The activity of creatine kinase in frog skeletal muscle studied by saturation-transfer nuclear magnetic resonance

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1. The activity of creatine kinase in intact anaerobic frog muscle at 4°C at rest and during contraction was investigated by using saturation-transfer ^{31}P n.m.r. 2. At rest, the measured forward (phosphocreatine to ATP) reaction flux was $1.7 \times 10^{-3} \text{ M} \cdot \text{s}^{-1}$ and the backward flux was $1.2 \times 10^{-3} \text{ M} \cdot \text{s}^{-1}$. The large magnitude of both fluxes shows that creatine kinase is active in resting muscle, so the observed constancy of [phosphocreatine] demonstrates that the enzyme and its substrates are at equilibrium. 3. The apparent discrepancy between the fluxes must arise largely from an underestimation of the backward flux resulting from interaction of ATP with other systems, e.g. via adenylate kinase. For purposes of further calculation we have therefore adopted $1.6 \times 10^{-3} \text{ M} \cdot \text{s}^{-1}$ as an estimate of both fluxes. 4. During contraction, when the creatine kinase reaction is no longer at equilibrium, the net rate of phosphocreatine breakdown, estimated directly from the change in area of the inorganic phosphate peak, was $0.75 \times 10^{-3} \text{ M} \cdot \text{s}^{-1}$. Saturation transfer indicates that the forward reaction flux remains at approx. $1.6 \times 10^{-3} \text{ M} \cdot \text{s}^{-1}$ and the backward flux decreases to about $0.85 \times 10^{-3} \text{ M} \cdot \text{s}^{-1}$. 5. The activity of creatine kinase during contraction is large enough to account for the well-established observation that, during contraction, the concentration of ATP falls by less than 2–3%. The reaction catalysed by creatine kinase is driven forward during contraction by the large relative increase in the concentration of free ADP, which is more than doubled. 6. The observation that the forward flux does not increase during contraction and that the backward flux decreases can most simply be explained on the basis of competition of reactants for a limited amount of enzyme.

The only known function of *P*-creatine in skeletal muscle is to regenerate the ATP that is broken down during contraction. This regeneration takes place by means of the reaction catalysed by creatine kinase (EC 2.7.3.2):

Extensive studies of the chemical changes that

Abbreviation used: *P*-creatine, phosphocreatine.

occur during contraction of amphibian skeletal muscles all show that very little net ATP breakdown occurs during normal contraction (e.g. Gilbert *et al.*, 1971). On the basis of these results it has been generally assumed that the creatine kinase reaction is always close to equilibrium in skeletal muscle, and that the forward and backward exchange fluxes are considerably faster than the rate at which ATP is hydrolysed during contraction. A direct test for these assumptions has not been feasible because of the difficulty of measuring the rates *in vivo* of reactions that are at equilibrium or in steady state.

The rate of interconversion of two chemical species in equilibrium can be measured by n.m.r. provided that the rate is of the same order of magnitude as the spin-lattice relaxation rates of the observed nuclei in the two molecules. Forsen & Hoffman (1963) developed the theory for the method, known as saturation-transfer n.m.r., and demonstrated its feasibility by measuring the rate of proton transfer in the isomerization equilibrium of salicylaldehyde. Subsequently, Redfield & Gupta (1971) used saturation-transfer n.m.r. to derive the rates of electron transfer between oxidised and reduced cytochrome *c*. More recently, Campbell *et al.* (1978) have described a variety of n.m.r. pulse methods for measuring exchange rates. The first rate analysis *in vivo* by saturation transfer was reported by Brown *et al.* (1977), who measured the unimolecular exchange rates between P_i and ATP catalysed by the dicyclohexylcarbodi-imide-sensitive ATPase of *Escherichia coli*.

^{31}P n.m.r. spectroscopy can be used to observe several key phosphorus-containing metabolites in muscle (Hoult *et al.*, 1974) and techniques have been developed for maintaining both frog skeletal muscle (Dawson *et al.*, 1977), and perfused mammalian hearts (Garlick *et al.*, 1977) in a physiologically stable condition in the n.m.r. spectrometer. We have already measured the interconversion rates between ATP and *P*-creatine in resting frog skeletal muscle and perfused rat hearts by saturation-transfer n.m.r. (Brown *et al.*, 1978). In the present paper we present the method in detail and report the results of measurements on the creatine kinase and ATPase reaction during contractions (duration 3 s) of frog gastrocnemius muscle; we analyse the results by using the computer language FACSIMILE (Chance *et al.*, 1977), designed to simulate complex chemical reactions. A preliminary account of this work has been presented to the Physiological Society (Brown *et al.*, 1980).

Experimental

The n.m.r. spectrometer was constructed in Oxford (Hoult & Richards, 1975), and was equipped with a wide-bore superconducting magnet operating at 73.8 MHz for ^{31}P . Frog (*Rana temporaria*) muscles (4–8), weighing about 2 g *in toto*, were placed in Ringer solution [composition (mM): NaCl, 111; KCl, 2.5; CaCl_2 , 2; Pipes (1,4-piperazine-diethanesulphonic acid) from Sigma, 15; adjusted to pH 7.2 at 4°C with HCl] at 4°C in a sample tube of length 60 mm and inner diameter 12 mm. The muscle tendons were tied firmly to two metal hooks fitted to the ends of the tube. The hooks also acted as electrodes, and the muscles were stimulated by the application of 50 Hz sine waves at $15 \text{ mA} \cdot \text{cm}^{-2}$ (Csapo & Wilkie, 1956). Since the muscles were too

thick to be maintained in a fully oxygenated state, they were made uniformly anoxic by adding 2 mM-NaCN to the Ringer solution. Anaerobic muscles quickly lose their *P*-creatine stores through handling; this problem was minimized by carrying out all dissections and mounting in the cold (0–4°C). Muscles whose initial spectra showed that they contained less than 90% of the *P*-creatine present in fresh, resting aerobic muscle were not used.

The sample tube was positioned horizontally within a seven-turn solenoidal radio-frequency coil, and an electrical screen was placed between sample and coil to eliminate dielectric radiofrequency losses (Gadian & Robinson, 1979). The signal-to-noise ratio was sufficient to enable the magnetic field homogeneity to be adjusted by observation of the free induction decay of the muscle n.m.r. signal, which at the beginning of each experiment was predominantly from the narrow *P*-creatine resonance. A field-frequency lock was not required because of the high stability of the field and frequency synthesizer.

