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EQUILIBRIUM OF NUCLEOTIDES IN FROG SARTORIUS MUSCLE DURING AN ISOMETRIC TETANUS AT 20° C

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SUMMARY

1. The concentrations of creatine, phosphorylcreatine (PC), ATP, ADP, AMP and IMP have been measured in frog sartorius muscles at 20° C during isometric tetani lasting from 0·5 to 12 sec. Each muscle was tetanized once only for the chosen duration. The muscles were poisoned with iodoacetic acid and nitrogen to prevent oxidative and glycolytic activity.

2. The rate of PC splitting decreased exponentially with the duration of the tetanus ($\alpha = 0{\cdot}16$ sec^{-1}). Net ATP splitting began after 2 sec, accompanied by an increase in AMP and ADP; inosine monophosphate (IMP) also appeared both earlier and faster than adenosine monophosphate (AMP).

3. On the basis of two equilibrium reactions, the Lohmann and myokinase reactions, the concentration of adenosine nucleotides should be a function of the ratio creatine/phosphorylcreatine.

4. The agreement between nucleotide concentrations predicted by this equilibrium hypothesis and those observed experimentally was good provided it was assumed that 90 % of the acid-labile ADP found in resting muscle was bound *in vivo* and remained so throughout the tetanus. The validity of this assumption is discussed.

5. The IMP concentration was an exponential function of the ratio creatine/phosphorylcreatine.

INTRODUCTION

Our present concepts of the chemical events occurring during muscular contraction derive largely from the work of Lohmann (1934), who discovered that muscle extracts only hydrolyse phosphorylcreatine in

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the presence of adenosine diphosphate (ADP). He proposed an hypothesis which stated two things.

(i) The immediate source of energy for muscle contraction is the hydrolysis of adenosine triphosphate (ATP) to ADP and inorganic phosphate (P_i).

$$ATP + H_2O \rightarrow ADP + P_i. \quad (1)$$

(ii) The first recovery reaction is a phosphoryl transfer between PC and ADP, yielding ATP and free creatine (C_F)

$$ADP + PC \rightleftharpoons ATP + C_F \quad (2)$$

now commonly known as the Lohmann reaction.

There is a considerable body of experimental evidence which supports this hypothesis (for a review see Mommaerts, 1969) and a net break-down of PC during contraction is easily demonstrated (Lundsgaard, 1930; Carlson & Siger, 1959; Maréchal & Mommaerts, 1963). Demonstration of a break-down of ATP (reaction 1) is more difficult as it is obscured by reaction (2). Cain & Davies (1962) inhibited reaction (2) with the poison 1 fluoro-2,4-dinitrobenzene and were then able to show that ATP was broken down during contraction without any change in the concentration of PC. Since reaction (2) is the only one known which uses PC, this was taken as final proof of the Lohmann hypothesis. A consequence of this hypothesis is that the concentrations of ATP, ADP, PC and free creatine (C_F) should be in accordance with a simple chemical equilibrium corresponding to reaction (2). We have tested this last point experimentally using muscles poisoned with iodoacetic acid (IAA) and nitrogen to prevent aerobic and glycolytic recovery processes.

METHODS

General procedures

The experiments were performed on sartorius muscles of *Rana temporaria* during a period from April to June. They were dissected out, their standard length (l_0) measured (Hill, 1952) and paired muscles, still attached to the pelvic bone, were stored overnight at 5° C in Ringer solution (NaCl, 113 mM; KCl, 2·0 mM; $CaCl_2$, 1·8 mM phosphate buffer: 1·9 mM, pH 7·3) gassed with air.

The following morning, each pair was poisoned with 0·4 mM-IAA for 30 min at 20° C, blotted to remove surface water and mounted on the rapid freezing apparatus (Maréchal, 1964). The mounted muscles were maintained at 20° C in N_2 for 10 min before stimulation. The force developed was recorded with strain gauge transducers. Stimuli were supramaximal condenser discharges of alternating polarity at 50/sec. The muscles were frozen on the last shock of the tetanus by rapid immersion in isopentane cooled to $-160°$ C with liquid N_2.

