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CONTRACTION AND RECOVERY OF LIVING MUSCLES STUDIED BY ^{31}P NUCLEAR MAGNETIC RESONANCE

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SUMMARY

1. Phosphorus nuclear magnetic resonance (^{31}P NMR) can be used to measure the concentrations of phosphorus-containing metabolites within living tissue. We have developed methods for maintaining muscles in physiological condition, stimulating them and recording tension while at the same time accumulating their ^{31}P NMR spectra. Experiments were performed on frog sartorii and frog and toad gastrocnemii at 4° C.

2. The NMR signals from ^{31}P (the naturally occurring phosphorus) is weak, and signal averaging is required. In order to follow the time course of reactions it is necessary to maintain the muscles in a steady state for many hours while they are undergoing repeated contractions. Signals were accumulated in separate computer bins according to time after initiation of contraction. By these means spectra were obtained which corresponded to the different intervals during the contraction and recovery cycle.

3. In the absence of stimulation, the spectra of frog sartorius muscles and of their extracts indicated concentrations of adenosine triphosphate (ATP), phosphoryl creatine (PCr), inorganic orthophosphate (P_i) and sugar phosphates (sugar P) which are in reasonable agreement with the values obtained by chemical analysis.

4. We have confirmed that unidentified resonances representing unknown compounds appear in the spectra of both frog and toad muscle; one of these is much larger in spectra from toad than from frog. We have found an additional small, unidentified resonance which appears to be specific to toad muscle.

5. Spectra accumulated during actual contractions (1 s tetani every 2 min) did not differ dramatically from those accumulated throughout the 2 min cycle of contraction and partial recovery.

6. Following 25 s tetanii, approximately 20% of the PCr had been

hydrolysed; it was then rebuilt exponentially with a half-time of about 10 min. The increase in [P_i] immediately after contraction and the time course of its disappearance corresponded to the changes in [PCr]. During the later half of the recovery period the concentration of P_i was reduced to below that in resting muscle. The [sugar P] remained very high ($\simeq$ 4 mmol kg^{-1}) throughout the 56 min interval between contractions.

7. When frog sartorii were tetanized for 1 s every 2 min, the changes in [PCr] and [P_i] between contractions could not be observed because too little signal was obtained from these small muscles. However, when toad gastrocnemii were similarly stimulated, the changes in these compounds could be readily detected and were even greater than expected.

8. The position of the P_i resonance can be used to monitor intracellular pH and changes in pH. Under the conditions of our experiments the average intracellular pH in unstimulated frog sartorius muscles was 7·5. After a 25 s tetanus this was observed to move in the acid direction by a few tenths of a pH unit and to return to its pre-stimulation value before the end of the recovery period. After a 1 s contraction of toad gastrocnemius the environment of P_i became slightly more alkaline for the first few seconds.

INTRODUCTION

A powerful physical method utilizing the naturally occurring form of phosphorus, ^{31}P nuclear magnetic resonance (^{31}P NMR), has recently become available for the non-destructive measurement of phosphorus compounds in intact muscle (Hoult, Busby, Gadian, Radda, Richards & Seeley, 1974; Bárány, Bárány, Burt, Glonek & Myers, 1975; Burt, Glonek & Bárány, 1976*a*; Seeley, Busby, Gadian, Radda & Richards, 1976). We have developed this technique further, for the study of phosphorus compounds in living muscle during rest, contraction and recovery.

The methods currently used to study living muscle, ultra-rapid freezing (Kretzschmar & Wilkie, 1969) followed by extraction and chemical analysis, have given results that cast serious doubt on the accuracy (or at least, the completeness) of the 'text-book' description of the situation under conditions in which the only known net chemical reaction proceeding during contraction is the hydrolysis of phosphocreatine (PCr). This reaction is insufficient to account for the concurrent heat production (Gilbert, Kretzschmar, Wilkie & Woledge, 1971; for later references see Dawson, Gower, Kretzschmar & Wilkie, 1975; Gower & Kretzschmar, 1976; Rall, Homsher, Wallner & Mommaerts, 1976; and reviews by Woledge, 1975; Wilkie, 1975). The hydrolysis of PCr during contraction is also smaller than would be expected from the subsequent oxygen consumption (Kushmerick & Paul, 1976*a*) and lactate production (Kush-

merick, 1977) on the basis of current biochemical theories. Since these theories are largely based on experiments with disrupted tissue the emergence of a completely independent non-destructive method for studying biochemical changes is especially welcome at the present time.

Many of the metabolites known to be involved in cellular chemical energy transfers, including the fuel, ATP, which powers contraction, contain phosphorus and occur free in solution at sufficiently high concentrations (about 0·5 mmol kg^{-1}) to be detectable by ^{31}P NMR. The simultaneous observation of all these compounds, rather than just those selected for chemical analysis, could permit detection of phosphorus metabolism not predicted by current theories and which might be missed by conventional methods.

In the ^{31}P NMR studies cited above, the specimen tube was simply packed with muscle, at around room temperature. Under these circumstances the muscles die from anoxia fairly rapidly (see Hoult *et al.* 1974, figs. 3 and 4). Nevertheless, a very useful body of information has been built up, upon which there is gratifying agreement between different laboratories. The chemical shifts reported for different compounds are very consistent and the quantitative estimates for these compounds agree reasonably with direct chemical analysis. Unexpected resonances are frequently observed in the phospho-diester region of the spectrum which are surprisingly large in semitendinosus muscle from the rabbit (Seeley *et al.* 1976) and in toad gastrocnemius (Burt *et al.* 1976*a*).

In this paper we report the development of the physiological techniques required to maintain muscles alive for long periods within the spectrometer, to stimulate them and to record tension development at the same time that ^{31}P spectra are being obtained. Using this technique, phosphorus-containing metabolites have been measured in frog sartorii and toad and frog gastrocnemii at rest and during contraction and the recovery processes after two different patterns of stimulation have been compared. A preliminary report of this study has been given to the Physiological Society (Dawson, Gadian & Wilkie, 1976).

METHODS

The use of NMR to measure tissue metabolites imposes constraints which are quite different from those encountered in conventional physiological or NMR experiments. In order to appreciate these constraints it is necessary to know something about the physical principles of NMR. All atomic nuclei have positive charge and mass and some of them, including ^{1}H, ^{2}H and ^{31}P, possess spin. The rotating charge causes the nucleus to behave like a tiny magnet and the spinning mass endows it with the properties of a gyroscope. Thus when an external magnetic field is applied the nuclear magnets do not line up with it in the same way that non-spinning magnets would. Instead, they precess like tops around the direction of the field at a

characteristic frequency, the Larmor frequency ν_L, which is directly proportional to the flux density B_0. For ^{31}P nuclei, $\nu_L = 1{\cdot}723 \times 10^7 B_0$.

Thus in the (vertical) 7·5 Tesla field of the superconducting Oxford magnet, the nuclei precess at 129·2 MHz. If a transverse magnetic field oscillating at this frequency is applied, the nuclei absorb energy and will continue to radiate after the exciting field is switched off at a frequency proportional to the *local* magnetic field experienced by the nucleus. This differs very slightly from the applied magnetic field B_0 because of the effects of local circulating electrons, neighbouring magnetic nuclei, etc., which are characteristic of different types of compound. The resulting frequency shift, the chemical shift, can be detected and used to identify the different phosphorus compounds present.

Unfortunately NMR is an insensitive technique: in our experiments only a weak signal, not visible in the noise, is emitted following the 40 μs pulse at 129·2 MHz used for excitation. This makes it necessary to average many signals or 'scans', typically several hundred, in order to obtain useful spectra. Although the signal decays within about 20 ms ($T_2 \simeq 10$ ms) the pulse can be repeated only every 2 s because it takes a relatively long time (determined by T_1, the spin-lattice relaxation time) for the ^{31}P nuclei to revert to their unexcited state. As a result, the accumulation of a spectrum can take several hours.

In studying muscular contraction and recovery the inherent slowness of the method is overcome by stimulating repeatedly and averaging the signals during preset time intervals phased with the stimulus, see Figs. 7–9.

Physiological techniques

The physiological problem is the counterpart of the physical situation. The muscles must be kept in a steady state at rest, or making a series of repeated contractions, for many hours; this requires adequate oxygenation. The choice of experimental preparation thus involves a compromise; thick muscles would give large signals but only thin ones can be adequately oxygenated by diffusion. They should, if possible, function at low temperature (see Hill, 1965). Accordingly, most of our experiments were performed on frog sartorii from *Rana temporaria* at 4° C. This temperature was chosen rather than 0° C to provide a margin of safety against accidental freezing.

Even with four sartorii only about a third of the sample cross-section is occupied by muscle: this means that to achieve a given signal-to-noise ratio approximately nine times as many scans are needed as would be the case if one could simply fill up the measuring tube with muscle. For certain experiments we have therefore used pairs of small gastrocnemii weighing about 150 mg each either from *Rana temporaria* or from the toad, *Bufo bufo*. These fill up appreciably more of the space but are not as well oxygenated.

Design of chamber. The glass NMR sample tube, 7·5 mm in diameter and 70 mm long was converted into an experimental chamber. The choice of materials, arrangement of electrodes, means of oxygenation and design of force transducer were critical to minimize interference with the delicate magnetic and radio-frequency (RF) circuits. After much trial and error, the design shown in Fig. 1 was finally arrived at. The support system is constructed entirely of teflon, glass and epoxy resin as these materials do not appreciably disturb either the homogeneity of the magnetic field or the tuning of the RF coils.

