EXCHANGE OF ^{3}HHO IN INTACT ISOLATED MUSCLE FIBER OF THE GIANT BARNACLE

IGNACIO L. REISIN* and GILBERT N. LING

The Pennsylvania Hospital, Department of Molecular Biology, Eighth and Spruce Streets, Philadelphia, Pennsylvania 19107

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SUMMARY

(1) Efflux curves of ^{3}HHO from intact isolated muscle fiber of the giant barnacle were studied by a continuous washout technique.

(2) The accuracy of the washout technique was analyzed by studying the efflux of ^{3}HHO from two models: a) microsacs of collodion and, b) cylinders of agar gel. In both cases the experimental efflux kinetics follow the predictions of the respective models: surface-limited diffusion for the microsacs of collodion and bulk phase-limited diffusion for the filaments of agar gel.

(3) The experimental efflux curves of ^{3}HHO from isolated muscle fiber can be described by a sum of at least two exponential terms. Several models have been analyzed to explain the distribution and kinetics of the ^{3}HHO in this preparation. Those models which consider the muscle water as distributed in two compartments, extracellular and intracellular water, require that at least 27% (two compartments in parallel) or 46% (two compartments in series) of the total water has to be located in the extracellular space. This fraction of water exceeds the maximum extracellular water content (9%) estimated on the basis of extracellular space measurements by a large margin.

(4) It has been found that a model of bulk phase-limited diffusion and desorption mechanism can describe the ^{3}HHO effluxes adequately. This model basically suggests that the rate-limiting step of ^{3}HHO exchange is not permeation through a surface barrier but diffusion through the bulk of the intracellular water. According to this model, the apparent diffusion coefficient for ^{3}HHO has been estimated to be 55% of that in free solution.

(5) The bulk phase-limited diffusion and desorption kinetics is compatible with recent evidence indicating that the cell water is in a different physical state than in free solution.

*Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas la República Argentina Present address: Department Biophysique, Centre Hospitalier Universitaire, Universite de Sherbrooke, Sherbrooke, Quebec, Canada.

INTRODUCTION

Recent studies of nuclear magnetic resonance spectra,[1-3] intracellular freezing patterns,[4,5] water vapor equilibrium,[6] kinetics of ^{3}HHO exchange,[7] and nonelectrolytes exchange* have indicated that the water in living cells may exist in a different physical state than water in dilute solutions. More specifically, studies of the influx and efflux of 'HHO into and from frog ovarian eggs have shown that the rate-limiting step for the exchange is not a surface barrier, but rather, that it reflects a more-or-less uniformly slower rate of diffusion in the bulk of the cell water' and the presence of an adsorbed fraction of water as a result of interaction with cell proteins.'

All of these results are in support of a hypothesis on the physico-chemical nature of the living cell that envisages cell water as existing in a state of multilayers adsorbed on the cell macromolecules.[10-14]

In 1969, Bunch and Edwards[15] reported their studies on the ^{3}HHO exchange of isolated single barnacle muscle fibers, concluding that this exchange is limited by the surface membrane. The muscle fibers were cut and ^{3}HHO was introduced into the cell by injection. Subsequent investigations have brought to light the fact that a profound disruption of the normal state of the water could follow cutting or other injury of one end of the muscle cell cytoplasm.[16,17]

We recently reinvestigated this ^{3}HHO exchange problem, using *intact* isolated single giant barnacle muscle fibers. The data to be presented below indicate that the efflux of water from this cell also follows a bulk phase-limited diffusion in conjunction with a desorption process, In agreement with the earlier conclusion from the study of frog eggs.

METHODS AND MATERIALS

All experiments were performed on single muscle fibers of the giant barnacle, *Balanus nubilus*, isolated from the depressor muscles in a barnacle Ringer solution kept at 6-8°C. The muscle fibers were isolated intact with a small piece (0.25 cm^2) of the shell attached. For the uptake experiments, several fibers attached to a common piece of shell were isolated together.

The isolated muscle fibers were approximately cylindrical in shape and their diameters were measured with the aid of an ocular micrometer. Fibers that contracted at any stage of the experimental procedure were discarded. The composition of the barnacle Ringer solution used was in millimolar concentrations as follows: NaCl, 450; KCl, 8; $CaCl_2$, 20; $MgCl_2$, 12; $NaHCO_3$, 10. All experiments were performed at 23-25°C.

Introduction of Extracellular Markers

Several single fibers attached to a common piece of shell were immersed in 6 ml of

barnacle Ringer solution containing 1.0 μCi/ml of sorbitol-^{14}C for 40 min or 1.0 μCi/ml of sucrose-^{14}C for 60 min. The duration of these periods of incubation exceeded, with an ample margin, the time it takes for a water-f&d cylinder 0.1 cm in radius to reach 99% of the equilibrium distribution of these extracellular space probes in an external solute (i.e., 17 min for sorbitol-^{14}C and 21 min for sucrose-^{14}C, calculated on the basis of a diffusion coefficient of 0.7 x 10^{-5} cm^2/sec for D-sorbitol and 0.55 x 10^{-5} cm^2/sec for sucrose, 25°C).

After loading with the labeled sorbitol or sucrose, the muscle fibers were cut from the shell, blotted on wet filter paper, and weighed. The tracers taken by the muscle fiber were extracted in 2 ml of 0.1 N HCI for 16 to 20 hours at room temperature. 0.5 ml of the extracts, as well as 0.5 ml of dilution of the loading solutions, were mixed with 5 ml of Bray's solution" and counted in a Packard 341E liquid p-scintillation counter.

The volume of extracellular space occupied by the tracers per gram of wet weight were calculated by the relation:

$$\frac{\text{ml (extracellular)}}{\text{g wet wt. (muscle}} = \frac{\text{counts/min (muscle)}}{\text{counts/min ml (soln)}} \times \frac{1}{\text{g wet wt. (muscle)}}$$

Water Content

Muscle fibers incubated in barnacle Ringer solution were blotted on wet filter paper and weighed (wet weight) as described before; the fibers were then dried at 98°C for 16 to 20 hours and weighed again (dry weight). The water content was taken as the difference between the wet weight and the dry weight and expressed in ml per gram of wet weight ± 1 S.E.

Efflux of ^{3}HHO from Muscle Fibers

Loading: A single isolated muscle fiber was incubated in 3 ml of barnacle Ringer solution containing 10 μCi/ml of tritiated water for 45 to 60 min. In some experiments, only the muscle fibers were exposed to the labeled solution. This was done by passing the muscle fiber through a hole (0.25 cm in diameter) in a Teflon disc held over the labeled solution. After loading, the fiber was carefully blotted on wet filter paper and introduced into the washout apparatus.

Washout Apparatus: Figure 1 shows a diagram of the chamber used. The washing Ringer solution flowed in a groove 3.8 cm long, 0.27 cm wide, and 0.6 cm deep and was collected at the outlet on the floor of the chamber in graduated 15 ml centrifuge tubes (0.1 ml division ± 2%). The total volume of the groove and the outlet was 0.64 ml. The average flow rate of the washing solution was 17 ml/min. The performance of the cham-

ber was analyzed by following the washout of a sample of ^{22}Na introduced into the chamber. This was done by tilling the chamber with 0.6 ml of water containing 0.08 μCi of ^{22}Na. The washout was started by opening the inlets and the outlet of the chamber simultaneously. The flux of the washing solution was between 12-13 ml/min, and samples were collected for periods of 5 seconds. Under these conditions the half time of dilution was estimated to be between 4 and 5 seconds.

