Phosphorus Nuclear-Magnetic-Resonance Studies of Compartmentation in Muscle

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1. Phosphorus nuclear-magnetic-resonance measurements were made on rat vastus lateralis muscle. 2. In the absence of oxygenation, the resonance from P_i broadens, as the 'energy pool' of the muscle gradually runs down. This, together with measurements of transverse relaxation times (T_2), indicate that the intracellular pH is not uniform within the muscle volume. 3. Incubation of the muscle with acetate buffer at low pH (5.2) results in splitting of the P_i resonance into two components; one corresponds to phosphate in a low-pH environment and the other to phosphate in its original environment. These observations indicate that P_i is distributed among different compartments in the muscle cell. 4. Compartmentation of sugar phosphate (mainly glucose 6-phosphate) is also indicated by this method, but no evidence has been obtained for this type of compartmentation of ATP and phosphocreatine.

X-ray diffraction and electron microscopy have provided a wealth of information about the structure of striated muscle. In contrast, little is known about the spatial distribution of the rapidly diffusing molecules that fuel the cell or catalyse many of its functions. Knowledge of this distribution is essential if we are to verify those theories of cellular metabolism and its control that are based on experiments performed *in vitro*. For example, the activity of phosphorylase, the key enzyme of glycogen metabolism, is partly controlled by the binding of metabolites, and is therefore dependent on the local concentrations of these molecules. The activity of phosphorylase in resting muscle is very small; it can be increased by a factor of over 1000 during contraction. To reconcile the low resting activity with the measured binding constants and total metabolite concentrations it may be necessary to postulate that the concentrations of some of the ligands near phosphorylase differ from their spatially averaged concentrations in the sarcoplasm. Thus compartmentation of the metabolites into different regions of the cell could play a significant part in the control of phosphorylase or other regulatory enzymes such as phosphofructokinase.

Phosphorus (^{31}P) n.m.r. can provide detailed information about the concentrations and environments of phosphorus-containing metabolites in intact tissues (Hoult *et al.*, 1974; Burt *et al.*, 1976; Dawson *et al.*, 1977; Colman & Gadian, 1976) and organisms (Salhany *et al.*, 1975). We reported that compartmentation of metabolites in muscle can also

be observed by ^{31}P n.m.r. (Seeley *et al.*, 1976). In particular, we demonstrated that P_i exists in a range of environments that are likely to reflect variation of pH within the muscle volume. We have extended these observations by bathing muscles in acetate buffer to perturb the intracellular pH, and the results in the present paper indicate the presence of discrete pools of metabolites in the rat vastus lateralis muscle.

N.M.R.: A Theoretical Introduction

We shall introduce in this section some of the concepts that we use in interpreting our results [A detailed account of n.m.r. theory is given by Dwek (1973) and Farrar & Becker (1971).]

Resonance linewidths

The frequency of a nuclear resonance signal, ν , is proportional to the magnetic field experienced by the nucleus:

$$\nu = (\gamma/2\pi)B_0(1-\sigma)$$

where γ is the magnetogyric ratio of the nucleus, B_0 is the magnetic flux density of the spectrometer's magnetic field, and σ is the 'chemical shift' of a particular nuclear environment and describes the chemical environment of the nucleus.

If the magnetic field varies across the sample for instrumental reasons, this inhomogeneity in B_0 (ΔB_0) will contribute an amount $\Delta\nu_{\text{inh.}}$ to the line width of the signal:

$$\Delta\nu_{\text{inh.}} = (\gamma/2\pi)\Delta B_0$$

At 129 MHz, an inhomogeneity of only 1 part in 10^7 would produce a line broadening of 13 Hz. Such inhomogeneity could result simply from introducing a sample tube containing muscle into the spectrometer.

