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Water Permeability and Its Activation Energy of Fertilized and Unfertilized Mouse Ova

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Summary. A photomicroscopic method has been used to determine the kinetics of water loss at various constant temperatures from fertilized and unfertilized mouse ova. Ova were transferred into hypertonic saline solutions, photographed, and their volumes calculated from their cross-sectional areas as a function of time after transfer. Curves describing the observed water loss have been compared to those calculated using a programmed version of the classical water permeability equation. The hydraulic conductivity, L_p, was determined by changing its value in the calculation until the majority of the observed cell volumes fell on or very close to the calculated curve of volume vs. time. In this way, fertilized and unfertilized ova were found to have respective hydraulic conductivities of 0.43 and 0.44 $\mu m^3/\mu m^2$-min-atm at 20 °C and respective activation energies for water permeability of 13.0 and 14.5 kcal/mol.

Water permeability is a fundamental property of living cells. Its specific coefficient, now commonly referred to as the hydraulic conductivity, L_p, is characteristic of a given cell type when exposed to nonisotonic solutions producing osmotic water flux. The measurement of cell volume changes, analysis of the data, and interpretation of the results have become a highly specialized branch of cell physiology (*see* reviews by Stein, 1967; Dick, 1971; House, 1974). The majority of both the older and the more recent studies have examined the movement of water across the cell membranes of lower organisms [e.g., sea urchin eggs (Lucké & McCutcheon, 1932), amphibian eggs (Tuft, 1965)], and mammalian erythrocytes (*see* review by Forster, 1971). Although there have been some studies using other types of mammalian cells, such as tumor cells (Hempling, 1960; DuPre & Hempling, 1978) and lymphocytes (Hempling, 1973, 1977), the water permeability of mammalian ova and embryos has not been measured previously.

The purpose of the present study was to determine the water permeability and its temperature coefficient for fertilized and unfertilized mouse ova. This study was initiated as part of a continuing investigation of the response of mouse ova and embryos to freezing and thawing (*see* Leibo, 1977*b*, for review). Some years ago, it had been theorized that three characteristics of a cell that primarily determine its freezing sensitivity are (1) the hydraulic conductivity, (2) its activation energy, and (3) the surface area-to-volume ratio (Mazur, 1963). It has now been clearly established that this is so (*see* Mazur, 1977, for review). However, to test the theoretical predictions rigorously for a given cell type requires knowledge of these three cellular characteristics. This study was conducted to acquire that knowledge for mouse ova.

The approach used is analogous to that of McCutcheon and Lucké (1932) in which they measured the diameters of *Arbacia* eggs bathed by a hypertonic solution of a nonpermeating solute. Just as with the ova of invertebrates and amphibians, mouse ova lend themselves well to such an approach for two reasons. First, their approximately spherical shape permits simple calculation of their volume from their cross-sectional area. Secondly, their large size ($\sim 75\,\mu m$ diameter) results in water loss at a sufficiently low rate for their cross-sectional areas to be determined rather easily as a function of time. In contrast, human erythrocytes having a diameter of $\sim 7.5\,\mu m$ and a biconcave discoid shape lose water at such a high rate that elaborate apparatus is required to obtain meaningful data. In this study, ova were placed into a hypertonic solution of NaCl at several constant temperatures and then photographed at various times. There is one significantly different feature of the approach used. The density of ova in isotonic solutions is less than that of a hypertonic saline solution. Utilizing that fact, I transferred ova from an isotonic solution into the bottom of a capillary mounted in a vertical position that contained a hypertonic solution. The ova rose in the capillary, like a

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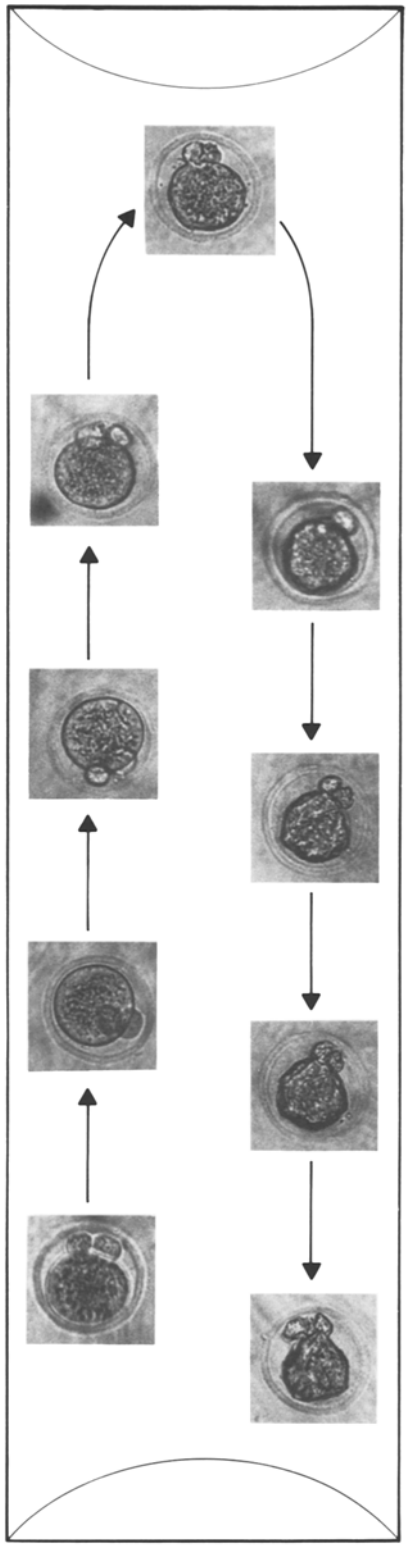

