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^{1}H NMR of intact muscle at 11 T

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^{1}H NMR spectra of intact frog, and chicken skeletal muscles, were recorded at 470 MHz with the Plateau and Guéron pulse sequence for the suppression of water [(1982) J. Am. Chem. Soc. 104, 7310]. Only a few transients were required to resolve the resonances from the protons of muscle metabolites. The previously unobserved exchangeable protons of muscles were also recorded and thereby phosphocreatine and creatine could be measured simultaneously. During aging of dissected frog muscle, changes in levels of phosphocreatine, creatine and lactic acid, and the decrease in the intracellular pH were followed by ^{1}H NMR.

^{1}H NMR *Water-suppression* *Muscle* *Phosphocreatine* *Lactic acid* *Intracellular pH*

1. INTRODUCTION

^{31}P NMR of muscle was the front runner of NMR application to biological systems [1–3]. ^{31}P NMR studies on the soluble metabolites of muscle, i.e., ATP, phosphocreatine, and P_i, contributed to our understanding of the energetics of muscle contraction [3,4], the mechanism of ischemia in heart [5,6], or the determination of intracellular pH in muscle [1–3,7]. Subsequent application of natural abundance ^{13}C NMR to muscle [8] provided the basis for the first direct determination of lactic acid in muscle [9] and opened the way for studying the mobility of phospholipids in membranes of intact muscle [10]. Authors in [11] pioneered in recording ^{1}H NMR spectra of frog and rat muscles at 2.35 T with selective saturation of the water signal. The resonances from total creatine, carnosine, lactate and lipids were well-resolved. They also suggested that ^{1}H NMR could be used for determination of the intracellular pH of muscle through the chemical shift changes of carnosine protons.

Recently, a new pulse sequence for the suppression of water was proposed in [12]. This method has several advantages over other methods aimed at eliminating the huge water peak: (1) the use of strong, non-selective pulses; (2) the ability to observe exchangeable protons; (3) the absence of baseline distortions. We have used the 'jump and return' pulse sequence of [12] for recording ^{1}H NMR spectra of intact muscle at 11.05 T. With this method, we have been able to observe the exchangeable protons of both phosphocreatine and creatine in dissected muscle. Furthermore, changes in the level of phosphocreatine, creatine, lactic acid, and the intracellular pH were followed simultaneously in muscles during aging.

2. EXPERIMENTAL

2.1. *Intact muscle*

Frogs (*Rana pipiens*) were chilled in ice prior to killing. The tibialis posterior and sartorius muscles were dissected and used for the NMR experiments

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immediately. The Ringer's solution added to the NMR tube contained 115 mM NaCl, 2.5 mM KCl, 2.0 mM $CaCl_2$, 2.15 mM Na_2HPO_4 and 0.85 mM NaH_2PO_4.

The pectoralis muscles from freshly killed chickens were stored at −20°C. Before the NMR experiments the muscles were thawed and cut to fit into the 5-mm sample tube.

2.2. *Subcellular fractions*

Muscles were homogenized with a Waring blender in 0.1 M KCl and 10 mM potassium phosphate solution (pH 7.0) at 4°C. The homogenate was centrifuged at 600 × *g* to obtain the myofibrils in the pellet. Centrifugation of the 600 × *g* supernatant at 7800 × *g* resulted in the mitochondrial fraction. Microsomes were isolated from the mitochondrial supernatant at 200000 × *g*. The supernatant of the microsomes was dialyzed against distilled water to obtain the soluble protein fraction, and another part of the fraction was treated with perchloric acid to obtain in the filtrate the metabolites of the cytosol.

Myofibrils, mitochondria, and microsomes were suspended in 0.15 M NaCl, dissolved in ^1H_2O, for ^{1}H NMR analysis. The soluble protein fraction was freeze-dried, and dissolved in 0.15 M NaCl containing ^1H_2O. The perchloric acid supernatant was neutralized with KOH, and was freeze dried after removal of the perchlorate precipitate; the lyophilized powder was dissolved in ^1H_2O.

