PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Studies of the Biochemistry of Contracting and Relaxing Muscle by the Use of <latex>^{31}</latex>P n.m.r. in Conjunction with Other Techniques

M. Joan Dawson, D. G. Gadian and D. R. Wilkie

Phil. Trans. R. Soc. Lond. B 1980 **289**, 445-455
doi: 10.1098/rstb.1980.0062

Phil. Trans. R. Soc. Lond. B **289**, 445–455 (1980) [445]
Printed in Great Britain

Studies of the biochemistry of contracting and relaxing muscle by the use of ^{31}P n.m.r. in conjunction with other techniques

By M. Joan Dawson, D. G. Gadian† and D. R. Wilkie, F.R.S.

Department of Physiology, University College London, London, U.K.
† *Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QR, U.K.*

When n.m.r. is applied to suitably chosen biological problems it yields a wealth of fundamental information unmatched by any other technique. By means of ^{31}P n.m.r. we have studied intact living muscle at rest, during contraction and during recovery from contraction. Phosphocreatine, ATP, inorganic phosphate, phosphorylated intermediaries of glycolysis, pH and the binding of Mg^{2+} to ATP are observed directly in the spectra. From the spectra can be calculated the concentration of free ADP, the free energy change for ATP hydrolysis, the production of lactic acid and the total ATP turnover. Changes in these quantities can thus be followed continuously *in vivo* and we have shown how they are related to the decline in force development and to the slowing of relaxation that occur during fatigue. Similar methods have been applied to study the control of glycolysis.

Almost all the work that we shall be describing is a result of collaboration between M.J.D. and D.R.W. at University College London (U.C.L.) and D.G.G. of the Department of Biochemistry, Oxford; we have made use of the unique n.m.r. facilities at Oxford. The development and testing of the physiological techniques was carried out, except in emergencies, at U.C.L.

We first began work together early in 1975 and, by the middle of that year, as a result of preliminary experiments, had decided that ^{31}P n.m.r. was a serious technique in the sense that one should no longer be astonished simply to *see* spectra from a living tissue, but that n.m.r., applied to appropriate problems in physiology and biochemistry, offered substantial advantages over conventional techniques.

Experimental methods

Accordingly, we set to work to solve the many problems, physiological and physical, involved in keeping muscles alive within the spectrometer for many hours, in stimulating them electrically and in recording their force development. We have worked with frog and toad muscles, partly from familiarity, but mainly because these function perfectly well at low temperature (we chose 4 °C). At low temperature, all the processes in the muscle are slowed down, which has the physical advantage of permitting us to average more scans and thus to achieve a better ratio of signal to noise, and the physiological advantage of facilitating oxygen supply by diffusion, since the oxygen requirement is considerably reduced.

In a brief article I must skip rather rapidly over many of the results that we have already published (see Dawson *et al.* 1977, 1978), since I wish to report also some of our new results and conclusions.

To give a concrete impression of what we did, I shall start by illustrating the muscle chamber

that we evolved to fit into the 7.5 mm diameter working space within the 7.5 T Oxford spectrometer (figure 1).

With it we were able to obtain the spectra of resting muscles, shown in figure 2, both panels of which show oxygenated muscles that survived without any deterioration over the 6 h period of the experiment.