Chemical procedures

Extraction. Each muscle was weighed and then placed in a plastic centrifuge tube (75 mm long × 7·5 mm internal diameter) kept in liquid nitrogen and containing 200 μl. 0·5 N perchloric acid. The muscle was ground up with a glass rod precooled in liquid nitrogen. When all the muscles had been so treated, the tubes were centrifuged at 10,000 rev/min for 30 min at 0° C. The contents of each tube were then mixed at 0° C with 100 μl. 1 N-KOH and the tubes centrifuged again (18,000 rev/min for 30 min at 0° C). The clear solution was decanted, kept on a bed of ice and divided into two parts:

(1) 50 μl. were added to 500 μl. of an alkaline solution containing 1·5 M-Na_2CO_3 and 1·5 M-NaOH. This was kept at 0° C for creatine analysis later the same day.

(2) 100 μl. were placed in a plastic centrifuge tube with 5 μl. of an acetate buffer (200 ml. 2 M sodium acetate + 33·7 ml. 3·5 N acetic acid: pH 4·2) at 0° C. The tubes were centrifuged for 20 min at 15,000 rev/min at 0° C and stored at −18° C for chromatographic analysis next day.

Analysis of creatine. Free creatine (C_F) was measured in duplicate by its reaction with diacetyl α-naphthol (Ennor & Stocken, 1948). After acid hydrolysis of phosphorylcreatine (10 min at 65° C in 0·4 N-HCl), and neutralization with NaOH, the total creatine ($C_T = C_F + PC$) was measured by the same reaction. An allowance of 11 % was made for creatinine formation during the acid hydrolysis of PC.

Chromatography. This was used to estimate the concentrations of adenosine and inosine nucleotides. 10 μl. of muscle extract was placed on the plate (Merck P.E.I. cellulose F) in 'spots' 10 mm long by 2 mm wide with a Hamilton microsyringe which delivered 0·5 μl. drops. The extract was duplicated on the same plate together with duplicates of a standard nucleotide solution prepared in the same way as the extract (10 μl. standard solution contained ATP 4·75 n-mole; ADP 4·76 n-mole; AMP 5·93 n-mole and IMP 4·5 n-mole).

The plates were developed in 1·5 M-NaCl for 15 min and dried in a stream of cold air. Reflectance measurements were made at 260 nm using an adapted Zeiss spectrophotometer (PMQ II). The areas under the reflectance curves were proportional to the amounts of the nucleotides, and the proportionality constants were checked with internal standards.

The absolute sensitivity of this chromatographic procedure corresponded to about 0·03 μmole/g for adenosine nucleotides and 0·06 μmole/g for inosine monophosphate. The results are expressed relative to the total creatine content of the muscle as this is a better index of muscle tissue extracted than is muscle weight (Wilkie, 1968). The mean total creatine of the muscles used in the experiments reported in this work was 33·9 μmole/g (S.D. = 2·8 mole/g; n = 144).

Statistical design

Because both batch and procedural effects can affect the results of this type of study, great care was taken with the experimental design. The results reported and discussed in this paper are only part (groups P_2 and P_{12}) of a much larger experiment designed to study energy balance during a tetanus. As it is not possible to discuss the design of only a part of this experiment, the detailed description of the design and analysis of the results is presented in the following paper (Canfield, Lebacq & Maréchal, 1973) and only a brief account included here.

Each experiment contained six pairs of muscles to which one of four stimulation treatments was to be applied (*a*, *b*, *c* and *d*). These four treatments were combined in all pair combinations (*ab*, *ac*, *ad*, *bc*, *bd* and *cd*) and the six treatment pairs so formed were randomly allocated to the six muscle pairs. The allocation of the two treatments

in a pair to the two muscles of that pair was also randomized. Each treatment was repeated three times in an experiment performed with this design and each experiment was repeated three times so that each treatment was repeated a total of 9 times. This formed one 'group' and two groups were used to obtain the results described here:

P_2 group: stimulation treatments with tetanus durations of 0, 0·5, 1 and 2 sec.

P_{12} group: durations of 0, 4, 8 and 12 sec.

It was not possible to use only one larger group to study all the required durations because this would have required twenty-one muscle pairs to form all the possible treatment pair combinations as required by this design and this number could not be handled at one time.