The muscle holder consists of a central glass capillary tube to which are attached two teflon bobbins. One of these serves as the top to the experimental chamber, and the other, a circular disk with two slots cut out, is fixed to the capillary tube approximately 1 mm from the bottom. The ends of the muscles are inserted into these slots

so that small attached pieces of bone form stops below the bobbin. Cotton tied to the tendon at the other end of the muscle is threaded through holes in the top of the chamber and tied over the force transducer which is fitted on to another teflon support above. The muscles are held in a vertical position parallel to the central glass tube and the magnetic field. Thus, as much muscle as possible is held within the

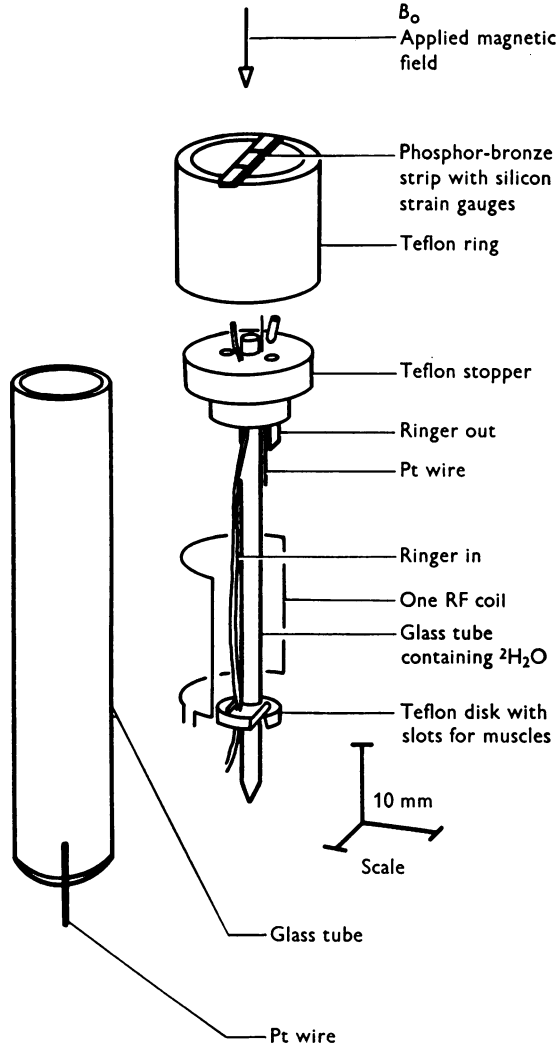

Fig. 1. Design of experimental chamber. The arrangements for stimulating, recording tension and perfusing oxygenated Ringer solution are further described in the text. The volume in which the NMR measurement is made is defined by the two single-turn radio-frequency (RF) coils, only one of which is shown in the diagram. They extend from 3 to 18 mm above the top of the lower teflon disk.

sample volume defined by the RF coils of the probe, while bone (which contains phosphorus), metal wires, etc., are held as far away as possible. The capillary tube serves an additional purpose, being filled with a solution of KCl in ^2H_2O. The KCl (134 g/kg ^2H_2O) is present to prevent the ^2H_2O from freezing at the temperatures of around 4° C at which the experiments are conducted (the freezing point of pure ^2H_2O is 3·81° C). The resonance from deuterium serves by a feed-back arrangement to hold the magnetic field steady to about one part in 10^8. This resonance is also used to optimize the spatial homogeneity of the field and although the axial arrangement of the ^2H_2O is not ideal we have not yet been able to devise a better arrangement. It is the spatial homogeneity of the field that limits the minimum line width in our spectra.

Stimulation and recording. The muscles are stimulated via two axially located platinum wires, one threaded through the teflon top and one sealed into the bottom of the chamber. This arrangement was found to be least disrupting to the field homogeneity; in addition, RF stoppers in the stimulating circuit immediately above and below the chamber were found to be necessary to isolate the radio frequency circuits from the damping effect of the stimulus leads. Stimulation was by 50 Hz sine waves at 7 volts r.m.s. (3 mA). This type of stimulus is very convenient since it is non-polarizing and the current can be monitored using a simple meter (Csapo & Wilkie, 1956). Gating for the various patterns of stimulation used (i.e. 1 s stimulation every 125 or 25 s every 56 min) was by a Devices 3290 digitimer which was externally triggered from the computer interfaced with the spectrometer.

The force-transducer consists of two silicon strain gauges forming a bridge circuit bonded above and below a narrow phosphor-bronze strip attached across a circular teflon support (see Fig. 1). It was necessary to insert RF filters and decoupling capacitors into the transducer and stimulation leads to prevent leakage of stray radiation from the laboratory into the probe. Muscle tension was recorded on a Sanborn 322 chart recorder, which was switched on and off automatically when the muscle was contracting by a relay operated by the digitimer.

Perfusion of oxygenated Ringer solution. Bubbling air or oxygen through the experimental chamber would result in severe disturbance of magnetic homogeneity; therefore, a re-circulating perfusion system was chosen as the method of oxygenation. Approximately 200 ml Ringer solution (composition in mmol l^{-1}: NaCl, 111; KCl, 2·5; $CaCl_2$, 2; Tris 10 (2-amino-2-hydroxymethyl-1, 3-propanediol) adjusted to pH 7·2 at 25° C with HCl) was bubbled with 100% oxygen at room temperature. This was pumped to and from the chamber with a Pharmacia P3 variable speed peristaltic pump. The outflow rate was made double the inflow rate to avoid any overflow into the electronic circuitry of the sample probe. Both inflowing liquid and the outflowing mixture of air and liquid were filtered with coarse gauge filter paper to remove particles which could clog the fine bore tubing. Normally the pump was run at its maximum inflow rate of 3·3 ml/min though this is much more than is needed for oxygen supply to resting muscles.

Preparation of muscle extracts. Extracts were made by soaking the frozen muscles for 4 days in 3 ml 1·25 mmol l^{-1} EDTA (ethylene diamine tetra-acetic acid) in 50% methanol, pH 7·6, at −30 °C. The fluid was freeze-dried and re-dissolved in the required volume of distilled water. This method extracts all the PCr present but only about 80% of the nucleotides (N. A. Curtin and R. C. Woledge, personal communication).

NMR spectroscopy

^{31}P NMR spectra were recorded at 129·2 MHz on a spectrometer constructed in the Oxford laboratory and interfaced with a Nicolet B-NC 12 computer. Details of this spectrometer have been reported by Hoult & Richards (1975). The spectrometer was

operated in the Fourier Transform mode and employed a deuterium field-frequency lock. Spectra were accumulated for periods of up to 1–10 h and stored on a magnetic disk. In high resolution NMR the sample is normally spun to improve the resolution. Unfortunately in our experiments this was impossible for reasons which should be obvious.

Identification of resonances. Identification of the muscle resonances is made by comparing the resonance positions (chemical shifts) with those of metabolites free in solution. In this way the resonances of ATP, PCr, P_i and sugar phosphates (sugar P) can be uniquevocally assigned (Hoult *et al.* 1974; Bárány *et al.* 1975). However, resolution of individual sugar P resonances is difficult as they are closely grouped. This region of the spectrum includes resonances from species of the form $-CH_2-O-PO_3^{2-}$ such as glucose-6-phosphate, fructose-6-phosphate, fructose 1-6 diphosphate, AMP and some triose phosphates. In the case of unidentified resonances it is necessary to show that a suspected compound not only has the correct chemical shift but also (by conventional methods) that it does indeed occur in the muscle.

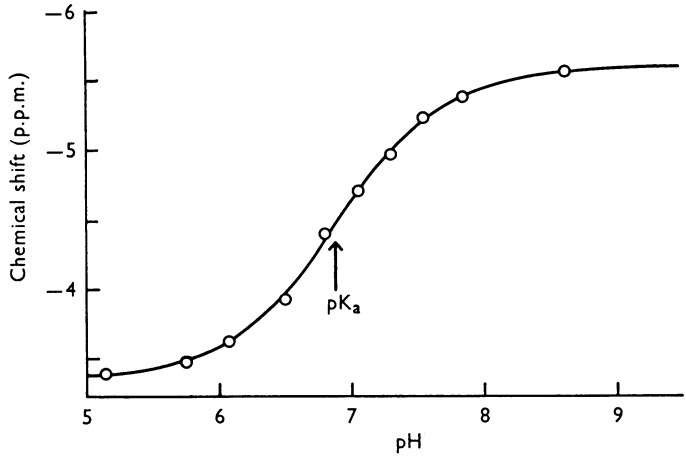

Fig. 2. The chemical shift of inorganic phosphate as a function of pH under physiological conditions of ionic strength and at 4° C. Abscissae, pH; ordinates, chemical shift in parts per million (p.p.m.) related to the resonance of phosphocreatine. The circles are experimental; the continuous line is drawn from the equation $pH = 6{\cdot}88 + \log_{10}(\sigma - \sigma_1)/(\sigma_2 - \sigma)$ where σ is the observed chemical shift and σ_1 and σ_2 are the shifts of $H_2PO_4^{1-}$ and HPO_2^{2-}, $-3{\cdot}35$ and $-5{\cdot}6$ respectively. For further details see text.

Chemical shift measurement and pH determination. The chemical shift of the P resonance is extremely sensitive to pH in the region of neutrality but insensitive to variations both of $[Mg^{2+}]$ and of ionic strength in the physiological range (see Colman & Gadian, 1976). Fig. 2 shows a pH titration of P_i in a solution of physiological ionic strength containing 10 Pi 5 PCr, 0·5 EDTA, 140 KCl (concentrations in mmol l^{-1}). Because PCr has a low pK_a (about 4·6), its resonance is quite insensitive to changes in pH above pH 6·0. It is therefore possible to determine the internal pH of the muscle fibres directly from the chemical shift difference between the PCr and P_i resonances. However, some caution is necessary in using the PCr resonance as a shift standard, because although the P_i resonance is not sensitive to physiological

concentrations of Mg^{2+} ions, the PCr resonance is slightly shifted by their presence (Burt *et al.* 1976*a*; D. G. Gadian and P. J. Seeley, unpublished). However, when frog gastrocnemius muscles were bathed in a magnesium-free solution of 10 PCr, 0·5 EDTA and 140 KCl (concentrations in mmol l^{-1}) at pH 7·4, the resonance from the solution coincided with that from intracellular PCr. Furthermore, experiments with extracts (Fig. 5*A* and *B*) have shown that even in the presence of 30 mmol l^{-1} Mg^{2+} the shift is only +0·15 p.p.m., which is almost undetectable in signals from intact muscle. We have therefore measured all our chemical shifts relative to that of the PCr resonance. We have followed the usual convention of calling negative a chemical shift towards a higher frequency.

Determinations of concentration. The areas of the various resonances are proportional to the quantities of metabolites within the volume enclosed by the RF coils, provided that the RF pulses are applied at time intervals much greater than the spin-lattice relaxation time T_1 of the resonances. However, in order to optimize the signal-to-noise ratio of the spectra, we normally applied 70° pulses at intervals of 2 s, which approximates to the T_1 values of the resonances. Under these conditions the areas of the resonances are reduced, an effect known as 'saturation', by factors determined by the T_1 values. In order to determine these factors, we accumulated several spectra of resting frog gastrocnemius muscles at pulse repetition intervals varying from 1 to 16 s.

