Is the Cell Membrane a Universal Rate-Limiting Barrier to the Movement of Water between the Living Cell and Its Surrounding Medium?

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ABSTRACT With the use of a newly introduced technique, the "influx profile analysis," we studied the diffusion of tritiated water in and out of frog ovarian eggs at 25°C. The results show that the rate-limiting step in the exchange of labeled water is not permeation through the cell membrane but diffusion in the bulk of the intracellular water.

Eighty-eight years ago Pfeffer postulated that the cell membrane is a *universal* barrier to the traffic of all solutes and water, i.e. all resistance to water movement lies in the cell membrane, and diffusion of water within the cytoplasm is instantaneous (1). Until recently studies of water movement into and out of living cells were interpreted on the basis of this postulate. In 1959, Dick pointed out that, in spite of the general acceptance of the assumption, there is no evidence in its favor (2, 3). Based on a correlation between the permeability coefficients of a large number of cell types and the surface/volume ratios of these cells, he concluded that cytoplasmic resistance to water movement is not insignificant but actually contributes to the "membrane permeability constants" reported in the literature. This finding reopens the basic question of whether the rate of water movement into and out of the living cells is determined by the cell membrane.

In this report we shall present the results of our study on the rate of permeation of tritium hydrogen oxide (THO)-labeled water into frog ovarian eggs. This study was made in the hope of obtaining a definitive answer to the above question. For this purpose we made use of a new method called the "influx profile analysis" which has been briefly introduced elsewhere by the senior author of this paper (4).

THEORY

The following is a brief examination of the theoretical basis of the influx profile analysis. The method permits determination of the rate-limiting step in the diffusion of substances in and out of a complex system such as a living cell.

I. MEMBRANE–LIMITED DIFFUSION

A. *Influx* According to the membrane theory, when a cell is introduced into a relatively large volume of solution containing a labeled substance, the ith, the rate of increase of its concentration inside the cell, is

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

where $[p_i]_{in}, [p_i]_{ex}$ are the concentrations of the ith substance in the intracellular water and the extracellular water respectively. k^i_{iw} and k^i_{ow} are defined as follows:

$$k^i_{iw} = \kappa^i_{iw}\frac{A_{cell}}{V_{cell}}, \tag{2}$$

$$k^i_{ow} = \kappa^i_{ow}\frac{A_{cell}}{V_{cell}}, \tag{3}$$

where A_{cell} and V_{cell} are the surface and volume of the cell. κ^i_{iw} and κ^i_{ow} are the inward and outward permeability constants of the cell membrane for the ith substance. Since $[p_i]^{t=0}_{in} = 0$, the solution of Equation 1 is

$$[p_i]^t_{in} = \frac{k^i_{iw}}{k^i_{ow}}[p_i]_{ex}[1 - \exp(-k^i_{ow}t)], \tag{4}$$

where $[p_i]^t_{in}$ is the intracellular concentration of the ith substance at time, t. When $t = \infty$,

$$[p_i]^\infty_{in} = \frac{k^i_{iw}}{k^i_{ow}}[p_i]_{ex}. \tag{5}$$

Combining Equations 4 and 5

$$\frac{[p_i]^t_{in}}{[p_i]^\infty_{in}} = [1 - \exp(-k^i_{ow}t)]. \tag{6}$$

Fig. 1 A (surface-limited curve) shows a plot of $[p_i]^i_{in}/[p_i]^\infty_{in}$ (as $\frac{M_t}{M_\infty}$, see legend) against $\sqrt{t}$, calculated from Equation 6. The curve is sigmoid. This

type of profile is shared by all single-compartment surface (membrane)–limited systems of transport, whatever the rate constants may be.

B. *Efflux* Consider the case when a cell previously equilibrated with labeled ith substance is washed in a very large volume of solution containing no labeled ith substance. The rate of decrease of labeled ith substance in the cell is

