THE NATURE AND ORIGIN OF CHEMICAL SHIFT FOR INTRACELLULAR WATER NUCLEI IN *ARTEMIA* CYSTS

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ABSTRACT We investigated the possible existence of chemical shift of water nuclei in *Artemia* cysts using high resolution nuclear magnetic resonance (NMR) methods. The results conducted at 60, 200, and 500 MHz revealed an unusually large chemical shift for intracellular water protons. After correcting for bulk susceptibility effects, a residual downfield chemical shift of 0.11 ppm was observed in fully hydrated cysts. Similar results have been observed for the deuterium and ^{17}O nuclei.

We have ruled out unusual intracellular pH, diamagnetic susceptibility of intracellular water, or interaction of water molecules with lipids, glycerol, and/or trehalose as possible origins of the residual chemical shift. We conclude that the residual chemical shift observed for water nuclei (^{1}H, ^{2}H, and ^{17}O) is due to significant water–macromolecular interactions.

INTRODUCTION

To gain further insight into the role of intracellular water in cellular function, a number of studies have been conducted on the physical properties of water in biological systems (1–5). Nuclear magnetic resonance (NMR) spectroscopy is one technique that has been widely used. It is well established that the relaxation times (spin-lattice relaxation time, T_1, and spin-spin relaxation time, T_2) and self-diffusion coefficients of cellular water protons, and other nuclei are reduced compared with the corresponding values for water in aqueous solutions (4, 5). These reduced NMR parameters for intracellular water nuclei have been considered evidence that water–macromolecular interactions play a significant role in cellular function (4). Little work concerning chemical shift has been reported for intracellular water protons or other nuclei in intact biological systems. Fritz and Swift have described a chemical shift of 0.05 ppm in frog sciatic nerve relative to the water proton signal in Ringer's solution (6). An upfield chemical shift of 0.1 ppm for protons in breast tumor culture cells has been reported by Frazer et al. (7). Although the origin of these shifts is not understood, they may be accounted for by bulk susceptibility effects (8, 9).

Cysts of the brine shrimp *Artemia* are capable of undergoing hydration–dehydration changes without losing significant viability (9–19). Thus *Artemia* is an ideal biological model for the investigation of the properties of water and the role of water in cellular function (9–19). In an earlier article (9), we reported the observation of a small residual downfield chemical shift of 0.11 ppm for intracellular water protons in *Artemia* cysts after correction for bulk susceptibility effects. We considered various interpretations for this residual chemical shift and concluded that it might be because of either specific water–macromolecular interactions or the interaction of water with paramagnetic metal ions in metalloproteins. In the current paper we present additional studies concerning the nature and origin of this shift using multinuclear NMR, electron paramagnetic resonance (EPR), and x-ray electron microprobe elemental analysis techniques. Our investigations suggest that the residual chemical shift observed for intracellular water nuclei may be attributed to specific water–macromolecular interactions in this intact living system.

MATERIALS AND METHODS

Processing of *Artemia* Cysts

Artemia have been well studied. This primitive crustacean produces encysted embryos or cysts with an average diameter of 0.2 mm that enter dormancy when dehydrated. Each cyst consists of ~4,000 closely packed, morphologically similar cells surrounded by a complex noncellular shell. The details of ultrastructure (13, 14), development (12), and biochemistry (11, 12, 15) are well documented. The cysts used in our studies were purchased from San Francisco Bay Brand, Menlo Park, CA and processed according to procedures described by Clegg (19).

Procedures for Hydration of Cysts

The hydrations reported here are generally >1 g of H_2O per g of dry cysts (hereafter referred to as g/g for the sake of brevity). For some of our

studies the cysts were hydrated overnight at 4°C and then dried in air for varying time periods. In other studies hydrations were obtained by immersing the cysts in NaCl solutions of different molarity. Because the cysts are impermeable to inorganic solutes and nonvolatile organic molecules, the water content in the cysts can be determined by water activity in NaCl solutions.

In one study the outer region of the shell enclosing the cysts, the 6-μm chorion layer was removed (dechorionated) by the established procedures of NaOCl treatment (11, 20). The procedure, conducted at ice bath temperatures, leaves the cells of each cyst intact and surrounded by a thin envelope (<2 μm) layer. The cysts with the shell (hereafter referred to as whole cysts) are brownish in color; with the removal of the chorion layer they are yellowish orange.

The procedures used for the study of D_2O and $H_2{}^{17}O$ were identical to those used in the H_2O studies. In the $H_2{}^{17}O$ studies the molecule was 50% enriched with ^{17}O.