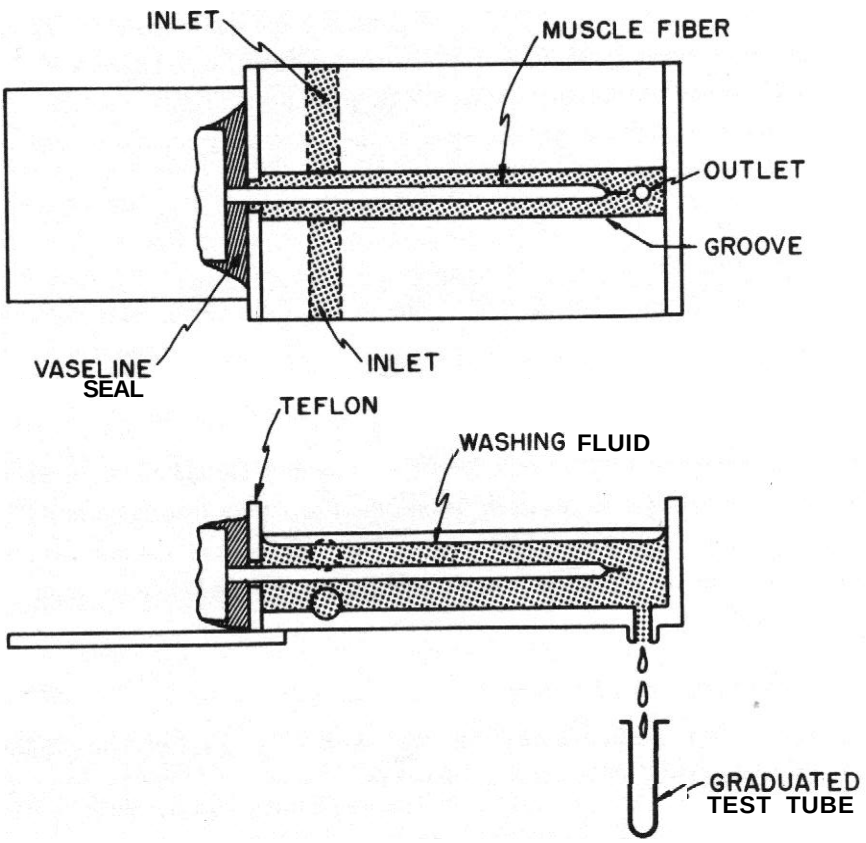

Figure 1. Diagram of the chamber for continuous washout of intact isolated muscle fibers. The isolated muscle fiber is washed by a continuous stream of barnacle Ringer solution that enters to the groove via two inlets. The position of the inlets assures that the muscle fiber is completely surrounded by the washing solution. The attached piece of basal shell remains outside the chamber and is covered with Vaseline. At the floor of the opposite end of the groove there is an outlet through which the washing solution drains and is collected. The samples were collected at intervals of 5, 10, 15 and 30 sec; the total time for changing from tube to tube (done manually) took no more than one sec. No washing solution was lost between samples.

Washout: The isolated muscle fiber was introduced into the chamber in the following manner: the Teflon wall of the chamber had a hole 0.25 cm in diameter through which a silk thread was pulled. The tendon of the fiber was tied to the thread; careful pulling of the thread brought the fiber into the chamber. (The piece of shell attached to the muscle fiber, as well as the external surface of the Teflon wall, were first covered with petrolatum.) The shell was then firmly pressed against the Teflon surface and fixed in that position by a rubber band.

Washing solution flowing through the chamber was collected without interruption in a series of 15 ml graduate centrifuge tubes. These tubes were changed at 5, 10, or 15 sec intervals during the first 3 min of the washout and every 30 sec thereafter; the total duration of the washout being 5 to 8 min. At the end of this period, the exposed part of the fiber was soaked in S-10 ml of barnacle Ringer solution for at least 4 hours to exchange fully the remaining ^{3}HHO water. The volumes of barnacle Ringer solution collected in centrifuge tubes were recorded; a 0.5 ml sample from each tube, as well as 0.5 ml of the final extraction solution, was mixed with 5 ml Bray's solutions and their radioactivity was assayed. Since the volume of the washing solutions collected in each time interval was known, the total counts/min lost by the muscle fiber in that period was readily calculated.

Efflux of Tritiated Water from Cylinders of *Agar* Gel and *Microsacs* of *Collodion*

Cylinders of Agar Gel: Cylinders of agar gel were prepared by dissolving 4 g of agar (Bacto-Agar, Difco) in 100 ml of hot distilled water. The mixture, still fluid, was aspirated into a length of polyethylene tubing. Once the agar had solidified, the agar gel fdament was squeezed out from the tubes by air pressure and stored in distilled water for a few hours before use.

Collodion Microsacs: Collodion microsacs were prepared according to the method of Ling (unpublished). Five grams of pyroxylin (Parlodion, Mallinckrodt Chem. Works) were dissolved in 100 ml of a mixture of 3 volumes ethanol and 1 volume ethylether. Thin glass rods, 0.1 to 0.15 cm in diameter, were dipped 3 to 4 times in the mixture, allowing partial drying each time. The collodion coating formed was removed from the glass rod mold, cut into 2.5 cm lengths, and each length was tied at one end with a thin silk thread. The microsac was filled with distilled water before the open end was also tied. Both tied ends were then sealed with a thin coat of Parlodion solution. The external diameters of these microsacs measured from 0.11 to 0.15 cm.

Efflux of Tritiated Water from Models: Agar gel filaments and the microsacs were incubated for at least 1 hour in 5 ml of distilled water containing 10.0 μCi/ml of tritiated water. At the end of the loading period the diameters of the agar gel filaments were measured. The filaments (and the microsacs) were gently blotted on wet filter paper and introduced into the efflux chamber described above. The procedure used to study the

efflux of tritiated water from these models was similar to that used in the study of isolated muscle fibers.

Analysis of the Efflux Curves

The efflux curves were obtained by adding up the collected, measured radioactivity in the washing solution and that of the residual tritiated water in the muscle fiber. The results were normalized by taking the total counts in the muscle at zero time as unity. All the efflux curves were analyzed on the assumption that the muscle fibers are cylinders whose lengths far exceed their diameters.

The efflux data are presented in two different ways: (1) in plots of the logarithm of remaining ^{3}HHO (as a fraction of the initial amount) against the duration of washing t, and (2) the fraction of the total "HHO already exchanged as a function of the square root of t. This type of plot more clearly displays the differences between surface-limited diffusion and bulk-limited diffusion process.[7,11] For the model in which the efflux of ^{3}HHO represents bulk phase-limited diffusion with desorption, tables of numerical solutions calculated with the aid of an IBM/360 computer were kindly provided by Dr. G. Karreman.

RESULTS

Efflux of Tritiated Water from Models

To analyze whether the washout method used had the requisite accuracy, efflux of ^{3}HHO from collodion microsacs and from agar gel filaments were studied. Both models were roughly of the same size and shape as the muscle fibers.

