# On the Density of Intracellular Water

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*ABSTRACT:* The density of individual *Artemia* cysts has been determined by sedimentation velocity measurements at unit gravity. Dried cyst (< 0.02 g $H_2O$/g dry weight) densities, $\rho_S$, were obtained by successive sedimentation in two nonpenetrating organic solvents. This removes geometric terms from the equation relating density to sedimentation velocity. Hydrated cysts (≈ 1.68 g $H_2O$/g dry weight) were sedimented in 0.0750 m NaCl to obtain their density ($\rho_C$). Values of $\rho_S$, $\rho_C$, and their ratios were found to be independent of cyst volume; therefore, the weight fraction of water in hydrated cysts is very nearly the same in cysts of greatly different size. It can be concluded that measurement of the water content of large populations of these cysts accurately reflects the water content of individual cysts, a point which has been assumed in previous work on this system. If $\rho_S$ does not change appreciably when dried cysts are fully hydrated then the density of their water, $\rho_W$, can be calculated to be 1.022 g/cm$^3$ (±0.0011 $\sigma$). That value is significantly higher than the density of pure water and is very close to estimates of $\rho_W$ in skeletal muscle and amphibian oocytes obtained by others. However, the assumption that $\rho_S$ is independent of hydration is open to serious criticism, for all these studies. Consequently, conclusions and interpretations derived from such measurements must be considered to be tentative and uncertain.

## INTRODUCTION

ALTHOUGH MOST RESEARCH on the physical properties of intracellular water has involved the use of nuclear magnetic resonance (NMR) spectroscopy (for recent reviews see Hazlewood, 1979; Finch, 1979; Mathur-DeVré, 1979; Ratković, 1981; and papers by Burnell, et al., 1981; Seitz, et al., 1981), a significant amount of information has also been obtained from microwave dielectrics (see Schwan and Foster, 1977; Grant, et al., 1978; Pethig, 1979; Foster, et al., 1980), differential scanning calorimetry (see Aubin, et al., 1978; Brown and Sturtevant, 1980) and solute distribution methods (see Paine and Horowitz, 1980; Hempling, 1981; Ling and Negendank, 1980; Ling, 1981; Garlid, 1979; Horowitz and Pearson, 1981). In spite of this research effort, the current status of the subject is one of considerable disagreement, and very different interpretations have been applied to the data, notably from NMR. These various views and their physiological consequences have been described in recent books edited by Keith (1979), Drost-Hansen and Clegg (1979), and Franks (1982).

Only two previous studies have been caried out on the density of cellular water: Pocsik (1967) who used rat skeletal muscle, and Hansson Mild, et al. (1979) and Hansson Mild and Løvtrup (1981), who studied amphibian oocytes.

It was concluded that the average density of cellular water in these systems exceeded that of pure water by about two percent. Such observations are important because they indicate that either all the water in these cells exhibits an increase in density compared with pure water, or that an appreciable fraction must do so.

The present paper will describe density measurements on single *Artemia* cysts, which, in principle, should also provide information on the density of their cell water. The results are very similar to those obtained from muscle and oocytes and, like those studies, can be interpreted to indicate the presence of water whose average density exceeds that of the pure liquid. However, such an interpretation requires that an important assumption be made about the density of the nonaqueous components in these systems; that assumption will be discussed in detail.

## MATERIALS AND METHODS

### Artemia Cysts

The cysts of *Artemia*, a primitive crustacean known as the brine shrimp, consists of about 4000 cells enclosed within a complex shell. The cells are capable of virtually complete but fully reversible dehydration, and they have been widely used as a model experimental system (Persoone, et al.,1980). When fully hydrated the cysts are spheres, roughly 225 $\mu$m in diameter for the population used in this study.

The outer layer of the shell, the tertiary envelope, can be removed by treatment with 0.5% sodium hypochlorite at $0^{\circ}$ C. (Nakanishi, et al., 1962) and all studies described here were done on such "decapsulated" cysts. The remaining cyst is essentially an inner cellular mass surrounded by a very thin protein-chitin cuticle which makes up less than 4% of the total volume, and even less of the total mass of the cyst. Therefore, the system beting studied here is an individual unit of about 4000 eucaryotic cells and the results can be interpreted in terms of cellular properties.