NMR Experiments

Three NMR spectrometers operating at 60, 200, and 500 MHz were used in this study. The 60-MHz instrument is a Varian EM-360 NMR spectrometer located at M. D. Anderson Hospital and Tumor Institute which uses a permanent magnet (Varian Associates, Instruments Division, Palo Alto, CA) at a probe temperature of 32°C in an unlocked mode. The 200-MHz high resolution proton NMR spectrometer is a Varian XL-200 Fourier transform (FT) NMR spectrometer (with superconducting magnet), located at Exxon Research and Engineering Company, Baytown, TX. The probe temperature was held at 20° or 32°C and the proton resonance frequency was 200.057 MHz. A pulse width of 1 μs (10°), an acquisition time of 2 s, a spectral width of 4,000 Hz, and line broadening of 0.5 Hz were used. (Insertion of the water sample before and after each experiment showed that the spectrometer–magnet system was stable to better than 1 Hz over several hours running unlocked.) The 500-MHz proton spectra were obtained on a NT-500 spectrometer (Nicolet Instrument Corp., Madison, WI) located in The Laboratory of Chemical Physics at The National Institutes of Health, Bethesda, MD.

The samples were contained in either 5-mm NMR tubes or flat-bottomed 5-mm outside diam coaxial tubes (Wilmad Glass Co., Inc., Buena, NJ). DSS (sodium, 2, 2-dimethyl-2-silapentane-5-sulfonate) dissolved in D_2O was placed in the outer coaxial tube and used as the external reference. The hydrated whole cysts or dechorionated cysts were packed closely in the coaxial NMR tubes, thus forming a cylindrical geometry and maximum packing.

^2H and ^{17}O High Resolution NMR Experiments. ^2H and ^{17}O high resolution spectra from the hydrated cysts were obtained at 30.711 and 27.122 MHz, respectively, using the Varian XL-200 high resolution FT NMR spectrometer. The whole or dechorionated cysts were packed closely in the inner tube of a coaxial cell (No. WGS-10 BL; Wilmad Glass Co.). In the case of the deuterium NMR experiments, the outer tube contained D_2O and the chemical shift of ^2H of intracellular water in the cysts was referenced with respect to ^2H in pure D_2O. However, the ^{17}O experiments had to be conducted differently; acetone was placed in the outer tube and the chemical shift of acetone relative to bulk water was determined (569.74 ppm). The chemical shift of $H_2{}^{17}O$ in the cysts was then determined relative to ^{17}O in the acetone.

EPR Experiments

EPR spectra in whole cysts (wet and dry), and dechorionated cysts have been recorded using an EPR spectrometer (model No. E-12; Varian Associates). The cysts (whole or dechorionated) are packed loosely in standard EPR quartz tubes (4-mm diam).

X-Ray Electron Microprobe Elemental Analysis

Elemental analysis on individual cysts was determined by x-ray microprobe analysis using a Bausch & Lomb Inc. (Rochester, NY) model SEM-Q. Elemental content was determined in selected regions (~20-m diam) of the cyst using standard procedures. We measured x-ray pulse height distribution in the range of 0–10.24 KeV, with a resolution of 10 eV/channel. The analysis was done on whole and dechorionated dry cysts and was standardless. Therefore the quantitative comparison between the whole and dechorionated cysts is relative.

RESULTS

Proton High Resolution NMR of Whole Cysts

The proton high resolution NMR spectra of whole cysts at 60, 200, and 500 MHz are displayed in Fig. 1. In an earlier article we described in detail the observed chemical shift of the cyst water at 60 and 200 MHz (reference 9, pp. 7–10).

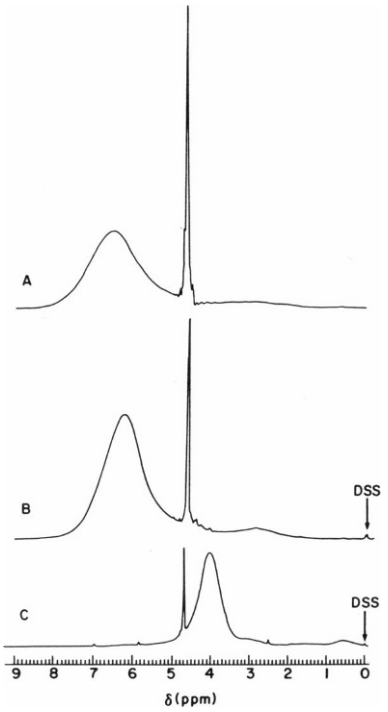

FIGURE 1 Proton high resolution NMR spectra at 32°C of *Artemia* cysts (with shell) hydrated in water (hydration 1.34 g/g) shown at (*A*) 500 MHz using a superconducting magnet, (*B*) 200 MHz using a superconducting magnet, and (*C*) 60 MHz using a permanent magnet. Spectra at 500 and 200 MHz were obtained using pulse Fourier transform techniques; spectra at 60 MHz were obtained using continuous wave techniques. Spectal parameters for the 60 MHz are as follows: RF power 0.05 mG; sweep time, 5 min; filter, open. The settings for the spectra at 200 MHz are as follows: spectral width, 4,000 Hz; acquisition time, 2.0 s; pulse width, 1 s; the number of transients, 200. The parameters for the 500-MHz spectra are similar to those used to obtain 200-MHz spectra. The spectra were obtained using 5-mm flat-bottomed coaxial NMR tubes; the cysts were contained in the inner tube while the outer tube contained a mixture of H_2O and D_2O dissolved with DSS.