Fig. 1. Diagrammatic representation of the method used to observe the water loss from mouse ova. The ova were inserted into the open bottom end of a capillary containing hypertonic NaCl solution. The capillary was mounted in the vertical position. The ova, initially less dense than the suspending solution, gradually rose in the capillary. When the ova lost sufficient H_2O to reach a density equal to that of the solution, they sank to the bottom of the capillary. Photographs of the ova were made at precisely measured times as they ascended and descended

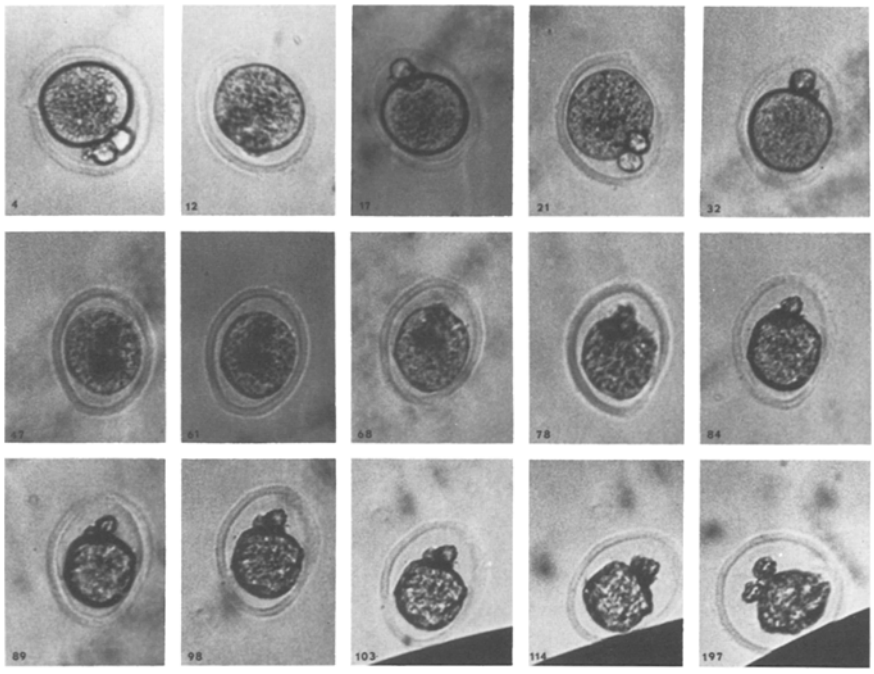

Fig. 2. A sequence of photographs of a fertilized ovum as it rose and sank in 0.5 M NaCl, as described in Fig. 1. The figures refer to time (sec) at which each photograph was made

small cork, until they lost sufficient water to reach density equilibrium with the bathing solution, at which point they gradually sank in the capillary. The ascending and descending ova were photographed at precisely measured times, and their volumes calculated from cross-sectional areas. The approach is illustrated diagrammatically in Fig. 1, and an actual timed sequence of photographs of a fertilized ovum is shown in Fig. 2.

Ova volumes were plotted as a function of time at several constant temperatures, and the plotted curves were fitted to the water loss calculated using the classical permeability equation. The hydraulic conductivity at 20 °C of both fertilized and unfertilized mouse ova is ~ 0.4 $\mu m^3/\mu m^2$-min-atm; their respective activation energies for water permeability are 13.0 and 14.5 kcal/mol.

Materials and Methods

Fertilized and Unfertilized Ova

Mouse ova were collected from superovulated B6D2F_1 female mice approximately 18 hr after the injection of human chorionic gonadotropin. Fertilized ova were collected from females mated with males of the same strain. The methods of superovulation, ova collection, and the assay of *in vitro* survival are standard pro-

Table 1. Variables, parameters, and constants for permeability equation

Symbol	Definition	Units	Values
L_p	Hydraulic conductivity	$\mu m^3/\mu m^2$-min-atm	Variable
V_1[a]	Isotonic cell water volume	μm^3	1.88×10^5
V_e[b]	Equilibrium cell water volume	μm^3	5.81×10^4
V_2	Cell water volume at time t	μm^3	Variable
t	Time	Minutes	Variable
A[c]	Cell Surface area	μm^2	1.82×10^4
R	Gas constant	μm^3-atm/mol/degree	8.205×10^{13}
T	Temperature	°Kelvin	273.15 + variable
N[d]	Osmoles solute in isotonic cell	Osmoles	5.38×10^{-11}
V_c[e]	Isotonic cell volume	μm^3	2.30×10^5
b[f]	Volume of cell solids	μm^3	4.18×10^4

[a] $V_1 = V_c - b$.

[b] Equilibrium cell water volume, V_e, is read from Fig. 3, relative cell volume at 0.5 M NaCl or 0.93 osmole. It was taken as $0.435\,V_c - b$.

[c] Surface area of ovum, A, is that of a sphere with a radius of 38 μm.

[d] Osmoles of solute, N, is 0.286 osmole $\times V_1 \times 10^{-15}$, where V_1 is given in μm^3.

[e] Volume of ovum, V_c, in isotonic solution is that of a sphere with a radius of 38 μm.

[f] Volume of cell solids, b, is read from Fig. 3, relative cell volume at infinite concentration, or $0.182\,V_c$.

cedures that have been used in this laboratory for several years (Whittingham, Leibo & Mazur, 1972; Leibo, Mazur & Jackowski, 1974; Leibo & Mazur, 1978).