RESULTS

(*a*) *The changes in high-energy phosphates during a* 12 *sec tetanus*

The isometric tension, PC and nucleotide concentrations during a 12 sec isometric tetanus are shown in Fig. 1. Neither AMP nor IMP were detected in the unstimulated muscles. During short tetani (< 2 sec) the only significant change was a fall in PC concentration; with longer periods of stimulation this was accompanied by a net decrease in ATP concentration and the appearance of AMP and IMP in the extracts. There was a slight increase in the ADP concentration during the tetanus and at all times the concentration of IMP was greater than that of AMP.

The presence of AMP and IMP suggested that two other reactions were occurring in addition to reactions (1) and (2):

$$\text{Myokinase: } 2\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}, \tag{3}$$

$$\text{Deaminase: } \text{AMP} + \text{H}^+ \rightarrow \text{IMP} + \text{NH}_4^+ \tag{4}$$

so that the simple equilibrium of reaction (2) could not be the sole determinant of the concentrations of C_F, PC, ATP and ADP. This is in agreement with other authors (Carlson & Siger, 1959; Seraydarian, Mommaerts & Wallner, 1962; Maréchal, 1964).

(*b*) *An equilibrium hypothesis*

We have therefore tested an equilibrium hypothesis in which the concentrations of C_F, PC, ATP, ADP and AMP are determined by reaction (2) (equilibrium constant K_2) and reaction (3) (equilibrium constant K_3). We have neglected any effect from H^+ or Mg^{2+} as these lead to a complex theory (Vincent & Blair, 1970), which cannot be tested by our experiments. Of the four main reactions occurring in these muscles during the tetanus, two are non-equilibrium reactions (1 and 4). An equilibrium can only be set by the other two (2 and 3) if these proceed more rapidly than the former (1 and 4). We can estimate the rates of reactions (1) and (4) from our results. The rate of ATP hydrolysis is about

