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Intracellular pH in human skeletal muscle by ^{1}H NMR

(carnosine/exercise/^{31}P NMR)

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ABSTRACT **We report here the *in vivo* observation of the imidazole protons (C-2 and C-4) of carnosine (β-Ala-His) by ^{1}H NMR at 4.7 T in human skeletal muscle. The relationship between the pH and chemical shift of the C-2 and C-4 resonances was determined analytically. These titration curves were used to measure the resting pH of human muscle *in vivo*, 7.01 × 0.04 (C-2 proton) and 6.97 ± 0.10 (C-4 proton). An *in vivo* titration curve of the C-2 proton resonance was determined by interleaving ^{1}H and ^{31}P NMR spectra after exhaustive exercise, during which muscle pH recovers from an acidic value of 6.1. We observed excellent agreement between the pH values as determined by the C-2 resonance of carnosine and that of inorganic phosphate. Carnosine provides an excellent pH indicator since (*i*) its concentration is relatively stable and (*ii*) it allows measurement of proton metabolites and pH to be achieved through the same coil, thus enabling a better signal/noise ratio and better localization.**

The imidazole protons of histidine have long been useful as pH indicators in NMR. In studies of proteins and intracellular pH of erythrocytes (1, 2), the chemical shift of the C-2 and C-4 ring protons with pH has yielded much information about the environment of the histidine residue. This method was extended by Yoshizaki *et al.* (3), who initially used the histidine-containing dipeptide carnosine (β-Ala-His) to determine pH in excised frog muscle. Subsequent analogous studies have been made of extracted human muscle (4) and *in vivo* rat muscle using anserine (β-Ala, methyl-His) (5, 6). We recently reported the *in vivo* observation of lactic acid production by ^{1}H NMR and its clearance in the human forearm muscle after exercise. Simultaneous tissue acidification was followed by ^{31}P NMR (7). Lactate was measured to have a clearance time of 10 min; however, parallel ^{31}P spectra showing recovery of muscle pH by using the pH-sensitive chemical shift of the P_i resonance was not always possible because of the acute drop in the concentration of P_i after exercise (8). Thus, an alternative method of *in vivo* pH determination would be very useful. We report here the *in vivo* observation of the C-2 and C-4 proton resonances of carnosine in human forearm muscle and show how they can be used to measure intracellular pH at rest and during recovery from exhaustive exercise.

METHODS

The relationship between pH and the chemical shift of the C-2 and C-4 protons was established by titrating a solution containing 150 mM KCl, 20 mM phosphocreatine (as a chemical shift reference), 30 mM carnosine, and, where specified, 1 mM $MgCl_2$. These analytic titration curves were determined on a Bruker AM-360 MHz wide-bore system using a 5-mm Helmholtz probe. Fully relaxed spectra were acquired with a spin-echo sequence with a 40-ms echo delay (all echo delay values in this paper are given as 1/2 of the entire spin-echo duration) with a total repetition time of 8 s. Water suppression was achieved by applying a single frequency presaturation pulse at 40 mW for 1 s and using carrier-suppressed 1–$\bar{1}$ and 2–$\bar{2}$ pulses (9) for both the excitation and refocusing pulses of the spin-echo sequence. The 90° pulse duration was 7 μs. Spectra were acquired at either 37°C or 21°C, as indicated by a Luxtron fluoroptic thermometer placed within the sample. pH measurements were obtained with a temperature corrected Corning 125 pH meter using an Orion glass electrode. A single pass through the pH range using 0.5 M HCl was taken to avoid large changes in ionic strength. Chemicals were obtained from Sigma or Baker.

A Bruker 4.7-T 30-cm bore Biospec system operating at 200.44 MHz for ^{1}H and 81.14 MHz for ^{31}P was used for all the human studies. A 2.5-cm single turn surface coil doubly tuned to ^{1}H and ^{31}P was placed over the flexor digitorum superficialis muscle. A plaster cast fitting the forearm was attached to the coil probe body to improve reproducibility and minimize movement. The muscle of interest was first localized by imaging on a 2.1-T 1-m-bore Biospec human system. NMR localization for the ^{1}H measurements was performed as described (10): the Π/2 duration in the volume of interest was determined to be 60 μs and localized shimming generally achieved a water width of $\delta\nu$ = 23 Hz. Subcutaneous fat resonances were suppressed by application of a seven-lobed sinc frequency selective 90° pulse in the presence of a field gradient linear in the axis perpendicular to the coil plane and dephased by application of pulsed-field gradients orthogonal to the selection axis. Further localization of the volume of interest was achieved by using a phase-cycled $\theta/3$ ($\pm x$) (11). Fat was also suppressed by application of a 1-kHz bandwidth hyperbolic secant inversion pulse centered on the upfield portion of the spectrum followed by a delay calibrated to null lipid resonances. Following the localization scheme, the acquisition sequence consisted of a spin-echo sequence with an echo delay time τ of 30 ms in the majority of the human experiments (sequence **1**) with a repetition time of 3 s. Water suppression was achieved by placing the carrier frequency on water and using a 1–$\bar{1}$ semiselective (9) pulse. The excitation maximum was optimized for either of the imidazole resonances by adjusting the interpulse delay τ_1 of sequence **1**