Each signal also has an intrinsic line width:

$$\Delta\nu_{\text{int.}} = 1/\pi T_2$$

where T_2 is a parameter known as the transverse relaxation time. It can be determined by using a sequence of radio-frequency pulses known as the Carr–Purcell–Meiboom–Gill sequence (Carr & Purcell, 1954). T_2 is dependent on the detailed motion of the nucleus and structural features of molecule concerned, and, in general, T_2 decreases (and intrinsic line width increases) when the rate of molecular motion decreases. A nuclear signal will therefore broaden when, for example, a small molecule binds to a slowly rotating macromolecule.

So-called ‘exchange effects’ can also broaden the signal. A nucleus in a molecule that exists in two or more forms participates in exchange. The two states of ionization of a phosphate group, for example, represent two chemical forms affecting a phosphorus nucleus. When the rate of exchange among different forms is very slow, separate signals are observable for each form. When the exchange rate between the two species (e.g. HPO_4^{2-} and H_2PO_4^-) is much greater than the frequency separation of their two signals, a single exchange-averaged resonance will be observed. Broadening occurs when the exchange rate is smaller than or similar to the frequency separation.

In the absence of exchange-broadening effects, the observed line width of a single resonance is:

$$\Delta\nu_{\text{obs.}} = \Delta\nu_{\text{inh.}} + \Delta\nu_{\text{int.}}$$

When $\Delta\nu_{\text{inh.}}$ and $\Delta\nu_{\text{int.}}$ are separately determined, and their sum is equal to $\Delta\nu_{\text{obs.}}$, we can conclude that the observed resonance consists of a single component. If there is a significant experimental discrepancy, the observed resonance must consist of several components of different frequencies, so that one observes the envelope of the individual signals. This is the basis of the arguments described below for the existence of a distribution of pH within muscle.

Magnetic-field inhomogeneity produces the same increment in line widths for all resonances. [It is conceivable that some metabolites that are segregated into distinct regions within the muscle experience greater field inhomogeneities than others. This is unlikely, since in the large number (approx. 10^3 – 10^4) of similar and small units of muscle (the fibres) metabolite compartmentation would be expected to occur on a microscopic scale (approx. $1\ \mu\text{m}$), whereas field inhomogeneity occurs on a macroscopic scale of millimetres.] The phosphocreatine signal is invariably the narrowest in the muscle spectra, and its line width therefore sets an upper limit to the broadening

produced by field inhomogeneity. It is also likely that inhomogeneities of magnetic susceptibility in the sample will affect all the resonances equally.

pH-dependence of resonance frequencies

P_i exists mainly as HPO_4^{2-} and H_2PO_4^- around neutral pH. In the absence of exchange, these two species would give rise to two resonances separated from each other by about 2.3 p.p.m. (300 Hz at 129 MHz). In solution, however, the two species exchange with each other extremely rapidly (approx. 10^9 – 10^{10}s^{-1}), and, as a result, the observed spectrum consists of a single resonance the frequency of which is determined by the relative amounts of the two species. The frequency of this signal as a function of pH gives a ‘standard’ titration curve. This resonance frequency of the phosphate signal in muscle can be used to determine intracellular pH (Hoult *et al.*, 1974). One must ensure that other factors (e.g. chelation by metal ions) produce negligible shifts in frequency. Control experiments demonstrated that the effects on the P_i signal of likely variation in ionic strength or metal-ion binding within the muscle are very small. The other prominent muscle phosphate signals are those of phosphocreatine and ATP. Because phosphocreatine has a very low pK_a (4.6), its resonance is very insensitive to pH changes around neutrality.

Materials and Methods

Treatment of muscle

Vastus lateralis muscles were excised from 200 g Wistar rats. The rats were normally killed by exposure to diethyl ether vapour for 5–10 min. A few experiments performed on the muscles of rats killed with sodium pentobarbitone confirmed that magnetic-resonance parameters were not grossly affected by the ether treatment.

