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# B. ACTIVATION OF SKELETAL MUSCLE PHOSPHORYLASE

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In the original work describing two forms of skeletal muscle glycogen phosphorylase, enzymatic conversion of phosphorylase *a* to phosphorylase *b* was clearly demonstrated (2, 3), and the existence of the reversal of this process was indicated by experiments carried out *in vivo* (1). Ten years later the conversion of phosphorylase *b* to *a* was achieved in a cell-free system (6), and the chemical nature of the interconversion reactions of phosphorylase was recognized as involving phosphorylation and dephosphorylation of the enzyme (7). Simultaneously, Sutherland and Wosilait (19) were showing that activation and inactivation of liver phosphorylase involved a similar mechanism. The enzyme catalyzing the phosphorylase *a* to *b* reaction, which had originally been designated as PR enzyme (2), was called phosphorylase phosphatase and the enzyme catalyzing the *b* to *a* reaction was referred to as phosphorylase *b* kinase (9).

Phosphorylase *a* has always been considered to be the more active form of phosphorylase physiologically (1), and it is customary to speak of the *b* to *a* reaction as "phosphorylase activation." This practice is followed here, although there is evidence that phosphorylase *b* may be active in tissue under some circumstances (11, 13). The activation and inactivation reactions of skeletal muscle phosphorylase can be written as follows:

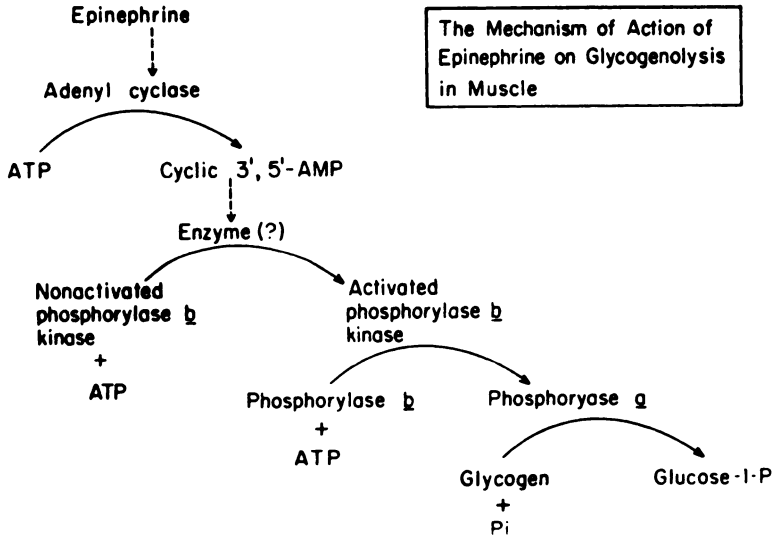
$$2 \text{ phosphorylase } b + 4 \text{ ATP} \xrightarrow{\text{phosphorylase } b \text{ kinase}} \text{phosphorylase } a + 4 \text{ ADP} \quad (1)$$

$$\text{phosphorylase } a + 4 \text{ } H_2O \xrightarrow{\text{phosphorylase phosphatase}} 2 \text{ phosphorylase } b + 4 \text{ Pi} \quad (2)$$

It is evident that the relative rates of reactions (1) and (2) determine which form of phosphorylase predominates in a tissue at a given time. Danforth *et al.* (4) deduced from kinetic data that the increased amount of phosphorylase *a*, which arises as a result of epinephrine (E) administration or muscle contraction, is due to increased activity of phosphorylase *b* kinase rather than inhibition of phosphorylase phosphatase. Experiments in our laboratory (9, 10) have supported the idea that the kinase is probably the more dynamic of the two converting enzymes, inasmuch as studies on the purified enzyme have revealed an elaborate mechanism for control of its activity. The present paper will be limited arbitrarily to a discussion of experiments on the kinase, and it will be tacitly assumed that activation of this enzyme is followed by activation of phosphorylase.

