Purification and Properties of Rabbit Skeletal Muscle Phosphorylase *b* Kinase*

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Phosphorylase *b* kinase was purified from fresh rabbit skeletal muscle by a procedure involving mild acid precipitation (*p*H 6.1), differential centrifugation, and free electrophoresis. The extent of purification achieved was 100- to 150-fold. In the procedure phosphorylase *b* was separated from the kinase by sedimentation of a glycogen–phosphorylase *b* complex at 80,000 × *g* for 1 hour. The kinase itself was sedimented when centrifuged at 100,000 × *g* for 3 hours, and there was evidence that the activity was associated with a component having a sedimentation coefficient in the range of 22–24 S. The form of phosphorylase *b* kinase purified in this preparation was referred to as nonactivated phosphorylase *b* kinase to distinguish it from activated kinase obtained by incubation of the enzyme with ATP or with trypsin. Nonactivated phosphorylase *b* kinase has a very high K_m (Michaelis constant) for phosphorylase *b* which was decreased in the presence of glycogen. The K_m for activated phosphorylase *b* kinase was much lower than that of nonactivated kinase. Activation of phosphorylase *b* kinase by incubation with ATP occurred more rapidly in the presence of adenosine-3′,5′-phosphate but did require this nucleotide. Glycogen and heparin also increased the rate of activation of phosphorylase *b* kinase by ATP. No gross changes in the sedimentation pattern of purified phosphorylase *b* kinase were caused by activation.

Muscle phosphorylase *b* kinase catalyzes the conversion of phosphorylase *b* to phosphorylase *a* according to the following equation (Krebs *et al.*, 1958):

$$2 \text{ phosphorylase } b + 4 \text{ ATP} \rightarrow \text{phosphorylase } a + 4 \text{ ADP}$$
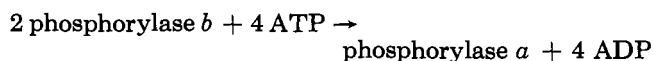
The terminal phosphate of ATP is transferred to a specific seryl residue (Fischer *et al.*, 1959) in the phosphorylase subunit, two of which are present in phosphorylase *b* and four of which are found in phosphorylase *a*. The latter has twice the molecular weight of phosphorylase *b* (Keller, 1955). Phosphorylase *a* formation can be followed conveniently by measuring phosphorylase activity in absence of AMP or by determining the extent of incorporation of ³²P into the protein (Krebs and Fischer, 1956; Krebs *et al.*, 1958). It is also possible to follow the reaction by coupling it with the pyruvic kinase–lactic dehydrogenase system, so that the ADP produced can be measured spectrophotometrically (Gonzales, 1962). No reversal of the kinase reaction has been demonstrated by any of several means employed (Krebs *et al.*, 1958).

Purification of muscle phosphorylase *b* kinase was undertaken to facilitate further studies on the mechanism of phosphorylase *b* to *a* reaction and as a part of a general program to resolve the numerous components involved in the complex system regulating glycogenolysis. It is known that glycogenolysis is accelerated in muscle under circumstances in which phosphorylase *a* formation is increased. Thus, when resting muscle containing phosphorylase predominantly in the *b* form (Krebs and Fischer, 1955) is stimulated electrically, phosphorylase *a* is formed and glycogenolysis ensues (Cori, 1956). Muscle glycogenolysis produced by epinephrine administration is also associated with phosphorylase *a* formation (Sutherland, 1951; Cori and Illingworth, 1956). The mechanisms underlying these physiological processes can be more readily understood when the various protein factors involved have been purified and are available for reconstruction of the systems *in vitro*.

Phosphorylase *b* kinase as extracted from resting skeletal muscle is present in a form that is essentially inactive at *p*H 7 and is only partially active at higher *p*H values (Krebs *et al.*, 1959). This form of the enzyme was originally called "*p*H 7–inactive" phosphorylase *b* kinase but will be referred to simply as nonactivated kinase in this paper. Several seemingly unrelated methods can be used to activate the kinase *in vitro*; the resulting preparation in each case exhibits greatly increased activity at *p*H 7 and some increase in activity at higher *p*H values. The methods for kinase activation (Krebs *et al.*, 1959; Krebs and Fischer, 1960) include: (1) incubation with Ca^{2+}; (2) incubation with ATP and Mg^{2+}; and (3) incubation with trypsin. The physiological significance of the various types of kinase activation is unknown, but Posner *et al.* (1962) showed that activation of this enzyme can be correlated with increased levels of phosphorylase *a in vivo*.

This paper presents a method for the purification of nonactivated phosphorylase *b* kinase from rabbit skeletal muscle. Certain physical and kinetic properties of the purified kinase are described together with detailed studies on activation of the enzyme by trypsin and ATP. The latter type of activation is affected by adenosine-3′,5′-phosphate (cyclic AMP), the nucleotide found to be an intermediate in the metabolic action of epinephrine (Rall and Sutherland, 1958). The following paper (Meyer *et al.*, 1964) will be concerned with activation of purified phosphorylase *b* kinase by Ca^{2+} and the separation of a protein factor required for this reaction.

MATERIALS AND METHODS

Phosphorylase b.—Crystalline rabbit skeletal muscle phosphorylase *b* was prepared as described previously (Fischer and Krebs, 1958) except that the third crystallization was carried out in the presence of 10^{-4} M AMP, 10^{-2} M $Mg(Ac)_2$ instead of 10^{-3} M AMP, 10^{-2} M $Mg(Ac)_2$. Use of the lower AMP concentration made it easier to remove the nucleotide and the yield of crystalline enzyme was almost as great at this stage as with

10-fold higher AMP levels. For the preparation of phosphorylase *b* solution to be used in the kinase assay, a portion of the crystalline suspension was centrifuged at 0°, and the protein was dissolved in sufficient neutral 0.015 M cysteine solution to give a concentration of 30–35 mg (48,000–56,000 units) per ml. This solution was clarified by centrifugation to remove small amounts of cystine that were present in the crystalline phosphorylase *b* suspension and was then treated with charcoal (Fischer and Krebs, 1958) to remove AMP. The solution was assayed for phosphorylase *b* and was diluted to a concentration of 40,000 units per ml. This solution was kept at 0° and was stable for several days.

Other Materials.—Sodium glycerophosphate, L-cysteine hydrochloride, and protamine sulfate were obtained from Nutritional Biochemicals, Inc. (Cleveland, Ohio). AMP and disodium ATP were obtained from Pabst Laboratories (Milwaukee, Wis.). Tris ("Sigma 121") and adenosine-3',5'-phosphate were purchased from the Sigma Chemical Co. (St. Louis, Mo.). Shellfish glycogen was obtained from Krishell Laboratories, Inc. (Portland, Ore.) and was purified by the Somogyi (1957) procedure. Samples of this glycogen and also of purified liver glycogen (Mann Research Laboratories, New York) were treated with Norit A (Pfanstiehl) to remove contaminating nucleotides. A sample of purified corn phytoglycogen was a gift from Dr. T. J. Schoch of the Corn Products Co. (Argo, Ill.). Crystalline trypsin and crystalline soybean-trypsin inhibitors were obtained from Worthington Biochemical Corp. (Freehold, N.J.). Purified heparin from a beef source was lot no. A-91800, kindly supplied by Eli Lilly Co. (Indianapolis, Ind.) and assayed 124 units per mg. Purified sodium hexametaphosphate was obtained from Fisher Scientific Co. (Fair Lawn, N.J.) and polyaspartic acid was purchased from Schwarz Bio Research, Inc. (Orangeburg, N.Y.). A sample of highly purified yeast nucleic acid was a gift from Dr. Milton P. Gordon.
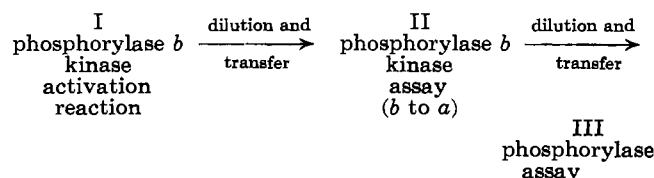
Determination of Phosphorylase b Kinase Activity and Definition of Units.—Since the original development of a method for assaying phosphorylase *b* kinase (Krebs and Fischer, 1956), several modifications have been introduced (Krebs *et al.*, 1959; Krebs and Fischer, 1962), and the necessity for controlling all conditions exactly, in order to achieve reproducible results, has become increasingly apparent. This is not surprising in view of the complexity of this determination. Activity determinations reported in this paper were carried out as follows:

A partial reaction mixture was made up containing 0.2 ml of 0.125 M Tris, 0.125 M glycero-P buffer either at pH 8.6 (final pH 8.2) or pH 6.8 (final pH 6.8), 0.2 ml of AMP-free phosphorylase *b* solution containing 8000 units of enzyme in 0.015 M neutral cysteine; and 0.1 ml of phosphorylase *b* kinase appropriately diluted in cold 0.015 M neutral cysteine. The phosphorylase *b* solution and the diluted kinase were kept at 0° until addition. Phosphorylase *b* was added no more than 15 minutes before starting the kinase reaction. The kinase was added no more than 5 minutes before the assay. The mixture was placed in a water bath at 30°, and after 1–2 minutes allowed for temperature equilibration, the reaction was started by the addition of 0.1 ml of 0.06 M Mg(Ac)₂, 0.018 M ATP, pH 7.0. At 5 minutes a 0.1-ml aliquot was removed and transferred to 1.9 ml of 0.04 M glycero-P, 0.03 M cysteine buffer, pH 6.8, at room temperature. Following this dilution, which effectively stopped the kinase reaction, assay for phosphorylase *a* was carried out by the method of Cori *et al.* (1943), modified slightly as described below (see also Illingworth and Cori, 1953). A control

reaction in which kinase is omitted was run along with each test.