Vastus lateralis muscles were used because they can be excised very rapidly (less than 4 min), and because they conveniently fit into the n.m.r. sample tubes (8 mm internal diameter). Under the conditions of our experiments it was not possible to maintain the resting concentrations of metabolites, and the amounts of high-energy phosphates were gradually depleted over a period of about 2–3 h. This is not a major drawback, as good spectra are obtained in as little as 3 min.

The Ringer solution contained 150 mM-NaCl, 6 mM-KCl, 2 mM- NaHCO_3 and 8 mM-glucose (pH 7.2). The acetate buffer contained 50 mM-NaCl, 6 mM-KCl, 2 mM- NaHCO_3 and 100 mM-sodium acetate and was adjusted to the required pH with HCl. Both solutions were bubbled with O_2 outside the spectrometer.

N.m.r.

^{31}P n.m.r. spectra were recorded at 129 MHz on a spectrometer constructed in this laboratory and interfaced with a Nicolet B-NC 12 computer. Details of this spectrometer have been reported elsewhere (Hoult & Richards, 1975). The spectrometer was operated in the Fourier transform mode and used an external $^2\text{H}_2\text{O}$ field-frequency lock. A 'stiff' magnetic disc was used for long-term storage of the accumulated free induction decays.

Metabolite concentrations were measured from signal areas on the basis of assignments made by Hoult *et al.* (1974).

To optimize the signal-to-noise ratio of the n.m.r. spectra, 60° radio-frequency pulses were applied at time intervals comparable with the spin-lattice relaxation time, T_1 , of the resonances. Consequently, the peak areas are only proportional to the concentrations of the metabolites if all their resonances have the same T_1 . To obtain the true concentrations of metabolites we must apply correction factors that are dependent on the T_1 values for each metabolite. To determine these factors spectra were accumulated at several pulse intervals, varying from 1 to 8 s. For pulse intervals of 2 s, the true quantities of metabolites can be obtained if the ATP, phosphocreatine and P_i resonance areas are multiplied by 1.0, 1.5 and 1.5 respectively. In the acetate experiments, the peak areas of the 'low-pH' pool of P_i must be multiplied by 1.25.

Absolute quantities of metabolites were determined from the resonance areas by assuming that in each muscle the quantities of phosphocreatine, P_i and sugar phosphate added up to $30\text{ }\mu\text{mol/g}$ wet wt. (cf. Fitch *et al.*, 1974; Beis & Newsholme, 1975). Determination of the absolute quantities could, if necessary, have been carried out by n.m.r. without this assumption, but this would have been extremely time-consuming, as a calibration would have been required for each muscle. Metabolite quantities determined by n.m.r. have been in good agreement with values from freeze-extraction studies (Fitch *et al.*, 1974; Beis & Newsholme, 1975).

Peak areas were determined by integration, by using the computer. For closely separated peaks the areas were determined with the aid of computer simulations of the peaks. Line widths were measured as the width at half-height of the peaks.

Chemical shifts were measured relative to the shift of a standard sample of 85% (v/v) H_3PO_4 . Experiments were performed at 15°C unless otherwise stated.

Transverse relaxation times (T_2) were measured by using the Carr–Purcell–Meiboom–Gill sequence of radio-frequency pulses: (90° , 1 ms; 180° , 2 ms; 180° , 2 ms; 180° , etc.). The train of 180° pulses (each $100\text{ }\mu\text{s}$) was stopped after a specified time and the residual

free induction decay observed. The areas of the resonances in the transformed spectra plotted as a function of the length of the 180° pulse train gave the T_2 values, i.e.:

$$A = A_0 e^{-\tau/T_2}$$

where τ is the length of the 180° pulse train.

Results

Fig. 1 shows ^{31}P n.m.r. spectra of a rat vastus lateralis muscle recorded at various times after excision of the muscle. The muscle remained in the closed n.m.r. tube throughout the period, and, owing to limitation of O_2 supply, the high-energy-phosphate reserve was gradually depleted; P_i was formed as a result of phosphocreatine breakdown. The width and shape of the P_i signal are particularly interesting. Initially it is about 40 Hz wide, but as the muscle ages the line width increases and the signal becomes asymmetric. The line width of the phosphocreatine signal remains constant at about 15 Hz. These results are typical of our many observations on aging muscles.