We reported our observation of the bulk magnetic susceptibility effect resulting from differing sample geometries and evaluated the correction term for intracellular water proton in whole cysts. After correction for the susceptibility effect, a residual chemical shift of 0.11 ppm downfield was observed. Additional experiments at 500 MHz have also shown a chemical shift of +1.70 ppm for intracyst water with reference to pure bulk water at 32°C and a line width of 1.21 ppm as measured on two 500-MHz spectrometers using superconducting magnets (Fig. 1 *A*). The nearly equal line widths for the intracellular water resonance lines at 200 and 500 MHz, in comparison with the line width of 0.64 ppm at 60 MHz (reference 9, Table 1), lend strength to the argument that the line width differences are due to broadening by magnetic susceptibility of the cysts rather than any other mechanism.

Proton High Resolution NMR of Dechorionated Cysts

To trace the origin and understand the nature of the chemical shift, we have investigated the proton high resolution NMR of decapsulated cysts. This is shown in Fig. 2; the full line width at half maximum ($\Delta \nu_{1/2}$), and the chemical shift data for intracellular water resonance from dechorionated cysts are displayed in Table I. As in the case of whole cysts (9), the water resonance line occurs at 0.66 ppm upfield with reference to bulk water resonance at 60 MHz, while at 200 MHz the water resonance occurs at 1.63 ppm downfield. Similar to whole cyst behavior, the line width (1.99 ppm) of the water resonance at 200 MHz is greater than that observed at 60 MHz (1.04 ppm). Another notable feature is that the water resonance of dechorionated cysts is much broader than that of whole cysts.

Using the correction equations presented in an earlier article (reference 9, p. 9), we evaluated the susceptibility correction for dechorionated cysts as shown in Table I.

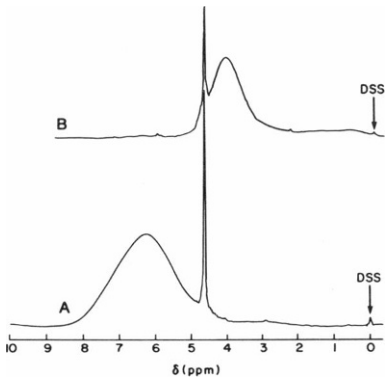

FIGURE 2 Proton high resolution NMR spectra at 32°C of dechorionated cysts (*A*) at 200 MHz and (*B*) at 60 MHz. Average hydration of the cysts in both experiments is 1.41 g/g. Other parameters are the same as in Fig. 1.

The $\Delta\Psi$ for dechorionated cysts was identical to that reported for whole cysts (9). Even in dechorionated cysts there was a residual chemical shift of +0.108 ppm for intracellular water protons after the susceptibility correction. The observed shift for intracellular water may arise from paramagnetic susceptibility effects and/or other interactions of water molecules with the cyst constituents within the cysts. The shell does not appear to contribute to the observed shift of the water proton signal.

EPR

To detect any paramagnetic metal ions (such as Fe^{3+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}) and/or paramagnetic free radicals contributing to the observed large chemical shift for water, we conducted a limited EPR study of the cysts. We were able to detect several EPR signals at room temperature. In Fig. 3, EPR spectra from cysts obtained in the $g = 4$ region are presented. In dried cysts, we detected a strong signal with a g-value of ~4.64 that was attributable to iron on the basis of similar signals for iron observed in tissues (21). The reduced signal in wet cysts is due to reduced sensitivity of the EPR cavity in the presence of water. In dechorionated cysts the EPR signal due to iron is barely detectable (Fig. 3 *C*). That this signal is due to iron is further supported by the x-ray microprobe elemental analysis, which is discussed later in this paper.

Fig. 4 shows the EPR spectra in the $g = 2$ region for these cysts. Two predominant EPR signals were detected in dry whole cysts, one was a six-line hyperfine pattern attributed to Mn^{2+}, and the other, a strong narrow signal with $g = 2.01$. The origin of this narrow signal could not be identified positively. It is tentatively thought to be due to ascorbic acid free radical because the cysts are known to contain significant amounts of ascorbic acid (22). Even in dechorionated wet cysts both the signal attributed to Mn^{2+} and the narrow signal could be detected, although it was reduced as compared with wet whole cysts.

