ON THE STATE OF WATER IN DEVELOPING MUSCLE: A STUDY OF THE MAJOR PHASE OF ORDERED WATER IN SKELETAL MUSCLE AND ITS RELATIONSHIP TO SODIUM CONCENTRATION

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Received for publication December 21, 1970

INTRODUCTION

The concentration of water in various biological tissues decreases during early postnatal development (1,2). In developing rat skeletal muscle, the decrease in water concentration (3) has been described as exponential (4), and is associated with changes in electrolyte concentrations and cellular potential (4,5,6,7). Attempts to understand the mechanisms of the age-dependent voltage changes and the associated changes in electrolyte concentrations led us to the conclusion that it was imperative to study further the physical chemical state of muscle water (8).

The application of nuclear magnetic resonance (NMR) spectroscopy in the study of tissues has produced evidence concerning the physical state of tissue water (9-16). NMR spectra show that muscle water exists in at least two order phases (14,15). These two phases are distinguished by the widths of their NMR signals and by deuterium exchange (15). The small (minor) phase of ordered water, defined operationally as that water in muscle tissue which does not exchange with D_2O in 24 hours, has been observed by using a wide line NMR spectrometer (15).

The larger (major) phase of muscle water, observed with high resolution NMR spectrometers, produces a signal ten times as broad as that of distilled water. Since the line width is a measure of the spin-spin relaxation time (T_2), the line broadening indicates that the correlation time of water molecules in the cellular environment is increased. This suggests that the motional freedom of the water molecules is restricted.

A number of artifacts can cause NMR line broadening, and these have been investigated in detail.

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1. Magnetic field inhomogeneity of the spectrometer. This was ruled out by checking the spectrometer with a pure water sample.
2. Nonuniform sample packing. This was observed to be a maximum of 2Hz, which is a small percentage of the total line width.
3. The mere presence of polymers dissolved in water. Investigations of agar and gelatin gels, blood plasma, protein solutions and denatured muscle samples produced NMR water signals comparable to those of pure water.
4. The adsorption of water molecules around free ions. Ionic solutions in physiological concentrations were found to have no effect on NMR water line width.
5. The presence of paramagnetic impurities. Water in muscle tissue can be exchanged with D_2O. The supernatant from minced muscle soaked in D_2O for extended periods of time showed no line broadening. In addition, denaturation of muscle samples caused a narrowing of the line width to almost that of pure water, indicating an insignificant concentration of paramagnetic material.

A detailed discussion of each of these potential artifacts has been published (15) and it has been concluded that the summation of all these effects is less than three Hertz (compared to 10-20 Hertz for various muscle tissues).

Evidence for the existence of ordered water also has been gathered using techniques other than NMR (17). By using a vapor equilibrium method, Ling (17) has studied the equilibrium water content of frog skeletal muscle over a wide range of relative vapor pressures. He concluded that 95% of muscle water is contained in an oriented multilayer fraction and that 5% exists in an even more tightly adsorbed fraction. Other data also are consistent with the concept of ordered water in skeletal muscle (18,19).

Previous work on developing rat skeletal muscle has suggested changes in cellular organization, particularly with respect to a protein-ion-water matrix (4,8,20,21). Joseph has shown that the dielectric constant for water (calculated from the chemical potential for sodium) decreased during normal postnatal development in tissues of various species (20,21). That is, with development (maturation), the tissue water should become more ordered. The present study of the NMR spectra of water in developing rat skeletal muscle leads us to suggest that there is an increase in the ordering of muscle water with maturation. We also report a correlation between the width of the NMR water signal and the tissue sodium concentration. A preliminary report of this work has been given (22).

METHODS

Houston Cheek rats used in this study were killed by cervical fracture. The hind limb musculature was removed and quickly dissected free of fat and gross connective tissue (23).

High Resolution NMR Method:

The muscle tissues were put in 5 mm diameter NMR sample tubes which were placed in the probe inserts of various high resolution NMR spectrometers made by

Varian.[3] Accurate measurement of changes in NMR line widths require the elimination of artifacts due to inhomogeneities in the muscles and in the packing of the tissue. All samples were placed in the NMR probe insert and spun, excluding the effects of inhomogeneities in the horizontal plane. Inhomogeneities along the axis of the sample tube were accounted for by obtaining two or more spectra at various positions along this axis. The width of the NMR water signals at one half amplitude was measured (15,24). The average value for each animal was taken then as the value for that age.

