Activation of Cardiac Phosphorylase \(b\) Kinase*

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**SUMMARY**

Nonactivated, partially purified bovine heart phosphorylase \(b\) kinase was activated by incubation with adenosine triphosphate and \(Mg^{2+}\) ion prior to assay. Adenosine \(3', 5'\)-cyclic phosphate increased the rate of activation, but there was no absolute requirement for the cyclic nucleotide. Activation was particularly prominent when the subsequent assay for kinase activity was carried out at pH 7.0, where the enzyme has low activity. Significant activation was also evident when the assay was performed at pH 8.2, where the nonactivated enzyme is partially active. The response to adenosine \(3', 5'\)-cyclic phosphate was linear from \(1 \times 10^{-4}\) to \(2 \times 10^{-7}\) M. Other ribonucleoside \(3', 5'\)-cyclic phosphates were without effect except at much higher concentrations.

Supramaximal doses of epinephrine (2 \(\mu\)g), when injected into perfused rat hearts, caused a modest but significant activation of phosphorylase \(b\) kinase as compared with controls. A 5-fold increase in enzyme activity occurred as determined by assaying \(30,000 \times g\) supernatant fractions at pH 6.8. Activation was also apparent, but proportionately less, when extracts were assayed at pH 8.2. Activation was characterized by a 3-fold increase in pH 6.8 to 8.2 activity ratios. The activation occurred rapidly, was maximal at 3 sec after epinephrine injection, and rose well ahead of the contractile response, which reached a maximum in 10 sec.

Phosphorylase \(b\) kinase, the enzyme which catalyzes the conversion of phosphorylase \(b\) to phosphorylase \(a\), can be extracted from skeletal muscle (1, 2) and cardiac muscle (3, 4) in a form which has very low activity when assayed at physiological pH (pH 6.8 or 7). At pH values above this, activity rises sharply to an optimum at pH 8.5. Krebs et al. (5) have referred to the enzyme with this pH profile as nonactivated phosphorylase \(b\) kinase. Nonactivated kinase from both skeletal muscle (1, 2) and cardiac muscle (3, 4, 6) is markedly activated by incubation with \(Ca^{2+}\) ion. Activation is especially marked when the enzyme is assayed at pH values of 6.8 and below and is less apparent when assays are conducted at pH 8.2. The ratio of activity at pH 6.8 to that at pH 8.2 has been used as a useful index of the degree of activation. The nonactivated enzyme is also activated by incubation with adenosine triphosphate and adenosine \(3', 5'\)-cyclic phosphate (cyclic adenosine monophosphate). Activation of the skeletal muscle enzyme by cyclic AMP has been extensively studied by Krebs et al. (5), and the reaction constitutes a sensitive method for the assay of the cyclic nucleotide in biological materials (7). The action of cyclic AMP on the cardiac enzyme has not been studied in detail although evidence is available (3, 4) that it may be similar to that of the skeletal muscle enzyme.

It is now established that epinephrine stimulates the formation of cyclic AMP in beating hearts (3, 8, 9) and causes the conversion of phosphorylase \(b\) to the \(a\) form. It has been assumed that increased phosphorylase \(a\) levels result from the activation of phosphorylase \(b\) kinase by the cyclic AMP formed by epinephrine stimulation. If this assumption is correct, it follows that epinephrine should activate phosphorylase \(b\) kinase when administered to beating hearts. Recently, Hammermeister, Yunis, and Krebs (3) have reported that epinephrine failed to activate the enzyme significantly in perfused rabbit hearts. This report describes the activation of purified cardiac phosphorylase \(b\) kinase by ATP and cyclic AMP. Evidence is provided that epinephrine causes a significant activation of the enzyme in perfused rat hearts.

**EXPERIMENTAL PROCEDURE**

**Materials**—Rabbit muscle phosphorylase \(b\), adenylic deaminase, glycogen, and glucose 1-phosphate were prepared or purified by methods used previously (4). Ribonucleoside \(3', 5'\)-cyclic phosphates, other than cyclic AMP (which was obtained from Calbiochem), were prepared by synthetic procedures (10, 11). Phosphorylase \(b\) kinase was purified from bovine hearts (6). The 100,000 \(\times g\) precipitate fraction was used in the present study and was stored in 50% glycerol at \(-18^\circ\). When necessary, it was suitably diluted in 15 mm neutral cysteine immediately before use.