The resonance areas of βATP, PCr and P_i and the three unidentified resonances around −3 p.p.m., were greater at 16 s than at 2 s intervals by factors of 1·0, 1·25, 1·8 and 1·3 respectively. A similar factor could not be determined for sugar P because their concentrations are too low in resting muscle to be observed by NMR. In experiments in which scans were repeated every 2 s, the relative concentrations of ATP, PCr, and P_i were obtained by multiplying their respective areas by 1·0, 1·25 and 1·8. The concentrations of sugar P were obtained from peak areas without correction for saturation; they may thus be underestimates. The peak areas were determined by integration using the computer.

Control of timing. A computer program was written to synchronize the accumulation of NMR data with the electrical stimulation of the muscle. A given experiment begins at time 0 with a pulse from the computer which, after a preset delay (typically 1 s), triggers the stimulator and causes the muscle to contract. Usually the RF pulses commence 2 s from time 0 and are repeated every 2 s (see previous section) throughout the course of the experiment. The first m scans are stored in bin 1 the next m scans in bin 2, etc., until n bins (mn scans) have been accumulated. A trigger pulse is then provided and the cycle recommences. The process is repeated many times to build up the required signal-to-noise ratio. By this means, although experiments may last many hours, the kinetics of reactions can be followed with a time resolution set by the minimal interval between successive bins, i.e. 2 s.

For the experiments involving 25 s stimulations, $m = 200$ and $n = 8$; so that eight bins, each of period 400 s are collected. The time interval between successive tetani is 3200 s.

For experiments with 1 s muscle stimulation, the program is modified to allow narrow time resolution during and immediately following the contraction but wider averaging after this crucial period. For example, in the first twelve bins m can be set to 1, while for the last six, $m = 8$.

Quantitative calibration. Quantitative calibration in absolute terms is complicated by the facts that the exact volume that contributes signal cannot be determined and that the sample contains both muscle tissue and an unknown amount of circulating Ringer solution. For these reasons a special calibration method was employed.

A resting spectrum was obtained in our normal Tris-buffered Ringer solution to confirm that the P_i level was low and thus that the muscles were in a steady resting

state. A spectrum was then obtained while circulating Ringer solution containing 10·0 mmol l^{-1} phosphate buffer (concentrations in mmol l^{-1}; NaCl, 111; KCl, 2·5; $CaCl_2$ 1·0; Na_2HPO_4, 10·0: pH adjusted with HCl to 7·2. The $CaCl_2$ was reduced to prevent precipitation of calcium phosphate). During the latter spectral accumulation (Fig. 3A) the ratio of peak areas (P_i/PCr) remained constant confirming that phosphate penetrates only slowly into the muscle fibres (Stella, 1928). Finally the

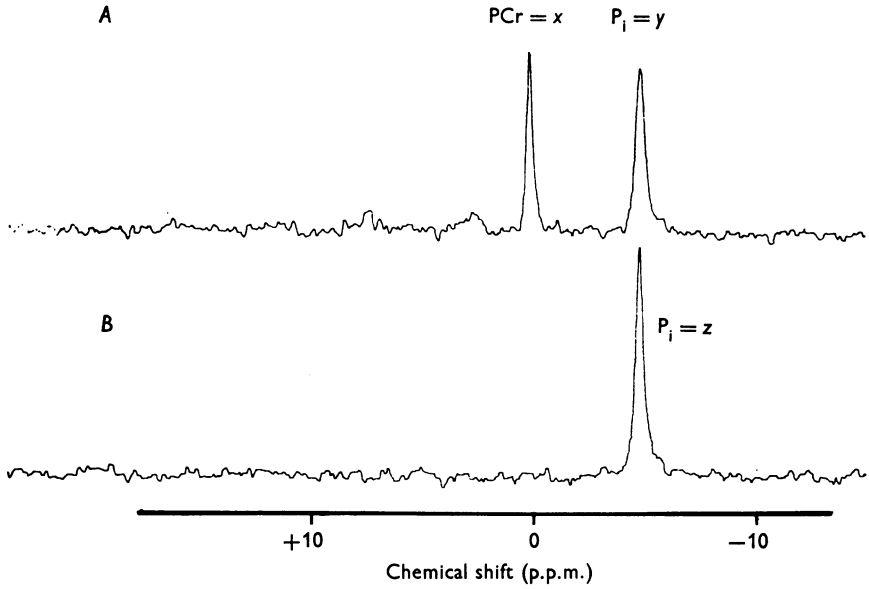

Fig. 3. Absolute calibration of peak area. See Fig. 4 for complete description of spectra. Spectrum *A* was obtained from four frog sartorii in phosphate-buffered (10 mmol l^{-1}) Ringer solution; spectrum *B* is from the sample tube filled with the same solution after removal of the muscles. The spectra are each accumulations of 238 scans. The repetition interval was 16 s, which is much longer than the T_1 of the resonances. The small letters represent relative peak areas, x for PCr and y and z for P_i. The concentration of PCr was estimated from

$$[\text{PCr}] = 10x/\{z-y+x(P_i/\text{PCr})_{\text{resting}}\} \text{ mmol(1 fibre volume)}^{-1}.$$

The resting value of (P_i/PCr) was obtained from a similar series of scans at 16 s intervals, not shown here. The concentration of PCr was calculated to be 23·5 mmol (1 fibre volume)$^{-1}$. The muscles weighed 0·3145 g frozen and 0·0588 g dry. For further details see text.

muscles were removed and a further spectrum (Fig. 3*B*) was obtained. The concentration of PCr within the muscle fibres was estimated from:

$$[\text{PCr}] = 10x/\{z-y+x(P_i/\text{PCr})_{\text{resting}}\}\text{mmol } l^{-1}$$

using the symbols shown in Fig. 3. The last term is a correction for the internal P_i of the resting muscle based on the first accumulation. This is not shown in Fig. 3,

but the P_i was small, with P/PCr $\triangleq$ 0·054. From Fig. 3, $x = 3{\cdot}5$, $y = 5{\cdot}1$, $z = 6{\cdot}4$, so [PCr] = 23·5 mmol (1 fibre)$^{-1}$.

This calibration procedure is expensive in machine time and cannot be repeated in each experiment. For many purposes the ratio of peak areas provides a sufficient basis for interpretation, as in Table 1.

RESULTS

We have considerably extended the findings of Hoult *et al.* (1974) and Bárány and co-workers (Bárány *et al.* 1975; Burt *et al.* 1976*a*) by maintaining our muscles under physiological conditions. We are therefore able to draw confident conclusions about the condition of the muscles at rest and about the metabolic changes during and following contractions.

Resting muscles

Frog sartorius

As Fig. 4*A* shows, it is possible to obtain in 6 h spectra from a single set of frog sartorii at 4° C with sufficiently good signal-to-noise ratio to detect concentrations of metabolites as low as 0·5 mmol (kg muscle)$^{-1}$. These spectra are remarkably consistent from one set of muscles to another: the only features that vary appreciably are the areas of the unidentified resonances around -3 p.p.m. The resonances are identified as described in Methods and the chemical shifts agree closely with those of Hoult *et al.* (1974) and Bárány and co-workers (Bárány *et al.* 1975; Burt *et al.* 1976*a*). The positions of the three phosphate resonances of ATP at $+16{\cdot}0$, $+7{\cdot}25$ and $+2{\cdot}45$ p.p.m. confirm that this substance is almost wholly (more than 95 %) bound to Mg^{2+}, a conclusion reached in the earlier studies. It may be noted that the α peak at $+7{\cdot}25$ p.p.m. is larger than the other two ATP peaks. Studies of muscle extracts (see Fig. 5) reveal overlap with another resonance probably from NAD (Burt *et al.* 1976*a*). In addition, the position of the P_i resonance peak in our experiments ($-5{\cdot}2$ p.p.m.) indicates an internal pH of 7·5 (s.d. = 0·06, $n = 5$) slightly higher than that reported by Burt *et al.* (1976*a*).

These spectra of living muscles do, however, differ in two significant ways from those of the earlier studies. The level of P_i and of sugar P was very low, and in many experiments we could not detect sugar P at all. Sugar P and P_i are all too readily produced by anoxia and under the conditions of previous experiments there were no facilities for providing oxygen. The three peaks at $-2{\cdot}7$, $-3{\cdot}05$ and $-3{\cdot}6$ p.p.m. are especially interesting since they do not correspond to any well-known metabolic intermediates. In Fig. 4*A* only the left-hand peak is at all prominent: the other two can just be made out in the magnified upper trace. We are sure of their presence only because we have seen all three in other experiments,

