Water Permeability of Isolated Muscle Fibers of a Marine Crab

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ABSTRACT This report deals with the diffusional and nondiffusional water fluxes of muscle fibers of the crab, *Chionoecetes bairdi*. Graphical analysis of the deuterium exchange indicates that two fiber compartments exist for water. The first, comprising about 60-70% of the fiber water, probably represents the sarcoplasm which is bounded externally by the plasma membrane. The second compartment might represent intracellular organelles. The ratio between the nondiffusional and diffusional fluxes is very much larger than that found earlier for erythrocytes and for the giant axon of the squid. A ratio of such size is unlikely to be caused by unstirred layers and more accurate determinations of the water flux must include study of the influence of the complex morphology of these muscle fibers.

INTRODUCTION

The study of water movements across cell membranes may yield information about the nature of the membranes. Parts of the membrane are said to resemble aqueous channels because the penetration of some molecules such as water is thought not to take place through the remaining, lipid phase of the membrane. Recently water fluxes have been used to estimate the size of these channels. The discovery that osmotic fluxes were much greater than diffusional fluxes across the frog skin (Hevesy et al., 1935) led Ussing (1952) to postulate that the mode of penetration was different in the two cases, that Poiseuille's law could describe the osmotic flux while Fick's law described tracer fluxes, and that the ratio of the two fluxes could be used to indicate the size of the channels. Similar assertions were made for capillaries by Pappenheimer and his colleagues (1953) and for a variety of cells and epithelia by Solomon and his coworkers (1959). Since the channel radius derived in this manner is one which is "equivalent" to the radius of a channel where the laws of Fick and Poiseuille are sufficient to describe the rate of water movement, the term

"Equivalent Pore Radius" (EPR) is used to emphasize the operational nature of these channels. The physical dimensions calculated in this manner for capillaries and erythrocytes are plausible since the results agree fairly well with independent estimates (Giebel and Passow, 1960; Pappenheimer, 1953; Solomon, 1968). However, additional studies of this type are needed because even though the results for the mammalian erythrocyte demonstrate the basic validity of this approach, a variety of other cells is available and other types of cells may allow comparison with estimates made with independent techniques. Nerve and muscle fibers offer an opportunity to compare the EPR with estimates of channel size arrived at with electrophysiological, osmotic, and solute transport studies. Such studies have been made on the isolated giant axon of the squid and an estimate of the maximum EPR has been obtained for that preparation (Villegas and Villegas, 1960).

In the case of isolated single muscle fibers, an estimate of the diffusional flux is available for the giant fiber of the barnacle (Bunch and Edwards, 1969) and there are reports of the nondiffusional water flux for fibers of the frog and crayfish (Hodgkin and Horowicz, 1959; Zadunaisky et al., 1963; Reuben et al., 1964) but, no studies have been made in which the same investigator determined both types of water flux and compared them.

I wish to report the results of a study of water fluxes in single, isolated muscle fibers of a marine crab. In order to determine tracer efflux I used a chamber which allowed both thorough stirring and frequent sampling. Deuterium, the tracer, was assayed with the Linderstrøm-Lang density gradient technique (1937). In order to determine the nondiffusional influx I recorded, with a photocell, the light transmission of swelling muscle fibers and correlated that transmission with fiber volume. A portion of these results was reported to the 24th International Congress of Physiological Sciences (Sorenson, 1968).

MATERIALS AND METHODS

The crabs, *Chionoecetes bairdi*, were dredged from Puget Sound, fed regularly, and kept in cold 10°C seawater. Single fibers from the extensor and flexor muscles of the walking legs were removed intact, essentially as described by Girardier et al. (1963). This dissection was made in seawater. The fibers were then transferred to crab saline, equilibrated there for at least 3 hr, and then thoroughly inspected for injury. The fibers were kept at about 10°C at all times during the dissection and the experimentation which followed.

These fibers are striated and are elliptical in cross-section. Their widths and thicknesses were measured with an ocular micrometer in a microscope. The widths ranged from 0.2 to 1.2 mm; the thicknesses averaged 63% of the width. The fiber volume and surface area were calculated by assuming a smooth elliptical cross-section and neglecting the increase in surface area of crab muscle fibers due to clefts and tubules (Peachey, 1967). If one assumes a uniform water permeability for all areas of the

muscle fiber surface, neglect of the tubule and cleft area will have no effect on the calculated EPR since a ratio of fluxes is used. But the calculated permeability coefficients will be in error and must eventually be adjusted with precise information about the extent of the true surface area of the fiber.

