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EXTRACELLULAR SPACE OF FROG SKELETAL MUSCLE *IN VIVO* AND *IN VITRO*: RELATION TO PROTON MAGNETIC RESONANCE RELAXATION TIMES

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SUMMARY

1. The Na and Cl distribution spaces of freshly isolated frog muscles are 16·7 and 12·6%, respectively. These values increase to 25·6 and 23·3%, respectively, on incubation.

2. The extracellular components of both Na and Cl efflux curves are significantly smaller in freshly isolated muscles (~ 12%) than in incubated muscles (~18%). The fast exchanging *A* component of the extracellular space is increased more by incubation than the more slowly exchanging *B* component.

3. The proton magnetic resonance (p.m.r.) transverse relaxation curve for the water of freshly isolated frog muscles did not show the long, slowly relaxing tail present in curves from muscles incubated in Ringer solution.

4. When muscles were incubated in hypertonic solutions the p.m.r. transverse relaxation curves could be resolved into three components whose sizes were consistent with the components present in the sodium and chloride efflux curves. The non-exponentiality of the p.m.r. transverse relaxation curve therefore appears to arise from water in both the *A* and *B* extracellular compartments of muscle.

5. Efflux analysis indicated that the cellular Na content of both freshly isolated and incubated frog muscle is similar to that predicted by others (Lev, 1964; Armstrong & Lee, 1971; Lee & Armstrong, 1974) from measurements of intracellular Na ion activity using Na-sensitive micro-electrodes. The remainder of the tissue Na was found in the more rapidly exchanging extracellular compartments. The results of these experiments are inconsistent with the presence of a substantial fraction of bound Na in frog muscle.

6. These experiments show that muscle extracellular space is smaller *in vivo* than *in vitro*. Efflux analysis is suggested as the most accurate method of assessing extracellular compartments.

INTRODUCTION

Boyle, Conway, Kane & O'Reilly (1941) showed that most of the Na and Cl present in freshly excised frog sartorius muscles was lost to an isotonic glucose solution within 1 hr. Making the assumption that these ions were present in a compartment whose ionic composition was equal to that of frog plasma, they found that the volume occupied by this compartment was about 12 ml./100 g muscle.

This value was similar to the inulin (9·6 ml./100 g tissue) and magnesium (8·2 ml./100 g tissue) spaces in frog sartorius muscles incubated in Ringer solution. For this reason Boyle and co-workers concluded that the Na and Cl compartment represented muscle extracellular space *in vivo*. They suggested that the small discrepancy between the Na and Cl spaces on the one hand, and the Mg and inulin spaces on the other, arose from vascular spaces (2·3 ml./100 g) within the muscle. Because of the confusion about the nature and size of the extracellular space in muscle (see Neville, 1979), this idea has persisted to the present, and the Cl space of muscle *in vivo* is often used to estimate the extracellular space *in vitro*.

In the preceding paper (Neville, 1979) we presented evidence that there are two extracellular compartments in frog sartorius muscle whose composition resembles that of the extracellular medium. During the course of these experiments we noted that when we loaded muscles with isotope *in vivo*, these spaces were smaller than when the muscles were loaded by incubation in Ringer solution *in vitro*. We further observed that the p.m.r. relaxation curve obtained from freshly isolated muscles was markedly different from that obtained from muscles incubated, even a short time, in Ringer solution. These observations led to the present studies in which we compared monovalent ion content, ^{22}Na and ^{36}Cl efflux and p.m.r. relaxation curves for muscles that were freshly isolated from the frog with similar observations in muscles incubated one-half hour or longer in Ringer solution. The results of these studies suggested that *in vitro* incubation leads to a major alteration of the extracellular compartments in muscle with very little effect on the cellular compartment.

METHODS

Materials

Four small leg muscles from northern *Rana pipiens*, the *sartorius*, *tibialis anticus longus*, *iliofibularis* and *semitendenosus* were dissected with all fibres intact and used for all experiments except efflux experiments where only the sartorius was used. For freshly isolated muscles, analyses were carried out without allowing the muscles to come in contact with Ringer solution. Incubated muscles were placed in frog Ringer solution (for composition see Neville, 1979) for half an hour or longer. ^{22}Na and ^{36}Cl were obtained from New England Nuclear.

