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HIGH DENSITY CELL WATER IN AMPHIBIAN EGGS?

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SUMMARY

The exchange of isotopic water, ^2H_2O and $H_2{}^{18}O$, has been studied in amphibian eggs. The experiments were carried out with ovarian and body cavity eggs of *Rana temporaria* and unfertilized eggs of *Ambystoma mexicanum*. The cytoplasmic diffusion coefficient for $H_2{}^{18}O$ was found to be $4{\cdot}6 \times 10^{-6}$ cm^2/s, somewhat higher than that for ^2H_2O, $3{\cdot}4 \times 10^{-6}$ cm^2/s. The total change in reduced weight, ΔRW, during the isotope experiments was compared with the total amount of water in the egg cell, m. The ratio $\Delta RW/m$ was significantly higher than would be expected from calculations using ordinary water density values. The results are discussed in terms of different phases of structured cell water.

INTRODUCTION

For many years it has been suspected that the water inside the living cell differs from bulk water with respect to its structure and various physical properties. The great interest in this problem is evidenced by a number of recent articles discussing the physiological role and importance of intracellular water (Walter & Hope, 1971; Hazlewood, 1973; Cooke & Kuntz, 1974; Wiggins, 1975; Cope, 1976; Kolata, 1976; Ling, 1977). In spite of all this attention, nobody has been able to provide a direct demonstration of the properties of the intracellular water in cells under normal physiological conditions. All that has been observed are a number of 'anomalies' in the properties of the living cells which can be accounted for on the assumption that part of the cell water is structured ('bound') in some way. The study of this problem is hampered by the fact that the living cell usually is so small that few analytical methods are applicable. This may be one reason why so many data on cell water concern amphibian eggs, these being among the largest cells known.

One anomaly in amphibian eggs is that they do not behave as perfect osmometers: not all of their water participates in the adjustment to changes in the osmolarity of the external medium. Thus, when a frog egg is placed in a isotonic solution only about 65% of the water content is osmotically active (Sigler & Janacek, 1969, 1971). It has also been found that the diffusion coefficient for water in cytoplasm does not

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vary smoothly as in bulk water, but exhibits a break at 16 °C (Hansson Mild & Løvtrup, 1974*a*). At this temperature, unexpected changes likewise occur in the internal hydrostatic pressure of eggs incubated in hypotonic solutions (Hansson Mild, Løvtrup & Bergfors, 1974) and in the apparent osmotic water permeability coefficient of frog ovarian eggs (Hansson Mild, Carlson & Løvtrup, 1974). Measurements of the relaxation times with NMR have shown a reduction relative to free water, which again suggests an increased structural order in the cell water in the frog egg (Hansson Mild, James & Gillen, 1972).

Several forms of structured water are known, at least thirteen polymorphic forms of ice exist, and all of them have densities different from that of ordinary water, one being lighter and the remaining ones heavier (Kamb, 1972). It might therefore be anticipated that if cell water is structured, then its density should differ from unity. In the present paper we present a method which, indirectly, may give information about the density of the cell water in the amphibian egg in the normal physiological state. By means of this method we have concluded that cell water, like most types of structured water, has a density higher than that of bulk water.

MATERIAL AND METHODS

Biological material

The experiments were carried out with ovarian and body cavity eggs of *Rana temporaria*, and ovarian and unfertilized eggs of *Ambystoma mexicanum*. The frogs were purchased from commercial dealers and kept at 5 °C until used. Ovulation was induced as described by Rugh (1952). The axolotls were raised in the laboratory, where they are kept at 16 °C. Spawning was induced by injecting the female with 150 i.u. of human gonadotropin.

The ovarian eggs of both species were obtained by withdrawing surgically a small portion of an ovarium and removing the follicle membrane mechanically with forceps. In the unfertilized eggs of *Ambystoma* the jelly was removed with forceps. All eggs were kept in Ringer solution. In all cases healthy looking eggs were chosen for experimentation. Prior to each experiment the radius of the egg was measured with an optical screw micrometer, an operation having a standard deviation of 1%. All experiments were carried out at 18 °C.