Electron Probe X-Ray Microanalysis

Elemental analyses were made on individual whole and dechorionated cysts to detect the presence of paramagnetic ions, such as Fe^{3+} and/or Co^{2+}, that could cause the observed chemical shift. The only paramagnetic metal ion that was detected in whole cysts was iron. Iron could not be detected in dechorionated cysts.

Deuterium and ^{17}O High Resolution NMR Studies

The deuterium high resolution NMR spectra of whole cysts hydrated in D_2O are presented in Fig. 5. In these preliminary studies, the spectra were obtained at 20°C on cysts at different hydrations. (Upon the suggestion of the referees, the D_2O study was repeated at 32°C some four years later. No temperature effect was observed.) As can

TABLE I
PROTON LINE WIDTH AND CHEMICAL SHIFT DATA FOR INTRACELLULAR WATER IN *ARTEMIA* (32°C) (DECHORIONATED CYSTS)*

Sample No.	Full width at half-maximum		$\delta_{obs} - \delta_{H_2O}$ at 200 MHz	$\delta_{obs} - \delta_{H_2O}$‡ at 60 MHz	$\Delta\Psi$§	$\delta_{corr} - \delta_{H_2O}$ for cyst water
	60 MHz	200 MHz				
	Hz (ppm)		*ppm*	*ppm*	*ppm*	*ppm*
1	63 (1.05)	394 (1.97)	+1.62	−0.70	+0.369	+0.072
2	59 (0.98)	374 (1.87)	+1.62	−0.60	+0.354	+0.141
3	65 (1.08)	427 (2.14)	+1.66	−0.68	+0.373	+0.11
						Average = +0.108 ppm

*Average hydration is 1.41 g/g for the cysts. The chemical shifts were measured with reference to pure water.
‡δ_{H_2O}, 4.70 ppm for pure bulk water at this temperature measured under similar conditions at both frequencies and with reference to DSS.
§$\Delta\Psi = \Psi_v^{ref} - \Psi_v$, the difference in the volume susceptibilities of the reference and the sample (i.e., the cysts).

be seen in Fig. 5 and Table II, the chemical shift for the deuterium nuclei of cyst water is similar to that observed for protons. The line widths are larger than for protons, as would be expected due to quadrupolar interactions of the deuterium nucleus. We assume that the same susceptibility correction obtained from our study of protons can be used for deuterium. Such an assumption is justified, because the bulk susceptibility effect would be the same for the different nuclei in the same molecule. Under these conditions we recorded a residual chemical shift of +0.084 ppm to +0.26 ppm. The difference in the chemical shift for the different hydrations is probably due to experimental uncertainty in the determination of the chemical shift of the broad resonances observed for quadrupolar nuclei.

The ^{17}O ($I = 5/2$) high resolution NMR spectrum from whole cysts hydrated with $H_2{}^{17}O$ (50% enriched) was obtained. The chemical shift of the ^{17}O nuclei in the cysts, relative to bulk water, is presented in Table III for four different hydrations. As has been observed for ^1H and ^2H resonances, the line width increases as the hydration is decreased. However, the line width for the ^{17}O nucleus is much greater than that observed for the ^1H and ^2H resonances because of the nonaveraging of the quadrupolar interactions of the intracellular water molecules with the cellular constituents. The chemical shift for the ^{17}O nucleus of the cyst water is ~2.70 ppm downfield from that of bulk water. The variation in the chemical shift for the four different hydrations is because of experimental uncertainty (±0.3 ppm) in the determination of the ^{17}O chemical shifts in the cysts. As has been observed for cyst water protons, the chemical shift is independent of the hydration in this range. Again, assuming the susceptibility correction to be the same for the ^{17}O chemical shift as for protons, we observed a residual chemical shift in the whole cysts of +1.16 ppm downfield from pure water.

The ^{17}O nucleus chemical shift for intracellular water in dechorionated cysts is presented in Table IV. It is to be noted that the line width of the ^{17}O resonance from dechorionated cysts is slightly larger than that from whole cysts, confirming the similar observation for cyst water protons.

The chemical shift for the ^{17}O nucleus in dechorionated cyst water uncorrected for bulk susceptibility effect is +1.89 ppm downfield from bulk water resonance. Using

