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Activation of Skeletal Muscle Phosphorylase *b* Kinase by CA^{2+} *

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The activation of rabbit skeletal muscle phosphorylase *b* kinase by preincubation with Ca^{2+} 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 Ca^{2+} in the presence of the kinase-activating factor were complicated by instability of kinase and kinase-activating-factor in the presence of Ca^{2+}; 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 Ca^{2+} 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 Mg^{2+} or Mn^{2+} 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,

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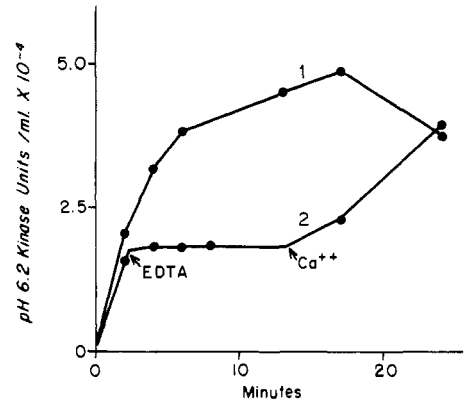

FIG. 1.—Activation of phosphorylase *b* kinase by Ca^{2+}. Identical activation reaction mixtures at 30° were made up containing: 0.25 ml of an acid-precipitate fraction of nonactivated phosphorylase *b* kinase in 0.05 M glycero-P, 0.002 M EDTA buffer at *p*H 7.2; 0.25 ml H_2O; and 0.5 ml of 0.003 M $Ca(Ac)_2$. Where shown by arrows, 0.02 ml of neutral 0.1 M EDTA or 0.1 M $Ca(Ac)_2$ was added to the reaction in curve 2. At the times indicated 0.1-ml aliquots were removed and diluted 1:1600 in cold 0.015 M cysteine buffer. Kinase activities were then determined at *p*H 6.2.

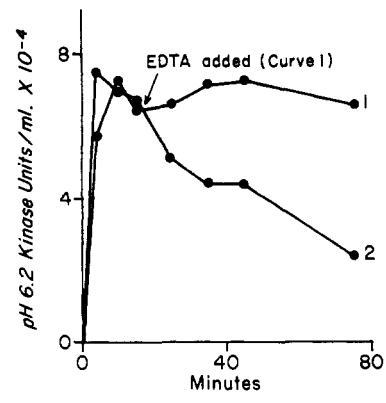

FIG. 2.—Inactivation of phosphorylase *b* kinase following activation by Ca^{2+}. Conditions were the same as in Figure 1 except that 0.004 M $Ca(Ac)_2$ was used. EDTA (0.02 ml, 0.1 M) was added where indicated by the arrow.

is in a form which has very low activity at *p*H 7 and possesses only part of its full activity at higher *p*H values; this form is referred to as nonactivated phosphorylase *b* kinase (Krebs *et al.*, 1964). Preincubation of the nonactivated kinase with various activating components leads to marked activation which is particularly evident when the enzyme is assayed at *p*H 7 or lower. Activation by trypsin and by ATP in the presence of adenosine-3′,5′-AMP (Krebs *et al.*, 1964) was discussed in the preceding paper. The present paper is concerned with activation by preincubation of the kinase with Ca^{2+} ions, a reaction which requires a protein factor distinct from phosphorylase *b* kinase. A preliminary report of these findings has been published (Meyer, 1962).

Materials and Methods

Phosphorylase b Kinase.—Nonactivated phosphorylase *b* kinase was prepared by the method of Krebs *et al.* (1964) and their nomenclature for the various fractions will be followed in this paper. All kinase fractions were handled and stored in 0.05 M glycero-P, 2×10^{-3} M EDTA buffer, *p*H 7.0–7.2, frozen, or simply in the cold when only a few days were involved. To make kinase essentially free of the kinase-activating factor (KAF),[1] the 40-precipitate fraction was diluted with the above-mentioned buffer to a protein concentration of 0.5%, and the solution was recentrifuged at 100,000 × *g* for 3 hours in a repetition of the step originally leading to this fraction. The pellet was taken up in buffer to a final protein concentration of 1–2%. The centrifugation step was sometimes repeated if appreciable amounts of the factor were still detectable. In addition to removal of the kinase-activating factor (KAF), the repeated centrifugation steps usually led to enhancement of the specific activity of nonactivated phosphorylase *b* kinase. In a typical experiment the original 40-precipitate fraction had an activity of 30,000 kinase units/mg at *p*H 8.2, which was increased to 50,000 units/mg by the recentrifugation procedure. On a few occasions the enzyme appeared incapable of withstanding repeated centrifugations and large losses of total and specific activity occurred.