Pulse NMR Methods:

The spin-lattice relaxation time (T_2) of water molecules in muscle was measured with the spin-echo technique. The muscle sample (approximately 0.1 gram) was placed in the gap center of a twelve inch magnet with a flux density of approximately 7 kilogauss. A series of high power radio frequency (30 M Hz) pulses were applied to excite the sample. Two kinds of pulses were used in the experiment: the so-called "90^o pulse" has a pulse width of about 40μ sec, and the "180^o pulse" is twice as wide as the 90^o pulse. The echoes, which are the signals generated by the nuclear spins of the sample, were amplified 10^4 times and recorded on photographic films. In our experiment the Carr and Purcell method B plulse sequence (Figs 1 and 2) was used to measure T_2 (25). The spin-spin relaxation time was directly deduced from the echo decay constant. T_1 was measured with a 90^o-90^o-180^o pulse sequence (25). All measurements were taken at room temperature ($28^o \pm 2^o$ C). The influence of diffusion on the echo decay was determined by applying a controlled magnetic field gradient (Fig 2). It is concluded that the contribution of error due to the diffusion effect is negligible for the existing homogeneity of magnetic field. A

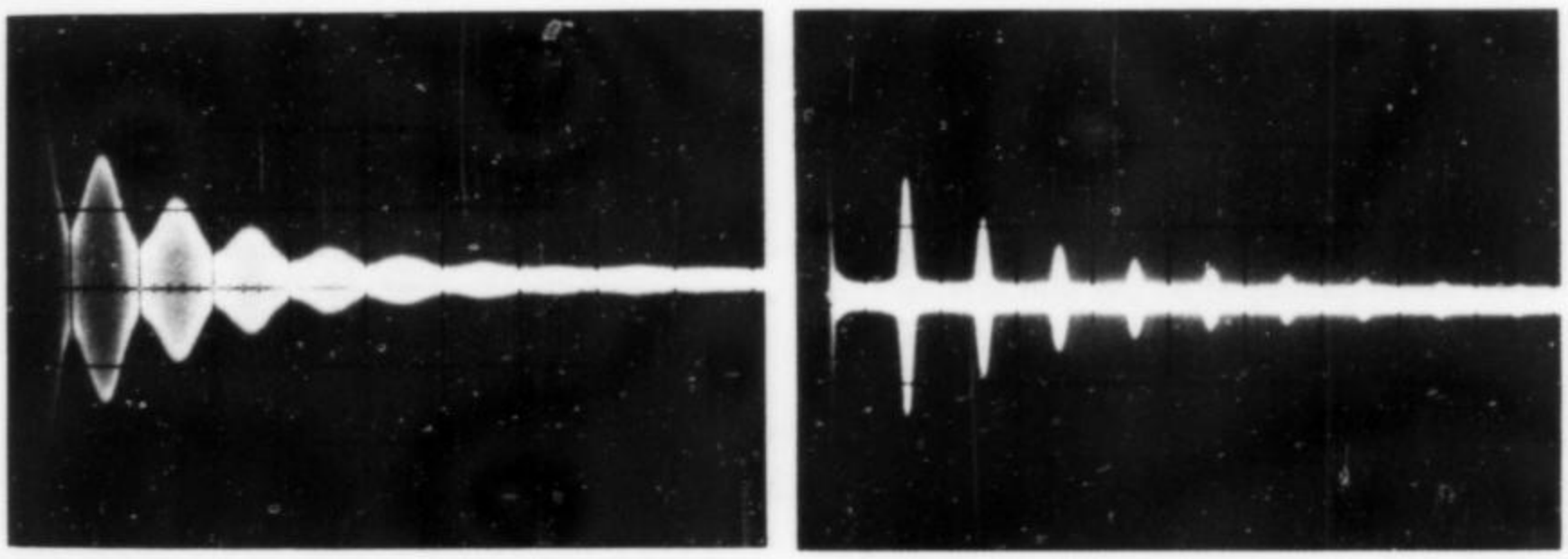

Fig 1. Echoes of muscle water with no applied magnetic field gradient. The first peak is a free induction decay. The other peaks are echoes. Between the echoes are the applied radio frequency pulses which are too narrow to be seen. Sweep Rate: 20 msec/division.

Fig 2. Same as Figure 1, except a 0.37 gauss/cm field gradient is applied.

[3]The high resolution NMR spectrometers were as follows: A60-A in the laboratory of Dr. Paul A. Srere, V. A. Hospital, Dallas, Texas; A60-A in the laboratory of Mr. N. F. Chamberlain, Esso Research and Engineering, Baytown, Texas; and A54/60-A and A60 in the Chemistry Department, Rice University, Houston, Texas.

detailed description of the pulsed NMR spectrometer employed in this study has been given by Chang (26).

RESULTS

NMR Study of Muscle Water:

The line widths of the NMR water signal were determined on skeletal muscles and plotted as a function of animal age. These data are presented in Figure 3. The signal widths increased from between 5 and 6 Hz at 2 to 4 days of age to 13 Hz between 30 and 40 days of age. A least squares analysis was performed to fit an exponential equation to these data. The dashed line in Figure 3 is the result.