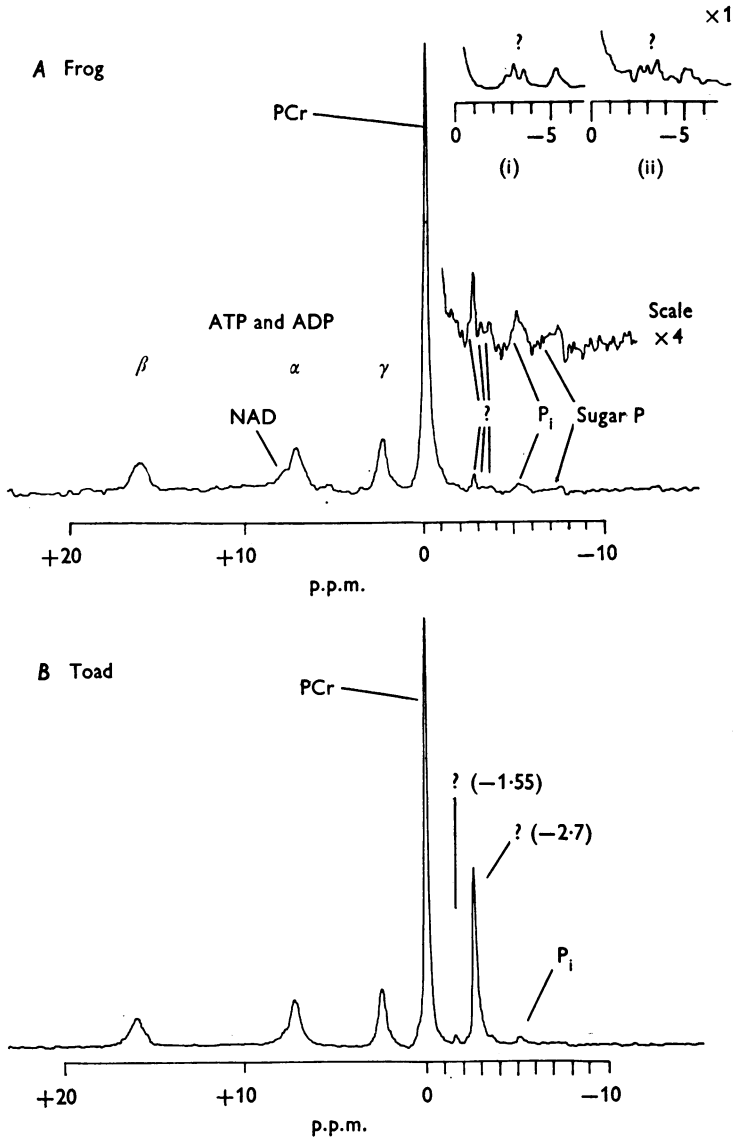

Fig. 4. Spectra from resting muscles at 4° C. Ordinates, signal; abscissae, chemical shift in p.p.m.; the frequency increases from left to right, the total span representing 5 kHz. *A*, four frog sartorii, May 19, 1976. Lowest line: Average of 10,000 scans at 2 s intervals, with peaks assigned as described in text. The short length of record on the right-hand side of the PCr peak has been enlarged 4 × vertically to show fine detail. Note that of the three unidentified peaks at −2·7, −3·05 and −3·6 p.p.m., only the left-hand one is conspicuous. The inset Figures show how these peaks vary in size, though not in position. (i) 16 November 1975; (ii) 29 April 1976. *B*, two toad gastrocnemii, 20 May, 1976. Average of 6000 scans at 4 s intervals.

as shown in the inserts to Fig. 4*A*. The chemical shifts of these three components are very consistent but their relative amounts evidently vary.

The spectrum shown in Fig. 4*A* was calibrated in terms of mmol l^{-1} as described in Methods. The muscles were then frozen in liquid nitrogen and their methanol extracts were analysed chemically. The PCr content determined by NMR was 23·5 mmol l^{-1} as compared to 21·2 mmol kg^{-1} obtained by chemical analysis. The correct basis for comparing these values

TABLE 1. Relative concentration of *P* compounds in resting frog sartorius as determined by NMR and by chemical analysis

		NMR	Chemical	*P*
PCr/βATP	$\bar{x}$	6·74	8·14	
	S.E.	0·309	0·744	n.s.
	n	6	18	
PCr/P_i	$\bar{x}$	16·02	13·10	
	S.E.	1·58	3·03	n.s.
	n	6	17	

The chemical analyses on perchloric acid extracts of resting muscles were kindly made available by Dr Curtin and Dr Woledge. They are from the control muscles of their recent paper (Curtin & Woledge, 1975). The NMR estimate of ATP is based on the area of the β peak since this peak is unique to ATP and would not be altered by the presence of ADP or NAD. Rough estimates of actual concentrations may be made by assuming that the resting PCr content is 27 mmol kg^{-1} (see Dawson *et al.* 1975). n.s., not significant.

will be dealt with in the Discussion. Since the absolute calibration method is too time consuming to be carried out as part of every experiment, another kind of comparison between NMR experiments and conventional measurements on resting muscles is given in Table 1. Here the ratios PCr/ATP and PCr/P_i are given as determined by the two methods. NMR tends to give a lower estimate for PCr/ATP and a higher one for PCr/P_i but the differences

Legend to Fig. 5.

Fig. 5. Spectra from extracts of resting muscles, May–June 1976. *A*, frog sartorii: the Mg^{2+} had been chelated with EDTA. Note that only two peaks can be seen in the −2 to −4 p.p.m. region, a prominent one at −2·7 and a small one at −3·7 p.p.m. The inset shows the same part of the spectrum from an extract made in December 1975. This shows peaks at −2·7, −3·1 and −3·7 p.p.m., the middle one being largest. *B*, frog sartorii: 30 mmol l^{-1} $MgCl_2$ had been added to the extract. The origin is at the same absolute frequency as in *A* and no longer coincides exactly with the PCr resonance. *C*, toad gastrocnemii: note the prominent peak at −2·7 p.p.m., with smaller ones at −3·05 and −3·6 p.p.m. There is also a small peak at −1·6 p.p.m. The frog sartorii were frozen in the 'hammer' quick-freezing apparatus (Kretzschmar & Wilkie, 1969) and the toad gastrocnemii were frozen using a single manually-operated aluminium hammer.

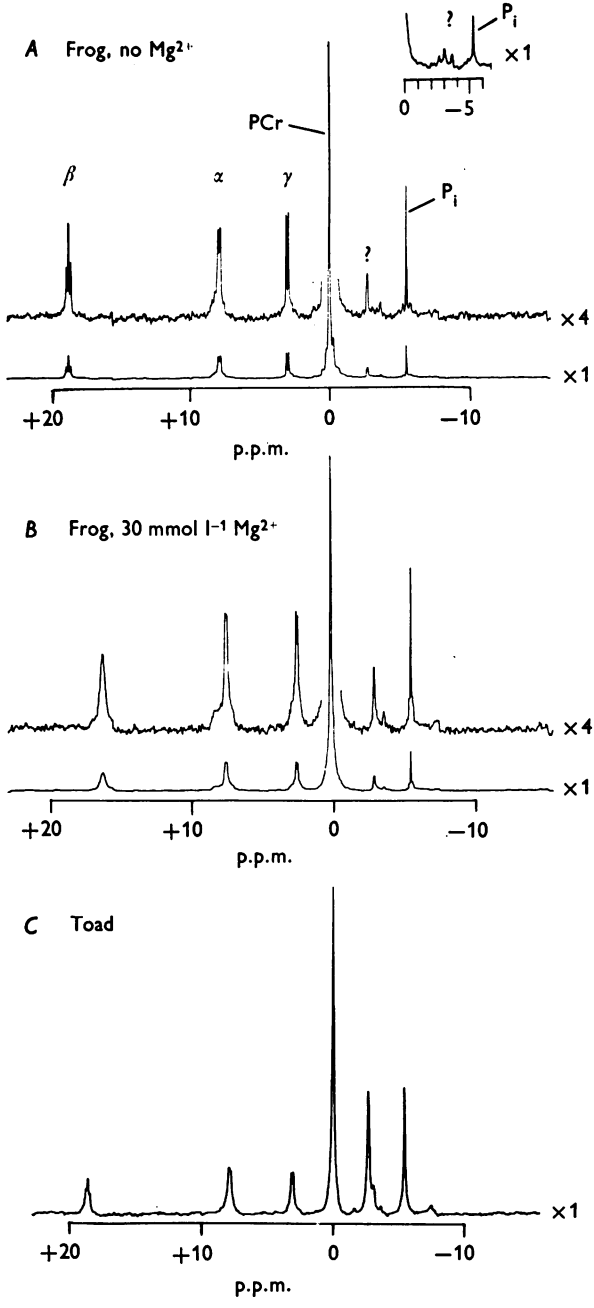

Fig. 5. For legend see opposite page.

are not significant, largely because of the high s.d. of the chemical determinations.

Toad gastrocnemii

As shown in Fig. 4*B* resting gastrocnemii from the toad closely resemble sartorii from the frog in having no observable sugar P and very little P_i. This indicates that at rest diffusion suffices to oxygenate these fairly large muscles. The toad muscles differ from frog muscles in one outstanding respect: as first shown by Burt *et al.* (1976*a*), the unidentified resonance at $-2{\cdot}7$ p.p.m is extremely large, almost half as big as that of PCr. There is also a small but definite peak at $-1{\cdot}55$ p.p.m. which cannot be seen in spectra from the frog. The positions of the adenosine P peaks are slightly different from those in the frog, with shifts of $+16{\cdot}25$, $+7{\cdot}4$ and $+2{\cdot}5$ p.p.m. respectively. The position of the P_i peak indicates an internal pH of 7·4.

Muscle extracts

Spectra from methanol extracts of muscle are shown in Fig. 5. The resonances are much sharper than those from intact muscle for various reasons. This makes it possible to see the tripling of the ATP β peak and the doubling of the α and γ peaks, which result from interactions between neighbouring P nuclei. The results from extracts are in every way consistent with those from intact muscles.

Frog sartorii

As Fig. 5*A* shows, exactly the same resonances are seen as in intact muscles (Fig. 4*A*), though the ATP peaks in the extract are at $+18{\cdot}6$, $7{\cdot}85$ and $3{\cdot}0$ p.p.m. rather than $+16{\cdot}0$, $7{\cdot}25$ and $2{\cdot}45$ p.p.m. The ratios PCr/βATP and PCr/P_i are 8·3 and 12, agreeing almost exactly with what is found in perchloric acid extracts by chemical analysis (Table 1). As in intact muscle, there is an unidentified peak at $-2{\cdot}7$ p.p.m. with a barely perceptible one at $-3{\cdot}65$ p.p.m. However, the inset shows that three peaks ($-2{\cdot}7$, $-3{\cdot}1$, $-3{\cdot}7$) have been seen in other extracts, inviting direct comparison with Fig. 4*A* (i).

Effect of magnesium. When 30 mmol l^{-1} $MgCl_2$ is added to the extract (Fig. 5*B*) the ATP β peak is broadened and all ATP peaks are shifted towards their values in intact muscle. The shift of the α peak uncovers the NAD peak to its left. As discussed under Methods, there is a slight effect on the PCr resonance, which is shifted to approximately $+0{\cdot}15$ p.p.m. The P_i resonance is not changed. These results agree with those of Burt *et al.* (1976*a*; see their Fig. 6).

Toad gastrocnemii

As Fig. 5*C* shows, the same resonances appear in muscle extract as in intact muscle (Fig. 4*B*). The chief difference is that there is much more P_i (PCr/P_i = 3), almost certainly because of the impossibility of freezing even small gastrocnemii really rapidly. The unidentified compound at −2·7 p.p.m. is present in large amounts (PCr/unknown = 1·7) and there are small resonances at −3·05, −3·6 and −1·6 p.p.m. This last resonance thus seems to be a characteristic feature of toad muscle.