Concentration measurements

Relative concentrations of phosphorus-containing metabolites were obtained directly from the measurement of peak areas (or intensities). In order to convert peak areas to absolute concentrations, it was assumed that the total concentration of *P*-creatine, ATP, P_i and sugar phosphates in solution within the muscle is $32.7 \text{ mmol} \cdot \text{kg}^{-1}$ (see Dawson *et al.*, 1980a). Any error in this assumption affects equally all the concentrations and fluxes quoted in this paper, and therefore does not significantly affect any of the conclusions that we reach. Concentrations of metabolites in the Results section are given as $\text{mmol} \cdot \text{kg}^{-1}$ wet weight of muscle, because this is the measurement actually made. In the Discussion, concentrations are expressed as $\text{mol} \cdot \text{litre}^{-1}$ (M) in order to facilitate comparison with studies *in vitro*. The conversion was made on the assumption that $1 \text{ M} \approx 1.0 \text{ mol} \cdot \text{kg}^{-1}$. This is a simplification; in fact $1 \text{ M} \approx 0.8 \text{ mol} \cdot \text{kg}^{-1}$ if all the muscle water participates, or $0.62 \text{ mol} \cdot \text{kg}^{-1}$ if only 77% is available (see, e.g., Dubuisson, 1954).

Measurement of saturation transfer

Resting muscles. Sixteen scans were accumulated with selective irradiation applied at the resonant frequency of the γ -phosphate of ATP, which is chemically shifted by -2.5 p.p.m. from the *P*-creatine signal. As a control measure, the irradiation was then switched to a frequency $+2.5 \text{ p.p.m.}$ from the *P*-creatine signal (i.e. to its high-frequency side), which happens to be the phosphodiester resonance position. Again, 16 scans were accumulated. The difference between the two spectra gave the percentage reduction in *P*-creatine signal intensity

resulting from saturation transfer. This procedure was adopted in order to minimize errors resulting from the imperfections in the selectivity of the irradiation.

Saturation transfer in the reverse direction was performed in a similar manner, with selective irradiation applied to *P*-creatine and at -5.0p.p.m. The positions at which selective irradiation was applied are illustrated in the Results section.

In all experiments other than the progressive saturation measurements, 90° radiofrequency pulses were applied at 12 s intervals, as slow pulsing considerably simplifies the analysis of the experimental data (see the Appendix).

Contracting muscles. After the above procedure, muscles were stimulated for 3 s every 204 s, and transfer of saturation in the contracting muscles was observed as described below and as shown diagrammatically in Fig. 1.

Selective irradiation was continuously applied to the *P*-creatine resonance (except that it was suppressed during the acquisition of data; see Fig. 1). Four scans were accumulated, and stored in one memory location of the computer. The fifth scan was stored in a second memory location, and coincided in time with the end of a 3-s electrical stimulation of the muscles. The next 12 scans were stored in a third memory location, and this routine of 4–1–12 scans (see Fig. 1) was repeated a further three times. In this way, over the course of four contractions, a total of 16 scans was accumulated just prior to contraction, four scans were accumulated at the end of 3-s contractions, and 48 scans were collected in the

periods following the contractions. The scans were collected continuously throughout this sequence at 12 s intervals. The three accumulations were stored on a magnetic disk, and the whole process was repeated three further times, so that each set of muscles underwent 16 contractions. The data for each set of four contractions were stored separately on the disk.

Observations were made on four sets of muscles, and the results from either the first four or the first eight contractions were used to measure the reaction fluxes during contraction. Two of the four sets of muscles described above underwent two additional contractions in which the γ -phosphate signal of ATP was selectively irradiated. This reverse experiment was also performed as the sole procedure on a fifth set of muscles which underwent ten contractions. Finally, control experiments were performed in which muscles underwent a series of 3-s contractions and spectra were accumulated in the absence of any selective irradiation.

The above experiments produced enough signal to measure the absolute saturation transfer on each set of muscles prior to the commencement of electrical stimulation, and permitted comparison of the transfer of saturation in the same muscles between and during contraction. The results, when considered together, enabled us to estimate the absolute magnitude of saturation transfer during contraction.

Measurement of T_1 values

In the same experiments, T_1 values were measured

Fig. 1. Diagram illustrating the timing of the saturation transfer experiments

(A). (a) represents the time for which selective irradiation was applied (11.67 s); (b) represents the 90° radio-frequency pulse, of duration $30\mu\text{s}$; (c) represents the acquisition time (0.33 s). (B). Radio-frequency pulses were applied throughout the experiments every 12 s. Data were collected in a routine of 4–1–12 scans, and stored in three different memory allocations. The thick vertical lines represent the electrical stimulation to the muscles, whereas the thin vertical lines represent the radio-frequency pulses. (d) represents the periods during which the blocks of four scans were accumulated; (e) represents the muscle stimulation, at the end of which one scan was collected; (f) represents the periods during which the blocks of 12 scans were accumulated.

by progressive saturation (Freeman & Hill, 1971), using the pulse sequence $90^\circ-\tau-90^\circ-\tau-$ etc. It was also necessary to estimate the T_1 value of the *P*-creatine and ATP γ -phosphate signals during contraction, as these might differ from those measured between contractions.

There are difficulties in measuring a T_1 value during a contraction lasting only 3 s. One problem is that the magnitude of the signal following the first in a train of pulses is not representative of the pulse interval that is employed, and in a short train this contributes significantly to the total signal. The following procedure, which overcomes this problem, was therefore adopted. Muscles were stimulated for 3.2 s every 208 s, over a period of about 30 min, and during this time 90° radio-frequency pulses were applied every 750 ms. The 90° pulses were synchronized with the stimulation by means of a computer program (Dawson *et al.*, 1977), so that five pulses were applied during each stimulation (see Fig. 2). However, the free induction decay following the first of these pulses was zeroed within the computer, so effectively only four scans were accumulated.

After each contraction, three further blocks of five scans, and two blocks of 129 scans were accumulated, and the first free induction decay in each block was zeroed. The cycle was repeated for eight contractions for each set of muscles, and results were summed for three sets of muscles. In this way, the intensities of the resonances under saturating conditions could be compared in muscles between and during contraction. From our measurements of the T_1 values in resting muscles, it was then possible to estimate T_1 values during and between contraction. Reaction rates could then be calculated from the data by using the theory developed by Forsen & Hoffman (1963), details of which are given in the Appendix.