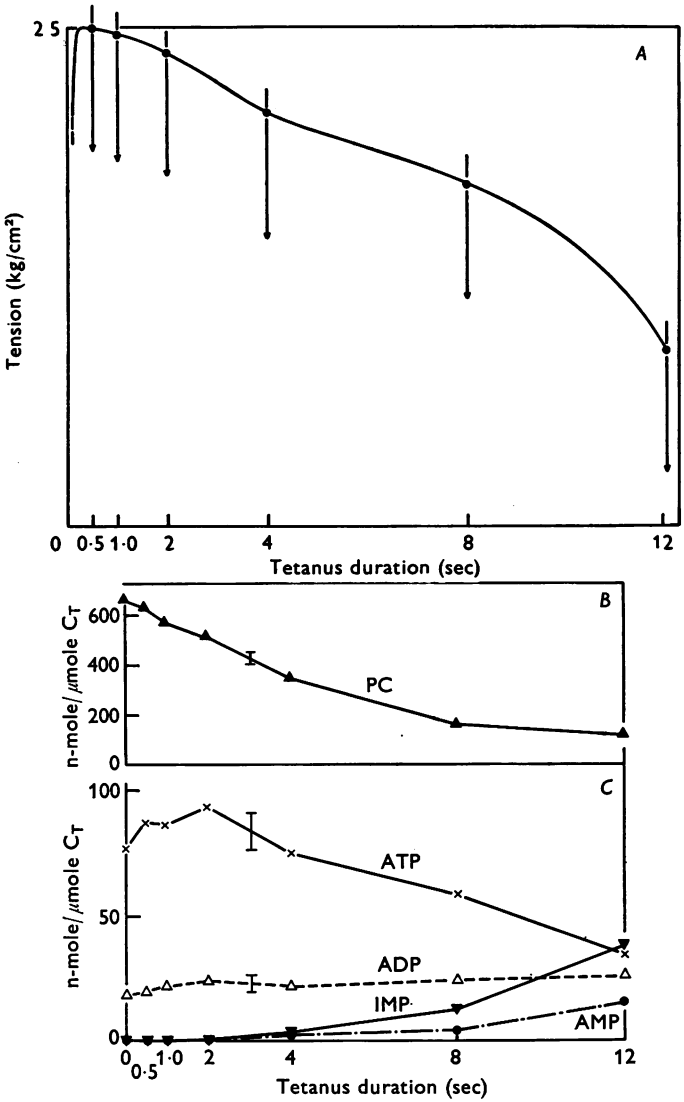

Fig. 1. *A*, mean isometric tension of frog sartorius muscle stimulated for up to 12 sec at 20° C. The vertical arrows represent the moments of freezing. The S.E. of the means is 0·07 kg/cm² ($n = 9$). *B*, phosphorylcreatine content of these muscles. *C*, nucleotide contents of these muscles. Note: it is a characteristic of the statistical design used that S.E. of all the means for a given substrate (e.g. ATP) are the same so that one may calculate an S.E. of the *difference* between any *two* means for that substrate. The vertical bars in *B* and *C* are such standard errors of differences between means.

3 μmoles/g muscle.sec (Fig. 3); that of IMP formation is maximal at the end of the 12 sec tetanus and equal to 0·3 μmole/g muscle.sec.

The rates of reactions (2) and (3) cannot be derived from our data, but some estimate may be obtained from the literature. The maximum rate of reaction (2) is 120 μmoles ATP synthesized/g muscle.sec (Kuby, Noda & Lardy, 1954). The maximum rate of the myokinase reaction (3) may be estimated from the myokinase content of muscle (4 mg/g muscle; Noda & Kuby, 1957) and its rate of reaction (2·1 M-ADP/min by 1 g myokinase; Bowen & Kerwin, 1955). It is thus equal to 140 μmole ADP/g muscle.sec. These figures apply to rabbit muscle at 38° C and it seems reasonable to assume that reactions (2) and (3) can also proceed faster than reactions (1) and (4) in frog muscle at 20° C.

The equilibrium constants for reactions (2) and (3) are:

$$K_3 = \frac{[\mathrm{ADP}]^2}{[\mathrm{AMP}]\,[\mathrm{ATP}]}\;; \quad K_2 = \frac{[\mathrm{C_F}]\,[\mathrm{ATP}]}{[\mathrm{PC}]\,[\mathrm{ADP}]}. \tag{5}$$

The sum of adenosine nucleotide concentrations, $\mathrm{A_T}$, is:

$$\mathrm{A_T} = \mathrm{ATP} + \mathrm{ADP} + \mathrm{AMP}. \tag{6}$$

Introducing eqn. (5) into (6):

$$\mathrm{A_T} = \left\{1 + \frac{\mathrm{C_F}}{\mathrm{PC}}\frac{1}{K_2} + \frac{(\mathrm{C_F})^2}{(\mathrm{PC})^2}\frac{1}{(K_2)^2\,K_3}\right\}\mathrm{ATP} = Z(\mathrm{ATP}). \tag{7}$$

This equation defines a new variable, Z, a function of K_3, K_2 and $\mathrm{C_F/PC}$; it is now easy to express the fractional concentrations of the adenosine nucleotides as a function of Z:

$$\left.\begin{aligned} \frac{\mathrm{ATP}}{\mathrm{A_T}} &= \frac{1}{Z}, \\ \frac{\mathrm{ADP}}{\mathrm{A_T}} &= \frac{\mathrm{C_F}}{\mathrm{PC}}\frac{1}{K_2}\frac{1}{Z}, \\ \frac{\mathrm{AMP}}{\mathrm{A_T}} &= \frac{(\mathrm{C_F})^2}{(\mathrm{PC})^2}\frac{1}{(K_2)^2\,K_3}\frac{1}{Z}. \end{aligned}\right\} \tag{8}$$

There are two advantages to this notation: eqn. (8) holds true, even if deamination reduces the total content of adenosine nucleotides, and they are independent of any variations in total creatine content.

Values for the constants K_3 and K_2 may be found in the literature: $K_3 = 0{\cdot}44$ (Eggleston & Hems, 1952) and $K_2 = 22$ (Carlson & Siger, 1959). It is then possible to construct curves (Fig. 2) which show the dependence of the concentrations of adenosine nucleotides on the ratio $\mathrm{C_F/PC}$. When the experimental data shown in Fig. 1 were plotted as a % $\mathrm{A_T}$ on the

curves shown in Fig. 2, a good agreement between experiment and theory was not obtained. The reason for this discrepancy is readily appreciated by reference to Fig. 1 which shows that, for resting or briefly stimulated muscle, ATP represents 75–80 % A_T and ADP some 15–20 % A_T. The hypothesis (curves in Fig. 2) predicts that, under these conditions, ATP should be more than 95 % A_T and ADP less than 5 % A_T. Obviously the data do not match the hypothesis.