Isotope exchange method

The isotopic water exchange method has previously been used to determine the diffusion coefficient of water in the cytoplasm and the water permeability coefficient of the plasma membrane of the amphibian egg (Hansson Mild & Løvtrup, 1974*a*, *b*). The theory of this method has been published earlier (Løvtrup, 1963; Bergfors, Hansson Mild & Løvtrup, 1970; Hansson Mild, 1972; Hansson Mild & Løvtrup, 1974*a*).

The exchange of isotopic water is followed by determination of the changes in the reduced weight (*RW*) of an egg placed in isotonic Ringer solution containing either 20% ^2H_2O or 20% $H_2{}^{18}O$, by means of the automatic diver balance (Bergfors *et al.* 1970). The exchange curve is registered in two ways, on a X–Y recorder, wher

The *RW* is plotted as a function of time, and on a paper tape with data points taken at 10 s intervals. The experimental curves are then compared with the theoretical expression for the exchange process (Hansson Mild, 1972) by a computer curve fitting procedure (see Hansson Mild & Løvtrup, 1974*a*).

The exchange process, started by loading the diver with an egg, causes a mechanical disturbance of the system and reliable readings are therefore obtained only after approximately 30 s. This means that the initial value of the curve, RW_0, the reduced weight at $t = 0$, and hence also the total change in *RW* during the isotope exchange process, must be calculated by the computer. We have estimated the error thus introduced by weighing the same eggs on two different balances, one floating in full strength Ringer solution made up with ordinary water, another in a medium consisting in part of isotopic water. On the first balance the value of RW_0 is obtained directly, and on the other one, as mentioned, indirectly. A series of experiments of this kind has shown that the error involved in the indirect method is less than 1 %.

After completion of the isotope exchange, the egg is removed from the diver and dried in an oven at 105 °C to constant weight (2 h), and the dry weight determined on a microbalance.

Calculations

The reduced weight, *RW*, of an object is its weight when submerged in a liquid medium, i.e. the wet weight minus the buoyancy. It is thus given by:

$$RW = V(\rho - \rho_m) = M - V\rho_m, \tag{1}$$

where V is the volume of the object (in our case an amphibian egg), ρ its density and M its wet weight, while ρ_m is the density of the medium. Rearranging the terms of equation (1) we obtain:

$$M = RW + V\rho_m. \tag{2}$$

The density of the medium is known from pycnometer determinations and *RW* from diver balance experiments. With the volume obtained from determinations of the radius, we may thus calculate the wet weight. The mass of water in the egg, *m*, is then obtained as the difference between the wet weight and the dry weight, *dw*. An analysis of the errors involved in the outlined procedure gives a total error of about 4 % in *m*.

The total change in reduced weight, ΔRW, during the isotope exchange process, is obtained from the experimental curve by the computer curve-fitting procedure mentioned earlier. The error involved in this determination of ΔRW is estimated to be about 3 %. An illustration of the steps involved in our method is given in Fig. 1, the reproduction of an experimental curve for a body cavity egg placed in Ringer containing 20 % $H_2{}^{18}O$.

The change in reduced weight (associated with an exchange experiment), ΔRW, can be expressed as

$$\Delta RW = M_i - M_o - \rho_m(V_i - V_o), \tag{3}$$

where the index i refer to the isotope-containing medium, and o to the ordinary Ringer solution, $V_i - V_o$ thus being the change in volume. In equation 3, the index o