Results

Resting frog gastrocnemii

The ^{31}P n.m.r. spectra of resting frog gastrocnemius muscles contain signals from the three phosphate groups of ATP, *P*-creatine, phosphodiester and P_i , as shown in Fig. 3(a). The spectra obtained with the signal from the γ -phosphate of ATP saturated (Fig. 3b) and with the saturating irradiation at 2.5 p.p.m. (close to the phosphodiester peak) providing the control (Fig. 3c) are also shown together with the difference spectrum (Fig. 3d) which was obtained by subtracting (b) from (c). The intensity of the *P*-creatine signal in Fig. 3(c) is only 7% smaller than in Fig. 3(a), indicating that the irradiation is sufficiently selective. Fig. 3(b) reveals a 17.8% decrease in the *P*-creatine signal intensity when compared with Fig. 3(c). This is best seen in the difference spectrum Fig. 3(d) which also shows

Fig. 2. Diagram illustrating the measurement of T_1 during contraction

Radio-frequency pulses (represented by the small vertical lines) were applied throughout the experiments every 0.75 s. (a) represents the muscle stimulation, of duration 3.2 s, during which one block of data was accumulated; (b) represents the interval between muscle contractions, during which five blocks of data were accumulated, as described in the text.

Fig. 3. ^{31}P n.m.r. spectra showing the effects of saturation transfer from the γ -phosphate of ATP to *P*-creatine (a) is a spectrum obtained in the absence of selective irradiation, whereas (b) and (c) are spectra obtained with selective irradiation applied at the frequencies indicated by the arrows. (d) is the difference spectrum obtained by subtracting (b) from (c). Each spectrum represents the sum of experiments performed on three sets of muscles; (a) was obtained from a total of 64 scans, and (b) and (c) were each accumulated over 24 scans. The Y axes have been adjusted so that total peak area per mol of phosphorus is the same in (a), (b) and (c). Chemical shifts were measured relative to the *P*-creatine resonance, which was used as an internal standard. The chemical shift scale follows the accepted convention whereby positive shifts are to higher frequency at the constant magnetic field employed in Fourier transform n.m.r. spectroscopy.

the expected positive peak from the γ -phosphate of ATP and the negative peak in the phosphodiester region. In the complementary experiments (Fig. 4) saturation of the *P*-creatine resonance results in a decrease of 29.7% in the intensity of the γ -phosphate signal of ATP.

Fig. 4. ³¹P n.m.r. spectra showing the effects of saturation transfer from *P*-creatine to the γ -phosphate of ATP. (a) and (b) are spectra obtained with selective irradiation applied at the frequencies indicated by the arrows. (c) is the difference spectrum obtained by subtracting (a) from (b). Above each spectrum the ATP peaks are shown scaled up by a factor of 4 for ease of observation. Each spectrum represents the sum of experiments performed on four sets of muscles, and was obtained by accumulating 96 scans *in toto*.

The measured T_1 values in the absence of saturating irradiation were 2.89 s for *P*-creatine and about 1.8 s for the γ -phosphate of ATP. The latter number is, however, only approximate, as the observed relaxation for ATP was not truly exponential. This effect arises because of the interconversion of ATP and *P*-creatine, which tends to average the true T_1 values, and it is more evident for ATP than for *P*-creatine essentially because their concentrations are so different. This problem is treated in the Appendix; the corrected T_1 values are calculated to be 3.2 s and 1.5 s respectively (Table 1). From the saturation transfer and T_1 values, together with the concentrations measured by n.m.r., the exchange fluxes can be obtained (Forsen & Hoffman, 1963).

Our results for the four sets of muscles (considered both separately and summed) are shown in Table 1. From the summed spectra, the rate of conversion of *P*-creatine to ATP is calculated to be 1.7 mmol · kg⁻¹ · s⁻¹, whereas the reverse rate is 1.2 mmol · kg⁻¹ · s⁻¹. Unfortunately, complications arise if other reactions of ATP or *P*-creatine have rates that are comparable with that of the creatine kinase reaction. There are no other known reactions of *P*-creatine in muscle (see Needham, 1971). However, ATP is a substrate for a wide range of other enzyme-catalysed reactions, notable amongst which is the adenylate kinase reaction

We do not yet have data for the rate of this reaction, and so we cannot quantify its effect on the observed saturation-transfer characteristics. All we can say is that its effect would be to reduce the extent of saturation transfer from *P*-creatine to the γ -phosphate peak of ATP. Therefore the estimated value of 1.2 mmol · kg⁻¹ · s⁻¹ for the rate of conversion of ATP to *P*-creatine represents a lower limit to the actual value. In resting muscle, the actual value must be equal to the rate of conversion of *P*-creatine to ATP, because otherwise the concentration of *P*-creatine would not remain constant. Since the

Table 1. Individual and summed results of saturation transfer and T_1 values

For individual sets of muscles, the T_1 values given are the measured T_1 values; the figures of 3.2 s and 1.5 s in the bottom row refer to the true T_1 values deduced from the theory of Forsen & Hoffman (1963). Each of the figures given in the bottom row has a coefficient of variation (s.d./mean) of about 10%.

Expt. no.	<i>P</i> -Creatine/ATP molar ratio	Reduction in signal from saturation transfer (%)		T_1 values (s)	
		<i>P</i> -Creatine	ATP	<i>P</i> -Creatine	ATP
1	6.1	14	27.5	2.9	1.9
2	5.5	—	34	—	—
3	5.4	19	25	2.9	1.8
4	6.2	19	30	3.0	1.7
Average (from summed spectra)	5.9	17.8	29.7	3.2	1.5

figure of $1.7 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$ for the conversion of *P*-creatine to ATP is subject to fewer uncertainties than the reverse rate, we consider that $1.6 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$ represents a good estimate for the forward and reverse rates. This is the figure used throughout the remainder of our paper.

Reaction rates during 3 s contractions

To obtain acceptable signal-to-noise ratios, it was necessary to accumulate data over a large number of contractions. For this reason, the spectra shown in Fig. 5 represent the summed data from four sets of muscles, and from the first four contractions undergone by each set of muscles. Thus the spectra were accumulated over 16 contractions.