There are reasons to believe that most of the ADP (90 %) found in the unstimulated muscle is bound to actin and other proteins and that it appears in the extracts because it is released by the perchloric acid treatment (see Discussion). Seraydarian *et al.* (1962) extracted muscle with

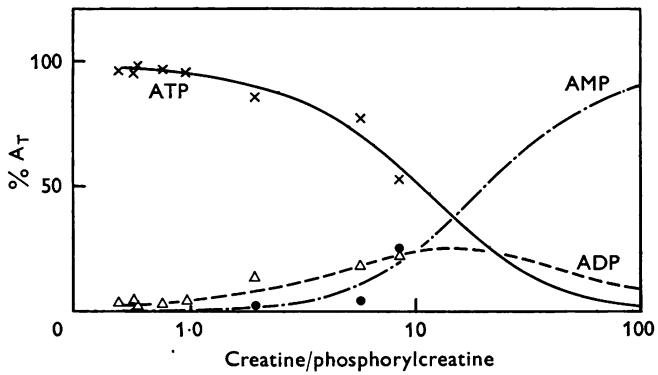

Fig. 2. Adenosine nucleotides as a function of the ratio creatine/phosphoryl-creatine (C_F/PC). The lines are the concentration (in % of total adenosine nucleotides, A_T) predicted if these compounds are in an equilibrium governed by the Lohmann and myokinase reactions. The points are the experimental observations, corrected for bound ADP (see text), from Fig. 1.

cold ethanol, and found that, whilst most of the ATP is solubilized, about 90 % of the ADP is not and remains bound. This bound ADP was released by further extraction with perchloric acid. They have also shown that ADP soluble in cold ethanol accumulates in muscles stimulated to an advanced state of fatigue (anaerobic, with or without iodoacetate); we too have found an increase in ADP, albeit small. What is perhaps more important is that they found that bound ADP (measured in perchloric acid extract of muscle residue previously extracted with cold ethanol) does not change when the muscles are stimulated (Table XI of *their* paper). Therefore we have assumed that 90 % of the ADP found in resting muscle is bound, and we have subtracted this quantity from all measurements of ADP and A_T for both resting and stimulated muscles. When corrected in this way, the experimental observations agree well with the curves predicted by the hypothesis (Fig. 2).

The best agreement is found when $K_3 = 0{\cdot}44$ and $K_2 = 22$, the literature values, but the data are compatible with other values of these constants, albeit over a limited range.

For example, a decrease of K_2 shifts the crossing points of the curves ATP/A_T and AMP/A_T to the left; the height of the crossing point remaining the same. For $K_2 = 19$, the crossing point shifts to $C_F/PC = 12{\cdot}6$ and the curves are still compatible with the experimental data. For $K_2 = 14$, the crossing point is at $C_F/PC = 8$, hypothesis and observations no longer agree, observed ATP/A_T being greater than predicted. For $K_2 = 40$, the crossing point is shifted to the right, at a value $C_F/PC = 23$. Again hypothesis and observations do not agree, observed ATP/A_T being less than the amount expected from the hypothesis. The effect of modifying K_3 is complex: it shifts the crossing point in the same direction as a change in K_2, but with a square root law; if K_3 is reduced to 0·18 (leaving $K_2 = 22$), the crossing point is at $C_F/PC = 8$ and the ratio ADP/ATP at the crossing point is reduced from 66 to 42 %; from the data a ratio ADP/ATP = 30 % was found; this is much lower than the predicted value. If K_3 is increased to 1, the three crossing points become one when $C_F/PC = 22$ and the concentrations of the three nucleotides are equal. From the experimental observations (Fig. 2) $ADP/A_T = AMP/A_T$ when $C_F/PC = 9$ so that observation and hypothesis do not agree when $K_3 = 1$.