For the phosphorylase *a* assay, 0.2 ml of diluted kinase reaction mixture was added to 0.2 ml of 0.032 M glucose-1-P, 2% glycogen, and the mixture was incubated for 5 minutes at 30°. A control reaction with buffer and substrate was also run. The reaction was stopped by addition of 8.2 ml of solution containing 0.5 meq of H₂SO₄ and 25 mg of ammonium molybdate. Inorganic phosphate analysis was then carried out by the procedure of Fiske and Subbarow (1925). For this it was necessary to add an additional 0.9 ml of 5 N H₂SO₄ and 0.5 ml of the aminonaphtholsulfonic acid reagent. A unit of phosphorylase *b* kinase was defined as before (Krebs and Fischer, 1956), i.e., that amount of kinase that gave rise to 100 units of phosphorylase *a* per ml of kinase reaction mixture in 5 minutes under the stated conditions. Kinase units were designated according to the pH of the reaction mixture. Phosphorylase *a* units were calculated as described by Cori *et al.* (1943). The amount of kinase used in the activity test was such that no more than 10% of the phosphorylase *b* was converted to phosphorylase *a* during the 5-minute interval of the test.

Activation of Phosphorylase b Kinase by ATP.—Activation of phosphorylase *b* kinase was carried out in a reaction mixture ordinarily made up as follows: 0.2 ml 0.125 M Tris, 0.125 M glycerol-P buffer, pH 6.8; 0.2 ml 6 × 10⁻² M Mg(Ac)₂, 1.8 × 10⁻² M ATP, pH 7.0; 0.4 ml H₂O or additions; and 0.2 ml of nonactivated kinase diluted in 0.015 M neutral cysteine solution or added without dilution according to the nature of the experiment. The activation was terminated after a timed interval of incubation at 30° by withdrawing an aliquot and diluting at least 1:20 in cold neutral 0.015 M cysteine. Kinase activities were then carried out in the usual manner. The sequence of events in this type of experiment is illustrated as follows:

I	dilution and	II	dilution and
phosphorylase *b* kinase activation reaction	⟶ transfer	phosphorylase *b* kinase assay (*b* to *a*)	⟶ transfer
		III phosphorylase assay	

In an attempt to avoid confusion, the term "stimulation" will be applied to describe the action of substances which increase the velocity of the phosphorylase *b* to *a* reaction when introduced directly into the kinase assay reaction mixture. The term "activation" will be reserved to describe those changes that nonactivated kinase undergoes when it is preincubated with various activating components. These changes are, however, also made manifest by an increased velocity of the phosphorylase *b* to *a* reaction.

Other Methods.—Protein determinations were carried out by the method of Robinson and Hogden (1940). Glycogen analyses were performed by the procedure described by Hassid and Abraham (1957). Chromatographic separation of cyclic AMP from other nucleotides was carried out with descending paper chromatography using isopropanol–NH₄OH–0.1 M H₃BO₃, 7:1:2. This system was kindly suggested by Dr. George Drummond.

RESULTS

Purification of Nonactivated Phosphorylase b Kinase. —Two 7- to 10-pound New Zealand white female rabbits were injected intravenously with 200–250 mg of
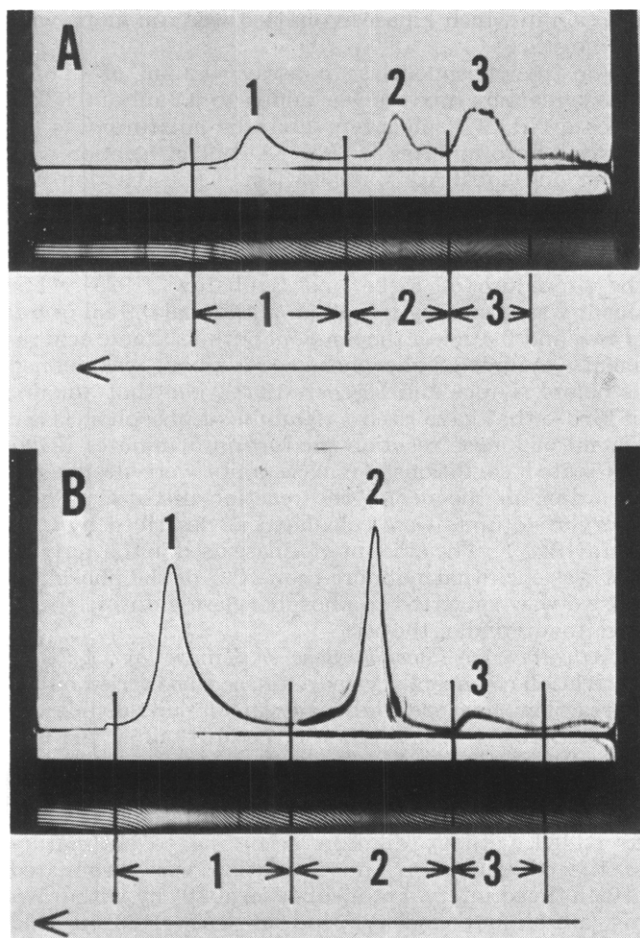
FIG. 1.—Free electrophoretic patterns of the 30-supernatant fraction (A) and the 40-precipitate fraction (B) of a phosphorylase b kinase preparation obtained in the ascending limb with the standard 11-ml cell of the Spinco Model H apparatus in 0.05 M glycero-P, 0.002 M EDTA, pH 7.50 at 5.7 v cm^{-1}. The 30-supernatant fraction was run for 11,820 seconds and the 40-precipitate fraction for 16,380 seconds. The mobilities ($\times 10^{-5}$ cm^2 volt^{-1} sec^{-1}) determined for the major components of the 30-supernatant fraction were: component 1, -5.9; component 2, -2.5; and component 3, -1.2. The limits of the zones used for counting Rayleigh fringes for the data in Table IV are indicated by the vertical lines drawn on the patterns. Protein concentration: A, 5 mg/ml; B, 8 mg/ml.

Nembutal and immediately bled through the jugular veins. The hind leg and back muscles were excised, chilled in ice, and ground with a meat grinder in the cold room. The ground muscle was weighed and immediately homogenized with 2.5 volumes (liters/kg) of neutral 4×10^{-3} M EDTA solution at 0° for 1 minute using a 5-quart capacity Waring Blendor. The homogenate was centrifuged at 0° for 40 minutes at 4000 × g, and the supernatant solution was decanted through glass wool to remove lipid material.

The pH of the cold extract, which was usually in the range of 6.5–6.7, was adjusted to 6.1–6.2 with 1 N HAc, whereupon it became moderately turbid. After standing in an ice bath for 5–10 minutes, the mixture was centrifuged at 0° for 30 minutes at 4000 × g. The sedimented material was taken up using an amount of 0.10 M glycero-P, 4×10^{-3} M EDTA solution, pH 8.2, approximately equal in volume to the protein pellets; then 0.05 M glycero-P, 2×10^{-3} M EDTA, pH 7.0 was added to a final volume of 165 ml per 1000 g of ground muscle used in the preparation. A final short homogenization at 0° was performed using a Teflon-pestle glass-tube homogenizer to assure complete mixing and

fine dispersion of the material, a portion of which dissolved. The final pH of the mixture was between 6.8 and 7.2.

The above suspension (acid-precipitate fraction) was centrifuged at 0° for 90 minutes at 30,000 rpm using the No. 30 rotor in the Spinco Model L preparative ultracentrifuge. The clear supernatant solution (30-supernatant fraction) was decanted from the pellets (30-precipitate fraction) and was stored in the frozen state unless the preparation was to be continued immediately.

The 30-supernatant fraction was centrifuged at 0° for 180 minutes at 40,000 rpm using the No. 40 rotor in the Spinco preparative ultracentrifuge, and the supernatant solution was decanted (40-supernatant fraction). The sedimented protein was taken up in 10–20 ml of 0.05 M glycero-P, 2×10^{-3} M EDTA buffer, pH 7.0. For this it was advantageous simply to cover the protein pellet in each tube with a small amount of the buffer and to allow 1–2 hours at 20–25° for gradual solution to occur without stirring. Gentle stirring and addition of the remainder of the buffer then effected complete solution with a minimum of foaming and denaturation. The final solution (40-precipitate fraction) was almost clear except for a slight opalescence. The spectrum showed a ratio of absorption at 280 mμ to absorption at 260 mμ of 1.65.