2.3. *NMR spectroscopy*

Spectra were collected by using the water suppression pulse sequence as in [12]. This sequence ($90°_y - \tau - 90°_{-y}$) decreased the water signal intensity by a factor of up to 250 under our experimental conditions. The radiofrequency is set on resonance with the water peak. The waiting time, τ, is such that the group of spins of interest precess by 90°, then the $90°_{-y}$ observed pulse is applied. In practice, the pulse length along the $-y$ axis is empirically adjusted to achieve a minimum ^1H_2O signal. A 180° phase difference occurs between the peaks upfield and downfield from the water peak. In our case, the phase of the spectrum was adjusted so that peaks upfield from the ^1H_2O peak are displayed as positive absorption peaks, while the peaks downfield from the ^1H_2O peak appear as negative absorption peaks. The spectra also display a frequency dependence of the effective pulse angle. For $\tau = 180\ \mu s$, this angle is 0° at the water frequency, increasing to 90° at ± 2.955 ppm from the water frequency, and decreasing again to 0° at ± 5.910 ppm from the water frequency [12].

^{1}H NMR spectra were recorded on a Nicolet NT-470 (5 mm probe) instrument operating in the Fourier transform mode. The irradiation frequency was 469.941 MHz and the sweep width was ± 3000 Hz. Radiofrequency pulses of 9.0 μs (90°) were used. The recycling time was 1.7 s and τ was 180 μs. Quadrature detection was used in collecting the 8 K data points. TSP (sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate) dissolved in ^2H_2O was used as an external standard (0.00 ppm). ^2H_2O was used as the external lock compound. The probe temperature was 20 ± 1°C. The number of transients accumulated for the various samples ranged from 1 to 256. For all the spectra the line broadening was 5 Hz. The resolution was enhanced by trapezoidal multiplication of the FID prior to Fourier transformation. The first 100 data points in each FID of the two quadrature channels were multiplied by an increasing linear function ranging from 0 to 1.0.

2.4. *The titration of carnosine*

The pK_a of the histidine ring of carnosine (β-alanylhistidine) was determined by following the pH dependence of the chemical shift of the C2-H, C4-H and N-H protons of the histidine ring. The model solution used for the titration experiments was 14 mM in carnosine and 0.15 M in NaCl in ^1H_2O. Spectra were recorded at 18 different pH values ranging between 3.6 and 10. The pK_a value was obtained by fitting the observed chemical shift, by means of a curve fitting program based on a non-linear metric minimization of χ^2 [13] to a Henderson-Hasselbach type equation:

$$\mathrm{pH} = \mathrm{p}K_a + \log\left(\delta_1 - \delta_{obs}/\delta_{obs} - \delta_2\right)$$

where δ_1 and δ_2 are the limiting chemical shifts for the protonated and unprotonated form, respectively, and δ_{obs} is the observed chemical shift at a certain pH. The pK_a value for the histidine ring of carnosine was found to be 7.06 ± 0.01. The limiting chemical shifts (in ppm) for the dissociated and protonated form, respectively, were: 6.920 and 7.260 for C4-H; 7.657 and 8.576 for C2-H; 7.980 and 8.224 for N-H.

3. RESULTS

3.1. *Chicken pectoralis*

Fig.1 compares the ^{1}H NMR spectra of intact chicken muscle (lower) with the cytosolic fraction of the same muscle (upper). This was the only subfraction that exhibited resonances expected to contribute to the spectra of the intact muscle. Furthermore, the cytosolic fraction showed the same resonances that were present in a perchloric acid extract of the muscle.

Based on the spectra of pure compounds, on the co-resonance of these standards added to the cytosolic fraction, and literature values for ^{1}H NMR chemical shifts, the following resonances, relative to the external TSP (0.000 ppm) may be identified: In the aliphatic region are seen the methyl (1.301 ppm) from lactic acid, the *N*-methyl (2.996 ppm) from creatine, the *N*-methylene (3.896 ppm) from creatine, the methylene protons from anserine (*β*-alanyl-1-methylhistidine) which generate two peaks (2.664 and 3.203 ppm) and contribute to another (2.996 ppm), the methyl of anserine (3.813 ppm), and the *N*-methyl of carnitine and choline which co-resonate (3.203 ppm). In the aromatic region are found the histidine ring protons C4-H, N-H and C2-H, of anserine (7.218, 8.291 and 8.550 ppm) and the 4 nitrogen-linked protons of the guanidino group of creatine (6.721 ppm).