The crab saline contained (mM) NaCl (304), Na propionate (159), KCl (8.0), $CaCl_2$ (10), $MgCl_2$ (39), and was buffered at pH 7.4 by Tris maleate (5). All changes in osmotic strength (calculated from osmotic coefficients [Robinson and Stokes, 1959]), were made by adding or deleting Na propionate. Because propionate is impermeant (Reuben et al., 1964), this practice permitted making changes in osmotic strength without changing the membrane potential or the K $\times$ Cl product of equilibrated fibers. D_2O (Matheson, Coleman, and Bell Co., Inc., Cincinnati, Ohio) replaced H_2O in the tracer efflux studies. No greater concentrations of D_2O than 10% were used in this study, even though Jenerick (1964) has shown that concentrations of D_2O as high as 99% had no effect on the electrophysiological properties of resting frog muscle fiber membranes.

The initial equilibration in crab saline causes a small opacity of the fiber which is transient and which is not indicative of injury since these fibers remained transparent and reversibly responsive, for up to 36 hr, to challenges by changes in the concentration of K^+ and to changes in the osmotic strength. Large replacements of permeant ions by impermeant species may cause fiber damage (Fatt and Katz, 1953; Hays et al., 1968), but such changes were avoided.

Measurement of Deuterium Efflux

The isolated fiber was first immersed for 30-60 min in a saline with 10% D_2O, rinsed quickly in several batches of normal saline, and then the efflux period was started.

The fiber was wrapped loosely around one end of a Plexiglas rod. Stainless steel clips on the apodeme and endoskeleton fragments held the fiber in place. The rod was held vertically in the efflux chamber and turned at 60 rpm. A small ledge on the rod supported the fiber from below. The chamber had a stopcock with one port for filling and one for draining; it also was coated inside with Siliclad (Clay-Adams, Inc., Parsippany, N. J.). Samples, which could be as small as 0.25 ml, were taken by draining the chamber. The chamber was then refilled with normal saline, exposing the fiber to air for about 2 sec.

Determination of Deuterium

The samples, collected in small test tubes, were weighed to within 20 mg and capped. Small aliquots of the sample were distilled overnight in Pasteur pipettes, modified in a way similar to those of Shaw (1955). Drops of this distillate were then analyzed for deuterium in the density gradient.

The preparation and use of these gradients have been described at length by others (Anfinsen, 1946; Friis-Hansen, 1954; Glick, 1961). Bromobenzene and kerosene were used to give a gradient range equivalent to 5% D_2O. The Plexiglas gradient chambers were rectangular with large reservoirs at each end and were placed in a large water bath at room temperature. The chamber was large enough to hold all the drops from one experiment. Drops of the distillates, about 1 mm in diameter, were placed in the

chamber at exact intervals of time and at specific locations. After 30 min, the vertical position of each drop was recorded using a cathetometer (Ole Dich, Hvidovre, Denmark) at exactly the same time intervals used for introduction of the drops into the gradient. The drop density was calculated by interpolation of the sample reading between the readings of two drops of known density.

Standard curves for two gradients are shown in Fig. 1. Measurements of duplicate and triplicate drops under normal conditions showed that the sensitivity of the determination of drop position was equivalent to ±0.0007 % D_2O. All but a few samples fell in the region on the standard curve between 0% and 0.05% D_2O and of those, more than 90% had densities greater than 0.007% D_2O—that is, 10 times the working sensitivity.

The Photocell Method

Parpart (1933) used a photocell to follow volume changes in muscle but no one appears to have utilized this technique for single fibers. In the present experiments a Clairex 705HL photoconductive cell (Clairex Electronics, Inc., Mt. Vernon, N. Y.), used in a DC Wheatstone bridge, was fixed in one eyepiece of a binocular microscope. A slit with a width in the image plane of about 75 μ was placed over the resistive surface. The light, furnished by a GE No. 1493 tungsten lamp and a car battery, was filtered by a 2% solution of $CuSO_4$ and a No. 92 Wratten filter. The transmission band was 6300-7100 A.

The bridge imbalance caused by the change in light flux was observed and photographed as a voltage change on an oscilloscope. The time constant of the system, in response to a step change, was always far less than 100 msec.

The muscle fiber rested on a hammock of nylon plankton netting and was held steady in the middle of the perfusion stream by loose loops of nylon monofilament. Irrigation of the fiber surface was incomplete only where the fiber rested on the filaments. The hammock was placed in a small Plexiglas chamber and sealed overhead by a coverslip, leaving a free volume of about 0.25 ml. This volume, as shown by experiments with dyes, was replaced within 1 sec.

Perfusion was directed lengthwise to the fiber and was continuous. The rate of perfusion, whether 15 or 60 ml/min, had no effect on the swelling rate. It is demonstrable, by visual inspection in the microscope, that the unstirred layer in the swelling experiments must be less than 30 μ since one can easily see pieces of connective tissue fluttering in the turbulence of the perfusion fluid. This movement is apparent at distances of 30 μ, and greater, from the fiber. For any experiment, the rate of delivery was constant and the temperature varied less than 0.2°C.