**Measurement of Kinase Activation**—Activation of purified kinase was followed by measuring the increase in enzyme units which occurred when the nonactivated enzyme was incubated together with ATP, \(Mg^{2+}\) ion, and cyclic AMP. The standard activation mixture contained 0.125 M \(1'\, 1'\)-Thio-0.125 M sodium \(\beta\)-glycerophosphate at pH 7.0, 0.05 ml; 50 mm magnesium acetate containing 50 mm theophylline, 0.05 ml; 5 mm ATP, 0.05 ml; 0.25 mm cyclic AMP (when present), 0.05 ml, in a final volume

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Wistar rats weighing 190 to 220 g were stunned and decapitated; the heart was rapidly removed, rinsed, and attached via the aorta to a glass cannula of a Langendorf perfusion system and perfused with oxygenated Tyrode's solution at 37° at a pressure of 50 cm of water. Contractile activity was measured by a Statham force displacement transducer attached to the apex of the ventricle and adjusted to provide an initial diastolic tension of 5 g to each heart. Recordings were made on a Grass polygraph. Hearts were perfused for a stabilizing period of 10 min before administering epinephrine. During this interval an epinephrine solution containing 10 μg per ml was prepared by diluting a stock solution (1 mg per ml in 0.1% sodium metabisulfite) with Tyrode's solution. The freshly prepared dilution (0.2 ml containing 2.0 μg) was injected through thin polyethylene tubing into the cannula immediately above the heart. For controls, 0.2 ml of Tyrode's solution was administered. All injections were delivered evenly over a 10-sec interval. At the desired time (measured from the instant of completion of injection), the heart was frozen by crushing between two aluminum blocks precooled in liquid N₂. Control hearts were frozen 10 sec after completion of Tyrode's injection. The frozen wafers were immersed in liquid N₂; ventricular tissue was chiseled from the remainder of the heart and stored in liquid N₂ until used (usually 1 to 3 days).

Preparation of Extracts—Each frozen ventricle was weighed (400 to 600 mg) and then homogenized in a Potter-Elvehjem homogenizer for 3 min at 4° with 10 volumes of a medium consisting of 50 mM Tris-HCl, 50 mM NaF, and 2 mM EDTA at pH 7.0. In initial experiments a portion (3.2 ml) of the resulting homogenate was centrifuged at 30,000 × g for 15 min. The supernatant fluid was removed and kept in ice. The precipitate was suspended in 1.0 ml of homogenizing medium. In later experiments, the entire homogenate was centrifuged at 30,000 × g for 15 min and the precipitate was discarded. All preparations were assayed for kinase activity at pH 6.8 and 8.2 within 5 min after centrifugation.

RESULTS

Activation of Cardiac Phosphorylase b Kinase by ATP and Cyclic AMP—Previous studies (4, 6) have shown that nonactivated cardiac phosphorylase b kinase possesses relatively low activity at pH 7.0 and below. Above pH 7.0, activity rises sharply to an optimum at pH 8.5. It has been previously reported that crude extracts of rabbit heart (3) and acid precipitate fractions from bovine heart (4) were activated by ATP and cyclic AMP. When the purified nonactivated enzyme was incubated with ATP and Mg²⁺ ion prior to dilution and assay at pH 7.0, significant activation occurred (Fig. 1, Curve B). Maximum activation occurred at 1 mM ATP, but a wide range of concentrations were effective. When cyclic AMP (5 × 10⁻⁴ M) was present in addition to ATP and Mg²⁺ ion, activation was considerably increased (Curve A). The cyclic nucleotide in the absence of ATP caused only slight activation; ATP was inactive in the absence of Mg²⁺ ion. Activation was most evident when the assay for kinase was conducted at pH 7.0 (Fig. 2), although significant activation was also apparent when assays were performed at pH 8.2. Usually a total activation of 6- to 9-fold was achieved when both ATP and the cyclic nucleotide were present in the activation reaction as manifested by kinase assay at pH 7.0.