The spectra of fig.1 were accumulated using 12 transients in order to detect possible minor

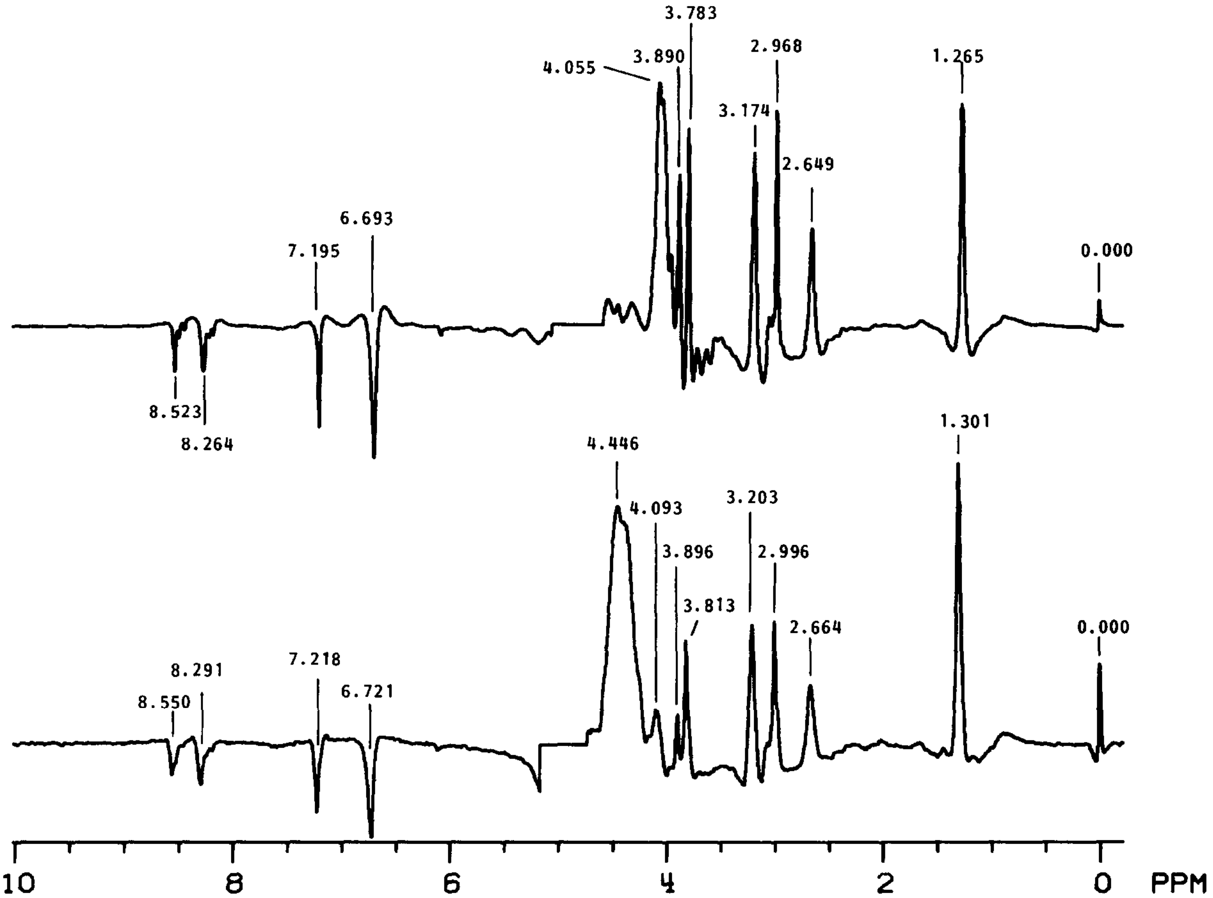

Fig.1. ^{1}H NMR spectra of intact chicken pectoralis (lower) and cytosolic fraction from chicken pectoralis concentrated twice (upper). Each spectrum is the result of 12 transients accumulated in a total time of 20.4 s.