Osmometric Behavior of Ova

An important corollary to any study of permeability is to determine the equilibrium osmometric behavior of the cell. This was done by transferring five groups of about 12 to 14 fertilized ova [from which the cumulus cells had been removed with hyaluronidase (Biggers, Whitten & Whittingham, 1971)] into 0.286 osmolal phosphate-buffered saline (PBS) contained in the wells of Microtest plates (Falcon No. 3034) covered with silicone oil (Dow Corning 200 fluid, 50 cs. viscosity) to prevent evaporation. The ova were photographed at 250× magnification on a Wild M-40 inverted microscope. They were then recovered from the PBS, washed twice in 2-ml volumes of five different concentrations of NaCl solutions without buffer, and then transferred into wells of Microtest plates containing the same NaCl solutions. The ova were held in the NaCl solutions for 60 min at room temperature before being photographed again. After all the groups of ova had been photographed, they were recovered from the wells, washed once in 2-ml volumes of PBS, and then cultured in SOF medium (Tervit, Whittingham & Rowson, 1974) at 37 °C under 95% air: 5% CO_2. The number of ova (×100) that developed into 2-cell embryos in culture divided by the total number of ova recovered from each NaCl solution was calculated as the percentage survival. The entire procedure was repeated once.

The negatives of 6 to 8 ova, first in PBS and then in each NaCl solution, were projected on a photographic enlarger at a total magnification of 1000×. That is, the projected images of ova in PBS had a diameter of ~75 mm. The images were traced, and their cross-sectional areas measured with a planimeter. Their volumes were calculated, assuming the cross-sectional area to be that of a sphere, using the relationship:

$$V = (4\pi/3)(C/\pi)^{3/2} \quad (1)$$

where V is volume and C is the cross-sectional area. The mean volume, in unitless dimensions, of each group of ova in a given NaCl solution was expressed as a percentage of the mean volume of that same group of ova in isotonic PBS, which was taken to be 100%. This method is similar to that used recently to determine the permeability coefficient of mouse ova to glycerol (Jackowski, Leibo & Mazur, 1979).

An analogous series of experiments was performed by Dr. W.F. Rall, who has kindly made his results available to me. In his experiments, Rall suspended fertilized mouse ova in various concentrations of buffered sucrose solutions and calculated their volumes in the same manner described above.

Permeability Measurements

The water loss of both fertilized and unfertilized ova was determined by photomicroscopy. After ova had been collected, and the cumulus cells had been removed by hyaluronidase, the ova were held at 0 °C until used. In most experiments, ova collected from two mice were pooled and used within 1 to 2 hr after collection. Each measurement was made on a single fertilized or unfertilized ovum.

The apparatus used consisted of a compound microscope mounted in a horizontal position on an optical bench. The sample chamber was a rectangular glass capillary tube, open at both ends, with optically flat viewing surfaces and an internal viewing path length of 100 μm (Microslide, Vitro Dynamics, Inc., Rockaway, N.J.). The outside dimensions of those capillaries used in this study for mouse ova were 0.3 mm deep, 1.2 mm wide, and 25 mm long. A fresh Microslide was used for each determination. PBS or a test NaCl solution was held in the Microslide by capillarity. In each experiment, a Microslide was mounted in a vertical position on the temperature-controlled plate of a thermoelectric microscope stage (Bailey Instruments, Inc., Saddle Brook, N.J.). This stage was in turn mounted on the mechanical stage of the horizontal microscope. Sample temperatures were measured with a 40-gauge copper-constantan thermocouple inserted into the Microslide with a micromanipulator. Temperature and time were recorded on an expandable scale oscillographic recorder (Sanborn Model 7700 Series). Observations were made at temperatures between 0 and 30 °C. Photographs of the ova at 100× magnification, using a long working-distance objective, were made using a motor-drive Nikon F2 camera. The shutter switch of the camera was connected to the recorder operating at a constant, calibrated speed. Therefore, during each determination, the sample temperature and the time at which each photograph was exposed were recorded simultaneously. To aid in introducing the ova into the Microslide, a stereomicroscope also mounted in the horizontal position was focused on the sample chamber.

The following sequence of steps was followed in each determination. An ovum was washed in PBS and then transferred with a drawn-out Pasteur pipet into PBS contained in a Microslide. It was photographed two or three times. The same ovum was then recovered from the sample chamber. A fresh Microslide was filled with a given NaCl solution and mounted on the stage to permit it to reach temperature equilibrium. Since the volume

contained in the Microslide was about 2.5×10^{-3} ml, it reached equilibrium very quickly. With the recorder running, the same ovum held in the very tip of a drawn-out pipet was carefully transferred into the bottom of the test solution. As the ovum was being observed through the stereomicroscope, the camera shutter was tripped just at the moment that the cell passed into the solution. This event marked on the recorder was taken to be 0 time. The mechanical stage was then manipulated so as to keep the ovum in focus as it ascended and then descended in the test solution. Usually, 15 to 30 photographs were made of each ovum, with the time of each photograph automatically recorded. After an ovum had sunk to the bottom meniscus of the solution, and it was judged (by eye) to have achieved a constant volume, the ovum was recovered from the sample compartment, washed in PBS, and photographed again. In no case did the volume of an ovum in PBS after exposure to NaCl differ from its initial volume in PBS by more than a few percent. At the conclusion of a determination, all fertilized ova were cultured in SOF to assay their ability to cleave in culture. Measurements of a given fertilized ovum were used only if the ovum developed to the 2-cell stage. Twenty of 23 fertilized ova used in this study developed in culture after being exposed to 0.5 M NaCl. A total of 13 unfertilized ova were also observed under comparable conditions, although without being cultured after the observation.

As was done for the equilibrium measurements, the negatives of an ovum in PBS before treatment, and of the same ovum in 0.5 M NaCl at various times, were projected and traced. The tracings were measured by planimeter and volumes were calculated using Eq. (1). These volumes were then expressed as a percentage of the isotonic volume (the mean of two or three measurements) of the same ovum.