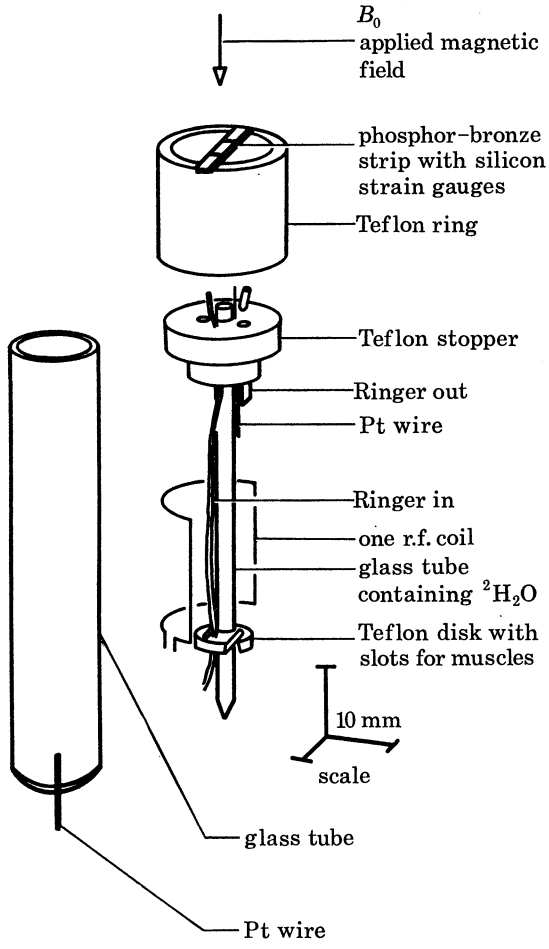

FIGURE 1. Design of experimental chamber. The arrangements for stimulating, recording tension and perfusing Ringer solution are described in Dawson *et al.* (1977). The volume in which the n.m.r. measurement is made is roughly defined by the two single-turn radio frequency (r.f.) coils, only one of which is shown in the diagram. They extend from 3 to 18 mm above the top of the lower Teflon disk. (Reproduced from Dawson *et al.* 1977.)

The spectra (figure 2) look similar to those seen in earlier contributions to this session and their excellent signal:noise ratio permits one to see a great deal of fine detail. In particular, note the very low level of inorganic phosphate (P_i) present; this is seen only under fully oxygenated conditions. The peaks marked (?) in the phosphodiester region present an intriguing problem, since the compounds from which they arise differ greatly in amount between different types of muscle (contrast figure 2*a*, *b*). The functional significance of these compounds has not been established. Perhaps the occurrence of similar peaks in the spectra from intact mitochondria will provide a clue (R. G. Shulman, unpublished).

To establish the validity of n.m.r. as a physiological technique, we went to great trouble to establish absolute calibrations, both for concentrations and for internal pH. The validation of

estimates of concentration is complicated by uncertainty about the volume of the sample tube that is 'seen' by the r.f. coils, and by further uncertainty concerning the fraction of this volume that is occuped by muscle fibres. Even when these difficulties are overcome (see Dawson *et al.* 1977), the comparison with direct chemical analysis must take account of the fact that muscles consist partly of muscle fibres and partly of extracellular space. Nevertheless, the two methods (chemical and n.m.r.) agreed surprisingly well, thus validating both. This was a relief to me as I had previously spent much time perfecting methods for ultra-quick freezing (Kretzschmar & Wilkie 1969) followed by extraction and conventional chemical analysis.

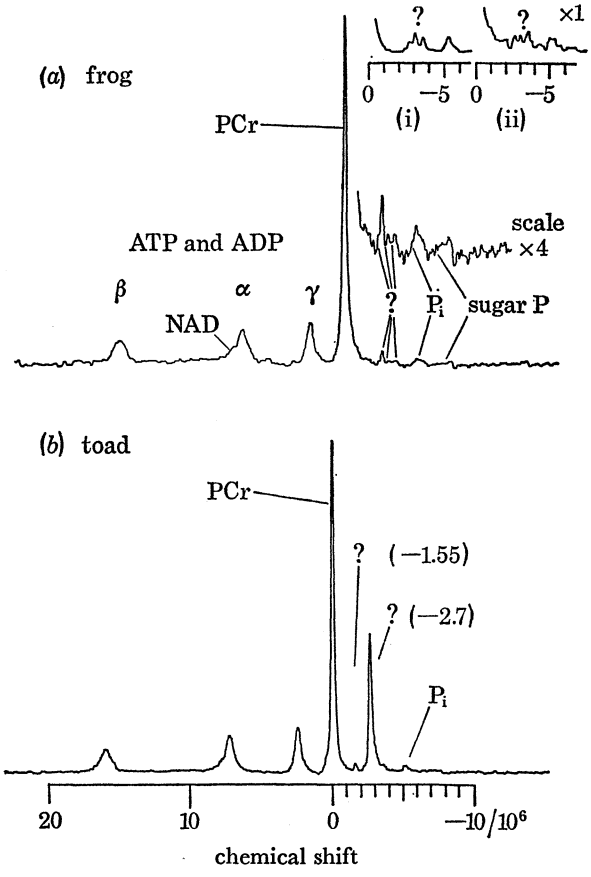

FIGURE 2. Chemical shift has been measured from the PCr peak. The frequency, at the constant field implicit in Fourier transform n.m.r., increases from left to right, the total span representing 5 kHz. (*a*) Four frog sartorii (19 May 1976). Lowest line: average of 10 000 scans at 2 s intervals. The 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 $-3.6/10^6$, only the left hand one is conspicuous. That this is not always the case is shown by the inset figures (i) and (ii), recorded on 16 November 1975 and on 29 April 1976, respectively. On different occasions the peaks differ in size, though not in position. (*b*) Two toad gastrocnemii (20 May 1976). Average of 6000 scans at 4 s intervals. The striking difference is that the peak at $-2.7/10^6$ is huge compared with that in the spectrum from the frog. There is also a small but definite peak at $-1.55/10^6$. (Reproduced from Dawson *et al.* 1977.)