The cysts were purchased from San Francisco Bay Brand, Newark, California (diploid, bisexual), the probable date of collection being the summer of 1978. They were washed, processed, and stored as previously described (Clegg, 1974, 1978). About 90% of the population is viable based on production of nauplius larvae in sea water for 72 hr. at 23°C. Decapsulated cysts can also be reversibly dehydrated, although the viability is often reduced to about 70%.

### Hydrated Cyst Density ($\rho_c$)

The apparatus used to measure the density of individual cysts is shown in Figure 1. The center tube of the Liebig condenser ($\approx$ 40 cm in length, and $\approx$ 1 cm inner diameter) was filled with a 0.0750 m NaCl solution. The cysts were equilibrated in that same solution at $0^{\circ}$ C prior to use, and their water content determined by a gravimetric method (Clegg, 1974, 1978). Temperature control was maintained at 25 $\pm$ 0.05$^{\circ}$ C by a Haake heater circulator and a Sargent refrigeration unit. Cyst diameters were measured to $\pm$0.5 $\mu$m by using a Bausch & Lomb micrometer disc (13-16-15) calibrated with an American Optical Company micrometer scale (2mm, in units of 0.01 mm) at 100X magnification. Single cysts were handled under a dissecting microscope with a

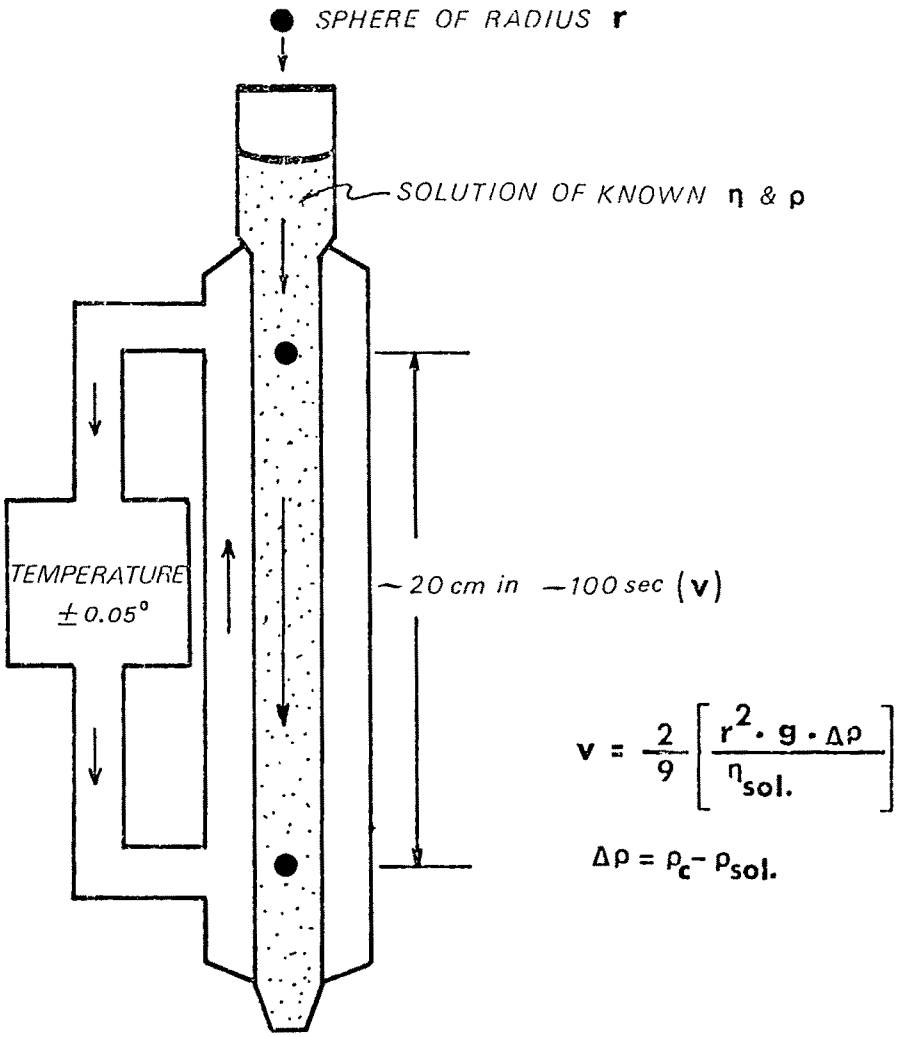


Fig. 1. Diagrammatic version of the apparatus used for sedimentation velocity measurements on *Artemia* cysts. The equation at the lower right yields the density of the cyst ($\rho_c$) using the velocity (v, in cm/s), the radius of the cyst (r, in cm). the force of gravity (g), the density ($\rho_{sol.}$, in g/cm$^3$) and the viscosity of the solution ($\eta_{sol.}$, in poise).

wood splinter, to which they adhere. Each cyst was transferred to the condenser inner tube and allowed to fall at least 5 cm through the solution before its sedimentation velocity (v) was measured using a cathetometer with a scale divided in units of 0.001 mm (Gaertner Scientific Corp., Chicago) and a stopwatch accurate to 0.05 s. Sedimentation velocity was shown to be constant as a function of distance over the 20 cm measured (Fig. 1). The density of individual cysts was calculated from the well known equation given in Fig. 1 which relates sedimentation velocity to sphere density. Values for the viscosity ($\eta_{sol.}$ = 0.00897 poise) and density ($\rho_{sol.}$ = 1.001 g/cm$^3$ of the NaCl solution were obtained from published values (Millero, 1970; Stokes and Mills, 1965), and g is the acceleration due to gravity, 980.7 cm sec$^{-2}$). Hence, the hydrated cyst density ($\rho_c$) was obtained by adding $\Delta\rho$ to the density of NaCl solution (Fig. 1).