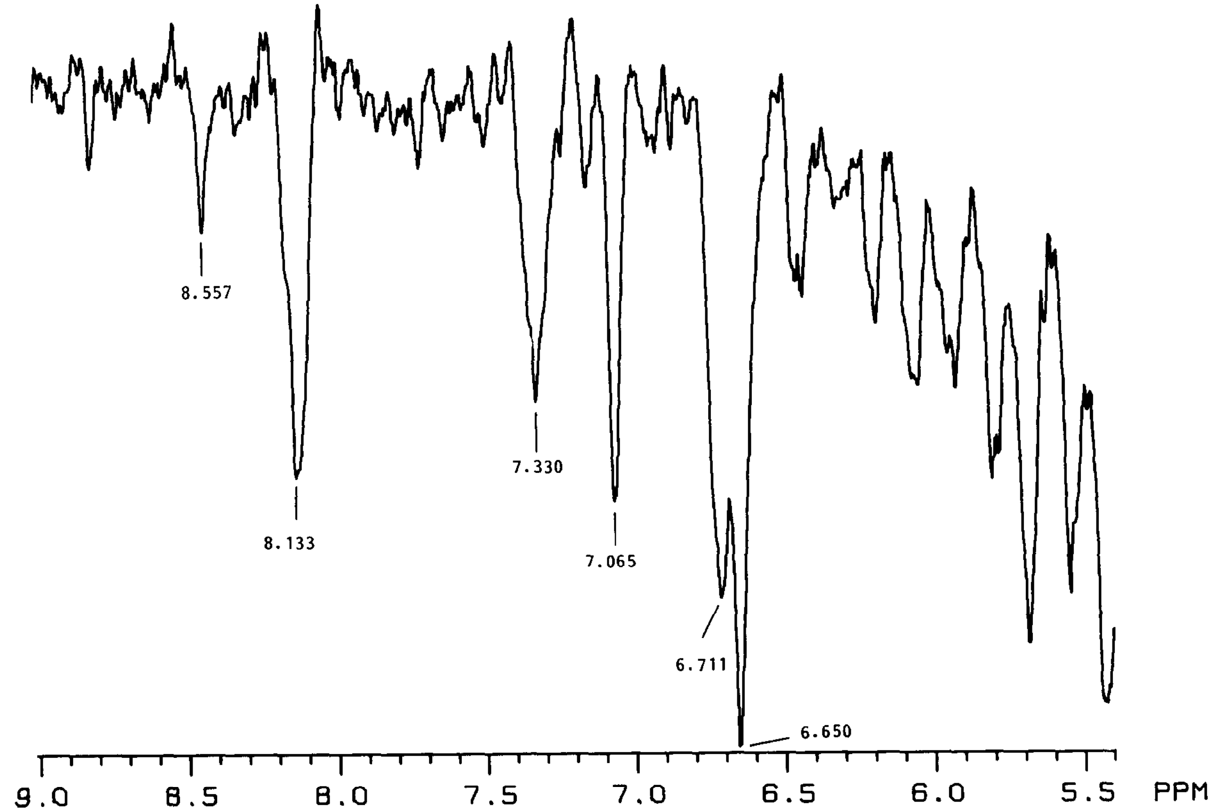

Fig.2. ^1H NMR spectrum of the aromatic region of fresh frog muscle. Three transients were accumulated in a total time of 5.1 s.

resonances. However, even with a single transient the signal : noise ratio was good enough to estimate the level of major metabolites, e.g., creatine or lactic acid.

3.2. *Fresh frog muscle*

All resonances in intact frog muscle originate from the cytosolic fraction, in agreement with results in [11] and as in the case of chicken muscle described above.