Permeability Calculations

The rate of osmotic water flow across a cell membrane depends on the difference between osmotic pressures of the intracellular and extracellular solutions. Starting from the relationship

$$\frac{dV}{dt} = L_p A(\pi_i - \pi_e) \tag{2}$$

in which V is the total cell volume, t is time in minutes, L_p is the hydraulic conductivity, A is surface area, and π_i and π_e are the osmotic pressures of the intracellular and extracellular solutions, respectively, Dick (1966, pp. 86-89) derived the following integrated expression:

$$L_p = \left[\frac{V_e - b}{V - b}\right]\left[\frac{1}{\pi A t}\right]\left[(V_e - b)\ln\frac{(V - V_e)}{(V_2 - V_e)} + V - V_2\right]. \tag{3}$$

The definitions and values used for the terms in this and subsequent equations are shown in Table 1. Converting cell volume to water volume, and substituting RTN/V_1 for π, one obtains the equivalent expression:

$$L_p = \frac{V_e(V_1 - V_2) + V_e^2 \ln\dfrac{(V_e - V_1)}{(V_e - V_2)}}{tANRT}. \tag{4}$$

In the experiments, cell volume was measured as a function of time. The corresponding term that is necessary to fit the observed behavior to the calculated one is V_2, the volume of cell water at any time, t. However, Eq. (4) cannot be solved in closed form for V_2, since it is transcendental. One procedure for obtaining an approximate solution of an equation of this form is the Newton-Raphson method (*see* Britton, Kriegh & Rutland, 1965, p. 515*ff*; Southworth and DeLeeuw, 1965, p. 183*ff*). This is an efficient method to find the root of a function, $f(x)=0$. It requires the first derivative of the function $f'(x)$. Then, assuming an initial value for the root x_1 and using the relationship:

$$x_2 = x_1 + \frac{f(x_1)}{f'(x_1)} \tag{5}$$

one obtains an improved approximation to the solution. Successive iterations are obtained by substituting x_2 for x_1 in this equation and solving for a new x_2. However, to assure that the iteration process will converge on the root, one must have the second derivative of the function $f''(x)$. The process will converge if the criterion

$$\frac{f(x_1)f''(x_1)}{[f'(x_1)]^2} < 1 \tag{6}$$

is satisfied. This was the method used to calculate values for V_2 for each t in Eq. (4).

To take the derivatives of Eq. (4), it was first simplified by the following algebraic manipulations. Letting $K = L_p tANRT$ and dividing by V_e^2 in Eq. (4), one obtains:

$$\frac{K}{V_e^2} = \frac{V_1 - V_2}{V_e} + \ln\frac{V_e - V_1}{V_e - V_2}. \tag{7}$$

Transposing and multiplying through by $(V_e - V_1)/(V_e - V_1)$, Eq. (7) can be converted to:

$$\frac{K}{V_e^2} = \frac{(V_e - V_1)}{V_e}\left[-1 + \frac{(V_e - V_2)}{(V_e - V_1)}\right] + \ln\frac{(V_e - V_1)}{(V_e - V_2)}. \tag{8}$$

Letting $x = (V_e - V_1)/(V_e - V_2)$ and $d = (V_e - V_1)/V_e$, Eq. (8) becomes:

$$\frac{K}{dV_e^2} = \frac{1}{d}\ln x + \frac{1}{x} - 1. \tag{9}$$

Multiplying by d, transposing terms, and letting $m = K/V_e^2 + d$

$$x \ln x = -d + mx. \tag{10}$$

In other words, the root of the equation:

$$f(x) = x \ln x + d - mx = 0 \tag{11}$$

can be used to find V_2 from:

$$V_2 = V_e - \frac{V_e - V_1}{x} \tag{12}$$

The first and second derivatives of the function are:

$$f'(x) = \ln x + 1 - m \tag{13}$$

$$f''(x) = \frac{1}{x}. \tag{14}$$

By assuming an initial value of V_2 only very slightly less than V_1 at $t=0$, one can calculate values for V_2 at successive times from Eq. (12) using the iteration shown in Eq. (5), the convergence criterion in Eq. (6), and the expressions for $f(x)$, $f'(x)$, and $f''(x)$ shown in Eqs. (11), (13), and (14), respectively. These equations were solved on a PDP11 Computer (Digital Equipment Corp., Maynard, Mass.) for cell water volume V_2 as a function of time t at constant temperature T. The total volume of a cell is equal to the calculated water volume plus the volume of cell solids b. The value of b was obtained from the graph of Relative Cell Volume *vs.* $1/\pi$ of the suspending solution by extrapolating to infinite concentration. The value used, $b = 18.2\%$ of the total cell volume, agrees rather well with the 20-21% figure determined by Rall using immersion

refractometry (personal communication). (*See* Ross, 1967, for a description of the method that Rall used.)

The method to determine L_p was to assume:

$$V_{2\,\text{at}\,t=0} = 1.879 \times 10^5 < V_1 = 1.880 \times 10^5$$

and also to assume some value for L_p at constant T. Eq. (4) was solved for V_2 as a function of t, V was calculated as $V_2 + b$, and the resulting curve was compared by eye to the experimental observations. The entire calculation was repeated using different values for L_p until the majority of the observed values fell on or very close to the calculated curve of V *vs.* t. Examples of this "curve fitting" are shown in Results.

Results

Osmometric Behavior

When fertilized mouse ova were suspended in unbuffered NaCl solutions for ~90 min at room temperature, there was relatively little effect on the developmental capacity of the ova up to a concentration of 0.5 M (Table 2). That is, 84% of 79 ova exposed to NaCl concentrations of 0.5 M or less developed *in vitro* to the 2-cell stage. There was a suggestion that higher concentrations may have a deleterious effect, since only 61% of ova in 0.7 M NaCl cleaved to the 2-cell stage.

