J. Mechanochem. Cell Motility
1974, Vol. 3, pp. 55–68

THE INCORPORATION OF RADIOACTIVE PHOSPHATE INTO ATP IN GLYCERINATED FIBRES STRETCHED OR RELEASED DURING CONTRACTION†

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(*Received February 20, 1973; in final form June 13, 1973*)

Bundles of glycerinated psoas muscles contracted in a solution containing 5 mM ATP-Mg, 1 mM ADP, and 1.67 mM sodium phosphate containing ^{32}P at a specific activity of 20 mC/mmole phosphate. Contractions were isometric, or isometric for 10 sec followed by a stretch or a release, or isotonic with zero load and lasted 13 to 40 sec. They were arrested quickly by freezing the bundle (with cold isopentane) or deproteinizing it with perchloric acid. ATP was separated from inorganic phosphate by a two step procedure: ATP was first adsorbed on charcoal; a subsequent separation was achieved by thin layer chromatography on cellulose polyethylenimine impregnated. The incorporation of ^{32}P into ATP increased with the duration of the contraction, and with the force held by the fibres. This was clearer when the phenomenon is corrected for the variations in bundle size. Stretched fibres incorporate more ^{32}P into ATP, but this may be explained by the higher tension supported by the fibre. The quantity of the ^{32}P incorporated into ATP was low; the equivalent chemical energy corresponding to this apparent "synthesis" of ATP was only 2% of the work done on the muscle. We found no evidence to support the hypothesis that mechanical work done on a fibre is used to synthesize ATP.

The total energy (work+heat) produced by a muscle which is slowly stretched is less than that produced during an isometric tetanus of identical duration (Abbott and Aubert, 1951). Corresponding differences in the amount of used chemical energy have been found (Maréchal, Mommaerts and Seraydarian, 1974).

The mechanism of this sparing effect is still unknown. Two hypotheses have been considered according to how the stretch would influence the rate of the ATP splitting reactions: either it is decreased (inhibition hypothesis) or it is reversed (reversal hypothesis). So far it has never been possible to detect a net synthesis of $\sim P$ at the end of a stretch of an intact muscle (Maréchal and Beckers-Bleukx, 1965; Infante, Klaupiks and Davies, 1964). This negative result does not rule out the possibility of the reversal because (i) the method of the balance sheet for $\sim P$ is inadequate to distinguish between "spare" and "resynthesis" of ATP; and (ii) stretches are usually applied to fully active muscle, *i.e.*, when a considerable splitting of ATP had already occurred; the amount of free energy usually provided by the stretch, even if completely transformed in $\sim P$, is not large enough to compensate for this preliminary ATP splitting and to bring the ATP level above its resting value.

In its simplest form, the reversal hypothesis can be described by the following equation:

$$ADP + P_i \xrightarrow[\text{Stretch}]{} ATP$$

Therefore the use of labelled inorganic phosphate (P_i) seemed to be a promising approach to test the occurrence of this reaction. In a previous paper (Maréchal *et al.*, 1974) $H_2{}^{18}O$ has been used to label intracellular P_i of intact muscle, but these experiments led to no firm conclusion because of many difficulties including the diffusion barrier of the muscle membrane, the various $\sim P$ pools and phosphorylating systems of the intact muscle fibre.

All these complications have been avoided in the experiments reported here by using glycerinated fibres. Moreover this preparation allows us to use the cheaper and more straightforward ^{32}P as a tracer for P_i.

In these conditions it has been possible to detect an incorporation of P_i into ATP at the end of a stretch of an active muscle. But the extent of this incorporation is very small in comparison with the free energy provided by the stretch. Further experiments have also shown that this incorporation is correlated rather with the force the muscle bears than with the stretch itself. Though the meaning of

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this force-induced incorporation has still to be elucidated, the results allow us to rule out that an appreciable resynthesis of ATP occurs during the stretch.

Preliminary results have already been communicated (Gillis and Maréchal, 1969, 1971).

METHODS

The results reported in this paper have been obtained from two series of experiments described in the section "results" and referred to as series I and II through the paper. As it will appear, the series differ in the conditions of contraction, in the way of stopping contraction and enzymic activity and in the method of extraction of the nucleotides.

Physiological Methods

a. *Material*

Strips of rabbit psoas were isolated and tied, at body length, on Perspex sticks. They were extracted, at 2–4°C, in 50% (v/v) glycerol solution buffered at pH 7.1 with 10 mM NaH_2PO_4/Na_2HPO_4. After 48 hours, the solution was renewed, and the muscles were stored at −20°C for at least 8 weeks before use. For the experiments, thin bundles of 1.5 cm long were dissected in the glycerol solution. In series I, the width of the bundles was about 200–250 μm; in series II, better standardization of the material was obtained by preparing 10 fibres per bundle. The average sarcomere length, measured by light diffraction, was between 2.30 and 2.50 μm. An average dry weight of 60 μg (measured with a Cahn electrobalance) was obtained from a batch of 5 bundles (length 1 cm, 10 fibres/bundle) specially dissected for this purpose.

The bundle was placed in a capillary tube (4.5 cm long, containing 10 μl) and passed around a nylon loop as shown in Figure 1. Both ends of the bundle were tied together to a thin nylon thread (Φ60 μm)