Effect of Time on Activation of Cardiac Phosphorylase b Kinase—

Perfusion and Epinephrine Stimulation of Rat Hearts—Female

Phosphorylase b Kinase Assay—The assay used was described previously (6) and is essentially that elaborated by Krebs et al. (5) for the skeletal muscle enzyme. Four times recrystallized phosphorylase b (10,000 Cori units) was used as substrate. Assays were conducted at 30° for 5 min at pH 6.0, 6.8, 7.0, or 8.2 as required. A final assay pH of 8.2 was achieved by using 10 volumes of a medium consisting of 50 mM sodium β-glycerophosphate and 30 mM cysteine, pH 6.8, to which had been added adenylic deaminase (200 μg of protein) (6). After 2 min at room temperature, a 0.2-ml aliquot of this diluted incubation mix was assayed for phosphorylase a (5). In studies involving purified kinase, a control containing phosphorylase b and all other components of the assay except phosphorylase b kinase was carried for each assay at each pH. When crude extracts of perfused rat hearts were assayed, a modified control was also used. This will be described later. The unit of phosphorylase b kinase was defined previously (6).

Perfusion and Epinephrine Stimulation of Rat Hearts—Female

Fig. 1. Activation of cardiac phosphorylase b kinase by ATP and cyclic AMP. Standard activation conditions were used except that ATP concentration was varied. Cyclic AMP concentrations was 5 × 10⁻³ M. Following the activation, the diluted mixtures were assayed for kinase at pH 7.0. Curve A, ATP plus cyclic AMP; Curve B, no cyclic AMP.

of 0.25 ml. Tubes were assembled in an ice bath, and then, after a 1-min equilibration at 30°, 0.05 ml of phosphorylase b kinase solution (500 pH 8.2 units) was added, and the tubes were incubated at 30° for 10 min. The reaction was terminated by the addition of 5 ml of ice-cold neutral 15 μM cysteine. The tubes were placed in ice and a 0.2-ml aliquot was used immediately for kinase assay at pH 6.0, 7.0, or 8.2. In order to avoid delay between the activation reaction and assay for kinase activity, tubes containing all components of the kinase assay were assembled during the activation reaction and were kept in ice.

Phosphorylase b Kinase Assay—The assay used was described previously (6) and is essentially that elaborated by Krebs et al. (5) for the skeletal muscle enzyme. Four times recrystallized phosphorylase b (10,000 Cori units) was used as substrate. Assays were conducted at 30° for 5 min at pH 6.0, 6.8, 7.0, or 8.2 as required. A final assay pH of 8.2 was achieved by using 10 volumes of a medium consisting of 50 mM sodium β-glycerophosphate and 30 mM cysteine, pH 6.8, to which had been added adenylic deaminase (200 μg of protein) (6). After 2 min at room temperature, a 0.2-ml aliquot of this diluted incubation mix was assayed for phosphorylase a (5). In studies involving purified kinase, a control containing phosphorylase b and all other components of the assay except phosphorylase b kinase was carried for each assay at each pH. When crude extracts of perfused rat hearts were assayed, a modified control was also used. This will be described later. The unit of phosphorylase b kinase was defined previously (6).

Perfusion and Epinephrine Stimulation of Rat Hearts—Female

Fig. 1. Activation of cardiac phosphorylase b kinase by ATP and cyclic AMP. Standard activation conditions were used except that ATP concentration was varied. Cyclic AMP concentrations was 5 × 10⁻³ M. Following the activation, the diluted mixtures were assayed for kinase at pH 7.0. Curve A, ATP plus cyclic AMP; Curve B, no cyclic AMP.

3.0
2.0
1.0

UNITS KINASE/ACTIVATION MIX x10⁻²

ATP [Μ]

0 -4 10⁻³ 10⁻²

0

NO ATP

NO 5,5 AMP

Activation of cardiac phosphorylase b kinase by ATP and cyclic AMP. Standard activation conditions were used except that ATP concentration was varied. Cyclic AMP concentrations was 5 × 10⁻³ M. Following the activation, the diluted mixtures were assayed for kinase at pH 7.0. Curve A, ATP plus cyclic AMP; Curve B, no cyclic AMP.
In the experiments just described, the activation reaction was terminated after 10 min. When ATP and cyclic AMP were present together, activation occurred rapidly, reaching completion essentially in 10 min, with very little further change occurring up to 60 min (Fig. 3, Curve A). When ATP and Mg++ ion were present alone, activation occurred at a slower rate (Curve B) but reached essentially the same maximum as when cyclic AMP was present. Thus, as with the skeletal muscle enzyme, there seems to be no absolute requirement for cyclic AMP; activation simply occurs at an enhanced rate when it is present.

