of their amino acid composition, and the data are given in Table III. A difference in relative lysine and arginine contents was anticipated because the two components differ in mobility on starch-gel electrophoresis. Large differences were also found in the serine, glycine, methionine, leucine, and tyrosine contents of fractions IIb_1 and IIb_2, but the significance of these cannot be discussed at this stage.

The extreme complexity of thymus histones, which was demonstrated by the starch-gel electrophoresis experiments of Neelin and Neelin (1960), has been emphasized by the studies on various chromatographic fractions of histone reported here. Only in the case of histone fraction IIb has the authenticity of the components indicated by starch-gel electrophoresis been established by a second electrophoresis as described by Neelin and Neelin (1960). The various chromatographic histone fractions contain a multiplicity of components, and the formidable task of establishing the difference or similarity of corresponding electrophoretic components in different chromatographic fractions is of importance in illustrating the true complexity of histone. Studies of partial enzymatic hydrolysates of various electrophoretic bands by the fingerprinting method of Ingram (1956) or the chromatographic procedure of Crampton *et al.* (1957) may provide the most convenient approach to this problem.

ACKNOWLEDGMENTS

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NOTE ADDED IN PROOF: Since the preparation of this manuscript, we have found that the unidentified compound in the hydrolysates of histone fractions IIaa, IIa, III, and IV resembles ϵ-*N*-methyllysine in its behavior on ion-exchange chromatography. We are grateful to Dr. F. Sanger for a gift of flagellin.

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Dialysis Studies. IV. Preliminary Experiments with Sugars*

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Thin-film dialysis experiments have been made with a variety of sugars. From the data it has been concluded that where adsorption does not play a role the factors which determine the rate of free diffusion also determine the rate of dialysis. However, in comparative studies with different solutes thin-film dialysis appears to amplify greatly the differences noted in free diffusion. The data also indicate that differences in effective molecular diameters of the order of 2% or less can be detected by dialysis.

The dialysis studies under way in this laboratory were originally undertaken with a rather limited objective in mind, that of making simple dialysis a more useful separation technique (Craig and King, 1955) for small-scale laboratory work. The inherent selectivity and nature of the process soon suggested other possibilities, however, and led us to broaden our objective considerably. The method now offers one of the most convenient and informative procedures for determination of the homogeneity (Craig *et al.*, 1957a) with respect to size of a given preparation. In addition, when such dialysis data (Goldstein and Craig, 1960) are supported by data from ultracentrifugation and other techniques, much can be learned in regard to the state of association in a given solution and of the changes in association and molecular shape that occur when the solvent is altered as

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shown by Craig *et al.* (1957b) in a variety of ways.

Ideally the method will be most informative when it is carried out in such a way that the maximum selectivity is achieved and under conditions that permit the selectivity to be clearly related to the size and/or shape of the molecule. Since in the method selectivity is recognized by the differences in the rates of dialysis of known molecules of slightly different size, it becomes important to have a series of reliable model substances of known size and shape for membrane calibration.

Thus far most of our model substances have been peptides or proteins well documented with respect to purity. For the most part, they have been charged substances amphoteric in nature, with several acidic and basic groups. Cellophane, the membrane of choice thus far in our work, probably does not carry many charged groups but can well be slightly acid owing to a relatively small number of free carboxyl groups. Although the data already published by Craig *et al.* (1957b) and Goldstein and Craig (1960) strongly indicate that charge plays a secondary role, it can be an effect which should not be overlooked.

In any case it seemed advisable to take up the study of the sugars as model substances for the calibration of membranes. Aside from lack of charge, many simple sugars can be obtained in a high state of purity. Data on free diffusion are available for a number of them (Longsworth, 1957). Many of them have a rigid and fixed shape, particularly the nonreducing sugars.

A series especially suited for use as model solutes for the study of dialysis can be found in the Schardinger dextrins. They are cyclic polymers of glucose joined through 1-to-4 linkages. The sizes and shapes of at least the lower members of the series are known (French, 1957).

Recently Pulley and French (1961) have developed a chromatographic procedure whereby all the members of the series containing from six to twelve glucose units can be isolated. These preparations have been available for the study reported here.

Definite differences in dialysis rates of various sugars with unmodified cellophane have been noted by other workers (Binkley, 1960; Templeman and Marshall, 1960), even though the porosity is far above that which would prevent diffusion. A less porous membrane should give higher selectivity. The recently published procedures of Craig and Konigsberg (1961) for reducing the effective pore size of cellophane membranes make it possible to study even the smallest sugars under conditions optimal for high selectivity.