Table I shows the results of a typical preparation of

TABLE I
PURIFICATION OF RABBIT MUSCLE
PHOSPHORYLASE b KINASE[a]

Fraction	Volume (ml)	Total Activity (units × 10^{-6})	Specific Activity (units/mg)
Extract	2500	27.3	454
Acid precipitate	206	24.5	3,750
Acid supernatant	2450	1.5	29
30-Supernatant	150	15.5	14,940
30-Precipitate	54	7.3	1,610
40-Supernatant	144	2.7	4,520
40-Precipitate	15	10.2	28,000

[a] Preparation procedure described in text. Phosphorylase b kinase activities were determined at pH 8.2 under standard conditions. Starting material: 1240 g of fresh rabbit muscle.

nonactivated phosphorylase b kinase carried through the steps described above. The 40-precipitate fraction represents a 60-fold purification on a protein basis starting with the muscle extract. Unless noted otherwise, the experiments reported in this paper were carried out on this fraction. Further purification was achieved by free electrophoresis (*vide infra*) or by recentrifugation at 100,000 × g (Meyer *et al.*, 1964). Attempts to fractionate the kinase using salting-out procedures, organic solvents, or chromatography on DEAE-cellulose were unsuccessful. The 40-precipitate fraction was free of phosphorylase phosphatase and ATPase activity which could interfere in the phosphorylase b to a conversion reaction. The kinase could be stored in the frozen state and was stable for at least 6 months. After freezing and thawing several times, the kinase sometimes developed a distinct turbidity; under these conditions the specific activity of the enzyme could be increased by a simple clarification spin. On very few occasions the ratio of activity at pH 6.8 to activity at pH 8.2 increased to 0.15–0.25 on prolonged storage of the 40-precipitate fraction.
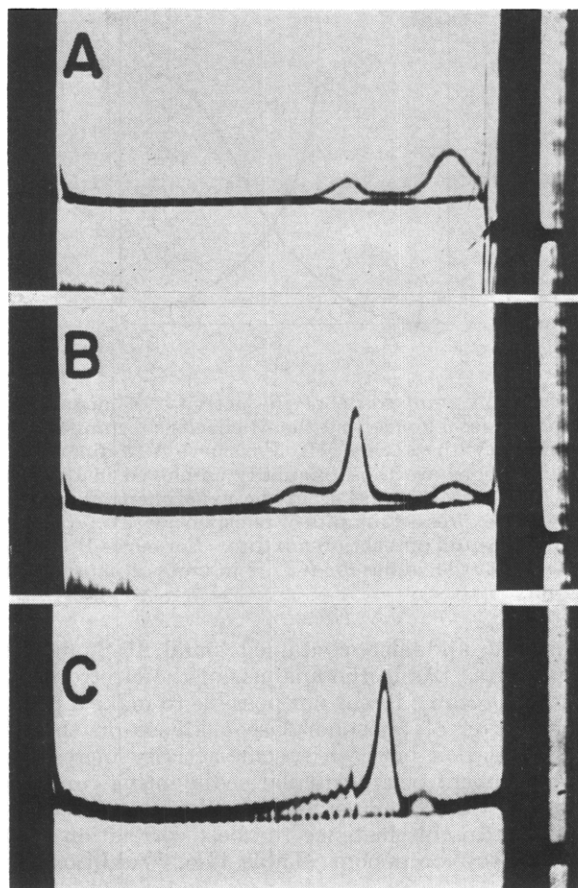
FIG. 2.—Sedimentation patterns of fractions from phosphorylase b kinase preparation. A and B = 30-supernatant and 40-precipitate fractions, respectively, from the kinase preparation shown in Fig. 1 (also preparation 1 in Tables II and III); C = fast component withdrawn from ascending limb after electrophoresis of a 40-precipitate fraction. All runs made in 0.05 M glycero-P, 0.002 M EDTA, pH 6.9. Duration of runs, 48 minutes at 39,460 rpm. Bar angle, 70°. Protein concentration (mg/ml): A, 5.0; B, 8.0; C, 10.0. The direction of sedimentation is from right to left in all cases. $s_{20,w}$ values for major components. A, 6.1 and 23.2 S; B, 7.8, 23.8, and 32.4 S; C, 1.96 and 24.0 S. No sedimentation constant calculated for heavy nondiscrete material in C.

FIG. 3.—Extent of phosphorylase b kinase activation during the phosphorylase b to phosphorylase a reaction at pH 8.2. Reaction mixtures were those regularly employed in the assay for phosphorylase b kinase activity. For curve E, pH = 8.2; for curve F, pH = 6.8. Kinase = 40-precipitate fraction, 0.4 μg/ml. Aliquots were removed from E at intervals and adjusted to pH 6.8 with a trace of 1 N acetic acid. Phosphorylase a activities were determined as shown. See text for further description.

The Occurrence and Distribution of Phosphorylase b in Fractions of the Kinase Preparation.—In the fractionation of nonactivated muscle phosphorylase b kinase, phosphorylase b itself proved to be a persistent contaminant in the various fractions. Since the latter enzyme is a substrate for the former and might logically be expected to bind to the kinase, it was of interest to study the distribution of both proteins in fractions from a typical preparation to see whether a constant ratio of kinase to phosphorylase was maintained. This did not occur. In the original muscle extract the ratio of phosphorylase b kinase to phosphorylase b activity was 4.6. In subsequent steps this ratio increased to 9.6, 81, and 470 in the acid-precipitate, 30-supernatant, and 40-precipitate fractions, respectively. It was apparent that much of the phosphorylase was being separated from the kinase in the centrifugation of the acid-precipitate fraction at 30,000 rpm. Sedimentation of phosphorylase b at this step was dependent on the presence of glycogen. When the acid-precipitate fraction was stored for several days at 0°, it was found that its glycogen content was greatly reduced, and under these conditions phosphorylase b stayed in the supernatant solution along with phosphorylase b kinase on centrifugation.
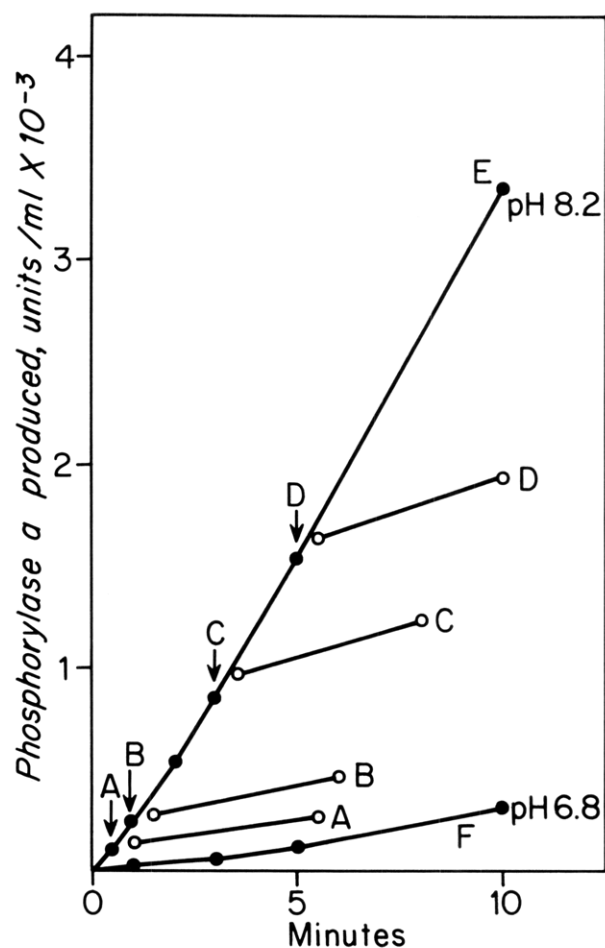
Electrophoresis of Nonactivated Phosphorylase b Kinase.—The 30-supernatant and 40-precipitate fractions when examined by free boundary electrophoresis showed three major components in both instances (Fig. 1). In an attempt to identify which peak corresponded to phosphorylase b kinase, the specific-activity change occurring between the 30-supernatant and the 40-precipitate fractions was compared with changes in the relative concentrations of individual components. For this, three mobility zones, each of which encompassed one of the major peaks, were marked off as shown in Figure 1; and the relative protein concentrations for each zone were determined by counting Rayleigh fringes. Results are shown in Table II. Although there was no exact correspondence between specific-activity increase and the relative amount of protein in a particular mobility zone, this condition was most nearly approximated for the most rapidly migrating component in zone 1. Isolation of that component by withdrawing it from the ascending limb of the electrophoresis cell resulted in a striking increase in specific activity of the kinase (Table II). Component 3, isolated by withdrawal from the descending limb of the cell, and a mixture of components 2 + 3 also obtained from the descending limb, showed no kinase activity. As a practical procedure in the preparation of phosphorylase b kinase, free electrophoresis was a
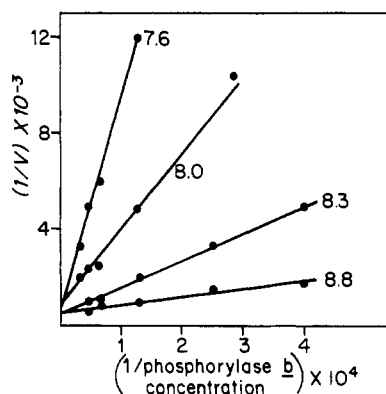
FIG. 4.—Reciprocal plots of velocity against phosphory-lase b concentration in the phosphorylase b to a reaction at different pH values using nonactivated phosphorylase b kinase. Reaction mixtures were those employed for the assay of phosphorylase b kinase except that the concentration of phosphorylase b was varied. Kinase = 30-supernatant fraction, 2.5 $\mu g/ml$. V = velocity in units of phosphorylase a activity per ml per 5 minutes. Phosphorylase b concentrations are expressed in units/ml.

TABLE II
COMPARISON OF CHANGES IN RELATIVE PROTEIN CONCEN-TRATIONS OF ELECTROPHORETIC COMPONENTS OF THE 30-SUPERNATANT AND 40-PRECIPITATE FRACTIONS WITH THE INCREASE IN SPECIFIC PHOSPHORYLASE b KINASE ACTIVITY[a]

| Prep-ara-tion | Fraction | Phorphor-ylase b Kinase Activity at pH 8.2 (units/mg) | Relative Protein Concentrations | | |
			Zone 1 (%)	Zone 2 (%)	Zone 3 (%)
1	30-Supernatant	14,500	32	28	40
	40-Precipitate	44,000	55	39	6
	Zone 1 from 40-precipitate	74,000			
2	30-Supernatant	26,000	31	23	46
	40-Precipitate	51,000	67	25	8
	Zone 1 from 40-precipitate	106,000			

[a] The values presented for preparation 1 in this table were obtained from the preparation used for the electrophoretic patterns presented in Figure 1. The values for preparation 2 represent a separate experiment. In each experiment the protein in zone 1 was withdrawn from the electrophoresis cell under direct visual control.

costly step since the yield was low. When electrophoresis with back compensation was employed in an attempt to improve the recovery, the enzyme obtained was much less stable than that obtained without compensation; no reason for this has been determined. Phosphorylase b kinase purified by free electrophoresis contained no phosphorylase b; the latter enzyme migrated with the mobility of component 2.