### Dried Cyst Density ($\rho_s$)

Following sedimentation in the NaCl solution each cyst was recovered from the bottom of the apparatus (Fig. 1) and dried in a small cup placed in an individual desiccator (scintillation vial containing "anhydrous" $CaSO_4$). The cysts buckle when they dry, assuming the shape of an irregular, thick-walled soup bowl. The initial dehydration was performed at 2° C, to suppress what little metabolism occurs during water loss, followed by equilibration at 25° C for 3 days. Gravimetric measurements of the water content of 100-mg quantities of these $CaSO_4$-dried cysts showed that they contained less than 0.02 g $H_2O$/g dry mass, referred to as "g/g" for brevity.

Each dried cyst was then sedimented at unit gravity in two organic liquids using the same apparatus as for hydrated cysts. Of the large number of liquids examined, 1-bromooctane and acetophenone proved to be the most suitable. Their densities and viscosities were measured at 25° C using 25-ml pyrnometers and Ostwald vicometers, respectively: 1-bromooctane, $\rho$ = 1.114 g/cm$^3$, $\eta$ = 1.329 cp; acetophenone, $\rho$ = 1.024 g/cm$^3$, $\eta$ = 1.661 cp. These values are in reasonable agreement with published values (Timmermans, 1956; Weast, 1976). Each cyst was air dried after the first sedimentation run and equilibrated over $CaSO_4$ as usual. Initial experiments showed that the sequence in which the two liquids were used had negligible effect on the ratio of sedimentation velocities. The data presented here were obtained by initial sedimentation in 1-bromooctane, then in acetophenone. Evidence will be presented to show that these two liquids neither penetrated the cysts nor interacted with them in a way that would alter the velocity of their sedimentation.

The reason for using two liquids is that it allows the geometric terms ($2r^2/9$) to be eliminated from the sedimentation equation (Fig. 1) which, in that form, applies only to spheres. Thus, one obtains two sets of sedimentation values for the same cyst, and it is easy to show that when the sedimentation equation is divided by itself (for these two sets of data) that

$$\frac{V_1}{V_2} = \frac{\eta_2 (\rho_5 - \rho_1)}{\eta_1 (\rho_5 - \rho_2)} \tag{1}$$

where subscripts 1 and 2 refer to the two liquids employed. All terms are known except $\rho_s$, the dried-cyst density, which is the desired quantity.