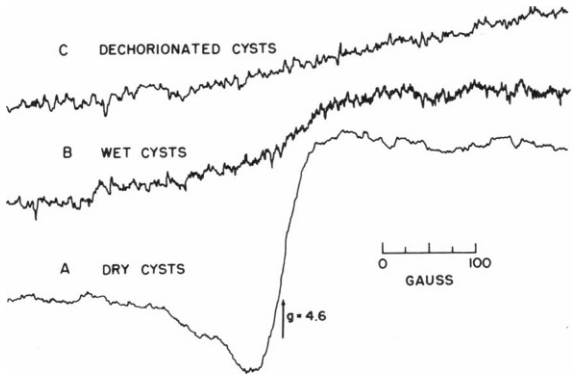

FIGURE 3 Electron paramagnetic resonance signal of the cysts at room temperature, 9.100 GHz (*A*) dry cysts, (*B*) wet whole cysts (hydration, 1.31 g/g), and (*C*) wet dechorionated cysts (hydration, 1.44 g/g). The field is calibrated with a weak pitch signal ($g = 2.0032$). The central field setting was 1,400 G with a modulation amplitude of 10 G and frequency of 10^2 KHz. A microwave power of 20 mwt was used. Receiver settings are as follows: (*A*) 5×10^3, (*B*) 10×10^3, and (*C*) 10×10^3.

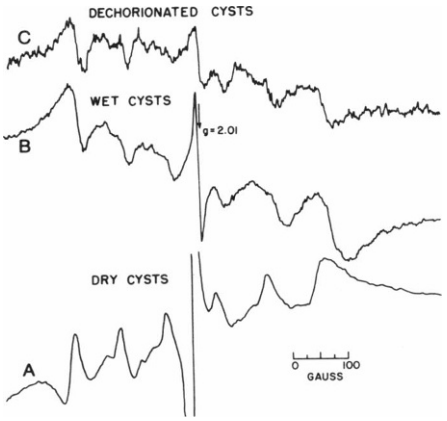

FIGURE 4 EPR signal of the same cysts as in Fig. 3 in the $g = 2$ region (central field set at 3,195 G). Receiver gain settings were (*A*) 1.25×10^3, (*B*) 5×10^3, and (*C*) 1.25×10^4.

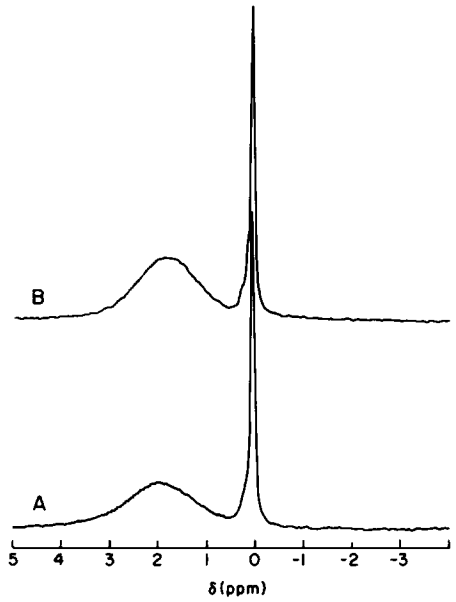

FIGURE 5 Deuterium NMR spectra at 30.71 MHz and 20°C in whole cysts hydrated in D_2O for hydrations (*A*) 1.01 *g* of D_2O/*g* of dry cysts and (*B*) 1.34 *g* of D_2O/*g* of dry cysts.

the correction term of $\Delta\Psi = +0.365$ ppm evaluated from ^{1}H-NMR experiments on dechorionated cysts, a residual chemical shift of +0.36 ppm downfield from that of bulk water resonance is obtained. However, difference in the residual chemical shift obtained for the ^{17}O resonance in the cyst water of whole and dechorionated cysts is probably not significant because of the large experimental uncertainty (±0.3 ppm) in the determination of the ^{17}O chemical shifts in this system.

TABLE II
DEUTERIUM NMR CHEMICAL SHIFTS AND LINE WIDTHS OF INTRACELLULAR WATER IN *ARTEMIA* CYSTS (20° and 32°C) AT 30.71 MHz

Sample No.	Temp.	Hydration	Line widths ($\Delta\nu_{1/2}$)	Observed chemical shift from pure D_2O
	°C	*g of D_2O/g of dry cysts*	*Hz (ppm)*	*ppm*
1	20	1.34	45 (1.47)	+1.62*
2	20	1.01	49 (1.67)	+1.80‡
3	32		46 (1.51)	+1.71§
4	32	1.4‖	47 (1.54)	+1.79§
5	32		46 (1.51)	+1.77§
6	32		46 (1.51)	+1.73§

*The chemical shift for deuterium nucleus in D_2O hydrated cysts corrected for susceptibility effects is +0.084 ppm. The correction is carried out using the same value of $\Delta\Psi = +0.367$ ppm evaluated from proton NMR experiments (9). Also Table I.

‡The chemical shift for deuterium nucleus in D_2O hydrated cysts corrected for susceptibility effects is +0.263 ppm. The correction is carried out using the same value as in the above footnote.

§The average shift for deuterium nucleus in D_2O hydrated cysts corrected for susceptibility effects is +0.213 ± 0.102 (95% confidence level). The calculation is carried out using the same value as in the first footnote.