As muscle fibers swell their light transmission increases and a decrease in transmission accompanies shrinkage or return from the swollen condition. The basis for these transmission changes is not a simple change in absorbance since dilution of the saline results in a negligible signal. Furthermore, Reuben et al. (1964) have reported that crayfish fibers form vesicles at the fiber surface when returned to a normal saline following a swelling in 50% saline. Similar changes must occur in crab muscle fibers since these fibers too show a delayed return to normal volume and an opacity which might also be attributed to light scattering by subcellular elements.

When the degree of this effect of vesiculation is held constant by measuring the light transmission of a single fiber only after the volume, measured by ocular micrometer, is steady the relationship between osmolarity and fiber light transmission is linear. Fig. 2 shows how the steady-state transmission of single fibers decreases upon successive transfer from 74% saline to 86, 100, and 117%. Measurements of fiber volume, taken at the same time, demonstrated that the relationship between osmolarity and fiber volume is also linear. The light transmission of single muscle fibers is then relatively insensitive to the influence of nonosmotic solids.

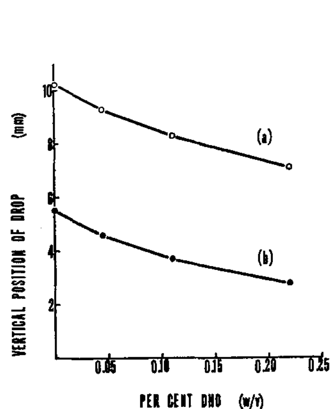

FIGURE 1

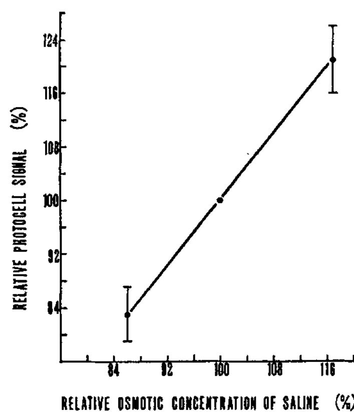

FIGURE 2

FIGURE 1. Standard curves for DHO in two separate density gradients. The value of the ordinate depends on an arbitrary setting of the cathetometer micrometer. The cathetometer can resolve two points 7μ apart.

FIGURE 2. The relationship between light transmission of single muscle fibers and the osmotic strength of the saline in which they are immersed. Six fibers were used to determine this relationship. The length of the bar represents one standard deviation. The results are normalized to the photocell signal in normal saline.

Measurement of the Swelling Rate

The fiber was mounted on the hammock, sealed in the chamber on the microscope stage, and perfusion was started. When the light transmission and fiber dimensions were steady, the perfusion fluid was changed to the experimental saline. After a delay, most of which was caused by the time taken for the saline to reach the chamber, the light transmission of the fiber began to increase. When the maximum signal was attained, the fiber dimensions were again recorded and perfusion with control saline was resumed. The change in fiber volume calculated from change in radius was correlated with the signal size for each fiber.

The nondiffusional permeability coefficient, P_{wf}, was calculated from

$$dV/dt = P_{wf}A(C_a - C_b),$$

in which C_a and C_b are the osmotic concentrations of normal and experimental salines,

A is the fiber surface area, and dV/dt is the initial rate of volume change obtained graphically as the slope of the linear portion of the swelling curve. This formula assumes nothing about the shape or geometry of the swelling object and its use makes possible, by graphical analysis, the study of swelling in highly complex objects in which a more exact mathematical analysis may be impossible.

RESULTS

The Swelling Curve

Most of the experiments on swelling were done with osmotic concentration gradients equivalent to dilutions of the normal saline by 10% or less, to minimize effects which might be due to more drastic changes in osmotic or ionic strength. A sample record is shown in Fig. 3. This figure was chosen to show the reproducibility which was routinely attained. Both figures are the result of five consecutive, identical, osmotic challenges of the same fiber. The group of swellings in Fig. 3 B was carried out 24 hr after that in Fig. 3 A.

The Nondiffusional Permeability Coefficient

The results obtained from 10 fibers, calculated according to the formula given in the methods section, gave an average P_{wf} of 98.7×10^{-4} cm/sec for an osmotic gradient of 2.0 atm (a change of about 10% in the osmotic concentration of the normal saline). The standard deviation was 52×10^{-4}. Values for P_{wf} for frog muscle fibers are 128×10^{-4} cm/sec (Hodgkin and Horowicz, 1959) and 223×10^{-4} cm/sec (Zadunaisky et al., 1963). The value reported by Reuben et al. (1964) for crayfish muscle fibers is 67×10^{-4} cm/sec. Considering the differences in species and techniques, the agreement is acceptable. These results, however, were calculated by assuming that the relationship between flux and driving force is linear.

