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REGULATION OF SKELETAL MUSCLE PHOSPHORYLASE PHOSPHATASE ACTIVITY

II. INTERCONVERSIONS

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SUMMARY

1. The incubation of the pigeon breast muscle homogenate at 37° resulted in a time-dependent decrease in phosphatase activity. This effect was stimulated by ATP, ADP, AMP, GTP, UTP, CTP or pyrophosphate.

2. Reactivation of an inactive phosphorylase *a* phosphatase preparation was obtained by incubation with ATP–Mg^{2+}. Phosphocreatine–Mg^{2+} or Mg^{2+} were also found to be effective in bringing about the reactivation of the enzyme.

3. 3′,5′-Cyclic AMP decreased the yield of the active enzyme when it was added either at the beginning or during the activation reaction.

INTRODUCTION

The evidence reported in the preceding paper[1] and in a preliminary report[2] indicated that pigeon breast muscle phosphorylase phosphatase has two interconvertible forms: one is active and the other appears inactive under the assay conditions.

As occurs in adrenal glands[3], the conversion of the inactive to the active form required ATP and Mg^{2+}. On the other hand, the reverse reaction, *i.e.* the conversion of the active to the inactive form, proceeded at a higher rate in the presence of ATP when no Mg^{2+} was added to the incubation mixture.

In this paper some properties of these phosphatase conversions are reported. In addition, the results obtained in experiments designated to investigate the effect of cyclic 3′,5′-AMP on the phosphorylase phosphatase conversions are given.

EXPERIMENTAL PROCEDURES

Phosphorylase phosphatase activity was assayed using ^{32}P-labeled rabbit-muscle phosphorylase *a* as substrate. Conditions of the assay were given in the preceding paper[1].

Phosphorylase *a* phosphatase was obtained from pigeon breast muscle. The tissue was homogenized with 2 vol. of 250 mM sucrose containing 50 mM glycylglycine buffer (pH 7.2) and was adjusted to pH 7.0. Aliquots of the homogenate (2 ml) were incubated for different periods at 37° without any addition. The samples were then passed through Sephadex G-25 columns (1 cm × 20 cm) equilibrated with 250 mM sucrose and 50 mM glycylglycine buffer (pH 7.2), and the first 1.5 ml of the colored effluent were collected. This effluent, "crude preparation", was used either directly as a source of enzyme in further incubations or was precipitated by the addition of 1.5 vol. of a neutralized $(NH_4)_2SO_4$ solution (saturated at 5° and containing 1 mM EDTA). The pellet obtained by centrifugation at 10 000 × *g* for 15 min was resuspended in 250 mM sucrose and 50 mM glycylglycine buffer (pH 7.2), and the suspension was passed through Sephadex G-25 columns, as described above, to obtain the "$(NH_4)_2SO_4$ preparation". In some experiments the Sephadex columns were equilibrated with 20 mM NaCl instead of with glycylglycine buffer; in these cases the eluates were adjusted to a final concentration of 0.05 M of *N*-ethylmorpholine buffer (pH 7.2) by the addition of the 1 M buffer solution. Protein determinations in the Sephadex eluate were carried out by the method of LOWRY *et al.*[4]. Conditions for the reactivation or inactivation of the phosphatase varied according to the type of enzymatic preparation. When the crude preparation was used, 0.25 ml of the enzyme was incubated at 37° with the additions indicated (see below) in a total volume of 0.3 ml. Reactions were stopped by dilution with 2.7 ml of a cold solution containing 10 mM mercaptoethanol, 5 mM EDTA and 40 mM glycerophosphate buffer (pH 6.8). The diluted samples were assayed for phosphatase activity. When the $(NH_4)_2SO_4$ preparation was used as a source of the enzyme, 0.05 ml was incubated at 37° with the indicated additions (see below) in a total volume of 0.06 ml. Reactions were stopped with 0.54 ml of the cold mercaptoethanol–EDTA–glycerophosphate buffer solution and assayed as indicated in the preceding paper[1].