The uncertainty over pH measurements arises from a lingering doubt whether the titration curve *in vivo* truly matches that *in vitro*, even when the latter determination has been matched in ionic strength etc. with the conditions thought to exist within the muscle fibre. Our confidence in the pH calibrations has been increased by recent experiments with use of simultaneous proton and phosphorus n.m.r. D. G. G. and R. Pitkethly have shown, in the aromatic region of

the proton spectrum of muscle, a pH-sensitive complex that resembles carnosine (β-alanyl histidine). If this identification proves correct, the internal pH indicated by this complex agrees exactly with that deduced from the position of the P_i peak from the same muscle. The experiment on minced muscle described by Hollis at this meeting still further validates the use of the P_i peak to measure internal pH.

Early results

Having developed and checked our methods, we then showed, in brief, that muscles could contract many times in the spectrometer and that they would recover completely if oxygen was available.

The most tedious experiment, because only one scan could be obtained per contraction and about 140 scans were needed to achieve a reasonable spectrum, was to examine sartorius muscles during contraction itself. No obvious change was visible in the spectrum. The fact that the area of the γ ATP peak is not visibly diminished during contraction, despite the fact that ATP is being briskly hydrolysed by the contractile proteins, shows that the creatine kinase reaction,

$$H^+ + ADP + \text{phosphocreatine} \rightleftharpoons ATP + \text{creatine},$$

can also run at high speed from left to right, without appreciable diminution of product concentration. This important conclusion had previously been reached, with greater precision but less certainty over timing, by more conventional methods (Gilbert *et al.* 1971; for a review of more recent work confirming the earlier conclusion, see Curtin & Woledge 1978). This experimental result is of vital significance in interpreting cross-saturation experiments such as those discussed by Brown (page 395, this symposium).

Muscular fatigue

Because of the difficulties inherent in oxygenating an active muscle by diffusion from its surface, we decided that an important physiological problem that could profitably be studied by n.m.r. was that of muscular fatigue under anaerobic, that is, oxygen-free, conditions. This permitted us to use large muscles and thus to reduce dramatically the time required to obtain acceptable spectra. As an illustration, figure 3 shows the spectrum that can now be obtained in a single scan, using 1.5 g of muscle in the new Oxford wide-bore spectrometer.

Anaerobic conditions also confer the biochemical advantage that the muscle is a thermodynamically closed system (i.e. one that exchanges energy, but not matter, with its surroundings). This simplifies considerably the interpretation of the chemical results.

Figure 4 shows the result of a typical experiment in which frog gastrocnemii were stimulated repeatedly for 5 s every 5 min. The records of force developed are shown on the right hand side. As is well known, the force progressively diminishes and relaxation becomes progressively slower. The last phase of relaxation is reasonably exponential and its time constant, τ (or the rate constant, $1/\tau$), is surprisingly unaltered by quite large mechanical interventions. This led Jewell & Wilkie (1960) to suggest that it reflected some underlying biochemical process. The corresponding changes in the spectra are shown on the left. Phosphocreatine (PCr) breaks down and P_i accumulates; only in extreme fatigue are the ATP peaks affected. The P_i peak also moves to the left, indicating the formation of lactic acid. During the course of interpreting our