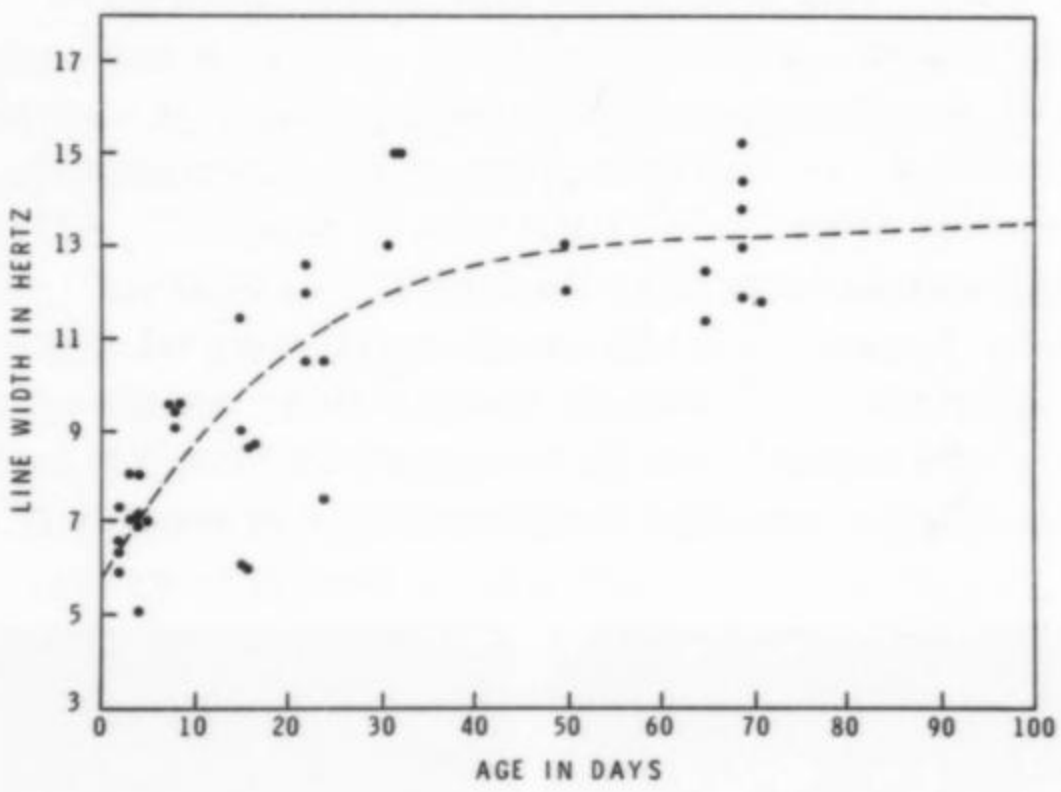

Fig 3. Ordinate: NMR line width in Hertz. Abscissa: animal age in days. The dashed line was determined from equation (1,b) and results from a least squares analysis.

In addition to the high resolution NMR absorption study, the water molecules were also investigated with pulsed NMR technique. The spin-lattice relaxation time (T_1) and spin-spin relaxation time (T_2) of proton in (cellular) muscle water were measured. The samples studied by this method are contained in the following categories: 1. skeletal muscle of rats whose age is less than ten days (this is referred as "immature muscle"). 2. skeletal muscle of rats whose age is larger than 40 days (this is referred to as "mature muscle"). The results of our measurements are given in Table I. The relaxation times of pure water were also measured and are listed in the same table. The results for pure water agree with the values published by Meiboom et al (27). Table I shows that T_1 and T_2 for both muscle samples are much shorter than the values for pure water. In addition, the relaxation times of immature muscle are on the order of two times longer than the relaxation times of mature muscle. These findings are consistent with the result of our high resolution NMR line width studies and agree in general with the notion that ordered water exists in muscle cell as suggested by other investigators (14,15,17).

TABLE I

Pulse NMR Measurements of Relaxation Times, T_1 and T_2, for Pure Water and Rat Skeletal Muscle Water

Sample	T_1 (sec)	T_2 (sec)
	(3)	(6)
Pure H_2O	2.97 ± 0.14	1.6 ± 0.11
	(6)	(6)
Mature Muscle	0.723 ± 0.049	0.047 ± 0.004
	(5)	(7)
Immature Muscle	1.206 ± 0.055	0.127 ± 0.009

NOTE: All values are given in seconds and are mean values ± standard error of the mean. Numbers in parentheses indicate number of samples analyzed.

Changes in Muscle Sodium with Development:

The changes in muscle sodium concentration have been reported in Table I of a previous publication (4). These findings are summarized in Figure 4.