$$-\frac{d[p_i]_{in}}{dt} = k^i_{ow}[p_i]_{in}. \tag{7}$$

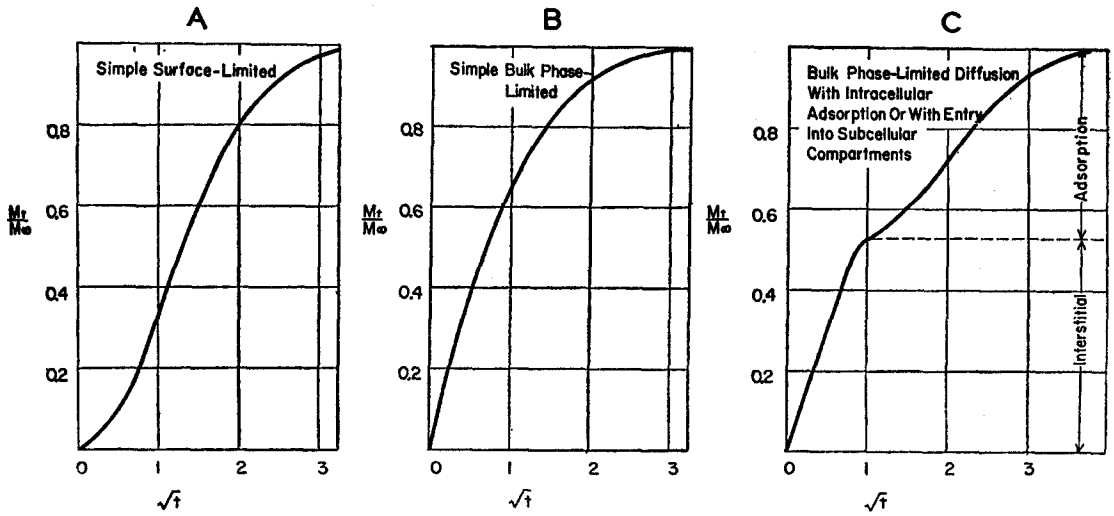

FIGURE 1. The time course of influx of a labeled substance into various systems with rate-limiting steps as indicated on each chart. The "influx profiles" are theoretically calculated. The ordinate represents the uptake, M_t, of the labeled material at time t as a fraction of the total amount of the material in the system (M_∞). The abscissa represents the square root of t.

The solution of Equation 7 is

$$\ln\left(\frac{[p_i]^t_{in}}{[p_i]^0_{in}}\right) = -k^i_{ow}t, \tag{8}$$

where $[p_i]^0_{in}$ is the initial concentration of ith substance. This predicts that a plot of log $([p_i]^t_{in}/[p_i]^0_{in})$ against t should yield a straight line (Fig. 2 A). It is possible to convert this efflux curve to an influx curve as follows: We first write Equation 8 in exponential form. Multiplying by -1 and adding 1 to both sides, we have

$$1 - \frac{[p_i]^t_{in}}{[p_i]^0_{in}} = 1 - \exp(-k^i_{ow}t). \tag{9}$$

Thus subtracting the value of log $([p_i]^t_{in}/[p_i]^0_{in})$, obtained in an *efflux study*, from unity, one obtains the value for the fractional uptake in an *influx study* of the same cell (Equation 6), t seconds after the introduction of the cell into a large body of the labeled medium. This simple *inversion method* is of considerable importance because it provides two ways of analyzing the same data, each offering a different set of discriminatory criteria (see below).

II. SIMPLE BULK PHASE–LIMITED DIFFUSION

When the cell membrane does not act as a rate-limiting barrier to the traffic of the ith substance, its rate of entry may be determined by its rate of diffusion in the bulk phase cytoplasmic water (and in certain cases at least, also by its rate of adsorption and desorption on certain sites).

For simple bulk phase–limited diffusion of the ith substance in the intracellular water of a spherical cell,

$$\frac{\partial[p_i]_{in}}{\partial t} = D_i\left(\frac{\partial^2[p_i]_{in}}{\partial r^2} + \frac{2}{r}\frac{\partial[p_i]_{in}}{\partial r}\right) \tag{10}$$

where r is the radius of the sphere; D_i, the diffusion coefficient of the ith substance in the cytoplasm. Setting

$$\mu_i = [p_i]_{in}r, \tag{11}$$

Equation 10 becomes

$$\frac{\partial\mu_i}{\partial t} = D_i\frac{\partial^2\mu_i}{\partial r^2} \tag{12}$$

The same equations govern both efflux and influx, with of course, different boundary conditions.

A. *Influx* The initial phase of uptake of the ith substance by a cell from a stirred solution of large volume plotted against $\sqrt{t}$ is approximately linear over a relatively large range as illustrated in Fig. 1 B.