Experiments conducted to test this assertion are summarized in Fig. 4 where the average initial swelling rate (dV/dt) of two single fibers is represented as a function of the osmotic gradient. Each point is the average of three swellings for each fiber. Seven similar experiments gave the same result; i.e., the apparent flux rate decreases as the osmotic gradient increases. The slope of this curve has the units of a permeability coefficient and it is apparent that the P_{wf} taken at points close to isotonic is about five times that taken between 21 and 22 atm. P_{wf} then, may be as large as 500×10^{-4} cm/sec.

Deuterium Efflux

The loss of DHO from the fiber is shown in Fig. 5 where the logarithm of the loss of tracer is plotted against time. One interpretation of curves such as these is that they reflect the exchange characteristics of a compartmented system (Solomon, 1960). Graphical resolution of the tracer loss shown in Fig. 5

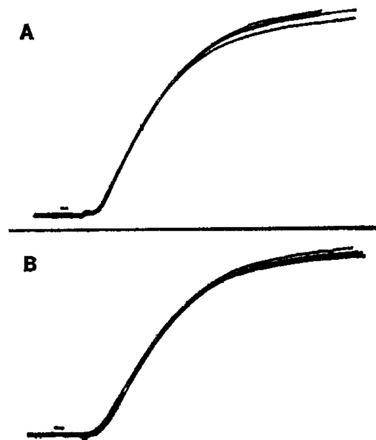

FIGURE 3

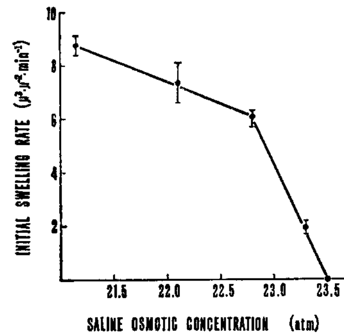

FIGURE 4

FIGURE 3. Records of swelling of a single muscle fiber as obtained by the photocell method. The displacement in the signal level at the left of the record is used as an event marker; its length represents 2.5 sec. At the cessation of the event marker, the flow of hypotonic saline (91.5%) was started. The delay before the change in light transmission is caused by the time required for the saline to reach the chamber from the perfusion flask and is not indicative of a delay in swelling caused by diffusion through unstirred layers at the fiber's surface. The vertical displacement of the event marker represents a signal of approximately 10 mv. Records A and B were taken from the same fiber, B being obtained after 24 hr had elapsed. Both records represent multiple exposures of a single frame showing five consecutive swellings of the fiber. The recovery of original volume in normal saline between swellings is not shown. Reproducibility can be improved by even more exact timing of the phases of the swelling and return to normal volume.

FIGURE 4. Initial rates of swelling at several different hypotonic challenges. Average results for two isolated fibers. Each fiber was swelled three or more times at each osmotic gradient. The length of the bar represents two standard deviations (averaged from the individual standard deviations for each fiber). The flux is uncorrected for the increment in surface area due to tubules, clefts, and invaginations in the surface membrane. Isotonic saline osmotic concentration is 23.5 atm. The permeability coefficient (slope of the curve) at small gradients is about five times that at greater gradients and the latter coefficient is less than half that obtained by assuming a linear relationship between the points at 21.2 and 23.5 atm.

requires two exponentials as follows:

$$S = C_1 \exp(-r_1 t) + C_2 \exp(-r_2 t). \qquad (1)$$

In this equation, S is the amount of tracer remaining in the fiber at time t. C_1, C_2, r_1, and r_2 are obtained from graphical analysis of the washout curve and represent, approximately, the compartment volumes (C) and rate constants (r). Resolution of this curve into more than two exponentials would not be justified in view of the paucity of points in the early phase of tracer loss and of the experimental variation between fibers.

The Diffusional Permeability Coefficient

Table I presents the results of this part of the investigation. The tracer permeability coefficient was calculated from: $P^*_{wd} = k_{23}(V_2/A)$, in which A is the surface area of the fiber, V_2 is the volume of the rapidly exchanging compartment, and k_{23} is the rate constant for efflux from that compartment. The model chosen for the purpose of the calculation is shown in Fig. 6. I will postpone discussion of the selection of models and the assignment of rate constants and

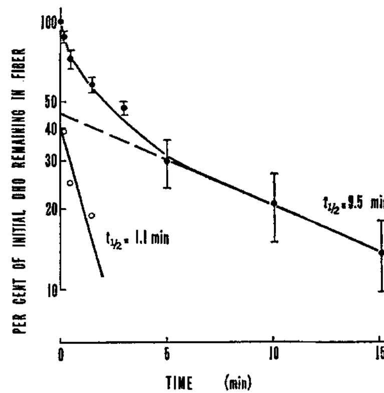

FIGURE 5. DHO loss from single crab muscle fibers. The circles and lengths of the bars represent the mean and one standard deviation of experiments from four fibers.