Experimental

The type of dialysis cell was the one with the removable inside tube reported in a previous publication by Craig and Konigsberg (1961). Stirring was employed in all cases. The solvent used throughout was deionized water. Solute concentrations were determined by microresidue weight determination (Craig, 1960) except in a few cases where the colorimetric determination with the anthrone reagent was convenient. The latter method was checked against weight determinations.

The cells used all provided approximately 50 cm^2 of dialyzing area. The inside or retentate volume varied slightly from cell to cell but approximated 0.6 ml. Thus the thickness of the retentate film was 0.1 to 0.2 mm. The diffusate volume also was different for each cell, from 4 ml to 7 ml. The amount of sample taken for each run usually was 5 mg, but in certain cases it was less because of the scarcity of the material. Thus the concentration of the sugar in the retentate usually approximated 1% at the start of the diffusion experiment.

A series of membranes of varying porosity were prepared from Visking casing as previously described (Craig and Konigsberg, 1961) by linear stretching to the limit and then acetylation in anhydrous pyridine with acetic anhydride. Table I gives the time of acetylation at an oil bath temperature of 65°. Cell No. 1 was the least porous, No. 5 the most porous.

Table I
Acetylation Times for Membranes

Membrane No.	From Visking	Acetylation Time (hr.)
1	23/32	2
2	18/32	3
3	18/32	2
4	20/32	3
5	20/32	2

With a few exceptions, all the data for a given porosity of membrane were obtained with the same cell. The membrane could thus be used repeatedly for several weeks without appreciable change in its porosity. Cells not in use were kept wet and stored in the cold room. When a membrane was found to leak, a new one with very similar porosity as measured by escape time for a standard solute could easily be prepared from the same roll of Visking.

Several different temperatures were studied: 25°, 40°, and 60°. The two higher temperatures were controlled by a small constant-temperature water bath. For the lower temperature, the work was done in a constant-temperature room held at 25°.

Results and Discussion

Membrane No. 1 was found to pass glucose at a rate that permitted considerable selectivity (3.5 hours half-escape time at 25°). When sucrose was tried, however, it was found to pass much more slowly, with a half-escape time approximating 30 hours (Fig. 1, lower pattern). The trisaccharide, raffinose, was found not to pass at all. Obviously, by making a membrane with slightly smaller pores, it would be possible to pass glucose but exclude sucrose.

That the selectivity is based on the dimensions of the molecule regardless of the size range is indicated by the published results of Craig and Konigsberg (1961). Ribonuclease and chymotrypsinogen (m.w. 13,600 and 25,000 respectively) in 0.01 N acetic acid were passed through an untreated 20/32 membrane. The half-escape time of ribonuclease was 3.5 hours and that of chymotrypsino-

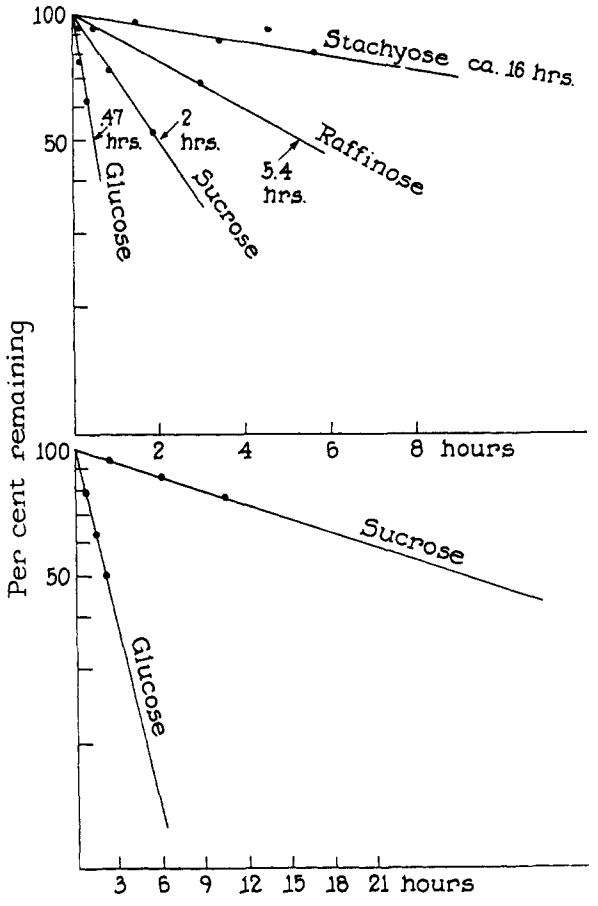

FIG. 1.—Escape patterns at 25° of various sugars through membrane No. 1, lower chart, and No. 2, upper chart.

gen 12 hours. In unpublished work still better selectivity has been achieved by use of a membrane with slightly reduced porosity.