B. *Efflux* For a cell previously equilibrated in a labeled solution and washed in a constantly changing solution containing no isotope, the equation for the labeled substance remaining at time t was given by Dünwald and Wagner (5). When t is sufficiently large,

$$\log\left(\frac{[p_i]^t_{in}}{[p_i]^0_{in}}\right) = \text{constant} - \frac{4.2865\,D_i\,t}{r^2} \tag{13}$$

Again one can obtain the fractional uptake, $[p_i]^t_{in}/[p_i]^\infty_{in}$ at time t by subtracting from unity $[p_i]^t_{in}/[p_i]^0_{in}$ from an efflux study, as described in the immediately preceding section.

III. BULK PHASE–LIMITED DIFFUSION WITH INTRACELLULAR ADSORPTION

In this case diffusion into a spherical cell follows an equation analogous to Equation 12

$$\frac{\partial[p_i]_{ins}}{\partial t} = D_i\left(\frac{\partial^2[p_i]_{ins}}{\partial r^2} + \frac{2}{r}\frac{\partial[p_i]_{ins}}{\partial r}\right) - \frac{\partial[p_i]_{ad}}{\partial t} \qquad (14)$$

$[p_i]_{ins}$ is the ith substance concentration in the cell water (interstitial), and

$$\frac{\partial[p_i]_{ad}}{\partial t} = \lambda_i\,[p_i]_{ins} - \mu_i\,[p_i]_{ad}\,, \qquad (15)$$

where $[p_i]_{ad}$ is the concentration of adsorbed ith substance; λ_i and μ_i are the rate constants for the adsorption and desorption processes. The solution for this case was obtained by Wilson (6) and by Crank (7). When the adsorption is very rapid, $[p_i]_{ad}$ is in constant equilibrium with $[p_i]_{ins}$. In this case the diffusion process is indistinguishable from simple bulk phase–limited diffusion. On the other hand if diffusion is very much faster than adsorption, the concentration of $[p_i]_{ins}$ is uniform throughout the cell; the rate of the whole process is then governed by the adsorption. When neither diffusion nor adsorption is much faster than the other, various influx profiles may be obtained. When $\frac{D_i}{r^2}$ is much larger than λ_i, but $\frac{\lambda_i}{\mu_i}$ is not much larger than 1, the diffusion profile consists of an essentially linear or slightly convex (upward) initial portion which starts to level off, forming a more or less distinct "shoulder" (Fig. 1 C). The position of the shoulder corresponds approximately to the time at which the diffusion process through the cytoplasmic water has reached equilibrium. The portion of the curve beyond the shoulder largely represents adsorption; the entire curve levels off asymptotically to a value of M_t/M_∞ corresponding to $\left(1 + \frac{\lambda_i}{\mu_i}\right)$. When $\frac{\lambda_i}{\mu_i} \gg 1$, the total concentration of the interstitial ith substance, $[p_i]_{ins}$, becomes insignificant. Since adsorption follows an equation similar to that for membrane-limited diffusion, the influx profile may not be distinguishable from that shown in Fig. 1 A.

Again in this case the influx profile of the ith substance can be deduced from the efflux curve by the inversion procedure described above.

MATERIALS AND METHODS

Single eggs or small clusters of mature ovarian eggs from leopard frogs (*Rana pipiens* Schreber) were used in all experiments. The bulk of the connective tissue attached to the eggs was removed leaving just enough to permit handling. After gentle blotting

on wetted filter paper, the eggs were incubated in a small Erlenmeyer flask of Ringer's-phosphate solution (for composition, see reference 7, Appendix H) containing tritium hydrogen oxide (THO) (New England Nuclear Corp., Boston, Mass., Lots No. 81-100-20 and No. 81-100A-31). The concentration of radioactivity varied from 0.04 to 0.5 mc/ml. The flasks were shaken in an Aminco constant temperature bath at the desired temperature ± 0.05°C, with a shaking rate of 120 oscillations per min, each excursion measuring 0.5 inch. After incubation, a single egg or a small cluster of eggs was picked up by its remaining bit of connective tissue with the aid of fine Dumont forceps and placed briefly on filter paper to allow drainage of adherent radioactive Ringer's solution.

Brief exposure of small egg clusters to ^{131}I-labeled human serum albumin (RISA), followed by rinsing, shows that the upper limit of the volume of external radioactive solution on the blotted eggs is 1.3% (Table I). A film of this volume has an average

TABLE I

VOLUME OF EXTERNAL RADIOACTIVE SOLUTION ADHERING TO EGGS

Small clusters of eggs were soaked in Ringer's solution tagged with RISA. Clusters were removed, blotted on wetted filter paper, and assayed for RISA. Eggs were then washed for 5 min in untagged Ringer's solution and RISA again determined.

