CONVERSION OF PHOSPHORYLASE *b* TO PHOSPHORYLASE *a* IN MUSCLE EXTRACTS*

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(Received for publication, January 17, 1955)

In 1943 rabbit skeletal muscle phosphorylase was isolated in the crystalline form by Green and Cori (1). The crystalline enzyme possessed 60 to 70 per cent of its maximal activity in the absence of AMP,[1] and in this characteristic differed from previously observed muscle phosphorylase that required AMP for activity. The type of phosphorylase represented by the new crystalline enzyme was designated as phosphorylase *a*, and phosphorylase with the absolute AMP requirement was designated as phosphorylase *b* (2). An enzyme (PR enzyme), which catalyzed a conversion of phosphorylase *a* to phosphorylase *b in vitro*, was discovered in muscle extract, and its action was studied extensively (2-4). Evidence was obtained that during muscle contraction phosphorylase *a* is changed to phosphorylase *b* and that during the recovery phase phosphorylase *a* is restored (5). Sutherland reported experiments showing phosphorylase *a* formation in the isolated muscle diaphragm (6).

In the preceding paper (7) it was found that phosphorylase as extracted from resting muscle is predominantly in the *b* form, *i.e.* requiring AMP. It has been determined that this enzyme can be converted readily to phosphorylase *a* in the cell-free extracts. The requirements for the reaction of phosphorylase *b* → *a* include a divalent metal ion and, under certain conditions, ATP.

Methods

The preparation of muscle extracts (Methods 1 to 5) and the determination of activity measurements and phosphorylase units are described in the preceding paper (7).

Reaction System for Conversion of Phosphorylase b to Phosphorylase a—1.0 ml. samples of muscle extract containing phosphorylase *b* are brought to 1.2 ml. final volume with water or various additions. After incubation

* Supported by the Initiative 171 Research Fund of the State of Washington and by a research grant (G-2399) from the National Institutes of Health, Public Health Service.

[1] The abbreviations or contractions used in the paper include AMP, adenosine-5'-phosphate; ADP and ATP, adenosine di- and triphosphate; EDTA, ethylenediaminetetraacetate.

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for specified intervals at 25°, samples are removed and diluted in 0.03 M cysteine-0.04 M glycerophosphate buffer, pH 6.0, and kept for 8 minutes at 30° prior to the activity test. The final dilution of the extract in the activity test system is 1:72.

Adsorption of Nucleotides with Norit—20 ml. of crude muscle extract were shaken with 1 gm. of Norit A (Pfanstiehl) for 5 minutes at 0° and the mixture was filtered through Whatman No. 42 paper.

Washing of Filter Paper—18 cm. Whatman No. 1 filters were washed with the following solutions added in succession: 50 ml. of 0.01 N HCl (per paper), 50 ml. of 0.01 N NaOH, 50 ml. of H_2O. The papers were allowed to dry at room temperature before use.

Filter Paper Extract—Twenty 18 cm. Whatman No. 1 filters were extracted with successive portions of 0.01 N HCl (total HCl = 1200 ml.). The extract was concentrated and dried *in vacuo*. 46.5 mg. of residue were dissolved in 10 ml. of H_2O and neutralized before use.

Ash of Filter Paper Extract—Half of the above extract before neutralization was dried in a platinum crucible (23.2 mg.) and ashed for 3 hours at 570°. The ash (17.6 mg.) was dissolved in 0.5 ml. of 1 N HCl and dried *in vacuo*. The residue (21.8 mg.) was dissolved in H_2O, adjusted to pH 7.0, and brought to 5.0 ml.

Results

Metal Requirement for Conversion of Phosphorylase b to Phosphorylase a—Fresh crude muscle extracts containing phosphorylase *b* were found to require only the addition of certain divalent cations and a short period of incubation to bring about complete conversion of the enzyme to phosphorylase *a*. Table I presents the data from a typical experiment in which the specificity of various metals for the reaction was determined. It can be seen that Ca^{++}, Sr^{++}, Ba^{++}, Zn^{++}, and Mn^{++} were all effective[2] in that the phosphorylase activity, as tested in the absence of added AMP, rose markedly. Metal ions, when added only to the test system, did not affect the relative concentrations of phosphorylases *a* and *b*. Total phosphorylase activity (measured with added AMP) also appears to be increased significantly in the transformation of phosphorylase $b \rightarrow a$; this finding will be discussed in a later section.

