Cat Heart Muscle *in Vitro*

V. *Diffusion through a sheet of right ventricle*

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ABSTRACT The rate of transfer of labeled molecules across a sheet of quiescent cat right ventricle separating two chambers containing chemically identical solutions was followed at 23°C. For the diffusion of sucrose, SO_4, and Na the experimental points fit the entire time course of the plot of the diffusion equation for a plane sheet. The tortuosity factor of the extracellular diffusion channel, λ, was 1.44 ± 0.05 (mean ± SEM) for sucrose and similar for SO_4 and Na. The fractional area available for extracellular diffusion, calculated from λ and the slope of the linear asymptote approached during steady state diffusion, was 0.17–0.23 for both impermeant species (sucrose, SO_4, Na) and permeant species (water, urea, glycerol). Permeant molecules showed a characteristic prolongation of the approach to the steady state, with an unexplained "hump" in the curve for water. The observed time courses for diffusion of permeant molecules are interpreted in terms of a model proposed by Fatt *et al.* for diffusion through linear porous media containing dead-end pore volume. Large molecules like inulin and dialyzed dextran (diameter 150 to 180 A) diffuse through the sheet. These molecules may have a reflection coefficient $\sigma > 0$. The fraction of muscle water occupied by the sucrose diffusion channel is significantly smaller than the 3 hr. mannitol, sucrose, and inulin spaces.

If a sheet of heart muscle separates two sides of a chamber, the rate of transfer of a radioactively labeled substance across the sheet may be used to study the properties of the extracellular compartment. When a radioactive tracer is initially added to one side, analysis of the rate of its accumulation on the other side permits calculation of the tortuosity factor for extracellular diffusion and of the area available for extracellular diffusion. The detailed shape of the curve describing the transfer of tracer across the sheet may then be used to differentiate between substances which enter cells and those which are excluded. Comparison of the extracellular diffusion channel with the equilibrium mannitol, inulin, and sucrose spaces indicates that the extracellular compartment is inhomogeneous, in agreement with our previous findings (1). Evidence is also presented as to the dimensions of the channels through which extracellular diffusion occurs.

METHODS

Experimental Procedure

The hearts of kittens or small cats anesthetized with pentobarbital were excised and placed in the dissection chamber of Page and Solomon (2). The right ventricular cavity was opened by careful dissection along its margin at the interventricular septum. A sheet of right ventricular myocardium was cut free from the heart and clamped between the two sides of a lucite chamber modified from that developed by Ussing and Zerahn (3) for measurements of ion fluxes across the frog skin. The heart muscle sheet divided the chamber into two equal conical halves. When the chamber was opened, the inside of each half had a volume of approximately 1 ml and a circular base having an area of 1.12 cm^2. The right ventricular sheet was impaled on a series of stainless steel spikes protruding from the carefully machined flat margin of one-half of the chamber. The spikes could be fitted into holes in a corresponding location on the margin of the opposite half of the chamber. A sharply defined and well sealed disc of heart muscle was produced by tightening an adjustable screw against the back wall of one chamber, thereby forcing the chambers together. Each chamber was connected by means of separate peristaltic action pumps (American Instrument Co., Model 5-8952) to a reservoir into which fluid was continuously pumped at the rate of 130 ml/min., the fluid being returned by gravity flow. The total volume of fluid on each side of the sheet was 20 ml, the height of the fluid columns on each side being identical to avoid a hydrostatic pressure gradient across the sheet. Water vapor–saturated gas (5 per cent CO_2, 95 per cent O_2) was bubbled through each reservoir and the muscle sheet was protected from the mechanical oscillations produced by the pump by baffle chambers inserted between the incubation chamber and the pump. The temperature of the bathing solution was kept at 22.5 — 23.5°C by water continuously circulated from a cooling reservoir through condenser jackets placed between the outputs of the pumps and the chamber. The fluid in the chamber was maintained at this temperature in order to lower the oxygen consumption of the tissue to the point at which a sheet of the thickness used (thickness of 96 sheets: 0.137 ± 0.002 cm [mean ± SEM]) could be oxygenated by diffusion, as well as to suppress spontaneous contractions which might interfere with analysis of the diffusion process. Selected experiments were also carried out at 8.5 — 10°C.