	A	B	C	D	E	F	G
Sample No.	Weight	Incubation time	Counts, external solution	Cell counts, before washing	Cell counts, after washing	ΔCounts (D-E)	$\left(\frac{F}{A \cdot C}\right)$
	mg	*min*					%
1	41.3	3	1.12×10^7	6481	817	5664	1.22
2	24.2	3	1.12×10^7	4445	1103	3342	1.23
3	20.0	1	1.12×10^7	2828	488	2340	1.04
4	20.3	0.5	1.12×10^7	2704	409	2295	1.01

thickness of 1×10^{-3} cm. It takes no more than 0.02 sec for 99% of the THO-labeled water in such a film to be removed by diffusion. In the series of experiments plotted in Fig. 5, the adherent fluid was removed by rinsing for 7 sec in a large body of cold Ringer's solution (0°C) before assay for THO content. No preliminary rinsing was done in other studies. Here the egg or egg cluster, after equilibration in labeled Ringer's solution and drainage, was rinsed in twenty or more successive 2 ml portions of nonlabeled Ringer's-phosphate solution for a total time of 2 or 3 hr. The washing solutions were shaken in a constant temperature bath (± 0.05°C) at the same rate and amplitude as in the preceding incubation procedure. For transfer from one solution to the next, the egg was either grasped by its connective tissue tag with fine forceps or handled by a piece of surgical thread tied to the connective tissue tag shortly after equilibration in the labeled solution. To prevent adherent fluid from being carried over to the next solution, the egg was dragged gently along the inner wall of the vial for a few seconds before transference. A final washing lasted 18 hr. After this the eggs were weighed and their diameter measured with the aid of an ocular micrometer. The small residual amount of THO in the egg was then extracted by

boiling in 2 ml of distilled water for 20 min. Aliquots (0.5 ml) of the washing solutions, the boiling water extract, and diluted samples of the incubating THO-Ringer solution (before and after egg incubation), were added to 10 ml of Bray's scintillation fluid (9). The THO contents were assayed on a Packard Model 314E liquid β-scintillation counter.

All theoretical curves following were computed on an IBM-7040 digital computer.

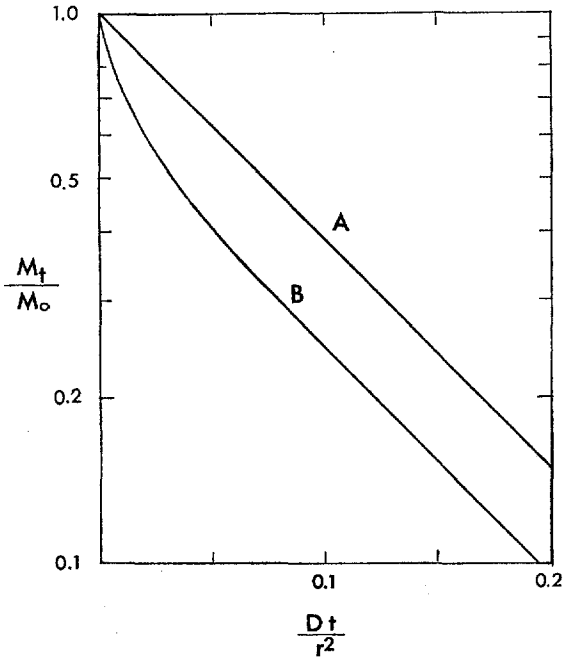

FIGURE 2. The time course of efflux of simple surface-limited diffusion (*A*) and simple bulk phase–limited diffusion (*B*) from a sphere. The ordinate represents the amount of labeled material remaining in the system at time t as a fraction of the total amount (M_0). The abscissa represents Dt/r^2 where D, the diffusion constant and r, the radius are constants.