Phosphorylase b Kinase Activity Tests.—Phosphorylase *b* kinase activities were determined as before (Krebs *et al.*, 1964). A given amount of nonactivated phosphorylase *b* kinase will be described in terms of kinase units at *p*H 8.2 where nonactivated kinase is partially active, but the practice of referring to kinase units at other specified *p*H values will also be followed.

Activation Reaction Mixtures.—In studies on the activation of phosphorylase *b* kinase, the nonactivated enzyme was preincubated with activating components under various specified conditions. The concentration of kinase in these activation mixtures was sufficiently high, so that the activating components were effectively diluted out prior to assays for kinase activity. Cold neutral 0.015 M cysteine was used as the diluent, and in all experiments in which activation times were critical it was established that dilution had effectively stopped the activation process. Assays were carried out at *p*H values between *p*H 6 and 7, since this is the region in which nonactivated kinase has very little initial activity (Krebs *et al.*, 1964). Activities are reported in terms of kinase units per ml in the activation reaction.

Results

Time Course of Phosphorylase b Kinase Activation by Ca^{2+}.—Phosphorylase *b* kinase in crude muscle extracts or in partially purified fractions of the enzyme is strongly activated by preincubation with Ca^{2+} (Krebs *et al.*, 1959). Time courses of such activation reactions at *p*H 7.2 are shown in Figures 1 and 2. The activation phase is followed by loss of activity; this is particularly evident in curve 2 of Figure 2, in which the experiment was continued for a longer period of time and at somewhat higher Ca^{2+} concentration than that of Figure 1. Both activation and inactivation of kinase were blocked by EDTA (curve 2 of Fig. 1 and curve 1 of Fig. 2) but the processes were resumed when Ca^{2+} was added in molar excess over the chelating agent (illustrated for the activation phase in curve 2 of Fig. 1). It is evident, therefore, that the two opposing effects on kinase activity, activation and inactivation, are dependent on free Ca^{2+} ions. In the preceding paper (Krebs *et al.*, 1964) it was shown that Mg^{2+} also destabilized phosphorylase *b* kinase even though it did not (in absence of ATP) activate the enzyme.

The Effect of Varying Concentrations of Ca^{2+} *on the Activation of Phosphorylase b Kinase.*—The effectiveness of different concentrations of Ca^{2+} in the activa-

[1] Abbreviation used in this work: KAF, kinase-activating factor.

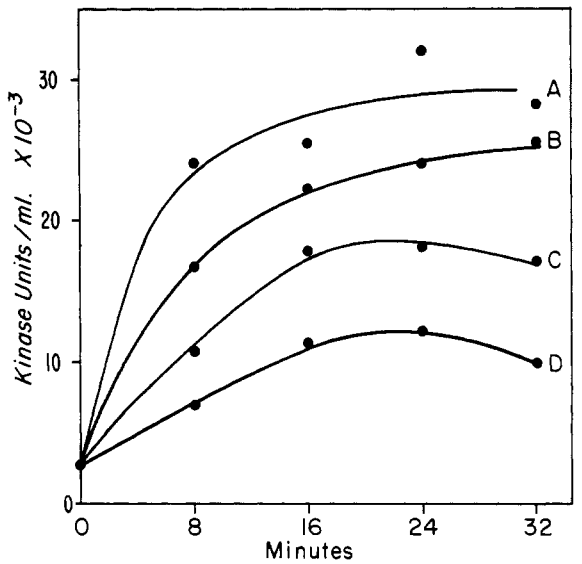

FIG. 3.—The effect of Ca^{2+} concentration on the activation of phosphorylase *b* kinase. Conditions were the same as in Figure 1 except that a different acid-precipitate fraction was used and kinase-activity tests were carried out at *p*H 6.8. $[Ca^{2+}]$ in excess over EDTA in the activation mixtures as follows: curve A, 1.4×10^{-3} M; curve B, 7×10^{-4} M; curve C, 5×10^{-4} M; curve D, 3×10^{-4} M.

tion of phosphorylase *b* kinase is shown in Figure 3. More rapid rates of activation and higher maxima were reached as the Ca^{2+} concentration was increased. In view of the biphasic nature of this process, it is probable that the relative plateaus seen in experiments of this type represent a balance between rates of activation and inactivation.