‖Samples 3–6 were taken from one large batch of cysts. The water content was determined on one sample from the same batch.

TABLE III
^{17}O LINE WIDTH AND CHEMICAL SHIFT* DATA FOR INTRACELLULAR WATER IN *ARTEMIA* (32°C) WHOLE CYSTS

Sample No.	Hydration	Full width at half-max ($\Delta\nu_{1/2}$)	$\delta_{obs} - \delta_{H_2}{}^{17}O$
	g of $H_2{}^{17}O$/g of dry cysts	*Hz (ppm)*	*ppm*
1	1.33	217 (8)	+2.94
2	1.14	271 (10)	+2.92
3	1.13	271 (10)	+2.60
4	0.97	325 (11.98)	+2.33
		Mean = +2.70 ppm	

*Chemical shift measured with reference to pure water. Using average $\Delta\Psi = +0.367$ ppm from ^{1}H-NMR experiments, one obtains $\delta_{corr} = +2.70 - 4\pi\Delta\Psi/3$. Hence $\delta_{corr} = 1.16$ ppm for ^{17}O nucleus in $H_2{}^{17}O$ of cyst water in whole cysts. (Downfield shift with reference to ^{17}O in pure water.)

DISCUSSION

It is evident from the multinuclear NMR studies described above that the water nuclei in the cysts exhibit a large chemical shift mainly due to bulk susceptibility effects. After correction for these effects, we observed net residual chemical shifts of +0.11 ppm for protons, +0.08 to +0.26 ppm for ^{2}H, and +0.36 to +1.16 ppm for ^{17}O. The residual chemical shift is downfield for all three nuclei, reflecting a true molecular interaction as the origin of the shift. The discussion that follows therefore addresses two questions: (*a*) What is the origin of the bulk susceptibility effect causing the chemical shifts for the nuclei of intracellular water, and (*b*) what is the nature and origin of the residual chemical shift observed for the nuclei after correction for the bulk susceptibility effect?

TABLE IV
^{17}O LINE WIDTH AND CHEMICAL SHIFT* DATA FOR INTRACELLULAR WATER IN *ARTEMIA* (32°C) (DECHORIONATED CYSTS)

Sample No.	Hydration	Full width at half-max ($\Delta\nu_{1/2}$)	$\delta_{obs} - \delta_{H_2}{}^{17}O$
	g of $H_2{}^{17}O$/g of dry cysts	*Hz (ppm)*	*ppm*
1	1.76	217 (8.0)	+1.63
2	1.24	243 (8.96)	+2.03
3	1.09	259 (9.55)	+2.03
		Mean = +1.89	

*Chemical shift measured with reference to ^{17}O nucleus in pure water. Using the average $\Delta\Psi = +0.365$ ppm for dechorionated cysts from ^{1}H-NMR experiments, one obtains $\delta_{corr} = 1.89 - (4\pi/3)\Delta\Psi$. Thus, $\delta_{corr} = +0.36$ ppm for ^{17}O nucleus in $H_2{}^{17}O$ of dechorionated cysts.

Bulk Magnetic Susceptibility Contribution to the Chemical Shift

As shown in Figs. 1, 2, and 5 and in Table I–IV, the bulk susceptibility effect to the observed chemical shift for nuclei is large; however, its origin is unclear. Bulk magnetic susceptibility effects may arise from the unusual diamagnetic susceptibility of cyst water and/or the paramagnetic susceptibility of cyst components. Since the chemical shift of the cyst water proton signal was shown to be independent of hydration, it has been suggested that diamagnetic effects are not operative (9). On the other hand, our EPR studies indicated the presence of iron, Mn^{2+}, and free radicals in the cysts (Figs. 3 and 4). Of these, iron is located primarily in the shell, as determined by our EPR analysis (Fig. 3) and our electron probe microanalysis. This conclusion is supported by other studies on elemental analysis using atomic absorption spectrometry and neutron activation analysis (23). Since the susceptibility effect is nearly equal in whole and dechorionated cysts (9), iron can be ruled out as a possible origin. This leaves the paramagnetic-free radicals and Mn^{2+} ions in the cellular mass of embryonic cysts as possible sources of the effect. The levels of other paramagnetic ions such as Ni^{2+} and Co^{2+} are about 3,000 times less in concentration as compared with that of iron (23). In solutions of Mn SO_4 it was found experimentally that a 1.23 mM solution gave a chemical shift of 0.1 ppm. This agreed with theoretical calculations based on the magnetic susceptibility data of the pure salt (CRC Handbook of Chemistry and Physics). We have two sources of data dealing with the concentrations of Mn^{++} in the brine shrimp. First comes from actual measurements of Mn^{++} concentration in a batch of brine shrimp from Dr. James S. Clegg. This atomic absorption analysis showed that cysts of the brine shrimp contains 185 μg Mn^{++}/g of Ash. This translates to 3.62 μg/g wet cysts or 66 μM Mn^{++} in the sample. The second piece of data comes from the analysis of the Mn^{++} signal in the EPR spectrum (Fig. 4 *B*) by Dr. Peter Gascoyne. From the area under the integrated spectrum it can be estimated that the *Artemia* cysts contain ~30 μM Mn^{++} for whole cysts. Making an $MnSO_4$ solution of the higher concentration (i.e., 66 μM Mn^{++}), we found that we could not experimentally detect a chemical shift. The calculations, on the other hand, predict a shift of 0.012 ppm, which is below our detection limit with these samples. Additionally in Fig. 4 it is clear that the bulk of the Mn^{++} resides in the shell. Removal of shell, however, had no effect on the chemical shift (see Table I and Fig. 2). From these measurements and calculations we can only conclude that Mn^{++} is not the source of the residual chemical shift.