Fig. 5(a) shows the spectrum obtained during the minute preceding contraction and Fig. 5(b) shows the spectrum obtained at the end of contraction, just as stimulation to the muscles is terminated. Both spectra were accumulated in the presence of selective irradiation of the *P*-creatine resonance, as described

Fig. 5. ^{31}P n.m.r. spectra of muscles obtained (a) before contraction, and (b) at the end of contraction, just as stimulation to the muscles is terminated

Both spectra were accumulated in the presence of selective irradiation of the *P*-creatine resonance. Spectrum (c) is the difference spectrum obtained by subtracting (a) from (b). The spectra represent the summed data from four sets of muscles, and from the first four contractions undergone by each set of muscles. (a) was accumulated over 64 scans, and (b) over 16 scans, and the Y axes have been appropriately adjusted.

in the Experimental section. The difference between the two spectra is shown in Fig. 5(c). The increase in intensity of the P_i signal corresponds to a formation of 2.25 mmol of $\text{P}_i \cdot \text{kg}^{-1}$; i.e. to a formation of $0.75 \text{ mmol} \cdot \text{kg}^{-1}$ per s of contraction (see below for a validation of this figure). This is therefore the average rate of hydrolysis of high-energy phosphate during these contractions. Earlier studies of repetitively stimulated anaerobic frog gastrocnemii lead us to expect the formation of about 1.0 mmol of $\text{P}_i \cdot \text{kg}^{-1}$ per s of contraction for the first four 3-s contractions [calculated from Fig. 4(b) and eqn. (12) of Dawson *et al.*, 1978). The value obtained in the present experiments is a little lower than expected, but is in reasonably good agreement with the earlier results.

The difference spectrum of Fig. 5(c) also indicates the possibility of a positive ATP γ -phosphate peak. Further improvement in signal-to-noise ratio was obtained by averaging over the first eight contractions for each set of muscles, and by comparing the spectrum obtained during contraction with the spectrum obtained by accumulating signal throughout the period between contractions. These spectra are shown in Fig. 6, and the difference spectrum of Fig. 6(c) reveals a significant increase in the ATP γ -phosphate peak during contraction. The γ -phosphate peak measured between contractions (Fig. 6a) has the same intensity as when measured in resting muscles prior to stimulation (Fig. 4a). Since the concentration of ATP remains unchanged throughout the experiment, we can conclude that saturation transfer from *P*-creatine to ATP results in the same decrease in the ATP γ -phosphate peak between contractions as is observed in the resting muscle prior to stimulation, i.e. 30% (Table 1). We can then conclude, from the spectra of Figs. 4 and 6, that saturation transfer results in a 17% decrease in intensity of the γ -phosphate signal during contraction as opposed to a 30% decrease in the quiescent muscle.

The difference between the figures of 17% and 30% for the decrease in intensity of the ATP γ -phosphate peak must reflect a change in the total rate at which ATP reacts, or a change in the T_1 of the ATP γ -phosphate peak, or both. An estimate of the T_1 of ATP during contraction was obtained by accumulating spectra during and between contractions by using pulse intervals of 750 ms (see the Experimental section). Comparison of the spectra obtained during contraction with those obtained between contractions revealed distinct changes in the intensities of the ATP signals, but far smaller percentage changes in the *P*-creatine and P_i signals. It is reasonable to interpret these changes in terms of a decrease in the T_1 values of the ATP resonances during contraction. From the T_1 measured before contraction, it is possible to estimate the T_1 values

Fig. 6. ^{31}P n.m.r. spectra of muscles obtained (a) throughout the period between contractions, and (b) at the end of contraction, just as stimulation to the muscles is terminated

Both spectra were accumulated in the presence of selective irradiation of the *P*-creatine resonance. Spectrum (c) is the difference spectrum obtained by subtracting (a) from (b). The spectra represent the summed data from four sets of muscles, and from the first eight contractions undergone by each set of muscles. (a) was accumulated over 512 scans, and (b) over 32 scans, and the Y axes have been appropriately adjusted.

for the ATP resonances during contraction. It was found that the T_1 of the ATP γ -phosphate peak is approx. 800 ms during contraction; this change in T_1 is sufficient to explain the decrease in transfer of saturation from *P*-creatine to ATP that is observed during contraction. Therefore, by the analysis shown in the Appendix, the total rate at which ATP reacts remains apparently the same during and between contractions.

The total rate at which ATP reacts is the sum of the backward reaction flux ($\text{ATP} \rightarrow \text{P-creatine}$) plus the rate of ATP hydrolysis to ADP and P_i . Between contractions the rate of ATP hydrolysis is negligible and therefore the total rate of reaction of ATP is equal to the forward reaction flux, which we have shown to be about $1.6 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$. During contraction, the rate of ATP hydrolysis was found to be $0.75 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$, and therefore the rate of conversion of ATP to *P*-creatine (the reverse reaction flux) must be $1.6 - 0.75 = 0.85 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$.

An estimate of the forward reaction flux during contraction can also be obtained from these data.

We know, both from control experiments in which no selective irradiation was applied, and from extensive studies of amphibian muscle (see the Discussion) that the concentration of ATP does not significantly change during contraction. Since ATP is lost at a rate of $1.6 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$ during contraction it must also be formed at this rate. Thus the forward reaction flux remains very similar during contraction to its value in resting muscle.

The forward reaction flux during contraction was estimated experimentally by comparing saturation transfer from ATP to *P*-creatine between and during contractions in the presence of selective irradiation of the ATP γ -phosphate signal, as described in the Experimental section. Quantitative interpretation of these results is difficult for three reasons. Firstly, the concentration of *P*-creatine is itself changing during contraction, and therefore intensity differences result from this, in addition to any possible changes in reaction rates. Secondly, because of the relatively long T_1 of the *P*-creatine resonance, if there are changes in saturation transfer resulting from contraction, it takes longer than 3 s for the new steady state of magnetization to be reached. Thirdly, the pseudo-first order kinetics scheme used to calculate the reaction fluxes (see Appendix) is not totally valid during contraction.

Nevertheless, our data suggest that any change in saturation transfer from ATP to *P*-creatine that may occur during contraction is small. Our T_1 data indicate that the T_1 of the *P*-creatine does not change significantly during contraction (and there is no reason why it should change significantly). Therefore, we conclude that the forward rate of the creatine kinase reaction remains approximately the same during contraction as between contractions; i.e. the rate is about $1.6 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$. Our data are internally consistent in that the rates we obtain are compatible with the experimental observation that the ATP concentration remains unchanged during contraction.