The experiments were designed to measure the self-diffusion of the substance being traced. The temperatures and hydrostatic pressures as well as the chemical concentrations of ions and uncharged molecular species were therefore identical on both sides of the chamber. Muscles were preequilibrated for a 1 to 3 hr. period with a solution of a given chemical composition. The solution on the side facing the epicardium was then changed to include a radioactive tracer, the chemical concentration on this side being adjusted so that the addition of carrier with the tracer did not produce a chemical concentration gradient. The reservoir above the chamber facing the endocardial side of the sheet was sampled with a 0.5 ml micropipette at appropriate intervals for 1 to 5 hrs., and the 0.5 ml volume replaced after each withdrawal by the same volume of non-radioactive solution. (In calculating the specific activity of this compartment, a correction was made for each interval to allow for the sample

withdrawn and for the subsequent dilution with non-radioactive medium.) At the end of the experiment muscles were removed from the chamber and lightly blotted. The disc of heart muscle across which diffusion had been measured was invariably sharply demarcated from the translucent and paper-thin surrounding area compressed between the margins of the two chamber halves. This disc was rapidly cut out with fine scissors, placed in a weighing bottle, and weighed.

Because of the long time which would have been required for preequilibration, experiments with dextran (molecular weight 60,000 to 90,000) were carried out with dextran initially in the radioactive side of the chamber only. Since inulin of the same chemical composition and distribution of molecular weights as commercially available inulin-C^{14}–carboxylic acid could not be obtained, diffusion of this substance was measured without preequilibration with non-radioactive inulin. The inulin had a stated molecular weight of 3000 to 4000, and was used at a chemical concentration of 0.1 gm/100 ml.

γ-Radiation from Na^{24} was counted in a well-type crystal scintillation counter, with appropriate corrections for decay. β-Radiation from S^{35}, C^{14}, and H^3 was assayed in a liquid scintillation counter with automatic sample changer and print-out (Packard Instrument Co., Inc., model 314-DC).

For determination of the extracellular space, muscles were equilibrated for 3 hrs. in solutions labeled with C^{14}–inulin (seven determinations), C^{14}–sucrose (six determinations), and C^{14}–mannitol (six determinations). After measurement of the wet weight, the discs from these muscles were dried overnight at 105–110°C in an oven and reweighed. They were then extracted by shaking for 48 hrs. with redistilled HNO_3 in 30 ml vycor microKjeldahl flasks and the extracts assayed for C^{14}–tracer as described by Page (1).

Solutions

Experiments were carried out in the physiological cat Ringer's solution previously described (2), the composition of which is, in mM: K 5.32, Na 178.5, Cl 163.1, Ca 1.40, Mg 0.50, HCO_3 22.0, HPO_4 0.59, H_2PO_4 1.45, and glucose 5.5. For self-diffusion experiments the chemical concentrations of the substances being studied were (in mM): sucrose 0.5, SO_4 0.5, urea 63, and glycerol 63.