TABLE I
HALF-TIMES, COMPARTMENT VOLUMES, AND TRACER PERMEABILITY COEFFICIENTS (P^*_{wd}) FOR CRAB MUSCLE FIBERS

Fiber No.	$T_{1/2}$ Fast	$T_{1/2}$ Slow	Volume of fast compartment	k_{23}	P^*_{wd}
	min	*min*	(% *of fiber* H_2O)	(sec^{-1}) × 10^2	(*cm/sec*) × 10^4
1	1.2	6.4	70	0.96	0.97
2	1.4	4.6	47	0.83	0.64
3	0.6	11.3	44	1.93	1.54
4	0.9	14.2	46	1.28	0.95
5a	1.0	10.0	83	1.16	1.31
5b	1.2	11.7	58	0.96	0.63
6	1.2	6.0	66	0.96	0.87
7	1.3	16.5	71	0.89	1.21
8	0.8	5.8	79	1.44	1.62
Mean	1.1	9.6	63	1.16	1.08
Standard deviation					0.36

Fibers 5a and 5b are a repeat experiment on the same fiber. The mean width for all fibers used in these experiments was 1.16 mm (range 1.05 to 1.34); the mean thickness was 0.64 mm (range 0.57 to 0.90).

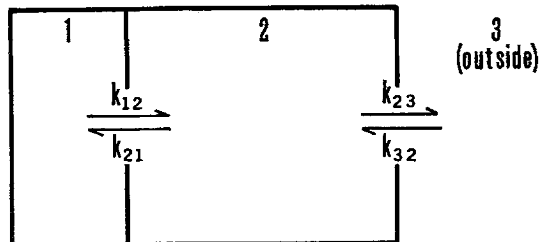

FIGURE 6. Three compartment model used for analysis of deuterium efflux from single muscle fibers. Compartments 2 and 3 are taken to be the myoplasm and extracellular fluid, respectively. The identity of compartment 1 is uncertain.

compartment volumes until the next section. The value for the tracer permeability coefficient is 1.08×10^{-4} cm/sec; after correction for self-diffusion effects (Villegas and Villegas, 1960), the diffusional permeability coefficient, P_{wd}, becomes 1.21×10^{-4} cm/sec. This can be compared with the value reported by Bunch and Edwards (1969) for barnacle muscle fibers, of 2.3×10^{-4} cm/sec.

Dainty (1963) has shown that the presence of unstirred layers of appreciable size can lead to underestimation of P_{wd} although P_{wf} is little affected. The external unstirred layer can be estimated for this system, and the influence of the lack of stirring in the myoplasm can be appraised. In both cases, in order to judge the influence of unstirred layers, it is the usual practice to calculate the rate of exchange which would be accomplished by diffusion alone, neglecting any influence of the cell membrane. If unstirred layers are to be unimportant in the system, exchange by diffusion alone must be faster than the exchange rate observed.

An estimate of the thickness of the external unstirred layer was obtained as follows. During rotation in the efflux chamber, the muscle fiber moved at 5 cm/sec. When a volume of saline equal to that in the efflux chamber moves past a stationary fiber at 5 cm/sec in the swelling experiment chamber, slack fibers are violently agitated. It was noted earlier that when the fiber was held steady in such a perfusion stream, the unstirred, nonturbulent layer was less than 30 μ. The half-time for water exchange by diffusion across a 30 μ sheet of seawater can be calculated from the following formula which describes the diffusional transfer into or out of an infinite plane sheet (Villegas and Villegas, 1960):

$$C_a/C_b = 1 - (8/\pi^2) \exp(-D\pi^2 t/4b^2) \quad (2)$$

in which C_a and C_b are the concentrations of the molecule in the plane sheet and in the medium, respectively, at time t, and b is the half-thickness of the layer. Using 1.57×10^{-5} cm²/sec for D, the self-diffusion coefficient for water at 10°C (Wang et al., 1953), and taking b as 15 μ, the time required for half-

exchange would be about 0.3 sec. The external layer is insignificant in these experiments since the average half-time observed for DHO efflux is 66 sec (Table I). Furthermore, this calculation shows that the diffusion exchange from the extracellular film would be too fast to be detected in the present experiments.