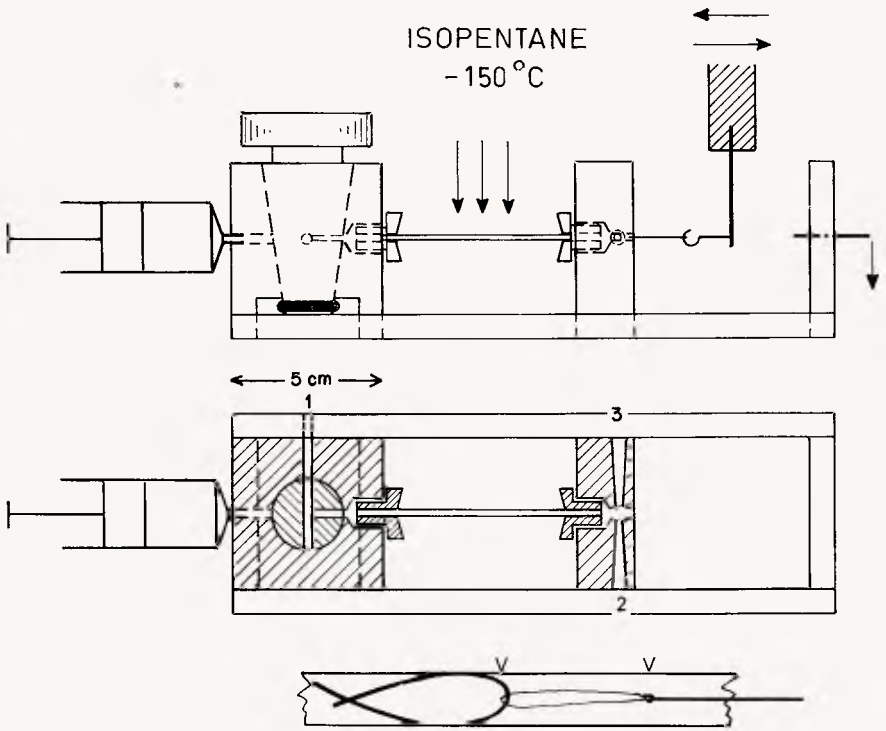

FIGURE 1 Device used to study $^{32}P_i$ incorporation into ATP in a glycerinated bundle. Lower part: capillary tube containing a glycerinated bundle passed across a nylon loop; the two "v" show the level of the diamond scratches made to break the tube after its content has been frozen. The capillary is mounted on small rubber stopper and placed in a trough (upper part, side view; in the middle, view from above). The bundle is tied to a force recorder (upper, right), which is movable (in order to stretch or release the bundle). Solutions flow from 1 through a three-way tap from left to right, through the capillary, and are sucked at 2 or at 3. Reactions are stopped either by pouring isopentane cooled to −150° C (first series), or by pushing perchloric acid in the capillary with the syringe.

connected to the force transducer. The capillary tube was then mounted on the perfusion assembly illustrated in Figure 1. Solutions entered the capillary tube from the left through a three-way tap and flowed into the second compartment where they were sucked out. In series II, the conical holes (2) and (3) were drilled in the wall between the two compartments and polythene catheters were inserted into them; solutions could be directed and collected by suction either through exit (2) or through exit (3).

b. *Solutions*

The capillary containing the fibres bundle was perfused in sequence with the following solutions: (1) 0.1 M KCl and 2 mM sodium phosphate buffer; (2) 0.1 M KCl, 1.0 mM $CaCl_2$, 7.0 mM Tris-HCl buffer, 1.67 mM sodium phosphate containing ^{32}P at specific activity of 20 mC/mmole phosphate, this solution was kept in contact with the bundle for 5 min; (3) contracting solution: solution (2) plus 5.0 mM Mg-ATP and 1.0 mM ADP. All solutions were adjusted to pH 7.1, and 0.1 mM dithiotreitol was added to protect -SH groups. In series II, oligomycin (2 μg/ml) and sodium azide (1.0 mM) were added to the solutions, for reasons to be discussed later.

c. *Contraction*

The bundle was connected to a thin steel beam which was mounted on a movable stand; the bundle could thus be stretched or released over an adjustable distance at controlled speed during contraction. Strain gauges glued on the beam were used to measure the force of the bundle which was recorded on a Sanborn 320 pen recorder. The compliance of the tension recorder was 0.22 micron/mg. The experiments were performed at room temperature ($\sim$20°C).

In order to detect any possible incorporation of ^{32}P into ATP produced by stretching the muscle fibres during contraction, quick stop of the underlying enzymic reactions was needed. Splitting of this newly labelled ATP would occur if too much time elapsed between the end of the stretch and the analysis of the muscle. Quick stop is also needed to study the exact temporal relationship between this incorporation and the mechanical state of the muscle.

In series I, the contraction was stopped by pouring isopentane (at about -150°C) into the compartment containing the bundle. The segment of the capillary tube containing the bundle was then broken off (at the level of two diamond scratches made in advance, see Figure 1) and placed into a cooled cartridge for the extraction (see below).

In series II, solutions were directed through exit (2) during contraction. The three-way tap was rotated by 90° to stop the perfusion and to connect the capillary tube to a syringe (S) containing 2 ml of cold 0.25 M perchloric acid. At the same time, suction was switched from exit (2) to exit (3). Contraction was stopped by flushing with the perchloric acid which was collected through (3) in a test tube. The capillary tube was then disconnected from the perfusion assembly and the fibres bundle with its nylon attachments was placed into the same test tube for the extraction of the nucleotides. This second method of stopping contraction simplifies the extraction procedure of the nucleotides, as it will appear below.

Chemical Methods

a. *Purification of the ^{32}P solution*

Radioactive phosphate from various commercial sources was found to contain up to five different components detectable by the chromatographic method described below. About 0.4% of the radioactivity of the solution was found near the ATP spot and 0.1% near ADP; the latter contaminant was identified as pyro-phosphate (the former contains probably polyphosphates). No radioactive compounds were found at the level of AMP or IMP. The elimination of these contaminants was absolutely required to detect the presence of radioactive ATP. The radioactive phosphate solution was purified as follows: 0.2 ml of carrier-free ^{32}P solution (10 mC/ml, in 1.0 M HCl) was diluted in 2.0 ml of 1.0 mM sodium phosphate and placed on a small column of an anion-exchange resin (Dowex 1×8, in the chloride form). The column was rinsed with 5 ml of distilled water and orthophosphate was eluted with 0.01 mM HCl; the first 20 ml were collected. Controls have shown that this solution contained only one radioactive component migrating as the orthophosphate. Nevertheless, new components reappeared as the solution was allowed to stand more than 24 hours at room temperature. The purification procedure was therefore repeated before each experiment (see RESULTS I, a).

b. *Extraction of the nucleotides*

Series I: the fragment of the capillary tube and its frozen content was placed in a stainless steel cartridge maintained in liquid nitrogen and containing a steel ball. The tube and the bundle were ground by violent shaking of the cartridge (3×15 sec) as described by Seraydarian, Mommaerts, Wallner and Guillory (1961) and by Maréchal (1964). Twenty μl of 5% v/v trichloracetic acid were then added for denaturation of proteins and 40 μl of 5.0 mM ATP was added as carrier for any radioactive ATP formed during contraction. The content of the cartridge was allowed to thaw and was transferred into a conical centrifuge tube; the cartridge was rinsed 5 times with 0.5 ml of distilled water. One ml of ether was added to the tube to remove TCA. After 5 min of centrifugation at 5000 rpm, the ether was removed and the aqueous phase was passed through a small column of cation-exchange resin (Amberlite 50, in the H^+ form) to fix traces of heavy metal removed from the cartridge during the grinding. This step was essential to avoid formation of metal-phosphate complexes which cochromatograph with the nucleotides.