Phosphorylase *b* kinase, as extracted from resting skeletal muscle, is almost inactive at pH 7 or below (curve A of fig. 1). This accounts for the predominance

and stability of phosphorylase *b* in muscle extracts at pH 6.5 to 7.0 (6) despite the presence of sufficient ATP and $Mg^{++}$ for conversion to phosphorylase *a* by equation (1). The kinase can be converted *in vitro* into a form manifesting the activity illustrated in curve B of figure 1 by several diverse mechanisms including incubation with 1) $Ca^{++}$ in the presence of a protein factor from muscle (9, 12), 2) trypsin (10) and 3) ATP. These transformations of the enzyme are referred to as "activation"; and, indeed, Posner *et al.* (15) have obtained evidence that under conditions in which phosphorylase *b* is converted to phosphorylase *a in vivo*, an increase in the ratio of kinase activity at pH 6.8 to activity at pH 8.2 is also seen. Such a change would take place, of course, if nonactivated kinase (curve A) were converted to activated kinase (curve B). Inasmuch as phosphorylase *b* kinase activation by ATP is accelerated by cyclic 3′,5′-AMP (8–10), it is probable that this method for activation represents the event which accounts for the action of E in the phosphorylase system. This can be illustrated as follows:




ACTIVATION OF PHOSPHORYLASE *b* KINASE IN CRUDE MUSCLE EXTRACTS

Activation of phosphorylase *b* kinase can be demonstrated by addition of cyclic 3′,5′-AMP to fresh rabbit muscle extract containing ATP (9). The reaction is rapid and is evidenced by a marked increase in kinase units as assayed at pH 7 or lower, while activity at high pH changes very little. Table 1 illustrates such an experiment and shows further that the activation appears to be reversible; however, after a prolonged period of incubation it is necessary to add ATP as well as cyclic 3′,5′-AMP to get a second burst of kinase activity. The changes seen in this type of experiment are due to modification of phosphorylase *b* kinase itself and not to the production of heat stable cofactors. In reference to the experiment of table 1 it should be pointed out that following treatment of the

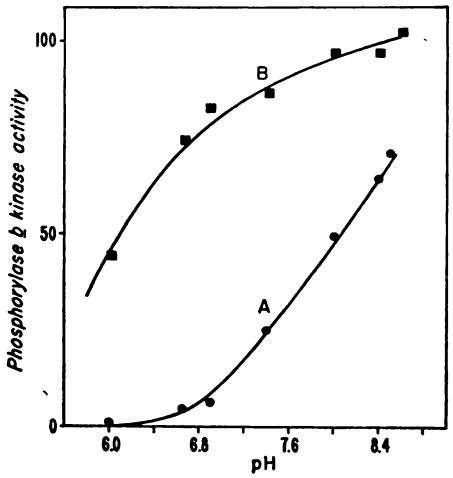



FIG. 1. The pH optima for nonactivated (curve A) and activated (curve B) rabbit skeletal muscle phosphorylase *b* kinase. Maximal activity is taken as 100.

TABLE 1

*The activation of phosphorylase b kinase in rabbit skeletal muscle extracts*

| No. | Addition | Incubation Time | Activity |
|---|---|---|---|
| | | *min* | *units/ml* |
| 1. | None | 0 | 150 |
| | None | 10 | 200 |
| | None | 70 | 100 |
| 2. | Cyclic 3′,5′-AMP | 10 | 3,100 |
| | Cyclic 3′,5′-AMP | 30 | 0 |
| | Cyclic 3′,5′-AMP | 60 | 0 |
| | Cyclic 3′,5′-AMP (readded at 60 min) | 70 | 400 |
| | Cyclic 3′,5′-AMP + ATP (readded at 60 min) | 70 | 3,400 |

Neutralized extract was incubated at 30°. [Cyclic 3′,5′-AMP] = $1 \times 10^{-4}$ and [ATP] = $2 \times 10^{-3}$ where added. Phosphorylase *b* kinase activities were determined at pH 6.8. Activity at pH 8.2 was 12,000 units/ml and constant.