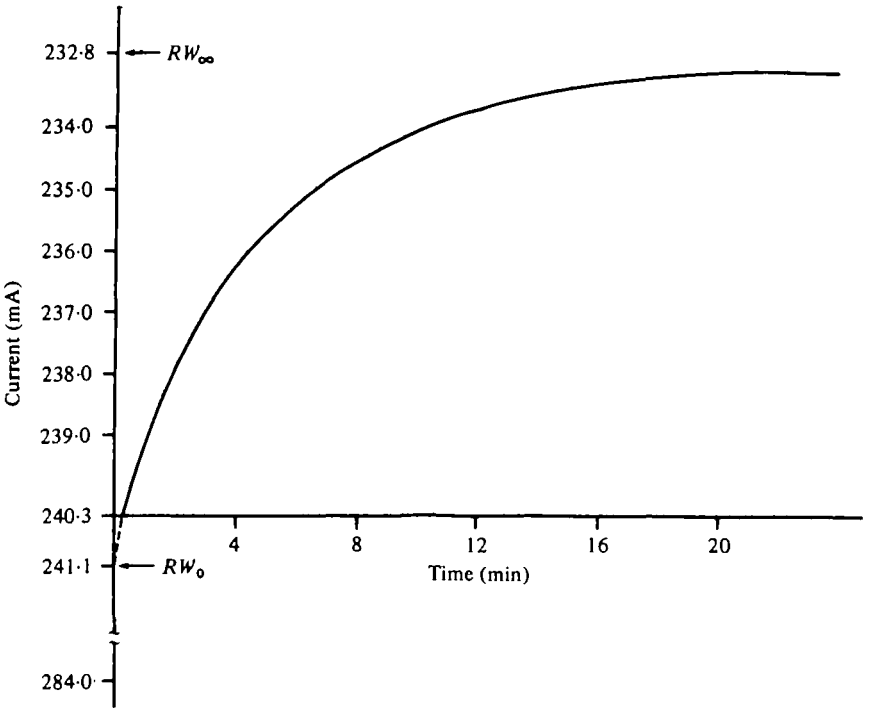

Fig. 1. Experimental curve from a body cavity egg of *Rana temporaria* in 100% Ringer solution containing 20% $H_2{}^{18}O$. The diver constant was 231 amps/g. The current in the coil with the diver unloaded was 284·0 mA and after loading the zero line of the recorder was shifted to 240·3 mA. From the computer fitting of the curve the current at time t = 0 was found to be 241·1 mA, thus giving a reduced weight, RW_0, of 186 μg. The current in the coil after completion of the isotope exchange was 232·8 mA, which corresponds to a change in reduced weight, ΔRW, of 35·9 μg.

may either mean zero, i.e. the value of the parameter at time t = 0, or the letter o, i.e. the value of the parameter in ordinary Ringer solution, either interpretation being equivalent in the following text. Furthermore, we have

$$M = dw + v\rho', \tag{4}$$

where the last term refers to the volume and the density of the cell water. Assuming the dry weight, as well as its associated volume, to be constant during the isotope exchange process, these parameters may be eliminated from equation (3), giving

$$\Delta RW = v_i.\rho_i' - v_o.\rho_o' - \rho_m(v_i - v_o). \tag{5}$$

If $v_i = v_o + \Delta v$, we arrive at

$$\Delta RW = v_o(\rho_i' - \rho_o') + \Delta v(\rho_i' - \rho_m). \tag{6}$$

The volume change, Δv, may have two sources, being associated either with the isotope-exchange experiment proper or with an osmotic imbalance between egg and medium.

The medium is assumed to be isotonic with the egg cytoplasm and, as might be expected, it is not possible with the optic method to demonstrate any volume change during periods which by far exceed the duration of an isotope experiment. If the

eggs are placed in a hypotonic solution, then an osmotic swelling, i.e. an uptake of water, takes place, but at a rate much slower than that of the isotope exchange process. To show this, we may employ the equation used in osmotic water permeability studies, giving the rate of change of the radius as follows:

$$\frac{dr}{dt} = P\bar{V}_W \Delta C, \tag{7}$$

where P is the osmotic water permeability coefficient, ΔC is the concentration difference across the membrane, and $\bar{V}_W$ the partial molar volume of water. Assuming a 5% difference in the tonicity between the ordinary Ringer and the isotope-containing solution gives $\bar{V}_W \Delta C = 2{\cdot}1 \times 10^{-4}$. P is of the order of 2×10^{-4} cm/s for a body cavity egg (Hansson Mild *et al.* 1974). For small change in radius equation (7) may be replaced by

$$\Delta r = \Delta t . P . \bar{V}_W . \Delta C. \tag{8}$$

If for Δt we insert the duration of an isotope exchange experiment (20 min) we get $\Delta r \sim 5 \times 10^{-5}$ cm. The corresponding volume change Δv is about 5×10^{-6} cm^3 (0·2%).