When exposed to hypertonic solutions of either an electrolyte (NaCl) or nonelectrolyte (sucrose), fertilized ova exhibit a "classic" osmometric response (Fig. 3). They shrink to a constant volume, relative to their isotonic volume, that is proportional to the reciprocal of the osmotic pressure of the suspending solution. Since these measurements were made ~60 min after the ova were suspended in the solutions, it appears that these cells do not undergo the "volume adjustment" that has been described for some other cell types. Extrapolation of cell volume in an infinitely concentrated solution indicates that the volume of cell solids, b, in fertilized mouse ova is 18% of the isotonic volume. The relative volumes of ova in NaCl and sucrose were calculated directly from the cross-sectional areas. The agreement between my results (ova in NaCl) and Rall's results (ova in buffered sucrose) demonstrates that the nature of the impermeant solute did not influence the results.

Permeability Measurements

A total of 20 fertilized ova, which developed into 2-cell embryos in culture after the treatment, were exposed to 0.5 M NaCl at various constant temperatures between 0 and 30 °C. Twelve unfertilized ova were exposed to 0.5 M NaCl at three temperatures, *viz.*, 10, 20, and 29.5 °C, and one was exposed at 2 °C.

The results in Fig. 4 show the observed water loss from fertilized ova as a function of time at four constant temperatures. As to be expected, the higher the temperature of exposure to hypertonic NaCl solutions, the faster the ova decreased in volume. In the examples shown, the ovum at 30 °C shrank to about 50% of its isotonic volume in ~0.6 min, the one at 20 °C shrank to that volume in ~1.3 min, and the one at 10 °C shrank to that volume in ~2.9 min. The ovum at 0 °C had not decreased to that volume even after 6 min of exposure. Even these raw data yield an estimate of the effect of temperature on the rate of water loss from the ova. Letting $\Delta V/t = K$ where K is an estimate of the rate of water loss, $\Delta V = 50\%$, and t = time in min, one obtains values of K for the three higher temperatures of 83.3, 38.5, and 17.2, respectively. Therefore, the Q_{10} (where $Q_{10} = K_T/K_{T-10\,°C}$) of water loss over the temperature range of 10 to 30 °C is approximately 2.2. The reproducibility of the method used is illustrated by the results in Fig. 5, which shows the water loss from three unfertilized ova exposed to 0.5 M NaCl at 10 °C.

Permeability Calculations

In calculating the L_p, each curve for an individual ovum was fitted separately. For the fertilized ova, the L_p's at 14 temperatures were tabulated separately, whereas for the unfertilized ova, several L_p's at each of three temperatures were pooled and the mean L_p at that temperature was calculated. The method for curve fitting to determine L_p is illustrated by the results in Fig. 6, in which the observed water loss from a fertilized ovum at 20 °C is compared to the water loss calculated using three values for L_p in Eq. (4). In the example shown, 8 of the 18 observed values lie almost precisely on the curve calculated for $L_p = 0.43$ $\mu m^3/\mu m^2$-min-atm, 4 of the values lie on the curve for $L_p = 0.38$, 1 of the values lies on the curve for $L_p = 0.48$; 5 of the values do not fit any of the calculated curves. On this basis, $L_p = 0.43$ was judged to yield a calculated water loss most closely approximating the observed water loss. The same approach was used to determine the L_p's for all sets of observations of fertilized ova.

In the case of unfertilized ova, each set of observations at a given temperature was fitted in the manner just described. Then the mean L_p was calculated for all sets at that temperature. An example is shown in Fig. 7 for unfertilized ova at 20 °C. The results in that figure show five sets of observations and the water loss calculated with the mean value of the five sets of fitted observations. In fact, the mean $L_p \pm$ standard error of unfertilized and fertilized ova

Table 2. Effect of hypertonic NaCl solutions on *in vitro* survival of fertilized mouse ova

[NaCl][a]	2-Cell/1-Cell[b]	%Survival
0.2	18/22	82
0.3	16/17	94
0.4	14/18	78
0.5	18/22	82
0.7	14/23	61

[a] Molarity.

[b] The figures show the number of 2-cell embryos that developed *in vitro* divided by the number of fertilized ova recovered after exposure to each NaCl solution for ~90 min at room temperature.

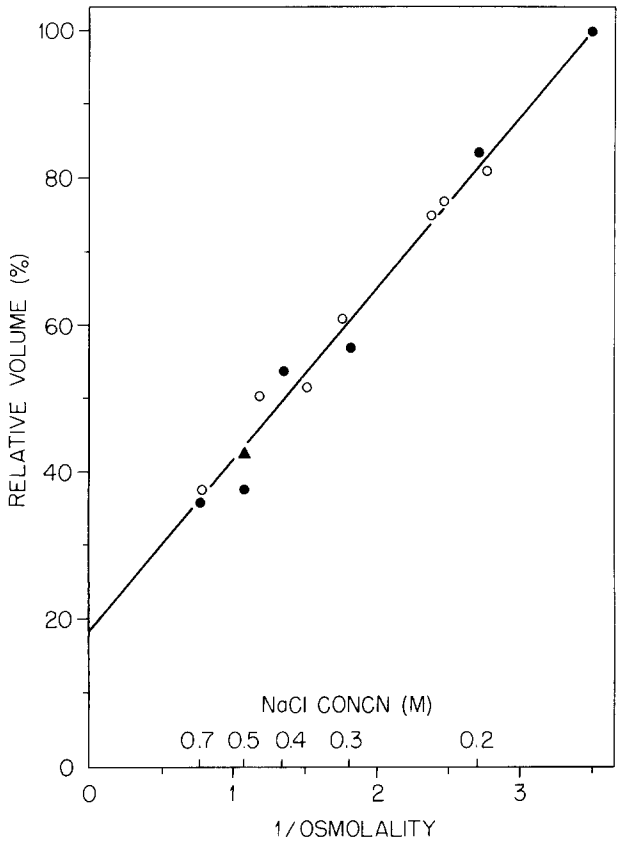

Fig. 3. The relative volume of fertilized ova as a function of the reciprocal of the osmolality of hypertonic solutions in which the ova were suspended for ~60 min at room temperature. The volumes are expressed as a percentage of the volume of ova in isotonic phosphate-buffered saline (PBS). Each solid point is the mean value for 6–8 ova suspended in NaCl solutions. These volumes were calculated from the cross-sectional areas of the ova, in the manner described in the text. The open circles are the data of Dr. W.F. Rall, who used a similar method to measure the volume of ova suspended in buffered sucrose solutions. The solid triangle is the mean final volume of all ova exposed to 0.5 M NaCl to measure the kinetics of water loss. The data were fitted by the method of least squares