RESULTS

Activation and inactivation of muscle phosphorylase phosphatase

The incubation of the pigeon breast muscle homogenate at 37° resulted in a time-dependent decrease in phosphatase activity (Fig. 1). This effect is not an irreversible inactivation of the enzyme, since the activity of the Sephadex eluate was restored by reincubation with ATP, phosphocreatine and Mg^{2+}. The opposite effect, *i.e.* a further decrease in activity, was produced by reincubation with ATP in the absence of Mg^{2+}.

Requirements for phosphatase activation

A partially inactive phosphorylase *a* phosphatase was obtained by incubating the homogenate for 40 min at 37°. Fig. 2 shows the time-course for the phosphatase reactivation in the presence of ATP, phosphocreatine and Mg^{2+}. The requirements for different substances in the activation of muscle phosphorylase phosphatase were studied in both crude and $(NH_4)_2SO_4$ preparations. The latter was employed in order to avoid possible artifacts determined by metabolite contaminations in the crude preparation. Activations were performed either in the presence of glycylglycine–NaOH, a Mg^{2+}-complexing buffer, or with *N*-ethylmorpholine buffer. The latter was

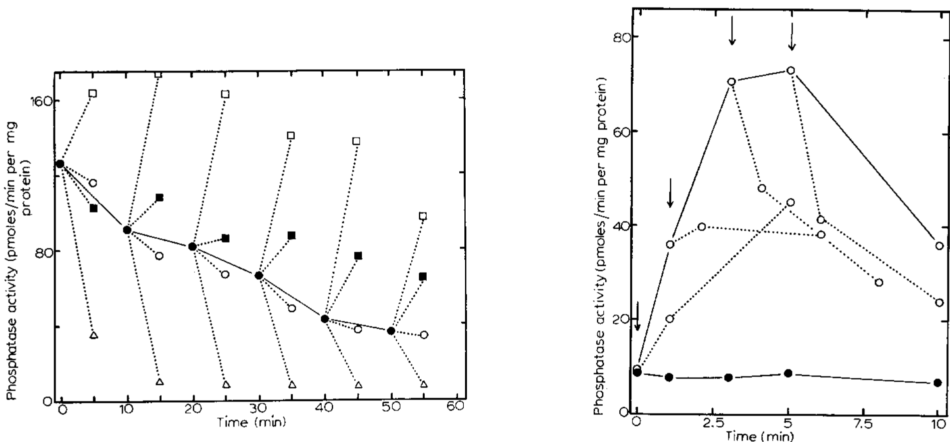

Fig. 1. Inactivation and reactivation of phosphorylase *a* phosphatase. The pigeon breast muscle homogenate was incubated at 37° and passed through a Sephadex column. The phosphatase activity in the eluate was measured after dilution and addition of theophylline as described in text (●). Aliquots of this eluate were further incubated for 5 min at 37° in the presence of 6.7 mM theophylline and 10 mM mercaptoethanol *plus* the following additions: (○) none; (■) 2.5 mM ATP–$MgCl_2$; (□) 2.5 mM ATP–$MgCl_2$ and 5 mM phosphocreatine–$MgCl_2$; (△) 2.5 mM ATP. Other conditions were as described in the text.

Fig. 2. Reactivation of phosphorylase phosphatase and effect of cyclic 3′,5′-AMP. A crude preparation was obtained from a homogenate incubated for 40 min at 37°. Reactivation mixtures contained 10 mM mercaptoethanol, 6.7 mM theophylline (●—●); or mercaptoethanol, theophylline *plus* 2.5 mM ATP–$MgCl_2$, and 5 mM phosphocreatine–$MgCl_2$ (○—○). Cyclic 3′,5′-AMP was added at the times indicated by the arrows at the final concentration of 0.017 mM. Dotted lines correspond to incubations containing cyclic AMP and full lines correspond to incubations not containing this nucleotide. Incubations were carried out at 37°. Other conditions were as described in the text.

used to avoid interference by magnesium chelators different from those used as substrates in the activation reaction. Using glycylglycine as the buffer system and in the presence of Mg^{2+}, ATP and phosphocreatine activated the phosphatase to a similar extent; Mg^{2+} alone also activated the enzyme, but more slowly (Table I, Expts. I and II).