## RESULTS

### Hydrated Cysts

Results from 47 hydrated cysts are shown in Figure 2. Several features are evident: there is no discernible dependence of cyst density on volume, as the density is independent of cyst radius; the densities are suprisingly uniform, the standard deviation being about 0.5% of the mean; the precision of the method is good. The value of 1.087 g/cm$^3$ for hydrated *Artemia* cysts is well within the range (1.02 to 1.12) observed for the densities of individual mammalian cells (Pretlow and Pretlow, 1974) and almost exactly the same as the density of unfertilized eggs of the amphibian *Ambystoma* at comparable water content (Hansson Mild, et al., 1979). This comparison further justifies the view that studies on decapsulated cysts can be considered to reflect properties of the cells themselves.

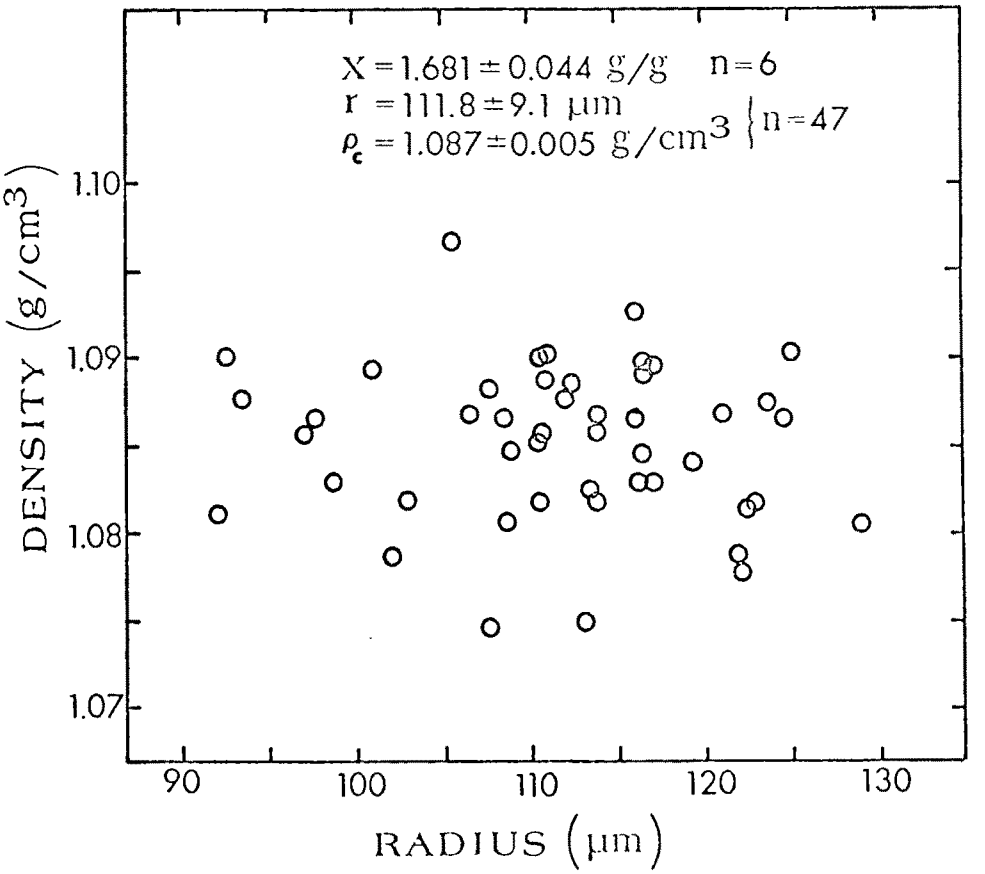


Fig. 2. The density of *Artemia* cysts as a function of cyst radius. Each point represents one cyst. At the top are shown the means (± their standard deviations) for cyst hydration (X), radius (r), and density ($\rho_c$).