Nucleotide Requirements for Conversion of Phosphorylase b to a—In crude muscle extracts aged for 24 hours at 3° or kept in the frozen state for several days, phosphorylase *b* could still be converted to phosphorylase *a*, but it was found that under these conditions addition of ATP[3] was required.

[2] The slight effects of Fe^{++} and Co^{++} could not be repeated in other experiments.

[3] In most of the experiments reported in this paper, Pabst disodium adenosine triphosphate was used. In later experiments crystalline ATP from the same source was used and was found to give identical results.

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Dialysis of muscle extracts or treatment with Norit also made the addition of ATP necessary for the conversion, as shown in Table II. Phosphorylase *b* is no longer converted to phosphorylase *a* by incubation with Mn^{++} alone, but the combination of Mn^{++} and ATP is effective.

TABLE I

Influence of Metal Ions on Conversion of Phosphorylase b to Phosphorylase a

1.0 ml. portions of muscle extract (Method 1) were brought to 1.2 ml. with water or additions and incubated for 20 minutes at 30°. 1×10^{-3} M ATP was present except in the first control. Activity measurements in the presence and absence of AMP were performed at a final 1:72 dilution of the extract. Units per ml. are calculated for undiluted extract in each case. Original activity of the extract before incubation, + AMP, 1300 units per ml.; − AMP, 20 units per ml.

Addition*	Activity		
	+ AMP	− AMP	$\frac{-\text{ AMP}}{+\text{ AMP}}$†
	units per ml.	*units per ml.*	*per cent*
Control (− ATP)	1260	70	6
" (+ ")	1400	180	13
K^{+}	1350	110	8
Mg^{++}	1320	120	9
Ca^{++}	1960	1730	88
Sr^{++}	2090	1840	88
Ba^{++}	1870	1230	65
Mn^{++}	2010	1840	92
Fe^{++}	1480	460	31
Co^{++}	1600	530	33
Ni^{++}	1710	80	5
Cu^{++}‡	1280	110	9
Zn^{++}‡	1710	1090	64
Hg^{++}‡	1430	140	10

* All salts were acetates or chlorides present at a final concentration of 1×10^{-3} M during the incubation.

† This quantity divided by 0.65 gives the percentage of phosphorylase *a* (1).

‡ 0.5×10^{-3} M during the incubation.

With the treated extracts (see above) ADP could be substituted for ATP with essentially identical results. AMP was also studied to determine whether it could serve to activate the conversion system of phosphorylase $b \rightarrow a$; inconsistent behavior of this nucleotide was noted, depending upon the treatment of the extract. In frozen extracts no conversion occurred on incubation with 1×10^{-3} M AMP and Mn^{++}, although ATP (or ADP) at the same concentration was completely effective. With a 10-fold increase in the AMP concentration partial conversion took place. In Norit-treated extracts AMP was completely ineffective, which made it

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appear unlikely that it could act as such in the reaction. Indeed, it was found that AMP could serve in Norit-treated extracts, if phosphocreatine was also added (Table III). As shown, phosphocreatine by itself was

TABLE II

Conversion System in Dialyzed or Norit-Treated Extracts

EDTA extract (No. I) from muscle (Method 4) was dialyzed for 3 hours at 5° against distilled H_2O and then incubated for 20 minutes at 25° with the additions indicated. Extract (No. II) obtained by Method 1 was treated with Norit and then incubated as above. Incubations with untreated control at 5°.

Extract	Ratio, phosphorylase activity (− AMP/+ AMP) after incubation with		
	No additions	3×10^{-3} M Mn^{++}	3×10^{-3} M Mn^{++} + 1×10^{-3} M ATP
	per cent	*per cent*	*per cent*
I. Before dialysis	9	86	86
" After "	8	2	72
II. Untreated	27	91	91
" Norit-treated	24	22	62

TABLE III

Ineffectiveness of AMP without Phosphocreatine

Muscle extract was obtained by Method 1 and frozen; it was thawed after 3 days and treated with Norit. Incubations carried out at 25°. 1×10^{-3} M Mn^{++} and 1×10^{-3} M Ca^{++} present in each incubation mixture. Phosphorylase activities carried out at 1:80 dilution of the incubation mixture.

