7	0.52	—

[a] Membrane No. 1 = 18/32 stretched and acetylated for 7 hours at 65°. [b] Membrane No. 2 = 23/32 stretched and acetylated 2 hours at 65°.

DISCUSSION

For comparative studies with the highest precision using thin-film dialysis, the data should all be obtained with the same cell. The modification procedure applied to the membrane usually does not give exactly matching porosities with different cells even though they are prepared in the same way. Some of this variation probably relates to the stretching procedure since the membrane is stretched (Craig and Konigsberg, 1961) while wet until it breaks at its weakest point. In spite of this, amazingly reproducible data can be obtained as was shown for sugars in a previous paper (Craig and Pulley, 1962). Here a selectivity was achieved which can be related to a difference of approximately 2 or 3% in effective molecular diameters. One molecule of the same shape as another but with twice the volume, i.e., 25% larger effective diameter, gave several-fold or more the 50%-escape time.

TABLE II

HALF-ESCAPE TIME IN HOURS AT 40° OF AMINO ACIDS IN MEMBRANE NO. 3

Amino Acid	Water	0.15 M $MgSO_4$
Tryptophan	5.3	8.3
Valine	14	
Glycine	5.8	8.5
Glutamic acid	20.5	
Glutamine	22.6	
Aspartic acid	13.6	
Asparagine	18	

Thus it was not surprising that considerable differences would be found for the different amino acids. It was expected that a less porous membrane would be required than for the sugars in order to show these significant differences. It so happens that membrane No. 2 of Table I is the same membrane or one of nearly equal porosity as the one of lowest porosity used in the sugar study (Craig and Pulley, 1962, membrane No. 1 of the earlier study). It was found to pass glucose with a half-escape time of 2.1 hours at 40°. This is 4-fold the time required for any of the amino acids. This difference is in agreement with a lower diffusion coefficient (Longsworth, 1957) in free diffusion and is probably caused by the known extensive hydration of the glucose molecule.

In any case this membrane is not sufficiently selective to reveal clearly the differences between the different amino acids. However, a more highly acetylated

TABLE III

POTENTIALS OBTAINED ACROSS VARIOUS MEMBRANES WITH DIFFERENT SOLUTES AND DIFFERENT CONCENTRATIONS ON EACH SIDE OF THE MEMBRANE

Solute	Concentration		Potential (mv)
NaCl	1.0 M/0.1 M	Liquid junction potential	+ 9.0
		Membrane 1	+ 9.0
		2	+10.0
		3	+12.3
NaCl	0.1 M/0.01 M	Liquid junction potential	+10.0
		Membrane 1	+ 9.2
		2	− 0.3
		3	− 8.9
Lysine (mono HCl)	0.1 M/0.01 M	Liquid junction potential	+27.3
		Membrane 1	+27.8
		2	+34.5
		3	Initially +24 but slowly rising to +32 in 15 min
Lysine (mono HCl)	0.01 M/0.001 M	Liquid-junction potential	+26.6
		Membrane 1	+33.0
		2	+ 7.5
		3	+8.0 (not steady)
Monosodium glutamate	0.1 M/0.01 M	Liquid-junction potential	−19.3
		Membrane 1	−26.5
		2	−42.7
		3	−44.0
0.1 M lysine (mono HCl) against 0.1 M sodium chloride		Liquid-junction potential	+ 7.0
		Membrane 1	+ 7.8
		2	+12.3
		3	+16.5