Study of Nonactivated Phosphorylase b Kinase Fractions in the Analytical Ultracentrifuge.—The preparative procedure for nonactivated phosphorylase b kinase (Table I) suggested that the activity was associated with high-molecular-weight material, since most of the enzyme was sedimented in 3 hours at 100,000 × g. Examination of the 30-supernatant fraction in the analytical ultracentrifuge showed the presence of a component with an $s_{20,w}$ value of 23 S[1] comprising 20% of the protein and a more abundant lighter component or group of components (Fig. 2A). The 40-precipitate fraction (Fig. 2B) showed an enrichment of the 23-S
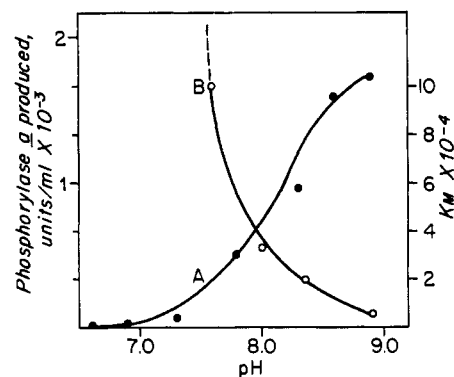

FIG. 5.—A comparison of the activity of nonactivated phosphorylase b kinase and the Michaelis constant for phosphorylase b with varying pH. For curve A (ordinate on the left) conditions are those regularly employed in the kinase assay. Kinase = same as in the experiment of Figure 4. Activity is expressed in units of phosphorylase a produced in 5 minutes per ml of reaction mixture. For curve B (ordinate on the right) the values for K_m are in units of phosphorylase b per ml.

component and also contained small 16-S and 32-S components. As in the analysis of the electrophoretic data (*vide supra*), it was not possible to make a positive identification of phosphorylase b kinase on the basis of a correlation between specific-activity increase and the enrichment of a particular sedimenting component in passing from the 30-supernatant to its 40-precipitate fraction. In this instance the best correlation existed for the 23-S component (Table III). Additional evi-

TABLE III
COMPARISON OF THE AREAS UNDER THE BOUNDARIES OF SEDIMENTATION PATTERNS OF 30-SUPERNATANT AND 40-PRE-CIPITATE FRACTIONS AND SPECIFIC ENZYMATIC ACTIVITIES[a]

| Prep-ara-tion | Fraction | Composition | | | | Activity at pH 8.2 (units/mg) |
		6–8 S (%)	16 S (%)	23 S (%)	32 S (%)	
1	30-Supernatant	80		20		14,500
	40-Precipitate	18	8	46	29	44,300
2	30-Supernatant	85		15		26,000
	40-Precipitate	26	6	68		51,000

[a] The sedimentation patterns for preparation 1 are shown in Figure 2A, B. Preparation 2 was from a separate experiment (not illustrated).

dence that kinase activity was associated with the 23-S component was obtained by examining a 40-precipitate fraction devoid of any 32-S material[1] using the Yphantis-Waugh (1956) separation cell; in this experiment more than 90% of the activity was recovered with the 23-S component. Examination of the fast-moving component obtained from the ascending limb of the cell after electrophoresis (see previous section) showed a major component with an $s_{20,w}$ = 24 S (Fig. 2C).

Time Course of the Phosphorylase b to Phosphorylase a Reaction with Nonactivated Phosphorylase b Kinase.—Previous studies (Krebs *et al.*, 1959) had shown that some degree of kinase activation occurred during the phosphorylase b to phosphorylase a reaction, when a crude preparation of nonactivated phosphorylase b

[1] Values for $s_{20,w}$ between 22.5 and 24.0 have been found for what is referred to as the 23-S component in the text. For the 32-S component the range of values has been from 30 to 33 S. The 32-S component has been absent in some preparations of phosphorylase b kinase but was present in others (see Figs. 2B and 13A).
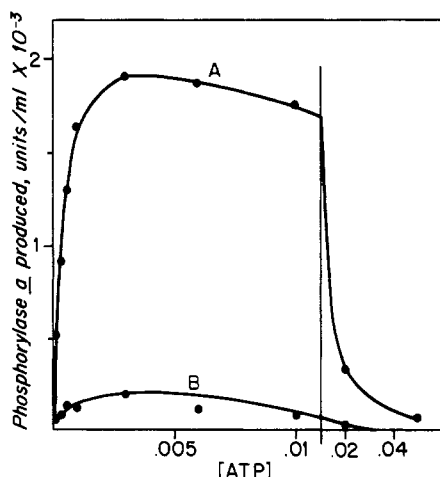
FIG. 6.—The activity of nonactivated phosphorylase *b* kinase with varying [ATP] in the presence of 0.01 M Mg²⁺ at pH 8.2 (curve A) and pH 6.8 (curve B). Conditions as in the regular kinase assay system except for varying [ATP]. Kinase = 40-precipitate fraction, 0.4 μg/ml.
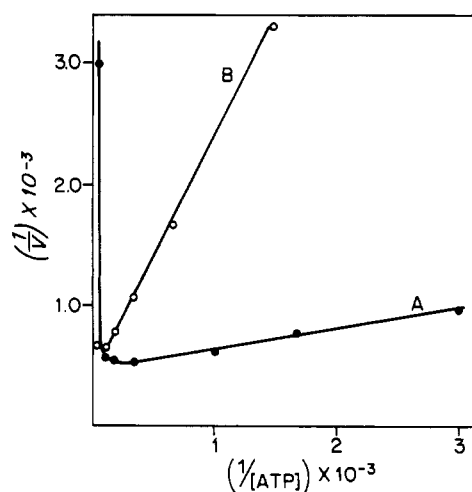
FIG. 7.—Reciprocal plots of velocity against ATP concentration in the phosphorylase *b* to *a* reaction using nonactivated phosphorylase *b* kinase at pH 8.2. Curve A from the data of Figure 6. V = velocity in units of phosphorylase *a* produced per ml per 5 minutes. For curve B the ratio [Mg²⁺] to [ATP] ratio = 3.33 for all points; otherwise, conditions as in regular kinase assay system. Kinase = 40-precipitate fraction, 0.3 μg/ml.

kinase was used as the enzyme. This had been anticipated, since the kinase is necessarily exposed to ATP in the *b* to *a* reaction, and it was known that preincubation with ATP caused activation of the enzyme. At the beginning of the present group of experiments, it was deemed desirable to reinvestigate this phenomenon using purified enzyme in order to determine the extent of activation that might be expected in assaying the nonactivated form of phosphorylase *b* kinase. In the experiment of Figure 3, the conversion of phosphorylase *b* to *a* by nonactivated phosphorylase *b* kinase at pH 8.2 was interrupted at intervals by abruptly lowering the pH of aliquots to 6.8 using a trace of acetic acid; reaction rates at the lower pH were then determined as shown. Between 30 seconds and 5 minutes in the reaction at pH 8.2 the kinase was activated slightly, as is shown by a doubling of the rate from A to D in the reaction at pH 6.8. As will be shown in subsequent sections, full activation of phosphorylase *b* kinase brought about by preincubation of the enzyme with ATP results in a 15- to 40-fold increase in the activity of the enzyme as measured at pH 6.8. It was concluded from these studies that, although it is impossible to assay nonactivated kinase without having some degree of enzyme activation in the test system, the extent of this activation is of little quantitative significance in relation to the activity changes seen by preincubation of the kinase under activating conditions.

The Michaelis Constant for Phosphorylase b with Nonactivated Phosphorylase b Kinase.—With purified nonactivated phosphorylase *b* kinase it was found that the rate of the phosphorylase *b* to *a* reaction was highly dependent upon phosphorylase *b* concentration, and particularly at low pH values, it was impossible experimentally to saturate the enzyme with respect to this substrate. Figure 4 shows reciprocal plots of the initial velocities of the reaction against phosphorylase *b* concentration at several pH values, and the Michaelis constants (K_m) derived from these data are plotted in Figure 5, curve B. Curve A of Figure 5 shows the activity of nonactivated phosphorylase *b* kinase as a function of pH. At pH 8.5 the value for K_m was 13,300 units of phosphorylase *b* per ml (3.3 × 10⁻⁵ M), which is equal to the concentration of this substrate commonly used in the kinase-activity test. Below this pH K_m increased rapidly, and at pH 7.6 which is almost the lower limit at which the constant can be determined with nonactivated kinase, a value of

100,000 units per ml (2.5 × 10⁻⁴ M) was found. The inverse relationship between activity and K_m suggests that the latter is an important factor accounting for the low activity of nonactivated phosphorylase *b* kinase at pH 7. The data of Figure 3 are not sufficiently precise to be used for determining the pH optimum for V_{max}, but it is apparent that great variations do not occur over the range studied in this experiment.