extract, a large dilution is carried out before the enzyme is introduced into the phosphorylase *b* kinase assay mixture itself. The latter in turn is diluted prior to carrying out assays for phosphorylase *a*. These stages can be illustrated as follows:

| Stage I | | Stage II | | Stage III |
|---|---|---|---|---|
| Phosphorylase *b* Kinase Activation Reaction | Dilution and Transfer → | Phosphorylase *b* Kinase Reaction | Dilution and Transfer → | Phosphorylase *a* Assay |

## ACTIVATION OF PURIFIED PHOSPHORYLASE *b* KINASE BY ATP AND CYCLIC 3′,5′-AMP

Purified phosphorylase *b* kinase is also readily activated by incubation with ATP and cyclic 3′,5′-AMP, but certain characteristics of the reaction are different from those found in crude extracts. Figure 2 shows an experiment with phosphorylase *b* kinase purified 65-fold from the muscle extract (10). Labeled ATP was used in the kinase activation reaction mixtures, and in addition to following the increase in kinase activity, incorporation of $P^{32}$ into protein was determined. As can be seen, in this case the enzyme was activated by ATP alone (curve B) or by ATP plus cyclic 3′,5′-AMP (curve A); only the *rate* of activation and not the final level of activity was higher in the presence of the latter nucleotide. During the activation reaction the kinase was phosphorylated to the extent of about 0.7 gram atoms of phosphorus per $1 \times 10^5$ g of protein. It will be noted that phosphorylation was stimulated by cyclic 3′,5′-AMP, but the net effect of the nucleotide was not as great as in the activation process. In experiments similar to that of figure 2, but of much longer duration, activity started declining after reaching its maximum at 30 min, but phosphate incorporation continued, reaching a plateau when approximately 2 gram atoms per $1 \times 10^5$ g of protein had been transferred from ATP. The activity lost during these long incubations could not be regained by addition of more ATP or cyclic 3′,5′-AMP and appeared to be due to an irreversible change rather than the reversible process seen in the short-term experiment with crude extract (table 1).

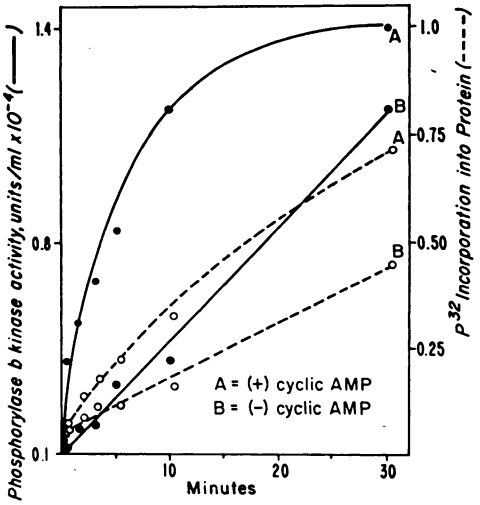



FIG. 2. Activation and phosphate incorporation into purified phosphorylase *b* kinase. Purified phosphorylase *b* kinase, *i.e.*, the 40,000 rpm precipitate fraction (10), is incubated at 30° with 0.01 M $Mg^{++}$ – $3 \times 10^{-3}$ M ATP-$\beta,\gamma$ $P^{32}$ at pH 7.0 in the presence and absence of $1 \times 10^{-6}$ M cyclic 3′,5′-AMP. Aliquots are diluted for activity assays at pH 6.8 or precipitated with trichloroacetic acid for counting. $P^{32}$ incorporation is given in gram atoms $P^{32}$ per $1 \times 10^5$ g protein. Kinase activities are in units/ml in the activation reaction mixtures.