Method of Data Analysis

We have found that an accurate efflux time course can be obtained from a single frog egg or a small cluster of eggs. Plotted as shown in Fig. 2, such data may indicate whether the process is membrane-limited or bulk phase–limited (4): If it is bulk phase–limited, the initial portion of the semilogarithmic plot is not linear. However, the departure from linearity may not be pronounced and thus it may be difficult to draw a definite conclusion from such a plot. On the other hand, the difference between membrane-limited and bulk phase–limited diffusion is prominently displayed by a plot of the influx against $\sqrt{t}$. However, direct influx studies usually do not yield accurate enough data because different cells must be used for each point on the curve. It is for this reason that the *inversion technique* mentioned above is of importance. With

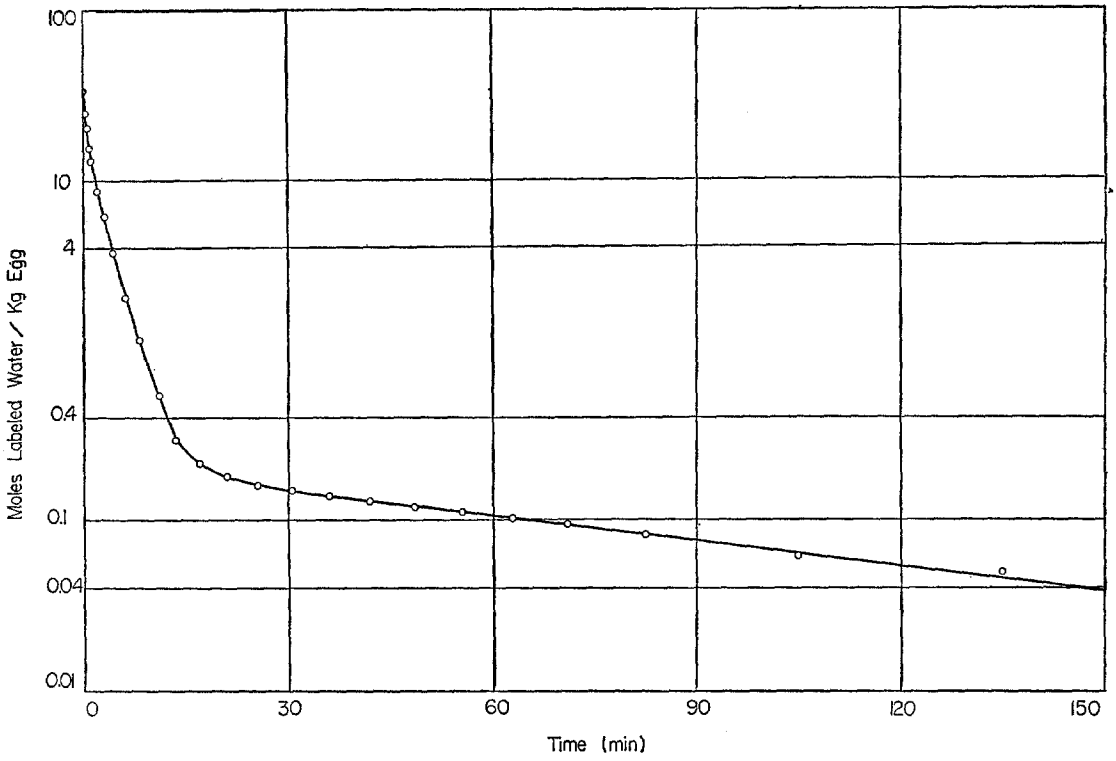

FIGURE 3. Efflux of THO-labeled water from egg cluster. Frog eggs were soaked in THO-labeled Ringer's-phosphate solution at 25°C. for 4 hr. THO activity in the incubation medium was 0.24 mc/cc. A cluster (five eggs) was washed successively in 2 ml portions of nonlabeled Ringer's-phosphate. Average diameter of eggs was 1.97 mm. (See Table II, No. G.)

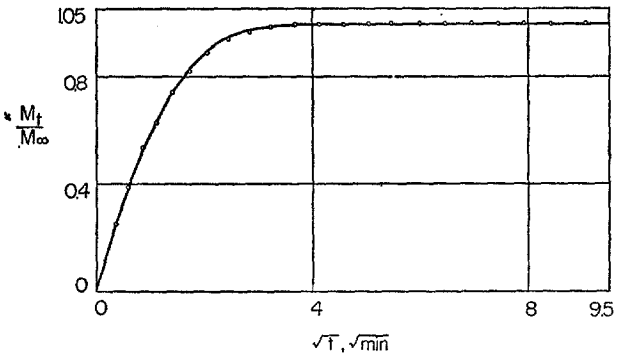

FIGURE 4. Influx profile of THO-labeled water into egg clusters. Data from Fig. 3 converted into an influx time course using the "inversion method" given in the text. The curve going through the points is theoretically computed on the basis of simple bulk phase–limited diffusion.

this technique the accuracy of the single egg study can be combined with the great discriminatory power of the influx profile analysis. This then is the method of analysis we finally adopted for the present series of experimental studies.