The efflux of ^{3}HHO from microsacs was expected to follow the kinetics of a surface-limited diffusion process, since the only significant diffusion barrier is the collodion wall. When the cylinders previously equilibrated with ^{3}HHO are washed in solution free of tracer, the efflux kinetics are governed by the equation:

$$\frac{d[C_i]_{in}}{dt} = -k^i_{ow}\,[C_i]_{in}\,, \tag{1}$$

where [C,], is the concentration of the ith substance in the interior of the bag and k^i_{ow} is the efflux rate constant of the ith substance. The solution to equation 1 is

$$\frac{[C_i]^t_{in}}{[C_i]^0_{in}} = \exp\,(-k^i_{ow}t)\,, \tag{2}$$

where the superscripts t and 0 indicate concentration of labeled water at time t and time zero, respectively. Equation 2 predicts that a plot of log $[C]_{in}^{t}/[C]_{in}^{0}$ against time should yield a straight line with an intercept of 1.0. The efflux described by equation 2 can be converted to an influx curve by writing equation 2 in an exponential form, multiplying it by (- 1) and adding one to both sides. If $1-[C_i]_{in}^{t}/[C_i]_{in}^{0}$ is plotted against the square root of the time, the curve obtained is sigmoidal (Fig. 2a).[7]

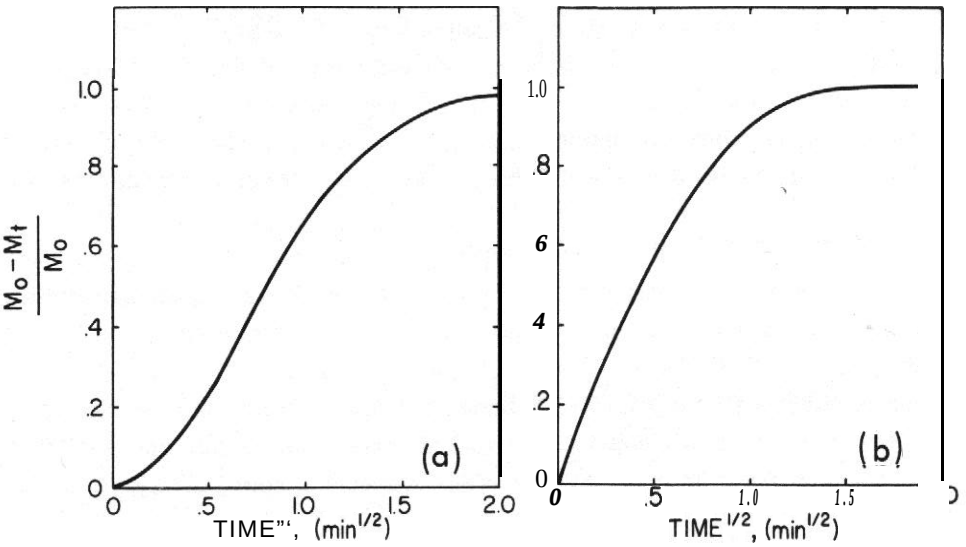

Figure 2. Theoretical efflux curves for surface and bulk phase-limited diffusion mechanisms. M_t and M_0 represent the amount of tracer in the cylinders at time t and time 0. The theoretical efflux curves are plotted as influx curves, as a function of the square root of time: a) the surface-limited diffusion mechanism was calculated by equation 2 with a rate constant of 1.045 min^{-1}; b) bulk phase-limited diffusion mechanism for a cylinder of 0.0475 cm in radius and an apparent diffusion coefficient of 1.29 x 10^{-5} cm^2/sec.

For the case of cylinders of agar gel, the rate-limiting step for the efflux of ^3HHO will be the diffusion of the tracer in the bulk of the cylinder. The efflux of ^3HHO from a cylinder of infinite length is described by the equation:[19,20]

$$\frac{[C_i]_i^t}{[C_i]_i^o} = 4\left\{\frac{\exp -\frac{Dv_1^2 t}{r_0^2}}{v_1^2} + \frac{\exp -\frac{Dv_2^2 t}{r_0^2}}{v_2^2} + \ldots\right\}, \qquad (3)$$

where D is the diffusion coefficient of the ith substance in the cylinder, ν_i^2 are the zeros of the Bessel function of zero order ($\nu_1 = 2.405$; $\nu_2 = 5.520$;. .), and r_0 is the radius of the cylinder. When t is sufficiently long, only the first exponential term is significant and equation 3 can be reduced to

$$\frac{[C_i]^t_{in}}{[C_i]^0_{in}} = 0.692 \exp \frac{-D\nu_1^2 t}{r_0^2} \tag{4}$$

Equation 4 shows that the last portion of a plot of the ℓn of $[C_i]^t_{in}/[C_i]^0_{in}$ plotted against time yields a straight line with an intercept on the ordinate equal to ℓn 0.692. The efflux kinetics can be transformed into an influx profile in a manner similar to that described above for a surface-limited diffusion process.[7,11] In a plot of $(1- [C_i]^t_{in}/[C_i]^0_{in})$ against the square root of time, the curve is approximately linear in the initial portion (Fig. 2b).

Efflux of ^{3}HHO from Microsacs of Collodion

Figure 3 presents the efflux of tritiated water from a microsac of collodion, showing that the efflux curve has the characteristics of a surface-limited diffusion process: a) the semilog plot is a straight line over two decades, and b) its extrapolation to zero time gives a value of $[C]^t_{in}/[C]^0_{in}$ (or M_t/M_0) close to unity (0.98). The average value of the extrapolated intercepts on the ordinate at time zero from four different experiments was 0.98 ± 0.01 (Table 1). Figure 4a shows the same efflux of Figure 3 plotted as an influx profile against the square root of time. It can be seen that the experimental curve, sigmoid in shape, fulfills the requirement of simple surface-limited diffusion, as described by equation 2.

Table 1. Intercepts of the ^{3}HHO efflux curves from microsacs of collodion. Intercepts are the extrapolation to zero time of the straight line obtained from a semilogarithmic plot of the fraction of initial ^{3}HHO activity as a function of time (± 1 S.E.).

Microsac	Intercept of Straight Line
a	0.97
b	1.00
c	0.96
d	0.98
Average	0.98±0.01

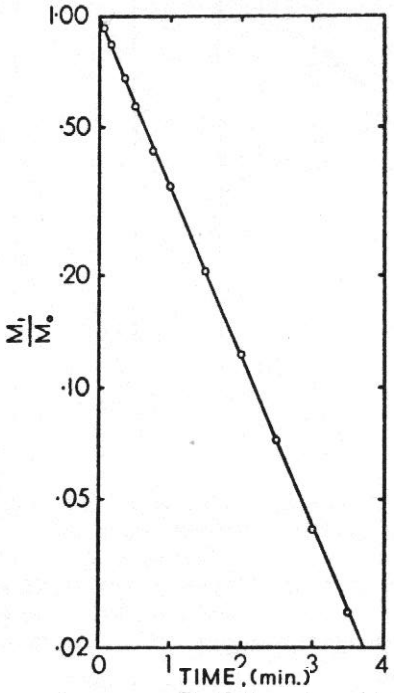

Figure 3. Efflux of ^{3}HHO from a microsac of collodion. Semilogarithmic plot of the amount of ^{3}HHO remaining in the sac at time t (M_t) as a fraction of the initial amount (M_0) as a function of time, t. Microsac d (Table 1). The extrapolation of the straight line is 0.98 and the rate constant 1.045 min^{-1}.