In another type of determination, which represents a cross between the experiments of table 1 and figure 2, activation of purified phosphorylase *b* kinase was studied in the presence of catalytic amounts of muscle extract (fig. 3). The latter, when added, contributed no more than 2% of the nonactivated kinase present in the activation reaction mixtures; hence, the ratio of kinase to other extract components was 50 times higher than in the experiment of table 1. Labeled ATP was used, and $P^{32}$ incorporation into the kinase as well as activity changes were followed. Several striking effects of extract upon kinase activation are apparent (fig. 3A). First, the effect of cyclic 3′,5′-AMP is very marked in the presence of extract; this is due to two factors: 1) an increased initial rate of activation in the presence of the nucleotide and 2) a greatly depressed rate in its absence. Second, extract appears to contain something which causes strong depression of the activation rate with time; this is apparent in that the controls overtake and pass the reaction with extract plus cyclic 3′,5′-AMP which initially was the most rapid. Lastly, boiled muscle extract inhibits the activation process; this phenomenon has been noted before (14). In comparing phosphate incorporation results (fig. 3B) with activity changes (fig. 3A), it is apparent that a remarkably similar pattern is present.

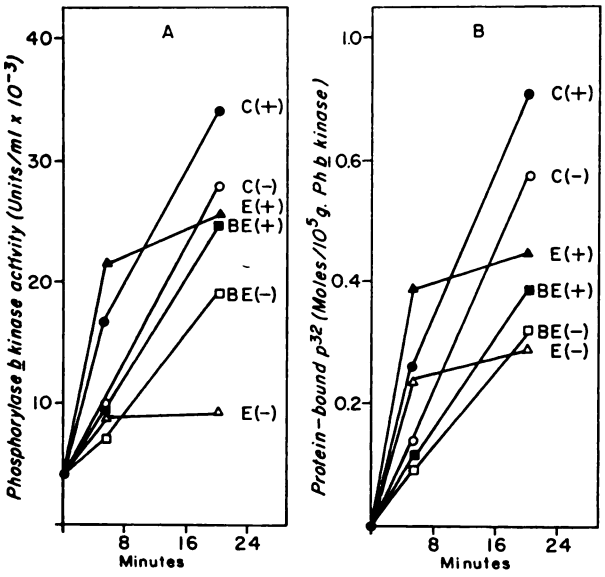



FIG. 3. Activation of purified phosphorylase *b* kinase in the presence of catalytic amounts of muscle extract. Kinase activation reaction mixtures are similar to those described in figure 2 except that the concentration of purified nonactivated phosphorylase *b* kinase is about 2 times greater. Muscle extract or boiled muscle extract, where added, constitutes one-tenth the volume of the mixture. C, E, and BE stand for control, added extract, and added boil extract respectively. (−) indicates without added cyclic 3′,5′-AMP and (+) indicates the presence of this nucleotide. Data on $P^{32}$ uptake are not corrected for slight contributions by phosphorylase *a* and possibly other proteins in the added muscle extract.

### KINASE II

Recently it was found that purified phosphorylase *b* kinase preparations, *i.e.*, the 40,000 rpm precipitate fraction (10) used in the experiments of figures 2 and 3, contains a second enzyme that will be referred to tentatively as Kinase II. This enzyme remains in the supernatant fraction when phosphorylase *b* kinase is precipitated at 30% saturation with ammonium sulfate. Kinase II has no activity with phosphorylase *b* as a substrate, but it does increase the rate of phosphorylation and activation of phosphorylase *b* kinase by ATP. Kinase II catalyzes the phosphorylation of casein by ATP, albeit at a very slow rate. As implied above, phosphorylase *b* kinase also catalyzes its own phosphorylation. This is shown using several times precipitated phosphorylase *b* kinase from which all of the Kinase II has presumably been removed. With such preparations phosphorylation and activation of the enzyme is autocatalytic. These results appear to indicate that two mechanisms exist for activation of nonactivated phosphorylase *b* kinase.