RESULTS

Establishment of the Inversion Technique

Fig. 3 shows the time course of efflux of THO-labeled water from a small cluster of eggs. The over-all shape of the curve is not the simple straight line predicted on the basis of simple membrane-limited diffusion. There is curvature in both the initial and the terminal portions of the curve.[1] Using the

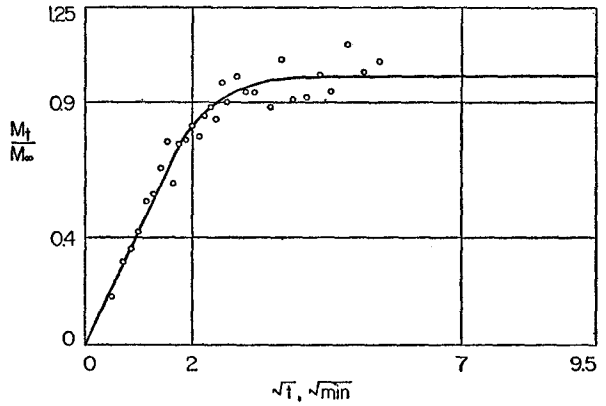

FIGURE 5. Influx profile of THO-labeled water into frog eggs. Clusters of five to seven eggs soaked in THO-labeled Ringer's-phosphate solution at 25°C. THO activity in incubation medium was 0.5 mc/cc. Egg clusters were removed at intervals and rinsed for 7 sec in a large body of cold, circulating, Ringer's solution (0°C). THO recovered by washing out in three 2 cc volumes of Ringer's solution followed by boiling water extraction for residual THO in eggs.

"inversion technique," the efflux data given in Fig. 3 can be converted into an influx time course as shown in Fig. 4. Fig. 5, on the other hand, shows an influx time course obtained by determining uptake directly: one egg cluster at a time was removed from a large number of clusters in a large body of labeled solution and the radioactivity assayed. Comparing the general profiles of Figs. 4 and 5 one finds that, except for the much greater scattering of data in Fig. 5, the two methods are in agreement.

Influx Profiles

Two types of influx profiles were observed. In one, illustrated by Fig. 4, the points could be fitted with a simple bulk phase–limited diffusion curve. The

[1] It is interesting to note that Prescott and Zeuthen's data show an initial point or points departing from the linear curve (10).

solid curve through the points has been theoretically computed on the basis of the solution of Equation 10 for simple bulk phase–limited diffusion. Two cases of this type were observed (see below). In the other type, in addition to the bulk phase–limited diffusion from the bulk of the cell water, a second more slowly exchanged fraction amounting to between 0.5 and 25% of the total cell water was observed. This type is illustrated in Fig. 6. The broken line is a theoretical simple bulk phase–limited diffusion curve which, however, only fits part of the experimental points. The solid line is a theoretical curve calculated according to Equations 14 and 15 for bulk phase–limited diffusion with

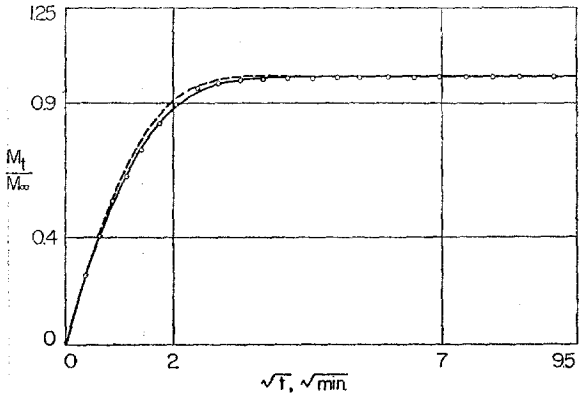

FIGURE 6. Influx profile of THO-labeled water into frog eggs. Experimental details the same as in Fig. 3, THO activity in incubation medium was 0.04 mc/cc. Influx time course obtained by the inversion method. The broken line curve shows a theoretical plot for simple bulk phase–limited diffusion. The solid line is a theoretical curve calculated according to Equations 14 and 15 for bulk phase–limited diffusion with intracellular adsorption. (See Table II, No. H.)

adsorption or entry into subcellular compartments; this curve gives a better fit.