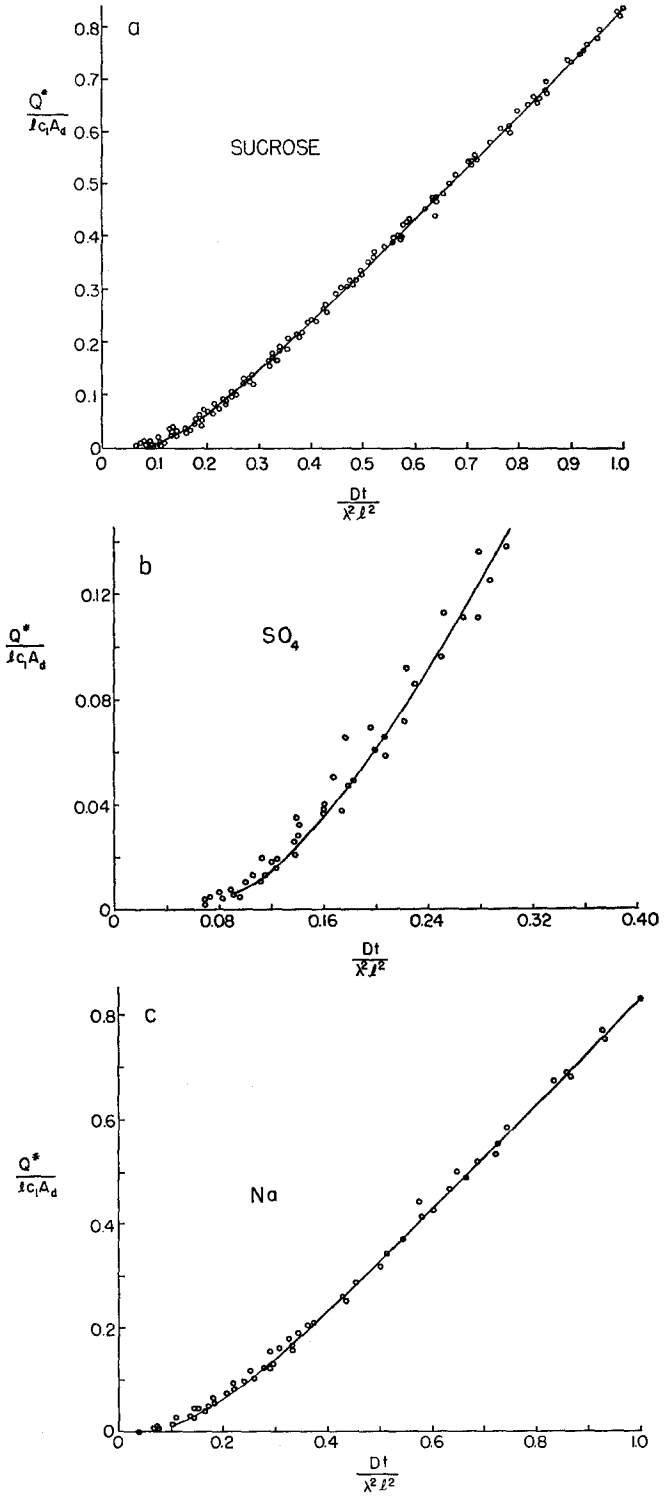
Sucrose-C^{14}, mannitol-C^{14}, glycerol-2-H^3, dextran-carboxyl-C^{14}, and inulin-carboxyl-C^{14} were obtained from New England Nuclear Corporation. The dextran was dialyzed overnight at 2–3°C against a large volume of Ringer's solution in order to remove low molecular weight contaminants.

RESULTS AND DISCUSSION

Theory

The solution of the diffusion equation for the time course of appearance of a radioactive substance diffusing across a uniform plane sheet from an infinite reservoir, given by Crank (4), is

$$Q^* = Dc_1At/l - lc_1A/6 - (2lc_1A/\pi^2)\sum_{n=1}^{\infty}[(-1)^n/n^2]e^{-Dn^2\pi^2t/l^2} \qquad (1)$$


a
SUCROSE
$\frac{Q^*}{\ell c_1 A_d}$
$\frac{Dt}{\chi^2 \ell^2}$
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
b
SO_4
0 0.04 0.08 0.12
0 0.08 0.16 0.24 0.32 0.40
c
Na
0 0.2 0.4 0.6 0.8
0 0.2 0.4 0.6 0.8 1.0

in which

Q^* = cumulative amount of radioactivity (in counts per minute) which has diffused across the sheet in time t,
l = thickness of the sheet,
A = total area of the sheet,
c_1 = counts per minute (CPM) per unit volume in the reservoir,
D = diffusion coefficient of the labeled species in free aqueous solution,
Index 1 denotes the reservoir, 2, the muscle sheet, and 3, the initially non-radioactive compartment.