The Effect of ATP Concentration on the Activity of Nonactivated Phosphorylase b Kinase.—When the concentration of Mg²⁺ was held constant at 0.01 M and ATP was varied, the activity of nonactivated phosphorylase *b* kinase at pH 8.2 reached an optimum at around 3 × 10⁻³ M ATP and fell off sharply when [ATP] exceeded [Mg²⁺] (Fig. 6, curve A). Very little activity was present at pH 6.8 under any condition (Fig. 6, curve B). The inhibition by excess ATP resembled that seen with fructokinase (Hers, 1952) and phosphofructokinase (Lardy and Parks, 1956). In earlier experiments using Mn²⁺ instead of Mg²⁺ (Krebs and Fischer, 1956) an excess of either ATP or the metal was found to be inhibitory. Using the linear portion of a reciprocal plot (Fig. 7, curve A) of the data of Figure 6, curve A, a value for K_m of 3.1 × 10⁻⁴ M ATP at pH 8.2 was derived. When the concentrations of both Mg²⁺ and ATP were varied, but the ratio of [Mg²⁺] to [ATP] was kept constant at 3.3, a K_m value of 4.3 × 10⁻³ M at pH 8.2 was obtained from a reciprocal plot (Fig. 7, curve B) of the data. Under these latter conditions slight inhibition was seen only at the highest concentration used, i.e., 0.1 M Mg²⁺, 0.03 M ATP. In an experiment (not illustrated) with 0.01 M Mg²⁺ the K_m for ATP at pH 7.4 was found to be 2.4 × 10⁻⁴ M. Since the value for K_m at pH 8.2 under these conditions was 3.1 × 10⁻⁴ M, it is apparent that no marked changes with pH occur as was found with K_m for phosphorylase *b* (*vide supra*).

The Effect of Components of the Phosphorylase Reaction on Nonactivated Phosphorylase b Kinase.—Binding between glucose-1-P and phosphorylase or between glycogen and this enzyme would be expected because of the role of these substances as substrates for the enzyme. Similarly, it would be anticipated that the cofactor, AMP, would bind to phosphorylase; and, indeed, the binding of this last substance as well as
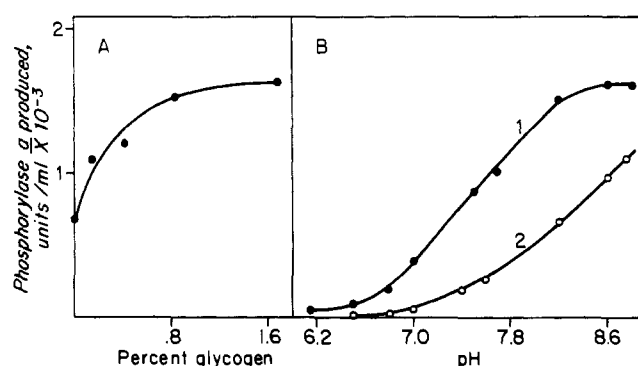
Fig. 8.—Glycogen stimulation of the phosphorylase b to a reaction using nonactivated phosphorylase b kinase. Part A, activity at pH 8.2 with varying concentrations of glycogen included in the regular kinase reaction mixtures. Kinase = 30-supernatant fraction, 0.7 μg/ml. Part B, activity at various pH values in the presence (curve 1) of 0.8% glycogen; curve 2, control without glycogen. Ordinate for parts A and B = phosphorylase a produced in 5 minutes.

that of glycogen to phosphorylase have been demonstrated directly by Madsen and Cori (1957, 1958). It appeared probable that one or more of these substances might affect the phosphorylase b kinase reaction in which phosphorylase serves not as a catalyst but as a substrate. Glucose-1-P, however, at concentrations up to 0.01 M, had no effect on the activity of nonactivated kinase when tested at either pH 6.8 or 8.2. AMP at concentrations up to 2 × 10^{-4} M also had no significant effect in the kinase reaction. Glycogen did have a marked stimulatory effect when introduced into the phosphorylase b to a reaction mixture (Fig. 8). At pH 8.2 with nonactivated phosphorylase b kinase half-maximal stimulation was found to occur at 0.3% glycogen (Fig. 8A). The extent of kinase stimulation varied depending upon the pH of the reaction's (Fig. 8B) being greatest on a percentage basis at points below pH 7.5. Pretreatment of a concentrated solution of nonactivated kinase with glycogen, followed by high dilution of the enzyme prior to testing its activity, resulted in no increase in reaction velocity; i.e., the glycogen had to be present in the phosphorylase b to a reaction mixture itself in order to be effective. Evidence that glycogen stimulation was not due to impurities was obtained by testing a variety of commercial shellfish- and liver-glycogen samples before and after purification with Norit as well as a sample of corn phytoglycogen; all had similar stimulating properties. By allowing kinase reactions to go to completion in the presence and absence of glycogen, it was determined that identical final levels of phosphorylase activity were obtained, thus showing that the stimulation of kinase activity by glycogen was not due to the production of a more active species of phosphorylase a in the presence of the polysaccharide. It was difficult to rule out all possibility that part of the stimulatory effect of glyco-gen might be owing to significant enzyme activation occurring in the phosphorylase b to a reaction, since, as will be shown later, glycogen does have an effect on kinase activation by ATP. Some insight into the mechanism of glycogen stimulation was gained by comparing the K_m for phosphorylase b determined in the presence of glycogen with that determined in its absence. In an experiment at pH 7.5 the K_m was found to be 4000 units of phosphorylase b per ml (1 × 10^{-5} M) in the presence of 0.4% glycogen and 50,000 units per ml (1.2 × 10^{-4} M) in the absence of the polysaccharide.[2] These data can probably be interpreted to mean that a

TABLE IV
EFFECT OF HEPARIN ON THE ACTIVITY OF
NONACTIVATED PHOSPHORYLASE b KINASE[a]

Heparin Concentration (mg/ml)	Phosphorylase a Produced in 5 Min at pH 6.8 ([units/ml] × 10^{-2})	Phosphorylase a Produced in 5 Min at pH 8.2 ([units/ml] × 10^{-2})
None	1.1	10.7
5.0	4.0	7.7
2.5	4.6	8.9
1.0	4.7	10.5
0.3	5.1	10.7
0.05	3.7	10.7

[a] Heparin at different concentrations was included in regular kinase reaction mixtures at pH 6.8 or 8.2. Kinase = 40-precipitate fraction, 0.2 γ/ml in reactions.

glycogen-phosphorylase b complex has greater affinity for the kinase than phosphorylase b alone.

The Effect of Heparin on Nonactivated Phosphorylase b Kinase.—Depending upon its concentration and the pH of the reaction, heparin was shown to stimulate or inhibit the activity of nonactivated phosphorylase b kinase (Table IV); particularly noteworthy were the stimulatory effects at low concentrations of the polysaccharide seen at pH 6.8. For these effects it was essential that the heparin be present in the kinase-reaction mixture itself; pretreatment of a concentrated solution of kinase followed by high dilution and assay had no effect on the enzyme. Other polyanionic substances tested, polyaspartic acid at 0.2%, hexametaphosphate at 0.16%, and yeast nucleic acid at 0.1%, had no stimulatory effect on the kinase at pH 6.8. At pH 8.2 the first two of these substances inhibited 30 and 64%, respectively.

Inhibitors of Nonactivated Phosphorylase b Kinase.— The most effective inhibitors of nonactivated phosphorylase b kinase include several chelating agents that will be discussed in the following paper (Meyer et al., 1964). Protamine, which is known to bind to phosphorylase b (Krebs, 1954; Madsen and Cori, 1954), caused 50% inhibition under standard assay conditions at pH 8.2 with a concentration of 0.16 mg/ml and complete inhibition at 0.8 mg/ml. Other cationic substances tested, spermidine and spermine (each at 0.005 M), had no effect. Among a variety of metabolites tested for possible effects on nonactivated kinase, glucose 6-P at a concentration of 0.01 M caused 45% inhibition at pH 8.2. Glucose (0.05 M) caused 25% inhibition under these conditions. Glucose-1-P (0.01 M) and UDP-glucose (0.002 M) had no effect on nonactivated kinase at pH 8.2; 0.01 M F$^-$ was noninhibitory at pH 8.2.

Activation of Phosphorylase b Kinase by ATP.— When purified nonactivated phosphorylase b kinase, i.e., the 40-precipitate fraction, was incubated with ATP and Mg^{2+} with or without added cyclic AMP prior to assaying in the phosphorylase b to a reaction, profound activity changes occurred (Fig. 9). This was particularly evident when the kinase assays were carried out at pH 6.8 (curves C and D), where the enzyme was essentially inactive prior to activation, but was also apparent when assays were performed at pH 8.2 (curves A and B),