Series II: the above procedure was simplified, the test tube containing the bundle was shaken during 5 min in perchloric acid; 50 μl of 5.0 mM ATP was added as carrier and ^{14}C-ATP was also added as internal standard to estimate the loss of ATP during the purification procedure.

c. *Separation of ATP, ADP and inorganic phosphate*

Standard procedures (paper chromatography or extraction of phosphomolybdate in an organic solvent) were found inadequate to achieve the high degree of separation of P_i from the nucleotides required by our experiments; with these methods, as much as 0.1% of the initial radioactivity associated with P_i was found in the nucleotides fraction. We used instead a two step separation procedure as proposed by Tsuboi and Price (1959): a first separation of P_i from the nucleotides was made by absorption of the latter on charcoal; further separation was achieved by thin layer chromatography. The final extracts obtained from experiments of series I and II were treated similarly.

Activated charcoal (25 mg) was added to the extract and after vigorously mixing for several minutes, the suspension was filtered on Millipore filter (pore size: 0.45 μm) previously covered with a thin layer of Celite to facilitate the removal of charcoal after the filtration. Charcoal was rinsed with 10 ml of distilled water, and then removed from the filter and mixed with 5 ml of an ethanol-pyridine-water mixture (1:0.2:1 v/v) for 90 min at room temperature to elute nucleotides. The charcoal suspension was then filtered on Celite and the filtrate was evaporated to dryness under vacuum at 30°C. The residue was dissolved in 0.2 ml of distilled water. This volume was too large for thin layer chromatography; it was fractionated in drops of 20 μl, placed side by side along the starting line. The chromatograms were made on polythene sheets precoated with polyethyleneimine (MN Polygram cell 300 PEI, from Macherey and Nagel, Düren). Before use, the sheets had been washed 24 hours with 1.0 M HCl, and rinsed thoroughly with distilled water. The chromatograms were developed 15 min in 0.1 M HCl. The observed Rf's were:

ATP = 0	Orthophosphate = 0.48
ADP = 0.15	Pyrophosphate = 0.15
AMP = 0.48	Metaphosphate = 0
IMP = 0.63	

The sheets were dried and examined in U.V. light (260 mμ). The presence of ATP and ADP was clearly ascertained in muscle extracts, but we never saw either AMP or IMP. The ATP and ADP spots were cut out and transferred directly into separate counting vials (series I). In series II, ATP was eluted with 2.0 ml of 0.4 M HCl before counting. At the end of this two step procedure, a very good separation of ATP and P_i was obtained as revealed by the blanks experiments made routinely through the entire course of the research. In these blanks experiments, the whole procedure described in this method section was made without any bundle in the capillary tube. In these conditions, the radioactivity of the spot of ATP was only 3.10^{-5} times that of the P_i initially present in the extract. The drawback of the method was a loss of ATP: the recovery was usually 20–30%, but in some cases, it was as low as 5%.

In a few experiments, detection of AMP and IMP was further attempted by cutting out a piece of the chromatograms at the level to which these nucleotides would have migrated, eluting them in normal HCl, and measuring the absorption of the eluate at 260 mμ or 248 mμ (IMP). No AMP nor IMP were detected. Therefore, in our experimental conditions, the activity of myokinase and AMP-deaminase were negligible.

The ^{32}P radioactivity of ADP was measured in all the experiments, and its ^{14}C activity was measured in the experiments of the second series.

These activities were equal to those of the blanks. These results confirm the absence of a sizeable myokinase activity, and, furthermore, demonstrate that hydrolysis of ATP does not occur to a sizeable extent during the purification procedure.

d. *Radioactivity assays*

Counting vials contained 10 ml of a liquid scintillator made of 8.25 g of diphenyloxazole and 0.15 g of 2,2′-p-phenylene-bis-4-methyl-5-phenyloxazone dissolved in 1.0 l of toluol to which 0.5 l of Triton X 100 had been added. ^{32}P and ^{14}C were counted simultaneously with a Tri-Carb Packard spectrometer with efficiency of 76 and 80% respectively. ^{32}P was counted up to 5000 counts to have the same standard deviation for all countings. For each experiment, an aliquot of the contraction solution was also counted for determination of the specific activity of P_i. The amount of P_i "incorporated" into ATP, for a given bundle, was calculated as follows:

$$P(\times 10^{-12} \text{ moles}) = \frac{\text{(radioactivity of the ATP spot, in cpm)}}{\text{(specific activity of } P_i\text{, in cmp}/10^{-12} \text{ moles) (fraction of ATP recovered)}}$$

RESULTS

SERIES I. The Incorporation of P_i into ATP and its Activation by the Stretch

a. *Design of the experiments*

The first series of experiments was designed to see whether stretching a contracted bundle in the presence of $^{32}P_i$ and ADP, produces $AT^{32}P$. In case of positive finding, the correct interpretation of the results requires adequate control experiments. It has been reported that myofibrils (Ulbrecht and Ulbrecht, 1957) and actomyosin (Dempsey, Boyer and Benson, 1963) incubated with $^{32}P_i$ and ATP did not show any incorporation of radioactive phosphate into ATP. We considered that we could not rely on these results only, and that we had to design control experiments more closely related to the experimental conditions of our stretch experiments. This has been achieved by releasing the muscle bundle during contraction instead of stretching it. Experimental factors like origin and size of the bundles, extent and speed of the movement and extraction-purification procedures of ATP were identical for the stretched and released muscles, the only difference being the direction of the movement.

Six to ten bundles were handled in one experiment; half of them were stretched and the other half were released; two blanks experiments (see Methods) were performed at the same time. Bundles which broke during the stretch were rejected. The results presented below were obtained with 39 bundles (19 stretched and 20 released).

b. *Choice of the mechanical parameters*

Absorption of negative work has been observed in intact muscles which were fully activated and slowly stretched. These two conditions are important, and they should be respected in glycerinated fibres as well.

Activation of glycerinated fibres is obtained by ATP (contraction solution), it is a rather slow process limited by the diffusion of ATP into the fibres. We assumed that activation was completed when the isometric tension developed by the bundle was maximum. In preliminary experiments, we found that 8 to 12 sec were needed to get the full tension. Therefore, as a standard procedure, the stretch began after an initial isometric phase of 10 sec.