Origin of the Residual Chemical Shift for Intracellular Water Nuclei

The residual chemical shift observed for water nuclei in these cysts could arise from the interaction of the water molecules with lipid molecules, paramagnetic ions, or macromolecules. The reliability of the measurement of the residual chemical shift observed for the water nuclei in the cysts has been evaluated in a prior communication and will not be discussed here (9).

Further support for the validity of Eqs. 1 and 2 in reference 9 for the *Artemia* system, and the reliability of the evaluation of $\Delta\Psi$ from these measurements is illustrated by the data shown in Table V for the lipid protons. The single broad peak corresponding to the lipid protons is broad and not as well defined as for the water proton peak. Despite this, the susceptibility correction term $\Delta\Psi$ evaluated from the chemical shifts measured at 60 and 200 MHz is shown in Table V and agrees well with the value of $\Delta\Psi$ (+0.367 ppm) for whole cysts evaluated earlier by us (9).

The chemical shift for water nuclei was measured using different spectrometers and using different sample configurations and experimental conditions. The observation of the same downfield shift for water nuclei under different conditions precludes systematic errors as the possible origin of the residual chemical shift. We also considered maladjustment of the phase as a possible source of error. It was found that such an error is within ±0.02 ppm for ^1H at 200 MHz where the lines are broad and within experimental variations for other nuclei. Because the water resonance line is very broad at 500 MHz, the residual chemical shift is calculated using the data at 60 and 200 MHz only. Thus a residual chemical shift due to molecular interactions of the water molecules with the cyst components seems to exist for the water nuclei in *Artemia* cysts.

Possible Mechanism for the Residual Chemical Shift

The water molecules in the cysts can interact with proteins (0.48 g/g dry wt), lipids (0.19 g/g dry wt), glycerol (0.04 g/g dry wt) and trehalose (0.12–0.15 g/g dry wt), all of which are major constituents of the cysts. The observed downfield shift for the water nuclei (^1H, ^2H, and ^{17}O) cannot be accounted for by the interaction of water

TABLE V
PROTON CHEMICAL SHIFTS OF THE PEAK CORRESPONDING TO LIPID PROTONS IN THE *ARTEMIA* CYSTS AT 32°C (WHOLE CYSTS)

Sample No.	$\delta_{observed}$		$\Delta\Psi$*	$\delta_{correct}$
	200 MHz‡	60 MHz		
	ppm		*ppm*	*ppm*
1	2.91	0.55	+0.376	1.34
2	2.90	0.53	+0.378	1.32
3	2.90	0.58	+0.370	1.35

*$\Delta\Psi$ has the same meaning as defined in Table I.
‡Chemical shift for the lipid proton peak measured at 500 MHz is 2.95 ppm.

molecules with lipids, because water molecules interacting with hydrocarbon chains exhibit upfield proton shifts (24, 25). Further, water molecules interacting with glycerol and/or with trehalose at the concentrations found in the cysts do not exhibit a chemical shift different from bulk water (unpublished results). This suggests that the observed downfield chemical shift may be due to the interaction of water molecules with macromolecules, which constitute the major constituents of the cysts (10). The evidence thus far obtained does not support the idea that the shift may be due to the interaction of water molecules with free cytoplasmic paramagnetic ions or paramagnetic ions bound to proteins. For example, the interaction of the water molecules with free paramagnetic ions in the cysts or in the metallo-enzymes seems to be ruled out because the chemical shifts of the cyst water peak were the same with or without shells. Therefore, the presence of iron in the shells did not affect the shift. Further, EPR and x-ray elemental analysis by the authors and extensive elemental analysis by Olney et al. (23) did not show the existence of significant levels of paramagnetic ions such as Ni^{2+} or Co^{2+}, which could also produce such a chemical shift. The levels of these ions are ~200–2,000 times lower than the levels of iron in the whole cysts. Therefore, the levels of paramagnetic ions found in these cysts do not appear to be sufficient to produce the observed residual chemical shift.