Equation 1 is applicable when the CPM in compartment 1 remain effectively constant, and the sum of the CPM in compartments 2 and 3 remains a negligibly small fraction of the CPM in 1. For diffusion through the extracellular space of a sheet of heart muscle Equation 1 takes the form

$$Q^* = Dc_1A_dt/\lambda^2 l - lc_1A_d/6 - (2lc_1A_d/\pi^2)\sum_{n=1}^{\infty}[(-1)^n/n^2]e^{-Dn^2\pi^2t/\lambda^2l^2} \qquad (2)$$

in which

A_d = an area associated with the diffusion channel[1] such that the quantity lA_d is the volume of the diffusion channel, and
λ = a tortuosity factor for the extracellular diffusion channel; *i.e.*, the increase in the mean diffusion path resulting from the fact that extracellular molecules must diffuse around rather than through muscle cells.

The thickness, l, may be obtained from the wet weight of the muscle disc and the specific gravity of mammalian muscle, taken as 1.055 (5). Experimentally it is convenient to plot Q^* against t. As t increases, Q^* approaches the linear asymptote

$$Q^* = Dc_1A_dt/\lambda^2 l - lc_1A_d/6 \qquad (3)$$

[1] The area available for diffusion might have been defined alternatively as A_d' in the relation

$$S = A_d' \frac{Dc_1}{\lambda l}$$

where S = the steady-state flux of tracer across the membrane. Hence $A_d = \lambda A_d'$.

FIGURE 1. Time courses of diffusion of three relatively impermeant species, superimposed on plot of Equation 2. (*a*) sucrose (ten experiments); (*b*) SO_4, non-steady state segment expanded (five experiments); (*c*) Na (five experiments). For each muscle, D/λ^2 was obtained from Equation 4, and the linear portion of the plot of Q^*/lc_1A_d against Dt/λ^2 was then superimposed on the linear portion of the theoretical plot by adjusting A_d as a scaling factor. The points of the non-linear portion of the experimental curve plotted in this way are observed to fall on the theoretical curve. The coordinates of the normalized plot are dimensionless.

When $Q^* = 0$, the asymptote intercepts the time axis at a point, L, given by

$$L = \lambda^2 l^2/6D \tag{4}$$

From equation 3, the asymptote has a slope, S,

$$S = Dc_1 A_d/\lambda^2 l \tag{5}$$

A_d may be calculated from Equation 5 after obtaining λ from Equation 4. If an independent value of λ is available, A_d may be derived directly from 5 without introducing the intercept.

Diffusion of Small Molecules and Ions Which Remain Extracellular[2]

D/λ^2 for sucrose, calculated from Equation 4, was $2.6 \pm 0.2 \times 10^{-6}$ cm²/sec. at 22.5–23.5°C and $1.64 \pm 0.26 \times 10^{-6}$ cm²/sec. at 8.5–10°C. Fig. 1 *a* shows the time courses for ten experiments on the self-diffusion of sucrose at 22.5–23.5°C, superimposed on the plot of Equation 2. Within the limits of experimental variation the values fall on the theoretical curve, a result consistent with previous studies which showed sucrose to be excluded from the cells (1). Figs. 1 *b* and 1 *c* give the time courses for self-diffusion of SO_4 and Na for which the fit is also good, in agreement with the previous finding that Na and SO_4 are relatively impermeant (1, 2).

The first column of Table I presents the values of λ calculated from Equation 4 for these three impermeant species. It is apparent that the tortuosity factor is the same for all three substances, consistent with their following the same extracellular diffusion path. Using the value of λ for sucrose in Table I, A_d/A for this molecule, computed from Equation 5, was found to be 0.17 ± 0.02. Since λ appears to be independent of the molecular species, it should be possible to compute A_d for SO_4 and Na by Equation 5 using λ for sucrose. The values of A_d/A obtained in this way for these two species agree well with each other, as shown in Table I, and do not differ significantly from the value for sucrose ($P > 0.05$. "Student" t test), thus indicating that A_d also does not depend on the nature of these small impermeant molecules.