Specificity for Metals in the Activation of Phosphorylase b Kinase. In addition to Ca^{2+}, Sr^{2+} and Ba^{2+} were also found to be effective in activating phosphorylase *b* kinase when they were incubated with partially purified fractions of the enzyme. Using reaction mixtures similar to those described in the legends of Figure 1 and comparing activation by Ca^{2+}, Sr^{2+}, and Ba^{2+} (all at 0.006 M) as determined at an arbitrarily chosen time (10 minutes), it was found that the latter two metals each gave 60% as much activation at Ca^{2+}. In the present study Mn^{2+} was found to be somewhat effective,[2] giving 20% as much activation as Ca^{2+} when compared at 0.005 M. Other metals tested, including Fe^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}, Mg^{2+}, and Ni^{2+}, were ineffective. The activation of kinase by metals was not influenced by the ionic strength of the incubation medium in the range from 0.02 to 0.15. The presence of Mg^{2+} did not antagonize activation by Ca^{2+}.

Attempts to Reverse the Activation of Phosphorylase b Kinase by Ca^{2+}.—No conditions were found which would reverse the activation of phosphorylase *b* kinase brought about by preincubation of the enzyme with Ca^{2+}. It is evident in the experiments of Figures 1 and 2 that simple addition of EDTA did not cause a reversal of the process. Prolonged dialysis of a Ca^{2+}-activated fraction against EDTA was also ineffective in this respect. A sample of activated kinase that had been dialyzed against EDTA to remove Ca^{2+} was mixed with a sample of nonactivated kinase and the

[2] In a previous study (Krebs *et al.*, 1959) Mn^{2+} ions were reported to be ineffective. No explanation for the difference in results is apparent, but the present findings are in keeping with the known ability of Mn^{2+} or Ca^{2+} to promote phosphorylase *a* formation in fresh muscle extracts (Fischer and Krebs, 1955).

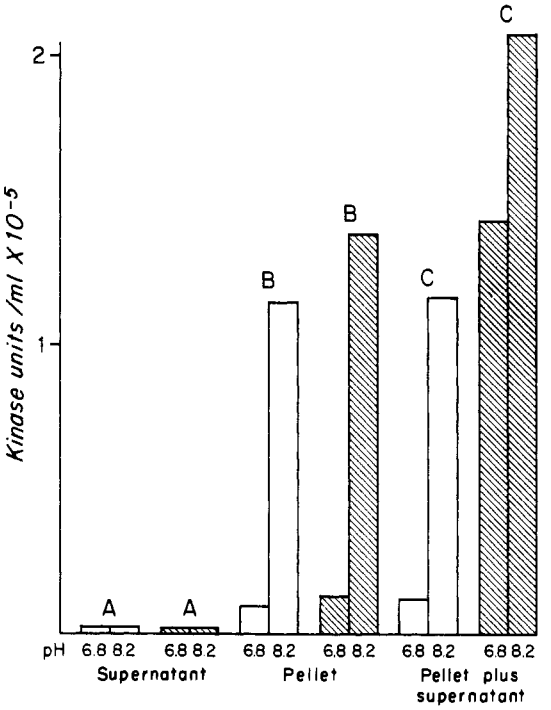

FIG. 4.—Separation of a factor needed for Ca^{2+} activation of phosphorylase *b* kinase. The preparation of the supernatant and pellet fractions is described in the text. Activation reaction mixture in A consisted of 0.1 ml of "supernatant" plus 0.1 ml 0.012 M $Ca(Ac)_2$ or H_2O; in B, 0.1 ml of "pellet" plus 0.1 ml 0.012 M $Ca(Ac)_2$ or H_2O; in C, 0.1 ml of "supernatant," 0.05 ml of "pellet," plus 0.05 ml of 0.024 M $Ca(Ac)_2$ or H_2O. Mixtures were incubated for 10 minutes at 30° and the reactions were stopped by dilution in cold 0.015 M cysteine, *p*H 7.0. Kinase assays were performed at the *p*H values indicated. Units are expressed per ml of "supernatant" in A and per ml of "pellet" in B and C. Open bars, controls without Ca^{2+}; shaded bars, with Ca^{2+}.

mixture was incubated for several minutes at 30°. Assays for kinase activity at *p*H 6.8 and 8.2 showed that the mixture contained a simple sum of the activities of the separate fractions.