Efflux of ^{3}HHO from Filaments of Agar Gel

Figure 5 shows the time course of tritiated water exchange for a filament of agar gel. The efflux is relatively faster at the beginning but later follows a straight line. The intercept of this straight line portion was calculated by regression using all values of M_t/M_0 less than 0.30. In these experiments, M_t/M_0 reached a value of 0.30 in about 20 to 40 sec. Table 2 presents the intercepts values of the straight line portion of the effluxes and the apparent diffusion coefficients for five experiments. No significant differences were found between the intercepts calculated for filaments b to e and the theoretical intercept expected for a bulk phase-limited diffusion process, only in one case (filament a, Fig. 5) the difference seems to be significant $(0.05>p>0.025)$. The apparent diffusion coefficient estimated is $D' = 1.44 \times 10^{-5}$ cm^2/sec. This value is lower than the diffusion coefficient

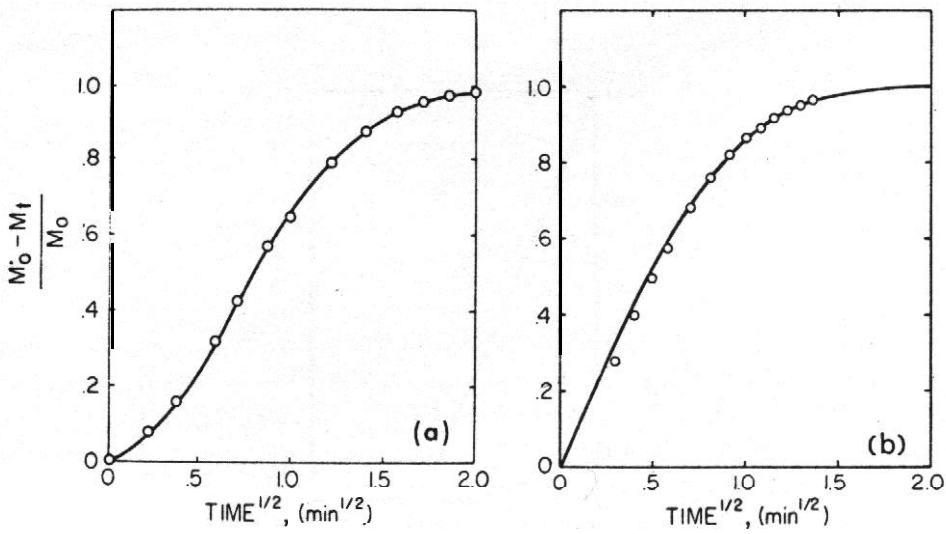

Figure 4. (a) Efflux of ^{3}HHO for a microsac of collodion. The efflux data are represented as an influx and plotted as a function of the square root of time showing a sigmoidal shape. The theoretical curve has been calculated from equation 2 with a rate constant of 1.045 min^{-1} (microsac d, Table 1); (b) Efflux of ^{3}HHO from a cylinder of agar gel. One efflux (filament a, Table 2) is converted and plotted as influx as a function of the square root of time. The continuous line was calculated far a pure bulk phase-limited diffusion mechanism. The radius used is 0.0520 cm and the apparent diffusion coefficient 1.28 x 10^{-5} cm^2/sec.

Table 2. Parameters of the ^{3}HHO efflux curves from agar gel filaments. The slopes and intercepts were obtained by regression. AU values of M_t/M_0 less than 0.30 were used. The apparent diffusion coefficient D' was calculated from the slope of the regression line, which is equal to $D' 5.784/r_0^2$, where r_0 is the radius of the filament. In four of the five filaments, the calculated intercepts were found to be not significantly different with respect to the theoretical value of $\ln 0.692$ (filament b, $0.9>p>0.8$; c, $0.9>p>0.8$; d, $0.8>p>0.7$; e, $0.3>p>0.2$; for filament a. $0.05>p>0.025$).

Agar Filament	Radius	Intercept of Straight Line		, D'
	cm	$\ln M_t/M_0$*	M_t/M_0	cm^2 sec^{-1} x 10^5
a	0.0520	-0.3189 ± 0.0166 (8)	0.727	1.28
b	0.0550	-0.3548 ± 0.0698 (5)	0.701	1.47
c	0.0475	-0.3526 ± 0.0812 (8)	0.703	1.28
d	0.0430	-0.3315 ± 0.1046 (5)	0.718	1.59
e	0.0533	-0.3218 ± 0.0346 (10)	0.725	1.58

* ± S.E.

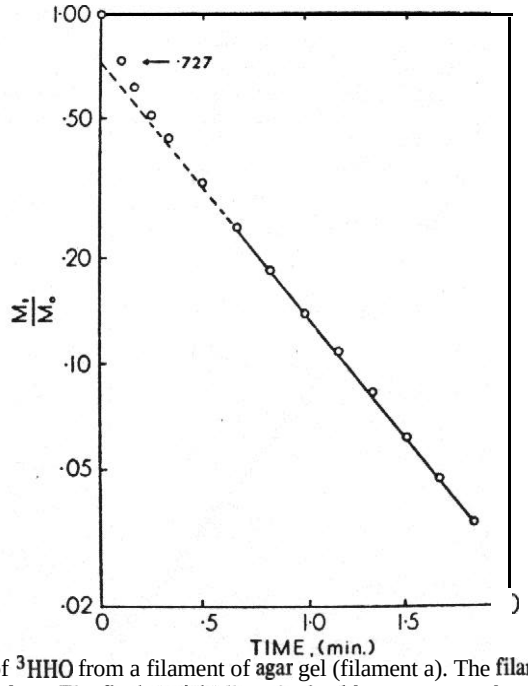

Figure 5. Efflux of ^{3}HHO from a filament of agar gel (filament a). The filament of agar gel 4% (w/v) is 0.0520 cm in radius. The final straight line obtained by regression has an intercept of $\ln$ 0.727.

of ^{3}HHO in pure water ($D_{^3HHO}$ = 2.44 x 10^{-5} cm^2/sec at 25°C"). This lowering of the diffusion coefficient has been attributed to the obstructive effects of the gel matrix." Nakayama and Jackson'" measured the apparent diffusion coefficients of tritiated water in agar gel with a varying agar concentration from 0.3 to 1 g/100 ml, showing that the apparent diffusion coefficient decreased as the percentage in the gel increased. Extrapolation of their data yields a $D'_{^3HHO}$ = 1.39 x 10^{-5} cm^2/sec for a 4% gel, which is close to the value obtained from the effluxes reported here.

All the efflux curves can be normalized by plotting them against $D't/r_0^2$ (Fig. 6). Except for the first 10-20 seconds of the washout, in which a departure from the theoretical curve of 6 to 10% can be observed, all efflux curves are in basic agreement with the expected kinetics. When the efflux is plotted as an influx, against the square root of time, the experimental values follow the theoretical curve for bulk phase-limited diffusion from a cylinder (Fig. 4b). This result indicates that extrapolation from the final straight line portion of the curves gives a quite accurate intercept, theoretically predicted. The efflux

of tritiated water from both models-microsacs of collodion and cylinders of agar gel-presents the characteristics theoretically predicted, indicating that efflux studies with this technique are accurate enough to yield meaningful analysis of the efflux of tritiated water from the single isolated barnacle muscle fibers.