Total muscle water, which constitutes 0.774 ± 0.001 of wet weight (twenty-one determinations), may be partitioned as follows on the basis of these measurements: The quantity, lA_d/(muscle water content), which gives the fraction of total water contained in the sucrose diffusion channel, was found to be 0.21 ± 0.02. This figure differs at the $P < 0.05$ level ("Student" t test) from the inulin space of 0.28 ± 0.03 determined on seven additional muscles by equilibration for 3 hrs. with C^{14}-inulin. It is very much smaller ($P < 0.01$)

[2] Results in Table I and text are expressed as mean ± standard error. Throughout these experiments the decrease in c_1 during the period of measurement did not exceed 4 per cent of the CPM at $t = 0$ and was usually less than 2 per cent.

than the mannitol space of 0.48 ± 0.02 and the sucrose space of 0.39 ± 0.03 also determined on the sheets. These results support our previous suggestion that the extracellular space in cat heart muscle may be kinetically inhomogeneous (1).

Diffusion of Permeant Molecules

A sheet of cat ventricle consists of multiple small cells (diameter 7 to 14 microns (6)). While no figure is available for the length of cat ventricular cells, Lindner (7) reports a figure of 12 to 40 microns for dog papillary muscle in which the cells are extended and parallel to one another. In the present

TABLE I*

λ, A_d/A, and D/λ^2 FOR VARIOUS MOLECULES AND IONS

Species	λ	A_d/A	D/λ^2	Reference for measurement of D
			$(cm^2/sec.) \times 10^6$	
Sucrose (10)	1.44±0.05	0.17±0.02	2.6±0.2	21
SO_4 (5)	1.45±0.05	0.22±0.03	3.8±0.3	22
Na (5)	1.38±0.06	0.23±0.04	7.1±0.6	23
Water (7)		0.20±0.03		24
Urea (5)		0.22±0.02		25
Glycerol (5)		0.22±0.04		26

* Figures in parentheses give number of experiments.
The values for D were determined in solutions whose composition often differed markedly from that of our Ringer's solution. Correction of D from the tabulated value at 25°C to the mean experimental temperature of 23° is negligible compared to the experimental error and is omitted. D for SO_4 was chosen from the graph of Nielsen, Adamson, and Cobble (22) for self-diffusion of $S^{35}O_4$ in Na_2SO_4 at the Na concentration used in the present experiments. The error due to the difference between the value of D obtained from the literature and its value in the experimental Ringer's solution is estimated to be 10 per cent or less.

preparation the cellular phase is not continuous in the direction from epicardium to endocardium, and the cells are not oriented parallel to the axis of diffusion. Instead each cell appears to be in contact with the extracellular compartment, as indicated by the unpublished observation of the senior author that when penetrating two successive cell layers with a microelectrode one invariably records the appearance and disappearance of the resting membrane potential. This finding persists for as many cells as can be penetrated without breaking the microelectrode. Moreover, the distance an electrode can be advanced with a micromanipulator without reentering the extracellular compartment is very small, suggesting that the extent of the myocardial cells in the axis of diffusion is likewise small.

Goodknight, Klikoff, and Fatt (8), Goodknight and Fatt (9), and Fatt (10)

have published a theory for diffusion through linear porous media containing dead-end pore volume which is useful in discussing diffusion of permeant molecules through the heart muscle sheet. These authors have shown that dead-end pores cause a prolongation of the non-linear portion of the diffusion time course, and a shift of the intercept to the right, the slope of the asymptote approached during steady state diffusion being unaffected by the dead-end pore volume. Since the cells bordering on the extracellular space of the right ventricular sheet are small relative to the thickness of the sheet, they may be considered as behaving like multiple dead-end pores with respect to permeant molecules. The effectiveness of the cells as "dead-end pores" would depend on the permeability of their cell membranes. As in the Fatt model, the attainment of the steady state would be delayed because of the time required to abolish the gradient of specific activity between the cells, cut by any plane parallel to the surfaces of the sheet, and the section of the extracellular space cut by the same plane. If this model is applicable, the slope of the asymptote approached during steady state diffusion should be the same for a permeant molecule as for a non-permeant molecule having the same diffusion coefficient. Accordingly, it should be possible to calculate A_d from the slope of the linear asymptote for permeant substances, using the same tortuosity factor as for impermeant substances.