The velocity of the stretch is the second critical point: myothermic and biochemical studies have shown that absorption of negative work is maximal when this velocity is low (about 0.2–0.4 cm/sec, for sartorius muscle at 0°C). Most probably those velocities are not adequate for glycerinated fibres as well because enzymic activities are much depending on the diffusion of the substrates. This difficulty was solved by analogy. When we say that a muscle is "slowly" stretched, we mean that the velocity of stretching is low compared to the velocities with which the muscle can shorten; on the other hand, if a muscle is released at a velocity equal to the "slow" velocity of stretch (*i.e.*, 0.2 to 0.4 cm/sec), the force maintained during the shortening is a large fraction (50 to 70%) of the maximal isometric tension. We may reason, by analogy, that the optimum velocity of stretch for glycerinated fibres should equal that velocity of shortening at which the bundle maintains a force equal to 50–70% of the maximal isometric tension. It was found equal to 0.3 mm/sec in trial experiments and therefore this velocity was chosen for our experiments. The same velocity was used for the released control fibres. Original records are shown on Figure 2. The magnitude of the movement was 1.0 mm, or about 10% of the length of the bundle. The average sarcomere length was about 2.4 μm before con-

traction; it was not measured at the end of the movement.

c. *The mechanical response*

Typical mechanograms of a stretched and a released bundle are shown in Figure 2. The maximal tension at the end of the initial isometric phase was around 550 mg, *i.e.*, about 1.5 kg/cm^2. During the movement, tension increased during a stretch while it dropped below the isometric level during a release. In many cases, such as these illustrated in Figure 2, freezing of the muscle occurred 1 to 1.5 sec after the end of the movement (see METHODS) at a time when the high tension of the stretched muscle has already decreased somewhat, and when

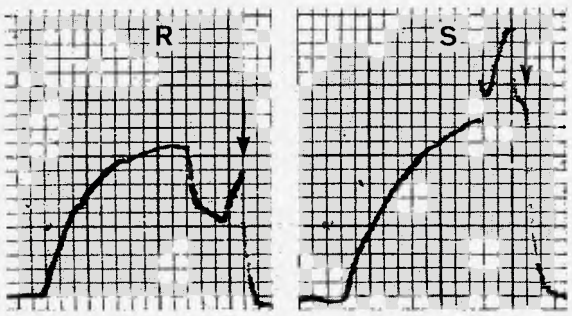

FIGURE 2 Actual mechanical records of the force developed by two different bundles. The isometric phase was followed either by a release (R) or by a stretch (S). The time of freezing is indicated by arrows; tension changes recorded after the arrows are artefacts due to the freezing. Scale: one large square is 5 sec (abscissae) or 190 mg (ordinates).

the tension of the released muscle has recovered. This is in fact very favourable for the absorption of the negative work by the contractile machinery: in intact muscle, an important fraction of the negative work is absorbed just after the stretch (Aubert, 1956) while the series elastic components are stretching the contractile system.

Table I shows the isometric tensions developed in the two groups of bundles before the movement, and the work done on or by the bundles during the movement. It is seen that the mean value and the standard deviations of the isometric tensions are similar in the two groups. This indicates that the amount of actively contracting material had been the same. As expected from the tension records, the work received by the stretched bundles was, in absolute value, much larger (1.6 times) than the work done by the released bundle. The same difference in the relative magnitude of the works, has been observed for intact muscles stretched or released at equal speeds (Maréchal, 1964).

TABLE I

Mechanical responses of glycerinated bundles

	Stretched n = 19	Shortened n = 20
Isom. tension (mg) ± S.D.	581 ±165	567 ±153
Work (g.cmx10^{-3})/ bundle ± S.D.	−72 ±18	46 ±16

The first two lines show the isometric force developed after 10 sec, just before the movement; the second two lines show the work done on or by the bundles during the movement.

d. *The incorporation of P_i into ATP*

The radioactivity of the ATP extracted from the muscle bundles was significantly higher than the blank values, but no significant increase could be detected in the radioactivity of the ADP. This observation will be referred here as "the incorporation of P_i into ATP". This expression is simply a description of the result and does not imply, so far, any assumption about its underlying mechanism. The mean values of this incorporation obtained from all the experiments of this first series are shown in Table II.

It is clear from the data of Table II that ATP extracted from the stretched bundles has a much higher radioactivity than the blanks and than the released bundles. This effect can be reasonably

TABLE II

Incorporation of $^{32}P_i$ into ATP

	Stretched (n = 19)	Shortened (n = 20)	Stretched in rigor (n = 3)	Blanks (n = 22)
Moles $P_i \times 10^{-12}$ incorporated /bundle (± S.E. of mean)	6.20 ± 0.90	3.20 ± 0.17	0.60 ± 0.23	1.17 ± 0.15
Difference Stretched—Shortened	3.00 ± 1.16 (0.02 < P < 0.01)			

The glycerinated bundles contracted in a solution containing 0.1 M KCl, 1.0 mM $CaCl_2$; 7.0 mM Tris HCl, pH 7.1; 0.1 mM dithiotreitol; 1.67 mM sodium phosphate containing ^{32}P at a specific activity of 20 mC/mmole; 5 mM Mg-ATP and 1.0 mM ADP. The bundles stretched in rigor were in the same solution; but without ATP. In the "blanks", the bundles were omitted (but not their nylon attachment).

ascribed to stretch because, except for the direction of the movement, all the other experimental factors were equal in the two groups as a result of the design of the experiments. This conclusion rests on the assumption that the specific activity of ^{32}P was constant in all cases. We shall examine this assumption in the discussion, after the data of series II (to which this limitation also applies) have been shown.

Fibres kept in rigor were also incubated in the presence of $^{32}P_i$ and stretched. In this case, carrier ATP was added to the cartridge after the denaturation of the muscle extract. In these conditions, no increase of the radioactivity of ATP above the blanks values could be detected (Table II). One can conclude that, in rigor, P_i is not incorporated into the acidosoluble protein bound nucleotides (see Janke, 1968) and that no incorporation occurs during the extraction-purification procedures when ATP and denaturated actomyosin are in contact for a long time.

A preliminary account of these results has been already presented (Gillis and Maréchal, 1969). They have been confirmed by Mannherz (1970) and by Ulbrich and Rüegg (1971). These authors used insect fibres and observed a significant P_i incorporation into ATP for isometric contractions; ATP radioactivity was markedly increased by stretch. This independent confirmation of our results is particularly important because different preparation and method have been used.

These convergent evidences clearly demonstrate that stretching a contracted glycerinated fibre increases the incorporation of P_i into ATP. Does it follow that ATP is resynthesized during a stretch, at the expenses of the mechanical energy received by the muscle from the outside world? We think that two main objections must be answered before drawing a definite conclusion of that kind:

1) The observed incorporation could be an exchange reaction between P_i and the terminal phosphate of ATP occurring in mitochondrial fragments which are still present in aged glycerinated fibres (Lardy, Johnson and McMurray, 1958).

2) The incorporation of P_i was also observed in the released muscle, though to a lesser extent. This result was rather unexpected as no incorporation had been detected with myofibrils and actomyosin (Ulbrecht and Ulbrecht, 1957; Dempsey *et al.*, 1963). As the released muscle has not received any energy but, to the contrary, has produced mechanical work the observed incorporation cannot be explained by a synthesis of ATP in the frame of the "reversal" hypothesis presented in the introduction. It follows that a sound interpretation of the stretch effect is not straightforward and must be delayed until the incorporation in the released muscle has been adequately explained.