The muscle fibers used in these experiments are large and since there is no stirring in the myoplasm, the thickness of this unstirred layer must be equal to the fiber radius or one-half the minor axis in the case of an ellipse. The following formula describes diffusional transfer into or out of a cylinder (Hill, 1928):

$$C_a/C_b = 4[\exp(-Dv_1^2t/r^2)v_1^2 + \exp(-Dv_2^2t/r^2)/v_2^2 + \cdots] \qquad (3)$$

in which C_a and C_b are the concentrations of the diffusing molecule inside and outside the cylinder at time t, D is the diffusion coefficient of water in the cylinder, r is the cylinder radius, and v_1 and v_2 are the zeros of the Bessel function of zero order (they equal 2.405 and 5.520, respectively) neglecting higher order terms. Taking 0.978×10^{-5} cm²/sec as the self-diffusion coefficient for water in a 24.5% solution of ovalbumin at 10°C (Wang et al., 1954) and taking r as 600 μ (Table I), the half-time for exchange by diffusion, as calculated using the graphical solution of equation (3) presented by Hill (1928), is found to be 6.3 sec. This value, too, is considerably faster than the observed half-time of 66 sec. Therefore, one can conclude that the internal unstirred layer is not important. The plasma membrane thus appears to be a site of finite resistance to DHO exchange in crab muscle fibers. This conclusion is not negated by the presence of tubules, invaginations, and clefts unless they contain extracellular substances which hinder free diffusion to a significant degree.

DISCUSSION

The Model for Compartmental Analysis of Deuterium Exchange

Crab muscle fibers apparently contain at least two major compartments for water. Morphological evidence would suggest that there may be even more. As already noted, the surface of crab muscle fibers is indented by invaginations and tubules and creased by longitudinal clefts. Therefore the extracellular space is morphologically complex. The morphology of the sarcoplasm is also complex, with at least three major components being apparent: myofibrils, mitochondria, and the sarcoplasmic reticulum. The following discussion will seek to reconcile the morphological evidence with that gathered in these physiological experiments; there are several models to be considered.

(*a*) TWO FIBER COMPARTMENTS IN PARALLEL One suggestion is that the extracellular space may be one compartment and the sarcoplasm the other. In

this model, the exchange kinetics may result from diffusion out of the surface invaginations and clefts, with a path length nearly equal in length to the fiber radius, discharging into the medium in parallel with the plasma membrane at the surface. Initially, both compartments would be at specific activity equilibrium and the preliminary washes with normal saline would remove the adherent surface deuterium but not that present in the surface clefts and invaginations. The efflux would then be the product of two compartments: an extracellular phase and an intracellular phase. Since a slow extracellular compartment would mask the appearance of a faster compartment, the rapidly exchanging compartment must reside, in this model, in the extracellular space. Thus the slower phase would be attributed to the plasma membrane and the sarcoplasm. This is the simplest possible model; several arguments suggest that it is not the simplest acceptable model.

(*a*) This model ascribes a major restriction to tracer movement to the physical dimensions of the tubules or invaginations. The diameter of the smallest element, the tubule, is at least 100 A so there would be little hindrance to free diffusion of the water molecule based on lateral restriction but the path length for diffusion out of the tubules and invaginations may be important. In order to evaluate this factor, consider the random movements of a tracer molecule near the end of a tubule. Assume that the length of the tubule and invagination is equal to the fiber radius and that in its permeability to water the fiber membrane is macroscopically homogeneous. Since water diffusion in myoplasm is nearly the same as in saline (Bunch and Kallsen, 1969), the two paths available to the molecule, the tubule or the myoplasm, present equal restrictions to diffusion since the molecule has to cross only one membrane. That membrane may be either at the tubule tip or at the external surface and the tracer molecule arrives in the external medium in a length of time independent of the path taken. The same argument would hold for water molecules placed anywhere along a tubule, cleft, or invagination. However, we have already seen that radial diffusion from a cylinder of dimensions similar to those of these crab fibers would be much faster than the fastest half-time observed, so path length appears to have no influence on the efflux kinetics unless the tracer permeability of the surface membrane differs from that of the tubules or invaginations.

(*b*) This model requires that the extracellular space constitute 60% of the total. Since these experiments dealt with single fibers in a well-stirred medium, the annulus of unstirred fluid was small, about 30 μ. The extracellular space of barnacle muscle fibers is about 6% of the fiber volume (Gayton and Hinke, 1968) and when compared with the observed value for the volume of the fast compartment of about 60% (or 72% calculated by the more exact method of Villegas and Villegas), it is improbable that the thickness of the annulus of unstirred fluid can account for this volume. In order that the volumes of a

cylinder and an annulus be 40 and 60%, respectively, the thickness of the annulus must nearly equal the radius of the cylinder. In this case, the external unstirred layer must be 300 μ, a value considerably in excess of that obtained by measurement.

(*c*) This model asserts that the appearance of two rate constants is the result of washout from the extracellular space. If so, the efflux kinetics should not be influenced by the time required to "load" the fiber with tracer. The shortest possible leading time would be by injection and there should still appear two compartments. But Bunch and Edwards (1969) found only a single rate constant in tritium efflux from fibers which had been injected with the tracer. Thus, loading time may be important. In this regard, Hahn and Hevesy (1941) found that a loading time of an hour was necessary to bring perfused frog muscle fiber water to tracer equilibrium. It is reasonable to assume that the compartment which requires longer loading times is intracellular.