Table I shows the values of A_d for the permeant molecules water, urea, and glycerol, calculated from the slope of the linear asymptote by Equation 5 using λ for sucrose. The values agree with those obtained for non-permeant species. These results, indicating that A_d is approximately constant for small molecules of different size, support the validity of the treatment above and imply that the dimensions of the diffusion channel are large relative to those of the diffusing species.

Fig. 2 *a*, a representative experiment for glycerol, illustrates that permeation alters the intercept but not the slope of the linear asymptote approached during steady state diffusion. The theoretical curve, calculated from Equation 2 using λ for sucrose and A_d from the slope of the asymptote, becomes linear earlier than the experimental curve. The corresponding plot of five experiments with urea was similar. In Fig. 2 *b* the non-steady state portion of the curve for three experiments with water is shown on an expanded scale. The figure illustrates a "hump," in this portion of the time course, which appeared regularly with water but only exceptionally with sucrose or urea. In spite of the hump, the over-all time for reaching the steady state was prolonged for water as for urea and glycerol. The interpretation of this phenomenon must await further experiments.

In connection with the theory of Fatt *et al.*, it is of interest to consider the effects on extracellular diffusion of the striking tubular invaginations of the cell membrane of heart muscle first described by Lindner (7) and examined in detail by Simpson and Oertelis (11) and Nelson and Benson (12). The time

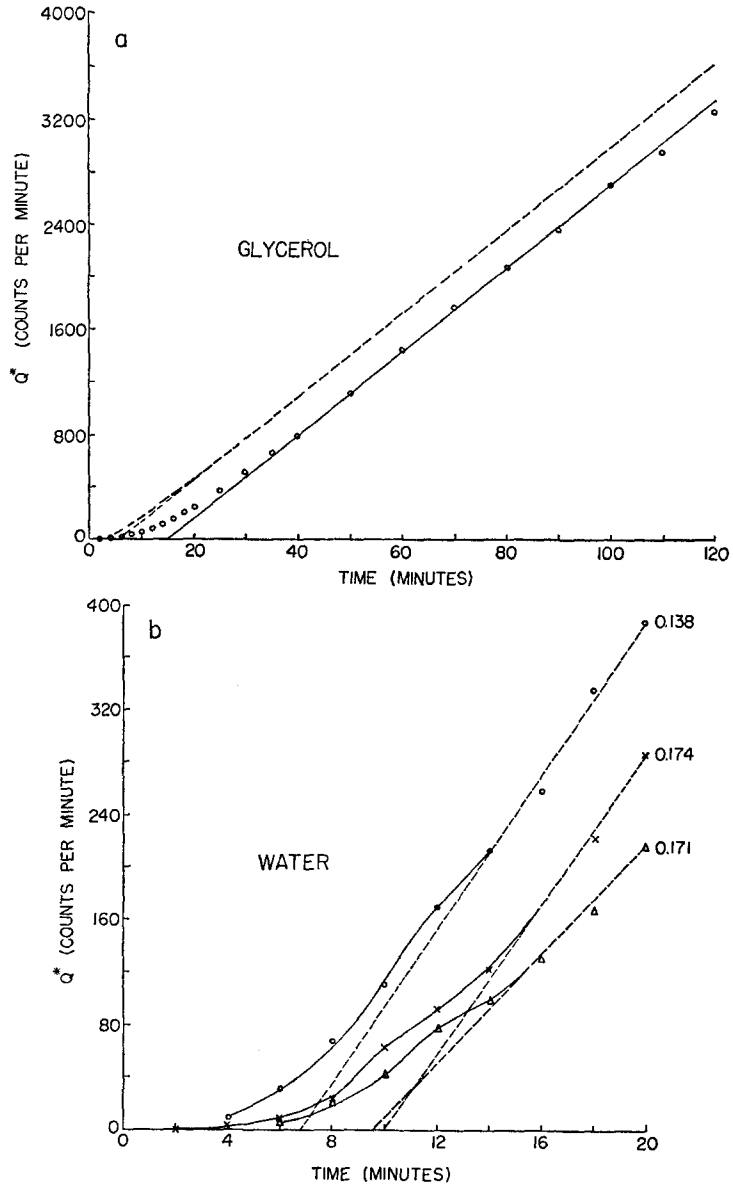


FIGURE 2. Diffusion complicated by permeation. (*a*) Plot of experimental time course (open circles) and asymptote (solid line) for a typical experiment with glycerol (D = 8.3 × 10^{-6} cm²/sec.) compared with the time course and asymptote predicted by Equation 2 (broken line). In this experiment l was 0.085 cm; (*b*) experimental time courses for three experiments with water, showing typical "hump" in non-steady state segment. The figures give the thickness of the sheet in centimeters.