Series II. The Influence of the Mechanical Tension on the Incorporation of P_i into ATP

The analysis of the results of the first series of experiments left some doubts as to the right way to interpret the effect of the stretch on the incorporation of P_i into ATP. As the tension during the stretch was much larger than during the release (Figure 2), one may wonder whether the tension as such could influence the magnitude of the incorporation.

a. *Design of the experiments*

Mechanical parameters. In the former experiments, the parameters of the contraction were the same for all fibre bundles, except for the direction of the movement. By contrast, a wide variety of mechanical conditions and of durations of contraction were used in this second series. The purpose was to obtain a large range of tension in order to correlate quantitatively the mechanical output with the incorporation of ^{32}P into ATP. In a first group of nineteen bundles, contraction was isotonic with zero load (the fibre was not connected to the tensiometer and the bundle, attached at one end, was freely floating in the capillary tube). For the other sixty-nine bundles, the contraction was isometric for the first 10 sec (*i.e.*, similar to those of series I); the tension reached a plateau; the bundles were then either stretched ($n = 17$) or released ($n = 18$) at various slow speeds (0.01 to 0.05 mm/sec), or they were maintained at the same length ($n = 34$); some bundles of the last case were mounted very slack so that the fibres contracted to about 65% of the "contraction" length (defined below) and produced low isometric tension. The duration of contraction was also varied for every bundle: contraction was stopped 13 to 40 sec after the beginning of the development of the tension. A schematic drawing of the various types of tension records, obtained in these experimental conditions, is shown in Figure 3. A quantitative analysis of the mechanical records requires that the observed tension is only the tension produced by actively contracting material. This was the case for isometric and released bundles, but there was a problem for the stretched ones. It is

possible that activation of glycerinated fibre bundles by external ATP solution is incomplete because of the limited rate of diffusion of ATP around and through the fibres (Hasselbach, 1952). Even when active tension is maximal, there may remain a rigid core of inactive fibrils surrounded by active ones. The relatively low tension obtained in our conditions(1.5 kg/cm^2) was a clear indication of the incomplete activation of the bundles, because single glycerinated fibres can developed as much as 4 kg/cm^2 (Weber, 1951) just like intact fibres (Lüttgau, 1963). Therefore an unknown fraction of the recorded tension might originate from the extension of myofibrils in rigor. In order to avoid this artefact, the procedure shown in Figure 4 was used. After dissection, the bundles were mounted with a resting tension of a few milligrams. The bundles were then straight: the length of the fibres, l_r, was the same as the distance between the points of attachment, L_r. Next, L_r was reduced by 1.0 mm (about 12%) to L_c. As l_r had not changed, the bundles were then slack. It was assumed that when ATP was perfused around the bundles, the activated fibrils shortened from l_r to a length l_c, equal to L_c, became straight and then developed tension at this "contraction" length l_c. On the other hand, myofibrils which were not activated by ATP, retained their original length, l_r, and remained slack. During the stretch, L_c increased to L_r; the rigid fibrils became straight again, but because their actual length had never changed, they made no contribution to the measured tension, which was produced only by the active fibrils extended from l_c to l_r.

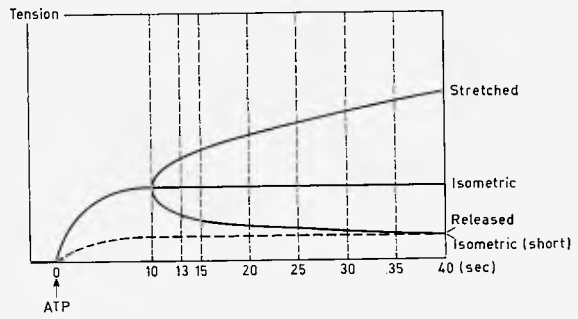

FIGURE 3 Schematic mechanical records for the various conditions used in experiments of series II. The contraction began with an isometric phase lasting 10 sec, either at a long or at a short length; the bundles were stretched or released at various speeds (of which only one is shown for each); the contraction was arrested after 13 to 40 sec by perchloric acid, at the times shown by the dashed vertical lines. Some bundles contracted isotonically under zero load.

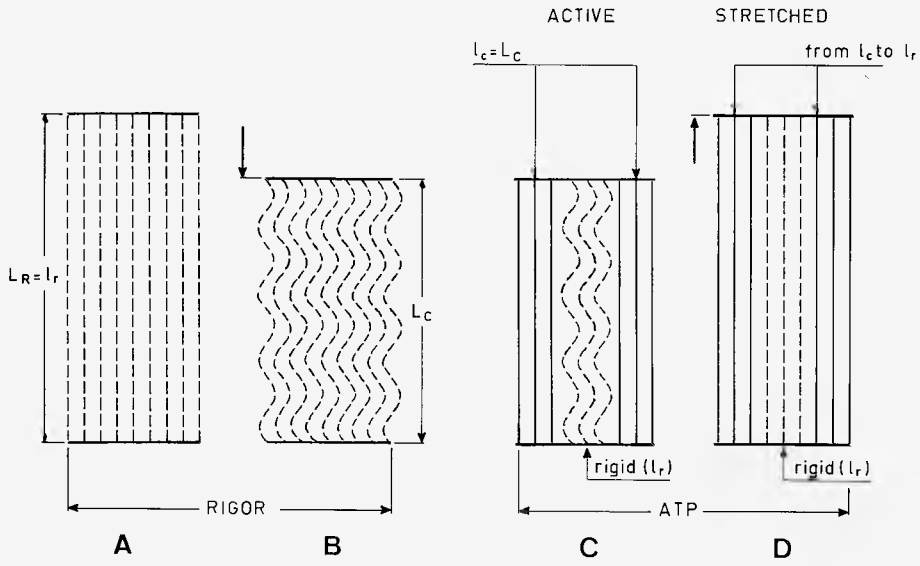

FIGURE 4 Schematic drawing of the length changes of bundles fibres during a stretch; dashed lines: fibres in rigor; continuous lines: fibres in contraction. A. Bundle in rigor at length L_R; the length of the fibres in the bundle, l_r, is equal to L_r. B. Bundle in rigor shortened to length L_C; the length of the fibres in the bundle is however still l_r. C. Bundle contracts in an ATP solution; the fibres in the periphery contract to the length l_c, equal to L_C; the fibres in the central core do not contract, and they remain in rigor, at length l_r. D. Bundle is stretched: the active fibres are stretched from length l_c to l_r; fibres of the rigid core are brought back to length L_R; however their length l_r does not change. Therefore, the tension measured during the stretch arises only from the active fibres.