Separation of Phosphorylase b Kinase and a Factor Required for Ca^{2+} *Activation of the Enzyme.*—During purification studies on the kinase it was noted that kinase sedimented in the preparative ultracentrifuge showed a poor response to Ca^{2+} but responded as well as an uncentrifuged fraction when the supernatant fluid was readded. A typical experiment, which established the presence of a kinase activating factor (KAF) in the preparation, was carried out as follows:

A 4-ml sample of the acid-precipitate fraction from the kinase preparation was diluted to 12 ml in 0.04 M glycero-P, 0.03 M cysteine buffer, *p*H 7.0, and centrifuged for 2 hours at 100,000 × *g*. The supernatant fluid was decanted, diluted to 12 ml, and recentrifuged under the same conditions. The supernatant fluid from the second centrifugation was used as "supernatant" in the experiment of Figure 4. The original pellet was suspended in a final volume of 12 ml and recentrifuged. This time the pellet was suspended in a volume of 3.5 ml and was used as "pellet" in the experiment of Figure 4. Kinase activities at *p*H 6.8 and 8.2 were determined after preincubation with Ca^{2+} or H_2O for the "supernatant" and "pellet" taken separately and in combination. The results in Figure 4 show that the recentrifuged "pellet" was not responsive to Ca^{2+} alone but did undergo activation when incubated with Ca^{2+} plus a small amount of "supernatant." The latter fraction contained almost no detectable kinase.

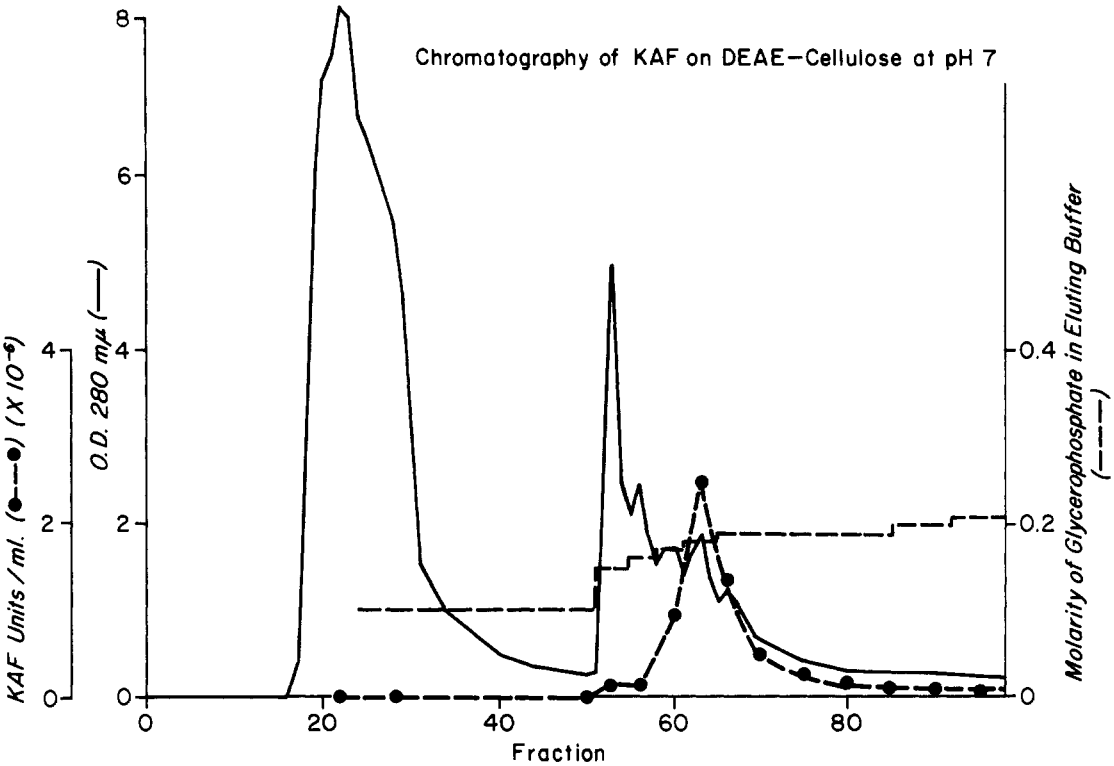

FIG. 5.—Chromatography of KAF on DEAE-cellulose. Aliquots from each fraction were analyzed for KAF activity as described in the text.

The response of the original acid-precipitate fraction to Ca^{2+} (not illustrated in Fig. 4) was almost identical to the combination of "pellet" plus "supernatant."

In the regular procedure for purification of phosphorylase *b* kinase (Krebs *et al.*, 1964) centrifugation of the 30-supernatant fraction leads to the separation of kinase and much of the KAF present in the preparation. Recentrifugation of the 40-precipitate fraction as described under Methods yields a KAF-free kinase.