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

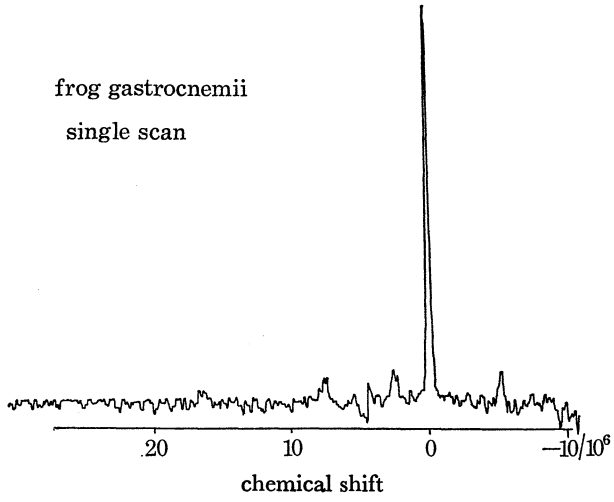

FIGURE 3. The spectrum now obtainable in a single scan from the new Oxford wide bore spectrometer with use of 1.5 g of frog gastrocnemii. This is a 'snapshot' of the muscle over an interval of about 10 ms. The main features of the scan are qualitatively visible, and only a few scans would need to be averaged for most quantitative work.

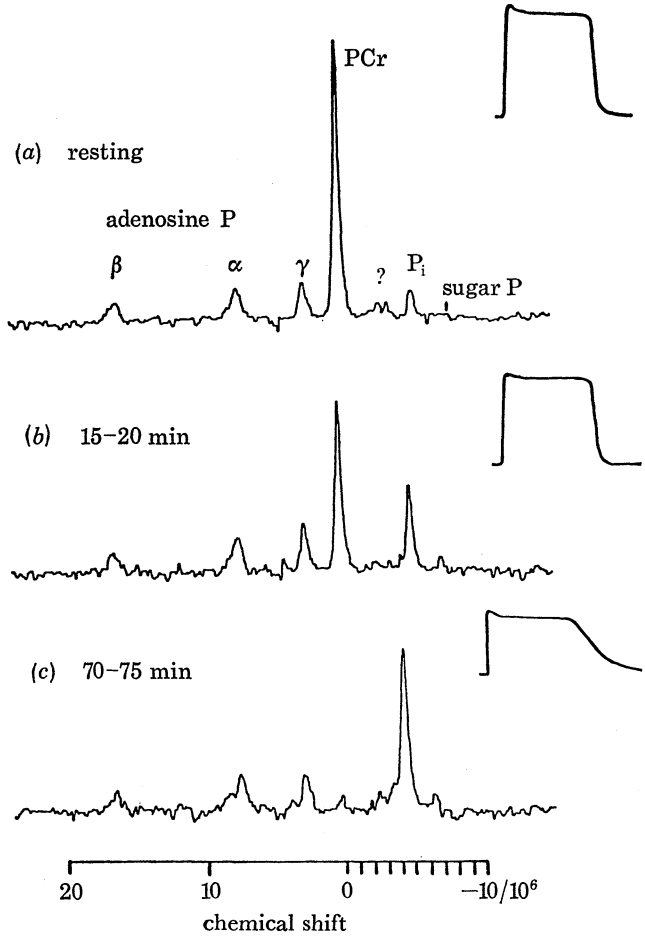

FIGURE 4. The spectra obtained from anaerobic frog gastrocnemii at 4 °C during a fatiguing series of 5 s contractions repeated every 5 min. On the right are shown mechanical records of the time course of isometric force development; note how force progressively declines and relaxation becomes slower. On the left are shown the corresponding ^{31}P n.m.r. spectra. (Reproduced from Dawson *et al.* 1980.)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

results (see Dawson *et al.* 1978) we realized that it was possible to calculate several interesting quantities that cannot be determined directly from the spectra. These included:

(1) The concentration of free ADP (most of the ADP found by chemical analysis is bound either to actin or to myosin).

(2) The free-energy change for ATP hydrolysis. This is important because it is equal to the maximum work theoretically available per mole of ATP split, either for mechanical performance or for active transport of solutes against a concentration gradient.

(3) The amount of lactic acid formed by glycogenolysis. This was calculated from the observed decrease in pH combined with the best available information about the internal buffers of frog muscle.

Recent preliminary experiments by D.G.G. & R. Pitkethly have shown that the production of lactic acid calculated in this way agrees reasonably with that observed directly in simultaneous proton n.m.r. spectra. Knowing the production of lactic acid, one can estimate the amount of ATP regenerated by glycolysis, and, in conjunction with the observed breakdown of PCr (and to a much lesser degree, of ATP), draw up a complete balance sheet for the total ATP breakdown over any specified time interval.