Ion and water content of muscles

Freshly dissected muscles were quickly removed from the frog, weighed on a Precision torsion balance and placed in vials for analysis. Incubated muscles were removed from solution and blotted between four sheets of filter paper moistened with Ringer solution prior to weighing and analysis. To obtain the water content the muscles were dried overnight at 100 °C and reweighed. For Cl^- analysis the dried muscles were dissolved in 0·6 N-NaOH; the solutions were deproteinized with $ZnSO_4$, subjected to akaline perborate oxidation and the chloride titrated using the electrometric method of Cotlove (1964). This method did not provide sufficient sensitivity for accurate measurement of the Cl content of freshly isolated muscles. The Cl from such muscles was extracted with a 5% solution of trichloroacetic acid (TCA) before titration. Blanks and standards also contained 5% TCA. If the TCA solutions were freshly made, they did not interfere with the electrometric titration of Cl ion.

Na and K were extracted from the muscles overnight with 2 ml. distilled water. Four ml. of a solution containing 7·5% TCA (to precipitate the proteins), 22·5 mM-LiCl (to reduce ionization enhancement) and 15% isopropyl alcohol were added. After thorough mixing, the protein precipitate was removed by centrifugation and the Na and K content of the supernatant determined by flame photometry. Standards contained corresponding concentrations of TCA, LiCl and isopropyl alcohol.

Na and Cl efflux

For freshly dissected muscles, ^{22}Na or ^{36}Cl was injected into the peritoneal cavity of a frog which was allowed to rest in a minimal quantity of water at room temperature for at least 16 hr. This was found to be sufficient time for complete equilibration of all leg muscles (see also Macchia, Polimeni & Page, 1978). A measure of specific activity for efflux experiments was obtained from the ratio of counts/min in an unwashed muscle to non-radioactive Na determined by flame photometry. *In vitro* muscles were incubated overnight at 0 °C in Ringer solutions containing ^{22}Na or ^{36}Cl 0·5 μc/ml. Measurement of specific activity of solution and muscles indicated that complete equilibration was achieved in this time. Efflux curves were obtained as described in the preceding paper (Neville, 1979).

P.m.r. techniques

In p.m.r. experiments the tissue sample is placed in a strong, steady magnetic field. A pulse of magnetization oscillating in the radiofrequency range is then applied to the sample at a right angle to the steady field. When the frequency of the oscillating pulse is equal to the resonant frequency of the magnetic dipoles induced in the hydrogen nuclei by the steady-state magnetic

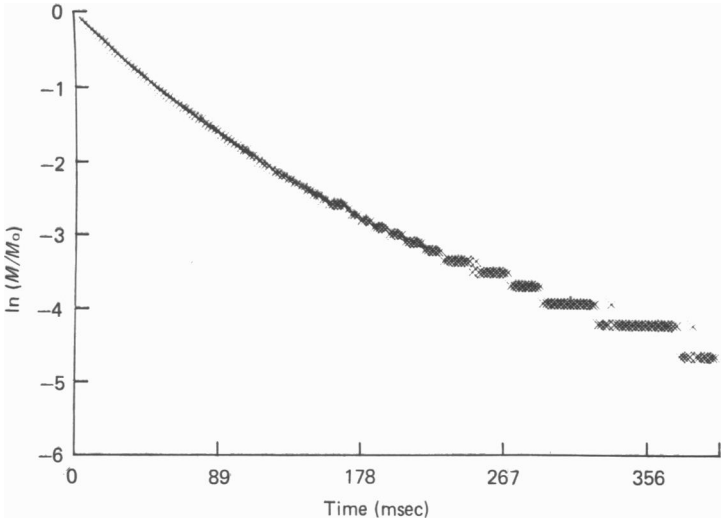

Fig. 1. Computer plot of raw data from transverse relaxation experiment on incubated frog muscle.

field, energy is absorbed. The energy absorption can be measured and gives rise to the p.m.r. signal. When the radiofrequency field is turned off, the order induced in the system decays exponentially at a rate (the relaxation rate) which depends in a complex manner on the molecular interactions of the protons with protons on the same and neighbouring molecules. The time scale of decay of magnetic resonance is such that rotational and diffusional motions have a major influence on the relaxation rates in liquids. In particular the type of relaxation with which we will be concerned here, i.e. the transverse (or spin-spin) relaxation time, T_2, decreases as the rate of motion of the molecules decreases. Generally T_2 is in the msec range and is proportional to the water content of the tissue (Cooke & Wien, 1973).