By chilling the frog on ice to minimize resting metabolism, dissecting the muscle on ice, transferring the sample into an NMR tube kept in ice, and recording the spectrum within 5 min, major resonances from phosphocreatine become visible (fig.2). The resonance at 7.330 ppm originates from the two protons linked to nitrogen in phosphocreatine ($H_2\overset{+}{N} = C\langle$), while the resonance at 6.650 ppm originates from the proton linked to the nitrogen that contains the phosphoryl group ($= C\langle^{NHPO_3^{2-}}$). These assignments are based on the ^1H NMR spectra of pure phosphocreatine. In fig.2, the resonance at 6.711 ppm originates from the 4 protons linked to two nitrogens in creatine ($H_2\overset{+}{N} = C\langle^{NH_2}$).

The resonance at 8.557 ppm in fig.2 is from the H8 proton in the heterocyclic ring of ATP. The

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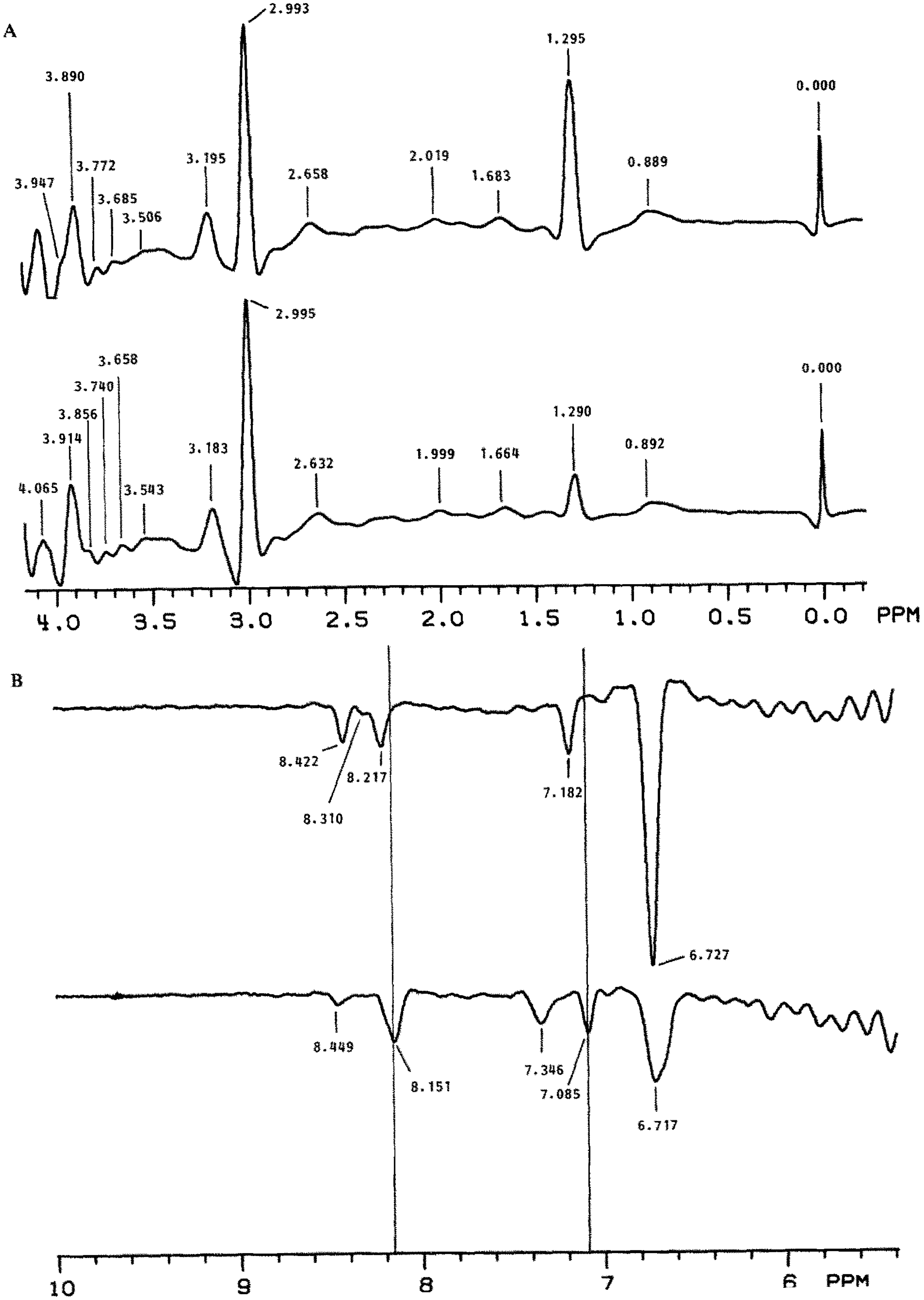
Fig.3. Aging of frog muscle: The lower spectrum shows the freshly dissected muscle and the upper spectrum shows the same muscle after 9.5 h in the NMR tube at 20°C. A, aliphatic spectral region; B, aromatic spectral region. Each spectrum was accumulated with 256 transients (7 min). The perpendicular lines in B show the downfield shift of C4-H, C2-H and N-H protons of carnosine in 9.5 h.