Fig. 7 is a composite showing six additional studies. We could find no significant difference between experiments using single eggs (*D*, *E*, and *F*) and those using small clusters of eggs (*A*, *B*, and *C*), nor did the dosage of radioactivity make any difference. Without exception, all curves show influx profiles of bulk phase–limited diffusion with and without adsorption.

Table II gives the pertinent data derived from these studies. The diffusion coefficient of THO in the cell water at 25°C ranges from 0.721×10^{-5} to 1.47×10^{-5} cm²/sec in comparison with the diffusion coefficient of THO in normal water at the same temperature, 2.44×10^{-5} cm²/sec (11). The experiments with the higher diffusion coefficients as a rule depart farther from simple bulk phase–limited diffusion; they have a larger slowly exchanging fraction (*R*, in Table II).

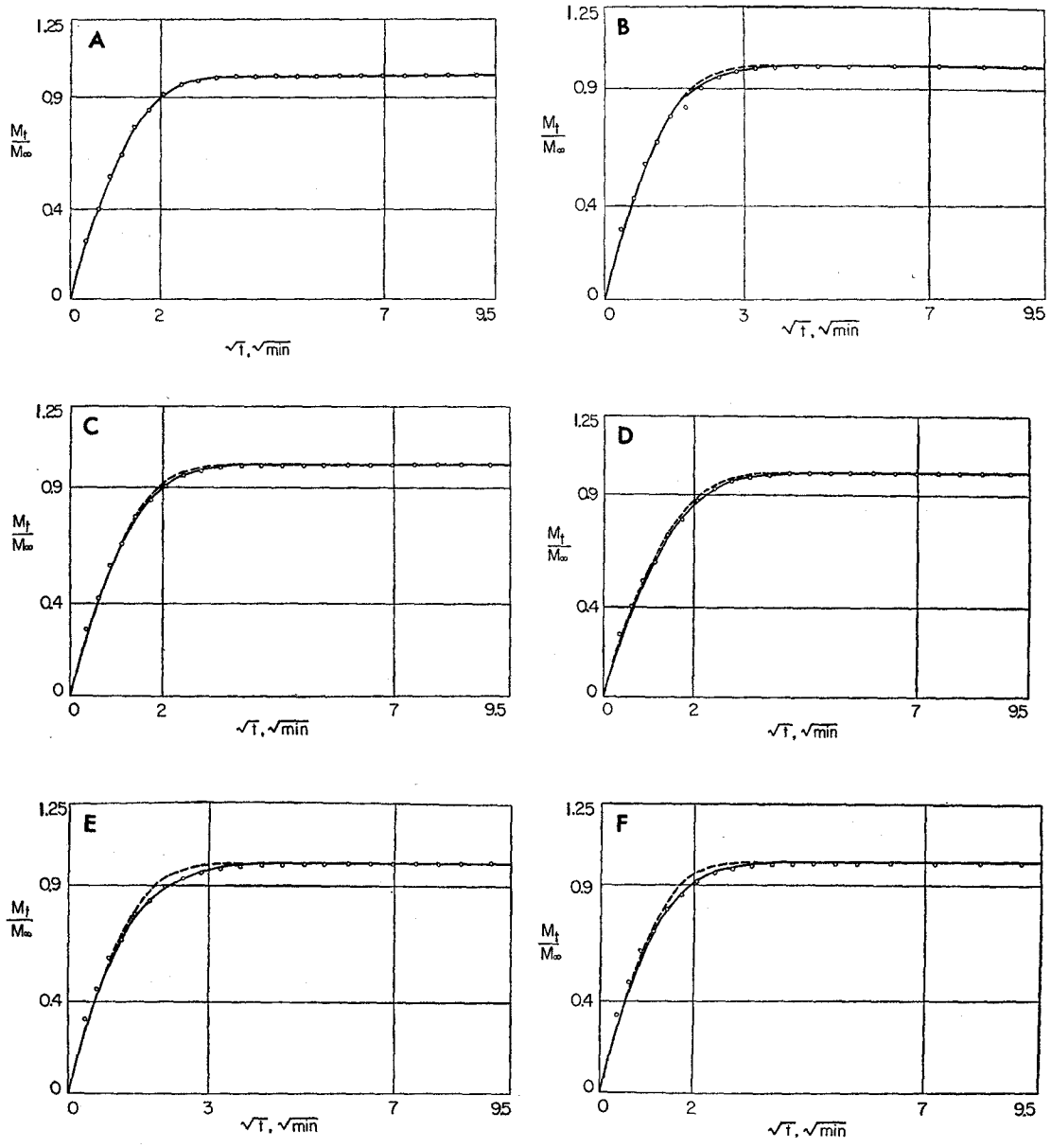

FIGURE 7. Influx profiles showing simple bulk phase–limited diffusion and bulk phase–limited diffusion with intracellular adsorption. Influx time course obtained by the inversion method. The solid curve in *A* and the broken line curves in figures *B* thru *F* show a theoretical plot for simple bulk phase diffusion. All other lines are theoretical curves for bulk phase–limited diffusion with intracellular adsorption.

DISCUSSION

The rapidity of water exchange in living cells is one of the major obstacles to direct study of water transport by living cells. Thus, even after Dick's discovery that the rate of water diffusion in the cytoplasm plays a significant role in determining the rate of water transport, he was careful to include alternate interpretations for his experimental data: one set of values, calculated on the basis of surface-limited diffusion, is given as permeability constants; another set, calculated on the basis of bulk phase–limited diffusion, is given as diffusion constants (see also Løvtrup, 20).

In the present studies the large size of amphibian eggs and the recently developed diagnostic techniques of the influx profile analysis and the inversion method have provided us with means to reach more unequivocal conclusions about the rate-limiting step of water diffusion in ovarian eggs of leopard frogs.

TABLE II

DIFFUSION COEFFICIENTS AND OTHER EXPERIMENTAL DATA

Experiment No.	Date	No. of eggs	r^2	R^*	D	$\frac{\mu r^2}{D}$
			cm^2		cm^2/sec	