Force development

Our experimental design involved three patterns of stimulation of increasing severity; 5 s/5 min, as shown in figure 4, 1 s/min and 1 s/20 s. Although all the changes occurred much more rapidly in the 1 s/20 s pattern, we found (see Dawson *et al.* 1978, figures 2, 3) that the relation between force development and all the substrate levels was an invariant one which seemed to be exactly the same in all three patterns of stimulation. Moreover, the force bore an interesting and simple relation to the total rate of ATP utilization (see Dawson *et al.* 1978, figure 5).

Relaxation rate

The results of a similar analysis of relaxation rate (Dawson *et al.* 1980) also show clearly that mechanical performance and the chemical status of muscle are very closely linked. Here, too, we found that the relations between relaxation rate constant ($1/\tau$) and all the substrate levels did not depend on the pattern of stimulation. The forms of the relations were quite different from those seen between force and substrate levels, and in several instances appeared to be discontinuous.

The most interesting relation to emerge, because of its apparent simplicity, is that shown in figure 5, between relaxation rate constant and the affinity for ATP hydrolysis ($\mathscr{A} = -\mathrm{d}G/\mathrm{d}\xi$, often mistakenly called $-\Delta G$; see *Quantities, units and symbols*, 1975, p. 16). This is a simple linear relation, a slight extrapolation of which would indicate that relaxation should fail entirely when $\mathscr{A}$ drops to about 40 kJ mol^{-1}.

It is believed, on very good grounds, that the biochemical processes of contraction are switched on by the release of Ca^{2+} from the sarcoplasmic reticulum (s.r., see figure 6) into the much larger volume of the sarcoplasm (sp.), which contains the contractile proteins actin and myosin, and also regulatory proteins, of which the key one for present purposes is troponin C; this has four sites for binding Ca^{2+} (see Endo 1977). The effect of the Ca^{2+} release is to raise the concentration of Ca^{2+} in the sarcoplasm from its resting value of about 10^{-7} M to about 10^{-5} M. This activates the contractile proteins and sets off ATP hydrolysis; the resulting free-energy change can manifest itself as mechanical force and work.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

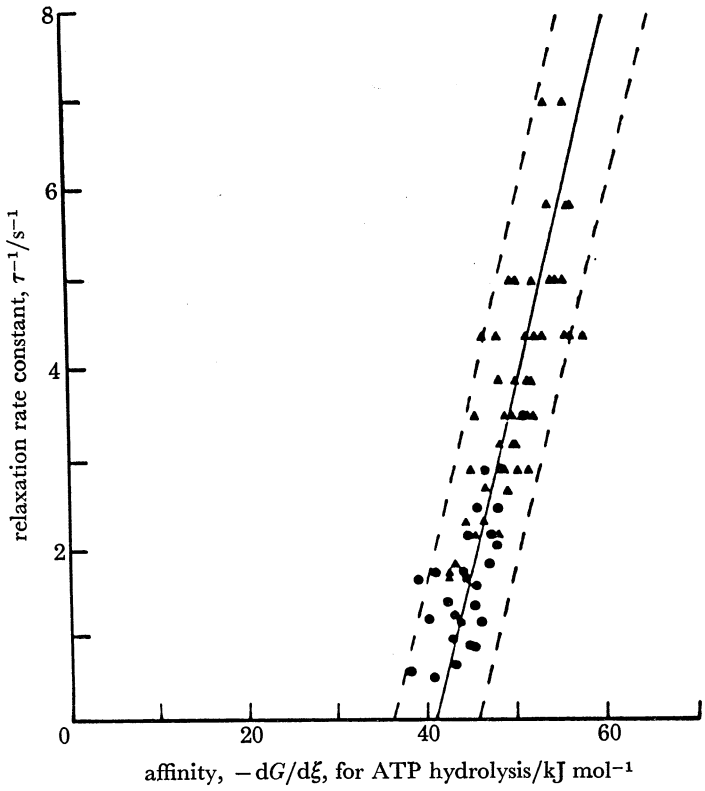

FIGURE 5. The relation between the relaxation rate constant and the affinity (negative free-energy change per mole) for ATP hydrolysis in series of contractions of 1 s/20 s, 1 s/min and 5 s/min. The triangles and circles separate points where the force developed was 40–50 % of its initial value. This is the region where the various discontinuities mentioned in the text occur, and also where PCr ceases to be a significant contributor to the rebuilding of ATP. See also figure 7. The interrupted lines are 95 % confidence limits. (Reproduced from Dawson *et al.* 1980.)