At 25°, membrane No. 2 passed glucose too readily to have high selectivity but passed raffinose slowly and the tetrasaccharide stachyose even more slowly, as shown in Figure 1, upper pattern.

Membrane No. 5 passed stachyose at 25° with a half-escape time of 0.68 hour. Cyclohexaamylose under the same conditions had a half-escape time of 1.1 hours. These data and others are given in Table II.

With membrane No. 1 the pentoses xylose and arabinose were found to dialyze more rapidly than glucose and galactose, as shown in Table II. Moreover, xylose dialyzed more rapidly than arabinose. The type of escape curve also was of some interest. Arabinose, xylose, and galactose gave the type of curve shown in Figure 2, which indicated the presence of more than one size or type of molecule. A reinvestigation of glucose, pursued until more than 70% of the solute had emerged, revealed that for glucose, too, there was a break in the curve at about the 50% escape point. The curves for lactose and cellobiose also showed breaks.

As experience with other sugars accumulated, it was found that all the reducing sugars gave a break in the curve while the nonreducing sugars did

TABLE II

RELATIVE DIALYSIS RATES OF VARIOUS SUGARS

	m.w.	Half-Escape Time (Hr.)	Curve Shape
	Membrane No. 1[a]		
Sucrose	342	30	Straight line
KCl	74	0.37	Curve positive
NaCl	58	0.44	Curve positive
Xylose	150	1.3	Break positive
Arabinose	150	1.9	Break positive
Glucose	180	3.5	Break positive
Galactose	180	4.8	Break positive
	Membrane No. 2[a]		
Sucrose	342	1.5	Straight line
Glucose	180	0.47	Break positive
Lactose	342	2.6	Break positive
Cellobiose	342	2.7	Break positive
Raffinose	504	5.4	Straight line
Stachyose	666	15	Straight line
	Membrane No. 5[a]		
Stachyose	666	0.68	Straight line
Cyclohepta-amylose	1152	1.1	Straight line

[a] For explanation of membrane numbers, see Table I.

not, as can be seen from Table II. This tentative finding should not be taken too seriously until a greater number of sugars have been studied.

In the case of galactose, the cause of the break

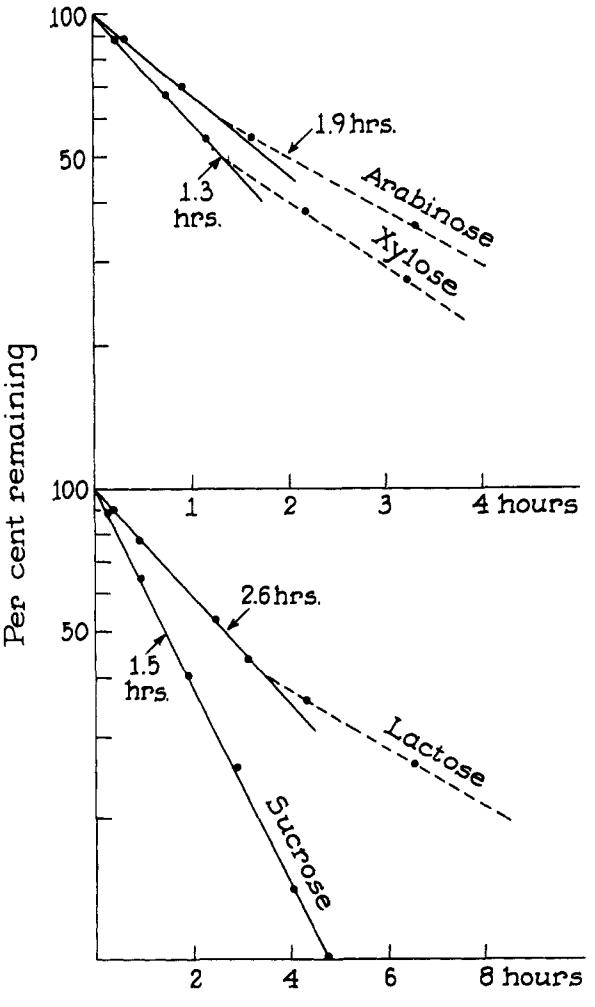

FIG. 2.—Escape patterns at 25° of four sugars through membrane No. 2, lower chart, and No. 1, upper chart.