A	5-13-66	8	9.22×10^{-3}	0.0049	7.21×10^{-6}	0.19
B	4-29-66	5	9.65×10^{-3}	0.144	11.82×10^{-6}	4.0
C	4-29-66	6	9.80×10^{-3}	0.0818	9.66×10^{-6}	5.23
D	4-29-66	1	9.65×10^{-3}	0.25	14.68×10^{-6}	3.0
E	4-29-66	1	9.70×10^{-3}	0.0818	7.60×10^{-6}	5.23
F	4-29-66	1	9.70×10^{-3}	0.144	13.28×10^{-6}	4.0
G	4-29-66	5	9.70×10^{-3}	0.006	7.27×10^{-6}	0.27
H	5-13-66	9	9.22×10^{-3}	0.0818	8.13×10^{-6}	5.23

* Fraction of intracellular adsorbed (or subcellular) THO-labeled water. It is equal to λ_i/μ_i.

The demonstration that the diffusion of THO-labeled water is bulk phase–limited in all or at least in the greater part of ovarian egg cytoplasm shows that the cell membrane is not a *universal* rate-limiting barrier to the traffic of all solutes and solvents between the cell and its surrounding medium. The cell can therefore no longer be considered to be surrounded by a *continuous* water-insoluble lipoid membrane; more likely, there are aqueous channels of molecular dimension in the cell surface (for a similar conclusion, see references 12-16). That the bulk of the cell water has a *uniform* diffusion coefficient for THO-labeled water different from that in normal water bespeaks a uniformity in the over-all physical properties of water throughout the bulk of the egg cell. This supports our earlier conclusion that the entire cell, including the cell membrane, consists of a heterogeneous protein-aqueous fixed charge system (4, 8, 16-18). In such a system aqueous channels of molecular dimension are not limited to the cell membrane but permeate the entire cell.

The present findings are in harmony with other experimental findings (4; 7, chapter 11; 16, 19) which show that diffusion of many nonelectrolytes and ions also follows a pattern of simple bulk phase–limited diffusion or bulk phase–limited diffusion with adsorption.

The present findings also agree with the conclusion reached from entirely different lines of evidence that the intracellular water exists in polarized multilayers (17, 18). This conclusion demands that the diffusion coefficient of THO be slower within the cell than in normal water. The water molecules most highly polarized and oriented by the cell proteins (and other macromolecules) will exchange with THO less rapidly as the appearance of a second fraction in most of the data of Fig. 7 suggests.

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