A
2.993
3.890
1.295
0.000
3.947
3.772
3.195
2.658
2.019
1.683
0.889
3.685
3.506
2.995
3.658
3.740
3.856
3.914
4.065
3.543
3.183
2.632
1.999
1.664
1.290
0.892
0.000
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PPM
B
8.422
8.310
8.217
7.182
6.727
8.449
8.151
7.346
7.085
6.717
10
9
8
7
6
PPM

resonance at 8.133 ppm is from the C2-H and NH protons of the histidine ring in carnosine, whereas that at 7.065 ppm is derived from the C4-H proton of carnosine. From this chemical shift of the C4-H proton a pH of 7.19 is estimated in the intracellular water of the fresh frog muscle.

3.3. *Aging of frog muscle*

We followed the aging of dissected frog muscle under anaerobic conditions up to 9.5 h at 20°C. Fig.3 shows the first and the last spectra. In the aliphatic region one observes the build up of lactic acid (1.295 ppm), whereas in the aromatic region phosphocreatine disappears (7.346 ppm) and the creatine level (6.7 ppm) increases. (Note that creatine contributes 4 protons, whereas phosphocreatine contributes only one proton to the 6.7 ppm peak.) The downfield chemical shifts of the C4-H, C2-H, and NH protons as a result of muscle aging are also illustrated in the aromatic region. The intracellular pH changes from 7.09 to 6.53 during the 9.5 h period.

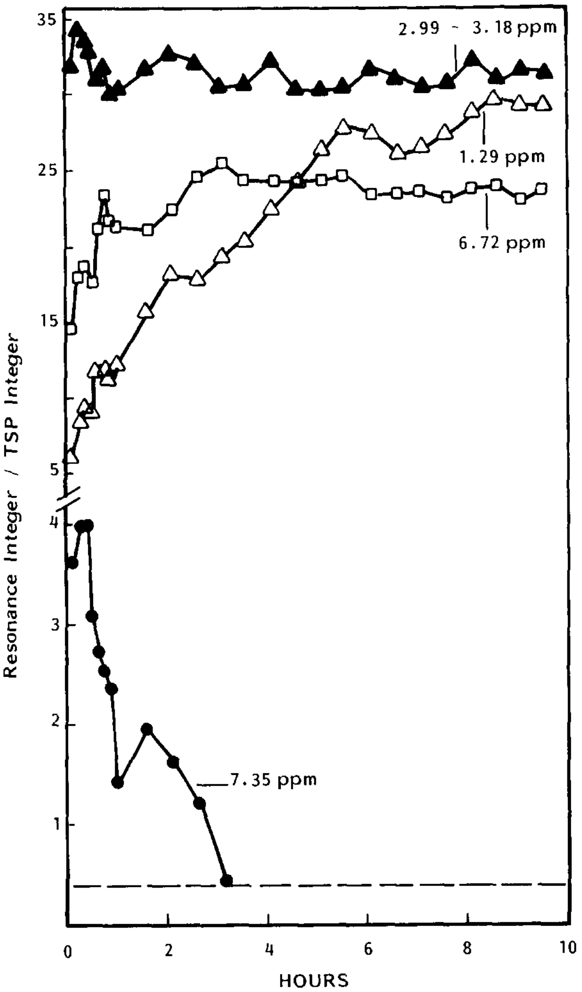


Fig.4. Changes in the relative levels of phosphocreatine (7.35 ppm) (●), creatine (6.72 ppm) (□), lactic acid (1.29 ppm) (△) and the sum of total creatine, carnitine and α-glycerophosphorylcholine (2.99–3.18 ppm) (▲) as a function of time of aging of freshly dissected frog muscle at 20°C. Each data point is derived from a spectrum accumulated with 256 transients (7 min). The dotted line at the bottom of the figure indicates the limit of detection.