We have measured the intrinsic line width of the P_i signal using the Carr–Purcell–Meiboom–Gill technique (see the Materials and Methods section). $\Delta\nu_{\text{int.}}$ for P_i is 3 Hz. The contribution of field inhomogeneity to the line width is unlikely to be greater than 15 Hz, the width of the phosphocreatine signal. Therefore the observed P_i line width, if the resonance is a single component, should be no greater than 18 Hz. Since the line width is always considerably greater than this (approx. 40 Hz), the P_i signal must consist of narrow (18 Hz wide), partially overlapping, components: the observed signal is the envelope of the individual components. This is confirmed by the resolution of several signals for P_i in some muscles. Fig. 2 shows spectra of such a muscle that was allowed to age at 25°C .

Since the frequency of the P_i signal is dependent on the pH of the solution near neutrality ($\text{p}K_{a2} = 6.9$) (see below), a simple explanation for our observations is that pH is not uniform within the muscle volume. The various components of the P_i signal therefore correspond to regions of differing pH, and the shape of the signal represents the weighting of the pH distribution (see the Discussion section).

Acetate experiments

The experiments described above suggest that the pH-dependence of the P_i signal can be used to examine the distribution of metabolites in muscle.

Fig. 3 shows spectra of a vastus lateralis muscle that had been bathed in acetate buffer, pH 5.2, for 20 min before being placed in the spectrometer. The interesting feature of these spectra is the gradually increasing amplitude of a new signal of chemical

Fig. 1. Series of ^{31}P n.m.r. spectra of a rat vastus lateralis muscle, recorded as a function of time after excision at 15°C . The muscle was in Ringer solution at 15°C throughout the course of the experiment. Each spectrum represents the accumulation of 400 scans repeated at 2 s intervals, and the times given are the midpoints of each accumulation. Peaks A, B and C arise from the β -, α - and γ -phosphorus atoms respectively of ATP; peak D arises from phosphocreatine, peak E from P_i and peak F from sugar phosphates.

Fig. 2. Series of ^{31}P n.m.r. spectra of a rat vastus lateralis muscle, recorded as a function of time after excision at 25°C . The muscle was in Ringer solution at 25°C throughout the course of the experiment. Each spectrum represents the accumulation of 100 scans repeated at 2 s intervals, and the times given are the midpoints of each accumulation. Peak assignments are as for Fig. 1.

shift (-1.05 p.p.m.); other features of the spectra are similar to those of muscle bathed in Ringer solution. Although the position of this new peak corresponds approximately to that of the phosphate diester reported to be present in some muscles by us (Seeley, 1975; Seeley *et al.*, 1976) and others (Burt *et al.*, 1976), it is easy to demonstrate that the signal that we observe here is from P_i . Thus in a muscle, freeze-clamped after acetate treatment, the HClO_4 extract only gave a single signal in the phosphate region (after neutralization of the solution). In contrast, extracts prepared from muscles that originally showed the phosphodiester signals showed this peak.

pH titrations of MgATP, phosphocreatine, P_i and glucose 6-phosphate are shown in Fig. 4, and, by comparing these titrations with the spectra in Fig. 3, we can conclude the following: ATP and phosphocreatine are present only in solutions of $\text{pH} \geq 5.7$; P_i is present in two types of solution, one of the usual muscle pH (approx. 7.0) and another of pH about 5.8.