For the p.m.r. measurements ten muscles (*ca.* 0·6 g) were placed in a flat-bottomed 10 mM-p.m.r. tube which was placed in the probe of a Bruker variable frequency spin-echo spectrometer BKR-322 operating in the diode detection mode. The temperature in the probe was maintained at $0 \pm 0{\cdot}5$ °C with cooled nitrogen gas. Triplicate base line readings were taken; the height (M_0) of the free induction signal following a 90 ° pulse (FID) was then measured in triplicate waiting at least $5 \times T_1$ between measurements. The reading was made 100 μsec after the 90 ° pulse to avoid including the rapidly decaying protons from the solids in the muscle. This produced an

underestimate of 0·8% in the height of the FID, an amount considered to be negligible in comparison to other sources of variability in the data. The Carr–Purcell pulse sequence (90°–180°–180°–...) with the Gill–Meiboom modification (Meiboom & Gill, 1958) was used to obtain transverse relaxation curves using 2 or 3 msec pulse intervals for a total of 400–600 msec. The height (M) of the echo was measured 1 or 1·5 sec after each 180° pulse using a gate time of 10 μsec. All signal heights were fed directly to a Burroughs 6700 computer through an 8 bit digitizer. The base line was subtracted and the quantity ln (M/M_0) calculated where M is the echo height and M_0 the height of the FID, both corrected for the base line. Fig. 1 shows a computer plot of the data as obtained from an incubated muscle. The quantity ln M/M_0 and the corresponding time were punched on IBM cards for further processing.

The diode detector, whose response was somewhat non-linear, was calibrated using 3×10^{-4} M-$MnCl_2$ in aqueous solution. Assuming that the true T_2 decay of the water protons in this solution was exponential, we found that the following empirical formula gave a suitable relationship between the observed (M_{obs}) echo height and the true (M_{cor}) echo heights:

$$M_{cor} = M_{obs} + A - B.M_{obs} - C.\exp(D.M_{obs}), \quad (1)$$

where A, B, C, and D are constants. A non-linear least-squares fitting routine based on the method of Marquardt (1963) was used with the data from the $MnCl_2$-doped solutions to obtain the best fitting parameter estimates for constants A, B, C, and D. These estimates were then used to correct the data from the muscle samples.

Data analysis

Both the p.m.r. transverse relaxation curves and the solute efflux curves reflect multi-exponential processes. A technique for resolution of the efflux curves was described in the preceding paper. P.m.r. relaxation curves were resolved into either two or three components using the same computer technique according to the equation:

$$M_t = \sum_{i=1}^{N} M_0(i) \exp(-t/T_2^i), \quad (2)$$

where M_t is the echo height at time t, $M_0(i)$ is the fraction of the FID arising from the ith component and T_2^i is the transverse relaxation time for the ith component. N is either 2 or 3. We found that use of all data points for curve resolution was prohibitively expensive as well as inaccurate because the voltage digitizer limited the precision of the points when the echo height was small (see Fig. 1). For this reason we used every third data point for the initial part of the curve, then the most accurate points, the last to give a particular value, for later times.

RESULTS

Monovalent ion contents of incubated and freshly isolated muscles

Column 4 of Table 1 shows the measured tissue content of Na, Cl and K of mixed frog muscles analysed directly after removal from winter frogs (freshly isolated). The results were compared with similar data from muscles incubated in Ringer solution 30 min before analysis (Table 1, Incubated). In the case of Cl the observed tissue content increased from 10·4 to 26·6 μmole/g on incubation. If we assume that the cell Cl is in electrochemical equilibrium with the extracellular Cl in both freshly dissected and incubated muscles, the Cl concentration in the cell water can be calculated from the Nernst equation (Adrian, 1961; Macchia *et al.* 1978). The remainder should be in the extracellular space. Its distribution space $[ECS]_{total}$ can be calculated from the expression

$$[ECS]_{total} = \frac{[Cl]_t - 0{\cdot}81[Cl]_i}{[Cl]_m - [Cl]_i}, \quad (3)$$

where $[Cl]_t$ is the Cl content of the muscle in μmole/g, $[Cl]_i$ and $[Cl]_m$ are the cell water and external concentrations in mM, and 0·81 is the water content of the muscle.

Eqn. (3) is derived by rearrangement of the following equation which expresses the fact that the total Cl in the muscle represents the sum of the intra- and extracellular Cl contents:

$$[Cl]_t = [ECS]_{total} \cdot [Cl]_m + (1 - 0{\cdot}19 - [ECS]_{total}) \cdot [Cl]_l,$$

where 0·19 is the fractional dry weight of the muscle.