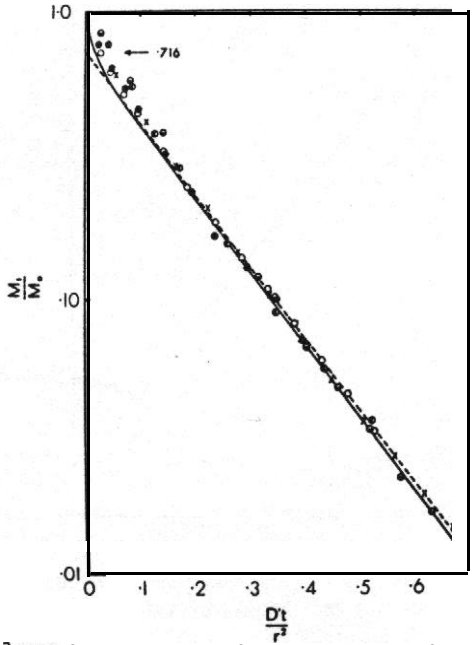

Figure 6. Efflux of ^{3}HHO from a filament of agar gel. AU data from five different filaments have been normalized by plotting them as a function of the nondimensional parameter $D't/r_0^2$; r_0 is the radius of the filament in cm (Table 2) and t, the time in seconds. The continuous line has been obtained from the data tabulated by A. V. Hill (ref. 19, Table IV). The dashed straight line is the regression line calculated by using all values of M_t/M_0 less than 0.30. The intercept ($\ln 0.716 = -0.3341 \pm 0.021$) was found not significantly different from the theoretical value of $\ln 0.692$ (-0.3682; $0.20 > p > 0.10$).

Efflux of Tritiated Water from Muscle Fibers

The efflux experiments were carried out after loading the muscle fibers for one hour in barnacle Ringer with ^{3}HHO. This period of incubation is sufficient for the ^{3}HHO to completely exchange with the cellular water (Table 3). The distribution of tritiated water was calculated from the uptake of ^{3}HHO and the specific activity of the loading solution, and is expressed in microliters per gram of wet weight. The water content was calculated from the wet weight of each muscle and the percentage water content (ml/g wet wt.) estimated on other muscle fibers from the same barnacle. The value of 1 for the ratio of the ^{3}HHO exchange over the water content indicates that the water of the muscle has been completely exchanged after one hour of incubation. Figure 7 shows two efflux curves of ^{3}HHO from intact isolated muscle fibers. The shape of the curve is not a simple straight

Table 3. Volume of ^3HHO distribution in intact isolated muscle fibers. The intact muscle fibers were incubated for 1 hour in barnacle Ringer solution containing 10 μCi/ml ^3HHO. The fibers were then cut from the basal shell, blotted, weighed, extracted and counted as described under Methods. The water content of the fibers was obtained by multiplying the wet weight of each muscle (column 2) by the fraction of water content determined separately on muscles of the same barnacle (0.787 ml/g wet wt).

Muscle	Wet Wt.	Volume of Distribution of ^3HHO	Water content	$\frac{\text{Volume } ^3HHO}{\text{Water content}}$
	gram	ml	ml	ratio
a	0.0322	0.0247	0.0253	0.98
b	0.0337	0.0268	0.0265	1.01
c	0.0311	0.0240	0.0245	0.98
d	0.0360	0.0287	0.0283	1.01
e	0.0287	0.0233	0.0226	1.03
f	0.0373	0.0304	0.0294	1.03
g	0.0345	0.0280	0.0272	1.03
h	0.0235	0.0174	0.0185	0.94
i	0.0261	0.0207	0.0205	1.01
j	0.0258	0.0192	0.0203	0.95
k	0.0199	0.0155	0.0157	0.99
l	0.0218	0.0182	0.0172	1.06
m	0.0215	0.0173	0.0169	1.02
Average				1.00±0.01

line. There is an initial fast fraction of ^3HHO that is completely washed out in the first minute. The following portion of the efflux curve represents a straight line. The complete efflux curves can be approximately described by a sum of two exponential terms:

$$\frac{[C_i]^t_{in}}{[C_i]^0_{in}} = Ae^{-\lambda_A t} + Be^{-\lambda_B t} \qquad (5)$$

where A, B, λ_A and λ_B are constants, the value of the parameters in equation 5 for eight efflux experiments are given in Table 4. It should be emphasized that obedience to equation 5 does not necessarily imply that each exponential term represents a different compartment of water in the muscle fiber.

Different models for the distribution of water in the muscle fiber will be analyzed next.

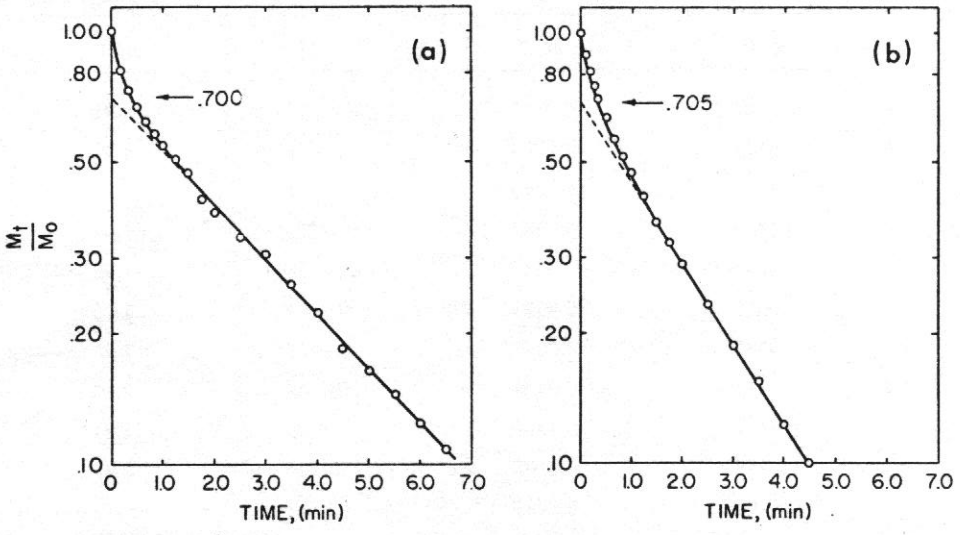

Figure 7. Efflux of ^{3}HHO from an intact isolated muscle fiber. Fig. (a): muscle a; and Fig. (b): muscle d (Table 4). The fibers were previously equilibrated for at least one hour in barnacle Ringer solution containing 10 μCi/ml of ^{3}HHO. The graphs are semilogarithmic plottings of the fraction of initial ^{3}HHO activity as a function of time. The experimental points do not follow a simple straight line. The intercepts of the final straight line are 0.700 and 0.705 respectively (Table 4).

DATA ANALYSES

I. Surface-Limited Diffusion

Assuming that the rate of exchange of ^{3}HHO in the sarcoplasm is limited by the surface membrane, two compartments would be expected to be observed in the efflux curves, each representing an extracellular and intracellular fraction. Furthermore, these two compartments would be considered to exchange with the washing solution independently (two fiber compartments in parallel) or, alternatively, the cellular water must pass through the extracellular compartment (two fiber compartments in series). For both models it is reasonable to assume that the extracellular compartment would exchange faster than the

intracellular compartment.