course for diffusion of the non-permeant species, sucrose, satisfactorily fits Equation 2, suggesting that no extracellular dead-end pore volume is included in the spaces equilibrating with these molecules. From the electron

micrographs of Simpson and Oertelis the transverse tubules would appear to have diameters in excess of 500 A; consequently these large structures may be readily accessible for the diffusion of the small sucrose molecule. If such invaginations of the cell membrane do not act as dead-end pores for sucrose, it is necessary to explain the difference between the sucrose diffusion channel and the equilibrium inulin, sucrose, and mannitol spaces in terms of an extracellular compartment equilibrating so slowly as not to affect diffusion across the sheet appreciably. This compartment might correspond to specialized regions of the transverse tubular system or to the sarcoplasmic reticulum (longitudinal tubular system) as described by Porter and Palade (13) and others (12).

Diffusion of Large Molecules

Since the present results indicated that the diffusion channel is large relative to the 9.0 A diameter of sucrose (14), it appeared important to estimate the dimensions of this channel, at least to an order of magnitude. Experiments with dextran, molecular weight 60,000 to 90,000 (0.8 gm/100 ml), showed that this substance passes readily through the muscle sheet. Senti *et al.* (15) have measured various physical properties of acid-hydrolyzed dextran fractions somewhat larger than those studied here. They related the root–mean–square radius R to the weight–average molecular weight M_w by the equation, $R = 0.66M_w^{0.43}$. This formula yields an estimate of 150 to 180 A for the diameter of the dextran diffusing through the sheet of heart muscle.

Reliable values of D are not available for the dextran and inulin used in the present experiments. The value of $A_d l$/(muscle water content) can, however, be obtained by combining Equations 4 and 5 to give

$$A_d l = 6SL/c_1 \tag{6}$$

The value of $A_d l$/(muscle water content) computed in this way for the five experiments with inulin was 0.09 ± 0.02, which is significantly lower ($P < 0.01$) than the corresponding figure of 0.21 ± 0.02 for sucrose. Two experiments with dextran yielded the still lower values of 0.06 and 0.01. Calculation of the fractional volume of the diffusion channel from Equation 6 assumes that the dimensions of the diffusion channel are large relative to the 30 A diameter of the inulin molecule (16) and the 150 to 180 A diameter of dextran. The observation that A_d is constant for molecules whose diameters range from that of water to that of sucrose (Table I) supports this assumption for these small species. With increasing size of the diffusing molecules, collisions with the orifice and "walls" of the channel become more probable. As pointed out by Pappenheimer *et al.* (17) and by Renkin (18), these interactions result in a progressive decrease in the apparent area available for diffusion. Moreover,

Ginzburg and Katchalsky (19) have shown that the tortuosity derived from diffusion measurements on swollen cellulose membranes decreases when the size of the diffusing molecule approaches that of the interstices through which diffusion occurs. These effects would tend to make the product $A_d l$ smaller.

The experiments with inulin and dextran, unlike those with the smaller molecules, were carried out under conditions of net diffusion. Restrictions to the net diffusion of large molecules may result in an osmotic flow of the solvent, water, in the opposite direction (from endocardium to epicardium). In the terminology of the thermodynamics of irreversible processes, the reflection coefficient, σ, for such large molecules is greater than zero (20).

The channels large enough to allow the diffusion of dextran most probably correspond to the interspaces between muscle cells and fiber bundles. Whether and to what extent other, more restricted, extracellular regions behave as dead-end pores with respect to dextran and inulin remains an interesting problem for future investigations.

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