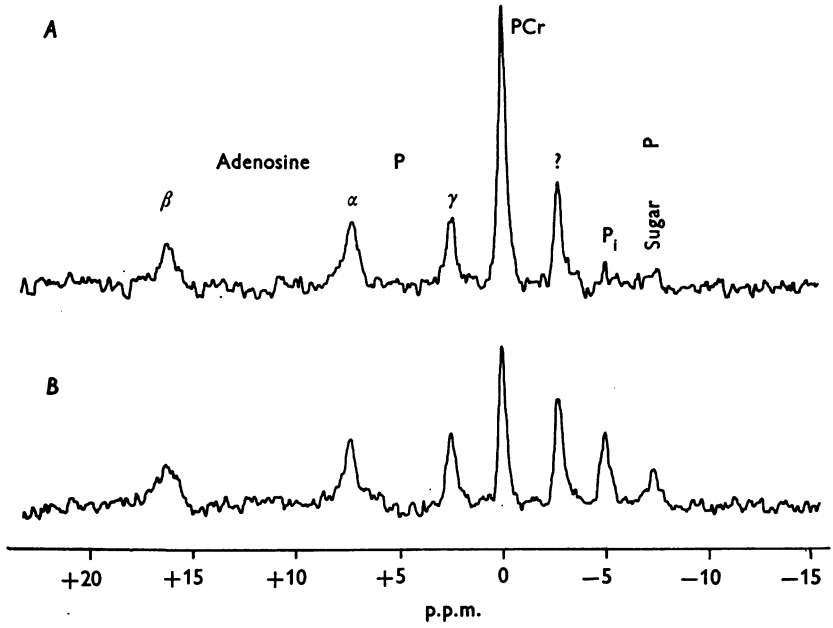

Fig. 6. The effect of prolonged stimulation on the ^{31}P spectrum of toad gastrocnemius. Spectrum *A* was obtained on a pair of small toad gastrocnemii in oxygenated Ringer solution at 4° C. The muscles were then stimulated for 35 s, during which time the tension fell from 1·58 to 0·63 N. The perfusion was turned off to retard recovery and spectrum *B* was accumulated. Each spectrum was obtained over a period of 7 min and represents an accumulation of 200 scans at 2 s intervals.

Stimulated muscles

Effect of a single prolonged contraction

Large changes in metabolite concentrations occur after prolonged stimulation. This is demonstrated in Fig. 6, which shows (*A*) a spectrum of a pair of resting toad gastrocnemius muscles, and (*B*) the spectrum of the

same muscles after a single tetanic stimulation lasting 35 s. In this experiment recovery was impeded by turning off the perfusion pump. The changes are as expected; PCr falls to about half its resting level and there are large increases in [P_i] and [sugar P]. [ATP] remains constant. The pH indicated by the P_i resonance before stimulation is 7·2; this does not change during the 7 min after relaxation.

The metabolic changes demonstrated by Fig. 6 are very dramatic, and confirm that it is possible to produce large metabolic responses to electrical stimulation and to measure them in a single experiment using ^{31}P NMR. It is, however, more interesting to study the smaller metabolic changes associated with shorter stimulations from which the muscles recover.

Contraction and recovery

Two different patterns of muscle stimulation were employed: 25 s stimulations every 56 min were used to study recovery after large metabolic changes, whereas 1 s stimulations every 2 min were used when accumulating spectra during contraction.

Each of these patterns is very near the maximum that the muscles can sustain without severe progressive deterioration. The muscles are stimulated for almost the same number of seconds per hour in the two cases; thus, any differences in the metabolic recovery processes must result from the different patterns, rather than the total amounts of stimulation.

Sartorius and gastrocnemius muscles of the frog and gastrocnemius muscles of the toad were used in both types of experiment. Despite our careful attention to oxygenation, the muscles are not completely able to recover metabolically between contractions as time goes on. In all cases there is a gradual decline in [PCr] over a period of several hours, accompanied by an increase in [P_i] and sometimes a gradual shift of pH in the acid direction. The problem is more acute when the relatively thick gastrocnemius muscles are used. The tension developed upon stimulation also

Legend for Fig. 7

Fig. 7. Recovery of frog sartorius from long contractions. Four frog sartorius muscles were repeatedly stimulated for 25 s every 56 min and spectra accumulated into eight bins of 7 min each. Spectrum *A* was accumulated from 0 to 7 min after the contraction and spectrum *B* was obtained throughout the last 28 min of recovery. The horizontal line at the PCr resonance in spectrum *B* indicates the height of this peak in spectrum *A*. Scans were made at 2 s intervals. The muscles gave seven virtually identical responses, during each of which the tension fell from 1·22 to 0·47 N. The graph (*C*) shows how PCr(+), ATP(0), $P_i(x)$ and sugar P(△) varied throughout the eight bins. The ordinates show the resonance peak areas as multiples of the mean area for the β ATP peak. The right-hand scale applies to PCr. The exponential curve drawn through the PCr points has a $T_{\frac{1}{2}}$ of 9·1 min.

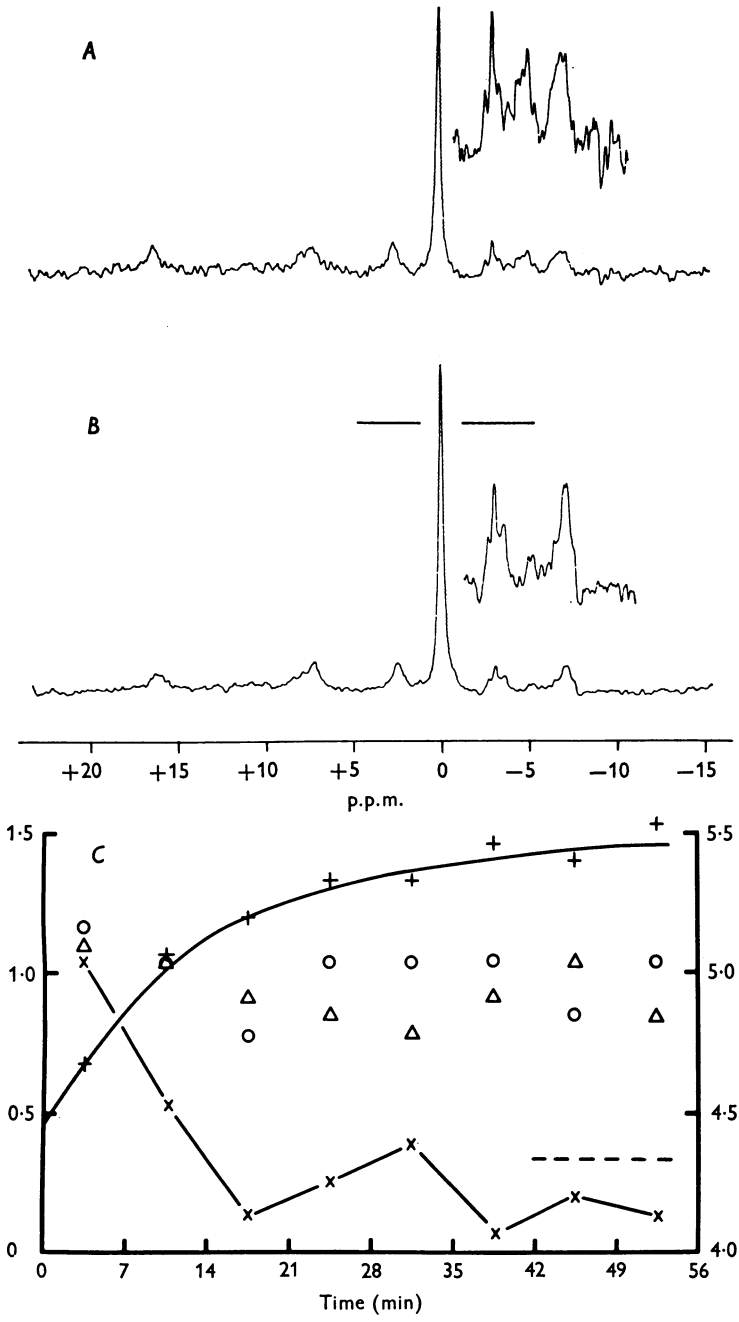

Fig. 7. For legend see opposite page.

decreased to a variable degree with time and the experiments were always terminated if tension dropped to half that observed initially.

Recovery after long contractions. Frog sartorii were stimulated for 25 s every 56 min and scans from the recovery period between contractions were accumulated in eight bins, each of 7 min duration. Spectra from the first bin (Fig. 7*A*) and the last four bins (Fig. 7*B*) are shown, and Fig. 7*C* shows the variations in metabolite concentration with time after contraction.

There is a clear difference in [PCr] between the two spectra shown in Fig. 7, indicating a large break-down of this compound during contraction, with subsequent regeneration. The rebuilding of PCr is roughly exponential, with a half-time of about 10 min; extrapolation to zero time indicates that 20% of the PCr is broken down during contraction. The [P_i] is increased about fourfold immediately after contraction, which is roughly equivalent to the phosphate released upon hydrolysis of PCr. The half-time of removal of P_i corresponds with that of the rebuilding of PCr. Interestingly, during recovery the P_i falls to a level which is significantly lower than that observed in resting, unstimulated muscles (compare Fig. 7*B* with Fig. 4*A*). In the experiment shown in Fig. 7 the average ratio PCr/P_i during the latter half of the recovery period was about 30 (as compared to 16 in resting muscle; Table 1), and in some experiments the [P_i] was so low that it was impossible to identify the peak with certainty.

The [sugar P] increases greatly after these long contractions and remains high throughout the recovery period. This is shown by both the spectra of Fig. 7, and Fig. 7*C* shows a high and constant level of these compounds, approximately 4 mmol l^{-1}. In contrast, very little sugar P is detectable in resting muscle (see Fig. 4*A*). In one experiment a spectrum was obtained 6 h after the last stimulation, at which time the sugar P had fallen by 70%, showing that these changes can be reversed in oxygenated muscle.

No significant changes in the unidentified resonances were observed during the course of the recovery period. However, in four early experiments the average total area of these three unknown resonances was about 45% larger after 25 s stimulation than in six other sets of resting muscles ($0{\cdot}1 > P > 0{\cdot}05$ by conventional t test). When a good resting spectrum was accumulated before stimulation in another experiment there was no difference in the area of the unknown resonances before and after stimulation, so perhaps the apparent effect of activity merely reflected the large variations observed in the unknown compounds from one set of muscles to another (see Fig. 4*A*).