A. Two Fiber Compartments in Parallel: For this simple case the fraction of extracellular and intracellular compartments are given respectively by the constants *A* (fast compartment) and *B* (slow compartment) of equation 5.

The extracellular compartment will then have a value of about 27% of the total muscle water, which far exceeds the extracellular space reported for isolated muscle fibers of barnacle (7% of fiber water).[24] Studies of D_2O exchange in single muscle fibers of a marine crab[25] have also shown that if a model of two fiber compartments, in parallel is applied, an extracellular space of 60% will be required. It might be asked whether this excess of ^3HHO in the fast compartment might not be due to a leakage of tracer from the piece of shell at the outside of the chamber (Fig. 1). This possibility is ruled out because the same efflux curves have been obtained when only the muscle was exposed to the loading solution (muscles g and h, Table 4).

$\lambda = k\ (1/sec)$

Table 4. Efflux of ^3HHO from intact isolated muscle fibers of barnacle (fitting parameters according to equation 5). The fraction of the initial ^3HHO activity as a function of time can be described by a sum of two exponential terms: $A \exp(-\lambda_A t) + B \exp(-\lambda_B t)$, where A, B, λ_A, λ_B are constants.

Muscle	Radius	Fast Component		Slow Component	
		Intercept (*A*)	Rate Constant (λ_A)	Intercept (*B*)	Rate Constant (λ_B)
	cm	% of total initial ^3HHO	min^{-1}	% of total initial ^3HHO	min^{-1}
a	0.075	30.0	2.376	70.0	0.288
b	0.106	13.9	3.326	86.1	0.280
c	0.060	35.5	2.310	64.5	0.397
d	0.067	29.5	3.465	70.5	0.440
e	0.075	32.8	3.465	67.2	0.472
f	0.061	15.0	5.198	85.0	0.538
8	0.051	26.5	5.198	73.5	0.708
h	0.045	29.0	4.377	71.0	0.611
Average	0.068 ±0.007	26.5 f2.8	3.714 ±0.398	73.5 f2.8	0.467 ±0.053

Another possible source of an extra amount of water in the fast compartment might be faulty procedure in blotting the muscle before the washout experiments. Let us assume that after blotting, 20% of the water adheres to the surface as a uniform annulus surrounding the muscle fiber. It can be calculated that the width of this annulus could be about 80 μ for an average fiber radius of 0.068 cm (Table 4). The half-time of exchange of this annulus of water can be estimated by the following equation?

$$\frac{[C]_t}{[C]_0} = \frac{8}{\pi^2} \exp - \frac{D\pi^2 t}{4b^2} , \qquad (6)$$

where $[C]_t$ and $[C]_0$ are the concentrations of "HHO at time t and zero respectively; D is the diffusion coefficient of ^{3}HHO in water (2.44 x 10^{-5} cm^2/sec at 25°C[21]); and, b the thickness of the annulus* (0.008 cm). The half-time of exchange for this adhering water would be 0.7 sec, a value one order of magnitude smaller than the fastest fraction observed. This is below the limit of resolution of the present experiments and can be considered instantaneous. Since the average half-time of exchange for the fast fraction is 12.1 sec, an annulus of loosely attached water, if it had existed, would have been too rapidly washed away to explain the high value of water in the first component.

However, to further clarify this question, we carried out experiments to determine the volume of the extracellular space in the muscle fibers under conditions similar to those in the efflux studies.

Muscles incubated in sorbitol-^{14}C for 40 min and sucrose-^{14}C for 60 min were blotted on a wet filter paper, in a manner similar to that used for the efflux experiments. For comparison, a third group of muscle fibers equilibrated in sorbitol-^{14}C were washed for 30 sec in an isotonic sucrose solution and blotted on a filter paper, following the procedure described by Hinke.[24] Table S shows the results. The sorbitol-^{14}C space of fibers that were rinsed and blotted (0.049 ml/g wet wt.) is 74% of the sorbitol space of fibers that were blotted but not rinsed (0.066 ml/g wet wt.). The sucrose-^{14}C space, despite the fact that it is a larger molecule than sorbitol-^{14}C, appears to be higher than the sorbitol-^{14}C space, although the difference is not statistically significant ($0.20>p>0.10$). A ceiling value of the volume of water in the extracellular space can be calculated by dividing the extracellular space per gram of wet weight by the fraction of water in the fresh tissue (0.775 ml/g wet wt., Table 5). For the fibers rinsed and blotted, the extracellular water

*When equation 6 is applied for describing the exchange of substances through both surfaces of a plane sheet b is taken as one half of the slab thickness. When the exchange taker place through one surface only the value of b has to be equal to the slab thickness.

Table 5. Sorbitol-^{14}C and sucrose-^{14}C spaces in the intact isolated muscle fiber of barnacle. Intact muscle fibers were incubated in barnacle Ringer solution containing tracer amounts of sorbitol-^{14}C or sucrose-^{14}C (1 μCi/ml). After incubation the muscles were cut from the basal shell and blotted as described under Methods. The fibers indicated as rinsed and blotted were washed for 30 sec in isotonic sucrose and then blotted, following the procedure used by Hinke.[24] The water content in the extracellular space (ECS) was calculated by dividing the volume of distribution of sorbitol-^{14}C or sucrose-^{14}C by the water content determined in other muscle fibers of the same barnacle (0.775 ml/gm wet wt.). The difference between the volume of distribution of sorbitol-^{14}C of muscles blotted only are not significant ($0.20 > p > 0.10$).

Tracer	Time of Incubation	Procedure	Vol. of Distribution of Tracer	Water in ECS/ Total Water	n
	min		ml/g wet wt.		
Sorbitol-^{14}C	40	blotted	0.066M.002	0.085M.003	(12)
Sorbitol-^{14}C	40	rinsed and blotted	0.049±0.003	0.063±0.004	(12)
Sucrose-^{14}C	60	blotted	0.070±0.002	0.090±0.003	(11)

represents 6.3% of the total water, in good agreement with the values reported by Hinke.[24] The percentage of total water in the extracellular space, for fibers blotted only, rises to 8.5 and 9.0% for sorbitol-^{14}C and sucrose-^{14}C, respectively. This space includes the volume of solution that adheres to the external surface of the fiber. Since the fibers used for the efflux experiments were blotted only, it would be expected that the fraction of the ^{3}HHO efflux representing the extracellular space should not be higher than 9% of the total water. This value is far below 27%, the fraction of the total water in the extracellular space predicted by the "two compartments in parallel" model. This discrepancy suggests that this model cannot be applied.

B. Two Fiber Compartments in Series: For applying the model of three compartments in series (where the three compartments are the cell water, extracellular water and the washing solution), it has to be assumed that the exchange of the slowest compartment representing the intracellular water washed out to the external solution through the extracellular compartment. In this case, the constants A and B in equation 5 do not represent the actual sizes of the intracellular and extracellular compartments, respectively, as in the preceding model, but rather can be calculated from the parameters in equation 5 by the relation derived by Huxley:[27]

$$P_{30} = \frac{AB(\lambda_A - \lambda_B)^2}{A\lambda_A^2 + B\lambda_B^2} \quad ;$$

$$P_T = P_{20} + P_{30}$$

where P_{20} and P_{30} represent the fractions of the total initial activity of ^{3}HHO, P_T, in the extracellular and intracellular compartments respectively; A and B the extrapolation at zero time of the fast and slow exponentials and λ_A and λ_B the rate constants. The average value of the water in the fast compartment predicted by this model is 46.2 ± 3.1% of the total water, increasing the discrepancy with the extracellular space directly measured.