Phosphate in extracellular fluid

The signal from a pool of P_i of pH close to that of the bathing medium suggests that some of the P_i may

Fig. 3. Series of ^{31}P n.m.r. spectra of a rat vastus lateralis, recorded as a function of time after excision and kept in acetate buffer at 15°C

The muscle was bathed in acetate buffer, pH 5.2, for 20 min, and then placed in this buffer into the spectrometer. The temperature was 15°C throughout. Each spectrum represents the accumulation of 200 scans repeated at 2 s intervals, and the times given are the midpoints of each accumulation. Peak assignments are as for Fig. 1, and the additional peak X is assigned to P_i at low pH.

have leaked into the extracellular fluid. To examine this possibility, we bathed the muscle in acetate buffer, pH 5.2, containing 2 mM- MnCl_2 . The Mn^{2+} ion is paramagnetic and therefore considerably broadens the n.m.r. signals of any phosphate mole-

cules that are accessible to it in the extracellular fluid. A solution of 20 mM- P_i containing 2 mM- MnCl_2 , for example, gives a phosphate signal of about 5 kHz width. No broadening (or intensity loss) of any of the muscle phosphate signals was observed in the

Fig. 4. pH titrations of (a) the α - (▼), β - (▲) and γ - (■) resonances of MgATP, and (b) the resonances of glucose 6-phosphate, P_i and phosphocreatine

The chemical shifts of the ^{31}P n.m.r. resonances were measured as a function of pH. The titrations were performed by adding HCl or NaOH to solutions containing (a) 5 mM-ATP, 6 mM-MgCl₂, 0.5 mM-EDTA, and (b) 5 mM-glucose 6-phosphate (■), 5 mM- P_i (▼), 5 mM-phosphocreatine (▲) and 1 mM-EDTA. Measurements were made on the spectrometer described in the Materials and Methods section.

presence of 2 mM-MnCl₂, indicating that none of the muscle phosphate is accessible to Mn²⁺. This demonstrates that no muscle resonances arise from extracellular phosphate, and therefore that both pools of P_i must be intracellular. Against this, one might wish to consider the possibility that Mn²⁺ is not accessible to that region of the muscle where P_i could be extracellular. Were this view to be correct, it would be necessary to assume that none of the extracellular P_i was accessible to Mn²⁺, whereas all of the extracellular phosphate was perturbed by acetate. We consider this possibility to be extremely unlikely.

Diffusion of acetate into muscle

Muscles were bathed for 50 min in acetate buffer, pH 5.2, and then placed in the same buffer in the n.m.r. spectrometer. The quantities of phosphocreatine and the two pools of P_i obtained from the n.m.r. spectra (see the Materials and Methods section) are given in Table 1. The final quantities of 'low-pH' and 'high-pH' P_i agree well with the values of 7.5 and 15.5 $\mu\text{mol/g}$ wet wt. obtained from a muscle bathed in acetate buffer for 80 min before being placed in the spectrometer.

To determine the time required for acetate to

Table 1. Quantities of phosphocreatine and the two pools of P_i , measured as a function of time in spectrometer. Muscles were bathed for 50 min in acetate buffer, pH 5.2, then placed in this buffer into the spectrometer. The quantities of the metabolites were obtained from the n.m.r. spectra as described in the Materials and Methods section. Results are expressed as means $\pm$ s.d. for observations made on four separate muscles.

Time in spectrometer (min)	Phosphocreatine ($\mu\text{mol/g}$ wet wt.)	P_i ($\mu\text{mol/g}$ wet wt.)	
		'Low pH'	'High pH'
5	8.4 $\pm$ 2.0	4.8 $\pm$ 1.2	12.5 $\pm$ 3.3
13	6.1 $\pm$ 1.2	6.3 $\pm$ 0.3	13.3 $\pm$ 2.9
21	4.1 $\pm$ 1.7	7.1 $\pm$ 0.3	15.1 $\pm$ 3.3

diffuse through the muscular volume, muscles were left in Ringer solution for about 90 min, by which time more than half the phosphocreatine had been hydrolysed. They were then bathed in acetate buffer, pH 5.2, for 20 min, and then placed in this buffer in the spectrometer. The average sizes of the two pools of P_i during the first 10 min in the spectrometer were