$$\theta/6\text{-}\tau_1\text{-}\theta/6[\pm x],\ \theta/2\text{-}\tau_1\text{-}\theta/2,\ \tau,\ \theta\text{-}\tau_1\text{-}\theta[\pm x,\ \pm y],\ \tau,\ \text{Acquire} \quad [1]$$

In most of the exercise experiments, ^{1}H spectra were accumulated with eight transients acquired in 30 s. Pulse acquire ^{31}P spectra were acquired with a tip angle of ≈30° in the volume of interest with a repetition time of 200 ms. Each ^{31}P spectrum represents 162 transients acquired over 35 s and arises from a volume that is ≈25% larger than that of the ^{1}H volume.

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The exercise protocol was performed within the magnet bore. It consisted of a supine subject lifting a 2-kg weight attached to the hand through a pulley system by flexion of the fingers until exhaustion. ^{31}P spectra were acquired during the exercise and interleaved ^{1}H and ^{31}P spectra acquired immediately after cessation of exercise.

RESULTS

Fig. 1 shows the titration curves obtained at 37°C from the model KCl/carnosine solutions with and without Mg^{2+} and at 21°C without Mg^{2+}. These data can be fitted to a three-parameter equation of the form

$$pH = pK + \log \frac{\sigma H - \sigma}{\sigma - \sigma A}. \quad [2]$$

Table 1 shows the pK values and limiting chemical shifts for the C-2 and C-4 carnosine resonances and the two temperatures. There is no significant difference between the pK_a values and limiting chemical shifts in the presence or absence of Mg^{2+}. The 37°C titration curves were used throughout the analysis of the *in vivo* experiments.

Fig. 2*a* shows a stacked plot of spin-echo *in vivo* spectra from human muscle showing the C-2 and C-4 carnosine resonances over a range of echo delays ranging from 8 to 15 ms. The pH values that are predicted by the two resonances are 7.01 ± 0.04 and 6.97 ± 0.10, respectively. The apparent T_2 values of the C-2 and C-4 resonances obtained from these and other spectra were calculated to be 39 ms and 11 ms, respectively. The relatively short T_2 of the C-4 resonance

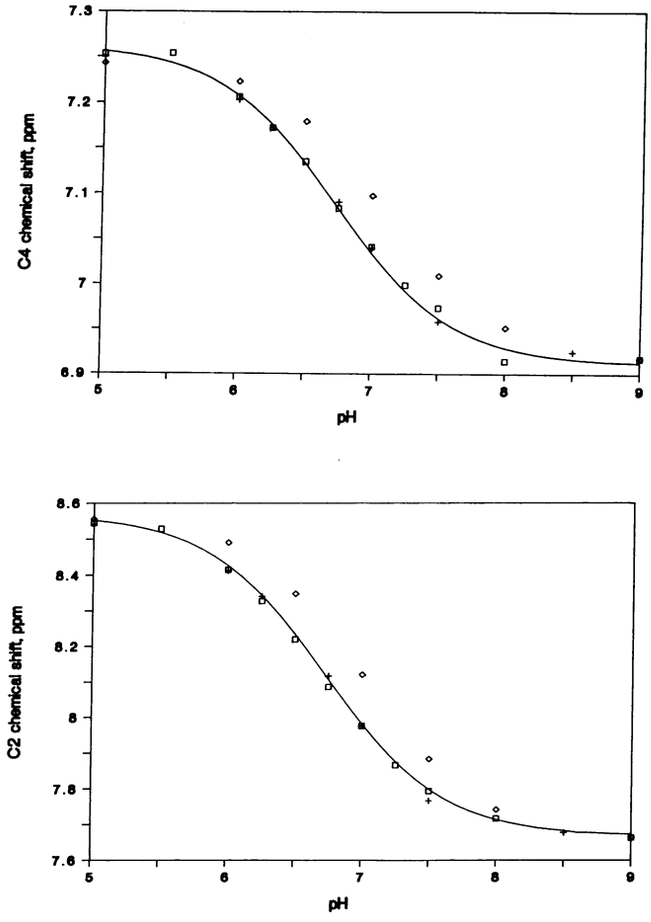

FIG. 1. Analytic titration curves of the C-2 (*a*) and C-4 (*b*) proton resonances carnosine. □, $-Mg^{2+}$, 37°C; +, $+Mg^{2+}$, 37°C; ◇, $-Mg^{2+}$, 21°C; solid line, fitted Henderson–Hasselbach equations.