at 20 °C are 0.44 ± 0.03 and 0.43 ± 0.01 $\mu m^3/\mu m^2$-min-atm, respectively.

Effect of Temperature on L_p

The observed curves for water loss at several temperatures for fertilized and unfertilized ova were fitted, and the activation energies for the hydraulic conductivity were calculated. The results are shown as Arrhenius plots in Fig. 8*A* and 8*B* for fertilized and unfertilized ova, respectively. The activation energies for fertilized and unfertilized ova are 13.0 and 14.5 kcal/mol, respectively. Despite the fact that the method used to estimate the activation energies differed in the two cases (i.e., individual values at many temperatures for fertilized ova; multiple values at a few temperatures for unfertilized ova), there is no significant difference between the two values.

Discussion

A photomicroscopic method has been used to determine the hydraulic conductivity and its activation energy of fertilized and unfertilized mouse ova. Curves of observed cell shrinkage were fitted to ones calculated using the classical permeability equation. The hydraulic conductivity for both stages of ova is about 0.4 $\mu m^3/\mu m^2$-min-atm at 20°, which, in these units, is equivalent to 9.0 μm/sec or 9.0×10^{-4} cm/sec[1]. The surface area/volume ratio (SA/V) of mouse ova is $\cong 0.079$ $\mu m^2/\mu m^3$ (*see* Table 1). In his tabulation of osmotic water permeability coefficients of animal cells, Dick (1966, pp. 90–91) lists three values for *Arbacia punctulata*, which has the same volume (Lucké et al., 1939)[2] and SA/V ratio as mouse ova. The coefficients that are shown for this cell type are 0.054 (Leitch, 1934)[2], 0.12 (Lucké et al., 1939)[2], and 0.17 $\mu m^3/\mu m^2$-min-atm (Lucké et al., 1951)[2]. The values that Dick lists for mammalian cell types, other than erythrocytes, range from 0.3 to 4.0 $\mu m^3/\mu m^2$-min-atm, corresponding to 6 to 89 μm/sec. More recently, Hempling (1967, 1973, 1977) and DuPre and Hempling (1978) have reported hydraulic conductivities of several different types of mammalian cells other than erythrocytes. These range from a high of 2.3 for Ehrlich ascites cells maintained in mice to about 0.4 for these same cells maintained in culture, to values of about 0.3 to 0.4 for a variety of leu-

[1] Hydraulic conductivity has been reported in many different units. The following useful collation of conversion factors by Stein (1967, p. 39) was used: 1 $\mu m^3/\mu m^2$-min-atm ≡ 22.4 μm/sec ≡ 2.24×10^{-3} cm/sec.

[2] Reference from Dick (1966).

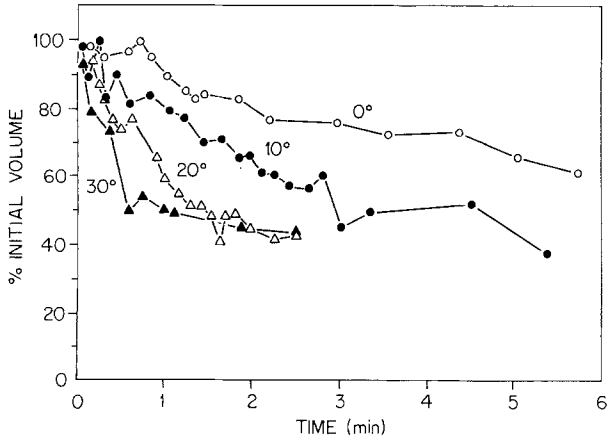

Fig. 4. The observed water loss, as a percentage of their initial volumes in PBS, of four fertilized ova as a function of time of exposure to 0.5 M NaCl at four temperatures. The ova were photographed and their volumes calculated from their cross-sectional areas, as described in the text

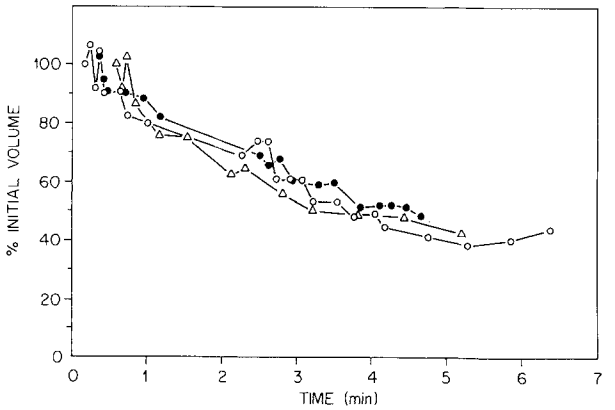

Fig. 5. The observed water loss, as a percentage of their initial volume in PBS, of three unfertilized ova as a function of time of exposure to 0.5 M NaCl at 10 °C