5 and 12 $\mu\text{mol/g}$ wet wt. These values are very similar to those obtained after bathing muscles in acetate for 50 min, indicating that the acetate had had its full effect after about 20 min. The acetate must therefore

Fig. 5. Quantities of phosphocreatine and the two pools of P_i plotted as a function of time after placing muscle into the spectrometer

'Time' represents time in spectrometer after 20 min incubation with acetate. ▲, Phosphocreatine; ▼, 'high-pH' P_i ; ■, 'low-pH' P_i . The quantities were determined from the peak areas of the spectra shown in Fig. 3, by using the correction factors as described in the Materials and Methods section.

diffuse through the muscle within a period of 20 min. When muscles are incubated with acetate for less than 20 min, the 'low-pH' and 'high-pH' P_i resonances are not well resolved, indicating that the 'low-pH' solution takes time to reach the final pH value of approx. 5.8. The conclusion that acetate diffusion reaches an equilibrium that is dependent on the rates of entry of acetate into different intracellular compartments (see the Discussion section) and not different extents of diffusion into the inner and outer volumes of the muscle is supported by detailed studies on muscles of different sizes, varying in weight from about 200 mg to 1 g. Irrespective of the total muscle volumes we essentially observed the same response of the muscle spectrum to acetate treatment.

Quantities of P_i in the two pools

From Fig. 3, we can determine the quantities of phosphocreatine and the two pools of P_i , and these values are plotted in Fig. 5. The ATP concentration stays constant at about 4 $\mu\text{mol/g}$ wet wt. We cannot estimate accurately the amounts of 'low-pH' and 'high-pH' P_i at the time of excision, but extrapolation of the plot back to the time at which the muscle was placed in the spectrometer gives the quantity of P_i in the 'low-pH' pool as only about 0.5 $\mu\text{mol/g}$ wet wt. Similar results were obtained in experiments performed on three further muscles. Values of 5 $\mu\text{mol/g}$ wet wt. and greater were obtained for the 'high-pH' pool.

Are there two pools of ATP and phosphocreatine?

Because MgATP and phosphocreatine have low pK_a values, the frequencies of their n.m.r. signals are

Fig. 6. ^{31}P n.m.r. spectra of a rat vastus lateralis muscle exposed to acetate buffer, pH 4.5

The muscle was bathed in the buffer for 20 min, and then placed in this buffer into the spectrometer. The temperature was 15°C throughout. The spectrum represents the accumulation of 200 scans, repeated at 2 s intervals, and the peak assignments are as for Fig. 3. This spectrum is typical of our observations of many muscles of different sizes bathed in acetate buffer of pH between 4.0 and 4.5.

Fig. 7. ^{31}P n.m.r. spectrum of a rat vastus lateralis muscle, exposed to Ringer solution containing $50\text{ }\mu\text{M}$ -adrenaline and $500\text{ }\mu\text{M}$ -ascorbic acid

The muscle was bathed in the solution for 20 min, then placed in this solution into the spectrometer. The temperature was 15°C throughout. The spectrum represents the accumulation of 200 scans repeated at 2 s intervals, and the peak assignments are as for Fig. 1.

not very responsive to pH changes above 5.5, and therefore the experiments that we have described do not totally exclude the existence of 'low-pH' pools of these compounds. However, even on bathing muscle in acetate buffer at pH 4.5, no shifts of the phosphocreatine or ATP resonances from their normal frequencies, or any splitting of these signals, were observed (see Fig. 6). The concentration of ATP was the same as in unperturbed muscles. The pH of the 'low-pH' solution, as determined from the frequency of the P_i signal, is less than 5.5, and is sufficiently low to permit resolution of signals from 'low-pH' and 'high-pH' pools of ATP and phosphocreatine should they exist. The quantities of ATP and phosphocreatine in the 'low-pH' environment are therefore within the noise level of our observations, i.e. less than $1\text{ }\mu\text{mol/g}$ wet wt.

Is there a 'low-pH' pool of sugar phosphates?