The volume change due to the isotope exchange proper can be calculated approximatively by assuming that the isotope-containing water has the same density in the cytoplasm as in solution, and furthermore that the exchange takes place on a mole–mole basis. This leads to $v_i = 1{\cdot}0007\, v_o$ for the ^2H_2O solution.

If we assume that both of the above mentioned volume changes act in the same direction, we thus have at most $\Delta v \sim 0{\cdot}3\%$ or about 7×10^{-6} cm^3. The density difference in equation (6), $\rho_i' - \rho_m$, is of the order 0·02 g/cm^3 and thus the contribution of a volume change is of the order 0·1 μg, as compared, with a total ΔRW of 40 μg. We therefore feel entitled to neglect the influence of volume changes on ΔRW.

Introducing the mass of the cell water, equation (6) may now be rearranged to

$$\Delta RW = m\frac{\rho_i' - \rho_o'}{\rho_o'}. \tag{9}$$

Inserting the bulk values for the densities we obtain for the factor $(\rho_i' - \rho_o')/\rho_o'$ the value $2{\cdot}17 \times 10^{-2}$ for the ^2H_2O solution and $2{\cdot}47 \times 10^{-2}$ for the $H_2{}^{18}O$ solution. These values are hereafter referred to as 'bulk values'.

RESULTS

The experiments with the isotope $H_2{}^{18}O$ in *Rana* oocytes gave an average cytoplasmic water diffusion coefficient of $4{\cdot}6 \times 10^{-6}$ cm^2/s in the ovarian eggs. The water permeability coefficient was too high to be measured with our method. In the body cavity eggs the membrane permeability is $2{\cdot}7 \times 10^{-4}$ cm/s. These results should be compared with the results previously obtained by us on these eggs, using heavy water as a tracer, where we found a diffusion coefficient of $3{\cdot}4 \times 10^{-6}$ cm^2/s in the ovarian eggs and a permeability coefficient of $1{\cdot}4 \times 10^{-4}$ cm/s in the body cavity eggs (Hansson Mild & Løvtrup, 1974*a*). Thus, the isotope $H_2{}^{18}O$ gives slightly higher values for the measured parameters, as might be expected since the self-diffusion coefficient for $H_2{}^{18}O$ is known to be higher than for ^2H_2O.

Table 1. *Representative values of egg density, wet weight, water content, and change in reduced weight. ΔRW, from isotope exchange experiments with 20% ^2H_2O and $H_2{}^{18}O$ in* Rana *eggs*

Isotope	Egg type	Egg radius (cm)	Egg density, ρ (g/cm³)	M (mg)	m (mg)	ΔRW (mg)	$\Delta RW/m$
^2H_2O	Oocyte	0·0831	1·115	2·680	1·548	0·0382	$2·47 \times 10^{-2}$
^2H_2O	Oocyte	0·0812	1·119	2·511	1·383	0·0369	$2·67 \times 10^{-2}$
^2H_2O	Body cavity	0·0849	1·115	2·859	1·593	0·0418	$2·62 \times 10^{-2}$
^2H_2O	Body cavity	0·0865	1·112	3·016	1·728	0·0434	$2·51 \times 10^{-2}$
$H_2{}^{18}O$	Oocyte	0·0850	1·119	2·877	1·613	0·0450	$2·79 \times 10^{-2}$
$H_2{}^{18}O$	Oocyte	0·0808	1·118	2·470	1·307	0·0356	$2·72 \times 10^{-2}$
$H_2{}^{18}O$	Body cavity	0·0883	1·120	3·231	1·724	0·0484	$2·81 \times 10^{-2}$
$H_2{}^{18}O$*	Body cavity	0·0804	1·117	2·431	1·325	0·0359	$2·71 \times 10^{-2}$

* See also Fig. 1.