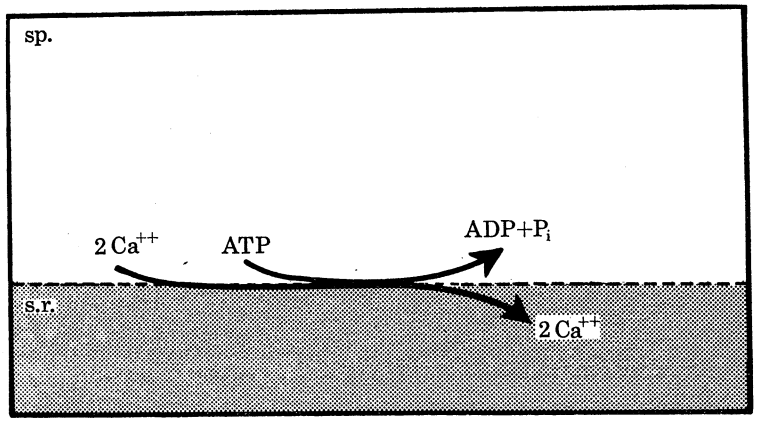

FIGURE 6. Diagram to illustrate in simplified form what is currently believed about the calcium-pumping system that controls contraction and relaxation. The diagram is not to scale; the sarcoplasm (sp.) actually occupies about 90% of the fibre volume, and the sarcoplasmic reticulum (s.r.) only about 10%.

Relaxation is produced by lowering the Ca^{2+} concentration by a process that involves an active transport system for which the fuel is again ATP. Two features of this calcium pump concern us here (see Hasselbach 1974). First, it has been shown that two Ca^{2+} ions are transported for every ATP molecule hydrolysed. Secondly, the pump is reversible both in the

normal sense (ATP can be synthesized from ADP by suitable manipulation of the concentrations of the participating reactants) and in the thermodynamic sense that the pump stops acting when the concentration ratio comes to equilibrium with the chemical process of ATP hydrolysis. This will occur when the affinity for ATP hydrolysis is given by

$$\mathscr{A} = 2RT \ln\{[\mathrm{Ca}]_{\mathrm{s.r.}}/[\mathrm{Ca}]_{\mathrm{sp.}}\}.$$

The concentration of Ca in the s.r. is thought to be held fairly constant at about 5×10^{-4} M by the presence there of calcium buffers. It would thus just become impossible to achieve a resting level of 10^{-7} M in the sarcoplasm when $\mathscr{A} = 39$ kJ mol^{-1}.