The intensity of the sugar phosphate signals can be increased by bathing muscles in Ringer solution containing one of three additives.

(i) 20 mM -2-Deoxyglucose. This compound is converted into 2-deoxyglucose 6-phosphate in the sarcoplasm, but cannot be metabolized further, since it is not a substrate for phosphoglucose isomerase. Deoxyglucose 6-phosphate therefore accumulates and can be observed in the n.m.r. spectra.

(ii) 2 mM -Iodoacetate. This alkylating reagent inhibits D-glyceraldehyde 3-phosphate dehydrogenase, and sugar phosphates formed at the head of the glycolytic pathway are consequently built up.

(iii) $50\text{ }\mu\text{M}$ -Adrenaline (+ $500\text{ }\mu\text{M}$ -ascorbic acid). Fig. 7 shows the spectrum obtained after bathing a muscle for 20 min in Ringer solution containing adrenaline. The main sugar phosphate signal has the frequency of glucose 6-phosphate at pH 7.0 and the smaller signal of slightly lower frequency corresponds to fructose 6-phosphate at this pH. (The pH of the muscle is 6.95, as determined from the P_i signal.)

The pH titration of glucose 6-phosphate (Fig. 4) indicates that a pool of the sugar phosphate at pH 5.2 would produce a resonance overlapping the 'high-pH' P_i signal. We therefore tested for the existence of a 'low-pH' pool of glucose 6-phosphate by bathing muscle in acetate buffer of pH 5.8. After incubating a muscle for 1 h in Ringer solution, and another for the same period in acetate at pH 5.8, the spectra of Figs. 8(a) and 8(b) were obtained: Fig. 8(c) was obtained by subtracting spectrum (a) from (b). Two positive peaks are evident, one of which corresponds to P_i at pH 6.2, and the other corresponds to glucose 6-phosphate at pH 6.1. The pH of the muscle in Ringer solution is 6.8. This experiment indicates the presence of two pools of glucose 6-phosphate, one at low pH and the other at the normal muscle pH.

Discussion

The perturbation technique that we have used, bathing muscles in 100 mM -acetate at relatively low pH, can potentially produce artifacts, and therefore our results must be treated with caution. Muscles bathed in acetate for 30 min lose their capacity to

Fig. 8. ^{31}P n.m.r. spectra indicating the presence of two pools of sugar phosphates

Spectrum (a) is of a vastus lateralis muscle incubated for 1 h in Ringer solution and then placed in this solution into the spectrometer. Spectrum (b) is of a muscle incubated for 1 h in acetate buffer, pH 5.8, then placed in this solution into the spectrometer. Both solutions contain $50\ \mu\text{M}$ -adrenaline and $500\ \mu\text{M}$ -ascorbic acid. Each spectrum represents the accumulation of 200 scans repeated at 2 s intervals, and the temperature was 15°C throughout. Spectrum (c) was obtained by subtracting, with the aid of the computer, spectrum (a) from spectrum (b). Peak A is assigned to P_i , and peak B to sugar phosphate, both at low pH. (Similar observations have been made in other experiments.)

contract in response to electrical stimulation, even though they have at this time a high complement of ATP and phosphocreatine. We have not yet investigated what stages in the activation process are adversely affected by acetate, but its presence does not appreciably alter the rate of breakdown of high-energy phosphate.

Aging muscle

A multi-component P_i signal is frequently observed in aging muscles. This suggests that muscular P_i experiences several pH environments, each of which gives rise to one of the components in the observed resonance. This interpretation is consistent with the narrowness of the resonance of phosphocreatine,

since its pK_{a2} is 4.6, and therefore the resonance frequency is insensitive to small variations in pH around neutrality. Furthermore, intracellular pH determinations from the P_i resonance frequency are entirely consistent with measurements of muscle pH by using weak-acid-distribution methods (Aicken & Thomas, 1976) and microelectrodes (Roos, 1975).