The values so calculated for the extracellular distribution spaces of freshly isolated and incubated muscles are given in the last column of Table 1. There is an apparent increase in the extracellular space of incubated muscle which amounted to approximately 10 % of the muscle volume. A similar result was calculated from the Na data when the intracellular Na was taken as 9 mM (see below).

TABLE 1. Na, Cl and K content of freshly dissected and incubated muscles from winter frogs. See Methods section for procedure. *Cl concentration from Nernst equation assuming a membrane potential of −90 mV; Na concentration obtained from results of efflux experiments in Table 2. **Theoretical value from average of values obtained from Na and Cl results. Intracellular water content of (0·81 − 0·104 =) 0·706 and (0·81 − 0·213 =) 0·607 used to calculate cell K concentrations in freshly isolated and incubated muscles, respectively

Ion	Treatment	Extracellular concentration (mM)	Tissue content (μmole/g)	Concentration in cell water (mM)	Apparent extracellular space %
Cl	Freshly isolated	79·2	10·4 ± 1·7	2·2*	11·2 ± 2·2
Cl	Incubated	114·0	26·6 ± 1·4	3·2*	21·7 ± 1·3
Na	Freshly isolated	103·8	17·3 ± 1·1	9·0*	9·6 ± 1·2
Na	Incubated	113·5	29·1 ± 1·5	9·0*	20·9 ± 1·4
K	Freshly isolated	2·5	87·8 ± 1·7	125 ± 2	10·4**
K	Incubated	2·5	82·7 ± 1·3	141 ± 2	21·3**

On the other hand, the K content decreased in incubated muscles as one would expect if a greater proportion of the muscle volume was occupied by extracellular space. However, the decrease was quantitatively less than would be predicted from the apparent increase in extracellular space calculated from the Na and Cl contents. Thus when the average extracellular spaces from the Na and Cl data (marked with**) are used in the calculation, there is an apparent increase in cellular K on incubation. As will be discussed further below, this increase probably results from shrinkage of the sarcoplasmic compartment because the Ringer used was slightly hypertonic to frog plasma.

Na and Cl efflux from freshly isolated and incubated frog muscle

In order to be certain that the apparent increase in extracellular space calculated from the change in ion content arose from an increase in the extracellular compartment and not an increase in the intracellular compartment, we injected summer frogs intraperitoneally with 10 μc of either ^{22}Na or ^{36}Cl. The frogs were allowed to rest overnight in a covered pan containing a minimum amount of water. The following day one sartorius muscle from each frog was dissected and immediately subjected to the washout procedure. The paired sartorius was analysed for isotope and ion content to obtain a measure of specific activity. Fig 2. shows an efflux curve from such a freshly dissected muscle compared with an efflux curve from a muscle loaded

with isotope by incubation *in vitro*. In the previous paper (Neville, 1979) we showed that two extracellular components (called *A* and *B* for convenience) are present in solute efflux curves from incubated muscle. Both components are also present in muscles loaded with isotope *in vivo*. This observation suggests that neither of the two extracellular components is the result of damage incurred during dissection.

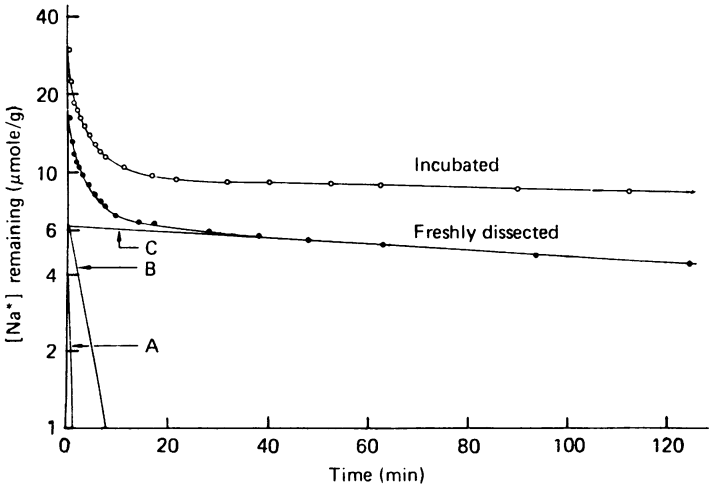

Fig. 2. Na efflux from freshly isolated and incubated frog sartorius muscles at 0 °C. The components obtained from resolution of the curve from the freshly dissected muscle are shown.