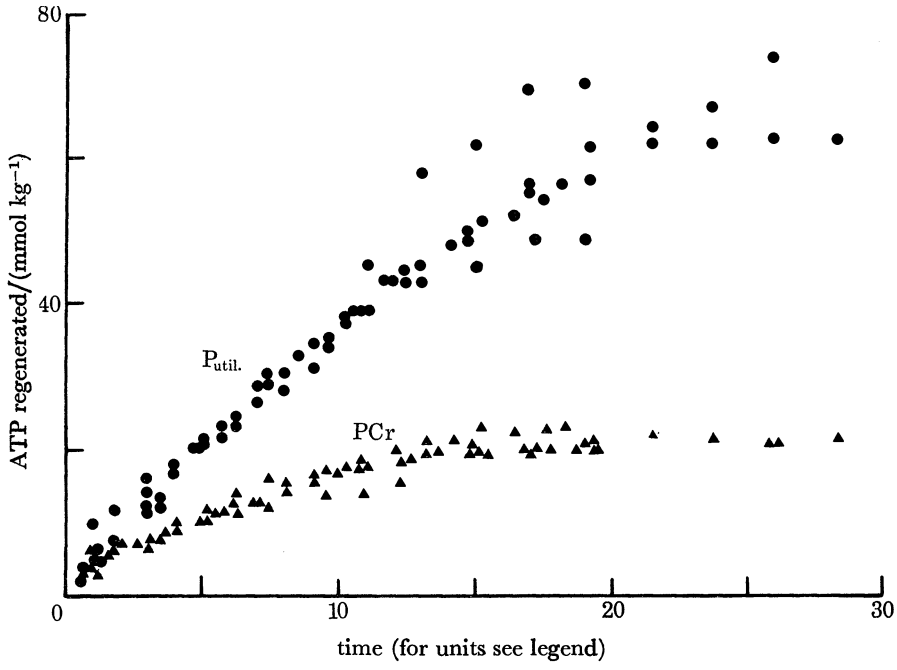

FIGURE 7. The relation between ATP regenerated and time in anaerobic muscles undergoing one of three different patterns of stimulation. One unit on the time scale denotes 1 min when the muscles were stimulated 1 s/20 s, 2.2 min when stimulated 1 s/min and 4.5 min when stimulated 5 s/5 min. Triangles: ATP regenerated from PCr. Circles: total ATP regenerated. The difference between triangles and circles shows ATP regenerated by lactic acid formation.

The closeness of this value to the intercept in figure 5 may be partly fortuitous since there is some uncertainty about the calcium concentrations, but it is, nevertheless, encouraging, and we are trying to find a theory to account for the rest of the linear relation. Here, too, there are problems about the precise role of calcium, notably concerning the relation between force during relaxation and the proportion of troponin sites that have Ca bound to them.

GLYCOLYSIS

The rates at which PCr is hydrolysed and lactic acid is formed are strongly dependent on the pattern of stimulation (see Dawson *et al.* 1978, figure 4). However, this difference is almost completely removed by suitable adjustment of the time scales. As shown in figure 7, at any given stage of fatigue a fixed proportion of the ATP regenerated has come from hydrolysis of PCr and a fixed proportion from lactic acid formation.

This clear connection between stimulation and PCr hydrolysis is perhaps not surprising, since PCr and ATP are so closely interdependent via the creatine phosphokinase equilibrium. The equally clear connection between stimulation and glycolysis must be more indirect since it depends on the activation of at least two enzymes, phosphorylase and phosphofructokinase (PFK). This subject has been much studied and the two main theories that have emerged seem to be:

(1) that the enzymes are activated by the products of ATP hydrolysis, notably AMP, which is formed from ADP by the enzyme (ubiquitous in muscle) adenylate kinase;

(2) that the enzymes are activated by the same agent that activates contraction, that is, a rise in the concentration of Ca^{2+}.

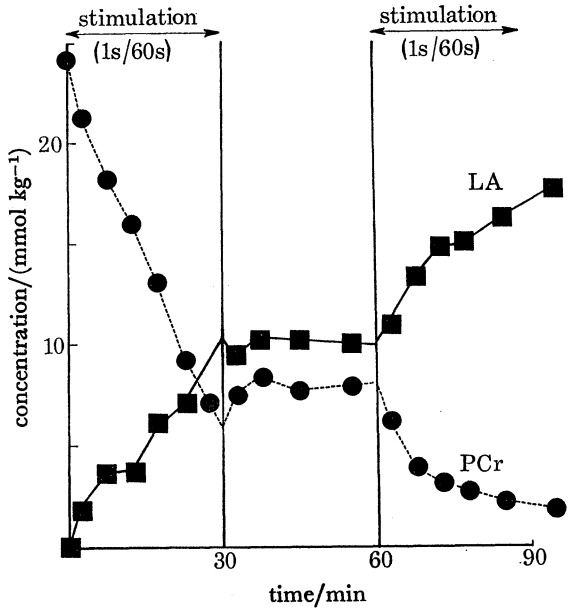

FIGURE 8. The time course of changes in phosphocreatine (PCr, circles) and lactic acid (LA, squares) in anaerobic frog gastrocnemii at 4 °C. The muscles were stimulated (tetanized) for 1 s every minute from 0–30 min and again from 60–90 min.

The experiment shown in figure 8 strongly supports the second theory. For the first 30 min the muscles were stimulated for 1 s/min, exactly as in the intermediate pattern of stimulation described above; this has led to the expected breakdown of PCr (circles) and formation of lactic acid (squares). Stimulation was then switched off. The continuation of glycolysis and the resulting rebuilding of PCr were both small in amount and brief in duration. Thereafter, neither PCr nor lactic acid changed for the rest of the 30 min period, despite the fact that the concentration of ADP, and presumably of AMP, must have been raised. It might be imagined that the glycolytic enzymes had been inactivated by some trivial cause, such as the low pH in the muscles; PFK in particular is known to be inactivated by low pH.