was investigated a little further. An initial run with three times the amount of sample was pursued until about 65% had escaped. At this time the majority of the faster-dialyzing component should have been removed. The retentate was then recovered by lyophilization and a run made on this material. A definite break was observed again, but a little earlier in the run. It thus would seem that at least two interconvertible species are indicated. Reducing sugars are known to be equilibrium mixtures of isomers, with the establishment of complete equilibrium requiring a certain time. An attempt to throw light on the problem by direct measurement of optical activity of the diffusates was not too conclusive because of the dilution and varying concentrations but was sufficient to indicate that the first diffusate had a rotation different from the last and from the retentate. Further study will be required to settle the type of isomerization responsible for the inhomogeneity on dialysis.

It is certainly reasonable to expect that different isomers or conformational forms could show different dialysis rates provided a sufficiently selective membrane is used. Here the selectivity of the membrane is already demonstrated by the results in Table II and perhaps more precisely demonstrated with the Schardinger dextrins as discussed below. In this connection also a basis for the selectivity could be the fact that one isomer could be hydrated to a different degree than the other. If so, this could give rise to detectable differences in rates of dialysis.

The effect of increasing the temperature can be studied very easily with the experimental procedure used in these experiments. The rate of escape can be measured at a particular temperature until about one third of the sample has dialyzed, then the cell can be placed in a water bath at a higher temperature. Thus as shown in Figure 3 temperature coefficients can be determined easily. With an ideal solute, the Stokes-Einstein equation, $D = kT/6\pi\eta r$, should hold and the rate of dialysis should be directly proportional to the absolute temperature and inversely proportional to the viscosity. From these data it can be calculated that an ideal solute in water should dialyze 1.44 times as fast at 40° as at 25° and about the same amount faster at 60° than at 40°. The ratios noted in Figure 3 are somewhat higher than this, as was found to be the case with other sugars. A likely explanation would seem to be that they are inclined to be less hydrated at the higher temperature. The effective size of the molecule—its diameter as far as dialysis is concerned—is the diameter of hydrated volume. Temperature effects will be treated in more detail in a following paper.

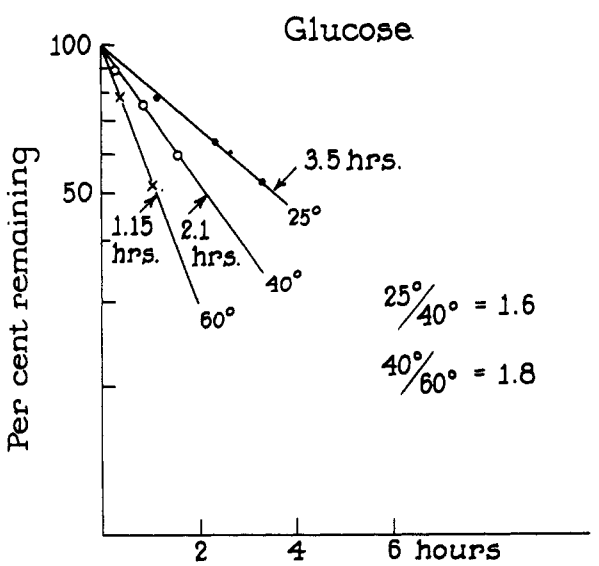

FIG. 3.—Effect of temperature on the dialysis rate of glucose.

In any case, with higher temperatures which give a more rapid rate of dialysis, it should be possible to use a membrane with an average pore size correspondingly smaller than is necessary at lower temperatures. Thus a higher inherent selectivity would be expected. In specific cases this might not hold because of the hydration behavior of the particular solutes involved. A corresponding tendency toward loss of hydration could affect the membrane also at the higher temperature. It has been established that a membrane calibrated against a known solute at 25°, then repeatedly used at 60°, will give the original calibration when returned to 25°.

The smallest Schardinger dextrin, cyclohexaamylose, was found to pass membrane No. 3 with a half-escape time at 40° of 6 hours. Half-escape times in this membrane of the hepta, octa, and nona cycloamyloses are plotted in Figure 4, right chart. Since the higher members of the series would not dialyze through membrane No. 3, data for the whole series up to cyclododecahexaamylose were obtained with membrane No. 5. These data are plotted on the same scale for comparison and also on an expanded scale in the left chart. Two points in a similar study with this membrane at 25° are given and also points with the tighter membrane at 60°, right chart. It is obvious from the data that the tighter membrane is considerably more selective.