Biochemical changes and reaction rates between 3 s contractions

During each 3 s contraction, about 2.25 of mmol *P*-creatine $\cdot \text{kg}^{-1} \cdot \text{s}^{-1}$ is broken down. The glycolytic recovery following each contraction is rapid [almost all reaction occurs within the first minute following contraction (Dawson *et al.*, 1980b)], but is incomplete. In the present experiments, only approx. 40% of the *P*-creatine that is broken down during each contraction is subsequently regenerated. As a result, the metabolic state of the muscle alters continuously, and it is for this reason that most of our conclusions are derived from only the first four or eight contractions of each set of muscles.

The pH of the muscles, as determined from the chemical shift of the P_i signal, is initially 7.5, remains

at this value over the first few contractions, but by the end of 16 contractions falls to about 7.1. The *P*-creatine concentration, initially $25 \text{ mmol} \cdot \text{kg}^{-1}$, falls to less than $5 \text{ mmol} \cdot \text{kg}^{-1}$ after 16 contractions. As indicated in the Discussion, between contractions the concentration of free ADP can be calculated from the creatine kinase equilibrium. We deduce that before the first contraction the concentration of free ADP is about $20 \mu\text{mol} \cdot \text{kg}^{-1}$; this increases progressively throughout the course of each experiment, and is approximately $50 \mu\text{mol} \cdot \text{kg}^{-1}$ after four contractions, $100 \mu\text{mol} \cdot \text{kg}^{-1}$ after eight contractions, and $180 \mu\text{mol} \cdot \text{kg}^{-1}$ after 16 contractions.

The intensity of the γ -ATP peak measured between contractions (with selective irradiation applied to the *P*-creatine signal) remains unchanged throughout the full course of at least 16 contractions undergone by each set of muscles. Since the concentration of ATP does not significantly fall until the *P*-creatine is almost totally depleted, we can conclude that the extent of saturation transfer to the ATP γ -phosphate peak from *P*-creatine between contractions remains approximately unchanged throughout the course of the experiment. We can then conclude that the rate of the creatine kinase reaction remains approximately constant as the concentrations of its reactants gradually alter. In further experiments on two of the sets of muscles, we measured saturation transfer in the reverse direction (from ATP to *P*-creatine), not only before the first stimulation, but also during rest periods between sets of stimulations. For one set of muscles, we found that the rate of reaction was initially $1.8 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$, and remained at about $1.8 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$ when the *P*-creatine concentration has declined to half its initial value. For the second set, the initial rate was $1.9 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$, and went up to $2.7 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$ when the *P*-creatine concentration was $18 \text{ mmol} \cdot \text{kg}^{-1}$ and $2.9 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$ when the *P*-creatine concentration was $2.9 \text{ mmol} \cdot \text{kg}^{-1}$. These results, when considered together, indicate that there is little increase in the rate of the creatine kinase reaction as the metabolic state of the muscle changes. This is of interest because the ADP concentration increases by a very large percentage of its initial value.

Transfer of saturation to P_i

It could be expected that the signal from the P_i that is formed during contraction should be decreased because the P_i is derived from the γ -phosphate of ATP, the signal of which is partially saturated in our experiments. However, the increase in area of the P_i peak was observed to be very similar, regardless of whether the ATP γ -phosphate peak was fully saturated or only partially (17%) saturated. This suggests that very little of the

saturation of the ATP γ -phosphate peak is in fact transferred to the P_i , and therefore that the figure of $2.25 \text{ mmol} \cdot \text{kg}^{-1}$ that we obtain for the formation of P_i during each contraction is correct.

It is interesting to postulate that the transfer of saturation to the P_i is small because there is an intermediate in the hydrolysis of ATP in which the resonance from the ATP γ -phosphate has a short T_1 . One possibility could be that in the course of hydrolysis of ATP, the ATP bound to the actomyosin is considerably distorted. The consequent increase in chemical shift anisotropy might considerably decrease the T_1 of the ATP γ -phosphate resonance, whose saturation would thus disappear before the P_i is formed. Such a mechanism might also account for the decrease in T_1 of the ATP γ -phosphate peak that is observed during contraction.

Possible saturation transfer involving phosphodiester

It must be considered that errors might arise in estimating the forward rate constant due to the presence of the phosphodiester peaks at $+2.5 \text{ p.p.m.}$ from the *P*-creatine peak, where saturating radiation must be applied to control for possibly imperfect selectivity in irradiating the γ -phosphorus of ATP at -2.5 p.p.m. Significant transfer of saturation from these phosphodiesters, whose function in muscle is unknown, to ATP and from ATP to *P*-creatine would affect the intensity of the *P*-creatine peak in the difference spectrum of Fig. 3(d) which is used in calculation of the forward rate constant. However, no difference is observed in the ATP peaks when the spectrum in Fig. 3(a) (in which no saturating irradiation is applied) is compared with that in Fig. 3(c) (irradiated at $+2.5 \text{ p.p.m.}$) indicating that no demonstrable transfer of saturation can occur by this route.

Discussion

Recent studies *in vitro* support the view that the mechanism of the creatine kinase reaction is probably of the random rapid-equilibrium type with two abortive complexes (see Watts, 1973 for a discussion of the literature and Opella & Lu, 1979, for recent n.m.r. contributions). Unfortunately there seem to be no comparable studies which involve the low concentration of free ADP that exists *in vivo*. Nevertheless, the above mechanism provides the best available basis for the analysis of the present results together with the results of n.m.r. studies *in vitro* on rabbit creatine kinase (J. Bland, E. M. Chance & G. K. Radda, unpublished results). However, this quantitative theoretical analysis is a project of major proportions and would be beyond the bounds of the present paper. Therefore, for the

time being, our results will be analysed only in so far as they relate to the following questions:

1. Is the creatine kinase system at equilibrium in resting muscle?
2. If so, what are the equal and opposite exchange fluxes?
3. Are these fluxes compatible with the fact that [ATP] does not fall detectably during contraction save in extreme fatigue?