TABLE 2. Resolution of curves for Na and Cl efflux from frog sartorius muscle (data on incubated muscles taken from Table 2. Neville, 1979). All data from summer frogs. * Results from freshly isolated muscles differ significantly from incubated muscles ($P < 0.01$)

			Distribution space		
Ion	Muscle treatment	*n*	*A*	*B*	*C*
^{36}Cl	Freshly isolated	4	4·2 ± 0·3 %*	8·3 ± 1·0 %	0·48 ± 0·04 μmole/g
	Incubated	4	9·4 ± 1·6 %	9·1 ± 1·8 %	0·65 ± 0·06 μmole/g
^{22}Na	Freshly isolated	10	4·9 ± 0·6 %*	6·1 ± 0·8 %	6·3 ± 0·8 μmole/g
	Incubated	15	10·4 ± 1·6 %	7·9 ± 0·3 %	6·2 ± 2·7 μmole/g
			Rate constant (min^{-1})		
			A	*B*	*C*
^{36}Cl	Freshly isolated	4	1·37 ± 0·3	0·17 ± 0·01	0·007 ± 0·001
	Incubated	4	0·90 ± 0·21	0·17 ± 0·03	0·010 ± 0·003
^{22}Na	Freshly isolated	10	2·0 ± 0·6	0·22 ± 0·02	0·0032 ± 0·0004
	Incubated	15	0·7 ± 0·19	0·17 ± 0·01	0·0029 ± 0·0008

In Table 2 the results of computer resolution of washout curves from fourteen muscles loaded *in vivo* are compared with the results obtained from efflux experiments on incubated muscles. The incubation procedure appeared to have no significant effect on the rate constants of efflux for either Na or Cl. Further, the sodium and chloride contents of the slowly exchanging components are similar in both freshly

isolated and incubated muscles suggesting that incubation does not affect the mechanisms which maintain cellular ion content. (The distribution space of component *C* for Cl gave an underestimate of the cellular Cl. When the efflux experiment is carried out in NO_3-Ringer to reduce exchange diffusion (Adrian, 1961; Neville, 1979), the size of this component increases). The distribution space for component *B* is slightly smaller in freshly dissected than in incubated muscles. However, the distribution space for component *A* in freshly isolated muscles is significantly smaller, with a value about one-half that observed in incubated muscles.

The sum of the distribution spaces of the *A* and *B* components in freshly dissected muscles from summer frogs is $12{\cdot}2 \pm 1{\cdot}0\,\%$ for Cl and $11 \pm 1{\cdot}0\,\%$ for Na, not significantly different from the values estimated in Table 1 for the extracellular space

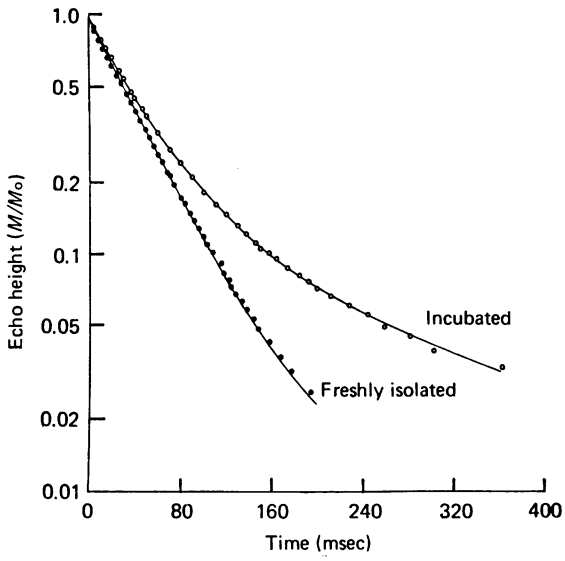

Fig. 3. Proton magnetic resonance transverse relaxation curves from freshly isolated and incubated frog muscle. Not all data points are shown (see Fig. 1).

of freshly isolated muscles from winter frogs. Similarly, for the incubated muscles the sum of the distribution spaces of the *A* and *B* components was $18{\cdot}5 \pm 2{\cdot}4\,\%$ for Cl and $18{\cdot}3 \pm 1{\cdot}6\,\%$ for Na, only slightly smaller than the values for the extracellular space of incubated muscles from winter frogs given in Table 1. We have shown previously that the extracellular component is larger in winter frogs (Neville, 1979). These data confirm the notion that the increase in the Na and Cl when muscles are incubated *in vitro* results from an increase in the extracellular space. They suggest that the greater part of this increase can be attributed to changes in compartment *A*.