It should be understood that the size range of the cysts examined in Figure 2 does not represent a random sampling of this cyst population. On the contrary, an effort was made to sample cysts across their entire size range. Thus, very few cysts in these populations exhibit the extremes of radius shown in Fig. 2; unpublished size distribution studies on large populations show that the mean decapsulated cyst radius is 105 $\mu$m at this water content, with a normal distribution about this mean.

## Density Measurements on the Same Cyst Hydrated and Dried

Figure 3 shows the outcome of studies in which the densities of hydrated and dried cysts were determined on the same individual. A total of 21 cysts was carried successfully through the three separate determinations required, one sedimentation velocity for the hydrated cyst on 0.075 m NaCl, and two for the dried ones in the organic liquids. The filled circles in Figure 3A and B represent a cyst whose ratio of $\rho_s/\rho_c$ was somewhat high (1.226) and is not represented in Fig. 3C; however, this cyst was included in calculations of the means and standard deviations also provided in this Figure.

Dried-cyst densities are evidently independent of their hydrated radius

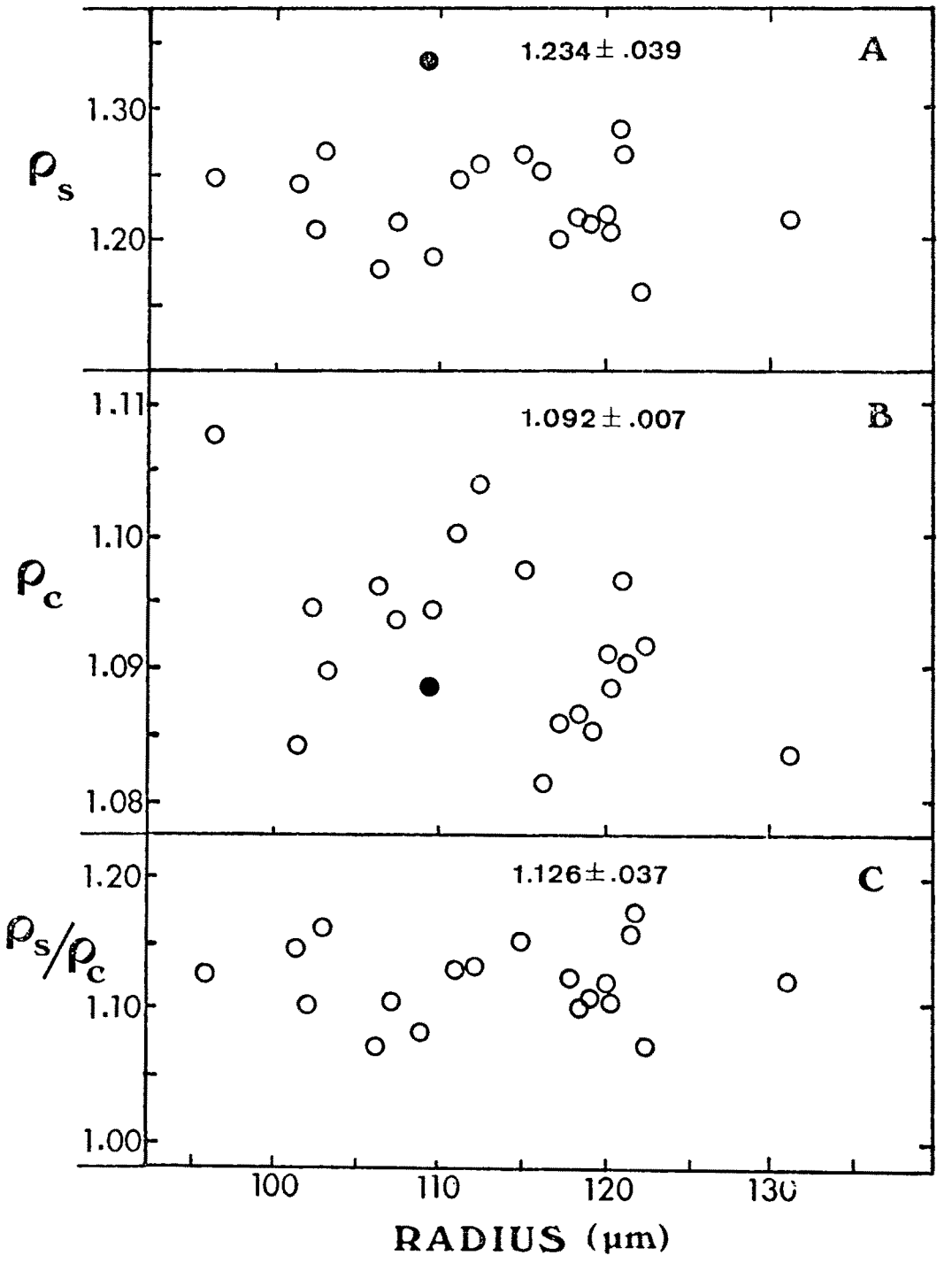


Fig. 3. Densities (g/cm$^3$) of the same dried ($\rho_s$) and hydrated ($\rho_c$) cyst as a function of the hydrated cyst radius. The numbers at the top refer to means ± standard deviations for 21 cysts. The filled circle represents a cyst whose ratio, $\rho_s/\rho_c$, was 1.226. That point is not shown in part C, but was included in the calculations.

(Fig. 3A). Of some significance is the observation that the ratio of dried to hydrated cyst density (Fig. 3C) is also independent of cyst size (i.e., hydrated radius). This demonstrates that the ratio of water mass to dry mass is much the same in cysts of very different size. For instance, the extremes in hydrated cyst volume for these data are 3.6 to 9.4 x $10^6$ $\mu m^3$, almost a threefold difference, but the ratio $\rho_s/\rho_c$ remains within a few percent of the mean throughout this volume range. Therefore, measurements of water content using large populations (hundreds of milligrams) accurately describe the water content of individual cysts.

## DISCUSSION

Because of their small mass—a dried cyst weighs roughly 3 $\mu g$, depending on the population (Clegg, 1974) -- it is not usually feasible to measure water contents of individual cysts unless $^3H_2O$ of high specific activity is used (Clegg, 1976). The latter study did indicate that measurements of water content on large populations of cysts reflect the hydration behavior of idividual cysts, but the errors were large and some uncertainty existed concerning "isotope effects" and