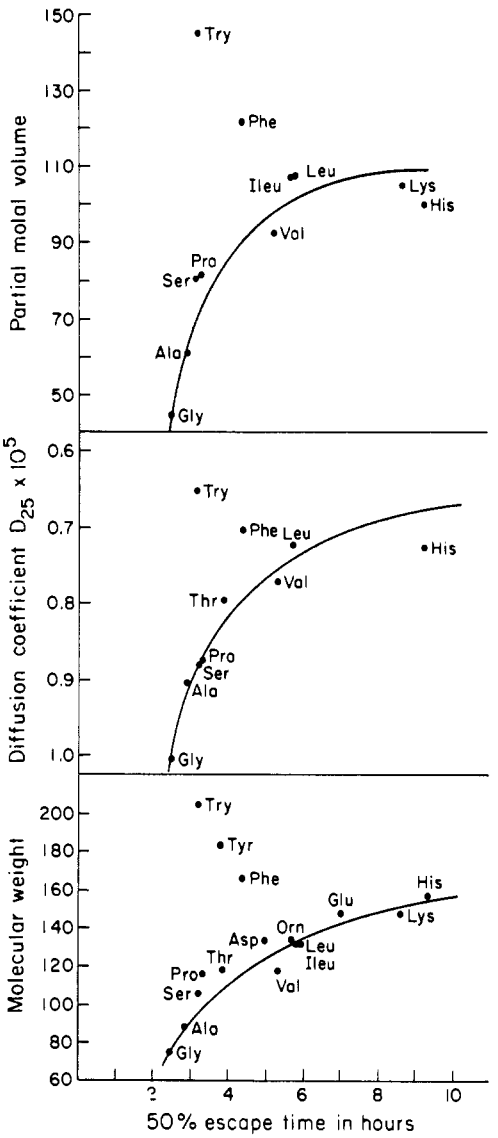

FIG. 1.—Curves comparing the correlation of escape rate with molecular weight, diffusion coefficient, and partial molal volume.

membrane makes this possible as the data in Table I show. Thus glycine diffuses through membrane No. 1 at between 2- and 3-fold the rate of leucine or isoleucine. Valine and alanine give intermediate rates as expected.

The data from Table I can be compared graphically as in Figure 1 by plotting 50%-escape times against molecular weight, against partial molal volume, or against diffusion coefficients. These comparisons clearly show that the rate of dialysis under these conditions does not correlate closely with molecular weight, molal volume, or diffusion coefficient for all the amino acids. A reasonably good correlation with published diffusion coefficients (Longsworth, 1957) is obtained if the aromatics are excluded. If only the unsubstituted aliphatic amino acids are considered, the correlation between partial molal volume and escape rate would seem to approximate a straight line. This, however, has no significance since the selectivity factor increases sharply as the limiting pore size is approached (Craig and King, 1962). The correlation thus should follow a curve as in Figure 1.

The correlation with molecular weight is nearly as good as with molal volume except, again, for the aromatic amino acids. Figure 1 also shows that the excess positive or negative charge on the basic and acidic amino acids is not a primary controlling factor. If fixed negative charges such as carboxyl groups were on the membrane, this should strongly retard the passage of the acidic amino acids as compared to the basic amino acids. On the contrary lysine passes more slowly than glutamic acid. This, therefore, speaks strongly against fixed charges on the membrane.

Thus it would appear that the earlier observations (Craig *et al.*, 1957) regarding the lack of effect of charge are correct. Apparently as far as membrane diffusion is concerned, the rate does not depend on which side of the isoelectric point the solution happens to be unless an appreciable change in shape occurs on going from one side of the isoelectric point to the other. Nonetheless it can be shown by suitable potentiometric measurements during the diffusion of certain salts through the membrane that a limited number of fixed charges negative in nature are actually present on the membrane. Data from measurements of this type are given in Table III.

When measurements were made of the liquid-junction potential between 1.0 M sodium chloride and 0.1 M sodium chloride, the concentrated solution was 9 mv more positive than the 0.1 M solution. However, when a cation-exchange membrane (a sulfonic acid–type resin imbedded in a plastic film matrix; obtained from National Aluminate Co.) was inserted, the passage of chloride ions was prevented and the concentrated solution registered a potential of −48 mv.

On the other hand, the No. 2 membrane gave a potential of +10.0 mv, a slight increase over the liquid-junction potential. The tightest membrane, the one offering the greatest differential resistance to the diffusion of Na^+ ion as compared to Cl^- ion, gave a still higher potential, +12.3 mv.