Transverse relaxation curves from freshly isolated and incubated muscles

Fig. 3. shows transverse relaxation curves obtained from freshly isolated and incubated mixed frog muscles. Examination of these curves indicates that the long, slow tail in the curve from the incubated muscles is small or missing in the curve from the freshly isolated muscles. These curves as well as curves from duplicate samples, were resolved into two exponentially decaying components as described in

Methods. The resultant components of the curve from incubated muscles are shown in Fig. 4 and the quantitative results given in Table 3. In both types of curve the sum of the initial heights of the two exponential components is less than 100 % of the total initial signal height by an amount designated 'remainder' in Table 1. This small, very rapidly decaying fraction was seen in all samples and has been noted by others as well (Belton & Packer, 1974; Belton, Jackson & Packer, 1972; Hazlewood, Chang, Nichols & Woessner, 1974). Preliminary experiments indicate that the protons in

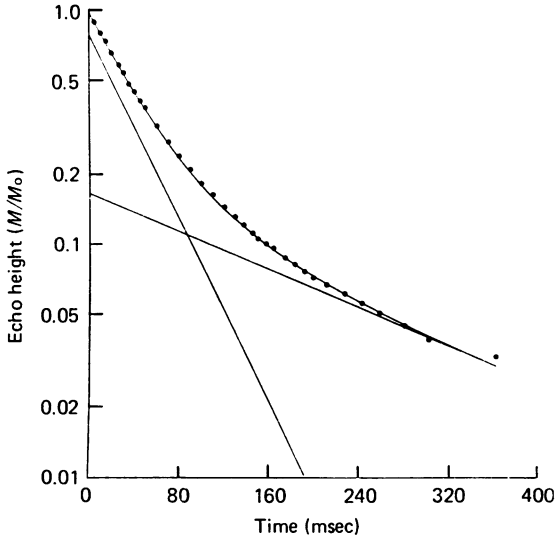

Fig. 4. Two component resolution of transverse relaxation curve from incubated muscle.

TABLE 3. Two component resolution of transverse relaxation curves from freshly isolated frog muscles and frog muscles incubated overnight at 0 °C in Ringer solution. n is the number of samples, each containing approximately ten mixed leg muscles. Results are given as mean ± S.E. of mean.

		'Fast-relaxing fraction'		'Slowly relaxing' fraction		
	n	Intercept (% M_0)	T_2 (msec)	Intercept (% M_0)	T_2 (msec)	Remainder % M_0
Freshly isolated muscles	3	85·7 ± 3·4	41·5 ± 1·3	10·1 ± 3·1	133 ± 37	4·2
Incubated muscles	4	80·7 ± 2·7	38·9 ± 2·0	15·0 ± 1·9	213 ± 5	4·3

this fraction do not exchange with D_2O, suggesting they represent protons in compounds other than water, such as those on mobile side chains of proteins (M. C. Neville, unpublished; K. J. Packer, personal communication). For the remainder of this discussion we will disregard this fraction and consider only the major fractions described in Table 3 as 'fast-relaxing' and 'slowly relaxing'.

The 'slowly relaxing' fraction has been noted by a number of investigators (Belton *et al.* 1972; Hazlewood *et al.* 1974); it has been suggested that it arises from water in the extracellular space. It averaged 15 % of the tissue water in incubated

muscles, somewhat smaller than the estimated value for the proportion of extracellular water in muscle from winter frogs (Table 1). In freshly isolated muscles the 'slowly relaxing' fraction appeared to occupy about 10% of the tissue water; this value is similar to the value of extracellular space given in Table 1. The rate of decay of this fraction was considerably faster than that of the corresponding fraction from incubated muscles. The relaxation time (T_2) of the larger, 'fast-relaxing' component of both freshly isolated and incubated muscles was approximately 40 msec, very similar to the value of 44·6 msec obtained by Hazlewood *et al.* (1974) for the major fraction of water in rat gastrocnemius muscle.