(*d*) Finally, if one ignores all preceding arguments and asserts that the fast phase is the extracellular phase while the slow phase represents the flux across the plasma membrane, the diffusional permeability coefficient will be nearly 10 times smaller. This result sorely aggravates an already troublesome situation, discussed in the next section, in which the discrepancy between the two types of permeability coefficients for water is already very large, leading to conclusions which are clearly in conflict with other studies.

(*b*) TWO FIBER COMPARTMENTS IN SERIES Since the parallel model is inappropriate, I have chosen the simple series model depicted in Fig. 6 to present my results. The main argument in favor of this choice is that it appears to be the simplest model offering plausible identification of the compartments. I have tentatively identified the fast compartment as the compartment which is bounded externally by the plasma membrane. This model ignores the presence of the surface irregularities but the qualitative conclusions of this report will not be affected. Quantitatively the permeability coefficient may be affected since the fast compartment itself must be composed of two elements: extracellular washout and membrane flux. The result is that the discrepancy between the diffusional and nondiffusional fluxes, already alluded to, may be even greater than it appears. This problem will be discussed later; meanwhile the identity of the slowly exchanging compartment is of interest.

The slow compartment, about 30-40% of the fiber water, could reside in membrane-limited compartments within the fiber or in "bound" water, or both. Some compartments of the first sort are nuclei, mitochondria, and the sarcoplasmic reticulum. In vertebrate skeletal muscle, the volume of the sarcoplasmic reticulum alone might amount to as little as 3% or as much as 20% of the fiber volume (Birks and Davey, 1969; Page, 1964; Peachey, 1965; Schiaffino and Margreth, 1969). A similar range of values can be found

for the volume of muscle mitochondria. No studies of these elements and their contribution to fiber volume are available for crustacean muscle fibers, but it is certainly possible that subcellular organelles constitute some or all of the slow compartment. In addition, since the physical state of water in muscle fibers is imperfectly known (Cope, 1967; Ling, 1965), a fraction of fiber water in a bound state may be an important portion of the slow compartment.

However, even though plausible arguments can be produced for identifying the compartments, positive identification is not yet available. Furthermore, this model ignores the complexities introduced by the surface specializations and it is likely that the magnitude of the diffusional permeability coefficient will be influenced by an arbitrary selection of a model for compartmental analysis.

(*c*) A SERIES-PARALLEL MODEL A more realistic model would allow the two fiber compartments to exchange not only with each other but also with a third, parallel, element represented by the surface specializations. The present experiments do not allow treatment by this model since better resolution of the early phase of the efflux is necessary. Until that resolution is attained the P_{wd} must be regarded as a composite value consisting of surface washout and membrane flux. It should be realized that the P_{wd} will need reestimation and representation by this more complicated model. Further exploration of the effect shown in Fig. 4 is also mandatory, for it would appear that P_{wf} may be even greater than thought. For the time being it must be sufficient to point out that a second compartment or group of compartments does indeed exist for water in single muscle fibers. The other main point of this communication deals with the existence of a discrepancy between the diffusional and non-diffusional fluxes.

Channels in Muscle Fiber Membranes

The permeability coefficients for water for *Chionoecetes* muscle fibers are compared with those for some other fibers and cells in Table II. The similarity of the values for the muscle fibers is quite good considering the variety of experimental conditions and species differences. Yet the value for the ratio of P_{wf} to P_{wd} is at odds with those found in careful studies on the erythrocyte and squid axon. This ratio can be used to estimate the EPR. The ratio of 2.4 given for the erythrocyte corresponds to an EPR of about 3.5 A; the EPR for muscle fibers of this crab, calculated according to the method of Paganelli and Solomon (1957), is about 35 A. The EPR for frog muscle fibers appears to be even larger.

The computation of an EPR from these data might be illusory; I have already noted that the P_{wf} needs further study and eventual reestimation and that the P_{wd} will depend directly upon the model chosen for its computation.

TABLE II
PERMEABILITY COEFFICIENTS FOR WATER FOR SELECTED ANIMAL CELLS

	P_{wf}	P_{wd}	P_{wf}/P_{wd}	Reference
	$(cm/sec) \times 10^4$	$(cm/sec) \times 10^4$		
Muscle				
Crab	98.7	1.21	81	This study
Frog, toad	128, 223	0.21	600–1000	Zadunaisky et al. (1963); Hodgkin and Horowicz (1959); Caputo and Villegas (1961)
Crayfish	67	—	—	Reuben et al. (1964)
Barnacle	—	2.3	—	Bunch and Edwards (1969)
Nerve				
Squid axon	11	1.4	8	Villegas and Villegas (1960)
Erythrocyte				
Human, adult	127	53	2.4	Paganelli and Solomon (1957)

However, it is relevant at this point to inquire into the validity of the conclusion that the discrepancy between P_{wf} and P_{wd} indicates the presence of aqueous pores in the muscle fiber membrane even though estimation of the size of the pores must await further investigation.