variation in the size of idividual cysts. The density measurements in the present paper (Fig. 3) remove this uncertainty. The point is not trivial because water contents enter heavily into calculations on the density of water in these cells, which will be considered later in this discussion. Equally important, a number of other studies have been carried out on the physical properties of water in this system using NMR (Seitz, et al., 1980, 1981), sorption isotherms (Clegg, 1978), calorimetry (Clegg, 1979; Crowe, et al., 1981), microwave dielectrics (Clegg, et al., 1982), and quasi-elastic neutron scattering (Trantham, 1981). These studies require the use of large cyst populations, and previous interpretations of the data have essentially assumed uniformity and equivalency of water content in all individuals in the large sample studied. That assumption has been shown to be correct.

It is evident that results on the dried-cyst density ($\rho_s$) are valid only if the organic liquids do not penetrate the cyst or otherwise interfere with its sedimentation (i.e., cause shape changes). It has been shown that dried whole cysts are impermeable to a wide variety of organic liquids including acetone, ethyl ether, and xylene (Tazawa and Iwanami, 1974), absolute methanol, ethanol, 1-propanol, and 1-butanol (Smith and Siegel, 1975), and *n*-heptane (Clegg, 1981). That conclusion is based on the ability of cysts to remain in these liquids for days or even months without measurable effects on viability. The permeability barrier has been identified to be the cuticle, a thin part of the shell that remains on decapsulated cysts (Morris and Afzelius, 1967; DeChaffoy, et al., 1978). If these liquids penetrated the cells it is inconceivable that they would survive (Clegg, 1981). Further support for lack of penetration was obtained by intentionally abrading dried cysts and then observing their sedimentation behavior in the liquids. In these cases the cyst would begin to fall, but then slow down and swell, often then moving upward in the tube (Fig. 1) indicating penetration of liquid. Indeed, this was also observed in some of the cysts that had not been intentionally damaged; presumably damage had occurred during handling, processing, and drying of the hydrated cyst. In other words, it was quite obvious when liquid penetration had taken place. Microscopic observation of dried cysts in the two liquids showed that no detectable alteration in shape occurred, and that they sedimented similarly in these solvents (i.e., fell with the same morphological orientation). Therefore, we conclude that the data in Fig. 3 are reliable measures of dried-cyst density. These results will now be considered in the context of the density of water in these cells at their maximum hydration.