Table 1. pK values and limiting chemical shifts for C-2 and C-4 carnosine resonances

t, °C	pK	σH, ppm	σA, ppm
C-2 37	6.73 ± 0.03	8.57 ± 0.01	7.67 ± 0.01
C-4 37	6.75 ± 0.06	7.26 ± 0.01	6.91 ± 0.01
C-2 21	7.04 ± 0.03	8.57 ± 0.01	7.66 ± 0.01
C-4 21	7.10 ± 0.06	7.25 ± 0.01	6.92 ± 0.01

results in significant losses in signal/noise when echo times are longer than 15 ms. Therefore, since the chemical shift of the C-2 resonance has a stronger dependence on pH and its T_2 is longer than that of muscle water and fat, we obtained the *in vivo* titration spectra on this resonance.

The concentration of carnosine within human skeletal muscle was approximated by comparison with the known creatine plus phosphocreatine concentration of 42.5 mM (12, 13), or 127.5 mM in methyl protons. Fig. 2*b* shows an exemplary ^{1}H spectrum from which we obtained relative ratios of carnosine and creatine. Correction factors predicted by (*i*) the magnetization vs. frequency profile resulting from pulse sequence **1** at the known rf intensity and by (*ii*) the relaxation rates of creatine and carnosine. Using creatine relaxation times of 1.05 s (T_1), 120 ms (T_2) (unpublished data of T_1 in human skeletal muscle and T_2 in postmortem rabbit muscle measured at 4.7 T) and a T_2 of 39 ms for the C-2 resonance of carnosine, we obtained from two volunteers an approximate carnosine concentration of 8.06 mM and 5.13 mM in forearm skeletal muscle. This is within the reported biochemical range of 2–20 mM (14) and supports our assumption that these downfield histidine resonances are indeed due to carnosine (15).

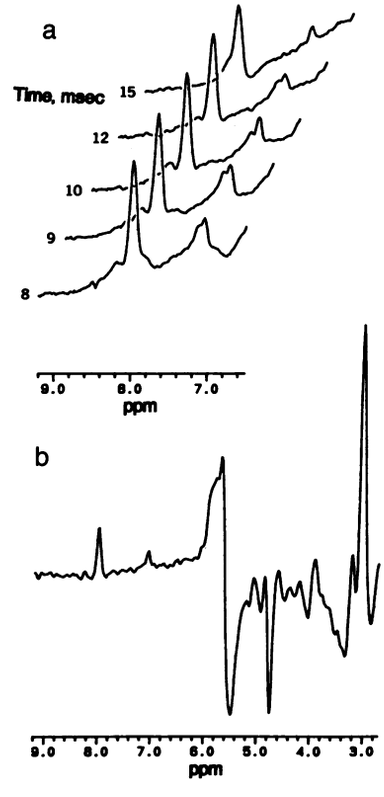

FIG. 2. (*a*) ^{1}H stacked human skeletal muscle spectra obtained *in vivo* by using sequence **1** in text showing the downfield carnosine resonances. Times indicate spin-echo delay τ_1. (*b*) *In vivo* human skeletal muscle spectrum where resonances are given in ppm: 8.0, C-2 carnosine; 7.0, C-4 carnosine; 6, olefinic protons of lipid; 3.2, choline and taurine CH_3; 3.0, creatine and phosphocreatine CH_3.