From these analyses, it becomes apparent that the partition of the fiber water in two compartments, in parallel or in series, requires at least 20% of the water to be contained in a compartment directly connected to the extracellular space. This compartment also has to be impermeable to molecules such as sorbitol and sucrose. A similar problem arises when the Na exchange in frog muscle is examined. In this case, the fast Na exchange fractions exceed the Na that can be located in the extracellular space?' Keynes and Steinhardt[29] have suggested that this fast fraction would include the Na in the sarcoplasmic reticulum. Since the same concept could be valid for the "HHO kinetics described here, it is appropriate to consider this alternative in some detail. Although morphological studies have shown that only the T system, accounting for only 0.2 to 0.4% of the total cell volume, is accessible to extracellular probes[30–32] and no evidence for direct continuity between the sarcoplasmic reticulum and the extracellular space have been found, the fraction of other small ions, besides Na^+, will also exceed their extracellular contents. Recent experimental evidence [33,34] indicates that the quantities of fast exchanging Br^- and K^+ agree well with their extracellular contents. These results do not support the idea of a direct communication between the sarcoplasmic reticulum and the extracellular space. The fact that the extracellular space measured by the Cl^- distribution[35] gives similar results than those obtained by using larger molecules suggests that a large intracellular space connected to the external fluid is hardly probable in this muscle fiber.

II. Bulk Phase-Limited Diffusion

A. Simple Bulk Phase-Limited Diffusion: If the cell water is entirely homogeneous, and if the rate of exchange of ^{3}HHO is not limited by its permeability through the surface

membrane but by the diffusion of the water in the bulk of the muscle water, then the efflux of ^{3}HHO should follow the kinetics described by equation 3.

In this case, the apparent diffusion coefficient for ^{3}HHO (equation 3) can be related to λ_B in equation 5 by

$$\lambda_B = \frac{D' \nu_1^2}{r_0^2} \tag{7}$$

where D' is the apparent diffusion coefficient, $\nu_1 = 2.405$, and r_0 is the radius of the fiber.

Figure 8 shows one efflux experiment represented as an influx profile plotted against the square root of time. It can be observed that during the first minute the experimental points do not precisely follow the theoretical curve. Similar deviations were observed for all the experiments presented here. The direction of the departure indicates that during approximately the first minute the fraction of ^{3}HHO exchanged is less than the fraction predicted for simple diffusion from a cylinder. This deviation cannot be attributed to experimental error, since effluxes of ^{3}HHO from cylinders of agar gel follow precisely the theoretical curve after the first 20 seconds of washout (Fig. 4b). It follows that a simple bulk phase-limited diffusion mechanism is not the most appropriate description of the efflux of ^{3}HHO from muscle fibers.

B. Bulk Phase-Limited Diffusion with Adsorption: If it is assumed that the cell water is not homogeneous but it is in two different physical states (adsorbed and interstitial water) and that the ^{3}HHO exchange is not rate limited by the surface membrane, the diffusion into or out of a cylindrical cell is described by the differential equation:*"

$$\frac{\partial [C_i]_{ins}}{dt} = \frac{1}{r} \frac{\partial}{\partial r} \left(rD \frac{\partial [C_i]_{ins}}{\partial r} \right) - \frac{\partial [C_i]_{ad}}{\partial t} \tag{8}$$

$$\frac{\partial [C_i]_{ad}}{\partial t} = \lambda [C_i]_{ins} - \mu [C_i]_{ad} , \tag{9}$$

where $[C_i]_{ins}$ is the concentration of water in the interstitial fraction, $[C_i]_{ad}$ the water concentration of the adsorbed fraction, D' the diffusion coefficient of the water in the intersticium, and λ and μ the rate constants for adsorption and desorption, respectively. In steady state, from equation 9 it follows:

$$R = \frac{\lambda}{\mu} = \frac{[C_i]_{ad}}{[C_i]_{ins}} , \qquad (10)$$

where ***R*** represents the ratio of adsorbed water over interstitial water. The solution for equation 8 was obtained by Wilson[36] and by Crank.[20] The characteristics of these kinetics were discussed in detail by Crank,[20] and their physiological applicability and significance by Ling[37] and Ling et al.' Figure 9 shows four different efflux curves plotted as influxes against the square root of time. The continuous lines are theoretical and were obtained from tables (see methods). All the experimental curves are in reasonable agreement with the prediction of this model; the parameters are given in Table 6. Since the average value of ***R*** is 1.1, the model also requires approximately one half of the total water to be more strongly adsorbed. The significance of these observations will be considered in the discussion

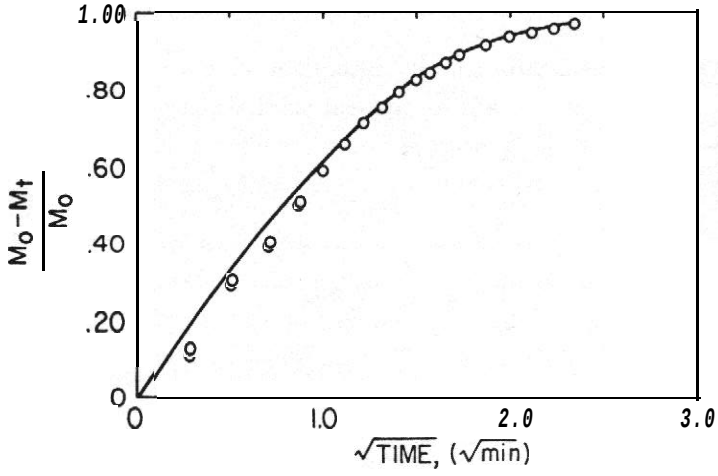

Figure 8. Efflux of ^3HHO from intact isolated muscle fibers. The efflux of ^3HHO from fiber h ('fable 4) is represented as an influx and plotted as a function of the square root of time. The theoretical curve for a bulk phase-limited diffusion process was calculated using a value of 0.36×10^{-5} cm^2/sec for the apparent diffusion coefficient and 0.045 cm for the fiber radius.