Equilibrium of the creatine kinase reaction in resting muscle

In resting frog muscle at 4°C the metabolic rate is very low (about 0.03% of that during activity). There are many reasons for believing that reaction (3) is then at equilibrium (see, e.g., Carlson & Wilkie, 1974), and the present n.m.r. studies provide direct evidence that this is indeed the case. The concentration of *P*-creatine remains constant for many hours; this can occur only if (a) it is metabolically or spatially isolated, or (b) is a part of a metabolic chain in a steady-state, or (c) is in equilibrium with another substance whose concentration remains constant. *P*-Creatine cannot be in a steady-state because only one enzyme in the muscle, creatine kinase, will accept it as a substrate (see Needham, 1971). The present n.m.r. studies show that in the resting muscle the enzyme is turning over between one-quarter and one-half of the terminal phosphate of the total ATP per second; therefore *P*-creatine is not isolated. Thus possibilities (a) and (b) are eliminated and only possibility (c), that the creatine kinase system is in equilibrium, remains. It is thus possible to calculate the free ADP concentration from the numerical values given in Table 2. The difference between this value and the total ADP concentration (about 450 μM) is consistent with the amount of ADP bound to actin and myosin (see p. 173 in Ferenczi *et al.*, 1978).

$$[\text{ADP}] = \frac{[\text{ATP}] \times [\text{creatine}]}{K \times [\text{P-creatine}]} = 2.04 \times 10^{-5} \text{ M} \quad (3)$$

The value of *K* used for the above calculation is derived from the experiments of Noda *et al.* (1954), which indicate that:

$$\frac{[\text{ATP}] \times [\text{creatine}]}{[\text{ADP}] \times [\text{P-creatine}] \times [\text{H}^+]} \approx 2 \times 10^9 \text{ M}^{-1} \text{ at } 25^\circ \text{C} \quad (4)$$

In estimating *K* it is necessary to make due allowance for the pH and for the Gibbs–Helmholtz correction to 4°C. *H* for this reaction is given as +14 kJ·mol⁻¹ by Curtin & Woledge (1978, see p. 722) and this value has been used directly in the temperature correction, though there are reasons, arising from deficiency in present knowledge about the control of intracellular pH, for supposing that this may lead to substantial over-correction (R. C.

Table 2. *Experimental information used in computer modelling*

The values of *F*₁, *F*₂, *F*₁', and *F*₂' are obtained from our saturation transfer experiments. *F*_c is the net flux arising from hydrolysis of ATP in the actomyosin ATPase system measured by the changes in area of the *P*_i resonance. The concentrations and pH values are typical under these experimental conditions (see Dawson *et al.*, 1978) and were obtained as indicated in the Experimental section.

At rest, before contraction	
[ATP]	4.2 × 10 ⁻³ M
[P-creatine]	25 × 10 ⁻³ M
[Creatine]	5.0 × 10 ⁻³ M
pH	7.5
<i>K</i>	$\frac{[\text{ATP}][\text{Creatine}]}{[\text{P-Creatine}][\text{ADP}]} = 41.20 \text{ at } 4^\circ \text{C and pH } 7.5$
<i>F</i> ₁	1.6 × 10 ⁻³ M·s ⁻¹
<i>F</i> ₂	1.6 × 10 ⁻³ M·s ⁻¹
<i>F</i> _c	0
During contraction	
<i>F</i> ₁ '	1.6 × 10 ⁻³ M·s ⁻¹
<i>F</i> ₂ '	0.85 × 10 ⁻³ M·s ⁻¹
<i>F</i> _c	0.75 × 10 ⁻³ M·s ⁻¹

Woledge, personal communication). In any case, the latest equilibrium measurements on creatine kinase, by Lawson & Veech (1979) indicate that the quotient in eqn. (4) should be 1.66 × 10⁹ M⁻¹ (at 38°C) rather than 2 × 10⁹ M⁻¹. Having regard to these unavoidable uncertainties, the estimate given in our Table 2 seems to be as good as can be made on the evidence currently available.

Constancy of [ATP] during contraction

The fact that no detectable decrease in [ATP] can be found during contraction was first demonstrated by Gilbert *et al.* (1971) using ultra-quick freezing (time uncertainty 60 ms), followed by chemical analysis; it has been frequently confirmed since (e.g. p. 359 of Curtin & Woledge, 1979). 'Detectable' in this context means about 2–3% of the ATP present, since the standard error is about 1% in sets of 80 determinations. A similar conclusion was drawn from ³¹P n.m.r. experiments on contracting muscles (Fig. 8 of Dawson *et al.*, 1977); in these experiments the precision was less but the time-resolution is, in some respects, better. The 'snapshot' taken of the muscle has an exposure of the order of *T*₂, about 10 ms.

It is an important test of our results that they should be compatible with these well-established facts. For the purposes of this test the reaction scheme shown below will be used as the simplest way to illustrate how our exchange flux measure-

ments relate to the concentrations of reactants during contraction. The conclusions reached on the basis of this simplified scheme are entirely consistent with those of the more complete analysis of the kinetics and mechanism of the creatine kinase reaction that will be published later.

The reaction catalysed by creatine kinase may be considered as being bireactant and expressed as

if it is borne in mind that the rate constants, k_1 and k_2 , incorporate information about the pH (effectively constant at 7.5 at the beginning of each experiment), and the effect of binding of Mg^{2+} to ATP and ADP. We have shown earlier the [free Mg] in frog muscle is large and effectively constant as far as reaction (5) is concerned, except in extreme fatigue (Dawson *et al.*, 1978). Q is a dimensionless variable that represents the deviation of the reaction from simple second-order kinetics. A single Q for both the forward and reverse exchange fluxes was found to be sufficient to account for our observations; Q is found to vary from the resting to the active muscle and to decrease as the experiment proceeds. The biochemical reasons underlying this variation will be described.

Saturation transfer measured during contraction

If the value of Q remained unchanged during contraction, both F_1 and F_2 would rise as a result of increases in the concentrations of reactants in both the forward and backward directions; there is a large relative increase in [ADP] with only a small drop in [P -creatine]; [creatine] increases while [ATP] remains virtually unchanged. In fact, as Table 2 shows, F_1' remains at $1.6 \times 10^{-3} \text{ M} \cdot \text{s}^{-1}$ and F_2' falls to $0.85 \times 10^{-3} \text{ M} \cdot \text{s}^{-1}$. According to the simplified reaction scheme (5), this observation must result from a decrease in Q . Simple algebra cannot predict the time-course of the changes in the metabolites throughout contraction, nor examine the detailed way in which Q varies with time. In order to make such complex calculations, the powerful computer language FACSIMILE (Chance *et al.*, 1977) has been employed.