Addition*	Ratio, phosphorylase activity (− AMP/+ AMP) after incubation for		
	0 min.	25 min.	120 min.
	per cent	*per cent*	*per cent*
None	10.4	9.7	13.7
Phosphocreatine	13.4	6.4	3.2
AMP	24.4†	31.7	33.8
Phosphocreatine + AMP	31.4†	75.5	91.8

* 1×10^{-3} M during incubation for each component.

† Norit-treated extracts contain almost no adenylic deaminase (8), and there is partial activation of phosphorylase *b* due to AMP carried over into the activity test system.

inactive. Assuming that a trace of ATP was still present in this extract, AMP could give rise to ATP in the presence of phosphocreatine through the myokinase and ATP-creatine transphosphorylase reactions.

Although it is difficult to determine the specificity of nucleotides in a crude system in which various transformations and interconversions may

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occur, the following nucleotides have also been tested: inosine mono- and triphosphate, uridine mono- and triphosphate, cytidine mono- and triphosphate,[4] AMP (3′), guanosine-3′-phosphate, and cytidine-3′-phosphate. Of these, only uridine triphosphate showed a slight effect in activating the conversion reaction of phosphorylase $b \rightarrow a$; the possibility of traces of ATP in the uridine triphosphate has not been excluded.

Extent of Conversion in Different Muscle Extracts—When fresh extract is incubated at 25° with added metal ion, the increase in the − AMP/+ AMP phosphorylase activity ratio takes place very rapidly, sometimes reaching

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TABLE IV

Extent of Conversion of Phosphorylase b to a in Rabbits under Various Conditions

The mean values are obtained by dividing the sum of the activity units per ml. of crude extract by the number of rabbits. Reaction system for conversion as described under "Methods;" the activities are determined after 30 minutes incubation at 25°.

Extract	No. of rabbits	Mean activities (units per ml.) after incubation with							
		No additions		10^{-3} M Mn^{++}		10^{-3} M ATP		10^{-3} M Mn^{++} + 10^{-3} M ATP	
		+ AMP	− AMP	+ AMP	− AMP	+ AMP	− AMP	+ AMP	− AMP
5°; Method 1	3	1680	345	1950	1500			2260	2190
24–28°; Method 1	8	1570	130	1840	1470	1730	150	2060	1830
Strychnine anesthesia; Method 5	2	1620	70	1800	1500	1580	135	2080	1590
Special anesthesia; Methods 3, 4	2	1160	125	1380	1020	1250	250	1390	1100

a maximum within less than 5 minutes. With continued incubation after conversion is complete, the activities remain relatively stable for several hours; then a definite decline in the ratio occurs. After overnight incubation a phosphorylase *b* picture usually results.

The extent of conversion of phosphorylase $b \rightarrow a$ after approximately 30 minutes of incubation was studied with fifteen different muscle extracts with no additions and with added Mn^{++} plus ATP (Table IV). In most of the experiments incubation was also carried out with ATP alone (omitting Mn^{++}). The extracts used are separated into four groups (Table IV) according to the temperature at which the animals were kept or to the

[4] Pabst Laboratories, Milwaukee. We are indebted to Dr. F. M. Huennekens for providing the samples.

procedure used in killing the rabbits and preparing the extracts. Although they all contained phosphorylase predominantly in the *b* form originally, conversion to phosphorylase *a* occurred in each case, even with extracts obtained from strychninized rabbits.

It will be noted that, after conversion, the phosphorylase activity measured without AMP exceeds the maximum of 60 to 70 per cent found for crystalline phosphorylase *a* (1). It is unlikely that this can be accounted for on the basis of a carryover of AMP from the crude extract into the activity test reaction mixture. In an experiment in which the − AMP/+ AMP activity ratio was 100 per cent after conversion of phosphorylase *b* to *a*, the reaction mixture was treated with Norit and filtered. The filtrate still showed a ratio of 92 per cent, although absorption at 260 mμ indicated that no detectable amount of nucleotides remained. 5×10^{-6} M AMP would be easily detected in the absorption method, and this would be equivalent to less than 1×10^{-7} M AMP in the activity test.