Effect of pH on Activation of Phosphorylase b Kinase—Routinely, the activation reaction was carried out at pH 7.0. An experiment was performed in which the pH of the activation reaction was varied. Following dilution of the activation mixtures, kinase assays were performed at pH 7.0. Fig. 4 shows that the rate of activation in the presence of ATP and Mg++ ion increased with increasing pH to an optimum in the area of pH 8.5 (Curve B). When cyclic AMP was present also (Curve A), the rate of activation again rose with increasing pH, but the increase caused by the cyclic nucleotide was less marked at higher pH. The greatest effect of cyclic AMP over that produced by ATP was seen at pH 7.0.

Effect of Cyclic AMP Concentration and Specificity of Activation —The cardiac enzyme, like the skeletal muscle enzyme, was found to be sensitive to very minute amounts of the cyclic nucleotide. In the presence of 1 mM ATP, the response to cyclic AMP was linear over the range 1 × 10⁻⁸ to 2 × 10⁻⁷ M. Concentrations as high as 10⁻³ M had no additional effect. Half-maximal activation occurred at approximately 5 × 10⁻⁸ M. The reaction appeared to be highly specific for cyclic AMP. When added to the activation reaction in the presence of 1 mM ATP, the ribonucleoside 3',5' cyclic phosphates of uridine, cytidine, guanosine, deoxyadenosine, deoxycytidine, thymidine, and deoxyguanosine were all inactive at 10⁻⁸ M. When present at 1 × 10⁻⁸ M, 3',5'-cyclic UMP, 3',5'-cyclic CMP, 3',5'-

Fig. 2. Effect of pH on activated cardiac phosphorylase b kinase. Standard activation conditions were used except that the pH of the activation mixtures was varied. Curve A, ATP and Mg++ plus cyclic AMP; Curve B, no cyclic AMP; Curve C, no ATP, no cyclic AMP. Following dilution of the activation reaction mixtures, kinase assays were conducted at pH 7.0.

Fig. 3. Effect of time on activation of cardiac phosphorylase b kinase. The concentration of cyclic AMP, where present, was 5 × 10⁻⁸ M; ATP, 1 mM. At the time periods noted, the activation mixtures were diluted and assayed for phosphorylase kinase immediately at pH 7.0. Curve A, ATP and Mg++ plus cyclic AMP; Curve B, no cyclic AMP; and Curve C, no ATP, no cyclic AMP.

Fig. 4. Effect of pH on phosphorylase b kinase activation. Standard activation conditions were used except that the pH of the activation mixtures was varied. Curve A, ATP and Mg++ plus cyclic AMP; Curve B, no cyclic AMP; Curve C, no ATP, no cyclic AMP. Following dilution of the activation reaction mixtures, kinase assays were conducted at pH 7.0.
cyclic GMP, and 3',5'-cyclic deoxy-AMP produced activation equivalent to 100, 80, 75, and 40%, respectively, of that produced by $1 \times 10^{-4} \text{M}$ cyclic AMP. This activation could possibly be due to trace amounts of cyclic AMP resulting from contamination of the parent 5'-nucleotides with minute amounts of 5'-AMP. When present with ATP and Mg$^{2+}$ ion, 5'-AMP, 5'-CMG, 5'-GMP, 5'-UMP, 5'-IMP, 5'-deoxy-AMP, 2',3'-CMP, and 5'-deoxy-GMP produced no activation at $1 \times 10^{-4} \text{M}$.

**Phosphorylase b Kinase in Perfused Rat Hearts Treated with Epinephrine**—It can be concluded that cyclic AMP facilitates the activation in vitro of cardiac phosphorylase b kinase in a manner similar to that of the skeletal muscle enzyme. Recently Robison et al. (8) and Cheung and Williamson (9) have shown that in rat hearts treated with epinephrine, cyclic AMP rose within seconds; peak levels were obtained before the contractile response reached a maximum. It was of interest, therefore, to determine whether phosphorylase b kinase was activated by epinephrine in beating rat hearts. To this end, extracts were prepared from control and epinephrine-treated perfused rat hearts and assayed for kinase activity at pH 6.8 and 8.2. A dose of 2 $\mu g$ of epinephrine was chosen for initial experiments. This dose is supramaximal with respect to contractile activity and is known to activate phosphorylase b almost fully in perfused rat hearts (12).