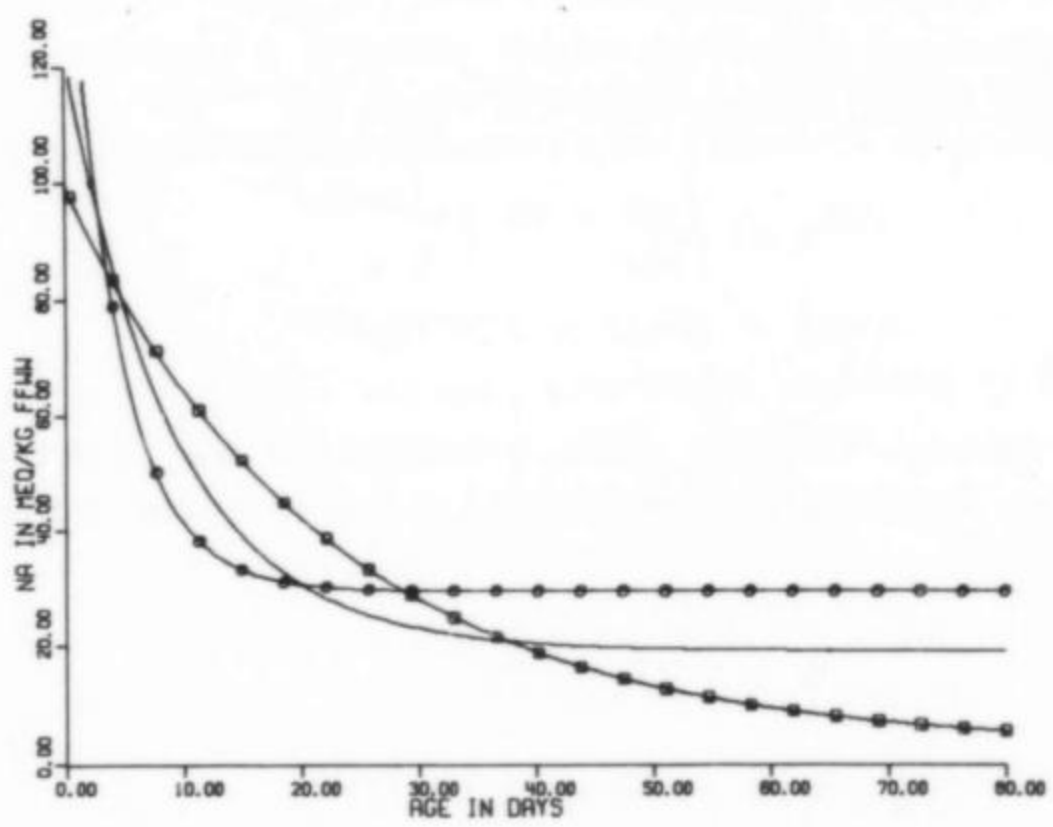

Fig 4. Ordinate: Muscle sodium concentration in milliequivalents of sodium per kilogram of fat-free wet weight. Abscissa: animal age in days. The solid line is the estimated line using equation (1,a). The line joining the circles is from equation (2,a). The line joining the squares is from equation (2,b).

Correlating Changes in the NMR Line Width of Muscle Water and Total Muscle Sodium with Age:

The NMR water signal line widths and sodium values for muscle change rapidly in the very young, then stabilize as the animal reaches maturity. The simplest mathematical model for this change is

$$y = a + be^{-\lambda t} \tag{1}$$

Where a, b and λ are derived constants and t is animal age in days. This model was fitted to the data in the following manner. The parameter λ was varied from a low of 0.01 in steps of 0.005 to unreasonably high values. With λ fixed, the problem becomes one of linear regression and least squares estimates of a and b are readily obtained. By this procedure we obtained an equation for total sodium in milliequivalents per kilogram of muscle:

$$(Na)_t = 19.18 + 103.65e^{-0.11t} \tag{1,a}$$

which is presented in Figure 4. The muscle water data are presented in Figure 5 and in equation (1,b) below:

$$W = 12.81 - 7.0e^{-0.55t} \tag{1,b}$$

In equation (1,b), W equals the signal width at one half amplitude in Hertz. Confidence intervals for the equations were then sought.[4] A method which compares the residual sum of squares of a particular curve to the minimum residual over all curves was used (28). Each mean value for an age group was counted as only one observation and the range of values for λ (equation 1) which gave acceptable curves by the above criterion was determined. If a value of 0.01 for λ was acceptable, then a linear model, $y = a + bt$, was tested. The linear model is obtained as λ approaches zero. The curves for muscle sodium concentration with the lowest and highest acceptable values of λ (equation 1) are presented by equations (2,a) and (2,b) and are reproduced in Figure 4.

$$(Na)_t = 3.04 + 96.37e^{-0.45t} \tag{2,a}$$

$$(Na)_t = 29.53 + 129.70e^{-0.24t} \tag{2,b}$$

In the case of the data on NMR line width, a straight line was an acceptable fit and the bounding curves are presented by equations (3,a) and (3,b) and are reproduced in Figure 5.

$$W = 7.77 + 0.08t \tag{3,a}$$

$$W = 11.13 - 11.29e^{-0.33t} \tag{3,b}$$

[4] A natural criterion for acceptability of equation (1) with particular parameters a, b and λ would be the residual mean square compared to the mean within age variance. We did not use this method because the animals at a particular age usually came from the same litter, so that the litter variation is added to the deviation from regression.