Total phosphorylase activity is also increased significantly during the conversion of phosphorylase $b \rightarrow a$ (Table IV), as was confirmed by statistical analysis. Although no definite explanation can be given at this time for this phenomenon, several factors might be involved.[5] Activity measurements are carried out at pH 6.0, *i.e.* below the pH optimum for phosphorylase *a* or *b*.[6] The two enzymes may have different pH dependence curves, and it is possible that the metal ion or the nucleotide added for the conversion affects these curves differently. In crude extracts, deamination of AMP by adenylic deaminase proceeds at such a rate at pH 6.0 that the nucleotide added in the activity test is partially destroyed before the end of the reaction. This might result in values too low for phosphorylase *b*, whereas phosphorylase *a* activity would not be affected. This is consistent with the fact that addition of fluoride, which inhibits the deaminase, increases the total activity of phosphorylase before conversion but not after. Likewise, when a Norit-treated extract is used for the conversion (from which most of the deaminase has been removed), a smaller increase in total activity is observed. Finally, several preparations of crystalline phosphorylase *a* showed an increased activity when tested after a short incubation in the presence of divalent metal ions (Ca^{++}, Mn^{++}). A similar activation of the enzyme by these ions could occur in the crude extracts and thus contribute to the observed result.

[5] Cori and Cori have found that under certain conditions there occurred a 23 per cent *decrease* in total phosphorylase activity at pH 6.8 when phosphorylase *a* was converted to *b* by PR enzyme action (9).

[6] The pH optimum for phosphorylase is around 6.7 to 6.8, but, in crude extracts, activation is measured at pH 6.0 to minimize mutase action. Activity at pH 6.0 divided by 0.7 gives activity at pH 6.8 (10).

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Properties of Conversion System—Although no definite proof has been obtained that the reaction of phosphorylase $b \rightarrow a$ is enzyme-catalyzed, it has been found that the presence of a protein fraction of the extract is necessary. If the pH of dialyzed muscle extract is lowered to pH 5.8, and the precipitate which forms[7] is removed, the phosphorylase *b* in the supernatant solution is no longer converted to phosphorylase *a* by incubation with Mn^{++} plus ATP. Addition of the fraction removed to the supernatant solution restores the reaction.

EDTA was found to block completely the reaction of phosphorylase $b \rightarrow a$, which was to be expected, since the system requires divalent metal ions. When calcium or manganous salts were added in molar excess over EDTA, the conversion system could be reactivated readily. Once phosphorylase *b* in the extracts had been converted to phosphorylase *a*, addition of EDTA had no effect on the phosphorylase activity itself.

Addition of F^- (0.04 M), arsenate (0.015 M), and cysteine (0.06 M) did not affect the degree of phosphorylase *b* to phosphorylase *a* conversion after 30 minutes of incubation of extract with Mn^{++} or Mn^{++} and ATP. In these experiments the extent of conversion at 30 minutes was essentially complete in all cases; hence slight effects on rate would not have been noticed.

Evidence of Phosphorylase a Formation during Conversion—It was important to establish beyond doubt that phosphorylase *a* was actually being formed and that the results observed were not due simply to AMP production and a carryover of this nucleotide into the activity test system. If this occurred, then naturally phosphorylase *b* could give the *a* picture. This possibility could be eliminated completely by several types of evidence.

If the total non-protein absorption of muscle extracts at 260 mμ is assumed to be due to AMP, the concentration of this nucleotide would be about 1.1×10^{-3} M in the extract. After dilution for determination of phosphorylase activity the concentration would be approximately 1.5×10^{-5} M AMP, *i.e.* a concentration capable of producing less than half maximal activation of phosphorylase *b* (11). Even in experiments in which adenine nucleotides were added to the conversion reaction mixture to concentrations of 1×10^{-3} M, the possible carryover of AMP would be too low to give half maximal activation.

The nucleotides present in the conversion reaction mixture after incuba-

[7] It is of interest that the fraction which is precipitated by this procedure contains the PR enzyme that catalyzes the reaction of phosphorylase $a \rightarrow b$ (2). Whether this means that the PR enzyme itself participates in the phosphorylase $b \rightarrow a$ process cannot be stated at this time, although it has been determined that purified PR enzyme alone does not catalyze the formation of phosphorylase *a* from phosphorylase *b* when incubated with Mn^{++} plus ATP.

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tion, as carried out for phosphorylase *a* formation, do not include amounts of AMP detectable by the method of Kalckar (12). Even when AMP itself was added to the incubation mixtures in high concentrations (to 6×10^{-2} M), it had all disappeared within 15 minutes, owing to the action of adenylic deaminase.