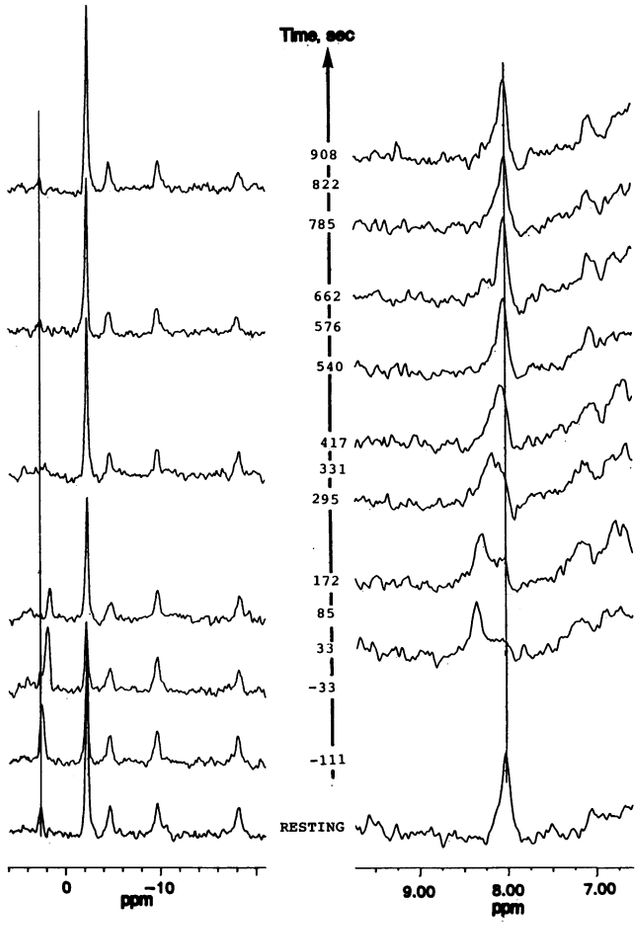

FIG. 3. Interleaved ^1H and ^{31}P spectra of an exemplary exercise study. ^{31}P spectra were obtained during exercise ($t < 0$); interleaved ^1H and ^{31}P spectra used for *in vivo* titration correlation obtained during recovery ($t > 0$). ^1H spectra acquired with 8 scans; repetition time, 3 s; ^{31}P spectra acquired with 162 scans; repetition time, 0.2 s. The ^1H spectra show a small contribution from the unshifted carnosine position and may represent signals from deeper unexercised muscle (flexor digitorum profundus).

The *in vivo* titration curve was determined over the acidic range (7.0–6.1) by obtaining interleaved ^1H and ^{31}P spectra after aerobically exercising to exhaustion. The acidification with exercise and subsequent pH recovery (as indicated by inorganic phosphate or carnosine) from pH = 6.1 to neutrality for a typical experiment is shown in Fig. 3. A plot of the recovery is shown in Fig. 4, in which we used a P_i titration curve at 1.36 mM free Mg^{2+} defined by σH = 0.94 ppm, σA = 3.33 ppm, and pK_a = 6.67 (16) to determine pH.

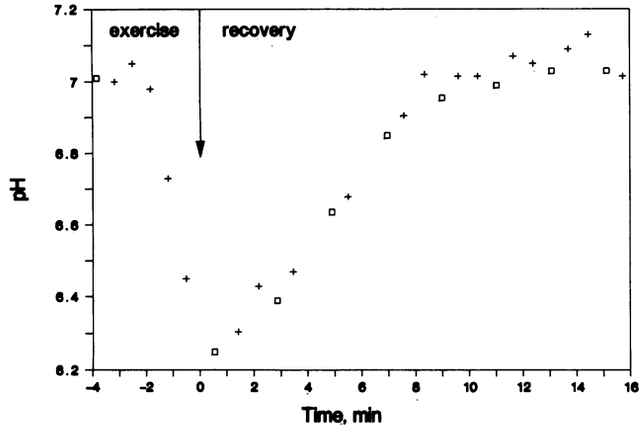

FIG. 4. Plot of pH recovery with time (min) as measured by spectra (□, carnosine, 8 ppm; +, P_i) shown in Fig. 3. Note that not all P_i spectra give measurable pH values.

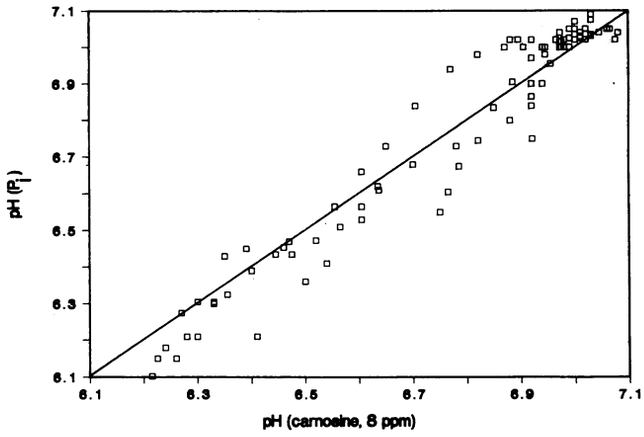

FIG. 5. Scatter plot of pH values as measured by the C-2 resonance of carnosine (x axis) or P_i (y axis). Perfect correlation (r^2 = 1.0) is indicated by the diagonal line.