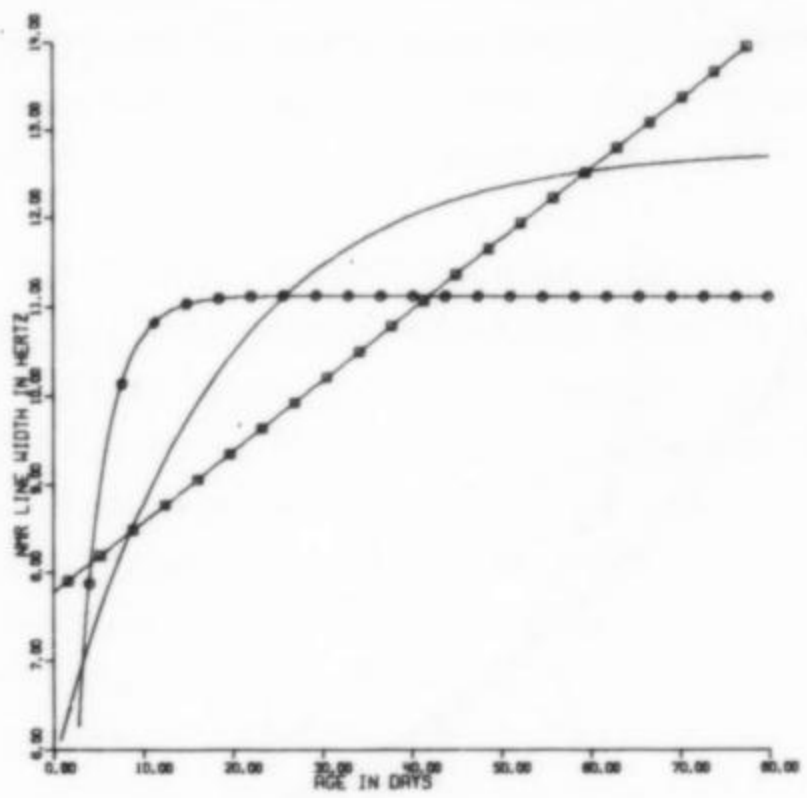

Fig 5. Ordinate: The NMR line width for skeletal muscle water in Hertz. Abscissa: animal age in days. The solid line is the estimated line using equation (1,b). The line joining the circles is from equation (3,b). The line joining the squares is from equation (3,a).

The Quantitative Relationship Between NMR Signal Width for Muscle Water and Total Muscle Sodium Concentration:

Assume that y and z change exponentially with time, that is,

$$y = a + be^{-\lambda t} \tag{4}$$

$$z = d + fe^{-gt} \tag{5}$$

We are interested in expressing z as a function of y. One solves equation (4) for t as a function of y and then inserts this expression into (5) and the desired relation is

$$z = d + f\,[(y - a)/b]^{g/\lambda} \tag{6,a}$$

If y is related linearly to t (y = a + bt) and z is as before (equation 5), then we derived

$$z = d + fe^{-(g(y - a)/b)} \tag{6,b}$$

Using equations (1,a) and (1,b) one can thus establish the expected relationship between muscle sodium and the width of the NMR signal for muscle water.

$$(Na)_t = 19.18 + 103.65\,[1.83 - 0.14\,W]^{0.2} \tag{7}$$

Similarly, by combining the values from equations (2,a) and (2,b) with (3,a) and (3,b) we obtain four curves which indicate reasonable variation in the relationship consistent with the data. These four extreme curves, along with the predicted curve are presented in

Figure 6. *In all cases, the sodium concentration varies inversely with the width of the NMR signal for muscle water.*[5]