It is possible that the residual downfield chemical shift of ~0.11 ppm could arise from a specific interaction of water molecules with the proteins that constitute the major constituents of the cysts. Specifically, a downfield shift of the magnitude that has been observed for intracyst water proton and deuterium nuclei could arise from increased hydrogen-bond interactions (26, 27) of the water molecules with the surface of the proteins. Control experiments were carried out at 500 MHz and under identical conditions on a solution containing D_2O, glycerol, and bovine serum albumin. At the concentration similar to that found in *Artemia* cysts, we established the existence of a downfield shift of 0.12 to 0.14 ppm for water protons interacting with proteins (unpublished results). This was further supported by our observation of a similar large downfield shift of ~1.2 ppm for whole cysts and ~0.36 ppm in dechorionated cysts for ^{17}O in the intracyst water. The difference in the residual chemical shift observed between whole and dechorionated cysts for ^{17}O is probably not significant due to large experimental errors in the determination of chemical shift as explained earlier. Nevertheless, the downfield shift observed for ^{17}O nucleus is significant and much larger than that observed for ^{1}H or deuterium in cyst water. The effect of hydrogen bonding on the ^{17}O chemical shift is known to be large and specific (28). Thus, the residual chemical shift observed for the ^{17}O nucleus in cyst water could be attributed to increased hydrogen bonding, confirming the similar shift observed for cyst water protons.

In the case of water–protein interactions, there are two possible explanations for the small residual chemical shift observed for water nuclei interacting with proteins. First, such a chemical shift could be experienced by all of the water molecules in the cyst due to the interaction of all of these molecules with proteins in the cysts. Second, the shift may occur as the result of fast exchange between a small fraction of water molecules interacting with proteins and experiencing a larger chemical shift and the bulk of the water molecules that do not interact with proteins. Our present NMR data do not allow us to distinguish between the two possibilities. However, the observation of a chemical shift in water protons independent of hydration at a level as low as 0.4 g/g (9) supports the former hypothesis.

Comparison of this Study with Results from Other Studies of Water in *Artemia* Cyst Water

The physical properties of water in *Artemia* cysts have been investigated using other NMR techniques and additional physical techniques such as quasi-elastic neutron scattering (17), microwave dielectric (18), and density (29) measurements (18). In earlier reports of NMR studies of the water protons in *Artemia* it was found that both the spin lattice (T_1) and the spin–spin (T_2) relaxation times were reduced (compared with bulk water) when in the fully hydrated state (30). Although multiple interpretations of these data were proposed, none were unique. Measurements of the diffusion coefficient, in addition to the relaxation times, helped reduce the number of possible interpretations. The self-diffusion coefficient (D) of water molecules in *Artemia* cysts was measured over a wide range of hydrations (16). It was found that the diffusion coefficient of fully hydrated cysts was reduced by a factor of 7 as compared with that of bulk water. These findings were further supported by quasi-elastic neutron scattering measurements (17); both the translational and rotational diffusion coefficients of cellular water were found to be reduced relative to bulk water. These results are consistent with a picture in which the majority of the cell water has strongly reduced translational and rotational diffusion coefficients that are not due to obstructions, compartments, or exchange with minor phases; rather they are an intrinsic feature of the intracellular water (1, 4). Similarly, dielectric studies (18) of cysts containing 1 g/g dry wt have indicated that a significant fraction of the total water in *Artemia* cysts exhibits dielectric properties different from that of bulk water. Our observations of the residual chemical shift for water nuclei in this system are consistent with the findings of the dielectric density and quasi-elastic neutron scattering studies; the intracyst water of *Artemia* exhibits properties different from that of bulk water.

The authors acknowledge the helpful suggestions and advice on the handling of the *Artemia* cysts as well as the supply of high grade cysts from Prof. J. S. Clegg, Bodega Marine Laboratory, University of

California, Bodega Bay, CA. The authors also thank Dr. Duane Ingram of Baylor College of Medicine for the x-ray microprobe elemental analysis and Prof. Joel Morrisett of Baylor College of Medicine for kindly extending us the use of the EPR facilities. The authors thank Dr. Peter Gascoyne of the M.D. Anderson Hospital and Tumor Institute for taking time to make the calculations of the Mn^{++} concentrations in our samples. The authors thank Drs. Edwin Becker, Jack Cohen, and Chi-Wan Chen of the Laboratory of Chemical Physics for the use of the 500-MHz spectrometer.

This work was supported in part by the Office of Naval Research contract N00014-76-C-0100 and by Robert A. Welch Foundation grant Q-390. The authors are grateful for a private donation given in the memory of Stanley T. Weiner.

Received for publication 23 December 1985 and in final form 20 April 1987.

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