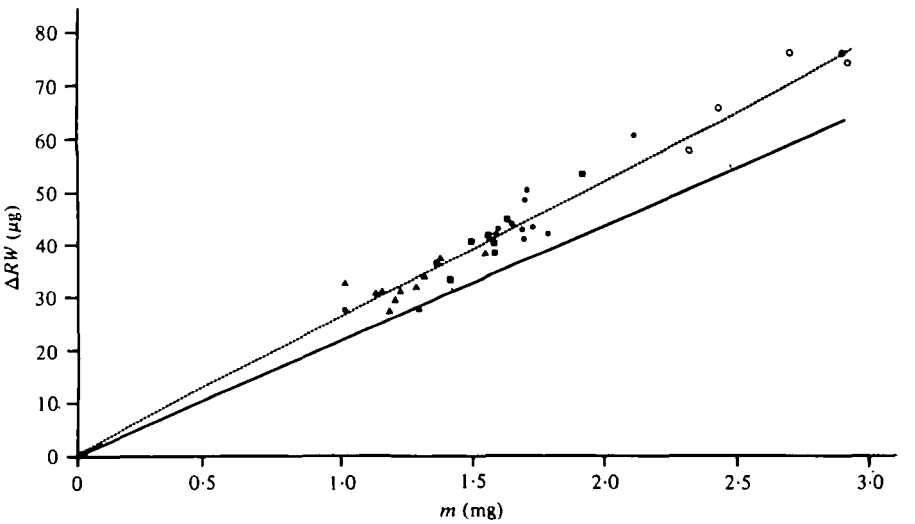

Fig. 2. The change in reduced weight, ΔRW (μg), as a function of the amount of water in the eggs, m (mg), in experiments with 20% ^2H_2O. The symbols used in the figure are as follows: ●, *Rana temporaria* body cavity eggs; ▲, *Rana temporaria* oocytes; ○, *Ambystoma mexicanum* unfertilized eggs; ■, *Ambystoma mexicanum* oocytes. The full line represents equation (6), $\Delta RW = km$, with $k = 2·17 \times 10^{-2}$ (bulk value) and the dotted line is obtained from regression analysis, giving $k = 2·6 \pm 0·2 \times 10^{-2}$.

In Table 1 are listed some representative experimental values of the water content and the change in reduced weight (ΔRW). Figs. 2 and 3 show plots of ΔRW versus m for the isotope exchange with ^2H_2O and $H_2{}^{18}O$, respectively. The average values for the ratio $\Delta RW/m$ were not significantly different between the two species and the different egg types employed in this study. The mean value for $\Delta RW/m$ was therefore calculated for all experiments with the isotope ^2H_2O and was found to be $2·6 \times 10^{-2}$ with a standard deviation of $0·2 \times 10^{-2}$ ($n = 36$). As shown above, the bulk value is $2·17 \times 10^{-2}$, and the difference between this and the experimental value is highly significant ($p < 0·001$). The mean value for the $H_2{}^{18}O$ experiment was $2·8 \times 10^{-2}$, standard deviation $0·2 \times 10^{-2}$ ($n = 16$). Also for this isotope there is a significant deviation from the bulk value $2·47 \times 10^{-2}$ ($p < 0·001$).