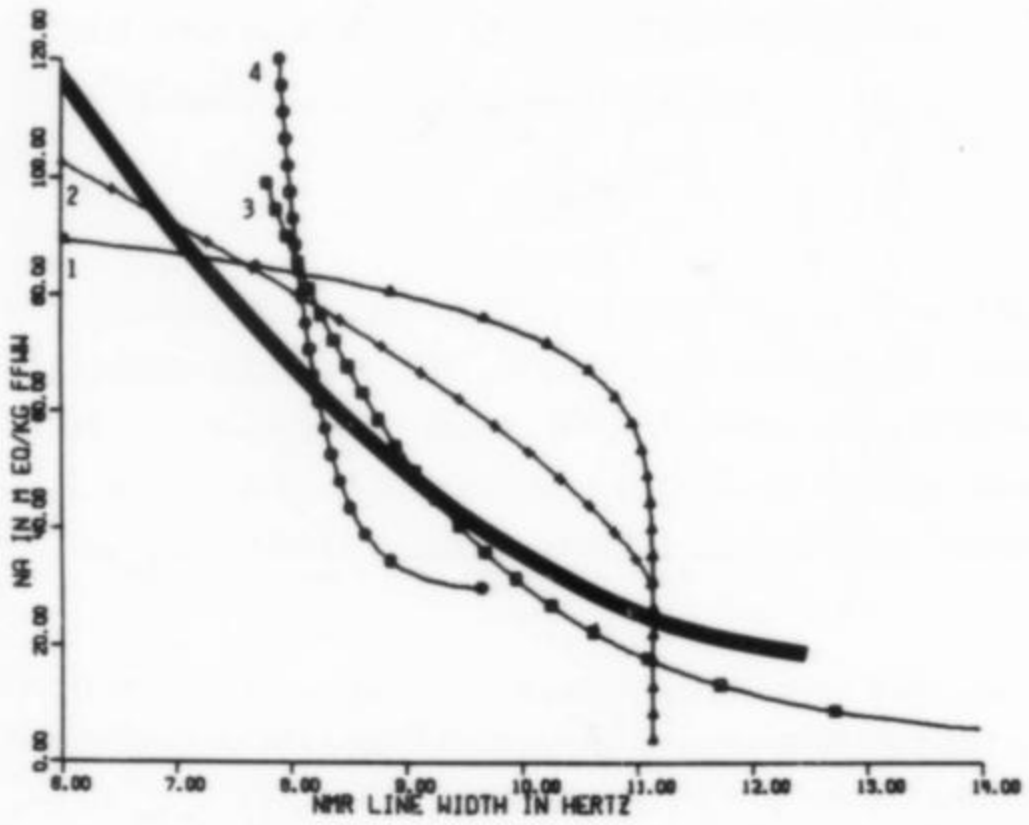

Fig 6. Ordinate: Skeletal muscle sodium concentration in milliequivalents per kilogram of fat-free wet weight. Abscissa: the NMR line width for skeletal muscle sodium in Hertz. The dark solid line represents the expected relation between muscle sodium concentration and the NMR width for muscle water and it results from equations (1,a) and (1,b). The four extreme curves representing the relationship between muscle sodium concentration and NMR line width for muscle water are as follows:

1. Equations (2,a) and (3,b).
2. Equations (2,b) and (3,b).
3. Equations (2a) and (3,a).
4. Equations (2b) and (3,a).

DISCUSSION

Water–Macromolecule Interaction:

The high resolution NMR signal for skeletal muscle water of mature rats is ten times as wide as that for pure water. This broadening (Table I) is due to the shortening of spin-spin (T_2) relaxation times (10,16). From our pulsed NMR study T_1 is also found to be shortened. The shortening of relaxation times can be due to two mechanisms. Firstly, the cellular macromolecules divide the water into small compartments which results in a large surface to volume ratio. The nuclear spins of the water molecules interact with the compartment walls (the macromolecules), and the relaxation is then greatly enhanced.

[5]A similar argument may be developed for the correlation between muscle water (NMR line width changes with age) and the changes in muscle potassium and resting potential. We did not develop the argument for potassium and for resting potential because, 1. the cellular changes in potassium concentration with normal development are not as clear as those for sodium (see reference 4 for detailed discussion of this point) and 2. the voltage changes represent a change in the cell surface and all our measurements of NMR line width for muscle water are on the whole tissue.

This effect is called *wall relaxation* and is well known in physical systems (29). Secondly, because of the *water macromolecule interaction*, some amount of cellular water is bound together in multilayers, resulting in longer correlation times between water molecules and therefore, shorter relaxation times (a broad, high resolution NMR signal) (29).

In the view of the authors, these two causes coexist, and each of them should contribute to the shortening of the proton relaxation times. It is well known among surface scientists that the structure of a liquid at the liquid-solid interface is very different from the structure of the bulk liquid. Since water is a polar substance and can easily form hydrogen bonding, the interaction between water molecules and certain hydrophilic atoms or polar sidechains of macromolecules is very strong. Because of this strong interaction, a lattice-like, ordered hydration shell would form at the water-macromolecule interface. The structure and thickness of this ordered hydration shell depends upon the geometry of the bonding sites on the surface of the macromolecule. The existence of this ordered water has been proposed by many investigators (30-37). Recently, Schultz and Asunmara (37) studied the ordered water in membranes used in water desalination. They estimated that the thickness of the ordered hydration shell on a cellulose acetate surface is about 20 Å. We can now see that the two mentioned causes of relaxation time shortening, the compartment effect and the structural change, are closely related because the compartment wall (macromolecules) surface promotes the lattice ordering in water. Furthermore, with the large surface to volume ratio of the compartment type arrangement of the macromolecules within the cell, the fraction of ordered water becomes quite large.