$$\underset{\text{(nonactivated)}}{\text{Phosphorylase } b \text{ kinase}} + \text{ATP} \xrightarrow{\text{Kinase II}} \underset{\text{(activated)}}{\text{Phosphorylase } b \text{ Kinase} - \text{P}} \quad (3)$$

$$\underset{\text{(nonactivated)}}{\text{Phosphorylase } b \text{ kinase}} + \text{ATP} \xrightarrow[\text{Kinase} - \text{P}]{\text{Phosphorylase } b} \underset{- \text{P (activated)}}{\text{Phosphorylase } b \text{ Kinase}} \quad (4)$$

### MECHANISM OF ACTION OF CYCLIC 3′,5′-AMP ON THE ACTIVATION OF PHOSPHORYLASE *b* KINASE

It has not been determined with certainty whether cyclic 3′,5′-AMP accelerates both of the reactions shown by equations (3) and (4) or just one of them. The reaction catalyzed by Kinase II cannot be studied independently, *i.e.*, without the reaction of equation (4) occurring simultaneously, and this makes it difficult to localize the site of action of the cofactor. The cyclic nucleotide does markedly enhance the rate of phosphorylation and activation of phosphorylase *b* kinase [equation (4)] using preparations of that enzyme from which Kinase II has been removed by the ammonium sulfate precipitation step. Since cyclic 3′,5′-AMP shows no direct effect on the phosphorylase *b* to *a* reaction itself [equation (1)] it would seem possible that its effect on the activation of phosphorylase *b* kinase by equation (4) may occur as a result of its making this enzyme a better substrate for its own phosphorylation rather than making it a better catalyst. It has not been possible, however, to demonstrate binding of cyclic 3′,5′-AMP to phosphorylase *b* kinase either in the presence or absence of $Mg^{++}$, nor has any protective action of the nucleotide against heat or urea denaturation been demonstrated. Kinase II is not available in sufficient purity to use at substrate levels, so that no direct binding studies of cyclic 3′,5′-AMP to this protein have been carried out.

Preincubation of cyclic 3′,5′-AMP alone with phosphorylase *b* kinase has been shown to have no effect on the activation reaction. If the cyclic AMP is destroyed

by the addition of skeletal muscle cyclic 3′,5′-AMP diesterase prior to the addition of ATP, no enhancement of activation rate is observed. The possibility of a direct action by cyclic 3′,5′-AMP as a phosphate-transferring coenzyme is being investigated. At least 95% of the nucleotide can be recovered as such from activation reaction mixtures and no exchange of $P^{32}$ from $\gamma$-$ATP^{32}$ into cyclic 3′,5′-AMP is observed.

### MUSCLE PHOSPHOPROTEIN PHOSPHATASE AND THE REVERSAL OF PHOSPHORYLASE *b* KINASE ACTIVATION

Since the activation of skeletal muscle phosphorylase *b* kinase by ATP is presumably due to its phosphorylation, it is reasonable to expect that the reversal of activation would be catalyzed by a phosphatase. Using $P^{32}$-labeled phosphorylase *b* kinase as a substrate, a $Mg^{++}$-requiring phosphoprotein phosphatase was detected in muscle; and after partial purification of this enzyme, its ability to reverse kinase activation was tested. Although this phosphatase would remove up to 60% of the bound $P^{32}$, it did not have any effect on kinase activity. It was concluded that the reversal of activation of endogenous phosphorylase *b* kinase seen in crude muscle extract (table 1) required other factors. In recent experiments using activated purified phosphorylase *b* kinase to which catalytic amounts of extract were added, reversal of activation accompanied by partial dephosphorylation of the enzyme has been achieved (table 2). Although it cannot be said with certainty that removal of phosphate was involved in this reversal of activation, it is possible that different phosphorylated sites on the activated kinase were being dephosphorylated as compared to those in the experiment with the purified phosphatase. The reversal of kinase activation is blocked by NaF.