Fig. 3 shows the effect of varying the concentration of ATP, phosphocreatine and Mg^{2+} on the activation rate. In the presence of ATP and Mg^{2+} at equimolar concentrations, the maximum effect was observed at 2.5 mM; at higher concentrations, the activation rate declined (Curve *a*). The addition of Mg^{2+} in a concentration over that of ATP increased the rate of the reactivation, and no inhibitory effect was observed at high concentrations of ATP (Curve *c*). In the presence of phosphocreatine and Mg^{2+} at equimolar concentrations, no inhibition was obtained at high concentrations of the phosphoric ester, and the activation rate increased up to 7 mM (Curve *b*). The maximal rate of activation was obtained by the association of ATP, phosphocreatine and Mg^{2+}. In this condition, the rate rapidly increased at low concentrations of ATP–Mg^{2+} *plus* phosphocreatine–Mg^{2+}, to reach a maximum at 2.5 mM ATP and 4.5 mM phosphocreatine (Curve *d*).

With *N*-ethylmorpholine–HCl as buffer, the activation in the presence of ATP–Mg^{2+} proceeded at rates significantly higher than those observed with glycyl-

TABLE I

REQUIREMENTS FOR DIFFERENT SUBSTANCES IN THE ACTIVATION OF MUSCLE PHOSPHORYLASE PHOSPHATASE

The final concentration of the additions were as follows: mercaptoethanol, 10 mM; theophylline, 6.7 mM; ATP–$MgCl_2$, 2.5 mM; phosphocreatine–$MgCl_2$, 5 mM; and $MgCl_2$, 5 mM. In Expt. I, the enzyme was a Sephadex eluate (crude preparation) obtained from a homogenate inactivated by incubation at 37° for 40 min, and in Expts. II and III the same preparation was precipitated with $(NH_4)_2SO_4$ ($(NH_4)_2SO_4$ preparation). Glycylglycine–NaOH buffer (pH 7.5, 0.05 M) was used in Expts. I and II. In Expt. III 0.05 M *N*-ethylmorpholine (pH 7.0) was used as buffer. Additions were made at zero time, and the incubations were carried out for the indicated times. Other conditions were as described in EXPERIMENTAL PROCEDURES.

Time (min)	*Additions*	*Activity (pmoles/min per mg protein)*		
		Expt. I	*Expt. II*	*Expt. III*
0	None	4.3	25.0	
0	Mercaptoethanol, theophylline		45.0	34
5	None	2.2	20.0	
5	Mercaptoethanol, theophylline	12.2	48.0	
5	Mercaptoethanol, theophylline, ATP–Mg^{2+}	60.0	72.0	
5	Mercaptoethanol, theophylline, phosphocreatine–Mg^{2+}	43.5	89.0	
5	Mercaptoethanol, theophylline, ATP–Mg^{2+}, phosphocreatine–Mg^{2+}	80.0	115.0	
5	Mercaptoethanol, theophylline, Mg^{2+}	23.5	62.0	
5	Theophylline, ATP–Mg^{2+}	64.0		
5	Theophylline, phosphocreatine–Mg^{2+}	49.0		
5	Theophylline, Mg^{2+}	30.0		
5	ATP–Mg^{2+}	19.3		
5	Phosphocreatine–Mg^{2+}	26.0		
5	Mg^{2+}	8.0		
1	Mercaptoethanol, theophylline			30.6
1	Mercaptoethanol, theophylline, ATP–Mg^{2+}			68.0
1	Mercaptoethanol, theophylline, ATP–Mg^{2+}, Mg^{2+}			106.0
1	Mercaptoethanol, theophylline, phosphocreatine–Mg^{2+}			44.0
1	Mercaptoethanol, theophylline, ATP–Mg^{2+}, phosphocreatine–Mg^{2+}			100.0
1	Mercaptoethanol, theophylline, Mg^{2+}			39.2