Use of metabolic inhibitors. After dissection, the muscle bundles were soaked, for 45 min at room temperature, in solution (1) (see methods) containing 10 mM sodium azide and 2 mg/l oligomycin in order to inhibit a possible ATP-P_i exchange reaction catalysed by mitochondrial fragments in the glycerinated fibres as was discussed at the end of series I.

b. *The relation between the incorporation of P_i into ATP and the mechanical response*

The amount of P_i incorporated into ATP for sixty-nine bundles has been plotted versus the duration of the contraction (Figure 5). First of all, it is clear that the inhibitors (azide + oligomycin) have not suppressed the P_i incorporation; this phenomenon is not explained by an exchange reaction in mitochondrial fragments. Although, the points are widely scattered, the trend of the incorporation to increase with time can be clearly observed. It is also clear that the incorporation was much higher for the bundles which develop tension than for those which shortened isotonically with zero load (crosses). This observation indicates that tension may perhaps influence the incorporation. Therefore, the analysis of the results was made using the hypothesis that the incorporation was *simultaneously* depending on tension and on duration of contraction. In a first step we have studied the relationship between the incorporation and the magnitude of the tension-time integral measured on the tension records. In Figure 6 each result of P_i incorporation, already shown in Figure 5, has been replotted versus the tension-time integral of the corresponding contraction. It is clear from Figure 6 that the incorporation of P_i and the tension-time integral are linked by a positive relationship which seems the same for the various types of contraction. A regression analysis has been performed on the results from the stretched, released and isometric contractions. The regression equation and its parameters are shown in Table III. The data from isotonic unloaded contractions were not used in this regression analysis. We thought that the significant shortening of these bundles and the change of their shape had modified the diffusion conditions for ATP and ^{32}P. It was therefore possible that the magnitude of the P_i incorporation was affected by these changes, so that it was not safe to use these results together

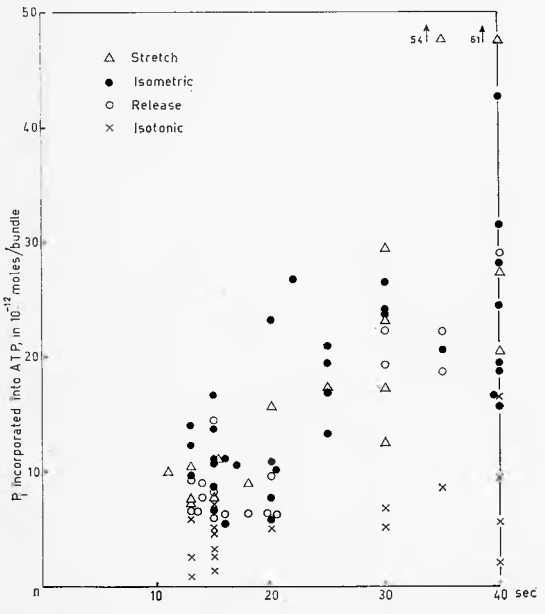

FIGURE 5 $^{32}P_i$ incorporated into ATP for all the bundles used, as a function of the duration of the contraction. X: isotonic with zero load; ●: isometric; △: stretched fibres; ○: released fibres.

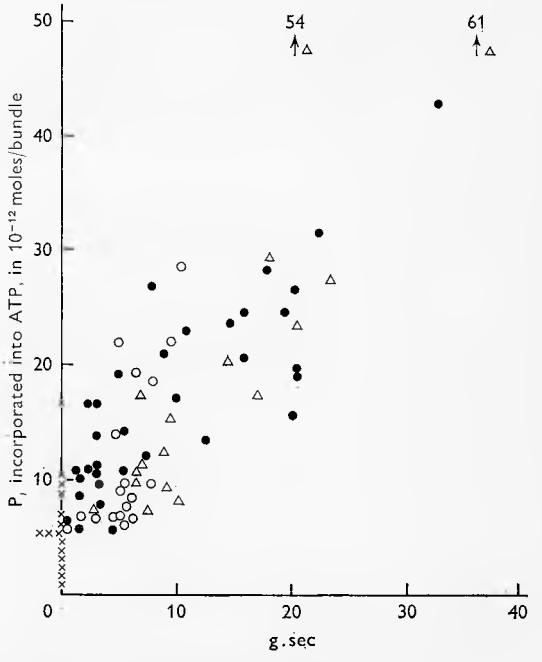

FIGURE 6 $^{32}P_i$ incorporated into ATP for all the bundles used, as a function of the integral tension-time of the contraction. X: isotonic with zero load; ●: isometric; △: stretched fibres; ○: released fibres.

with those from the other types of contraction in the same statistical analysis. Nevertheless, it is interesting to note that the mean values of the incorporation observed in isotonic contraction was 6 pmoles P_i/bundle while by the regression analysis it is predicted that the incorporation should be 5.8 pmoles when the tension-time integral is zero.

TABLE III

Analysis of the incorporation of P_i into ATP (I) as a function of the tension-time integral (S)

	I_0	b
All data n = 69	5.77	1.11±0.09
Restricted data* n = 27	6.86	0.95±0.24
Isometric n = 34	8.86	0.84±0.09
Release n = 18	1.20	1.88±0.51
Stretch n = 17	−1.19	1.58±0.22

The data were analysed by a regression analysis of the type $I = I_0 + bS$; I is the incorporation of $^{32}P_i$ into ATP (in pmole/bundle); I_0 is the incorporation for a zero tension-time integral; S is the tension-time integral. The regression coefficient b has the dimension pmole $P_i \cdot g^{-1}$, sec^{-1} per bundle. On the first line, all the data were used for the regression. On the second line, the regression was computed using the results from bundles whose weight was within 9.5% that of the mean weight of the bundles (restricted data, see text). In the three following regression analyses, the data were segregated according to the treatment given to the bundles.

The apparent importance of the tension for the magnitude of the incorporation which can be deduced from the Figure 6 may however be fallacious. One could argue that tension is an index of the amount of contractile material involved, so that this amount, and not the tension as such, is the true important factor. The fact that the size of the bundles was standardized by dissection is a partial answer to this objection. As already discussed for the results of the first series of experiments, another acceptable way to check the homogeneity of the bundles is to measure the maximal isometric tension after the first 10 sec of contraction, before the onset of the movement (Figure 3). The distribution of the isometric tensions recorded at this moment of the contraction is shown in Figure 7. It is seen that 47.3% of all the measurements, are contained in the central bloc whose limits are only ±9.5% of the mean (525 mg). We assumed that the amount of active contractile material was similar for the bundles of this bloc and the regression analysis of the P_i incorporation on the tension-time integral was calculated again with the results from this more homogeneous group of bundles. The parameters of the new equation are shown in Table III ("restricted data"); they do not differ from those previously calculated with the whole set of data.

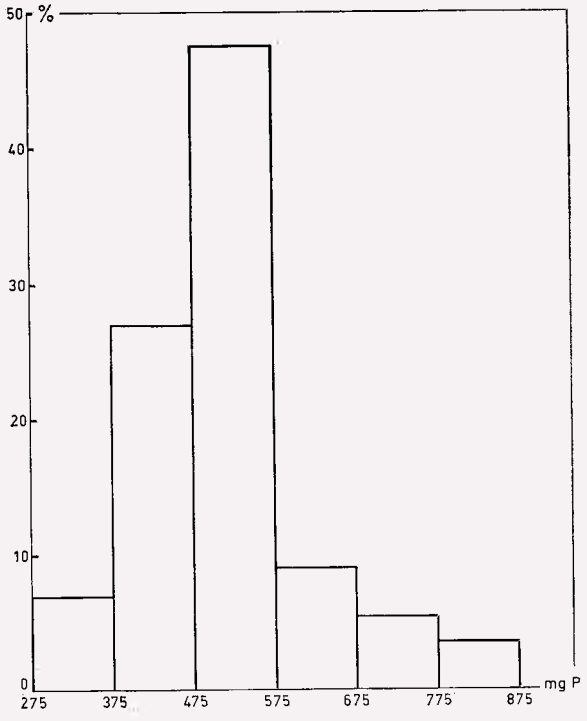

FIGURE 7 Histogram of the force (P, in mg) developed by the bundles in an isometric contraction lasting 10 sec. Stretches and releases were preceded by such an isometric phase, all the bundles (n = 69) were used for this figure, with the exception of those where the contraction was isotonic with zero load.

Finally, the same analysis was performed separately on the data from the stretched, released and isometric conditions. The parameters of the equations are also shown in Table III. This separation of data in three groups increased the residual error for each regression, so that the intercepts, though looking different, are not so on a statistical basis. For the stretched and the released bundles, *i.e.*, in cases of contractions with movement, the slopes (b) are similar, but they are significantly different from that calculated for the isometric contractions.

The relationship between the P_i incorporation and the tension-time integral provides a likely explanation for the results of the first series of experiments. The difference between the incorporation of the stretched and the released bundles (Table II) can probably be explained by the difference of the tension-time integral of the two groups of bundles (Figure 2). The effect of the stretch is thus not a specific one but is due to the high tension obtained.

At this stage of the analysis, the observations which indicate that tension influences the P_i incorporation are the following: (i) the relationship between the incorporation and the tension-time integral, (ii) the results of the first series of experiments which showed that for contractions of equal duration, the higher P_i incorporation was found for the bundles which developed the higher tension, (iii) the low incorporation for the bundles which contracted without tension (isotonic contraction with zero load). Nevertheless, a definite demonstration that tension *per se* had an influence on the incorporation can be obtained by the second step of the analysis of the results where the method of multiple variables regression analysis was used. It was assumed that the P_i incorporation was determined by two variables, tension and time, whose effect simply add up, according to the linear equation $I = aP + bt$, where I is the incorporation, P, the tension, t, the time, and a and b are the regression coefficients. Therefore, a significant coefficient for P would clearly demonstrate the role of the tension.

As tension was continuously changing during contractions with stretch or release, the mean tension developed during the whole contractions was used for calculation; it was obtained by dividing the tension integral already measured by the total duration of the contraction. The same procedure was used for the isometric contractions.

The analysis of the regression of the P_i incorporation on the mean tension developed *and* on the time of contraction, was made with the sixty-nine results from the stretch, released and isometric contractions; it yielded the following equation:

$$I = 21.1(\pm 4.0)P + 0.63(\pm 0.09)t$$

where I is in pmoles P_i/bundle; P, in g and t, in sec. The standard errors of the coefficients are shown within brackets. As both regression coefficients are highly significant (<0.001), it can be concluded that tension and time are each determining factors of the P_i incorporation.

DISCUSSION

Our results show that ^{32}P is found in ATP extracted from glycerinated fibres which contract in the presence of ATP, ADP and P_i with $^{32}P_i$ as a tracer. This incorporation is proportional to the mean tension supported by the fibre and to the duration of the contraction.

a) The incorporation of P_i into ATP: a fact or an artefact?

The radioactivity recovered in the purified ATP might be due to several factors as, for example, the incomplete separation from P_i or the spontaneous occurrence of phosphate compounds having a chromatographic behaviour similar to ATP, or even a synthesis of ATP in bacteria contaminating the solutions. All these possible artefacts were taken into account in the *blanks* experiments which show a very low incorporation, far below the level observed in the presence of muscle fibres.

i) The *site* of the incorporation might be located in the myofibrils, the mitochondria, or in the sarcoplasmic reticulum. Exchange reactions between the terminal phosphate of ATP and P_i have been observed in mitochondria. These reactions are blocked by oligomycin and azide (Lardy *et al.*, 1958). As these inhibitors did not affect the P_i incorporation in the experiments of Series II, mitochondria can be excluded as a site of incorporation in our conditions.

Synthesis of ATP can occur in the sarcoplasmic reticulum when calcium is discharged along the concentration gradient (Makinose, 1971). Preloading of the sarcoplasmic reticulum with Ca is thus required and hence an undamaged structure. This is probably not the case here after a prolonged glycerol-water extraction. This synthesis is inhibited by prenilamine (Makinose, 1971) and it has been reported to us by Miss M. Ulbrich and Prof. J. C. Rüegg that prenilamine does not affect the P_i incorporation in glycerinated insect fibres. Therefore the sarcoplasmic reticulum can be also excluded as a possible site.

Also of course, it is rather difficult to see how any reaction catalysed by mitochondria or sarcoplasmic reticulum could depend on the mechanical response as we have found here.

In conclusion, the P_i incorporation into ATP, if genuine, occurs inside the contractile apparatus.

ii) The *magnitude* of this incorporation however is very small; it does not represent more than 10^{-4} times the amount of $^{32}P_i$ present in the bundle. Such a small effect requires a high degree

of separation of ATP and P_i. Failure to achieve it may explain the negative results of Ulbrecht and Ulbrecht (1957) and Dempsey *et al.* (1963). Is this smallness genuine or is it due to experimental conditions foreign to the fibres, like those discussed below?

First, the incorporation is probably not limited by the small number of P_i molecules available at the catalytic sites, since the estimated P_i/myosin ratio inside the bundle is about 20. The same conclusion applies for ADP whose concentration was close to that of P_i.

The next problem concerns *the specific activity of* ^{32}P inside the fibres. We have assumed it to be the same as that of the external solution (see METHODS). As this assumption is very critical for a correct estimation of P_i incorporation into ATP, it must be carefully discussed. The specific activity of $^{32}P_i$ inside the fibres was certainly equal to that of the solution before contraction when the fibres were equilibrated for a few minutes in the radioactive solution, before admitting ATP. But during contraction the internal specific activity certainly fell because of the dilution of the radioactive $^{32}P_i$ by the unlabelled $^{*}P_i$ produced by the ATPase activity. The largest error introduced by neglecting this factor can be estimated in an extreme situation: if all the P_i produced during 40 sec of contraction remains in the bundle, its concentration would rise to about 10 mM, *i.e.*, about six times, leading to corresponding underestimation of the P_i incorporation. Obviously, the real increase was much lower because P_i diffused out into the surrounding solution. Although an exact figure is difficult to evaluate, it is clear that the correction for this concentration effect will not change the order of magnitude of the result. The incorporation is thus a very small effect and the conclusion drawn from this fact (see b, (ii) below) remains valid in spite of the imprecision inherent in the results.

It is also important to examine how the assumption of equal internal and external specific activities affects the comparison between the P_i incorporation observed in various mechanical conditions. The P_i produced could have been different for the various types of experiments, and therefore, the dilution of the radioactive tracer could have varied accordingly. The consequences of neglecting this possible effect can be roughly estimated: in Series I, the largest part of the contractions occurred in identical mechanical conditions: a 10 sec isometric contraction. Stretches or releases were applied only during the next 3 sec. Therefore, even if the P_i production stopped in one case (say the stretch one), the difference of the local P_i concentration between the two types of bundles would be about 15%. This effect is far too small to explain the difference of P_i incorporation actually observed. In Series II, an error would affect the present values of the slopes of the P_i incorporation versus the tension-time integral, so that only semi-quantitative comparisons between the results of the various types of experiment will be valid. On the other hand, this error would not invalidate the conclusion that, for each mechanical condition investigated, a P_i incorporation has been found which depended on time and tension.

The importance of this dilution effect on the specific activities was certainly reduced by diffusion as already discussed. It remains to see how the diffusion process itself might have been specifically affected by the various experimental conditions: factors like change of the diffusion path and "stirring" effects produced by stretch or release movements must be considered.

In contractions where either stretches or releases were applied, the radii of the fibres were consequently modified. Diffusion processes in or out cylindrical objects depend on the square of the radius (r) (Meyerhof, 1930). The relative change of this factor to the relative change of the fibre length is given by

$$\frac{dr^2}{r^2} = \frac{dl}{l}$$

In our experiments, the largest value for dl was 0.1 (l: the isometric length). The differential effect of stretch and release on the diffusion process is thus at most 20%, a value too low to explain the results of Series I. Moreover, an increase of the P_i incorporation produced by stretch has been observed in glycerinated insect fibres where the length change was only 0.01 l (Mannherz, 1970; Ulbrich and Rüegg, 1971).

Contractions with movements might also produce some "stirring" of the solution in the extracellular space or even within the fibres themselves: equilibrium with the perfusion solution would then be facilitated. Our results provide perhaps a possible evidence of such an effect: the slopes relating the P_i incorporation to the tension-time integrals (Table III) are larger for contractions with stretch or release than for isometric conditions. But, noticeably enough, this increase depends only on the movement, not on its direction. Therefore, a

"stirring" effect is probably not involved in the difference of the P_i incorporations in the stretched and released fibres of Series I.

It is therefore clear from the above discussion, that the assumption about the internal specific activity of $^{32}P_i$ is acceptable provided that the results are considered as a first approximation of the actual incorporations. Nevertheless, this restriction does not affect the main conclusions of the paper as they are presented below.

b) The significance of the P_i incorporation into ATP.

The aim of these experiments was to check the "reversal hypothesis" (see Introduction) as an explanation of the reduced use of ATP by a muscle forcibly stretched during contraction.

The incorporation of P_i into ATP which was found in these conditions cannot be taken as a support for this hypothesis for the following reasons:

i) the incorporation occurs also in isometric (see also Ulbrich and Rüegg, 1971) and isotonic conditions. It is proportional to the force the muscle bears, whatever the sign of the length change. Stretch and hence free energy coming from outside is not the important factor but the stress applied on the structure;

ii) There is a very large discrepancy between the economy of ATP observed in intact stretched muscle and the amount of P_i incorporated found in our stretch experiments: in Series I, the differences in $^{32}P_i$ incorporation between stretched muscles and released ones was 3 pmole/P_i/bundle. Assuming -11 kcal/mole for ATP hydrolysis, the synthesis of 3 pmoles of ATP increases the energy stores of the muscle by 34.10^{-9} cal. The free energy received by the muscle during stretch was 72×10^{-3} gcm, or 1.68×10^{-6} cal. The efficiency (chemical energy stored/mechanical energy received) equals 0.02: it is much lower than that observed in intact muscle where it can amount up to 0.5–0.9 (Maréchal, 1964).

As far as the "reversal hypothesis" is concerned the conclusion of this paper is thus that neither our results support it nor do the biochemical studies (Maréchal, 1964; Infante *et al.*, 1964; Maréchal and Beckers-Bleuckx, 1965). The only evidence remains the myothermic data of Hill and Howarth (1959), which have never been repeated. Moreover, our own results indicate that if some ATP resynthesis occurs, it is a minor feature, much too small to account for the marked effect of stretch on the intact muscle content of high energy phosphate compounds. The alternative hypothesis, that stretch reduces the ATPase activity of actomyosin (inhibition hypothesis) remains thus a more likely explanation for the reduced energy expenditure of stretched muscles.

However, the demonstration of a P_i incorporation into ATP during contraction might give some information about the mechanism of the actomyosin-ATPase. Present reaction schemes (see Taylor, 1972) incorporate a step where the terminal phosphate group is detached from ATP and which is considered as irreversible. Our results might indicate that a reverse reaction is present. Moreover, by comparing P_i incorporation with P_i production one can have a rough idea of the ratio of the two reaction rates: for a bundle of 10 fibres (about 60 μg dry weight) the ATPase activity should have split 2 to 20 nmole ATP for an average contraction of 20 sec, while in the same conditions, P_i incorporation amounts only to 20 pmoles, *i.e.*, 100 to 1000 times less. Even so our measurements give probably an underestimated value of the actual rate of P_i incorporation since any $AT^{32}P$ formed in a contracting fibre will have a certain probability to be split shortly after its formation. The importance of this factor is unknown.

The effect of tension on this P_i incorporation, though clearly demonstrated by our results, remains to be elucidated.

ACKNOWLEDGEMENTS

We are grateful to Prof. E. W. Taylor for his comments on the manuscript. We wish to acknowledge the excellent technical assistance of Mrs Claire Vercruysse-Meganck.

The expenses of this research were supported in part by "Le Fonds National de la Recherche Scientifique de Belgique".

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