TABLE 2

*Activation of purified phosphorylase b kinase and its reversal*

| Addition | Incubation Time | Kinase Activity | Protein-bound $P^{32}$ |
|---|---|---|---|
| | *min at 30°C* | *units/ml* $\times 10^{-5}$ | *Moles/$10^5$ g* |
| None | 0 | 0.34 | 0 |
| ATP, cyclic AMP ($10^{-4}$ M) | 20 | 4.68 | 0.74 |
| Hexokinase, glucose (added at 20 min) | 34.5 | 3.92 | 0.68 |
| Crude extract (added at 35 min) | 40 | 0.89 | 0.42 |
| NaF (0.05 M), ATP, cyclic AMP (added at 47 min) | 67 | 3.19[a] | 0.81 |

Purified nonactivated phosphorylase *b* kinase, 40,000 rpm precipitate fraction (10), was incubated at 30° with $P^{32}$-labeled ATP and cyclic 3′,5′-AMP followed by addition of glucose and hexokinase to remove the excess $ATP^{32}$. Muscle extract was then added and the mixture incubated 5 min. $ATP^{32}$, cyclic 3′,5′-AMP, and NaF were then added and the mixture was incubated to test for reversal of inactivation.

[a] This value represents full reactivation as compared to a control containing no extract but otherwise the same.

### THE BOUND PHOSPHATE IN ACTIVATED PHOSPHORYLASE *b* KINASE

In the conversion of phosphorylase *b* to phosphorylase *a* the enzyme is phosphorylated by ATP on a specific serine residue in the phosphorylase subunit (5). When the phenomenon of phosphorylase *b* kinase activation by ATP was first discovered, it was assumed by analogy with phosphorylase that a similar situation would exist for this enzyme. Experiments were undertaken to examine the chemical nature of the binding of $P^{32}$ in activated kinase, and, if possible, to determine the sequence of amino acids at the binding site. A study of conditions for hydrolysis showed that all of the bound phosphate in the kinase was labile in dilute alkali but relatively stable in acid, as is characteristic of serine-bound phosphate. A large amount of $P^{32}$-labeled kinase was then prepared and attacked exhaustively with trypsin and chymotrypsin for peptide studies; between 20 and 30 different phosphopeptides were released. A number of these were purified and their amino acid compositions determined, but none was obtained in sufficient quantity for sequence determination. From the composition alone, however, it was apparent that the kinase, unlike phosphorylase, is phosphorylated in more than one site during the activation reaction. Further support for this concept has come from the finding that the pattern of $P^{32}$-labeled peptides derived from partially phosphorylated kinase is different from that found with fully phosphorylated enzyme. As noted earlier, studies on the reaction of phosphorylated kinase with muscle phosphoprotein phosphatase are also in keeping with there being more than one phosphorylation site in the enzyme.

### DISCUSSION AND SUMMARY

An investigator in the field of endocrinology is ever faced with the fascinating and challenging problem of locating the point of action of a given agent. Sutherland and Cori (17) were the first to determine that the principal site of action of epinephrine in its glycogenolytic role was at the level of phosphorylase. Later Rall *et al.* (16) and Sutherland and Rall (18) determined that this effect was mediated by cyclic 3′,5′-AMP. The present contribution represents a continuation of this same general theme. We would like to know, in as specific terms as possible, what component of the phosphorylase system actually combines with cyclic 3′,5′-AMP and is responsible for its function. As has been pointed out (9, 10) the site of action appeared not to be on phosphorylase itself but rather on the enzyme, phosphorylase *b* kinase, which activates phosphorylase. Now, as a result of further investigation, the evidence is accumulating that the nucleotide may act even earlier in this complex array of regulatory components and reactions. Further work on the mechanism of phosphorylase *b* kinase activation will be required before the site of action of cyclic 3′,5′-AMP can be pinpointed with greater accuracy.