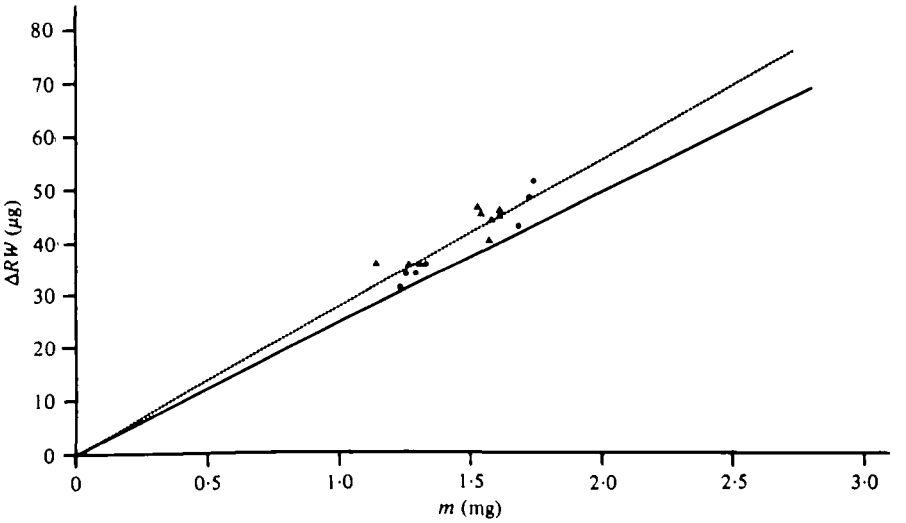

Fig. 3. The change in reduced weight, ΔRW (μg), as a function of the amount of water in the eggs, m (mg), in experiments with 20% $H_2{}^{18}O$. The symbols used in the figure are the same as in Fig. 2. The full line represents equation (6) with $k = 2{\cdot}47 \times 10^{-2}$ (bulk value) and the dotted line is obtained from regression analysis giving $k = 2{\cdot}8 \pm 0{\cdot}2 \times 10^{-2}$.

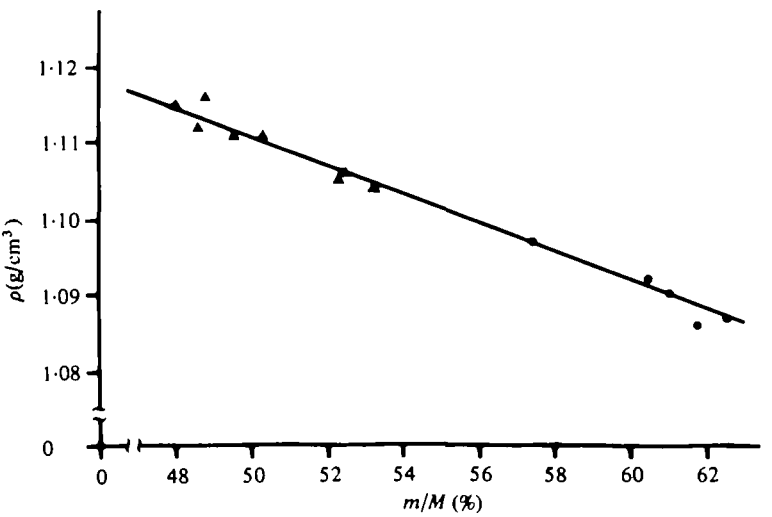

Fig. 4. The density of ovarian (▲) and unfertilized (●) eggs of *Ambystoma mexicanum* as a function of the relative water content, m/M. The line represents equation (12), $\rho = 1{\cdot}208 - 0{\cdot}193\,(m/M)$ obtained by regression analysis.

In *Ambystoma* the water content varies from about 48% in ovarian eggs to 60% in unfertilized eggs. This provides an opportunity to establish the relationship between egg density and water content, as shown in Fig. 4. The line is the least-square regression line (correlation coefficient = 0·99). Assume that the egg density is given by

$$\rho = \frac{dw}{M}\rho_s + \frac{m}{M}\rho_o, \tag{10}$$

where s refers to the solids. Equation (10) can be rearranged to

$$\rho = \rho_s - X(\rho - \rho_o), \tag{11}$$

where X is the water content (m/M). The regression analysis of the data from Fig. 4 gives the equation

$$\rho = 1{\cdot}208 - 0{\cdot}193X \tag{12}$$

and thus by comparing equations (11) and (12) we get $\rho_s = 1{\cdot}208$ and $\rho_o = 1{\cdot}015$ g/cm^3.