The observation of the envelope of several components, rather than an exchange-averaged line, indicates that P_i exchanges between its various environments with a rate of approx. 50 Hz or less. Since the viscosity of the sarcoplasm is not sufficiently high to account for the slowness of this exchange rate (Kushmerick & Podolsky, 1969; Seeley, 1975), it seems probable that the environments are separated from each other by membrane barriers.

It is thus feasible that different fibres have slightly different internal pH values. This might arise, for example, from a decreased accessibility to oxygen of the inner fibres and a resultant build-up of lactate in the centre of the muscle. Alternatively, the different metabolic properties of red and white fibre types could generate a pH distribution. Another explanation is that a distribution of pH exists within each cell. We cannot yet distinguish between an intra- and an inter-cellular distribution of pH, but at present we favour an interpretation of our results in terms of a distribution of pH among the different fibres.

Acetate-treated muscle

This interpretation of the spectra led us to examine the effects of perturbing the intracellular pH with acetate buffer. In the perturbed system two pools of P_i are observed. The time course of the appearance of the 'low-pH' pool suggests that its magnitude is not dependent on the extent to which acetate has penetrated macroscopically through the muscle volume; a steady state is reached when approx. 35% of P_i is in the 'low-pH' compartment and 65% in the 'high-pH' compartment. Also, the acetate effect is independent of the size of the muscle that we treat, implying that the observations do not arise from inadequate diffusion of acetate into centre of the muscle.

There are two possible explanations of these observations: (i) the two environments correspond to two types of fibre; (ii) each cell contains two types of compartment, which respond differently to acetate. The observation that the ATP and phosphocreatine signals are unaffected by acetate, even when the pH is sufficiently low to allow resolution of two pools of these compounds, argues against the first explanation. We therefore suggest that there are two types of compartment in the cell, which are distinguished by their response to added acetic acid. It then follows that, in the acetate-perturbed muscle, when the amount of P_i is low (similar to that in resting muscle) it exists predominantly in the pH-insensitive pool, but as the amount of P_i increases the pH-sensitive pool is

gradually filled. ATP and phosphocreatine exist predominantly in the compartment that is not titrated by acetate, whereas glucose 6-phosphate is present in both compartmental types.

The experiments do not define the compartments or show that metabolites are distributed the same way in the unperturbed muscle.

As a working hypothesis we suggest that the two compartments are the sarcoplasm and the sarcoplasmic reticulum.

It is believed that the sarcoplasmic-reticulum membranes are highly permeable to various small molecules and ions in the region of their junctional surface with the T-system tubules (Birks & Davey, 1969; Regus & Zierler, 1973).

Changes in external osmotic pressure by using uncharged molecules such as sucrose and ions such as Na^+ and Cl^- may be shown by electron microscopy to alter the volume of the sarcoplasmic reticulum (Birks & Davey, 1969). In addition, measurements of the wash-out of $^{24}Na^+$ from rat muscle into the extracellular space indicate a far greater flux from the sarcoplasmic reticulum than from the sarcoplasm (Regus & Zierler, 1973). It has been therefore suggested that the sarcoplasmic reticulum can be regarded as an extension of the extracellular space. It seems plausible that acetate too can penetrate into the sarcoplasmic reticulum, and therefore that the volume enclosed by the sarcoplasmic-reticulum membranes contains the acid-titratable pool of P_i and sugar phosphates. (We must postulate that the rate of entry of acetic acid into the sarcoplasm, either directly through the cell membrane or indirectly via the reticulum, is too slow to affect the pH in this region during the period of our experiments.) The molecules that are not titrated by acid are possibly located in the sarcoplasm.

It is thought that glycolytic activity may be associated with the sarcoplasmic reticulum (Roodyn, 1965; Karpatkin, 1967), and our suggestion regarding the compartmentation of P_i and glucose 6-phosphate is compatible with this view. Our results are also consistent with other reports of compartmentation of sugar phosphates (Moses & Lonberg-Holm, 1966; Landau & Sims, 1967).

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