A typical output is shown in Fig. 7; it is compatible with the fluxes F_1' and F_2' remaining constant throughout contraction at the values given in Table 2. Q has been made to vary throughout contraction in the manner required to maintain the fluxes constant; it falls first rapidly, then progressively to the value of 0.32 times its initial value. After the end of contraction, it rises again rapidly but only to the value to 0.63 times its initial value; the reason for this will be discussed later. The curve of [ADP] shown in Fig. 7 (solid line) can readily be

analysed into two terms, one of which (the broken line in Fig. 7) represents the value $[\text{ADP}]_{\text{eq}}$, that would be found if the system were in equilibrium. The second term is equal to the difference between [ADP] and $[\text{ADP}]_{\text{eq}}$, and it is this additional input of ADP which results from the hydrolysis of ATP by the actomyosin system that drives the reaction forward during contraction.

The rate of hydrolysis of ATP is assumed to be constant throughout contraction, as indicated by the constant rate of production of P_i shown in Fig. 7. The [ATP] diminishes by about 1% during contraction, then recovers rapidly almost to its resting value. The most striking change is in [ADP], which increases substantially in relative terms during contraction, returns rapidly towards its initial value, but ends up more than 50% above it. Under the anaerobic conditions employed in our experiments, recovery is incomplete and reaction products such as ADP accumulate (Dawson *et al.*, 1978). We have shown more recently (Dawson *et al.*, 1980b) that after a wide variety of patterns of stimulation the recovery of P -creatine that results from the formation of lactic acid is rapid, ending after about 1 min, but incomplete. Thus in successive contractions, creatine, ADP, (and H^+) progressively accumulate in the muscle. The raised [ADP] that is seen at the right-hand side of Fig. 7 is in consequence only partially returned towards its original value during the brief recovery period.

After the brief recovery period mentioned above, the concentrations of metabolites remain unchanged until the muscle is stimulated again, even though this may not be for 20 or 30 min. The saturation-transfer measurements during the quiescent period (see the Results section) show that, although there may be a small increase in the fluxes, Q remains depressed and is similar to its value immediately after contraction.

Conclusions

The forward and backward fluxes measured by saturation transfer are consistent with the assumption that the creatine kinase reaction is in equilibrium in resting muscle. We estimate that these equal and opposite fluxes are about $1.6 \text{ mM} \cdot \text{s}^{-1}$, that is about twice the rate of ATP hydrolysis during contraction. In contracting muscle the forward flux remained the same as at rest whereas the backward flux decreased to about half its resting value. Nevertheless, computer analysis (Fig. 7) shows that the measured fluxes are large enough to maintain [ATP] virtually constant, in agreement with previous well-established observations.

The findings that the forward flux did not increase during contraction and that the backward flux decreased is reflected by the decrease in the value of Q that appears in our eqn. (5). In general terms, this could be explained either by an allosteric inhibition

Fig. 7. Result of a calculation using FACSIMILE of the changes resulting from a 3 s contraction

Left ordinate: $[ADP]$ (μM) and Q [see eqn. (5)]. For convenience of labelling, Q is here expressed as a percentage of its initial value. Right ordinate: $[ATP]$ and $[P_i]$, (both mM). Abscissae: time (s); the muscle was contracting from 0–3 s. The calculation was made in such a way that the exchange fluxes at rest and during contraction correspond with the experimental values shown in Table 2. It has been assumed that the rate of hydrolysis of ATP and equal production of P_i are constant throughout contraction (see Gilbert *et al.*, 1971). The concentration of ATP hardly changes at any time, whereas $[ADP]$ alters markedly. The difference between the lines for $[ADP]$ and $[ADP]_{eq.}$ shows how much extra $[ADP]$ is needed to drive the creatine kinase reaction from left to right. The significance of Q is explained in the text.

of creatine kinase in active muscle or by competition of reactants for a limited amount of free enzyme. We know of no evidence for allosteric control of this enzyme and such a mechanism for inhibiting creatine kinase during contraction when its highest rates of activity are required would not make sense from a physiological point of view. The limited amount of total enzyme does make sense in relation to conservation of solvent capacity (see Atkinson, 1977). It is entirely plausible that a decrease in the value of Q during contraction should arise from a redistribution in the proportion of enzyme in its various bound forms. This would result from the observed concentration changes, particularly the large relative increase in $[ADP]$. Such a hypothesis fits well with current knowledge of creatine kinase, the postulated random-rapid equilibrium mechanism, and the occurrence of the abortive complex, enzyme-ADP-creatine (Morrison & James, 1965; Watts, 1973). Moreover, the fact that Q remains low in the inactive fatigued muscle is in keeping with this hypothesis. Thus Q is a variable that depends on the enzyme mechanism and on the relative concentrations of free enzyme, enzyme-reactant and abortive complexes. These relationships are emerging from the studies currently being carried out using

the FACSIMILE language in conjunction with n.m.r. experiments *in vitro*. Therefore the combination of these methods yields the details of the enzymology of creatine kinase *in vivo*.

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APPENDIX

In this section, we shall summarize the theory underlying our experiments. We should stress that the theoretical basis behind our studies is not new; it was thoroughly covered in a series of much earlier articles by Forsen & Hoffman (1963). However, it is appropriate to describe here those aspects of saturation transfer n.m.r. that are relevant to our experiments, if only to underline the possible pitfalls in experiments of this type.

Resting muscles

The reaction under consideration is:

If the metabolite levels are in a steady state, we can express:

$$k_{\text{rev.}} = k_2 [\text{creatine}] \quad (2)$$

$$k_{\text{for.}} = k_1 [\text{ADP}][\text{H}^+] \quad (3)$$

where $k_{\text{rev.}}$ and $k_{\text{for.}}$ are pseudo-first order rate constants which are applicable to the steady state. Therefore:

$$\frac{d[P\text{-creatine}]}{dt} = k_{\text{rev.}}[\text{ATP}] - k_{\text{for.}}[P\text{-creatine}] \quad (4)$$

Similarly:

$$\frac{d[\text{ATP}]}{dt} = k_{\text{for.}}[P\text{-creatine}] - k_{\text{rev.}}[\text{ATP}] \quad (5)$$

The modified Bloch equations that take into account this chemical exchange are:

$$\frac{dM_P}{dt} = \frac{M_{OP} - M_P}{T_{1P}} - k_{\text{for.}}M_P + k_{\text{rev.}}M_A \quad (7)$$

where M_{OP} is the equilibrium *P*-creatine magnetization that would exist in the absence of exchange, and M_P is the actual *P*-creatine magnetization at any given time. M_{OA} and M_A are the corresponding magnetizations for the γ -phosphate of ATP. T_{1P} and T_{1A} are the inherent T_1 values of the *P*-creatine and ATP that would be observed if no exchange took place.