The compatibility of the data with the hypothesis also rests on the amount of ADP assumed to be bound. If only 50 % of the ADP extracted from resting muscle is bound, then the concentration of ADP/A_T increases to 10–16 %, and ATP/A_T decreases to 85–90 %. When C_F/PC is in the range 0·4 to 2 the experimental data are absolutely incompatible with the curves of Fig. 2, and the fall of ATP/A_T as a function of C_F/PC is much steeper for C_F/PC values above 2. It was impossible to fit the data with the curves of Fig. 2 under these conditions even if the equilibrium constants were modified. However, if 80 % of the ADP is bound, the data remain compatible with the hypothesis.

It seems reasonable to conclude that our data are compatible with an equilibrium hypothesis involving only two reactions, the equilibrium constants of which are equal to those reported in the biochemical literature. These experiments do not prove that such an equilibrium indeed exists and other interpretations may be possible.

(c) *Reconstruction of nucleotide changes during a tetanus*

It is possible to obtain a complete mathematical description of the nucleotide changes during a tetanus from eqn. (8) if the time course of the change in the ratio C_F/PC is known. This was found to be a simple exponential function of the tetanus duration. The PC data of Fig. 1 are replotted in Fig. 3 (filled circles); an exponential curve has been fitted to them with a rate constant $(\alpha) = 0{\cdot}16$ sec^{-1}, the initial value $PC_0 = 666$ n-mole PC/μmole total creatine. From this, the time course of C_F/PC is readily computed as

$$\left(\frac{C_F}{PC}\right)_t = \left(\frac{C_F}{PC}\right)_o \exp(\alpha t) - 1. \qquad (9)$$

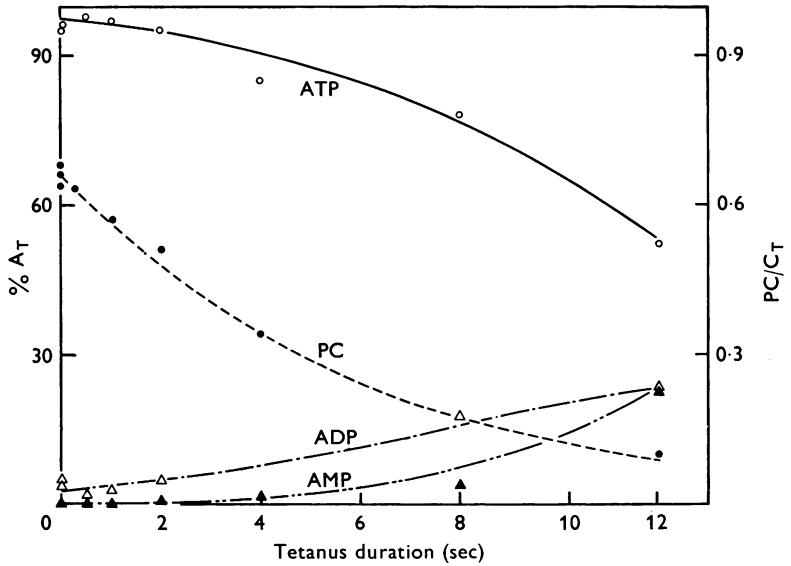

Fig. 3. Changes in adenosine nucleotides as a per cent of total adenosine nucleotides (A_T) with duration of the tetanus. The lines are drawn using the equilibrium hypothesis (Fig. 2) and the exponential relationship between PC content and duration of tetanus. The points are the experimental observations, corrected for bound ADP (see text), from Fig. 1.

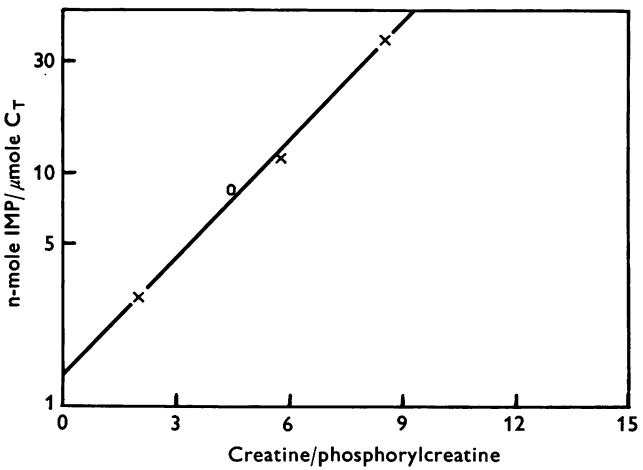

Fig. 4. Relationship between the ratio inosine monophosphate/total creatine content (IMP/C_T) and the ratio creatine/phosphorylcreatine (C_F/PC). The one point from unpoisoned muscles in oxygen (open circle) is discussed in the text.