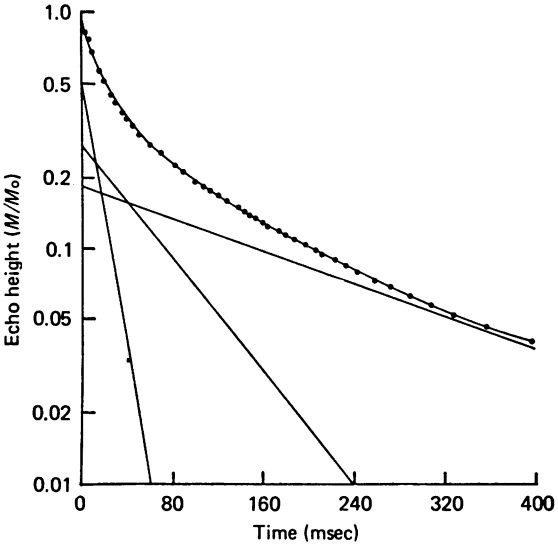

Fig. 5. Proton magnetic resonance transverse relaxation curve from muscles incubated 16 hr at 0 °C in hypertonic Ringer solution containing 300 mM-sucrose. Points experimental; not all points included (see Methods). Curve theoretical from summation of the three components shown. The components were obtained by computer resolution of the experimental data.

Effect of hypertonic sucrose on the p.m.r. transverse relaxation of water in frog muscle

For the analysis of the transverse relaxation curves of incubated and freshly isolated frog muscle above (Table 3), the assumption was made that there were only two major components in the relaxation curve. Because solute efflux curves were found to have three components, we attempted to detect three components in the p.m.r. relaxation curves as well. Although this proved possible for muscles incubated in isotonic solution, the uncertainty in the parameter estimates obtained rendered the analysis meaningless (see Table 4, 0 sucrose). In an attempt to circumvent this difficulty, 300 and 450 mM-sucrose was added to the Ringer solution and the muscles allowed to equilibrate at 0 °C overnight before p.m.r. analysis. Sucrose enters the extracellular spaces but not the sarcoplasm, bringing about shrinkage of the cellular compartment while the extracellular spaces retain their normal volume or possibly swell (Neville, 1979, Table 1). Because T_2, the transverse relaxation time, is proportional to the water content (Cooke & Wien, 1973), we reasoned that the hypertonic solutions might allow us to differentiate the sarcoplasmic component of

the relaxation curves more clearly. The prediction was correct, as is illustrated by the curve in Fig. 5 and the quantitative parameters obtained from three component resolution of curves from four such experiments (Table 4).

TABLE 4. Results of three component resolution of transverse relaxation curves from muscles incubated in isotonic and hypertonic solutions. Values shown are average of best parameter estimates ± S.E. from the computer resolution for duplicate determinations in each medium

	Sucrose added to Ringer (mM)	Slowly relaxing fraction	Intermediate fraction	Rapidly relaxing fraction	Theoretical size cellular fraction
Fraction size (% cell water)	0	5·9 ± 6·4	39·1 ± 11·0	56·9 ± 16·3	68·3
	300	20·7 ± 4·6	26·1 ± 2·9	53·3 ± 1·8	56·7
	450	21·3 ± 5·2	28·5 ± 3·6	50·3 ± 1·2	50·8
Relaxation time (T_2, msec)	0	545 ± 932	83·8 ± 42·8	33·1 ± 4·5	—
	300	270 ± 53	76·0 ± 15·2	15·3 ± 0·8	—
	450	228 ± 28	75·3 ± 12·7	12·6 ± 0·7	—

Considering the fraction sizes shown in Table 4, if we make the assumption that the sarcoplasmic compartment behaves as a perfect osmometer in hypertonic solutions (Dydynska & Wilkie, 1963) and the extracellular spaces retain the volumes they had in isotonic solution, we can predict that about 57 % of the tissue water will reside in the sarcoplasmic compartment in the presence of 300 mM-sucrose and 51 % in the 450 mM-sucrose (last column, Table 4). These predicted values are about the same as the observed average sizes of the component, 53 and 50 %, respectively, which were obtained from the transverse relaxation curves. While it is tempting to relate the slowly relaxing and intermediate components of the p.m.r. relaxation curves to components *A* and *B* of the Na efflux curves, present evidence does not allow this assignment to be made with any certainty.

DISCUSSION

A major conclusion to be drawn from the results of these experiments is that the size of the extracellular space of frog skeletal muscle is small *in vivo* (~ 10 % of muscle volume) and increases in size when the muscle is removed to Ringer solution. The increase in the sodium and chloride space observed by Boyle & Conway (1941) on incubation of frog muscle was the result, not of altered permeability properties of the cell membrane as they suggested, but rather of an increase in the volume of the extracellular space. A similar increase in extracellular fluid volume in rat brain slices has been documented by Møller, Møllgard, Lund-Anderson & Hertz (1974).