One may expect that since there is ordered water as well as bulk water in muscle tissue, two different values of T_2 (or T_1) should be observed in the experiment. This, however, is not the case. Because of the exchange effect, no compound echo decay has yet been observed in the pulsed NMR measurements of skeletal muscle water. In addition, only a single absorption curve is found in high resolution NMR studies. The width of the high resolution NMR signal can be altered by adding pure H_2O or D_2O to the tissue sample (15). As pure water is added to a muscle sample, the line width becomes narrower, yet only one spectrum is observed. This is evidence for the exchange of bulk water molecules with water molecules existing in the ordered state (15). It is very difficult to determine the exact volume of ordered water in the muscle tissue because of this exchange averaging.

We would like to point out that the concept of ordered water is not only consistent with our observation of shortened relaxation times in muscle tissue but also it is supported by the correlation between sodium concentration and proton NMR absorption line-width in the tissues of developing animals. One immediate consequence of the enhanced ordering of water molecules is a decrease in solubility of electrolytes. This is easily deduced from the fact that as an aqueous salt solution freezes, the solute is rejected from the ice. Horne et al (35) in a study of the electrical conductivity of dilute aqueous electrolyte solutions within particulate solid system, concluded that the water near an interface is highly structured and excludes electrolyte. In order to explain the desalination property of certain porous membranes, Sourirajan (38) postulated that a layer of demineralized water is present at the interface of the membrane. Hinke (39) utilizing cation selective electrodes, has concluded that 25% of the analyzable water in the myoplasm of Barnacle muscle does not act as aqueous solvent. These findings provide strong evidence for the notion that the fraction of ordered water in muscle tends to exclude the

electrolytes. When the fraction of ordered water increases, as indicated by our NMR data for skeletal muscle water in developing rats, the measured tissue sodium concentration will decrease because more sodium will be excluded. Certainly the changes in muscle sodium concentration and changes in the NMR line width for muscle water are correlated within the same time scale (Fig 6).

The increase in the relative amount of ordered water as the animal becomes mature can also be understood from the consideration of the known cellular development of muscle tissue. In the newborn, the sarcotubular system is disorganized; it then develops very rapidly (40-42). The contractile protein also increases rapidly during early postnatal life (43-46). The build up of contractile proteins, and the proteins (47) and mucopolysaccharides (or glycoproteins) (41-48) of the sarcoplasmic reticulum of developing skeletal muscle could easily increase the surface to volume ratio necessary to order water molecules. It is known that the concentration of macromolecules (dry solids) of skeletal muscle increases from about 12% in the newborn to about 23% in the mature rat (4). The surface area of the water macromolecule interfaces could also be expected to increase by a factor of two, resulting in a proportional change in the fraction of ordered water. (Table I). In fact, the changes associated with development in the NMR line width (Fig 3), and relaxation times (Table I) for muscle water are of the same order.

Proposed Model for Ion-Water-Macromolecule Interactions in Developing Skeletal Muscle:

In order to explain the changes in electrolyte and water composition and in cellular potential of developing skeletal muscle, one should take into consideration the recent findings of water-ion-macromolecule interaction. Several theories propose that cellular adsorption of ions and water occurs to a significant extent in skeletal muscle (30,31,49,50). In a previous report we successfully used the association-induction hypothesis of Ling to explain voltage and electrolyte changes in developing rat skeletal muscle (8). Since then, several publications have described the association-induction hypothesis in detail (31,51) and reported considerable evidence supporting it (14-24, 51-59).

The fundamental point of the association-induction hypothesis is that the cellular ions and water are not in free solution. The intracellular electrolytes are considered to be composed of a fraction dissolved in the cellular water, which is called the interstitial ion concentration, and an adsorbed fraction (31,51). The interstitial ions are dissolved in the cellular water and the adsorbed fraction is associated with charges fixed to macromolecules. The intracellular concentration of solutes is described by the following equation:

$$[S]_i = \alpha\ [S]_{int} + [S]^1_{ad} + [S]^2_{ad} + \ldots [S]^n_{ad} \qquad (8)$$

Where $[S]_i$ is the intracellular concentration of a given solute in milliequivalents per kilogram of cells, α is the percentage of water in the cell, $[S]_{int}$ is the interstitial concentration of a given solute in the cell water, and $[S]^1_{ad}$, $[S]^2_{ad}$, and $[S]^n_{ad}$ represent the solute, in milliequivalents per kilogram cells adsorbed to a number of specific sites. The different types of adsorption sites (1 through n) represent different cellular macromolecules that may adsorb a specific solute S.

The adsorption of a specific ion is determined by the interaction of that ion with the fixed charge on a macromolecule. The interaction of that ion with the fixed charge on the macromolecule varies with the physical-chemical state of that macromolecule and the neighboring macromolecules. Through alterations in the physical-chemical state of the macromolecules, the specific ion adsorbed may be changed. Therefore, the intra- and extracellular distribution of ions reflects both the selectivity of charges fixed to the macromolecules and the solubility of the ions in the cellular water (30,31,51,56-58,60). This model has gained appreciable support from recent investigations. Cope and Swift have shown that in both frog and rat skeletal muscle, sodium is divided into two fractions (52-53,61); one that is NMR visible and one that is NMR invisible.[6] Ling and Cope (31) have also shown that skeletal muscle depleted of potassium accumulates proportional amounts of sodium. The adsorbed (NMR invisible) fraction of sodium approximated the quantity of potassium lost.