Hammermeister, Yunis, and Krebs (3) have reported that a considerable amount of rabbit heart kinase was not readily extractable and appeared in the precipitate after sedimentation of a homogenate at $10,000 \times g$. Moreover, the sedimentable enzyme appeared largely active as manifest by high pH 6.8 to pH 8.2 ratios of activity (0.53 and 0.57 as compared with 0.11 and 0.14 for $10,000 \times g$ supernatant fractions). In the kinase assay used by the above authors, control tubes contained phosphorylase b but no heart extract (see Reference 2). In earlier experiments (4) we have shown that erroneous values were obtained when partially purified heart kinase preparations were assayed because of the conversion of ATP to AMP by contaminating enzymes. These errors could be minimized by treating the diluted kinase reaction mixtures with adenylie deaminase. The possibility existed that values obtained in the assay of uncentrifuged homogenates and particulate preparations with the use of phosphorylase alone in the control could be considerably in error. In the present experiments, therefore, two controls were prepared for each experimental tube at each pH. The first (Control A) contained phosphorylase (equivalent to that in the experimental) and all other components of the kinase assay except heart extract (2). In the second (Control B), heart extract and all components of the kinase assay except phosphorylase were present during the incubation interval. After dilution (which effectively terminates the kinase reaction) an amount of phosphorylase equivalent to that in the experimental tube was added (0.1 ml of a 1:6 dilution of the stock enzyme solution). Control tubes were then carried through the phosphorylase a assay as usual. Apparent kinase activity in each heart fraction was calculated applying each control as a correction. The results are shown in Table I. Apparent kinase activity differed markedly depending on which control was used as a correction. Kinase values calculated with Control B were much lower than those calculated with Control A, especially at pH 6.8. This resulted because of the much higher levels of inorganic phosphate present in the Control B tubes. Specifically, with Control B, kinase activity of the uncentrifuged homogenate at pH 6.8 was only 23% of that calculated with Control A, and in the $30,000 \times g$ precipitate fraction it was only 9%. The increased inorganic phosphate present in Control B tubes was not due to phosphorylase present in the heart extract. Thus, when a third set of controls was prepared in which heart extract was present during the incubation and phosphorylase subsequently omitted, values for inorganic phosphate were insignificantly above the phosphorylase reagent control. The data indicated that during the kinase incubation heart extract caused the formation of some factor or factors which when carried over into the phosphorylase assay resulted in phosphorylase b activity. It was shown chromatographically that ATP was largely degraded (to the extent of 60%) by the uncentrifuged homogenate and the $30,000 \times g$ precipitate fraction during the kinase incubation interval. The products of this degradation were ADP and AMP. The discrepancy was not likely to be due to AMP since in these assays

<table>
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<th>Table I</th>
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**Apparent phosphorylase b kinase in extracts of control and epinephrine-treated perfused rat hearts**