glycine (Table I, Expt. III). Fig. 4 shows the effect of varying ATP and Mg^{2+} at equimolar concentrations on the activation rate (Curve *a*). The maximal rate was observed at 2.5 mM; at higher concentrations the activation declined. The effect of the addition of Mg^{2+} or phosphocreatine *plus* Mg^{2+} (at equimolar concentrations) to the ATP–Mg^{2+}-containing mixture was also studied (Curves *b* and *c*, respectively). It can be seen that these additions elicited a marked stimulation, higher than those observed with ATP–Mg^{2+}. However, no differences were observed between Mg^{2+} and phosphocreatine–Mg^{2+} in the presence of ATP–Mg^{2+}. Fig. 4 also shows the effect of varying ATP in the presence of a fixed concentration of Mg^{2+} or phosphocreatine and Mg^{2+} at equimolar concentrations (Curve *c*). It can be seen that the activation declines sharply when the ATP concentration is higher than that of Mg^{2+} or phosphocreatine–Mg^{2+}.

Mercaptoethanol was added as a standard component in the activation mixtures. However, the omission of this substance did not modify the rate of the phosphatase activation (Table I, Expts. I and II).

Several substances were tested, in the presence of Mg^{2+}, for the ability to replace

TABLE II

EFFECT OF DIFFERENT NUCLEOTIDES AND OTHER METABOLITES IN THE ACTIVATION OF MUSCLE PHOSPHATASE

The final concentration of $MgCl_2$, nucleotides and other metabolites was 2.5 mM. Other conditions were as those of Expt. II of Table I.

Time (min)	*Additions*	*Activity (pmoles/min per mg protein)*
0	None	23.5
0	Mercaptoethanol, theophylline	55.0
3	None	21.0
3	Mercaptoethanol, theophylline	48.4
3	Mercaptoethanol, theophylline, ATP–Mg^{2+}	122.0
3	Mercaptoethanol, theophylline, phosphocreatine–Mg^{2+}	64.2
3	Mercaptoethanol, theophylline, ADP–Mg^{2+}	76.0
3	Mercaptoethanol, theophylline, AMP–Mg^{2+}	38.4
3	Mercaptoethanol, theophylline, UTP–Mg^{2+}	50.4
3	Mercaptoethanol, theophylline, GTP–Mg^{2+}	62.5
3	Mercaptoethanol, theophylline, TTP–Mg^{2+}	36.9
3	Mercaptoethanol, theophylline, CTP–Mg^{2+}	57.0
3	Mercaptoethanol, theophylline, pyrophosphate–Mg^{2+}	21.8

ATP in the phosphatase activation. Table II shows that ADP, GTP and CTP were partially effective in bringing about activation of the enzyme.

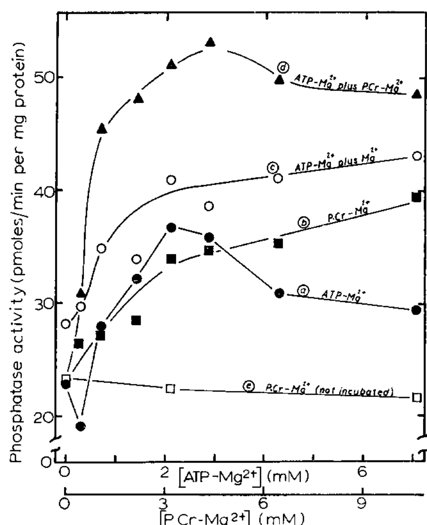

Fig. 3. Phosphorylase phosphatase activation varying ATP–Mg^{2+} or phosphocreatine–Mg^{2+} (P.Cr–Mg^{2+}) concentration using glycylglycine–NaOH as buffer system. Curve *c* contained in addition to the concentrations of ATP–$MgCl_2$ indicated in abcissae, 4.7 mM $MgCl_2$. Activation reactions corresponding to Curves *a*, *b*, *c* and *d* were carried out for 2 min at 37°. Those of Curve *e* were not incubated. Other conditions were indicated in the text of Fig. 2.