It is difficult to make accurate estimates of the variations in internal pH during recovery because in the first half of the recovery period the P_i resonance is fairly broad, while in the latter half it is very small. The

broadness probably reflects a distribution of pH within the muscle (see Discussion). However, the average pH seems to drop to about 7·0 during the first 20 min and then returns to 7·5 after about 40 min.

Brief contractions, frog (*1s/2min*). Fig. 8*A* was accumulated during actual contractions of sartorius muscles, just before they began to relax.

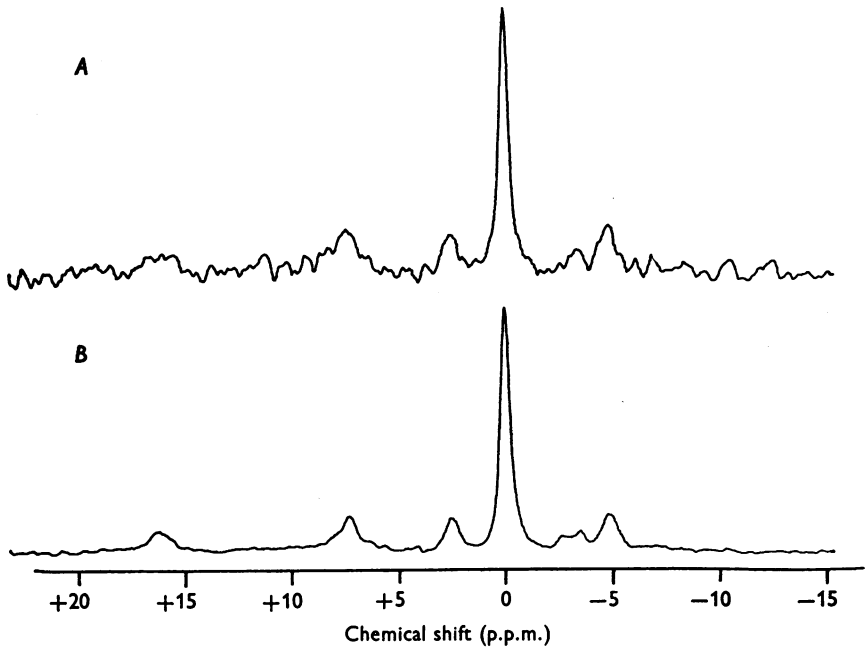

Fig. 8. Frog sartorius in a steady-state of activity. Four muscles were stimulated for 1 s every 125 s. The spectra shown here were accumulated during the first 137 out of 422 contractions. Maximum tension was 1·48 N in the first contraction and this fell to 0·92 N at the 137th. The interval between scans was 5 s because the program described under Methods had not yet been perfected. *A*, during contraction: this spectrum was an accumulation of 137 scans which were timed to occur after 0·8 s of contraction. *B*, average spectrum: in this spectrum all of the scans throughout the contraction and relaxation cycle were added together. Total number of scans was $137 \times 24 = 3288$. The ordinate is divided by 24 for direct comparison with spectrum *A*.

This spectrum does not differ in any dramatic way from the spectrum accumulated throughout the whole contraction and recovery cycle (Fig. 8*B*). Both of the spectra shown in Fig. 8 differ from the spectrum of resting muscle in having a high $[P_i]$ (approximately 5 mmol l^{-1}). The $[P_i]$ rose still further to 7 mmol l^{-1} at the end of a further 285 contractions. By then, the tension development had fallen to a quarter of its original value.

Few differences were observed when the various bins *between* contraction were compared. There were no changes in [ATP] and changes in [PCr] and [P_i] were too small to estimate accurately. Any changes in pH were very small ($< 0{\cdot}1$ pH unit) and we have not as yet been able to quantify them. In the experiment shown in Fig. 8 [sugar P] was observed to increase 5 s after contraction to about 1·5 mmol l^{-1}; during the following recovery period it rapidly decreased back into the noise. In the same bin there seemed to be a corresponding decrease in the unidentified resonances but the signal-to-noise ratio did not permit us to say this with certainty.

The short-lived increase in [sugar P] was not enough to affect the composite spectrum representing the sum of all the bins. Fig. 8*B* indicates a virtual absence of sugar P, as in resting muscle (Fig. 4), and is in marked contrast to the situation after long contractions (see Fig. 7).

Brief contractions, toad (1 *s*/124 *s*). Similar experiments were performed on toad gastrocnemii because their larger size leads to a better signal-to-noise ratio, which facilitates observation of small changes. Moreover, toad muscle contains large amounts of the unknown compound resonating at $-2{\cdot}7$ p.p.m. so more information might be obtained about the function of the unknown compounds. These advantages are to some degree offset by the fact that gastrocnemii are too thick to remain fully oxygenated during activity and their tension development falls to half after only about sixty stimulations. For this reason, Fig. 9 was made by adding together the results of five separate experiments.

Fig. 9 shows the spectra obtained during the first 16 s following stimulation (*A*) and the last 32 s of recovery (*B*), immediately before the next stimulation. The [PCr] in both these spectra is only about half that observed in the resting toad gastrocnemius (Fig. 4*B*) and there is a corresponding increase in [P_i]. The [sugar P] is also higher than in resting muscles (approximately 4 mmol l^{-1}) in both these spectra. Both spectra show the resonance at $-1{\cdot}55$ p.p.m. which appears to be characteristic of toad muscles (see Figs. 4*B* and 5*C*).

In order to reveal small differences between the two spectra, Fig. 9*A* was subtracted from Fig. 9*B* in the computer. The difference, scaled up fourfold, is shown in Fig. 9*C*. The most outstanding features of this difference spectrum are the two peaks at 0 and $-5{\cdot}0$ p.p.m., showing the break-down and subsequent rebuilding of about 10% of the steady-state PCr content, corresponding roughly (see Discussion) to a break-down of 1·5 mmol kg^{-1}. It is interesting that the chemical shift of the negative P_i peak in the difference spectrum corresponds to a pH of 7·3, whereas the average pH as obtained from the P_i resonance in Figs. 9*A* and *B* is 7·1. This will be dealt with in the Discussion. The other feature of interest in the difference spectrum is the possible negative peak in the region of the

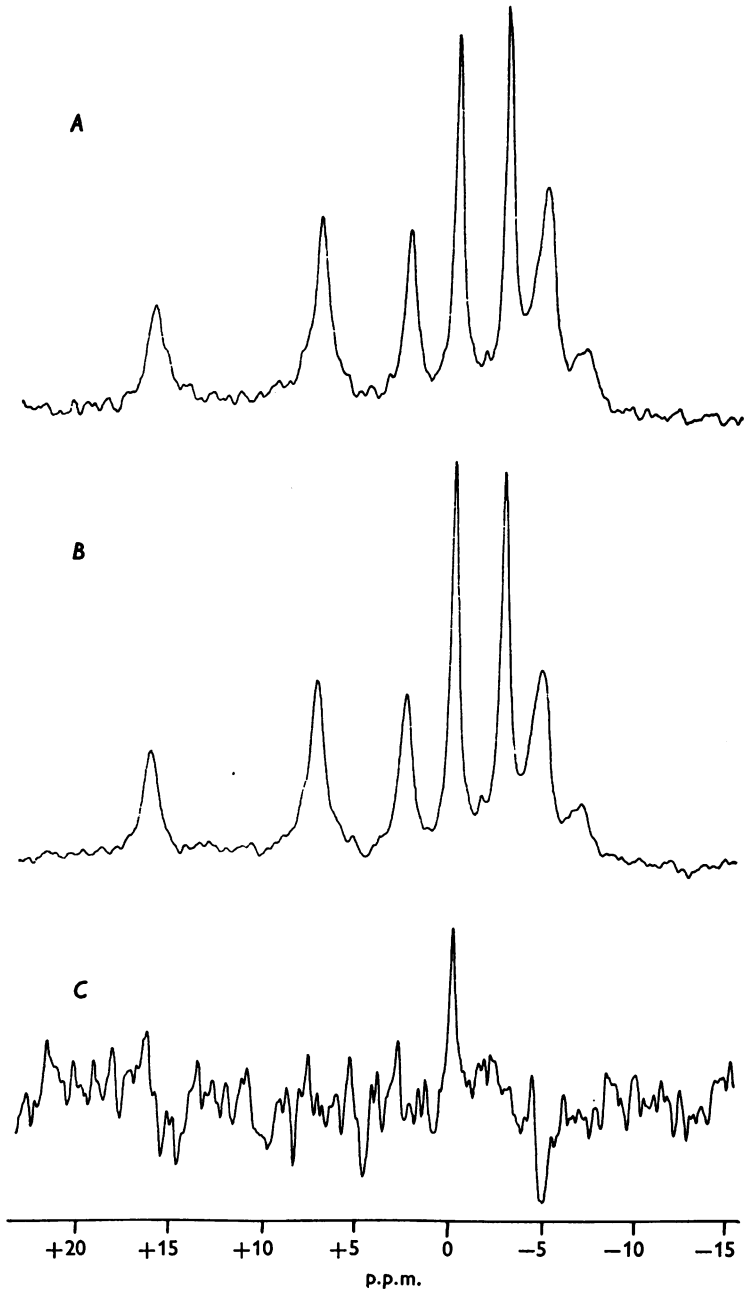

Fig. 9. Brief repeated contractions of toad muscle (1 s/124 s). These spectra were accumulated in five separate experiments, each on a pair of gastrocnemii. There was a total of 244 contractions; scans every 2 sec. *A*, first 16 s following relaxation; $244 \times 8 = 1952$ scans. *B*, last 32 s of recovery, before the next contraction; $244 \times 16 = 3904$ scans. The ordinate is divided by 2 for direct comparison with *A*. *C*, spectrum *A* subtracted from spectrum *B* in the computer; the ordinate is magnified four fold compared with *A* and *B*.

βATP resonance. Further experiment is required to establish whether or not this peak is significant.

In individual experiments, the unknown resonance at $-1{\cdot}55$ p.p.m. has seemed to disappear and that at $-2{\cdot}7$ to increase in the period shortly after contraction. However, these effects have not been consistently reproducible. The spectrum accumulated during the actual contractions of toad muscle is not shown because no unique features were observed.