[2] The absolute values for K_m were often found to vary somewhat when different nonactivated kinase preparations were used, but with any one preparation the changes in K_m brought about by inclusion of glycogen in the kinase reaction mixture or by activation of the kinase were always consistent.
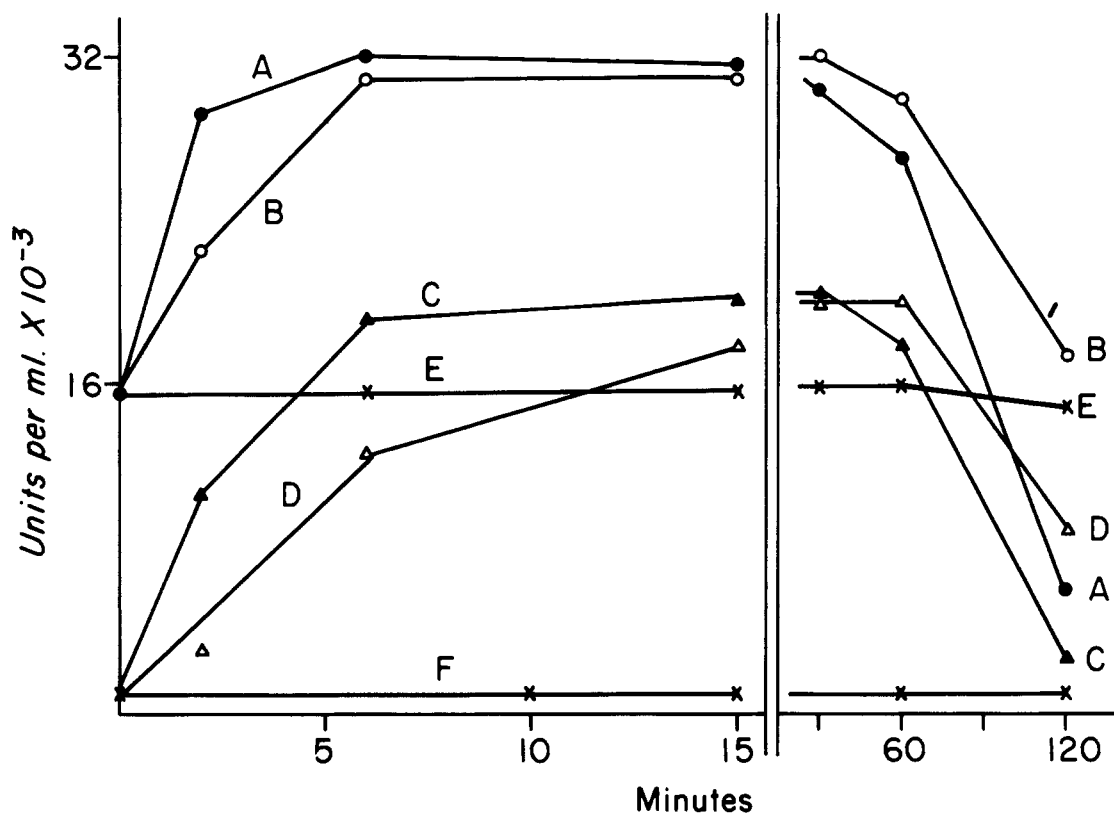
FIG. 9.—Time course of the activation of phosphorylase *b* kinase by 3.6 × 10⁻³ M ATP in the presence (curves A and C) and absence (curves B and D) of 1 × 10⁻⁴ M cyclic AMP. Activation reaction mixtures were made up as described under Methods. Kinase used = nonactivated 40-precipitate fraction at a concentration of 0.92 mg/ml in the activation reactions. Kinase assays were carried out on diluted aliquots of each reaction mixture at pH 6.8 (curves C, D, and F) and at pH 8.2 (curves A, B, and E). The reaction mixture represented by curves E and F contained no ATP, Mg²⁺, or cyclic AMP. Ordinate = units of phosphorylase *b* kinase activity (at pH 6.8 or 8.2) per ml in the activation reaction mixtures.

where the kinase was partially active initially. Following a period in which activity increased there was a gradual decline. When cyclic AMP was present in addition to ATP and Mg²⁺ (curves A and C) the rate of activation was greater than in the activation mixture without the cyclic nucleotide (curves B and D); as can be seen in Figure 8, the decline in activity was also faster in the presence of cyclic AMP. Under control conditions in which the kinase was incubated in buffer alone (curves E and F) no significant changes in activity occurred. It was also shown that ATP alone, cyclic AMP alone, or ATP and cyclic AMP in the absence of Mg²⁺ had no effect on the kinase.

The experiment of Figure 9 showed that there was no absolute dependence on cyclic AMP in the activation of purified phosphorylase *b* kinase, since the extent of activation was just as great with Mg²⁺ and ATP alone, although the process occurred more slowly under these conditions. It was important to determine that there was no hidden source of cyclic AMP in these reactions. The possibility that the ATP might be contaminated was ruled out by chromatography, and no cyclic AMP could be detected in heated samples of the kinase. No adenyl cyclase activity (Sutherland *et al.*, 1962) was detectable in phosphorylase *b* kinase fractions beyond the acid-precipitation step, so there appeared to be no possibility that the compound was being produced during the activation reaction.

The marked fall in activity that followed activation as seen in the experiment of Figure 9 was due to the lability of phosphorylase *b* kinase in the presence of Mg²⁺ ions and did not represent a reversal of the activation process. EDTA added in excess over the metal at the peak of activation stabilized the enzyme

almost completely. No reactivation occurred when additional ATP and cyclic AMP were added after inactivation in the absence of EDTA.

Specificity for ATP and Metals in the Activation of Phosphorylase b Kinase by ATP.—The activation of nonactivated phosphorylase *b* kinase by preincubation with ATP and Mg²⁺ was found to be specific for ATP. No activation was obtained with any of the following nucleotides tested together with Mg²⁺: 5′-AMP, 3′-AMP, cyclic 2′,3′-AMP, CMP, CDP, CTP, UMP, UDP, UTP, GMP, GDP, GTP, IMP, IDP, and ITP. Some activation was obtained when the kinase was incubated with ADP and Mg²⁺, but this was less than that found with ATP and the metal. Moreover, the kinase fraction used in this experiment (the 30-supernatant fraction) was known to contain some myokinase.

Mn²⁺ could substitute for Mg²⁺ in the activation of phosphorylase *b* kinase by ATP with or without cyclic AMP and the two metals appeared to be essentially equivalent. Zinc was tested in the system but was found to cause complete inactivation of the kinase at a concentration of 0.001 M. Tests with Ca²⁺ and ATP were difficult to interpret, because the kinase fraction available at the time when these experiments were carried out contained traces of kinase-activating factor (Meyer *et al.*, 1964). For this reason Ca²⁺ alone caused some kinase activation; mixtures of Ca²⁺ plus ATP gave less activation than with Ca²⁺ alone.

The Effect of Cyclic AMP Concentration on the Activation of Phosphorylase b Kinase by ATP; Glycogen and Heparin Effects.—In studying the effect of varying amounts of cyclic AMP on the activation of phosphorylase *b* kinase by ATP, it was found that the
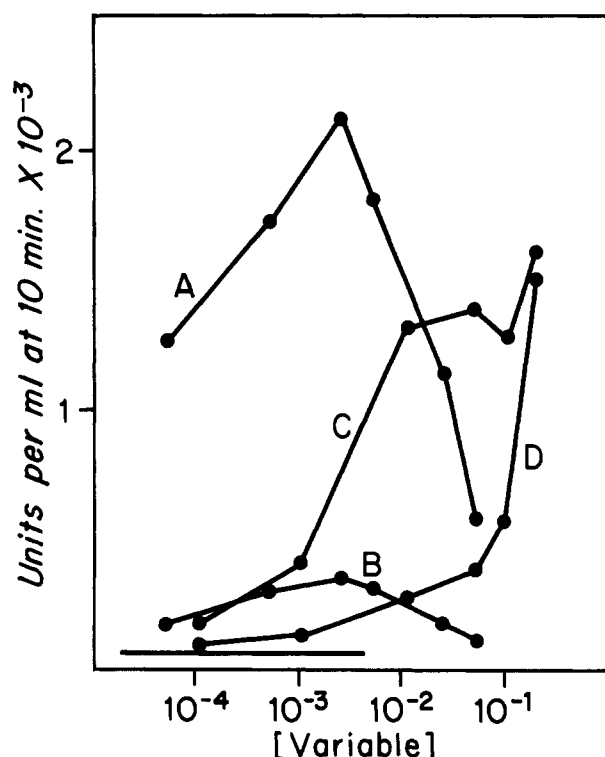
Fig. 10.—Phosphorylase *b* kinase activation with varying [ATP] and [Mg^{2+}] in the presence (curves A and C) and absence (curves B and D) of 1 × 10^{-5} M cyclic AMP. Activation-reaction mixtures were made up as described under Methods except for variations in concentration of ATP and Mg^{2+}. Kinase used = nonactivated 30-supernatant fraction, 0.24 mg/ml in the activation reactions. Initial activities: 0.1 × 10^3 units/ml at *p*H 6.8 (horizontal line) and 4.3 × 10^3 units/ml at *p*H 8.2. The curves show activities at *p*H 6.8 after 10 minutes' incubation at 30°. In curves A and B [Mg^{2+}] is constant at 0.01 and [ATP] is varied. In curves C and D, [ATP] is constant at 0.01 and [Mg^{2+}] is varied.