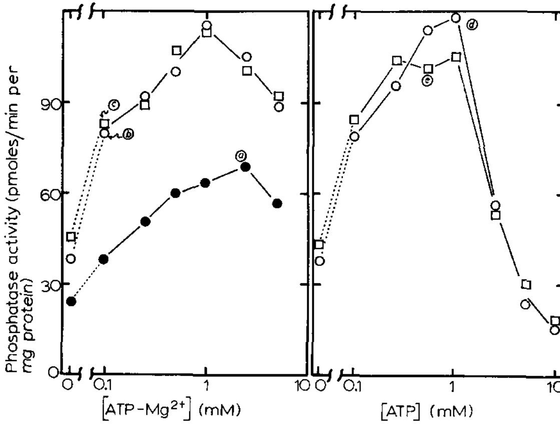

Fig. 4. Phosphorylase phosphatase activation varying [ATP–Mg^{2+}] or [ATP] using *N*-ethylmorpholine–HCl as buffer system. In Curves *b* and *d* the activation mixtures contained in addition 5 mM of $MgCl_2$ and those activation mixtures corresponding to Curves *c* and *e* contained in addition 5 mM phosphocreatine–$MgCl_2$. Activation reactions were carried out for 1 min at 37°. Other conditions were those of the Expt. III of Table I.

The effect of cyclic 3′,5′-AMP

Cyclic 3′,5′-AMP decreased the yield of the active enzyme when it was added either at the beginning or during the activation (Fig. 2). The maximal effect was observed at $1 \cdot 10^{-5}$ M, but a significant response to the cyclic adenylate was obtained at concentrations of this metabolite between $5 \cdot 10^{-7}$ M and $2 \cdot 10^{-6}$ M (Fig. 5). Since phosphorylase *a* concentration depends on the net balance between the rates of its formation by phosphorylase *b* kinase and on its degradation by phosphorylase *a* phosphatase, it might be thought that the effect of cyclic 3′,5′-AMP is due to the

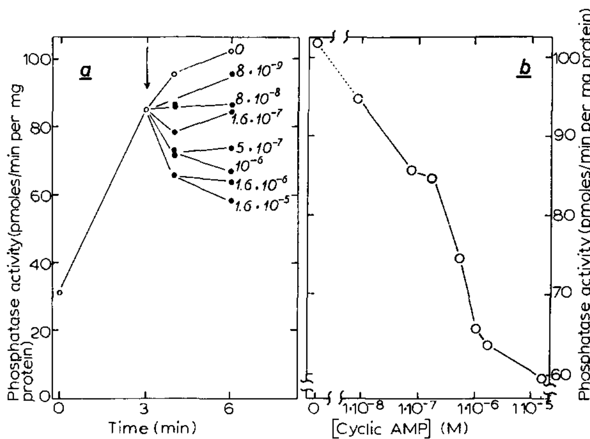

Fig. 5. Effect of varying the concentration of cyclic 3′,5′-AMP on phosphatase inactivation. a. The conditions were those of Fig. 2. At the time indicated by the arrow, cyclic 3′,5′-AMP was added at the indicated final concentrations. b. Concentration-dependence of the cyclic 3′,5′-AMP effect. Phosphatase activities corresponding to the incubations carried out for 3 min in the presence of the cyclic adenylate were plotted as a function of the concentration of this metabolite.