As mentioned, density measurements have been performed previously on skeletal muscle by Pócsik (1967) and on amphibian oocytes by Hansson Mild, et al. (1979) and Hansson Mild and Lǿvtrup (1981). In both cases the average density of the water in these systems was estimated to exceed that of pure water by one or two percent. If correct, that could have far-reaching cellular consequences. However, these authors employed calculations that either assumed, additivity of densities between the dry mass of the cells and the water present in them (Pócsik, 1967) or were based on indirect and uncertain values for $\rho_S$ (Hansson Mild and Lǿvtrup, 1981). Thus, it is well known that in the *absence* of interactions between components of a mixture (that is, when solutes are dissolved, two solutions are mixed, etc.), the following equation relates the densities of the components to that of the mixture (Moelwyn-Hughes, 1965):

$$\rho_C = \rho_S + (1 - \frac{\rho_S}{\rho_W}) \rho_C W_f \qquad (2)$$

We will use notation that applies to *Artemia* cysts: $\rho_W$ = the density of water present, $W_f$ = the weight fraction of total water, and the other densities apply to hydrated (c) and dried (s) cysts. Using experimental values and errors for $\rho_S$ and $\rho_C$ (Fig. 3) and a weight fraction of water 0.626 (= 1.672 g/g) we can calculate $\rho_W$ to be 1.022 g/cm$^3$ (± 0.011, accmulated $\sigma$). That value is significantly higher than bulk water density at 25° C (0.997 g/cm$^3$) and almost the same as $\rho_W$ for amphibian oocytes at comparable water content, also calculated by use of Eq. 2 (Hansson Mild and Lǿvtrup, 1981). However, it is clear that the assumption of additivity is open to very serious objections and that such calculations, and conclusions derived from them, are severely constrained as a result. For example, $\rho_W$ obviously depends upon the value of $\rho s$ that is used in the calculation. If the density of the nonaqueous components changes when the cysts are hydrated then the value of $\rho_S$ used in Eq. 2 will not be correct and will yield the wrong value for $\rho_W$. In fact, only a 5% increase in $\rho s$ upon hydration of the cysts will give $\rho_W$ equal to that of pure water at 25° C (0.997 g/cm$^3$ ). On the other hand, an increase in $\rho_S$ would indicate $\rho_W > 1.022$ g/cm$^3$. Without evidence for either increases or decreases in $\rho_S$, and in view of the fact that "volumes of mixing" can be positive for some solutes in water (expansion) and negative for others (contraction) and clearly of the order of 5% in both directions (Moelwyn-Hughes, 1965), no unambiguous conclusion can be drawn on the density of cell water in any system using such experimental values of $\rho_S$ and $\rho_C$.

The dehydration susceptibility of *Artemia* allows us to examine the problem in another way. If $\rho_C$ is measured as a function of water content (weight fraction) then a plot of $\rho_C$ vs. $\rho_C$ ($W_f$) should be linear over the entire range only if additivity applies (Moelwyn-Hughes, 1965). In that case, Eq. 2 would be valid and the value of $\rho_W$ thus obtained is likely to be correct. On the other hand, any nonlinearity in this relationship will reveal interactions that, for example, could result from hydration of the nonaqueous components. These studies are in progress. For the moment, however, it seems necessary to conclude that firm evidence for the value of the average density of water in any living cell has yet to be obtained.

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