Combining eqn. (9) with eqn. (8) yields the theoretical changes in $\frac{ATP}{A_T}$, $\frac{ADP}{A_T}$ and $\frac{AMP}{A_T}$ as a function of tetanus duration. These curves together with the experimental points have been drawn in Fig. 3. The fit between the curves and the data is excellent.

(*d*) *IMP production*

The IMP production is not an equilibrium reaction and it must be excluded from the equilibrium hypothesis. However, we have found an empirical correlation between IMP concentration (n-mole/μmole C_T) and C_F/PC:

$$IMP = 1{\cdot}4 \exp (0{\cdot}26\ C_F/PC).$$

The data are shown in Fig. 4 (crosses).

Unpoisoned muscles in O_2 also produce IMP in a 12 sec tetanus at 20° C, as shown by the circle in Fig. 4. This point is on the line obtained for IAA muscles. While this agreement may be coincidental, it suggests that there might exist some mechanism which controls IMP production according to the amount of PC split.

DISCUSSION

(*a*) *The equilibrium hypothesis*

The hypothesis that creatine, PC, ATP, ADP and AMP should be in a chemical equilibrium in the living muscle has previously received some experimental support (Carlson & Siger, 1959; Maréchal, 1964), although there are also reports to the contrary (Hohorst, Reim & Bartels, 1962; Kirsten & Kirsten, 1969). There are numerous papers in which some of these compounds have been measured, but simultaneous estimates of all five have been uncommon. Kirsten & Kirsten (1969) have analysed rat pyramidalis (a red muscle) and adductor magnus (a white muscle) for these five compounds and followed the concentration changes when the muscles were tetanized for several minutes. Their data roughly fit our hypothesis, if a slight change of K_2 from 22 to 16 is allowed, and if it is assumed that bound ADP in these muscles is about 0·5 μmole/g. It is impossible to make a more exact comparison of our hypothesis with these authors' results as they stimulated the muscles *in situ* so that glycolysis and oxidative recovery were not inhibited. Furthermore, there is no experimental evidence regarding the extent of ADP binding in these two muscles of the rat. P. Cerretelli & G. Ambrosoli (personal communication), from data on the metabolic changes resulting from contraction of frog gastrocnemius,

confirmed the validity of our hypothesis for this muscle. (*Some* of this data has been published; Cerretelli, di Prampero & Ambrosoli, 1972.)

We believe that Fig. 2 should prove helpful for future analyses of the relationships between these five compounds. It is perhaps a lack of a convenient graphical representation which has previously impeded work on this subject. Analysis of Fig. 2 suggests several interesting deductions. For example, a break-down of 0·28 μmole PC/g during a twitch of a muscle containing about 25 μmole total creatine/g should entail a decrease of 0·009 μmole ATP/g if the chemical equilibrium hypothesis is valid. Such a small change in ATP concentration cannot be measured by chemical methods; it is however compatible with the amount of ADP which reaches the mitochondria after a twitch (0·009 μmole/g), as estimated by fluorometric measurements (Chance & Connelly, 1957). If the ratio C_F/PC changes from 0·1 to 2, the decrease in ATP concentration would be only 10% of A_F. In this respect, it is unimportant that the ratio PC/total creatine in resting muscle is 0·66 (as here) rather than 0·86 (Wilkie, 1968), or that this ratio may show seasonal variations (Maréchal, 1964).

(*b*) *The bound ADP*

The amount of acid-soluble ADP found in muscle extracts is always higher than that required by the chemical equilibrium hypothesis, and, accordingly, we hypothesized that most of the acid-soluble ADP found in extracts from resting muscle was bound. The evidence for this should be discussed.