TABLE III

EFFECT OF DIFFERENT SUBSTANCES ON THE ACTIVATION OF MUSCLE PHOSPHORYLASE *b* KINASE

Phosphorylase *b* kinase was prepared from pigeon breast muscle. An "acid precipitate" fraction was prepared by the method described in a previous paper[7], and it was used as the source of phosphorylase *b* kinase and activating enzymes. The activation reaction mixture contained 25 mM *N*-ethylmorpholine–HCl (pH 7.0), 20 mM mercaptoethanol, enzyme (0.04 ml) and other additions in a total volume of 0.1 ml. Other conditions were as indicated previously[7].

Time (min)	*Additions*	*Kinase activity measured at pH 6.8 (nmoles/min per ml)*
0	None	23.4
2	None	20.0
0	ATP (5 mM), $MgCl_2$ (5 mM)	24.0
2	ATP (5 mM), $MgCl_2$ (5 mM)	29.8
2	ATP (5 mM), $MgCl_2$ (5 mM), cyclic 3',5'-AMP ($2 \cdot 10^{-6}$ M)	38.0
2	$CaCl_2$ (5 mM)	51.0
0	None	9.6
1	ATP (5 mM), $MgCl_2$ (5 mM)	9.9
1	ATP (5 mM), $MgCl_2$ (5 mM), cyclic 3',5'-AMP ($2 \cdot 10^{-6}$ M)	14.0
1	ATP (5 mM), $MgCl_2$ (5 mM), cyclic 3',5'-AMP ($2 \cdot 10^{-5}$ M)	14.7
1	ATP (5 mM), $MgCl_2$ (5 mM), cyclic 3',5'-AMP ($2 \cdot 10^{-4}$ M)	16.2

activation of phosphorylase *b* kinase (E.C. 2.7.1.38) rather than to the inhibition of the phosphorylase *a* phosphatase. However, this supposition can be discarded, since under the assay conditions (absence of Mg^{2+}), phosphorylase *b* kinase is completely inactive.

TABLE IV

EFFECT OF DIFFERENT NUCLEOTIDES, OTHER METABOLITES AND EDTA IN THE INACTIVATION OF MUSCLE PHOSPHORYLASE PHOSPHATASE

The final concentration of nucleotides, metabolites and EDTA was 5 mM. Conditions were as those of Expt. III of Table I, except that the homogenate was not incubated at 37°.

Time (min)	*Additions*	*Activity (pmoles/min per mg protein)*
0	Mercaptoethanol, theophylline	56
2	Mercaptoethanol, theophyline	53
2	Mercaptoethanol, theophylline, ATP	13
2	Mercaptoethanol, theophylline, ADP	0.3
2	Mercaptoethanol, theophylline, AMP	0
2	Mercaptoethanol, theophylline, UTP	17
2	Mercaptoethanol, theophylline, CTP	18
2	Mercaptoethanol, theophylline, carbamyl-*P*	49
2	Mercaptoethanol, theophylline, creatine-*P*	46
2	Mercaptoethanol, theophylline, GTP	8.1
2	Mercaptoethanol, theophyline, PP_i	2
2	Mercaptoethanol, theophylline, P_i	56
2	Mercaptoethanol, theophylline, EDTA	51