Let us consider transfer of saturation to *P*-creatine. If the ATP γ -phosphate signal is selectively saturated, $M_A = 0$, and so:

$$\frac{dM_P}{dt} = \frac{M_{OP} - M_P}{T_{1P}} - k_{\text{for.}}M_P \quad (8)$$

Therefore, if the *P*-creatine magnetization is in a steady state ($dM_P/dt = 0$) we find:

$$M_P = \frac{M_{OP}}{1 + k_{\text{for.}}T_{1P}} \quad (9)$$

i.e. the magnetization of the *P*-creatine is decreased by a factor $(1 + k_{\text{for.}} T_{1P})$ as a result of selective irradiation of the ATP γ -phosphate signal. This decrease arises from transfer of saturation, and is the parameter measured in our experiments.

If the *P*-creatine magnetization does not reach a steady state, we must use the full solution to eqn. (8). If $M_P = 0$ at time $t = 0$, the solution is:

$$M_P = \frac{M_{OP}}{1 + k_{\text{for.}} T_{1P}} \left[1 - \exp\left(-\left(\frac{1}{T_{1P}} + k_{\text{for.}}\right)t\right) \right] \quad (10)$$

i.e. following a 90° pulse, the magnetization tends to its steady state value

$$\frac{M_{OP}}{1 + k_{\text{for.}} T_{1P}}$$

with a time constant T_{1S} given by

$$\frac{1}{T_{1S}} = \left(\frac{1}{T_{1P}} + k_{\text{for.}} \right).$$

Clearly, the relationship between M_P and M_{OP} in the absence of a steady state is dependent not only upon $k_{\text{for.}}$ and T_{1P} , but also upon pulse angle and pulse interval. For this reason, we decided to ensure that M_P had acquired a steady state before application of each radio-frequency pulse, and therefore we applied pulses at intervals of 12 s, rather than at intervals of 0.5–2 s as is normally done to achieve optimum signal-to-noise ratios. When pulses are applied every 12 s, it is totally justifiable to use the steady state relationship shown in eqn. (9).

It is then necessary to determine T_{1P} in order to establish a value for $k_{\text{for.}}$. The problem here is that T_{1P} is the T_1 value for *P*-creatine that would be observed if there were no chemical exchange with the ATP. However, the process of exchange tends to average the T_1 values of the *P*-creatine and ATP γ -phosphate resonance; thus the measured T_1 values of the exchanging nuclei are closer in magnitude than the inherent values the two nuclei would have in the absence of exchange.

The magnetization that should be observed in a progressive saturation experiment is obtained from the theory of Forsen & Hoffman (1963), and is given, for the conditions of our T_1 measurements, by the following equations:

$$M_P = M_{OP} + C_1 e^{-\lambda_1 t} + C_2 e^{-\lambda_2 t} \quad (11)$$

$$M_A = M_{OA} + C_1 \left[\frac{1}{T_{1P}} + k_{\text{for.}} - \lambda_1 \right] [k_{\text{rev.}}]^{-1} e^{-\lambda_1 t} + C_2 \left[\frac{1}{T_{1P}} + k_{\text{for.}} - \lambda_2 \right] [k_{\text{rev.}}]^{-1} e^{-\lambda_2 t} \quad (12)$$

where λ_1 and λ_2 are given by the two roots to the quadratic equation:

$$\lambda^2 - \lambda \left[\frac{1}{T_{1P}} + \frac{1}{T_{1A}} + k_{\text{for.}} + k_{\text{rev.}} \right] + \frac{1}{T_{1P}} \frac{1}{T_{1A}} + \frac{k_{\text{for.}}}{T_{1A}} + \frac{k_{\text{rev.}}}{T_{1P}} = 0 \quad (13)$$

and

$$C_1 = \frac{M_{OP}(1 - \lambda_2 T_{1P})}{(\lambda_1 - \lambda_2) T_{1P}} \quad (14)$$

$$C_2 = \frac{M_{OP}(1 - \lambda_1 T_{1P})}{(\lambda_1 - \lambda_2) T_{1P}} \quad (15)$$

These expressions can be used to determine $k_{\text{for.}}$, $k_{\text{rev.}}$, T_{1P} and T_{1A} by an iterative procedure from our experimental observations.

There is an alternative, perhaps simpler, method for determining these parameters. This is to measure the T_1 values in the presence of selective irradiation. From eqn. (10) the relaxation time T_{1S} of the *P*-creatine resonance in the presence of selective irradiation of the ATP γ -phosphate peak is given by $1/T_{1S} = 1/T_{1P} + k_{\text{for.}}$. This parameter, together with the value of $(1 + k_{\text{for.}} T_{1P})$ measured by saturation transfer, permits both T_{1P} and $k_{\text{for.}}$ to be measured. It was not found necessary to use this method in our experiments because the exchange rates were sufficiently slow to ensure that the observed and inherent T_1 values were fairly similar.

The equations for saturation transfer to the ATP γ -phosphate resonance are totally analogous to those for saturation transfer to *P*-creatine that are given above.

Effects of contraction

During contraction, we may write the reaction as

The modified Bloch equation for ATP is now:

$$\frac{dM_A}{dt} = \frac{M_{OA} - M_A}{T_{1A}} - k_{\text{rev.}} M_A - k_c M_A + k_{\text{for.}} M_P \quad (17)$$

If we saturate the *P*-creatine resonance, $M_P = 0$, and in the steady state we find:

$$M_A = \frac{M_{OA}}{1 + (k_{\text{rev.}} + k_c) T_{1A}} \quad (18)$$

In comparing this expression with the situation in

resting muscle, we find that it is simply necessary to replace $k_{\text{rev.}}$ by $(k_{\text{rev.}} + k_c)$, which is the pseudo-first order rate constant representing the total rate at which ATP reacts. A problem is that the pseudo-first order rate constant is a constant only if both the magnetization and the chemical exchange are in a steady state. As shown in Fig. 7, the creatine kinase reaction is not in a steady state when the data are collected at the end of each 3 s contraction.

The question also arises as to whether 3 s is

sufficient for the magnetization to achieve a new steady state. The new steady state for ATP magnetization is achieved with a rate constant of $(1/T_{1A} + k_{\text{rev.}} + k_c)$ and under the conditions of our experiments is very nearly reached in 3 s. This is not the case for *P*-creatine, and for this and the other reasons described in the text, we feel that we can provide only a very approximate estimate of the forward rate of the creatine kinase reaction during contraction.