### REFERENCES

1. Cori, G. T.: The effect of stimulation and recovery on the phosphorylase *a* content of muscle. J. biol. Chem. **158**: 333-339, 1945.
2. Cori, G. T. and Cori, C. F.: The enzymatic conversion of phosphorylase *a* to *b*. J. biol. Chem. **158**: 321-332, 1945.
3. Cori, G. T. and Green, A. A.: Crystalline muscle phosphorylase. II. Prosthetic group. J. biol. Chem. **151**: 31-38, 1943.

4. DANFORTH, W. H., HELMREICH, E. AND CORI, C. F.: The effect of contraction and epinephrine on the phosphorylase activity of frog sartorius muscle. Proc. nat. Acad. Sci., Wash. **48:** 1191–1199, 1962.
5. FISCHER, E. H., GRAVES, D. G., CRITTENDEN, E. R. S. AND KREBS, E. G.: Structure of the site phosphorylated in the phosphorylase *b* to *a* reaction. J. biol. Chem. **234:** 1698–1704, 1959.
6. FISCHER, E. H. AND KREBS, E. G.: Conversion of phosphorylase *b* to phosphorylase *a* in muscle extracts, J. biol. Chem. **216:** 121–132, 1955.
7. KREBS, E. G. AND FISCHER, E. H.: The phosphorylase *b* to *a* converting enzyme of rabbit skeletal muscle. Biochim. biophys. Acta **20:** 150–156, 1956.
8. KREBS, E. G. AND FISCHER, E. H.: The role of metals in the activation of muscle phosphorylase. Ann. N. Y. Acad. Sci. **88:** 378–384, 1960.
9. KREBS, E. G., GRAVES, D. J. AND FISCHER, E. H.: Factors affecting the activity of muscle phosphorylase *b* kinase. J. biol. Chem. **234:** 2867–2873, 1959.
10. KREBS, E. G., LOVE, D. S., BRATVOLD, G. E., TRAYSER, K. A., MEYER, W. L. AND FISCHER, E. H.: Purification and properties of rabbit skeletal muscle phosphorylase *b* kinase. Biochemistry **3:** 1022–1033, 1964.
11. LYON, J. B. AND PORTER, J.: The relation of phosphorylase to glycogenolysis in skeletal muscle and heart of mice. J. biol. Chem. **238:** 1–11, 1963.
12. MEYER, W. L., FISCHER, E. H. AND KREBS, E. G.: Activation of skeletal muscle phosphorylase *b* kinase by $Ca^{2+}$. Biochemistry **3:** 1033–1039, 1964.
13. MORGAN, H. E. AND PARMEGGIANI, A.: Regulation of glycogenolysis in muscle. II. Control of glycogen phosphorylase reaction in isolated perfused heart. J. biol. Chem. **239:** 2435–2439, 1964.
14. POSNER, J. B., HAMMERMEISTER, K. E. AND BRATVOLD, G. E.: The assay of adenosine-3′,5′-phosphate in skeletal muscle. Biochemistry **3:** 1040–1044, 1964.
15. POSNER, J. B., STERN, R. AND KREBS, E. G.: Effects of electrical stimulation and epinephrine on muscle phosphorylase, phosphorylase *b* kinase, and adenosine-3′,5′-phosphate. J. biol. Chem. **240:** 982–985, 1965.
16. RALL, T. W., SUTHERLAND, E. W. AND BERTHET, J.: The relationship of epinephrine and glucagon to liver phosphorylase. IV. The effect of epinephrine and glucagon on the reactivation of phosphorylase in liver homogenates. J. biol. Chem. **224:** 463–475, 1957.
17. SUTHERLAND, E. W. AND CORI, C. F.: Effect of hyperglycemic-glycogenolytic factors and epinephrine on liver phosphorylase. J. biol. Chem. **188:** 531–543, 1951.
18. SUTHERLAND, E. W. AND RALL, T. W.: Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. J. biol. Chem. **232:** 1077–1091, 1958.
19. SUTHERLAND, E. W. AND WOSILAIT, W. D.: Inactivation and activation of liver phosphorylase. Nature **175:** 169–170, 1955.