TABLE V
EFFECT OF CYCLIC AMP, HEPARIN, AND GLYCOGEN ON THE RATE OF PHOSPHORYLASE *b* KINASE ACTIVATION BY ATP[a]

Cyclic AMP (M × 10^7)	Increase in Activity at *p*H 6.8 ([units/ ml] × 10^{-2})	Heparin (mg/ ml)	Increase in Activity at *p*H 6.8 ([units/ ml] × 10^{-2})	Glyco-gen (mg/ ml)	Increase in Activity at *p*H 6.8 ([units/ ml] × 10^{-2})
0	0.7	0	0.7	0	0.7
0.2	0.9	0.05	4.6	1	1.2
0.5	1.6	0.1	5.3	2	1.3
1.0	2.9	0.5	5.2	4	2.1
10	5.4	1.0	4.9	8	3.5
100	5.4				

[a] Activation reaction mixtures were made up containing 0.012 M Mg^{2+} and 3.3 × 10^{-3} M ATP with added heparin or glycogen as shown. A 30-supernatant fraction was used as a source of kinase at a concentration of 0.24 mg protein per ml in the activation reactions. The enzyme showed 80 kinase units per ml at *p*H 6.8 and 3600 units/ml at *p*H 8.2 before activation. Kinase activities at *p*H 6.8 were determined after 10 minutes' incubation at 30°.

reaction was sensitive to very low concentrations of this substance. A half-maximal increase in the rate of activation over that caused by ATP alone was brought about by 0.5–1.0 × 10^{-7} M cyclic AMP (Table V). In this type of experiment a somewhat greater maximal effect of cyclic AMP was seen with the 30-super-
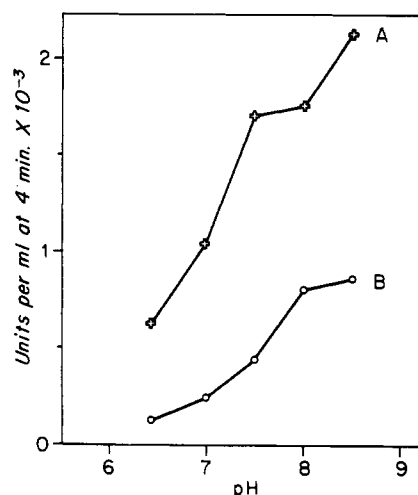

Fig. 11.—Optimum *p*H for activation of phosphorylase *b* kinase in the presence (curve A) and absence (curve B) of 1 × 10^{-5} M cyclic AMP. Activation reaction mixtures made up as described under Methods except for variation in the *p*H of the buffer. Kinase used = nonactivated 30-supernatant fraction, 0.5 mg/ml in the activation reactions. Initial activities = 0.13 × 10^3 units/ml at *p*H 6.8 and 3.1 × 10^3 units/ml at *p*H 8.2. Kinase activities determined at *p*H 6.8 after 4 minutes' incubation at 30°.

natant fraction as the source of nonactivated phosphorylase *b* kinase rather than the more-purified 40-precipitate fraction. The reasons for this are unknown, but it is of interest that Sutherland and Rall (1960) noted that cyclic AMP effects in the liver phosphorylase-activating system were also less prominent with highly purified fractions than with crude enzyme preparations, and they spoke of the removal of a "restraining factor" that could "magnify the action of cyclic AMP."

Glycogen and heparin, each of which stimulates the activity of nonactivated phosphorylase *b* kinase when included in the phosphorylase *b* to *a* reaction mixture (Fig. 8 and Table IV), were both found to have a marked effect on the rate of phosphorylase *b* kinase activation by ATP (Table V). In this experiment kinase activities were carried out at a 1:100 dilution of the activation reaction mixtures, so that even at the highest levels of heparin or glycogen used the amount of either of these substances carried over was below a significantly stimulatory level in the kinase reaction itself. No activation of the kinase occurred when Mg^{2+} and ATP were left out of preincubation mixtures. The glycogen effect was shown not to be due to contaminating cyclic AMP, since boiling with strong alkali did not diminish its effectiveness; whereas cyclic AMP was completely destroyed under these conditions. Moreover, the glycogen and cyclic AMP effects were shown to be additive. No tests for purity of the heparin were carried out, but the sample used (see Materials and Methods) had a high specific heparin activity, and in view of its effectiveness at low concentrations in the kinase activation reaction it is doubtful that its reaction could have been due to contaminating cyclic AMP.

Anticipating that the effect of cyclic AMP on the rate of activation of phosphorylase *b* kinase could be usefully employed as an assay system (Posner *et al.*, 1964), it was considered desirable to screen a large number of tissue components to determine whether they would have an effect on the response of kinase activation to cyclic AMP. To this end a method was established whereby the component in question was added to a kinase-activation mixture containing an

amount of cyclic AMP giving half-maximal stimulation of kinase-activation rate, i.e., around 5×10^{-8} to 1×10^{-7} M cyclic AMP. Under these conditions an enhancement or inhibition of activation could be noted. The following substances assayed at a final concentration of 1×10^{-3} M were without any effect: AMP, ADP, cyclic 2',3'-AMP, adenosine, CMP, CTP, UMP, UDP, UTP, GMP, GTP, IMP, ITP, UDP-glucose, NAD, glucose, glucose-1-P, glucose-6-P, lactic acid, glutathione, carnosine, carnitine, and inositol. Having no effect at a final concentration of 10^{-4} M were: creatinine, creatine, creatine phosphate, and anserine; these substances were not tested at higher concentrations. Acetyl choline at 10^{-5} M had no effect. As will be discussed in another paper (Posner *et al.*, 1964), boiled tissue extracts contain trypsin-sensitive material of unknown composition which is strongly inhibitory to activation of phosphorylase *b* kinase by ATP and cyclic AMP.

Effects of ATP and Mg^{2+} *Concentrations, pH, and Salts on the Activation of Phosphorylase b Kinase by ATP.*—With the concentration of Mg^{2+} held constant at 0.01 M the effect of varying [ATP] on the rate of activation of phosphorylase *b* kinase was determined in the presence and absence of cyclic AMP (curves A and B, respectively, of Fig. 10). In either case the optional [ATP] was about 3×10^{-3} M. As the ATP concentration approached and exceeded the Mg^{2+} concentration the activation rate fell off sharply. A determination of the effect of varying $[Mg^{2+}]$ with [ATP] held constant at 0.01 M is shown in curve C (with cyclic AMP) and curve D (without cyclic AMP) of Figure 10. In the absence of cyclic AMP very little activation was seen with increasing Mg^{2+} until the concentration of Mg^{2+} exceeded that of ATP; at 0.1 M Mg^{2+} there was a sudden increase in activation rate. In the presence of cyclic AMP the activation process appeared to be somewhat less hindered by low Mg^{2+} concentrations; the notch in curve C was reproduced in a second experiment and would appear to indicate that Mg^{2+} is involved in more than one action in this system. The effect of cyclic AMP was very small at the highest Mg^{2+} concentration.

The rate of activation of phosphorylase *b* kinase by ATP rose with increasing pH in the range from pH 6.5 to 8.5 with or without cyclic AMP. This is shown in the experiment of Figure 11. Activation of the kinase was strongly inhibited by increasing the ionic strength of the reaction mixture, and even the buffer ordinarily employed in the activation reaction, 0.025 M Tris–0.025 M glycero-P, was somewhat inhibitory. With a reaction mixture at pH 7.0 containing only 0.01 M Mg^{2+}, 0.003 M ATP, 0.01 M cysteine, and the kinase, the activation reaction was inhibited 55% by addition of 0.01 M glycero-P without cyclic AMP and 40% with cyclic AMP. With 0.075 M glycero-P the inhibition was 90% without cylic AMP and 60% with this nucleotide. Inhibition was very nearly the same when NaCl was substituted for Na glycero-P at the same ionic strength. The rates of the kinase activation reaction and the kinase reaction itself were identical in a medium in which K^+ was the dominant monovalent cation rather than Na^+.

The Effect of Activation of Phosphorylase b Kinase on the K_m *Values for Phosphorylase b and ATP.*—Activation of phosphorylase *b* kinase by preincubation with Mg^{2+}, ATP, and cyclic AMP resulted in a lowering of the Michaelis constant for phosphorylase *b* in the kinase reaction. In a typical experiment using nonactivated kinase the values for K_m at pH 7.5 and 8.2 were found to be 50,000 units/ml (1.25×10^{-4} M) and 16,000 units/ml (4×10^{-5} M), respectively.[2] After activation the constant was 15,000 units/ml ($3.7 \times$
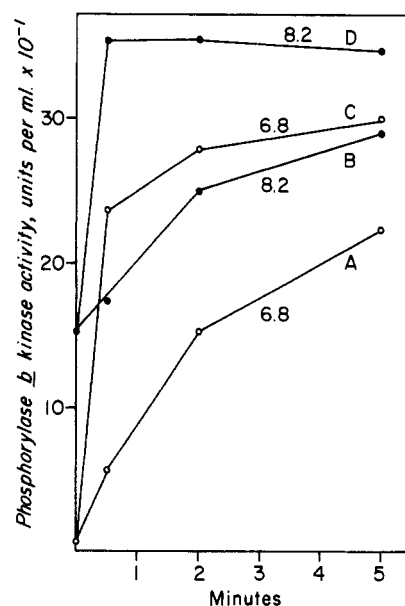

Fig. 12.—Time course of phosphorylase *b* kinase activation by two concentrations of trypsin. Reaction 1 (curves A and B), trypsin concentration = 0.0015 mg/ml. Reaction 2 (curves C and D), trypsin concentration = 0.024 mg/ml. Each reaction contained 0.04 M glycero-P buffer, pH 7.0, and 3.2 mg/ml nonactivated phosphorylase *b* kinase (30-supernatant fraction). Incubations at 30° for time shown. Aliquots removed and diluted 1:30 in 0.015 M cysteine buffer containing 0.05 mg/ml soybean-trypsin inhibitor and then diluted an additional 1:10 in buffer without inhibitor before kinase assays at pH 6.8 (curves A and C) or 8.2 (curves B and D).

10^{-5} M) and 7000 units/ml (1.7×10^{-5} M) at these pH values. Activation of phosphorylase *b* kinase did not result in a lowering of the K_m for ATP in the phosphorylase *b* to *a* reaction; instead the constant was somewhat higher for the activated enzyme. At pH 7.5 with 0.01 M Mg^{2+}, K_m was equal to 2.4×10^{-4} M ATP for nonactivated kinase and 3.8×10^{-4} M ATP for the activated enzyme.