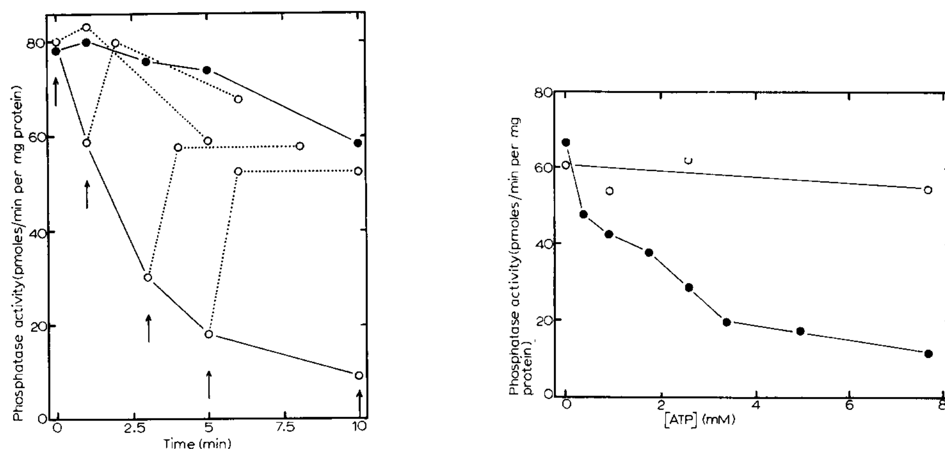

Fig. 6. Inactivation of muscle phosphorylase phosphatase. A crude preparation was obtained from a homogenate that was not incubated at 37°. Reaction mixtures containing 10 mM mercaptoethanol and 6.7 mM theophylline were incubated at 37° in the presence (○—○) or absence (●—●) of 2.5 mM ATP. At the time indicated by the arrow, phosphocreatine-$MgCl_2$ and $MgCl_2$ were added to the final concentration of 5 mM. Other conditions were as described in the text.

Fig. 7. Effect of varying the concentration of ATP on the inactivation of muscle phosphorylase phosphatase. Conditions were those of Fig. 6. The mixtures were incubated (●—●) or not (○—○) for 2 min at 37° with the indicated concentrations of ATP.

Some experiments were carried out to test the possibility that the cyclic adenylate also operates in the pigeon breast muscle at the level of the phosphorylase *b* kinase activation. The results of these experiments are shown in Table III. As can be seen, kinase activation was negligible at concentrations of ATP equal or higher than Mg^{2+}. Under these conditions, cyclic 3′,5′-AMP increased the activation of phosphorylase *b* kinase. It can also be observed that Ca^{2+} activated the kinase.

Requirements for phosphatase inactivation

Incubations of different preparations of pigeon breast muscle phosphorylase phosphatase with ATP (in the absence of Mg^{2+}) resulted in a time-dependent inactivation of the enzyme (Fig. 6). The presence of mercaptoethanol was not a requirement for this inactivation (Table I, Expts. I and II). Fig. 6 also shows that the addition of phosphocreatine and Mg^{2+} reverted the ATP effect. As can be seen in Fig. 7 the inactivation increased with the ATP concentration. Several substances were tested (in the absence of Mg^{2+}) for the ability to replace ATP in the phosphatase inactivation. Table IV shows that ATP, ADP, AMP, UTP, CTP, GTP and PP_i were effective in bringing about the inactivation of the phosphatase; EDTA was found to be ineffective.

Reversibility of the inactivation and reactivation reactions

Figs. 1, 2 and 6 show that inactivation and reactivation of pigeon breast muscle phosphorylase phosphatase were readily reversible processes. A further proof of their reversibilities is shown in Table V. An active phosphorylase phosphatase, assayed after elution from a Sephadex G-25 column, was inactivated at 37° for 40 min. The product of this incubation was passed again through a Sephadex G-25 column and