Seven rat hearts were used as a control and seven were injected with epinephrine (2 $\mu g$). The epinephrine-treated hearts were frozen at 9 sec when contractile response was at a maximum. Uncentrifuged homogenates and $30,000 \times g$ supernatant fractions (see "Experimental Procedure") were diluted 1:4, and the $30,000 \times g$ precipitate fractions were diluted 1:3 with 15 mM neutral cysteine and 0.2 mM aliquots used in the kinase assay. Both controls were carried for each experimental tube at each pH. All assays as well as all controls were performed in duplicate. Values are expressed as units of phosphorylase b kinase per ml of undiluted fraction.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Control method A (phosphorylase alone)</th>
<th>Control method B (phosphorylase added after kinase reaction)</th>
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<tbody>
<tr>
<td></td>
<td>pH 6.8</td>
<td>pH 8.2</td>
</tr>
<tr>
<td>Control hearts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncentrifuged homogenate</td>
<td>69.1 ± 4.3</td>
<td>191.3 ± 8.6</td>
</tr>
<tr>
<td>30,000 $\times g$ supernatant</td>
<td>17.1 ± 0.88</td>
<td>130.0 ± 4.3</td>
</tr>
<tr>
<td>30,000 $\times g$ precipitate fraction</td>
<td>49.1 ± 6.0</td>
<td>70.7 ± 9.4</td>
</tr>
<tr>
<td>Epinephrine-treated hearts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncentrifuged homogenate</td>
<td>91.7 ± 7.1</td>
<td>239.9 ± 12</td>
</tr>
<tr>
<td>30,000 $\times g$ supernatant</td>
<td>30.2 ± 1.7</td>
<td>169.0 ± 5.5</td>
</tr>
<tr>
<td>30,000 $\times g$ precipitate fraction</td>
<td>70.2 ± 11.4</td>
<td>99.4 ± 15.2</td>
</tr>
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adenylc deaminase was present in the dilution buffer, and it was shown chromatographically that the AMP produced was converted entirely to IMP. The latter nucleotide has no effect on phosphorylase b activity at the concentrations that would be present here. In other experiments, control tubes containing heart homogenate were placed in a boiling water bath for 2 min after the kinase incubation interval and prior to dilution and addition of phosphorylase. These tubes gave final inorganic phosphate values identical with the unboiled controls. The contaminating material thus appeared to be heat-stable. Other experiments indicated that it might be ADP. It was estimated from chromatograms that sufficient ADP was formed during the kinase incubation to yield a final carryover of \(5 \times 10^{-4} \text{ M}\) into the phosphorylase a assay. In the presence of \(2.5 \times 10^{-4} \text{ M}\) Mg++, ion (concentration of carryover), \(5 \times 10^{-3} \text{ M}\) ADP added to the phosphorylase a assay caused a rise in phosphorylase b activity sufficient to account for the inorganic phosphate levels of Control B. It is clear that controls employing phosphorylase alone do not constitute an adequate correction when assaying crude heart extracts for kinase activity, especially at pH 6.8, where the enzyme has low activity. Much of the apparent activity is an artifact, due to the formation of substances which increase phosphorylase b activity. A much more reliable control is that of Method B, in which this action of crude heart extracts is compensated for. It should be emphasized that the discrepancy between the two methods is considerably less for the 30,000 x g supernatant fraction in which most of the particulate material has been removed. Our data indicate that kinase of heart muscle is fully soluble by the homogenizing procedure used here and that no insoluble form of the enzyme exists. The small amount of activity remaining in the 30,000 x g supernatant fraction rose from 7.36 units per ml in control hearts to 28.8 units per ml in epinephrine-treated hearts. Increase in activity was also evident at pH 8.2, especially in the 30,000 x g supernatant fraction. However, a quite different picture emerged when the activities were calculated with Control B. Phosphorylase b kinase activities in the 30,000 x g supernatant fraction rose from 7.36 units per ml in control hearts to 28.8 units per ml in epinephrine-treated hearts. Increase in activity was also evident at pH 8.2, but the proportion of this increase was less so that the pH 6.8 to 8.2 ratios rose from 0.057 in the control to 0.183 in the epinephrine-treated hearts. It can be seen in Table 1 that, when Control A was used, epinephrine appeared to produce a small but unimpressive increase in apparent kinase activity, as evidenced by increased pH 6.8 to 8.2 ratios, especially in the 30,000 x g supernatant fraction. However, a quite different picture emerged when the activities were calculated with Control B. Phosphorylase b kinase activities in the 30,000 x g supernatant fraction rose from 7.36 units per ml in control hearts to 28.8 units per ml in epinephrine-treated hearts. Increase in activity was also evident at pH 8.2, but the proportion of this increase was less so that the pH 6.8 to 8.2 ratios rose from 0.057 in the control to 0.183 in the epinephrine-treated hearts. Although this degree of activation is not maximal which might be predicted from pH 6.8 to 8.2 ratios, it is highly significant statistically. Differences between 0.8 values and between pH 6.8 to 8.2 ratios for control and epinephrine-treated hearts were subjected to statistical analysis. As determined by the Student t test, P values in each case were less than 0.001. A larger dose of epinephrine (5 µg) produced no greater activation of the enzyme.