DISCUSSION

In order to understand the initiation and control of cellular processes, it is necessary to know how these processes occur in intact cells under physiological conditions. NMR is being increasingly exploited for this purpose. In addition to the study of intact muscle, ^{31}P NMR has been used to follow metabolic changes in systems such as aging whole blood and intact red cells (Henderson, Costello & Omachi, 1974) and in developing amphibian embryos (Colman & Gadian, 1976). In these studies, metabolic changes occurring over long time intervals were reflected by changes in relative concentration of phosphorus-containing compounds. In the present study muscles are stimulated within the spectrometer and tension is recorded under conditions resembling those *in vivo*. Thus, both physiological and biochemical processes are studied while the muscles are performing their natural function.

The poor time resolution of NMR measurements has been improved by averaging signals from repeated contractions and changes occurring in a few seconds have been analysed. The length of our Methods section reflects the number of technical difficulties we have overcome and the results presented here illustrate only a small fraction of the experimental possibilities thus created.

Comparison of NMR spectra with chemical analysis of resting muscle

The nature of the information yielded by NMR differs slightly from that obtained by conventional chemical analysis. *First*, NMR spectra can be obtained on intact living tissue, while chemical analysis requires prior freezing and extraction. Thus, conventional methods may be subject to artifacts due to incomplete extraction or to accidental break-down of unstable metabolites. On the other hand, quantitative interpretation of NMR spectra is subject to its own ambiguities, such as possible overlap or saturation (see Methods) of resonances. *Secondly*, while total extractable metabolite is measured by chemical methods, the NMR resonance linewidth is dependent upon molecular mobility, so that highly immobilized compounds give rise to resonances which may be too broad to observe.

In our spectra the linewidths suggest that the resonances arise almost exclusively from compounds which are mobile and are therefore either in free solution or bound to molecules that are themselves mobile. Thus, when the results from the two methods differ, there are numerous possible reasons for the discrepancy. But when the results of carefully performed experiments agree, much confidence can be placed in the values obtained as representing the total concentration of mobile metabolite in intact tissue.

Determination of PCr content on a set of four frog sartorii (see Methods, Fig. 3) showed reasonable agreement between the estimate by NMR (23·5 mmol l^{-1} fibre volume) and by chemical analysis (21·2 mmol kg^{-1} frozen muscle). However, a precise comparison between these two numbers cannot be made because chemical methods do not directly yield intracellular concentrations, but must be corrected for density and extracellular space. The density of our muscles can be estimated as 1·060 from the formula given by Hill (1965, p. 245); thus the chemical estimate is 22·5 mmol l^{-1} frozen muscle. An unknown fraction of frozen muscle (from 10 to 35%, see Dydyńska & Wilkie, 1963) consists of extracellular fluid, so the chemical estimate of intracellular [PCr] is higher than that obtained by NMR and is also less certain. It should be noted that both methods agree in showing that this particular set of muscles contained appreciably less PCr than is usual (27 mmol kg^{-1}; Dawson *et al.* 1975). A more precise comparison can be made when the results are expressed as the ratio of other phosphorus-containing metabolites to [PCr]. Table 1 shows that there is no significant difference between the values obtained in this way by NMR and by chemical methods. There is, however, a fairly large s.d. associated with the results from chemical methods, so the small quantitative differences which are observed may in the future prove significant.

In spite of the possible minor discrepancies indicated above, there is no doubt that the *in vivo* NMR measurements and chemical measurements agree in their description of properly oxygenated resting muscle. In particular, it contains little P_i and even less sugar P. The NMR analyses of frog muscle extracts (Fig. 5*A* and *B*) show that the relative quantities of metabolites are similar to those in resting muscle. However, it is clear from the comparison of Figs. 5*C* and 4*B* that even slight delay in freezing the thick gastrocnemius muscles leads to the break-down of PCr and the appearance of P_i. This phenomenon is all too well known to those engaged in the study of muscle metabolism.

We conclude that these NMR studies confirm, by an independent method, the essential correctness of the chemical methods now in use to measure the known metabolites in muscle. This is a great relief, since the quantitative accuracy of these analyses is becoming increasingly important,

particularly in studies aimed at comparing energetic changes with breakdown of phosphorus compounds.

Compartmentation. It has been reported that considerable proportions of PCr (Hill, 1962) and adenine nucleotide (Hill, 1959, 1960*a*, *b*) are constrained within particular portions of muscle sarcomeres in fixed tissues (see also Engström, 1944). The present work shows that it is unlikely that these findings can be accounted for by binding of these metabolites to macromolecules in the living tissue. ^{31}P NMR studies of rat skeletal muscle now in progress do suggest that P_i and sugar P are distributed within muscle fibres in a different manner from ATP and PCr (Seeley *et al.* 1976; Busby, Gadian, Radda, Richards & Seeley, 1977).

pH measurement by NMR

The measurement of intracellular pH involves both theoretical and practical difficulties. The two most commonly used techniques are: insertion of a pH-sensitive micro-electrode directly into the cell; or analysis of the distribution of weak acids, from which pH can be calculated. Both of these methods have well documented disadvantages (see Waddell & Bates, 1969).

^{31}P NMR provides an alternative method, which is more direct than either of those discussed above. Since P_i has a pK close to neutrality, the intracellular pH greatly affects the relative amounts of $H_2PO_4^-$ and HPO_4^{-2}. A single resonance is obtained for P_i, with a chemical shift determined by the relative amounts of the two species present. Thus, a pH titration curve can be constructed (Fig. 2) on the basis of the P_i resonance position. Such a measurement directly reflects the ability of the hydrogen ion to undergo a binding reaction under intracellular conditions. The width of the P_i resonance can reflect the distribution of pH within the muscle; this is an additional advantage of ^{31}P NMR over other methods, which yield only a single value for intracellular pH.

Resting muscle. The internal pH of the resting frog sartorius muscle under the conditions of our experiments was determined from the position of the P_i resonance peak to be 7·5. Muscle pH is dependent upon a number of factors, including the P_{CO_2} and the pH of the external medium as well as upon mechanical activity and metabolic state. In these experiments Tris-buffered Ringer solution was adjusted to pH 7·2 at 20 °C and then perfused into the experimental chamber at 4 °C. Due to the great temperature dependence of the Tris buffer, the pH of the Ringer solution during the experiment was 7·7. Since no CO_2 was added to the Ringer solution and that formed in the muscle was removed with the circulating Ringer solution, the P_{CO_2} was negligible.

Of the many studies of muscle pH in the literature, two recent ones, which include conditions similar to ours are those of Aickin & Thomas (1976) and Roos (1975). Using micro-electrodes, Aickin & Thomas found the internal pH of mouse soleus muscle to be 7·42 in CO_2-free Ringer solution buffered to pH 7·4 with HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid). From the distribution of DMO (5,5 dimethyl-2-4-oxazolidinedione), Roos found the internal pH of rat hemidiaphragm to be 7·55 when the external pH was adjusted to 7·90 in 5 mM Tris, and the P_{CO_2} was 1·5 mmHg.

Due to the high external pH and lack of CO_2 in our experiments, it is very likely that the pH of these muscles was somewhat greater than would be found *in vivo*. The difference between our results and those of Burt *et al.* (1976*a*), whose NMR studies indicated a pH of 7·2 in frog sartorii, probably arises from the fact that CO_2 and lactic acid were retained in their preparation.

It should be noted that in all of the spectra shown, the P_i resonance is broader than that of PCr. Part of the broadening is probably due to the existence of a range of internal pH environments, as has been postulated for mammalian skeletal muscle (Seeley *et al.* 1976). However, this cannot be the whole explanation since the right-hand side of the peak often extends beyond $-5{\cdot}6$ p.p.m., a shift which cannot be achieved by raising the pH (see Fig. 2).

Contraction and recovery. The measurement of muscle pH during contraction and recovery can provide useful general information concerning the rates and extents of metabolic and mechanochemical processes. Dubuisson and Distèche (see Dubuisson, 1939; Distèche, 1960) have measured changes in pH by placing a glass electrode on the surface of active muscle. Although variations of approximately 0·001 pH unit can be detected by this method, it is not clear exactly how these variations relate to the actual intracellular pH changes. In the present study, direct evidence has been obtained of variations in intracellular pH following both 25 s and 1 s contractions.

In studies of 1 s contractions of toad muscle both the spectrum obtained immediately before contraction and that obtained immediately after indicate the same average intracellular pH of 7·1 (see Fig. 9*A* and *B*). However, in the difference spectrum (Fig. 9*C*) the peak corresponding to P_i is shifted by 0·2 pH unit to a slightly more alkaline position. This may indicate that there is, in fact, an alkaline shift immediately after contraction but that it is too small to alter the observed average pH. Such a result is consistent with the finding of Distèche (1960, fig. 65) that there is an alkaline shift of $< 0{\cdot}01$ pH unit at the muscle surface during a 1 s tetanus of frog sartorius at 2° C; this was attributed to the fact that the hydrolysis

of PCr absorbs about 0·35 mole protons per mole of hydrolysis. Alternatively, the alkaline shift in the difference spectrum may possibly indicate that the P_i liberated during contraction is in a more alkaline environment than the average for the muscle.

In our experiments on recovery from long contractions, large, but ill-defined, variations in pH were observed. The pH was found to go increasingly acid during the first 20 min of recovery, changing by a total of about 0·5 pH unit, before returning to its pre-contraction value. The acid phase following a contraction is generally attributed to the formation of lactic acid (Dubuisson, 1939), so our observations can be related to the time course of formation and loss of this compound under the conditions of these experiments. The return of pH to resting levels should follow efflux of lactate from the muscle; the factors influencing this rate are complex (Mainwood & Worsley-Brown, 1975).

The unidentified resonances

In this and in previous ^{31}P NMR studies of skeletal muscle (Bárány *et al.* 1975; Burt *et al.* 1976*a*; Seeley *et al.* 1976) unexpected resonances have been observed in the region between the PCr and P_i peaks. The same resonances appear in the spectra of muscle extracts (Burt *et al.* 1976*a* and Fig. 5 of this paper) indicating that they cannot arise from the same compound in different intracellular environments, but must result from the presence of several previously unsuspected compounds occurring in large quantities (0·5–15 mmol l^{-1}).