Alternative explanations for the discrepancy between the two permeability coefficients are available. Dick (1966) for example, contends that diffusion within the cytoplasm is very slow and he concludes that P_{wd} is actually measuring the mutual diffusion coefficient of water and intracellular organic molecules. This appears not to be the case for muscle fibers, since Bunch and Kallsen (1969) found that the diffusion coefficient of tritium was the same in myoplasm as it was in saline. Another explanation for the discrepancy has been advanced by Dainty (1963). Dainty feels that the presence of unstirred layers can explain the difference between P_{wf} and P_{wd}. An unstirred layer must always be present, but its size depends on the experimental procedures. I have already presented calculations from diffusion equations which suggest that tracer exchange of deuterium appears to be membrane-limited since the exchange rate is slower than would be expected from diffusion. In addition, the discrepancy between P_{wf} and P_{wd} is too large to be accounted for by the presence of external layers of unstirred saline solution adjacent to the muscle fiber. Using equation (48) given by Dainty, I find that the thickness of an unstirred layer required to cause a discrepancy as large as this is 500 μ whereas my measurements indicate that the actual thickness is closer to 30 μ. The third major challenge to the idea that $P_{wf}:P_{wd}$ indicates the size of membrane pores comes from Harris (1956) who suggests that the presence of long, narrow pores, in which "single-file" diffusion is necessary, can account for the results. This explanation has been disputed by Hirsch (1967) and Lifson et al. (1960).

It seems reasonable to retain the idea that the discrepancy between P_{wf} and P_{wd} indicates the presence of pores in the cell membrane. It is less certain

that the data can be used to estimate the size of channels in the membranes of muscle fibers. Some of the assumptions involved in such calculations deserve examination (Solomon, 1959)—in particular, the assumption that the areas of membrane available for diffusion and filtration are equal. This assumption is probably an oversimplification (Koefoed-Johnsen and Ussing, 1953; Collander, 1937) and I would suggest that bulk water movements may flow through both porous and nonporous membrane areas as a result of the intensity of the driving force (the hydrostatic pressure) while the tracer flux is largely confined to the aqueous channels. If so, P_{wd}, calculated as done here, would be decreased relative to P_{wf} and the resultant EPR would be large. Since the ratio of P_{wf} to P_{wd} for muscle fibers ranges from 80 to 1000, then, by this reasoning, the proportion of porous membrane would be less than 1%, a feasible value. Another assumption is that of isoporosity. It is possible that there are two populations of channels with regard to size, but the fraction of large channels required to account for the magnitude of the difference between the two fluxes is too large to be consistent with electrophysiological data.

Three final suggestions should be mentioned even though the state of development of theoretical and experimental studies may not yet be adequate to deal with them. First, the actual water permeabilities of the surface and tubular membranes may be different. This could explain the obvious "compartmentation" of water in fibers in quite a different manner; studies of tracer exchange of water in fibers in which the tubule system is swollen (Brandt et al., 1968) or from which the plasma membrane has been removed may be of interest. Second, the existence of an electro-osmotic water flux has been demonstrated for crayfish muscle fibers (Reuben et al., 1964), and it may be of importance in the study of osmotic water movements. This effect deserves further attention. Finally, there may be an element of water flux which is actively transported; a flux ratio analysis of tracer exchange would be valuable in this regard (Ussing, 1952).

The strongest case in favor of the existence of aqueous channels is found in studies on the frog skin (Koefoed-Johnsen and Ussing, 1953). Experimental treatments which modify the osmotic flux while the diffusional flux is little affected have been carried out only on the frog skin. Since the osmotic flux is expected to be strongly influenced by the radius of the channel while the diffusional flux is not, this is a critical point in establishing whether or not equivalent pores are likely to exist as morphological entities. Until such experiments can be performed on muscle fibers, the usefulness of the ratio of P_{wf} to P_{wd} as an indicator of channel size must remain in doubt even though the discrepancy between them seems real. Furthermore, detailed information concerning the water permeability of different areas of the muscle fiber membrane must be made available by further experimentation. The mammalian erythrocyte remains the best studied and most consistent example. On the other hand, muscle fibers exhibit facets of physiological behavior which sug-

gest strongly that they should be used to investigate further the nature of the forces present during diffusional and osmotic water movements, the nature of membrane responses to changes in osmotic or ionic strength, and the influence of membrane heterogeneity on water and solute fluxes.

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