DISCUSSION

The experimental values presented in Figs. 2 and 3 indicate a linear relationship between ΔRW and m. A comparison shows that the experimental values of the ratio $\Delta RW/m$ are significantly larger than those obtained from equation (9) when the 'bulk values' are used. The slope k of the lines in Figs. 2 and 3 is

$$k = (\rho_i - \rho_o)/\rho_o = \Delta\rho/\rho. \tag{13}$$

Inserting the experimental values for k into this equation, and taking into account that we are dealing with 20% isotope solutions, we obtain the following relations between the densities of pure isotope water and ordinary water isotope in the cell cytoplasm:

$$\rho_{^2H_2O} = 1{\cdot}129\rho_{H_2O} \tag{14}$$

and

$$\rho_{H_2{}^{18}O} = 1{\cdot}138\rho_{H_2O}. \tag{15}$$

Our data pertaining to the density of cell water are represented by equations (13)–(15). Unfortunately, these are given as ratios and differences, a circumstance that evidently limits the interpretation. In interpreting equation (13) we first assume that $\Delta\rho$ is the same as for bulk solutions; inserting the experimental values of k we thus obtain $\rho_o = 0{\cdot}85$ and $0{\cdot}89$ for the ^2H_2O and $H_2{}^{18}O$ experiments, respectively. These values are unlikely for two reasons: (1) the value for ordinary ice is $0{\cdot}92$ and (2) the values should be identical, and their difference exceeds that allowed for by the errors of our experimental method. It must therefore be assumed that $\Delta\rho$ is larger for cell water than for bulk water. The smallest acceptable value for $\Delta\rho$ is obtained if it is assumed that water has its normal bulk density in the egg cytoplasm. On this assumption equation (13) gives a density of $1{\cdot}129$ for ^2H_2O in the cell cytoplasm, as contrasted to the bulk value at $1{\cdot}105$. The corresponding values for $H_2{}^{18}O$ are $1{\cdot}139$ and $1{\cdot}124$, respectively.

There is one possible way to estimate the density of the cell water, namely, from the regression line in Fig. 4 according to equation (12). We are, of course, aware of that the value of $1{\cdot}015$ thus obtained is highly uncertain. Nevertheless, if it is accepted, the value for cytoplasmic ^2H_2O is $1{\cdot}146$ and for cytoplasmic $H_2{}^{18}O$ it is $1{\cdot}156$. And it should be recalled that these values are the averages for the total water content, which implies that if our values represent phases of structured water these should have even higher densities.

The only value for the density of intracellular water recorded in the literature is that published by Pocsik (1967). Working with frog muscle, this author estimated

the density as a function of the water content, registering an increase from 1·01 at 80% water to 1·33 at 5% water. The former value agrees with our result. However, it should be observed that these experiments were not carried out on living tissue.

A number of observations have been made on the effect of ^2H_2O on biological systems. For example Ussing (1935) found that the development of frogs eggs was retarded at 20% ^2H_2O and inhibited at concentrations higher than 30%. Garby & Nordqvist (1955) noted a decrease in the conduction velocity in isolated frog nerves. Gross & Spindel (1960*a*, *b*) studied the influence of ^2H_2O on cell division of sea-urchin eggs and found that it was blocked at concentrations higher than 75%. If the cells were not kept too long in the deuterium-rich medium, the block was reversible. In a study of the temperature-tolerance of adult *Drosophila* flies, Pittendrigh & Cosbey (1973) found that ^2H_2O increased the heat resistance of the flies. All these experiments have been interpreted as the result of a stabilizing effect of ^2H_2O on various biological macromolecules. If this stabilization follows from the isotopic water being more structured, more strongly bound to cytoplasmic components, and if this structuring is associated with an increase in density, then our result showing a relatively higher density for ^2H_2O than for H_2O constitutes a corroboration of the interpretation of these previous observations.

In several articles (e.g. 1971, 1973, 1976) Drost-Hansen has discussed the structural and functional aspects of water near biological interfaces. The properties of this vicinal water appear to be distinctly different from those of bulk water, reflecting structural differences. He suggests that possible candidates for this vicinal water are clathrate hydrates and high pressure ice polymorphs, all of which have densities higher than 1. In his opinion these vicinal structures extend from a minimum of 0·01 up to 0·1 μm from the interface.

Many indirect observations thus suggest that cell water has properties different from bulk water and that it is structured. If this is true, it might have a density different from bulk water. We have tested this possibility and found it corroborated. But we are the first to admit that our method as well as our results are open to severe criticism. However, we feel justified in publishing this work since it is the first attempt to measure the density of water in a living cell.

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