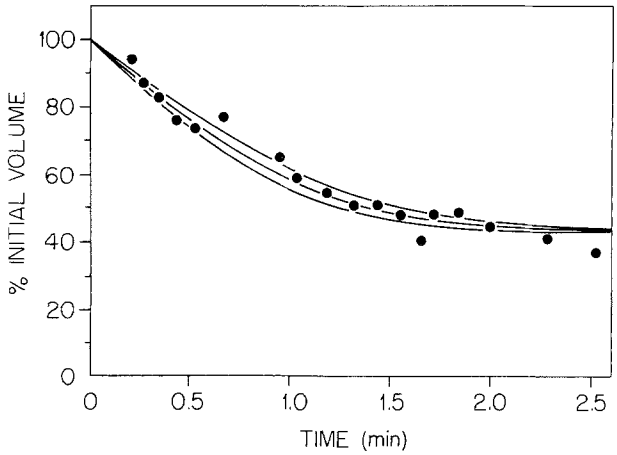

Fig. 6. Comparison of the observed water loss, expressed as a percentage of initial volume, of a fertilized ovum with the water loss calculated with three values for the hydraulic conductivity, L_p. The values used for L_p for the smooth curves, reading from top to bottom, were 0.38, 0.43, and 0.48 $\mu m^3/\mu m^2$-min-atm. The calculation was performed using a programmed version of Eq. (4) (*see* text). On the basis of this comparison, a value of $L_p = 0.43$ $\mu m^3/\mu m^2$-min-atm was judged to yield the "best fit" of calculation to observation

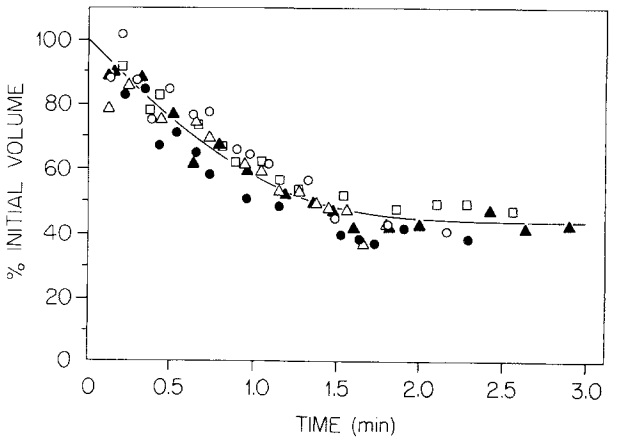

Fig. 7. Comparison of the observed water loss from five unfertilized ova with the calculated water loss (solid line) using the mean of the five sets of $L_p = 0.44$ $\mu m^3/\mu m^2$-min-atm. Each symbol refers to the results for an individual ovum. Each set of observations was fitted by the method described in the text to obtain an individual value for L_p

cocytes, all in units of $\mu m^3/\mu m^2$-min-atm at 20 °C. Therefore, mouse ova seem somewhat more permeable than an invertebrate cell of the same size, and approximately as permeable as a variety of mammalian cell types. But ova are substantially larger than all other types of mammalian cells, and have a SA/V ratio that is about one order of magnitude smaller than most mammalian cells.

With respect to the effect of temperature on water permeability, mouse ova display rather large activation energies of 13 to 14.5 kcal/mol, similar to that found for sea urchin eggs (McCutcheon & Lucké, 1932). Those investigators found the activation energy for the permeability of fertilized *Arbacia* eggs (20 kcal/mol) to be substantially higher than that of unfertilized eggs (15 to 16 kcal/mol). In contrast, fertilization apparently does not significantly alter the membrane of the mouse ovum in this respect (*cf.* Fig. 8*A* and 8*B*). Parenthetically, it has been found that the fertilized mouse ovum is some three times more permeable to glycerol than the unfertilized ovum, and that the latter stage has an activation energy for glycerol permeation of 28 kcal/mol *vs.* 19 kcal/mol for the former ovum stage (Jackowski, Leibo & Mazur, 1979). Other cell types for which high activation energies for hydraulic conductivity have been reported include chicken erythrocytes (11.4 kcal/mol; Farmer & Macey, 1970), Ehrlich ascites tumor cells (9.6 kcal/mol; Hempling, 1960), cultured