When the concentration gradient was 0.1 M against 0.01 M, a different result was obtained. Here a reversal of the sign of the potential was experienced, a result which could be due to the meager fixed charges on the membrane.

Lysine monohydrochloride gradients in the same molar concentration gave liquid-junction potentials of the same sign but of considerably higher potential. Moreover, the reversal of the sign when the membrane was inserted with the 0.1/0.01 M gradient was not noted. At a gradient of 0.01/0.001 M an effect in this direction was registered, but the measurements were less steady.

When monosodium glutamate was substituted for lysine hydrochloride, a gradient of 0.1/0.01 M gave the expected reversal in sign.

Finally when a gradient of 0.1 M lysine monohydrochloride was studied against 0.1 M sodium chloride, the liquid-junction potential was +7.0 mv on the lysine side when membranes were interposed. This potential increased as the membrane became less porous.

These results are all consistent with the concept of an increased selectivity due to differences in diffusional resistance as the pores of the membrane become smaller. The fixed charges on the membrane appear to play a role only with the dilute solutions.

One of the more interesting observations from the data concerns tryptophan. Although it has almost three times the molecular weight of glycine it diffuses through the membrane almost as rapidly, and much more rapidly than leucine or isoleucine. Tyrosine and phenylalanine also diffuse more rapidly than leucine and isoleucine.

Aromatic amino acids are known to be adsorbed on surfaces to a greater extent than the aliphatic amino acids. In order to test this property as a possible ex-

planation, the standard diffusion experiment was performed with tryptophan except that the experiment was interrupted when only half the solute had passed through the membrane. The inside solution was then removed and the membrane was quickly rinsed. When the total recovery was measured by optical density measurement, it was found that only about two-thirds of the tryptophan initially taken could be accounted for. This contrasted with the recovery of 90–95% of leucine or isoleucine under similar conditions. However, with tryptophan the loss could be recovered by leaching the membrane for an additional hour or more with two changes of fresh solvent. A similar but somewhat less striking behavior was noted with tyrosine and phenylalanine. Peptides containing tryptophan and tyrosine were found to be adsorbed also but to a lesser degree.

In spite of this adsorption, tryptophan gave a straight line with satisfactory recovery when the run was continued until 90% or more of the sample had passed. Apparently the concentration of the aromatic amino acids inside the membrane itself can be higher than on either side because of adsorption. Curiously enough the net effect is to accelerate the passage through the membrane.

Accelerated diffusion of gases through porous material because of adsorption has been noted before (Gilliland *et al.*, 1958) and indeed forms the basis of a very interesting separation method.

As far as solutions are concerned, it has been noted with gel filtration (Gelotte, 1960; Porath, 1960), a method based on the relative rates of diffusion into gel interstices, that aromatic amino acids and particularly tryptophan are retarded on the column. The form of the band tends toward the somewhat skewed form obtained where adsorption plays a role. If Sephadex adsorbs aromatic amino acids, it might be expected that cellophane, a similar material, would behave likewise.

With the tighter membrane used in Table II, the relative rates were in the same order as in membrane No. 1 but with somewhat larger differences owing to still greater selectivity. Again tryptophan showed anomalous behavior and diffused at the same rate or a little more rapidly than glycine. It was interesting that asparagine diffused through more slowly than aspartic acid and glutamine, and perhaps also a little slower than glutamic acid. This could well be a matter of conformation in solution or hydration but could also be due to the effect of the rapidly diffusing hydrogen ion on the negative larger ion.

In this membrane at 40°, 0.15 M $MgSO_4$ was found to slow the diffusion of both tryptophan and glycine. Any salt in this concentration seems to slow somewhat the rate of diffusion through cellophane of most solutes, even sugars. This probably is due to its effect on the gel structure of the membrane as well as on the conformation of the solute. This relatively small effect contrasts sharply with that found for certain of the longer peptides and proteins where considerable change in conformation can play a role. For instance, as will be reported in a later communication, addition of sodium chloride (0.2 M) can change the half-escape time of ACTH from 1 to 8 hours.

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