Norit treatment of phosphorylase *a* after the conversion reaction removed all detectable traces of AMP, but did not alter the high ratio of − AMP/+ AMP activity.

Final proof of phosphorylase *a* formation resulted from its isolation in the crystalline form after conversion: To a crude muscle extract, prepared by Method 1 of the preceding paper (7), sufficient 0.5 M EDTA solution,[8] pH 7.0, was added to make the final concentration 0.001 M EDTA. One 450 ml. portion (Sample I) of this extract was carried through the regular steps in the preparation of phosphorylase *a* (1, 10), with the exception that the filtration step through paper was omitted. Dialysis prior to the removal of PR enzyme was carried out against distilled water.[9] Upon dialysis of the $(NH_4)_2SO_4$ precipitate, no protein separated from solution within 24 hours. Ultracentrifugal analysis on a similar fraction from a different preparation showed the presence of a single component with s_{20} = 8.83 in 0.03 M cysteine-0.04 M glycerophosphate buffer, pH 6.8. A second 450 ml. portion (Sample II) of the extract containing EDTA was treated with manganous acetate (final concentration of Mn^{++} in extract = 3×10^{-3} M) and ATP (final concentration = 5×10^{-4} M) and allowed to stand for 2 hours at 0° before proceeding with the preparation. The next steps were carried out as described above. With this portion a heavy precipitate of phosphorylase *a* developed, when the $(NH_4)_2SO_4$ precipitate was dialyzed. The enzyme was recrystallized twice in the usual manner. The microscopic appearance of the crystals resembled that shown for phosphorylase *a* (1). Table V shows the recoveries in the different steps of the preparation. A considerable loss of phosphorylase occurred in the isoelectric precipitation of PR enzyme, but this was compensated for by dissolving the 41 per cent saturated $(NH_4)_2SO_4$ fraction in only a small volume of water.

The twice recrystallized enzyme was contaminated with traces of PR enzyme, which made ultracentrifugal analysis difficult; however, the major component found (60 per cent of the total) had an s_{20} of 13.27 which is in

[8] The EDTA was added so that the control portion of the extract (Sample I) could be carried through the preparation without risk of conversion to phosphorylase *a* by chance contamination with metals. Dialysis was carried out against distilled water rather than tap water (1) for the same reasons.

[9] In 1945 Cori noted an apparent increase in the concentration of phosphorylase *a* following dialysis of frog and rabbit muscle extracts (5).

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close agreement with the known value for phosphorylase *a* ($s_{20,w}$ = 13.2) (4). A second component with an s_{20} of 8.47 comprised 38.2 per cent of the total; this was presumably phosphorylase *b* ($s_{20,w}$ = 8.2) (4).

TABLE V

Phosphorylase Purification with and without Conversion to Phosphorylase a

Original extract obtained by Method 1 and EDTA added to final concentration of 0.001 M. Extract divided into two portions: Sample I, no conversion procedure; Sample II, converted to phosphorylase *a* as described in the text.

Step	Sample I				Sample II			
	Volume	+ AMP	− AMP	Total units	Volume	+ AMP	− AMP	Total units
	ml.	*units per ml.*	*units per ml.*	× 10^{-3}	*ml.*	*units per ml.*	*units per ml.*	× 10^{-3}
Original extract (with EDTA)	450	2,360	20	1062	450	2,360	20	1062
After incubation with Mn^{++} + ATP					450	2,450	2,350	1102
After dialysis	460	1,990	200	916	465	2,000	1,750	930
" removal of isoelectric ppt.	460	700	0	324	462	1,020	800	471
After dialysis of $(NH_4)_2SO_4$ ppt.	6.0	36,000	100	216	6.4	52,000	33,500	332

TABLE VI

Filter Paper Effects on Muscle Extract

Phosphorylase activities were determined on portions of muscle extract obtained by Method 1 and treated as indicated. Original activity of muscle extract before any treatment, + AMP, 1800 units per ml.; − AMP, 20 units per ml.