At least four such unidentified resonances can be distinguished by their different chemical shifts. Resonances at −2·7, −3·05 and −3·6 p.p.m., in the phosphodiester region of the spectrum, occur to varying extents in mammalian and amphibian muscles (Burt *et al.* 1976*a*; Seeley *et al.* 1976). A resonance at −2·7 p.p.m. also appears in the spectrum of dystrophic, but not of normal, chicken pectoral muscle (Burt *et al.* 1976*a*, indicated in their work as +0·5 p.p.m. from their standard, 85% phosphoric acid). The resonance at −3·05 p.p.m. from several different striated muscles of various species has been assigned to glycerophosphorylcholine (Burt, Glonek & Bárány, 1976*b*); a similar conclusion was reached by Seeley and co-workers (1976) regarding the large resonance in the same position in the spectrum of rabbit semitendinosus. In this present study an additional small resonance has been observed at −1·55 p.p.m. in the spectra of toad gastrocnemii; compounds such as phosphoenolpyruvate and 1,3-diphosphoglycerate resonate in the region of −1·5 to −2·0 p.p.m. at neutral pH.

The observation of these resonances is consistent with earlier detection

in muscle of phosphorus compounds of unknown functional significance. In pioneering studies using paper chromatography, unexpected compounds were reported in frog (Caldwell, 1953), turtle (Caldwell, 1953; Roberts & Lowe, 1954) and human (Caldwell & Prankerd, 1954). One such compound was identified in turtle muscle as the *O*-phosphodiester of L-serine and ethanolamine (Roberts & Lowe, 1954); the others remain unidentified. The fraction of the total phosphorus found in these compounds agreed roughly with what is now indicated by the unidentified resonances in ^{31}P NMR spectra. It is thus likely that at least some of the same compounds are being studied.

Once the structures and function of these compounds has been established there may be little reason to discuss them together. At present, however, so little is known about them that only a generalized treatment can be offered. They do have in common a great variability in concentration, not only between different species and muscle types but also, though to a much lesser extent, between individual muscles of the same type. For example, the resonance at $-2{\cdot}7$ p.p.m. is over ten times greater in the spectrum of the toad gastrocnemius (representing more than 10 mmol l^{-1} of the compound) than it is in the spectrum of the frog sartorius (representing less than 1 mmol l^{-1}). The relative areas of the three unknown resonances centred at $-3{\cdot}1$ p.p.m. vary from batch to batch in frog sartorius muscle (see Figs. 4*A* and 5*A*), and M. Bárány and C. T. Burt and T. Glonck (personal communication) have observed a seasonal variation with respect to the total amounts present of these three unknown compounds.

In the present study we have focused particularly on the question whether the size of these unknown resonances changes as a result of muscle contraction. Our results so far have been ambiguous and unfortunately have shed little light on the possible function of these compounds. In some experiments there appeared to be small changes in the areas of these peaks; however, they were not reproducible from one experiment to another.

NMR studies of stimulated muscle

NMR is a particularly useful method for studying metabolic changes during and following contraction. Using conventional methods it is necessary to interrupt chemical reactions by freezing; thus to study the time course of metabolic changes during recovery by conventional methods it is necessary to do many experiments, terminating them at different times after stimulation and to do enough at each point in time for a statistical analysis. This always involves many weeks of work. Using ^{31}P NMR, metabolism can be followed throughout the contraction and recovery period in a single experiment lasting just a few hours and

information is gained about the physical state of metabolites as well as their changes in concentration.

During contraction. The spectra accumulated during the actual contraction of frog sartorius or toad gastrocnemius were not different in any apparent way from those accumulated during the recovery interval between contractions. Only a moderate signal-to-noise ratio was achieved in these experiments, because only one scan can be made during each contraction but the results do rule out any *dramatic* changes in concentration of metabolites or in any of those physical characteristics which are observable by ^{31}P NMR. Chemical changes resulting from contraction can, however, be detected by comparison of spectra accumulated from the larger number of scans obtained during the recovery period between contractions, as explained below.

Recovery from 1 s contractions every 2 min. We were unfortunately not able to achieve a sufficient signal-to-noise ratio to show clearly changes in [PCr] and [P_i] between 1 s contractions in the frog sartorius. However, experiments with the larger toad gastrocnemius permitted observation of clear and consistent changes between contractions. Fig. 9*C* shows a rebuilding of about 10% of the steady-state [PCr] between 10 and 108 s following stimulation. Taking the composition of resting toad muscle from Table 1 of Burt *et al.* (1976*a*), and assuming that the rebuilding of PCr occurs at a steady rate throughout the 124 s cycle, this corresponds to about 1·5 mmol kg^{-1} PCr break-down during each contraction.

No measurements have been reported of the chemical changes during contraction of toad gastrocnemius at 4° C and for technical reasons it is difficult by chemical methods to examine individual contractions in a steady-state of repeated contractions. However, the value obtained by NMR (although it requires further verification) indicates that in a steady state of contraction at 4° C, the toad gastrocnemius breaks down some two-and-a-half to five times more PCr per second than do frog sartorii in a single brief contraction at 0° C (see Gilbert *et al.* 1971; Homsher, Rall, Wallner & Ricchiuti, 1975; Curtin & Woledge, 1975; Kushmerick & Paul, 1976*a*). As indicated in the Introduction, the break-down of PCr in brief single contractions of frog and mammalian muscle is insufficient to account for the energy produced and the deficit must be attributed to an unknown process. The present results raise the possibility that during a steady state of repeated contractions this deficit might disappear.

Mechanical response and chemical change. When frog sartorius muscles are stimulated within the spectrometer at a rate of 1 s every 2 min, tension declines slightly with each contraction for approximately the first half hour and then remains nearly constant for many hours. This agrees with earlier observations, such as those of Jewell & Wilkie (1958) and of Aljure

& Borrero (1968) on tension response of sartorius muscles to similar patterns of stimulation. The spectrum accumulated during the course of this steady state of mechanical activity (Fig. 8*B*) is very similar to that of a resting sartorius muscle (Fig. 4*A*), except with regard to [P_i] which increases as the experiment progresses. Thus, these muscles in a steady state of mechanical activity also maintain a steady state with respect to most of the measured metabolites, which are at or near their resting levels. It will be interesting in future experiments to determine whether the long term increases in [P_i] are related to the gradual fall in tension development.

Recovery from 25 s contractions. Approximately 20 % of the PCr (roughly 5 mmol l^{-1}) is broken down as a result of a 25 s contraction and this is approximately equalled by the increase in [P_i]. This result agrees with the observation that about 1 % of PCr is broken down per second of contraction (see Gilbert *et al.* 1971). Both the [PCr] and [P_i] recover with a half-time of approximately 10 min. The same value for the half-time of recovery of PCr was reported by Dydyńska & Wilkie (1966) when studying 30 s contractions of the same muscle under similar conditions. Kushmerick & Paul (1976*b*) also found a half-time of about 10 min for recovery of PCr and P_i as well as for recovery oxygen consumption in frog sartorii undergoing 20 s contractions at 0° C. An unexpected result of the present experiments, however, was that while [PCr] returned to approximately its resting value, the [P_i] fell below that in resting muscle.

Another unexpected finding is the maintenance of sugar P at a high and constant level throughout the recovery period following these long contractions. High levels of sugar P are not observed in resting muscles nor in muscle undergoing repeated short contractions. Indeed, such an event is generally associated with anoxia and muscle deterioration. For this reason it must be emphasized, as a glance at Fig. 7 will show, that these muscles are not depleted of ATP and PCr during the latter half of the recovery period, nor is their mechanical response declining.

An explanation of the high and constant level of sugar P following long contractions must await further experiment, including identification of which sugar phosphates contribute to the increased height of this peak. This finding can, however, be satisfactorily explained if the data of Danforth, Helmreich & Cori (1962) on activation of phosphorylase in frog sartorius muscle under anaerobic conditions can be applied to our studies. According to their work, the half-time for conversion of phosphorylase to its active *a*-form during contraction at 4° C should be 30 s (based on their Table 2 and their Q_1 of 3·5 for phosphorylase activation). Thus, very little phosphorylase *a* should be formed during our 1 s contractions and it should be virtually completely re-converted to the inactive *b*-form during the 2 min

recovery period. On the other hand, during a 25 s contraction a high percentage of phosphorylase *a* can be expected for many seconds, resulting in a large formation of sugar P. In the experiments by Kushmeric & Paul (1976*b*) less than 6–9 % of the PCr was regenerated directly by the glycolytic pathway in aerobic recovery of frog sartorius at 0° C. If the same is true under the conditions of our experiments. one would expect only about 0·2 mmol l^{-1} of sugar P to be metabolized during the 56 min recovery period following a 25 s contraction. This amount could not be detected in our experiments and would explain the observed constant concentration of these compounds.

The present role and future prospects of NMR in the study of living muscle

NMR is a complicated and expensive technique and its use must be justified by the information it yields. Our *in vivo* measurements on living contracting muscle using NMR provide a completely independent confirmation of the validity of the methods of quick freezing, extraction and chemical analysis that have been painstakingly evolved over many years. Furthermore, our studies of the changes occurring during and following contraction have been useful in that they have shown both what does occur and what does not. Speculation can now be laid to rest concerning effects which we would have seen, had they existed.

The greatest problems encountered in the present study have arisen from the low sensitivity of NMR and the consequent poor time resolution. In studies of living muscle severe constraints are imposed on the sample size (see Methods), resulting in smaller signals than is usual in NMR studies. We cannot predict the extent to which sensitivity of NMR will be increased by future technical advances; however, the present situation may well be improved by application of statistical methods to detection of small signals in noise. With improved signal detection, the time resolution could be increased by an extension of the method of accumulating spectra during preset time intervals phased to repeated contractions. The ultimate limit to time resolution, about 20 ms, is set by the T_2 of the phosphorus resonances.

The usefulness of NMR and of chemical analysis are complementary. At present, small changes in concentration of known compounds, particularly over short time intervals, are more amenable to examination by chemical analysis. However, destruction of the tissue for each analysis necessitates extensive replication and gravely limits the range of possible experiments. When relatively slow changes, such as those occurring during recovery from contraction are of interest, information can be obtained in a single afternoon using NMR which would require weeks of effort using other methods. Thus, NMR will greatly facilitate future investigation of such

problems as the action of metabolic inhibitors and the consequences of the enzyme defects that characterize some muscle diseases.

Note added in proof. Recent experiments have shown that the 'T_1 correction factor' for sugar P (see Determination of concentration; Methods) is approximately 1·5 when the concentration of sugar P has been raised to 12 mol Kg^{-1} by metabolic inhibitors. Our estimates of [sugar P] should thus be multiplied by this factor.

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