That this is not the reason becomes evident when stimulation is resumed, for both PCr splitting and glycolysis start up promptly at roughly their previous rates and the muscle becomes even more acid than before. During this second period of stimulation the relation between force developed, relaxation rate constant and the various substrate levels is the same as during the first period. This reinforces the conclusion given above, that the mechanical characteristics are

functions of the substrate concentrations as such and are independent of the historical path by which these concentrations were attained.

An analogous result, but with better time-resolution, is given by the experiment shown in figure 9. The muscles were tetanized for 30 s, leading to a smaller but much more rapid breakdown of PCr than in the experiment shown in figure 8. The subsequent production of lactic acid and rebuilding of PCr starts rapidly and ends after about 2 min, despite the fact that the

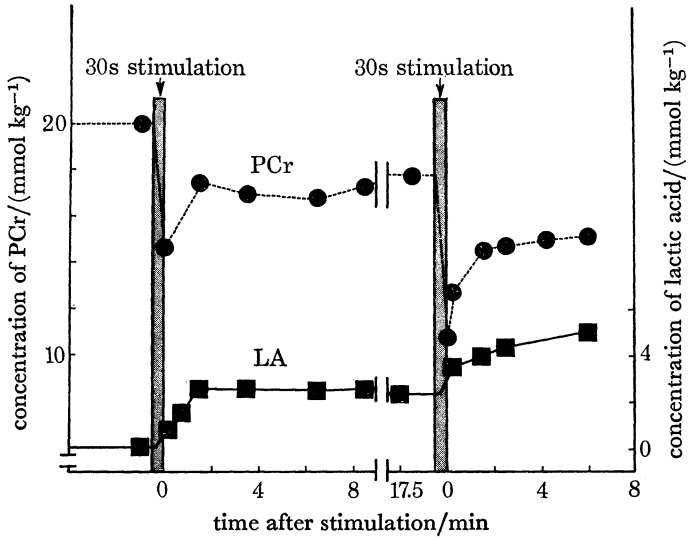

FIGURE 9. The time course of changes in PCr and lactic acid resulting from two 30 s tetani in anaerobic muscles. Note the breaks in the left ordinate and abscissal scales.

PCr has not been rebuilt to its initial level. Thereafter, no further glycolysis occurs until the muscle is stimulated again, when the previous sequence repeats itself. It seems a strange quirk of the control system that the muscles do not take the opportunity to recharge their PCr store when they apparently have the substrates and enzymes potentially available to do so. We are currently investigating the free-energy changes and the ADP/ATP ratio to see if these give a clue to what is happening. Nevertheless, the conclusion seems sure, that whatever it is that switches glycolysis on is very closely associated with contraction, and its effects last only a short time after relaxation is completed.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

References (Dawson *et al.*)

Curtin, N. A. & Woledge, R. C. 1978 Energy changes and muscular contraction. *Physiol. Rev.* **58**, 690–761.

Dawson, M. J., Gadian, D. G. & Wilkie, D. R. 1977 Contraction and recovery of living muscles studied by ^{31}P nuclear magnetic resonance. *J. Physiol., Lond.* **267**, 703–735.

Dawson, M. J., Gadian, D. G. & Wilkie, D. R. 1978 Muscular fatigue investigated by phosphorus nuclear magnetic resonance. *Nature, Lond.* **274**, 861–866.

Dawson, M. J., Gadian, D. G. & Wilkie, D. R. 1980 Mechanical relaxation rate and metabolism studied in fatiguing muscle by phosphorus nuclear magnetic resonance (^{31}P N.M.R.). *J. Physiol., Lond.* **299**, 465–484.

Endo, M. 1977 Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* **57**, 71–108.

Gilbert, C., Kretzschmar, K. M., Wilkie, D. R. & Woledge, R. C. 1971 Chemical change of energy output during muscular contraction. *J. Physiol., Lond.* **218**, 163–193.

Hasselbach, W. 1974 Sarcoplasmic membrane ATPase. In *The Enzymes* (ed. P. D. Boyer), vol. 10, 3rd edn, pp. 432–465. New York: Academic Press.

Jewell, B. R. & Wilkie, D. R. 1960 The machanical properties of relaxing muscle. *J. Physiol., Lond.* **152**, 30–47.

Kretzschmar, K. M. & Wilkie, D. R. 1969 A new approach to freezing tissues rapidly. *J. Physiol., Lond.* **202**, 66–67P.

Quantities, units, and symbols (2nd edn) 1975 London: The Royal Society.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES