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STUDIES ON THE BIOCHEMISTRY OF CONTRACTION AND RELAXATION IN GLYCERINATED MUSCLE

THE EFFECTS OF PHOSPHOENOLPYRUVATE

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INTRODUCTION

In 1949 SZENT-GYÖRGYI[1] introduced a new approach to the biochemical study of muscle by extracting whole fibers in cold 50% aqueous glycerol. With this procedure, the basic structure of the contractile apparatus remains essentially unaltered, while some constituents are removed. The effects of the removal of these constituents, as well as of returning them individually to the muscle, may be observed. SZENT-GYÖRGYI showed that rabbit psoas fibers extracted in the above manner contract

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upon the addition of adenosine triphosphate (ATP) in the presence of KCl and $MgCl_2$.

At first it appeared that this contraction could be reversed only under apparently non-physiological conditions such as the presence of salyrgan[2], pyrophosphate[2,3,4], urea[3], high concentrations of KCl[3,5], or abnormally high concentrations of ATP (greater than 10 to 20 mM)[3,6,7,8]. However, BOZLER[9] observed that with the addition of 20 mM phosphocreatine relaxation occurred even at physiological concentrations of ATP (about 5 mM) which alone would produce only sustained contraction. In independent studies of the relaxing effect of phosphocreatine GOODALL AND ANDREW G. SZENT-GYÖRGYI[10] were able to demonstrate repeated contraction–relaxation cycles by varying the pH between about 6.6 and 6.2 in the presence of 10 mM phosphocreatine and 4 mM ATP. These authors also observed that if bicarbonate was added to the glycerol extraction medium, the ability of the fibers to relax with phosphocreatine was destroyed in a few days, but it could be restored by adding a protein-like substance obtained from extracts of muscle. This factor was soon identified by LORAND as ATP-creatine transphosphorylase[11]. His finding supported the idea, considered earlier in various forms[9,12,13], that as part of the relaxation process, adenosine diphosphate (ADP) must be rephosphorylated.

A parallel line of investigation into relaxation was initiated by MARSH [14,15], who observed that a muscle extract causes the sediment of a centrifuged muscle brei to swell in the presence of ATP. This extract was shown by BENDALL[16,17] to bring about relaxation in glycerinated muscle fibers after isotonic contraction in ATP. Furthermore, he noted that if the fibers had been extracted only briefly, a spontaneous relaxation followed the ATP-induced contraction, even without the addition of the above muscle extract. Later[18], he presented evidence that the relaxing effect of the muscle extract was due to myokinase, and he attributed the spontaneous relaxation of briefly-extracted fibers to intrinsic myokinase. These observations further strengthened the hypothesis that rephosphorylation of ADP is involved in relaxation, and therefore it was proposed by LORAND AND ANDREW G. SZENT-GYÖRGYI to investigate the pyruvate phosphokinase system, which catalyses the reaction:

$$\text{ADP} + \text{phosphoenolpyruvate} \rightleftharpoons \text{ATP} + \text{pyruvate}.$$

The results of the present work show that the addition of phosphoenolpyruvate (PEP) does indeed cause relaxation of glycerinated muscle fibers after development of isometric tension in 4 mM ATP. On the other hand, PEP appears to play a dual role, since, at lower ATP concentration, it promotes contraction.

A brief account of some of this work has already been published[19].

MATERIALS AND METHODS

Glycerinated psoas

Fibers were prepared by the method of SZENT-GYÖRGYI[1] by extracting the two psoas of a rabbit in about 500 ml of 50% glycerol solution at 0° C for 2 days. They were stored in the deep freeze at —20° C in a smaller volume of 50% glycerol. For use in experiments these bundles of "stock fibers" were then further divided, in a dish of cold 50% glycerol, into strips ¼ to ½ mm in diameter. Where further washing was employed, groups of these smaller fibers were tied to glass rods and placed in the appropriate extraction medium in test tubes held horizontally on a mechanical shaker.

Contraction–relaxation experiments

The tension developed by the muscle fibers was measured by means of an isometric lever and recorded on a kymograph. (The figures presented in this paper were enlarged from kymograph

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records.) The lever system was of such dimensions that a fiber 3.5 to 4.0 cm in length shortened about 0.5% for each gram of tension developed. Fibers were mounted horizontally and could be immersed in a shallow bath. The solution was stirred by a vibrating glass rod, and small electrodes at one end permitted verification of the pH of the medium.

In all cases except where otherwise noted, the 5 ml bath contained 0.1 *M* KCl, 4 m*M* $MgCl_2$, and 0.01 *M* NaH_2PO_4-Na_2HPO_4 buffer at pH 7.0. Generally, the final concentration of ATP was 4 m*M*, and that of PEP was 10 m*M*. The bath was prepared with all reagents except ATP and PEP, and the fiber was then mounted on the isometric lever and immersed in the bath. All experiments were carried out at room temperature (22–25° C), and none lasted longer than about 15 minutes.

Preparation and assay of PEP

The silver-barium salt of PEP was synthesized from β-chlorolactic acid by the method of BAER[20], and the material was then converted to the sodium salt as described by KACHMAR AND BOYER[21]. The silver-barium salt was triturated in a slight excess of cold 1 *N* HCl, and an equivalent amount of 0.5 *M* Na_2SO_4 was then added. The suspension was centrifuged and the precipitate washed twice with small amounts of water. The pH of the combined supernatant and washings was adjusted to 7.0 with NaOH, and any traces of precipitate were filtered out. This solution was stored frozen at —20° C after dilution to a concentration of 0.20 *M*.

PEP preparations were assayed by determination of the liberated pyruvate and phosphate after hydrolysis with mercuric acetate[22]. A 1.0 ml aliquot of approximately 4 m*M* solution was mixed with 0.1 ml 1% mercuric acetate and 0.9 ml 0.2 *N* HNO_3. After standing at about 25° C for at least 5 minutes, 0.1 ml aliquots were assayed for inorganic phosphate and for pyruvate as described below. Using the digestion procedure recommended by LEPAGE[23], total phosphorus was also determined. A sample containing approximately 4 μmoles total phosphorus was heated with 0.4 ml 10 *N* H_2SO_4 in an oil bath at 130–160° C for 1 hour. Two drops of 30% H_2O_2 were added and the digestion mixture was returned to the oil bath for an additional 20 minutes. One ml of water was then added and the tube placed in a steam bath for 10 minutes, after which the solution was diluted to 2.0 ml with water, and 0.1 ml aliquots were assayed for inorganic phosphate as described below.

The sodium PEP solutions used in this work always gave values of total phosphorus and mercuric acetate-labile pyruvate and phosphate which agreed within 5%. Furthermore, elementary analysis of a sample of the silver-barium salt of PEP (kindly performed by Miss H. BECK of this department) showed the ratio of total carbon and phosphorus to be that expected for this compound, indicating the absence of organic contaminants.

Pyruvate assay

The dinitrophenylhydrazone method described by KACHMAR AND BOYER[21] was used for the estimation of pyruvate. A sample containing up to 0.4 μmoles pyruvate was diluted to 3.0 ml with water, and 1.0 ml of a solution containing 0.025% 2,4-dinitrophenylhydrazine in 2 *N* HCl was added. The tube was incubated 10 minutes in a 37° C water bath, after which it was removed, and 6.0 ml 0.5 *N* NaOH were added. The optical density was measured at 510 mμ after 5 to 10 minutes. The color is fully developed at this time and is relatively stable, declining only slightly in an hour. The method was standardized against redistilled pyruvic acid (b.p. 55–60° C at 8–10 mm Hg).

Inorganic phosphate assay

Phosphate was determined by a modification of the method described by KIRK[24], which is based on the butanol extraction procedure of BERENBLUM AND CHAIN[25]. A 0.1 ml sample, containing up to 0.3 μmoles inorganic phosphate, was added to 1.0 ml of a solution containing 1.25% ammonium molybdate in 0.75 *N* H_2SO_4. Then 0.5 ml *n*-butanol was added and the mixture shaken vigorously for a few seconds, after which 0.4 ml of the butanol layer was removed and mixed with 0.3 ml of diluted $SnCl_2$ solution (10 g $SnCl_2 \cdot 2H_2O$ in 25 ml conc. HCl, diluted daily with 200 volumes 1*N* H_2SO_4). To this mixture, 10 ml 95% ethanol were added, and after a few minutes the color was measured at 660 mμ. The blue color was stable for many hours. The procedure was standardized against KH_2PO_4.

Preparation of pyruvate phosphokinase

Pyruvate phosphokinase was prepared from rabbit muscle by the method of BÜCHER AND PFLEIDERER[26], which involves ammonium sulfate fractionation of a water extract of the muscle. The "crystalline" enzyme was dissolved in 0.2 *M* phosphate buffer, pH 7.0, and freed of ammonium sulfate by dialysis against 0.005 *M* phosphate buffer, pH 7.0. After filtration, the resulting solution was frozen and stored at —20° C. This preparation is referred to hereafter as the stock enzyme solution. Using the assay procedure described below, 1 ml of the stock enzyme would liberate about 2.5 millimoles of pyruvate per minute.

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Pyruvate phosphokinase assay

Pyruvate phosphokinase activity was determined by following the liberation of pyruvate from PEP in the presence of ADP[21]. The final reaction mixture contained 2.5 m*M* ADP, 0.15 *M* KCl, 5 m*M* $MgCl_2$, 1.4 m*M* PEP, 0.04 *M* tris(hydroxymethyl)aminomethane–HCl buffer, adjusted to pH 7.4 at 37° C, and 0.1 ml of the diluted enzyme sample, in a total volume of 0.5 ml. All components except the enzyme were combined and brought to 37° C in a water bath, and the reaction was then started by the addition of the enzyme. After agitation at 37° C for the desired length of time, 1.0 ml dinitrophenylhydrazine solution was added, together with 2.5 ml of water, and the estimation of pyruvate was carried out as described above. The enzyme concentration was chosen so that the reaction reached about half completion in 15 to 20 minutes. The reaction rate was usually nearly constant up to about 40% of completion. Blanks were run simultaneously using tubes in which the enzyme sample was replaced with water or boiled enzyme.

Total nitrogen

The Nesslerization procedure given by UMBREIT[27] was used for the estimation of total nitrogen. Samples containing up to 50 μg total nitrogen were digested with 1.5 ml 2*N* H_2SO_4 overnight in a sand bath at 150–200° C. Two drops of 30% H_2O_2 were then added, and the tubes returned to the sand bath for at least one more hour. After cooling, 3 ml of water, 3 ml of modified Nessler reagent[27] and 4.5 ml 2 *N* NaOH were added, and 15 minutes later the color was measured at 490 mμ. The method was standardized with $(NH_4)_2SO_4$, and cross-checked against a sample of crystalline serum albumin (Armour).

Other reagents

Solutions of crystalline disodium ATP ($Na_2H_2ATP \cdot 3H_2O$, Sigma Chemical Co.) and sodium ADP ($NaH_2ADP \cdot 2H_2O$, Sigma Chemical Co. or Pabst Laboratories) were prepared at a concentration of 0.05 *M* after neutralization with NaOH to pH 7.0 These stock solutions were stored at —20° C. All other chemicals were reagent grade, and the water used throughout was distilled water which had been passed through a column of Amberlite MB-3 ion-exchange resin.

RESULTS

Spontaneous relaxation with ATP

When stock fibers were used directly without further washing, the addition of ATP caused only partial development of tension, followed by a spontaneous relaxation which could be reversed by the addition of $CaCl_2$ (Fig. 1, 0 min). Even fibers stored for several months at —20° C sometimes showed this phenomenon, which appears to be similar to that observed by BENDALL[17] and attributed to intrinsic myokinase[18]. In order to study the effect of PEP, it was necessary first to obtain fibers which would not relax spontaneously, and this was accomplished by further washing of the stock fibers in 20% glycerol solution at room temperature (22-25° C). Fig. 1 shows the gradual loss of spontaneous relaxing activity with extraction of the fibers in 20% glycerol, and it can be seen that fibers washed for about one hour are suitable for the study of the relaxing effects of PEP. These fibers were used in most of the following experiments.

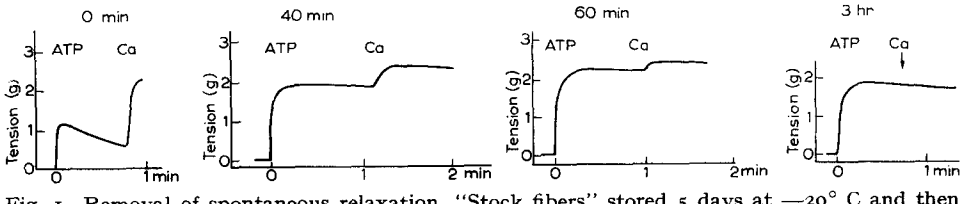

Fig. 1. Removal of spontaneous relaxation. "Stock fibers" stored 5 days at —20° C and then washed in 20% glycerol at ~25° C for the indicated times (0 min, 40 min, 60 min, 3 hr). Original bath contained 0.1 *M* KCl, 4 m*M* $MgCl_2$, 0.01 *M* Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0. At "ATP" 4 m*M* final conc. of ATP added. At "Ca", 1 m*M* final conc. of $CaCl_2$ added.

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Effect of PEP and Mg^{++}

If 10 m*M* PEP is added to the bath after such a fiber has developed tension in 4 m*M* ATP, a striking relaxation occurs, which can be reversed by the addition of a small amount of Ca^{++} (Fig. 2). The effect requires the presence of Mg^{++}; increasing the $MgCl_2$ concentration up to about 2 to 4 m*M* enhances relaxation. In the experiments described here, a concentration of 4 m*M* was used.

As shown in Fig. 3, about 10 m*M* PEP is required for the maximum relaxing effect, although some relaxation occurs with as little as 1 m*M*. Repeated contraction–relaxation cycles may be obtained by immersing the fiber alternately in two baths which are identical except that PEP is present in only one of them (Fig. 4). After the first few cycles, in which the fiber appears to become equilibrated in some way, there is no loss of tension from one contraction to the next.

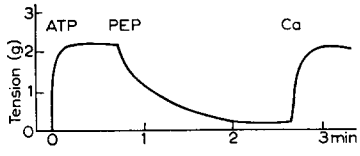

Fig. 2. Relaxation with PEP. "Stock fibers" stored 8 days at —20° C and washed 55 min in 20% glycerol at ~25° C. Original bath contained 0.1 *M* KCl, 4 m*M* $MgCl_2$, 0.01 *M* Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0. At "ATP", 4 m*M* final conc. ATP added. At "PEP", 10 m*M* final conc. PEP added. At "Ca", 1 m*M* final conc. $CaCl_2$ added.

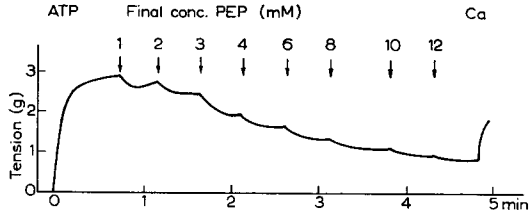

Fig. 3. Effect of PEP concentration. "Stock fibers" stored 17 days at —20° C and washed 1 hour in 20% glycerol at ~25° C. Original bath contained 0.1 *M* KCl, 4 m*M* $MgCl_2$, 0.01 *M* Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0. At "ATP", 4 m*M* final conc. ATP added. PEP then added in small increments, bringing final conc. to indicated levels. At "Ca", 1 m*M* final conc. $CaCl_2$ added.

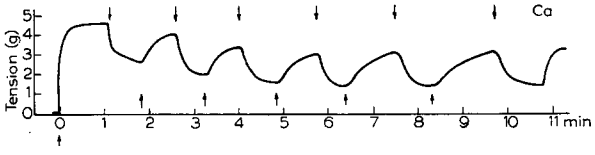

Fig. 4. Contraction–relaxation cycles. "Stock fibers" stored 14 days at —20° C and washed 1 hour in 20% glycerol at ~25° C. At ↑ fiber immersed in bath containing 0.1 *M* KCl, 4 m*M* $MgCl_2$, 0.01 *M* Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0, and 4 m*M* ATP. At ↓ fiber immersed in bath containing, in addition to the above, 10 m*M* PEP. At "Ca", 1 m*M* final conc. $CaCl_2$ added.

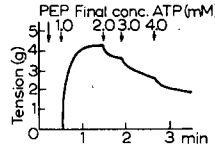

Fig. 5. ATP requirement for relaxation. "Stock fibers" stored 6 weeks at —20°C and washed 90 min in 20% glycerol at ~25° C. Original bath contained 0.1 *M* KCl, 4 m*M* $MgCl_2$, 0.01 *M* Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0. At "PEP", 10 m*M* final conc. PEP added. ATP then added in small increments, bringing final conc. to indicated levels.

ATP dependence

The relaxation requires a minimum ATP concentration of about 2 m*M*, and is enhanced by further increases in ATP concentration (Fig. 5). However, at very low levels of ATP, the addition of PEP has the effect of enhancing contraction. In fact, as shown in Fig. 6, at ATP concentrations which normally cause almost no contraction, the addition of PEP causes development of high tension. Utilizing these effects of ATP concentration, it is possible to obtain repeated contraction–relaxation cycles by

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immersing the fiber alternately in two baths, both of which contain 10 mM PEP, but only one of which contains 4 mM ATP. In such a case, presumably, the small amount of ATP needed for contraction in the presence of PEP is carried over with the fiber into the bath which initially lacks the nucleotide.

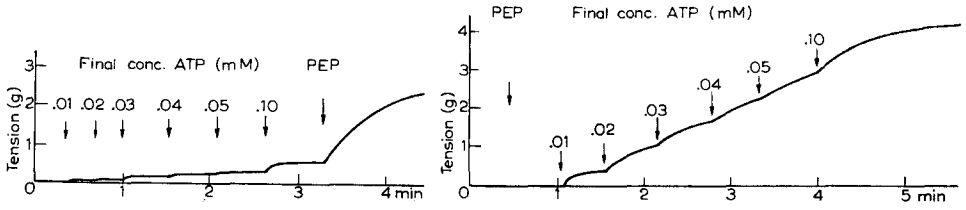

Fig. 6. Contraction with PEP at low ATP concentration. "Stock fibers" stored 6 weeks at —20° C and washed 90 minutes in 20% glycerol at ~25° C. Original baths contained 0.1 M KCl, 4 mM $MgCl_2$, 0.01 M Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0. ATP added in indicated increments of final conc. At "PEP", 10 mM final conc. PEP added. In left hand figure, ATP added before PEP as a control. In right hand figure, ATP added after PEP.

Calcium reversal

In the experiments reported here, the $CaCl_2$ concentration used to reverse the relaxation was 1 mM. The minimum concentration giving any observable effect is about 0.01 mM, and the extent of the recontraction increases with $CaCl_2$ concentration up to about 0.1 mM, after which further additions have no apparent effect (Fig. 7).

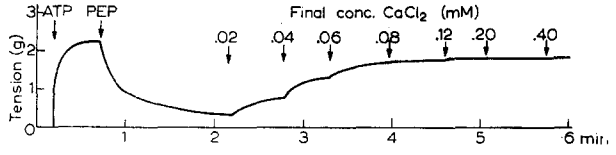

Fig. 7. Recontraction with calcium. "Stock fibers" stored 4 weeks at —20° C and washed 80 min in 20% glycerol at ~25° C. Original bath contained 0.1 M KCl, 4 mM $MgCl_2$, 0.01 M Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0. At "ATP", 4 mM final conc. ATP added. At "PEP", 10 mM final conc. PEP added. $CaCl_2$ then added in small increments, bringing final conc. to indicated levels.

Potassium requirement

In view of the possibility that PEP produces relaxation through the action of pyruvate phosphokinase present in the fibers and the fact that this enzyme has a requirement for K^+ [21], the effect of this cation on PEP-relaxation was studied. For this purpose, the bath was prepared initially with NaCl in place of the usual KCl, and the latter was added in small increments after contraction with ATP and subsequent addition of PEP (Fig. 8). It can be seen that in the absence of K^+ only a slight relaxation occurs, and small amounts of this ion bring about the normal course of relaxation, even in the presence of relatively large amounts (0.1 M) of Na^+.

Although the presence of K^+ is obligatory for relaxation, the effective K^+ concentration under these conditions varies considerably from fiber to fiber, over the range from about 2 to 20 mM, indicating that perhaps the fibers contain varying amounts of bound K^+. The K^+ content of the bath and the fibers was therefore estimated using a flame photometer. It was found that the initial bath contains no more than $5 \cdot 10^{-5}$ M K^+. The K^+ in the fibers was determined using homogenates

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directly, and also by dissolving fibers in a small amount of concentrated H_2SO_4 and assaying the clear solution after appropriate dilution. Calculating the fiber volume from wet weight, assuming a density of 1.0 g/ml the K^+ concentration within the fibers is roughly 2 to 10 m*M*. Since this is the same order of magnitude as the concentration of KCl that must be added to the bath for full relaxation, it may be supposed that the variations in the concentration of KCl that must be added to obtain full relaxation are due to the presence of variable amounts of bound K^+ within the fibers.

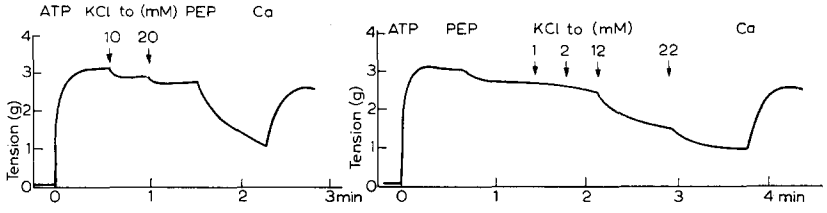

Fig. 8. Potassium requirement for relaxation. "Stock fibers" stored 14 days at —20° C and washed 1 hour in 20% glycerol at ~25° C. Original baths contained 0.1 *M* NaCl, 4 m*M* $MgCl_2$, 0.01 *M* Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0. At "ATP", 4 m*M* final conc. ATP added. At "PEP", 10 m*M* final conc. PEP added. At "Ca", 1 m*M* final conc. $CaCl_2$ added. KCl added in small increments, bringing final concentration to indicated levels. In left hand figure, KCl added before PEP as a control. In right hand figure, KCl added after PEP.

Effect of prolonged washing

If stock fibers are washed in 20% glycerol at room temperature (22–25° C) longer than the usual period of approximately one hour that is needed to remove the spontaneous relaxation as described above, the relaxation produced by the addition of PEP also gradually diminishes. This deterioration of relaxing ability usually starts after 1 to 2 hours of washing and, while it sometimes proceeds slowly with washing periods as long as 24 hours, in other cases it is complete after 4 to 6 hours. A similar effect is obtained with washing in 0.1 *M* KCl containing 4 m*M* $MgCl_2$, at room temperature.

An effort was made to restore the relaxing ability of these extensively washed fibers at all stages of the washing, by the addition of purified pyruvate phosphokinase. Stock enzyme solution was added in amounts of 0.1 to 0.5 ml to the bath at the beginning of the experiment, and the fiber was incubated in this mixture for 10 minutes with stirring before the addition of ATP. It was found that the added enzyme did not consistently cause any appreciable restoration of relaxing ability after washing either in 20% glycerol or in 0.1 *M* KCl–4 m*M* $MgCl_2$.

Intrinsic pyruvate phosphokinase activity

Experiments were then carried out to determine whether pyruvate phosphokinase activity is present in fibers which relax with PEP, and whether the loss of relaxing ability with washing is associated with a loss of pyruvate phosphokinase activity. A series of fibers, washed in 20% glycerol at room temperature for varying times, was tested for relaxation with PEP; each fiber at the end of the experiment was briefly washed in water to remove the bath reagents, and was then homogenized in water with a glass homogenizer. The suspensions were assayed for pyruvate phosphokinase activity and for total nitrogen, and specific activities were calculated. It was found that a considerable amount of pyruvate phosphokinase is indeed present in such fibers; however, the loss of relaxing ability is not associated with a decrease in enzymic

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TABLE I

EFFECT OF EXTRACTION OF FIBERS IN 20% GLYCEROL, 25° C, ON PEP-RELAXATION AND ON INTRINSIC PYRUVATE PHOSPHOKINASE ACTIVITY

Duration of washing (h)	*Specific activity of intrinsic phosphokinase, in μmoles/min/mg N*	*Estimated relaxation with PEP (% drop of tension)*
0.1	12	*
0.5	16	50
1.0	12	55
1.5	19	45
2.0	20	55
3.0	24	30
3.5	26	none
4.0	12	50
5.0	27	none

* Relaxation with PEP obscured by spontaneous relaxation.

activity (Table I). In fact there appears to be an increase in the latter with washing, although the differences may not be significant. If the increase is real, it is probably due to the removal of a substance which either inhibits the enzyme or interferes indirectly in its determination, although it may also be due simply to the removal of non-enzyme protein, thus increasing the specific activity of pyruvate phosphokinase. Another series of fibers was washed in 0.1 *M* KCl containing 4 m*M* $MgCl_2$ at room temperature, and was treated similarly, and in this case there was a depletion of enzymic activity with washing (Table II).

TABLE II

EFFECT OF EXTRACTION OF FIBERS IN 0.1 *M* KCl–4m*M* $MgCl_2$, 25° C, ON PEP-RELAXATION AND ON INTRINSIC PYRUVATE PHOSPHOKINASE ACTIVITY

Duration of washing (h)	*Specific activity of intrinsic phosphokinase, in μmoles/min/mg N*	*Estimated relaxation with PEP (% drop of tension)*
0.5	9.1	50
0.8	5.8	40
1.1	4.3	40
1.4	2.6	44
2.0	1.7	24
2.2	1.7	21
2.7	2.2	9
4.5	0.7	none
24	0.4	none

In an attempt to learn more about this bound enzyme, further investigations were carried out concerning its extractability from muscle in water and in dilute KCl solution. Stock glycerinated fibers were homogenized in 50 to 100 ml per gram wet weight, and the suspensions were assayed for pyruvate phosphokinase activity. Then, after extraction at 0° C for about 10 minutes and centrifugation for 30 minutes at 30,000 r.p.m. in the cold, the activities of the supernatants and sediments were determined. It was found that if the homogenate was prepared in water, the entire activity of the original suspension was recovered in the sediment while the super-

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natant was practically inactive. However, when 0.1 *M* KCl, containing 0.02 *M* phosphate buffer at pH 7.0, was the suspending medium, 95% of the activity of the homogenate appeared in the supernatant, only 5% remaining unextracted in the sediment.

Similar experiments were also carried out on psoas muscle which had not been glycerinated. The tissue was homogenized, immediately after removal from the rabbit, in 10 volumes of cold water with a Waring Blendor, and after standing at 0° C for 15 minutes, the suspension was centrifuged 30 minutes at 30,000 r.p.m. The sediment was blended again with the original volume of water, extracted for 15 minutes, and centrifuged as before. This second sediment was then homogenized in 0.1 *M* KCl, containing 0.02 *M* phosphate buffer at pH 7.0, using a volume corresponding to that of the original homogenate. After centrifugation, the third sediment was resuspended in the salt solution. All fractions were assayed for pyruvate phosphokinase activity, and the results are summarized in Fig. 9. It can be seen that roughly one third of the total activity of the muscle is not extractable with water but can be removed by 0.1 *M* KCl.

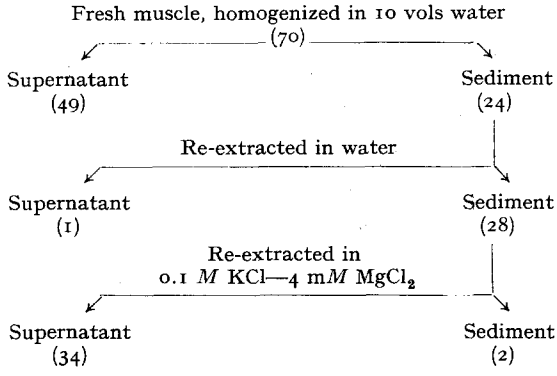

Fig. 9. Extraction of pyruvate phosphokinase from fresh rabbit psoas. All centrifugations were carried out for 30 min at 30,000 r.p.m., and re-extraction of sediments was accomplished by suspending in a volume corresponding to that of the original homogenate and extracting for 15 min at 0° C before centrifuging. Total enzymic activity of each fraction is given in brackets, in μmoles pyruvate liberated per min per ml.

In order to determine whether this portion of the enzyme is insoluble in ion-free water after its extraction from the muscle, a sample of the 0.1 *M* KCl extract of glycerinated muscle was dialyzed exhaustively against water in the cold for several days. A considerable amount of precipitate appeared and was centifuged out, but it had very little enzymic activity and was insoluble in 0.1 *M* KCl. Determination of pyruvate phosphokinase activity and total nitrogen in the original extract and in the supernatant after dialysis showed that, although the total activity had decreased by about 30%, the specific activity of the solution was slightly increased by the dialysis. Thus the pyruvate phosphokinase which cannot be extracted from the muscle with ion-free water becomes soluble in this medium after it has been extracted from the muscle with dilute KCl.

DISCUSSION

It has been found here that after development of isometric tension in 4 m*M* ATP,

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some glycerinated muscle fibers may be relaxed by the addition of 10 mM PEP in the presence of 4 mM Mg^{++}. The addition of a small amount of Ca^{++} generally causes immediate recontraction. The relaxation shows a requirement for K^+, suggesting that PEP exerts its effect through the action of pyruvate phosphokinase, since a requirement for K^+ is a rather singular property of this enzyme[21]. In fact pyruvate phosphokinase activity has been shown by direct assay to be present in considerable amounts in all fibers which relax with PEP, and the enzyme appears to be bound in such a way that, although it is soluble in ion-free water, it can be extracted from the muscle only by dilute salt solutions.

Altogether, now, the following three enzyme systems, naturally occurring in muscle, are implicated in the relaxation of glycerinated muscle fibers: ATP-creatine transphosphorylase[11], which catalyses the reaction:

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

myokinase[18], which is involved in the dismutation of ADP:

$$2\text{ADP} \rightleftharpoons \text{adenylic acid} + \text{ATP},$$

and finally, pyruvate phosphokinase, catalyzing the reaction:

$$\text{ADP} + \text{PEP} \rightleftharpoons \text{ATP} + \text{pyruvate}.$$

Although the phosphate donors in the reactions shown are rather different, the three systems have in common the ability to rephosphorylate ADP, suggesting that their relaxing effects are expressed through the resynthesis of ATP from ADP, and that this process is a basic step in relaxation in glycerinated muscle fibers, as has been proposed earlier by several workers[9,11,12,13]. Recently, GOODALL[28] has reported that carnosine phosphate can also produce relaxation, and he suggested a mechanism involving transphosphorylation to ADP.

It is interesting to note, however, that if the ATP concentration is lowered, the ATP-creatine transphosphorylase and pyruvate phosphokinase systems cause an enhancement of contraction rather than relaxation. In fact, in the presence of either of these systems, strong isometric contraction may be obtained at ATP concentrations which alone would produce little or no tension. BOZLER[9] found that 20 mM phosphocreatine produces considerable tension in the presence of ATP concentrations as low as 10^{-6} M. Similar observations have been made by PERRY, working with isolated myofibrils[29], and by MOOS, using artificial actomyosin fibers[30], with the addition in both cases of purified ATP-creatine transphosphorylase. Now we have shown that in the presence of 10 mM PEP, an ATP concentration of 10^{-5} M produces appreciable contraction (Fig. 6). All these observations indicate that the ability of a small amount of ATP to produce contraction becomes much enhanced if the nucleotide is maintained continually as the triphosphate by means of a transphosphorylase system functioning within the fiber. However, if the transphosphorylating system is present together with the higher ATP concentration (2 to 4 mM), relaxation may occur. Thus the two transphosphorylase "relaxing factors", ATP-creatine transphosphorylase and pyruvate phosphokinase, act as such only at ATP concentrations not much below 4 mM, while at lower levels of ATP (0.1 to 1 mM) they have the opposite effect, and enhance contraction.

If the properties of glycerinated muscle bear a direct relationship to the func-

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tioning of live muscle, one can envisage a variety of ways in which the contraction–relaxation cycle may be controlled, on the basis of the various parameters which have been found to influence the behavior of glycerinated muscle in the presence of the three "relaxing factor" systems. One of these parameters is the *ATP concentration*. As discussed above, a low ATP concentration in the presence of a transphosphorylase system brings about contraction, whereas higher levels of ATP cause relaxation. Assuming that muscle normally contains free ATP in a concentration of the order of 2 to 4 m*M* (which seems plausible in view of MOMMAERTS' recent observation that a total of 2 to 3 μmoles of ATP per gram are present in muscle[31]), the "relaxing factors" present would be expected to keep the system relaxed. This picture, of course, would explain the fact that microinjection of ATP into intact muscle fibers produces no contraction[32], since a further increase in ATP concentration would, if anything, promote relaxation, as shown in Fig. 5. Under these conditions, a decrease in the free ATP concentration would initiate contraction. Conceivably a decrease in ATP could be brought about locally in the muscle either by rendering the ATP inactive through binding it in some way, or by depletion of the ATP through the sudden activation of some enzyme system. In order to re-establish the relaxed state, the ATP concentration would have to be restored, since all three of the above-mentioned enzyme systems can cause relaxation only at higher ATP concentrations[10, 17] (see Fig. 5). This could be accomplished in three ways: through the transphosphorylating activity of the enzymes themselves, by the release of ATP from a bound state, or by the movement of free ATP into the active region from elsewhere in the muscle structure.

Another possible mechanism for triggering the contraction of relaxed muscle might be the *sudden release of* Ca^{++}, which would cause immediate contraction in the presence of about 4 m*M* free ATP and any of the three "relaxing factors". This is the only mechanism for which direct evidence has been obtained in intact muscle, in which the microinjection of Ca^{++} elicits contraction[33, 34].

It should be noted here that the mechanism by which Ca^{++} reverses relaxation in glycerinated fibers is still obscure. The possibility that it might act by complexing with ATP, thus rendering the latter inactive, is unlikely in view of the stoichiometry of the effect. Maximal recontraction is obtained with 0.1 m*M* Ca^{++}, even in the presence of 4 m*M* ATP (Fig. 7). Nor does it seem to act directly by blocking the enzymic activity of the "relaxing factors", since it is not known to be an inhibitor of the activity of either myokinase[35] or ATP-creatine transphosphorylase[36], and it inhibits pyruvate phosphokinase only partially even at a concentration of 2 m*M*[21].

While ATP and Ca^{++} changes produce very striking effects in glycerinated fibers, there are other parameters whose effects on the glycerinated system might also have a bearing on the functioning of intact muscle. One of these is the *Mg^{++} concentration*, which was shown to be a critical factor in the reversal of actomyosin "superprecipitation" by the ATP-creatine transphosphorylase system[37]. The myokinase relaxing system studied by BENDALL[17] requires 2 to 4 m*M* Mg^{++} to cause relaxation, while at concentrations of 0.1 to 0.5 m*M*, the relaxing effect is not observed, and contraction occurs. Similarly we have found here that the relaxing effect of PEP requires 2 to 4 m*M* Mg^{++} for its expression.

The *pH* and *K^+ concentration* also have effects on glycerinated muscle fibers. The former has been studied in detail mainly with the ATP-creatine transphosphorylase

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relaxing system, in which it was found that contraction and relaxation can be brought about merely by raising the pH to 6.6 and lowering it to 6.2 respectively[10,11]. A similar pH dependence was reported for the relaxation induced by carnosine phosphate[28]. K^+ has been shown here to be required for the relaxing effect of PEP; however, it seems unlikely that the intracellular K^+ concentration in living muscle could be suddenly reduced to the extent needed to block the relaxing activity of PEP and thus trigger contraction.

Thus there is a variety of parameters which may control the activity cycle of muscle, and many more, no doubt, remain undiscovered at present. Moreover, it is likely that none of these factors alone is the key to the control of muscle function; their effects may be interdependent in some complex way.

Returning now to a consideration of relaxation in glycerinated fibers, it is clear that the rephosphorylation of ADP does not completely explain the phenomena observed in studies on relaxation. Fibers that have lost the ability to relax with PEP as a result of extensive washing are apparently not restored to activity by the addition of purified pyruvate phosphokinase. Moreover, if the washing is carried out in 20% glycerol, the loss of relaxing ability is not accompanied by any depletion of the intrinsic pyruvate phosphokinase activity, indicating that the relaxation may be dependent on some other factor or factors in addition to the transphosphorylating system. If unknown factors are extracted or destroyed by the washing procedure, it might be possible to restore the relaxation by adding back a muscle extract. Experiments along this line are under way. Of course, it is also possible that the washing produces some apparently irreversible structural alteration which prevents relaxation.

In connection with these observations, the recent work of KUMAGAI, EBASHI, AND TAKEDA[38] is of interest. These authors found, using an isotonic technique, that extensively washed glycerinated fibers fail to relax with the myokinase or ATP-creatine transphosphorylase systems unless certain other factors are present. EBASHI and co-workers[39] believe that the magnesium-activated ATPase of KIELLEY AND MEYERHOF is necessary for relaxation with both these systems. However, at least in the case of relaxation with the myokinase system, they showed that still other, as yet unknown, factors are required.

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SUMMARY

Psoas muscle fibers extracted in 50% glycerol at 0° C for two days, and subsequently washed in 20% glycerol for about one hour at 25° C, contract upon the addition of adenosine triphosphate (ATP), and no spontaneous relaxation occurs. If 10 m*M* phosphoenolpyruvate (PEP) is now added, an immediate relaxation follows, which may be reversed by the addition of 0.1 m*M* $CaCl_2$. The optimal conditions for this relaxation are the presence of 4 m*M* $MgCl_2$, 4 m*M* ATP, and 10 m*M* PEP. It is interesting to note that K^+, which is known to be required for the enzymic activity of

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pyruvate phosphokinase, is obligatory for relaxation with PEP. This fact, coupled with the presence, as shown by direct assay, of considerable amounts of pyruvate phosphokinase in the glycerinated fibers, suggests that PEP causes relaxation through the action of this enzyme, or in other words, through the rephosphorylation of adenosine diphosphate (ADP). Thus there are now three enzymic "relaxing factors": ATP-creatine transphosphorylase, myokinase, and pyruvate phosphokinase. All have in common the ability to rephosphorylate ADP, which supports the hypothesis that the resynthesis of ATP is an essential step in the relaxation process.

However, with fibers which have been extracted in 20% glycerol or 0.1 m*M* KCl for many hours, PEP causes no relaxation, even though pyruvate phosphokinase is either present intrinsically in the fibers, or added in purified form. This may suggest that other as yet unknown steps are involved in relaxation in addition to the rephosphorylation of ADP.

It was also observed that, in the presence of a trace of ATP (0.1 m*M* or less), which alone causes only slight contraction, the addition of PEP elicits development of high tension. This observation is analogous to similar observations that have been reported using the ATP-creatine transphosphorylase system, and indicates that if the ATP is maintained continuously as the triphosphate, it can cause contraction in much lower concentrations. It appears, then, that these transphosphorylase "relaxing factors" have a dual role; at low ATP levels (0.1 m*M*) they enhance contraction, whereas at higher ATP concentration (4 m*M*) relaxation is promoted.

An incidental observation is the fact that the enzyme pyruvate phosphokinase appears to exist in muscle in two states, one of which is readily extracted when the tissue is homogenized in ion-free water, while the other requires the presence of salts for its removal, although it, too, is soluble in ion-free water after it has been extracted from the muscle in 0.1 *M* KCl.

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BEWIRKT DAS SYSTEM PHOSPHOKREATIN-PHOSPHOKINASE DIE ERSCHLAFFUNG DES LEBENDEN MUSKELS?

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I

5 Beobachtungen haben zu der Meinung geführt[1,2,3,4,5], die Restitution des ATP nach der Spaltung sei ein wesentlicher Faktor der Muskelerschlaffung. Es handelt sich um die folgenden Beobachtungen:

1. BOZLER[1] findet, dass kontrahierte, recht dicke Fasermodelle des Skelettmuskels auf Kreatinphosphatzusatz (CP) bei pH 7 erschlaffen, wenn sie sehr kurz extrahiert sind und wenn die vorhergegangene Kontraktion in einem ATP (Adenosintriphosphat)-Bad von physiologischer Konzentration ($4 \cdot 10^{-3} M$) stattgefunden hat.

2. Die Schule von SZENT-GYÖRGYI[2,3] findet, dass solche Faserbündel auf Zugabe von CP und CPase (Kreatinphosphokinase) zwar nicht bei pH 7, aber bei pH 6 erschlaffen, wenn sie vorher bei pH 7 zur Kontraktion gebracht sind.

3. BENDALL[4] kommt bei den Versuchen, den "relaxing factor" aus Muskelfasern zu isolieren, zu dem Ergebnis, dass Myokinase und "relaxing factor" identisch seien.

4. Das CP-System kann als System der Erschlaffung auch durch Phosphopyruvat und Pyruvatkinase ersetzt werden (LORAND[5]).

5. Auch mit Phosphocarnosin erschlaffen Modelle (GOODALL[6]).

Alle die genannten Systeme restituieren gespaltenes ATP. Es ist aber schwer einzusehen, warum die Restitution des gespaltenen ATP nicht nur neues ATP für die Kontraktion zur Verfügung stellt, sondern auch gleichzeitig Erschlaffung bewirken soll.

Deshalb wird in dieser und der folgenden Mitteilung überprüft und analysiert, worauf die erschlaffende Wirkung eines ATP restituierenden Systems, des CP-Systems, unter den Bedingungen der Versuche von BOZLER einerseits und der SZENT-GYÖRGYI Schule andererseits beruht. In einer weiteren Mitteilung wird gezeigt, dass die Myokinaseaktivität eines Muskelextraktes durch Abtrennung der Muskelgranula nicht abnimmt, während die ganze erschlaffende Wirkung sich in den Granula wiederfindet.

Literatur S. 482.