The most direct evidence is that of Seraydarian *et al.* (1962). A fraction of the ADP in frog sartorius muscle was extracted with cold aqueous ethanol, the average yield being 0·25 μmole/g. There was some indication that about 0·15 μmole/g was bound reversibly to various enzymes, and that only 0·10 μmole/g was free ADP. An additional fraction, 0·4 μmole/g, could be extracted by an aqueous solution containing 2 mM *p*-chloromercuric benzoate: a corresponding quantity was also found in fibrils, and was regarded as the ADP bound to F-actin. The two fractions were released by perchloric acid extraction. According to these authors, the free ADP is about $0{\cdot}1 \pm 0{\cdot}05$ μmole/g, and the total acid-soluble ADP is about 0·65 μmole/g. Free ADP is thus 15 ± 7% of the total ADP. Our adopted figure of 10% is thus in good agreement with their analysis.

Less direct evidence is provided by Valin & Charpentier (1969). They studied the post-mortem catabolism of nucleotides in beef muscle and found that ATP was rapidly dephosphorylated to AMP, and then deaminated to IMP; catabolism progressed further to inosine and hypoxanthine. However, acid-soluble ADP did not change, even after 15 days, at a time when practically all nucleotides were catabolized to hypoxanthine. This

indicates that most of the acid-soluble ADP (about 0·5 μmole/g) is bound and cannot be destroyed by sarcoplasmic enzymes.

The last piece of evidence comes from experiments on muscles poisoned with 1-fluoro-2,4-dinitrobenzene (Cain, Infante & Davies, 1962). This blocks the Lohmann reaction whilst the myokinase reaction remains active. The concentrations of ATP, ADP and AMP (given in *their* Table 5) enable the calculation of values for K_3 (*our* eqn. (5)). The values obtained are too high by an order of magnitude. Recalculating these on the assumption that 0·6 μmole/g ADP was bound, gives values compatible with the true value of $K_3 = 0{\cdot}44$ (Eggleston & Hems, 1952) (values for K_3 calculated from the data of Cain *et al.* 1962, assuming 0·6 μmole/g ADP bound; 0·32; 1·54; 0·11 and 1·15).

(*c*) *The deaminase activity*

AMP-deaminase is a very active enzyme, and, in the current view exemplified by Bendall (1960), AMP can never accumulate in muscle because it would be deaminated to IMP. It is true that we have observed an increase in IMP which is both earlier and faster than that of AMP (see Fig. 1). Nonetheless AMP concentration does increase appreciably during a prolonged tetanus. This phenomenon must surely be of importance in the control of glycolysis, because several key enzymes are sensitive to AMP concentration.

Our theory predicts a large increase (Fig. 2) of AMP/A_T if PC is depleted (ratio $C_F/PC > 10$) and one may question the lack of data in this region. The reason for this lack is that, at such low PC and ATP concentrations, the muscles go into rigor. In this state there is no accumulation of AMP in muscle. Bendall (1960), in a thorough review on rigor, barely mentioned AMP at all. We have confirmed this in a few experiments on muscles in rigor: the concentration of AMP does not increase beyond that of ATP. For this reason we have restricted our experiments to muscles which could still relax completely at the end of the tetanus. The equilibrium hypothesis thus breaks down for muscles in rigor. The reason is that AMP is deaminated to IMP as fast as it is produced (Bendall, 1960). Deaminase activity is such that we may no longer neglect reaction (4) in the study of the equilibrium of the nucleotides. As resting muscles have no detectable amount of IMP, deaminase activity must be very low in these muscles. The progressive acceleration of IMP production during the tetanus indicates that deaminase activity is increasing. The mechanism of this activation is probably complex. We have found an empirical correlation between C_F/PC and the logarithm of IMP concentration (Fig. 4). This suggests that PC inhibits AMP deaminase. Ronca-Testoni, Raggi & Ronca (1970) have indeed found that PC is an allosteric inhibitor of AMP

deaminase *in vitro*. They have also shown that ADP and AMP are powerful activators of this enzyme. At the end of a long tetanus, most of the PC is hydrolysed, whereas ADP and AMP have increased: these conditions would favour a stimulation of deaminase activity, up to a point where the equilibrium between reactions (2) and (3) would be disturbed.

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