The correlation between pH values as determined by carnosine and P_i is shown in Fig. 5 from a number of experiments. When fitted to a $y = x$ line, the two methods of pH determination agreed with each other to r^2 = 0.94. Ideal correlation (r^2 = 1.0) is indicated by the solid line.

The signal/noise ratios of P_i and the 8 ppm carnosine resonance obtained in Fig. 3 are shown in Table 2. As shown in this table and Fig. 3, there are several pH points in which the signal/noise ratios and concentration of P_i drops severalfold below resting levels. This is in agreement with previous studies showing a physiological undershoot of P_i after aerobic exercise to exhaustion (8). The uncertainty in pH values at these time points is thus large. This highlights the utility of carnosine since its concentration is not observed to change with recovery from exercise. Also shown in Table 2 are the values for the minimal pH change measurable (δpHmin) at each pH value. This quantity is calculated from the signal/noise ratio, shift in hertz of the pH-sensitive resonance defined by the analytic titration curve, and its apparent line width. Finer resolution of the pH value is possible with a smaller δpHmin; thus, under the given acquisition conditions, the 8 ppm resonance of carnosine enables a measurement of the change in pH of at least 0.043 pH unit at all pH values.

DISCUSSION

We have presented a means of pH determination in human skeletal muscle by ^1H NMR that uses the pH sensitivity of the C-2 proton resonance of carnosine. Analytic titration curves

Table 2. Signal/noise ratios (S/N) after exercise at various pH values

Carnosine			P_i		
pH	S/N	δpHmin	pH	S/N	δpHmin
6.25	5.5	0.043	6.31	4.67	0.058
6.39	5.5	0.037	6.44	2.67	0.087
6.64	5.5	0.033	6.8	2.0	0.10
6.85	6.25	0.029	6.94	1.8	0.12
6.96	8.0	0.024	6.99	2.0	0.11
6.99	8.5	0.023	7.01	2.0	0.12
7.03	7.5	0.027	7.03	2.3	0.10
7.03	8.25	0.024	7.04	2.3	0.097

comparing KCl/carnosine solutions with and without Mg^{2+} did not display any significant differences. This is in contrast to P_i, where both the pK_a and limiting chemical shifts of P_i change with the concentrations of free Mg^{2+} (16). Our values for the limiting chemical shifts and pK (at 21°C) agree well with reported values (4, 5). The pK depends on the temperature, as seen by a shift in the pK by 0.35 from 37°C to 21°C.

In vivo, the C-2 resonance of the carnosine imidazole ring has an apparent T_2 of 39 ms. The C-4 resonance has a shorter apparent T_2 of 11 ms. This may be due to paramagnetic cation binding effects, since ESR and NMR studies (17) of Cu^{2+}–carnosine complexes have shown imidazole proton interactions with the metal ion. In these studies, Mg^{2+} did not have an effect on the NMR spectrum of carnosine, supporting our *in vitro* observations that Mg^{2+} does not appear to affect the pK_a and chemical shifts of the histidine protons.

The signal/noise ratio of the 8 ppm resonance of carnosine may be further improved by several means. Composite pulses can be used to increase the size of the sensitive volume and may be expected to give a 40% increase in the signal/noise ratio of the metabolite signal (18). Furthermore, these experiments were performed with a ^1H preamplifier in which the noise figure could be improved 3-fold. Finally, shorter echo delays can be used such that magnetization decay due to transverse relaxation is decreased. Adequate water suppression is obtained with sequence **1** until very short echo times ($\leq$4 ms) are used, in which $1\text{–}\bar{3}\text{–}3\text{–}\bar{1}$ or presaturation techniques may also be used.

The usage of carnosine as a ^1H NMR pH indicator is complementary to and distinct from P_i in several ways. First, it enables an independent noninvasive comparison of tissue pH with excellent agreement between the two methods. Second, carnosine may be extremely useful in studies in which the concentration of P_i drops acutely—e.g., muscle exercise studies, or obscured by other resonances—e.g., 2,3-diphosphoglycerate. In exercise studies, carnosine can thus substantially improve the time resolution. Finally, more precise volume localization is enabled when measuring both pH and metabolites by the same nucleus. Using one surface coil at the same frequency and power for both measurements ensures identical radio frequency penetration into the sample and thus provides for the observation of equivalent sample volumes.

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