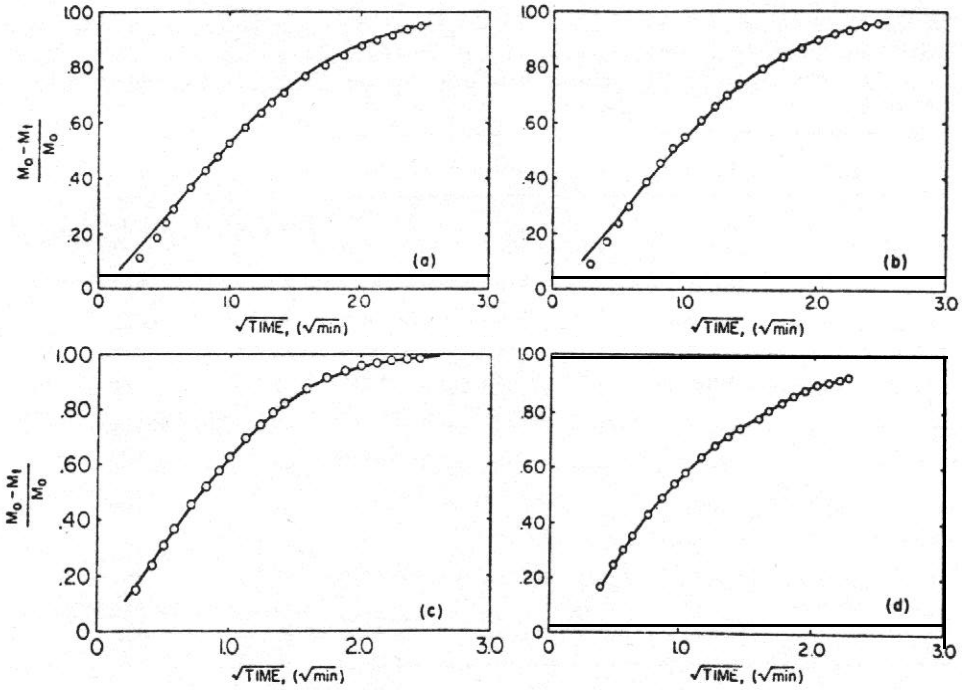

Figure 9. Effluxes of ^{3}HHO from intact isolated muscle fibers. The efflux data are represented as influxes as a function of the square root of the time of washout. Tbe continuous curves are theoretical for the bulk phase-limited diffusion and desorption (see text). The parameters of these curves are given in Table 6. Fig. (a): muscle d: Fig. (b): muscle e; Fig. (c): muscle f; and Fig. (d): muscle c.

DISCUSSION

The interpretation of the efflux experiment in terms of bulk-limited diffusion with concurrent adsorption-desorption is compatible with the idea that the cell water exists as polarized multilayers.[6,10–14] This model is supported by other experimental evidence.

Studies of nuclear magnetic resonance have consistently shown a broadening of the water signal in nerve' and in muscle.[2,3] This observed broadening of the water signal or decrease of relaxation times-which has been put on a more rigorous foundation from subsequent studies using spin echo techniques-has been interpreted as an indication that the cell water is more restricted in its rotational and translational motions than normal liquid water.[38–41] This, in turn, could be the result of the partial immobilization of the water molecules due to protein-water interactions.[2,3,11,13]

Table 6. Fitting parameters for the efflux curves of ^3HHO from intact isolated muscle fibers for a bulk phase-diffusion and desorption mechanism. The best theoretical curve for each efflux of ^3HHO was selected from tables (see Methods). Each of these theoretical curves corresponds to a specific value of R and of $\mu r^2/D'$.

Muscle	Radius	R	$\mu r^2/D'$	D'	μ
	cm			$cm^2/sec \times 10^5$	sec^{-1}
a)	0.075	1	10	1.18	0.021
b)	0.106	2	10	2.36	0.021
c)	0.060	1	10	1.15	0.031
d)	0.067	1	10	1.38	0.031
e)	0.075	1	10	1.85	0.033
f)	0.061	1	10	1.07	0.029
g)	0.05 1	1	10	1.05	0.040
h)	0.045	1	10	0.76	0.038
Average	0.068 ±0.007	1.125 ±0.125	10	1.35 ±0.18	0.031 ±0.002

Hansson Mild, James and Gillen[42] by using spin echo techniques have recently provided further evidence indicating that approximately 67% of the water in frog ovarian eggs is relatively more immobilized. These results give strong support to the hypothesis referred to above.' Furthermore, their measured self diffusion coefficient of frog ovarian egg, 6.8 $\times 10^{-6}$ cm^2/sec is actually lower than the average values Ling et al.reported(9.96 $\times 10^{-6}$ cm^2/sec), but close to the lower values 7.2 $\times 10^{-6}$ cm^2/sec. Since the value they obtained by NMR technique is independent of cell membrane barrier, this general agreement supports the conclusion of Ling et al.[7] that the cell membrane of frog ovarian egg poses no specific barrier to the diffusion of THO in and out of the cell. Indirectly, this agreement also strengthens the conclusion of the present paper.

Vapor equilibrium studies also support this model. Thus, Liig and Negendank[6] have shown that 95% of the water in frog sartorius muscles equilibrated at different vapor pressures follows the Bradley isotherm.[43] According to this model, the first layer of water strongly interacts with the protein adsorption sites;'" this layer in turn reacts with the next layer of water molecules; and so on. The energy of interactions among the different layers will decrease as the distance from the first layer increases. In this particular type

of adsorption the translational freedom of the water molecules in the cell will be more restricted than in free solution. Conversely, the water molecules located in layers far from the first one will have the highest mobility, although they also will be more restricted than in free solution. The apparent diffusion coefficient for ^{3}HHO obtained from the experimental efflux curves (1.35 x 10^{-5} cm^2/sec) is 55% of the diffusion coefficient of ^{3}HHO in free solution (2.44 x 10^{-5} cm^2/sec), reflecting primarily the diffusion properties of the more loosely adsorbed water molecules.

The value of the apparent diffusion coefficient of ^{3}HHO in the interstitial water of the barnacle muscle is in the same range as that reported by Ling et al.' for the diffusion coefficient for ^{3}HHO in frog eggs (from 0.72 to 1.47 x 10^{-5} cm^2/sec).

The apparent diffusion coefficient reported here is in keeping with that established by Rorschach, Chang, Hazlewood, and Nichols[44] using spin echo nuclear magnetic resonance techniques. These authors have found that in rat gastrocnemius muscles the diffusion coefficient of water is 42% lower than in free solution, suggesting that this lower value can be attributed to a more organized state of the cell water and not merely to the obstructive effect of the solid matter of the cytoplasm.

The water molecules that directly interact with the protein sites (first layer) and those immediately next to them suffer the greatest translational restriction. The diffusion in those layers of polarized water will tend to be slower than in water molecules farthest from the protein molecules.

The model of multilayer adsorption also offers an interpretation for the solubiiity properties of the cellular water. Hinke[24] has reported that 25-26% of the water of the muscle fiber of the giant barnacle is osmotically inactive. The remaining fraction of the cellular water was considered to have the same activity as that of the external Ringer solution. The separation of the cellular water into two completely different kinds of water, though it could be operationally useful, does not necessarily reflect the actual state of the water in these muscles. As it is, this model cannot account for the fact that the distribution of nonelectrolytes in frog muscle shows a continuous spectrum of steady state distribution values from 0.18 to near unity.[13,34]

Alternatively, if the model of multilayer adsorption for the state of water is considered, it would be expected that a gradation of the properties of the water would depend on the energy of interactions among the different molecules and hence, in the activity of the cell water. The distribution of the different nonelectrolytes will depend fundamentally on the relative energy and/or rotational freedom that a certain molecule will have in the multilayer water structure with respect to its freedom in the free solution (entropic exclusion"[“]). Consequently, in steady-state, the ratio of internal to external concentration of different nonelectrolytes can be distributed over a wide range, depending upon the properties of the particular nonelectrolyte in consideration and the physical state of the cellular water.

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