Treatment	Activity		
	+ AMP	− AMP	$\frac{-\text{AMP}}{+\text{AMP}}$
	units per ml.	*units per ml.*	*per cent*
Filtration through unwashed paper	2200	1700	77
" " washed paper	1740	20	2
Incubation with filter paper extract	2660	1420	53
" " " " ash	2340	1470	63

Effect of Filtration of Muscle Extracts—In the preceding paper (7) it was noted that rabbit muscle extracts containing only phosphorylase *b* could serve, nevertheless, for the isolation of crystalline phosphorylase *a*, when the method of Green and Cori (1) was followed. It was found that the *a* form of the enzyme appeared following filtration of the extract through

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Whatman No. 1 paper. If the filtration step was omitted, partial conversion of the original phosphorylase *b* was found to occur during dialysis[9] against cold tap water, but not during dialysis against distilled water. As presented in Table VI the effect of filtration can be accounted for by extraction of metals present in the paper. Washing of the paper abolishes the effect. When filtration is omitted and filter paper extract or ash is added, phosphorylase *a* is formed after a short incubation period. Incubation with no additions does not change the original activity.

Kritskiĭ and Kuvaeva (13), using a procedure based largely on that of Green and Cori (1) but in which filtration through paper and dialysis were avoided, claimed to have obtained a crystalline material showing a phosphorylase *b* type of activity.

Cori found that muscle extracts from rabbits killed with strychnine would not serve for the preparation of crystalline phosphorylase *a*. In the present study it has been found that the conversion of phosphorylase $b \rightarrow a$ does occur in this type of extract when divalent metal ions are added immediately (see Table IV); however, the pH of these extracts (pH 6.0 to 6.1) is lower than those obtained with barbiturate anesthesia and drops rapidly on standing to a point (pH 5.8) at which no conversion occurs with metal alone. When filtration or dialysis serves as the sole source of metal ions, it is possible that they may be introduced too late to effect a conversion.

DISCUSSION

In this study, evidence has been presented showing that the conversion of phosphorylase *b* to *a*, as it occurs in cell-free muscle extracts, requires a nucleotide containing high energy phosphate, in addition to a divalent metal ion.[10] Whether this implies that during conversion there is a direct phosphorylation of the enzyme or the formation of an "active" intermediate cannot be stated at this time. It is also possible that the function of ATP is concerned with the synthesis of a prosthetic group.

No definite information is available as to whether phosphorylase *b* present in crude muscle extract (7) is identical with the phosphorylase *b* produced from crystalline phosphorylase *a* with purified PR enzyme. Keller and Cori (4) have found that in this process a halving of the molecular weight

[10] Rosenfeld and Petrova (14) have reported a conversion of phosphorylase *b* to *a* in muscle extracts that were autolyzed for 48 hours at room temperature, stored for several weeks in the refrigerator, and finally alkalinized. It is very difficult to interpret what may have happened under their conditions. In the present study, no spontaneous conversion has been noticed during storage of the extracts. However, on a few occasions fresh crude extracts showed a slight increase in phosphorylase *a* when made alkaline.

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of phosphorylase *a* occurs. Recent experiments that will be reported in detail in a subsequent publication show, however, that phosphorylase *b*, obtained by the action of PR enzyme on crystalline phosphorylase *a*, is also readily converted back to phosphorylase *a* in the conversion system described in this paper.

It is of interest to recall that the dimerization of another muscle protein, that of G-actin to F-actin (15), also requires a divalent metal ion and ATP. In this system the monomer units are thought to be linked together through the nucleotide prosthetic group and the metal. With phosphorylase the possibility of two monomer (phosphorylase *b*) units being linked together through a metal ATP complex appears to be unlikely, since very little adenine is found in crystalline phosphorylase *a* (16). Tracer techniques are being used in order to determine whether the metal or any portion of ATP is incorporated during the conversion of phosphorylase *b* to *a* in a purified system. Sutherland and Cori (6, 17) have presented evidence that epinephrine exerts its glycogenolytic effect by causing an increased concentration of active phosphorylase in tissues. This increase is due presumably to the conversion of phosphorylase *b* to *a*. Since the requirements for this reaction have now been established, a study of the influence of epinephrine on the mobilization of the various components involved may give a clue to the mode of action of this hormone.

The authors wish to thank Mr. Roger Wade for carrying out the ultracentrifugal analyses and Mrs. Jeanne Dills and Mr. Charles Beaudreau for their technical assistance.

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SUMMARY

The conversion of phosphorylase *b* to phosphorylase *a* has been accomplished in cell-free muscle extracts.

Requirements for the reaction include a protein fraction of the extract, divalent metal ions, and ATP.

Crystalline phosphorylase *a* can be obtained from phosphorylase *b* after conversion of phosphorylase $b \rightarrow a$ *in vitro*.

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