In a recent review of the chemistry of the Schardinger dextrins, French (1957) has published photographs taken of space-filling models of these interesting substances. From the photographs with a scale in Angstroms attached, it is possible to approximate[1] rather closely their size and shape, excluding the water of hydration. The Schardinger dextrins resemble a doughnut with the smallest dimension approximating 7.8 A. Assuming the ring does not tend to crumple as its size is increased in the higher homologues, this dimension would remain constant. The outside diameter of the ring would then measure approximately 13.7, 15.3, and 16.9 A, respectively, for the hexa, hepta, and octa homologues. It would be expected that the ring would assume a round shape rather than an elliptical one. The increase in effective size due to hydration is not known, but since these dextrins are all 1-to-4 polymers of glucose, their escaping

[1] In a personal communication, Dr. French has stated that a misprint in the manuscript had occurred and that each square of the background in the published chart should correspond to 4.5 A.

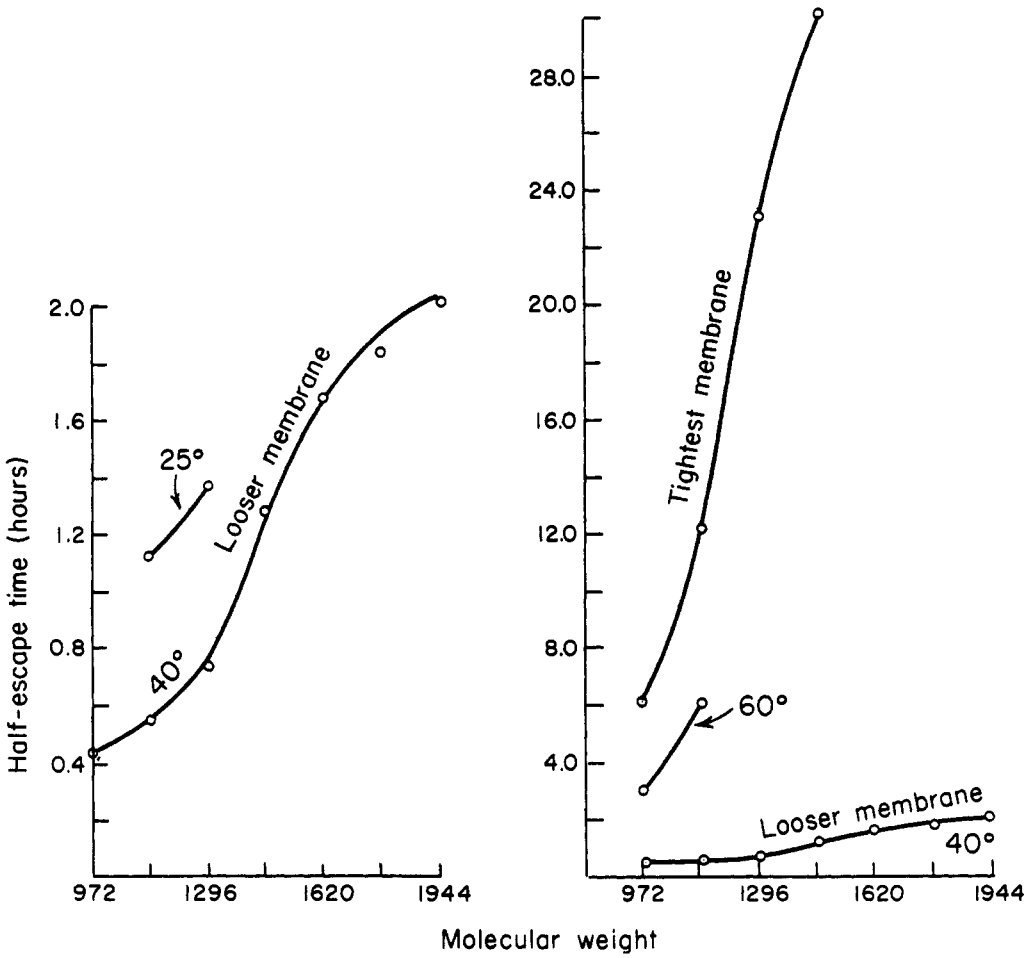

FIG. 4.—Comparative dialysis rates of the Schardinger dextrins with two membranes. In this set of experiments the tightest membrane was No. 3, the looser one, No. 5. For further details, see text.

tendency should be decreased a constant amount by hydration of each glucose unit.