Activation of Phosphorylase b Kinase by Trypsin.—Investigation of trypsin as a possible activator of nonactivated muscle phosphorylase *b* kinase was suggested by the work of Rall and Sutherland (1958), who found that this enzyme mimicked the action of cyclic AMP in causing the formation of active liver phosphorylase in their system. In this study it was found that low concentrations of trypsin caused strong activation of purified muscle phosphorylase *b* kinase (Fig. 12). Activation was manifested by a sharp increase in kinase activity determined at pH 6.8 (curves A and C) and also by an increase in activity at pH 8.2 (curves B and D) similar to that seen with ATP and Mg^{2+} (Fig. 9). With higher concentrations of trypsin than those employed in the experiment of Figure 12 or with longer periods of incubation, activity falls off.

Effect of Activation on the Sedimentation Behavior of Phosphorylase b Kinase.—To determine whether activation of phosphorylase *b* kinase resulted in any changes in its sedimentation coefficient, aliquots of a single preparation were activated by incubation with trypsin, ATP, or Ca^{2+} and kinase-activating factor[3]; and each sample was examined in the ultracentrifuge and compared to the nonactivated control (Fig. 13). It is apparent that no gross changes occurred. There was some increase in the amount of material lighter than the

[3] Details of activation by Ca^{2+} are discussed in the following paper (Meyer *et al.*, 1964).
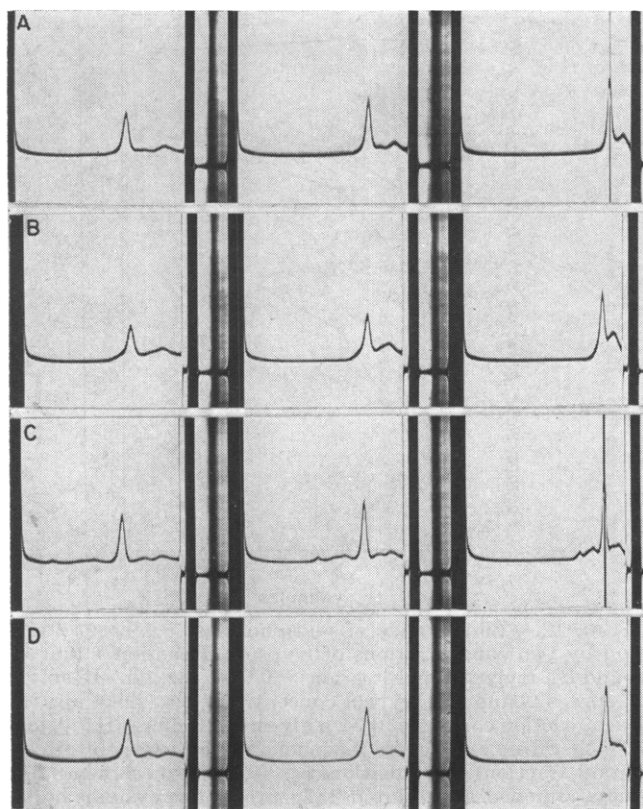
FIG. 13.—Sedimentation patterns of nonactivated and activated 40 - precipitate fraction of phosphorylase *b* kinase. (A) Nonactivated kinase in 0.05 M glycero-P, 0.002 M EDTA, pH 7.0, 8.0 mg/ml. (B) The same kinase preparation activated by incubation for 5 minutes at 30° with 2.5 μg trypsin per ml followed by addition of 2.0 μg soybean-trypsin inhibitor per ml (C) The same kinase activated by incubation for 60 minutes at 30° with 0.007 M Mg (Ac)$_2$ and 0.003 M ATP. (D) The same kinase activated by incubation for 15 minutes at 30° with 0.01 M CaCl$_2$ and 0.4 mg/ml of purified kinase-activating factor (Meyer *et al.*, 1964) followed by chelation of the excess Ca^{2+} with EDTA. Sedimentation analyses at 5–7° were performed immediately after the activation reactions without preliminary dialysis; i.e., the activation-reaction mixture served as the solvent. Sedimentation coefficients of the major components, corrected to the viscosity of water at 20° but not for solvent effects, are: 21.0 S in (A), 26.4 S in (B), 22.3 S in (C), and 21.0 S in (D). The direction of sedimentation is from right to left. The frames are 16, 32, and 48 minutes after reaching a speed of 39,460 rpm.

major 23-S component after trypsin treatment (Fig. 6B) and a number of small rapidly sedimenting components were present after activation by ATP (Fig. 6C).

DISCUSSION

The purification procedure for nonactivated phosphorylase *b* kinase described in this paper utilizes mild acid precipitation and differential centrifugation, and in many ways it bears a closer resemblance to the methods employed in isolating cellular organelles than to those commonly used in purifying soluble glycolytic enzymes. The 40-precipitate fraction contains a major heavy component with a sedimentation constant of 23 S and further purification leads to an enrichment of the component, suggesting that it indeed represents phosphorylase *b* kinase. The possibility exists that a group of similarly sedimenting components are present and that the kinase is only one of these. There is also the possibility that the 23-S component represents a complex of enzymes, only one of which is phorphorylase

b kinase. An initial impression that phosphorylase *b* itself, a protein that might logically be expected to bind to phosphorylase *b* kinase, was part of such a complex is not supported by the data in this paper. Phosphorylase *b* is tightly bound to glycogen particles, which accompany the kinase through the acid-precipitation step, but this enzyme is separated from the kinase in the subsequent centrifugation steps.

Nonactivated phosphorylase *b* kinase is activated by preincubation with ATP in the presence of Mg^{2+}. The activation reaction occurs at a more rapid rate in the presence of cyclic AMP, but this nucleotide does not appear to be essential in the process, and the same final levels of kinase activity are obtained with or without this substance when experiments are carried out with highly purified enzyme as in Figure 9. With less-pure preparations of the kinase the effect of cyclic AMP in the activation process is more pronounced but again no absolute requirement for the cyclic nucleotide exists. Activation of phosphorylase *b* kinase by ATP appears to involve phosphorylation of the enzyme (Trayser *et al.*, 1962) and it is possible that cyclic AMP acts by changing the configuration of the kinase so that it is more readily phosphorylated. With very high levels of Mg^{2+}, cyclic AMP has very little effect on kinase activation, so it is possible that this metal is exerting the same structural influence on the enzyme as that caused by low concentrations of the cyclic AMP. In this connection it is of interest that Mg^{2+} destabilizes the kinase and that this effect is more pronounced in the presence of cyclic AMP. Although phosphorylase *b* kinase activation probably involves a molecular change in the enzyme, the sedimentation patterns of nonactivated and activated kinase do not support the idea that such a change is accompanied by any gross alterations in molecular weight of the enzyme.

Activation of phosphorylase *b* kinase results in greater affinity of the enzyme for its substrate as is evidenced by the decrease in K_m. The available evidence would point toward this change as being of paramount importance in regulating the activity of the kinase, since the nonactivated form has an extremely low affinity for phosphorylase *b* in the physiological range of pH around neutrality; this is seen by extrapolating curve B of Figure 5. Rabbit skeletal muscle contains about 3 mg of phosphorylase per g of tissue (Fischer and Krebs, 1958) or 4800 units per g (1.2 × 10^{-5} M) calculated as phosphorylase *b*. Even though the enzyme is probably nonuniformly distributed in the muscle cell, it would seem unlikely that it would be localized to such an extent that its concentration would become nonrate-limiting in the phosphorylase *b* to *a* reaction. The effect of glycogen on the affinity of phosphorylase *b* kinase for phosphorylase *b* is also of interest in this connection, particularly since phosphorylase is probably associated with glycogen in the cell. Under different experimental conditions than those employed in the present study, Mansour and Mansour (1962) found variations in the affinity of phosphofructokinase for its substrate, when the enzyme was activated in a system involving cyclic AMP.

Attempts to demonstrate the reversal of phosphorylase *b* kinase activation have been hampered by the instability that activated kinase exhibits in the presence of Mg^{2+}; irreversible loss of activity occurs under these conditions. In crude muscle extracts (Krebs *et al.*, 1959) partial activation appeared to be followed by a true reversal of the process but this has not been duplicated in a purified system.

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Activation of Skeletal Muscle Phosphorylase *b* Kinase by CA²⁺ *

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The activation of rabbit skeletal muscle phosphorylase *b* kinase by preincubation with Ca²⁺ requires a separate protein factor removed during purification of the kinase. This factor, designated as kinase-activating factor, was purified about 700-fold by acid precipitation, ammonium sulfate fractionation, and column chromatography using DEAE-cellulose. Studies of the kinetics of kinase activation by Ca²⁺ in the presence of the kinase-activating factor were complicated by instability of kinase and kinase-activating-factor in the presence of Ca²⁺; although the activation reaction resembled an enzymatic process in many respects, some of the experiments suggested that a stoichiometric reaction was occurring between kinase and kinase-activating factor. No proteolytic activity was found in kinase-activating factor preparations. Phosphorylase *b* kinase was inhibited by certain chelating agents, particularly those that are effective in binding alkaline earth metals. Addition of Ca²⁺ was effective in relieving this inhibition.

The conversion of phosphorylase *b* to phosphorylase *a* was observed for the first time in crude skeletal muscle

extracts after they had been passed through unwashed filter paper (Fischer and Krebs, 1955). At that time it was established that this effect was due to the extraction of metals (probably calcium) from the paper. The phosphorylase *b* to *a* reaction itself was found to require Mg²⁺ or Mn²⁺ ions along with ATP (Krebs and Fischer, 1956), but a role for calcium was discovered in the activation of phosphorylase *b* kinase, which catalyzes the phosphorylase *b* to *a* reaction (Krebs et al., 1959; Krebs and Fischer, 1960).

Phosphorylase *b* kinase, as extracted from muscle,