Rate of Activation of Phosphorylase b Kinase by Epinephrine—In the previous experiment, hearts were frozen at 9 sec after injection of epinephrine, when the contractile response reached a maximum. In order to further clarify the nature of the activation, additional hearts were injected with this dose of epinephrine and frozen at 3, 9, 16, 38, and 65 sec after completion of the injection. During this interval the contractile response rose to a maximum and then receded. A 30,000 x g supernatant fraction from each heart was prepared and assayed for phosphorylase b kinase at pH 6.8 and 8.2 with Control B described above. The data in Fig. 5 show that a 5-fold increase in phosphorylase kinase activity occurred in the epinephrine-treated hearts over the control as determined by assays at pH 6.8. Activation was also apparent in the assays at pH 8.2 but of lesser total magnitude as compared with the control. The result was a 3-fold increase in the pH 6.8 to 8.2 ratios. Moreover, these changes occurred with striking rapidity and were maximal at 3 sec when the first hearts were frozen. The contractile response did not reach a maximum until 10 sec after the injection and then fell rapidly. Kinase activity remained maximal until after 10 sec and then decreased somewhat more slowly than the contractile response. It should be noted that this supramaximal dose of epinephrine causes a
latent depression of perfused rat hearts which results in an initial rapid decline in contractile activity. At no time was phosphorylase kinase fully activated as could be predicted from pH 6.8 to 8.2 ratios, i.e., these ratios never approached 1. The maximum pH 6.8 to 8.2 ratios obtained were 0.18. The changes, however, are highly significant statistically. All differences between the control (pH 6.8 values, pH 8.2 values, and pH 6.8 to 8.2 ratios) and epinephrine-treated hearts at the 3-, 9-, and 10-sec intervals were subjected to statistical analysis by the Student t test. In every case the P values obtained were less than 0.001.

**DISCUSSION**

The data presented establish that nonactivated cardiac phosphorylase b kinase is activated by preincubation with ATP and Mg++ ion. The rate of the activation reaction was increased by cyclic AMP, but there was no absolute requirement for the cyclic nucleotide. Activation was especially apparent when the assays were conducted at pH 7.0, where the nonactivated enzyme has low activity, but was also evident when assays were performed at pH 8.2. The activation process is, therefore, characterised by an increase in pH 7.0 to 8.2 activity ratios. The response to cyclic AMP was linear over the range, 1 x 10^-8 to 2 x 10^-7 m. The reaction was highly specific for cyclic AMP; other cyclic nucleotides produced no activation above that of ATP at concentrations below 1 x 10^-4 m. In all respects, the activation of cardiac phosphorylase b kinase is qualitatively similar to the activation of the enzyme from skeletal muscle (5).

There are obvious difficulties in assaying phosphorylase b kinase in crude heart extracts especially at pH 6.8. The difficulties arise from contaminating enzymes in these crude extracts which produce a substance or substances which when carried over into the phosphorylase assay stimulate phosphorylase b activity. Because of this, crude heart extracts cannot be reliably assayed with phosphorylase alone in the control. In the present study, a control was used in which heart extract was present during the kinase incubation and phosphorylase was added after dilution to stop the kinase reaction. The data obtained with this control clearly give a more reliable measure of phosphorylase kinase activity in crude heart extracts, especially in assays conducted at pH 6.8. With the use of this procedure, pH 6.8 to 8.2 activity ratios of about 0.06 were obtained in control perfused rat hearts. One can conclude that epinephrine causes a modest (5-fold at pH 6.8) but highly significant activation of phosphorylase b kinase in perfused rat hearts. Activation relative to the control was greater at pH 6.8 than at pH 8.2 so that pH 6.8 to 8.2 activity ratios increased 3-fold. The activation was maximal at 3 sec after injection of epinephrine when the first hearts were frozen. The contractile response reached a maximum after 9 sec. Evidence exists that phosphorylase a levels rise later than the contractile response and lag well behind the rise in cyclic AMP level in hearts injected with supramaximal doses of epinephrine (8, 9). It is generally felt that phosphorylase activation is dissociated from the contractile response. The rapid activation of phosphorylase b kinase would suggest that this reaction follows closely the rise in cyclic AMP levels (8) and clearly precedes the contractile response. Although the dose of epinephrine chosen was deliberately supramaximal (with respect to contractile response), the data lend support to the idea that epinephrine-induced phosphorylase activation in the myocardium may result from activation of phosphorylase b kinase by cyclic AMP. Additional studies with physiological doses of epinephrine are needed to define more clearly the relationship between phosphorylase b kinase activation, increased cyclic AMP levels, and the inotropic response. Such a study is the subject of the succeeding report (13).

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