If the reasoning above is accepted, there is an increase in diameter of about 11% for each homologue, or somewhat less if there should be a tendency for the larger ring to crumple. From Figure 4 it can be seen that this difference doubles the 50%-escape time required. Since a 20% increase in effective dialysis rate can certainly be recognized it follows that a 2% increase in effective diameter should be clearly recognizable, perhaps even a smaller difference. This conclusion emphasizes the importance of hydration effects and changes in conformation in any selective separation process depending on size.

In connection with the latter factor, it seemed of interest to study maltohexaose, the linear analogue of cyclohexaamylose. It, however, is a reducing sugar. A half-escape time of 7.5 hours was found at 40° in membrane No. 3. Redetermination of the escape rate of recrystallized cyclohexaamylose immediately afterward for comparative purposes gave a half-escape time of 5.5 hours. A somewhat larger difference than this was expected, but perhaps the linear polymer in solution tends to assume a conformation approaching that of the cyclohexaamylose. This possibility will be investigated further.

It is of interest to compare Longworth's data (1957) on free diffusion for certain of the sugars studied here. His data were obtained at 25°. While direct comparisons cannot be made, the order of decrease in the rate of dialysis is the same as the decrease in the diffusion coefficient with the exception of the arabinose-xylose pair. The data in Table III are arranged for comparison. In the 4th and 6th columns are given ratios for diffusion coefficients and 50%-escape times, respectively, of the solutes on the lines above and below. In the case of the 50%-escape time ratios, the size of the ratio will reflect the selectivity of the particular membrane. The number in parentheses in the 5th column refers to the membrane number in Table I. In spite of this variation, it can be seen from Table III that where for a given pair a difference in diffusion coefficient approximating 10% is noted, about a twofold difference in rate of dialysis is found. Where the diffusion coefficients for a pair are more similar, the ratios of half-escape time are also smaller.

The reason for the discrepancy in the case of the xylose-arabinose pair cannot be given without further work. It may result from the fact that the diffusion coefficients are relatively close together and were obtained at 25°. The half-escape time values were determined at 40°.

TABLE III

COMPARISON OF RELATIVE DIFFUSION COEFFICIENTS (D) IN FREE DIFFUSION WITH RELATIVE DIALYSIS RATES[a]

Substance	m.w.	D × 10⁶	D Ratios	Half-Escape Time[b] (hr.)	Half-Escape Time Ratios
			Membrane No. 1[c]		
Xylose	150	7.462		1.3 (1)	
			0.98		1.46
Arabinose	150	7.599		1.9 (1)	
			1.13		1.84
Glucose	180	6.728		3.5 (1)	
			1.01		1.37
Galactose	180	6.655		4.8 (1)	
			1.28		6.2
Sucrose	342	5.209		30 (1)	
			Membrane No. 2[c]		
Sucrose	342	5.209		1.5 (2)	
			1.03		1.73
Lactose	342	5.076		2.6 (2)	
			1.01		1.04
Cellobiose	342	5.039		2.7 (2)	
			1.16		2.0
Raffinose	504	4.339		5.4 (2)	
			1.13		2.8
Stachyose	666	3.839		15 (2)	
			Membrane No. 5[c]		
Stachyose	666	3.839		0.68 (5)	
			1.19		1.64
Cycloheptaamylose	1152	3.224		1.1 (5)	
			Membrane No. 3[c]		
Cyclohexaamylose	972	3.443		6 (3)	
			1.07		2
Cycloheptaamylose	1152	3.224		12 (3)	
			1.07		1.91
Cyclooctaamylose	1296	3.000		23 (3)	

[a] See text for further details. [b] The last three half-escape time values are at 40°; all other data refer to 25°. [c] See Table I for explanation of membrane numbers.

Diffusion coefficients have always been considered fundamental constants with well-known implications (Longsworth, 1955) regarding solvation, molecular symmetry or asymmetry, and states of association. While thin-film dialysis has the limitation that it permits only comparisons, it does appear, from the data given here and from additional data soon to be published, that thin-film dialysis amplifies greatly the differences usually observed in free diffusion. It often permits effective separations to be made, and since only 1 to 5 mg of sample is required, an almost ideal tool is provided for the study of the purity of a given sample with respect to molecular size.

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