A possible explanation for the small extracellular space *in vivo* can be found in the negative tissue pressure hypothesis of Guyton (see Guyton & Coleman, 1968, for review). Guyton and his co-workers presented evidence that under normal conditions the hydrostatic pressure in the interstitital spaces is about −7 mmHg. This negative pressure is maintained by the combined action of the lymphatics, which remove protein from the extracellular spaces lowering the colloid osmotic pressure, and the high colloid osmotic pressure of the plasma which draws fluid back into the vascular

system. When the muscles are removed from their *in vivo* situation, the mechanisms which normally remove extracellular fluid are no longer present and incubation medium is now free to enter the extracellular space. This interstitial swelling may be enhanced by the colloid osmotic pressure of the proteins within the extracellular compartment.

It is worth noting that entry of Ringer solution into the extracellular space *in vitro* has probably resulted in the use of somewhat hypertonic solutions for incubation of frog muscle and possibly other tissues. Thus, one criterion used in determining the osmolarity of an isotonic incubation solution is that the tissue concerned not gain weight on incubation. However, in the case of muscle, as with most tissues, the total weight includes both intra- and extracellular compartments. If the extracellular compartment expands by 10 % of the tissue volume, since the weight stays constant, the intracellular compartment must contract by an equivalent amount. Such a contraction is consistent with the apparent 10·5 % increase in the cellular K ion concentration (see Table 1) observed when frog muscles were placed in Ringer solution. We suggest that a Ringer solution which is truly isotonic with frog muscle would have an osmolarity of about 212 m-osmole rather than the 236 m-osmole solutions commonly used.

A number of authors have suggested, on the basis of evidence obtained using NMR spectroscopy (Cope, 1967) and cation-sensitive micro-electrodes (Lev, 1964; McLaughlin & Hinke, 1966), that a substantial portion of the sodium in muscle is complexed to intracellular macromolecules. Alternative interpretations of both types of data are available: Berendsen & Edzes (1973) pointed out that the NMR evidence of Cope and others could result from quadrupolar interactions of Na rather than from binding to intracellular components. Caldwell (1968) suggested that the inability of Na-sensitive micro-electrodes to sense all the intrafibrillar Na resulted from sequestration of the ion in a special region of the muscle fibre not readily accessible to large extracellular markers such as inulin. The evidence on both sides has been well summarized by Lee & Armstrong (1974).

The average cellular Na content of 6·2 μmole/g found in these experiments for both freshly isolated and incubated muscles corresponds to a sodium concentration in the fibre water of about 10 mM. The activity of Na in the cytoplasm of frog muscle measured with micro-electrodes (Lev, 1964; Armstrong & Lee, 1971; Lee & Armstrong, 1974) ranged between 5·5 and 7·7 mM. From these figures we can calculate an average activity coefficient of 0·66 for the Na in the cytoplasm. Given the variance in the data, this value is not significantly different from the activity coefficient of about 0·77 for 0·01 M solution of mono-mono-valent salts (Weast, 1969) suggesting that most cellular Na is unbound. Since the remainder of the Na in muscle has a much faster time constant of efflux (e.g. is found in the *A* and *B* compartments of the extracellular space), we suggest that the muscle does not contain a significant portion of bound Na.

A final conclusion which can be drawn from these studies is that the non-exponentiality of the p.m.r. transverse relaxation curves from freshly isolated frog muscles arises from water in both compartments *A* and *B* of the extracellular space. In incubated muscles three components appear, although satisfactory resolution of the curves is only possible when the incubation solution is hypertonic. The compart-

ment sizes derived from the p.m.r. relaxation curves are in reasonable quantitative accord with compartment sizes predicted from other types of experiments. This suggests that, since the major portion of the non-exponentiality of the curves arises from heterogeneous anatomical compartments, the contribution of the slow diffusional exchange between water in different physical states, e.g. water of hydration and free water, as postulated by Diegal & Pintar (1975) must be relatively minor. The multiplicity of the anatomical compartments in muscle which affect p.m.r. relaxation times suggests that muscle is at best a poor choice of tissue in which to study basic n.m.r. relaxation mechanisms in biological systems.

On the basis of the studies reported here and in the preceding paper (Neville, 1979) it is apparent that the most accurate method for assessing the amount of any solute residing in the extracellular space is efflux analysis under conditions where solute leaves the extracellular compartments much more rapidly than the cellular compartments. Often lowering the temperature of the efflux experiment will achieve this. In circumstances where efflux analysis is prohibitively complex or time consuming, the sucrose, inulin and dextran spaces all provide adequate measures of the size of the extracellular compartment(s), provided that care is taken that the isotopes contain no contaminants and sufficient time is allowed for equilibration.

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