Fig.4 shows the changes in the relative levels of phosphocreatine, creatine, and lactic acid as a function of the aging time of the muscle. A rapid decrease in phosphocreatine occurred, so that after 3 h phosphocreatine was not detectable with certainty. Concomitant with the decrease of phosphocreatine was the increase of creatine with a plateau after 3 h. On the other hand, the level of total creatine, carnitine, and α-glycerophosphorylcholine (3–3.2 ppm) remained constant, as expected. The curve depicting lactic acid showed a continuous rise during the 9.5-h period.

4. DISCUSSION

Our results confirm and extend those in [11] on the feasibility of ^{1}H NMR to investigate intact muscle. The main advantage of ^{1}H NMR over ^{31}P or ^{13}C NMR, of course, is the very short time needed to obtain a usable spectrum, indeed at 11 T even a single transient or 0.7 s may be sufficient. With this time resolution, ^{1}H NMR is by far the best candidate for detecting changes in metabolites during a single muscle contraction–relaxation cycle. Another important aspect is the small sample size. Thus, in a 5 mm sample tube only one frog sartorius is required for ^{1}H NMR as compared with the 4 sartorii for ^{31}P NMR [3]. Furthermore, a small piece of human muscle may be adequate for characterization of a disease by ^{1}H NMR as compared with a larger amount of muscle when ^{13}C NMR is used [14].

In our experience, the sequence in [11] for water suppression is superior to any other method tried previously for recording intact tissue ^{1}H NMR spectra. First, the suppression of water peak is at least 250-fold. Second, the baseline is smooth allowing the detection of minor resonances. Third, the exchangeable protons, e.g., those bound to nitrogen, are detected; this allowed us to resolve phosphocreatine resonances separately from creatine resonances within the same muscle. In this respect, ^{1}H NMR advances the study of muscle energetics, since ^{31}P NMR measures only phosphocreatine and ^{13}C NMR measures the total creatine content of muscle. With the technique of selective saturation of the water signal, authors in [11] were only able to determine the total creatine in muscle by ^{1}H NMR alone and had to use ^{31}P NMR on the same muscle to estimate creatine phosphate and creatine individually. We also observed the exchangeable, nitrogen-bound proton of carnosine not noted in [11]. In addition to the large differences in the magnetic field used in the two laboratories (2.35 *vs* 11.05 T), their failure to resolve these peaks may have resulted from the presaturation pulse technique [11]. This technique can produce cross saturation and the disappearance of resonances from protons that exchange with water.

Following the idea in [11] we have used the chemical shift of carnosine protons to determine the intracellular pH. From the pH dependence of the chemical shifts of the C4-H, C2-H and N-H protons of pure carnosine at physiological ionic strength we determined an NMR titration curve fitted to a Henderson-Hasselbach type equation (section 2.4). Our estimates for the intracellular pH of fresh frog muscle based on this curve range from 7.09 to 7.39 (depending on the sample), while for a muscle aged 9.5 h we found a value of 6.53. Authors in [11] reported an initial intracellular pH value of 7.2 for frog muscle and a value of 6.9 after 5 h of anaerobic incubation. With ^{31}P NMR authors in [2] estimated the intracellular pH of fresh frog muscle to be 7.2 whereas those in [3] estimated this pH to be 7.5. It appears that ^{1}H NMR is an alternative method to ^{31}P NMR for the measurement of muscle pH.

The simultaneous measurement of levels of lactic acid, phosphocreatine, creatine and intracellular pH illustrate the potential of ^{1}H NMR for studying muscle metabolism.

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