TABLE V

REVERSIBLE INACTIVATION AND ACTIVATION OF MUSCLE PHOSPHORYLASE PHOSPHATASE

A crude preparation was obtained as indicated under EXPERIMENTAL PROCEDURE from a homogenate that was incubated (inactive crude preparation) or not (active crude preparation) for 30 min at 37° and assayed for phosphatase activity. The inactive crude preparation was then incubated for 10 min at 37° in the presence of 10 mM mercaptoethanol, 2.5 mM ATP–$MgCl_2$ and 5 mM phosphocreatine–$MgCl_2$ in a final volume of 0.3 ml. After incubation the mixture (inactive-reactivated crude preparation) was assayed for phosphatase activity and then passed through a Sephadex G-25 column (0.7 cm × 10 cm) equilibrated with 0.25 M sucrose containing 0.05 M glycylglycine buffer (pH 7.2). The eluate was then incubated for 40 min at 37° without additions and assayed for phosphatase activity (inactive-reactivated-inactivated crude preparation). After this the enzyme was incubated with ATP–Mg^{2+}, phosphocreatine–Mg^{2+} as indicated above (inactive-reactivated-inactivated-reactivated crude preparation). All the samples to be assayed were previously diluted with 9 vol. of 40 mM glycerophosphate buffer (pH 6.8) containing 5 mM EDTA and 10 mM mercaptoethanol. Phosphatase assays were carried out as indicated under EXPERIMENTAL PROCEDURES in the absence of theophylline.

Crude preparation	*Activity (pmoles/min per mg protein)*
Active	36.8
Inactive	3.6
Inactive-reactivated	13.6
Inactive-reactivated-inactivated	5.5
Inactive-reactivated-inactivated-reactivated	12.0

was activated with ATP, phosphocreatine and Mg^{2+}. A further passage through a Sephadex column, and incubation of the eluate at 37° for 40 min again led to the inactivation of the enzyme. In another set of experiments (not shown here), enzymes isolated after inactivation with ATP or cyclic 3′,5′-AMP were fully reactivated by reincubation with ATP, phosphocreatine and Mg^{2+}.

DISCUSSION

From these experiments it can be concluded that phosphorylase phosphatase in pigeon breast muscle has at least two interconvertible forms.

Conversion of the phosphatase to the "active form" is associated with the presence of Mg^{2+}, ATP and phosphocreatine. This type of conversion provides an adequate mechanism for the regulation of glycogen deposition according to the levels of "high energy phosphates" and Mg^{2+}. However, it is difficult to determine the contribution of each metabolite to the conversion *in vivo* of the phosphatase. In fact, the relative activating effects of ATP–Mg^{2+}, phosphocreatine–Mg^{2+} alone vary according to the buffer system used in the conversion reaction. In the presence of a Mg^{2+}-chelating medium, activation by ATP–Mg^{2+} seemed to be depressed. On the other hand, the effect of phosphocreatine and other high-energy phosphates in the presence of Mg^{2+}, appears to be independent of the ATP-generating capacity since, in the $(NH_4)_2SO_4$ preparation, even a small contamination by ADP could be excluded. As was observed with rabbit skeletal muscle phosphorylase *b* kinase, Mg^{2+} also activated the phosphatase when no ATP was added to the conversion mixture.

Some other problems arose on the nature of the mechanism responsible for the

phosphatase inactivation. Cyclic 3',5'-AMP clearly depressed the levels of the enzyme measured after activation. This effect was not observed in the absence of ATP–Mg^{2+}. The action of the cyclic adenylate could be explained either in terms of an activation of the enzyme(s) responsible for the phosphatase inactivation or, in turn, by an inhibition of the enzymatic system that activates the phosphatase. Since the pioneer work of the SUTHERLAND group[5] on the effect of cyclic 3',5'-AMP on different hormonal-regulated systems, few attempts were made to elucidate the nature of the mechanism of action of this substance. The studies carried out by KREBS and co-workers[6] on the mechanism of activation of skeletal muscle phosphorylase *b* kinase by the cyclic adenylate, provide the only evidence available. It appears that cyclic 3',5'-AMP acts as a positive modifier of a protein kinase. Accepting this as the general mechanism for the action of cyclic 3',5'-AMP, it can be supposed that the enzyme(s) responsible for the phosphatase conversions also possesses active and inactive forms. Conversions between these forms should be mediated through cyclic 3',5'-AMP-stimulated kinase.

The work described in this paper proves that pigeon breast muscle phosphorylase phosphatase is interconvertible *in vitro*. However, up to now, no evidence of the existence of a similar mechanism was found *in vivo*.

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