The correlation between the cellular sodium concentration and the ordered water, which has been shown in this study, can be elucidated better by the following formulation. In the first section of the discussion we conclude that

$$(H_2O)_i = (H_2O)_i^o + (H_2O)_i^b , \tag{9}$$

where $(H_2O)_i$ is the total amount of intracellular water for one kilogram wet weight muscle tissue, $(H_2O)_i^o$ is the amount of ordered cellular water and $(H_2O)_i^b$ is the amount of cellular water in bulk phase. Let us define a parameter, γ, as the fractional ratio of ordered water, that is,

$$\gamma \equiv \frac{(H_2O)_i^o}{(H_2O)_i} \tag{10}$$

then, substituting equation (10) into equation (9), we have

$$(H_2O)_i^b = (1 - \gamma)(H_2O)_i \tag{11}$$

From the association-induction hypothesis,

$$(Na)_i = (Na)_{int} + (Na)_{ad} \tag{12}$$

where $(Na)_i$ is the total amount of intracellular sodium in one kilogram of wet weight tissue, $(Na)_{int}$ is the amount of interstitial sodium, and $(Na)_{ad}$ is the amount of sodium adsorbed to fixed ionic sites. Again, we can define a parameter

$$\beta \equiv \frac{(Na)_{ad}}{(Na)_i} \tag{13}$$

[6]The muscle NMR signal for sodium has been quantified and only 30-40% of the total muscle sodium can be observed. That is, 60-70% of the sodium produces a signal so broad that it cannot be observed except under special conditions (53,61).

as the fractional ratio of adsorbed sodium. If one computes the intracellular sodium concentration by taking the total amount of sodium inside the cell and dividing it by the amount of intracellular water, then

$$[Na]_i = \frac{(Na)_i}{(H_2O)_i} \tag{14}$$

$[Na]_i$ is the "total" intracellular sodium concentration. That is, the numerator contains both adsorbed and interstitial fractions of sodium and the denominator contains both ordered and bulk fractions of fiber water. The "effective" intracellular sodium concentration, which is important in the biochemical processes, is the fraction of sodium dissolved in the solvent fiber water and is described by equation (15)

$$[Na]_{int} = \frac{(Na)_{int}}{(H_2O)_2^b} \tag{15}$$

From equation (12) and (13) we have

$$(Na)_{int} = (1 - \beta)\,(Na)_i \tag{16}$$

Substituting equation (16) and equation (11) into equation (15), we have

$$[Na]_{int} = \frac{(1 - \beta)\ (Na)_i}{(1 - \gamma)\,(H_2O)_i} \tag{17}$$

From equation (14), equation (17) may be rewritten as

$$[Na]_{int} = \frac{1 - \beta}{1 - \gamma}\,[Na]_i \tag{18}$$

Solving equation (18) for the "total" intracellular sodium concentration, we have

$$[Na]_i = \frac{1 - \gamma}{1 - \beta}\,[Na]_{int} \tag{19}$$

We find that the "total" intracellular sodium concentration is determined by the fraction of adsorbed sodium and the fraction of ordered cellular water. The observed increase in the fraction of ordered water (γ) with development will favor the decrease of $[Na]_i$ according to equation (19). The exact behavior of $[Na]_i$ with development however, cannot be determined until we have quantitative data on the fraction of adsorbed sodium.

SUMMARY

Between birth and 40 days of age, the sodium concentration of muscle tissue in rats decreases almost exponentially. At the same time the NMR absorption line width of protons in muscle water becomes larger. This correlation is interpreted as evidence for the

existence of ordered water in the muscle fiber cell. We propose that because of the interaction between water and the cellular macromolecules, significant ordering of water molecules is promoted near the water-macromolecular interface. This highly structured water has a tendency to exclude electrolytes. In the postnatal development of skeletal muscle, the fraction of ordered water increases. This means that part of the fiber sodium is gradually excluded and that the average tissue sodium concentration decreases as the rat matures. A simple mathematical formulation of this model is attempted.

ACKNOWLEDGMENTS

Parts of this work were supported by the Medical Research Foundation of Texas, The Robert A. Welch Foundation, and the following USPH Research Grants: RR-00188, FR-00259, FR-00254, FR-5425 and CA-11430.

We also extend our thanks to Dr. R. L. Vick, Associate Professor of Physiology, Baylor College of Medicine and to Dr. H. E. Rorschach, Professor and Chairman of Physics, Rice University, for their critical review of the manuscript.

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