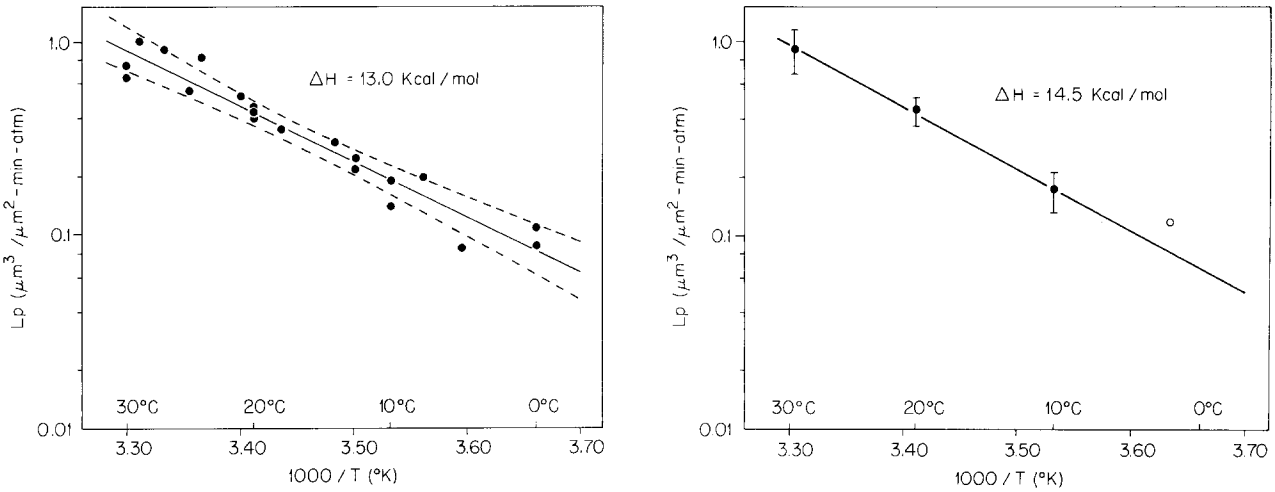

Fig. 8. Arrhenius plots of the hydraulic conductivities of fertilized (8*A*) and unfertilized (8*B*) mouse ova. The graph for fertilized ova shows the values of L_p for individual ova at a given temperature, ±the 99% confidence limits of the line. The graph for unfertilized ova shows the mean value of $L_p \pm$SD at a given temperature, calculated from 3 to 5 observations at that temperature. The data for each graph were fitted by least squares analysis. The open symbol in Fig. 8*B* is the L_p of a single ovum at 2 °C, and was not included in the regression analysis

ascites cells (8.7 kcal/mol; DuPre & Hempling, 1978), and a variety of leucocytes (13.1 to 18.4 kcal/mol; Hempling, 1973). Almost all mammalian erythrocytes have activation energies of roughly 3 to 5 kcal/mol (*see* tabulations by Forster, 1971, and House, 1974, for examples). Thus, it appears as if the low activation energy of mammalian erythrocytes, compared to that of other types of mammalian cells, may be the exception rather than the rule.

No attempt was made in this study to determine the role of the zona pellucida on water permeability of mouse ova. The reason was that the purpose of this study was to examine ova as they are used in most physiological studies, such as metabolism, and as used in cryobiology. A circumstantial argument can be made, however, that the zona plays only a negligible role. A number of studies have shown that fertilization causes a variety of changes in both the properties (*see* review in Gwatkin, 1964; Austin, 1965; Inoue & Wolf, 1975) and structure of the zona (Jackowski & Dumont, 1979), but there was little difference between fertilized and unfertilized ova with respect both to their hydraulic conductivities and to their activation energies. Therefore, one must conclude either that the changes in structure and properties of the zona that have been observed play no role in water permeability, or that the zona itself plays no role. A somewhat more definitive answer to this question might be obtained by studying the permeability of ova from which the zona had been removed. In that case, however, the methods used to remove the zona might be critical. For example, identifying artifactual effects on water permeability of an enzyme used to remove the zona might be difficult. There are data that show that the permeability to glycerol of fertilized and unfertilized mouse ova apparently is not affected by the removal of the zona (Jackowski, Leibo & Mazur, 1979).

From the vantage point of cryobiology, three characteristics of mouse ova are significant. Ova have a low surface/volume ratio (SA/V), a relatively low hydraulic conductivity (L_p), and a large activation energy (ΔH) for water permeability. Mazur's (1963) theoretical analysis of the kinetics of water loss at subzero temperatures identified these three cell characteristics as being of fundamental importance with respect to a given cell's freezing sensitivity. Briefly, he demonstrated mathematically that a low SA/V or a low L_p or a high ΔH would each reduce the cooling rate that would produce intracellular ice in a cell during freezing. It has been shown for a variety of cell types that cells killed by too high a cooling rate were killed by intracellular ice formation (*see* Mazur, 1977, and Leibo, 1977*a*, for review). Collectively, the effect of SA/V, L_p, and ΔH on freezing sensitivity means that optimum cooling rates for maximum survival of different cell types may vary by 2 to 3 orders of magnitude (Mazur et al., 1970; Leibo & Mazur, 1971; Leibo, 1977*a*; Mazur, 1977). For example, the first successful preservation of mouse ova and embryos demonstrated that their optimum cooling rate was ~0.5 °C/min, a rate significantly lower than is found for almost all other mammalian cell types (Whittingham, Leibo & Mazur, 1972). It is now clear that ova possess characteristics that reduce their rate of water loss with decreasing temperature much more than that of erythrocytes, for example. This means that ova must be cooled at rates significantly lower

than erythrocytes for maximum survival. A more detailed discussion of this argument has already been published (Leibo, 1977*b*). In that paper, the values that I reported for the L_p (0.3 $\mu m^3/\mu m^2$-min-atm) and ΔH (12 kcal/mol) of mouse ova were preliminary ones, and are somewhat lower than those reported here.

The method used in this study to observe water loss from mouse ova differs from those used in most recent studies of water permeability. Most such studies utilize elaborate mechanical and/or electronic apparatus to record volume changes of small cells ($\sim 10\,\mu m$ diameter) using samples of 10^6 cells or greater. In contrast, since the collection of even 200 mouse ova from superovulated females requires about 1 hr, it is not possible to use large numbers of ova for each determination. Furthermore, mouse ova have a diameter of 75 μm (volume of $2.3 \times 10^5\,\mu m^3$), and are easily damaged if forced through a narrow orifice, as would be true in a stop-flow apparatus or an electronic cell sizer, for example. Finally, the characteristics of mouse ova that determine their permeability to water result in volume changes that occur in minutes or fractions of minutes (*see* Fig. 4). In contrast, erythrocytes exhibit comparable volume changes in msec (*see* Forster, 1971, for discussion). The present method ought to be applicable to study volume changes of any large cell type. A single cell yields a complete curve of volume change as a function of time at a constant temperature. One unanticipated advantage followed from "tracking" the cells as they rose and fell in the capillary. That is, the ova "tumbled" in the solution as they responded to the hypertonic solution (note the position of the polar bodies relative to the cell itself in Fig. 2). This tumbling caused the ova to be photographed from many aspects rather than being viewed from a single one. The problem caused by estimating volumes of a large cell when viewed from a single aspect has been analyzed for toad oocytes by Dick, Dick and Bradbury, (1970).

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