Purification of KAF.—It was found that under properly chosen conditions there was a linear relationship between the amount of KAF and the degree of phosphorylase *b* kinase activation occurring in a given time, so that it was possible to set up an assay system and purify the factor to some extent. For the assay, reaction mixtures were made up containing 0.1 ml 0.03 M $Ca(Ac)_2$ in 0.1 M glycero-P, 0.1 M Tris buffer, *p*H 7.8; 0.1 ml of KAF-free nonactivated phosphorylase *b* kinase (2500 *p*H 8.2 units) in cysteine buffer; and 0.1 ml KAF diluted in cold neutral 0.015 M cysteine.[3] The mixture was incubated 30 minutes at 15° and the activation reaction was stopped by diluting an aliquot 1:200 in the cysteine solution. Phosphorylase *b* kinase activity was then assayed at *p*H 6.8. For convenience a KAF unit was defined as that amount of KAF causing an increase of one kinase unit in the activation reaction mixture.

Although KAF was originally detected in partially purified kinase fractions as described earlier, it became apparent that most of the factor remained in the supernatant fraction of muscle extracts following acid precipitation of kinase at *p*H 6.1 but could be precipitated at *p*H 5.1. Taking advantage of the fact that precipitation of proteins at the latter *p*H is a regular step in rabbit muscle phosphorylase *b* preparations routinely carried out in this laboratory, it was convenient to use the *p*H 5.1 precipitates from this fractionation procedure (Fischer and Krebs, 1962) as the starting point for KAF preparation. These precipitates were homogenized using 50 ml of 0.1 M glycero-P, 0.004 M EDTA buffer, *p*H 8.2, per kilogram of muscle used in the preparation; the *p*H of the mixture was then adjusted to 7.0 with 1 N NaOH and it was stored in the freezer until used.

A typical KAF preparation utilizing 1290 ml of *p*H 5.1 precipitate fraction derived from phosphorylase *b* preparations in which 21 kg of rabbit muscle was used was carried out as follows:

Step 1.—The thawed material was centrifuged for 1 hour at 40,000 × *g*, and the clear supernatant fluid (1015 ml) was adjusted to *p*H 4.5 with dropwise addition of 175 ml of 1 N HCl with stirring at 0°. The resulting thick creamy suspension was centrifuged at 1200 × *g* for 30 minutes and the packed precipitate was suspended and partially dissolved in 400 ml of 0.1 M glycero-P, 0.001 M EDTA, *p*H 8.5 buffer and the *p*H was adjusted to neutrality by addition of 1 N KOH. After storage overnight at 0° this material was centrifuged for 30 minutes at 25,000 × *g* and the clear supernatant solution with a volume of 383 ml (*p*H 4.5, precipitate fraction) was decanted.

Step 2.—The *p*H 4.5 precipitate fraction was brought to 50% saturation (0°) with solid ammonium sulfate and the resulting precipitate was collected by centrifugation and taken up in 200 ml of 0.25 M glycero-P, 0.001 M EDTA buffer, *p*H 7.0. This turbid solution was dialyzed 24 hours against several changes of the same buffer and was then clarified by centrifugation at 78,000 × *g* for 3 hours at 0°. One hundred seventy-five (175) ml of supernatant solution (ammonium sulfate precipitate fraction) was obtained.

Step 3.—A portion (100 ml) of the ammonium sulfate-precipitate fraction was applied to a 64 × 2.5-cm DEAE-cellulose column packed under 10 lb pressure and previously equilibrated at 30° with 0.1 M glycero-P, 0.001 M EDTA buffer, *p*H 7.0. Elution was

[3] Cysteine was included in the reaction mixture because it increased the rate of phosphorylase *b* kinase activation by Ca^{2+} severalfold.

started with a flow rate of 450 ml per hour using buffers at *p*H 7.0, 0.001 M in EDTA, with glycero-P concentration as indicated in Figure 5. Fractions 62–67, containing the major amount of KAF activity, were combined and the proteins were precipitated by addition of solid ammonium sulfate to 0.75 saturation. After centrifugation the precipitate was dissolved in 0.02 M glycero-P, 0.001 M EDTA buffer at *p*H 7.0 and dialyzed against several changes of the buffer to remove salt. The solution was clarified by centrifugation at 150,000 $\times$ *g* for 3 hours. The clear supernatant (chromatographic fraction) had a volume of 8.4 ml.

The preparation of KAF described above is summarized in Table I. Data are also given for the acid-

TABLE I
PURIFICATION OF KAF FROM RABBIT SKELETAL MUSCLE[a]

Fraction	Volume (ml)	Total Activity (units) $\times 10^{-6}$	Specific Activity (units/mg) $\times 10^{-3}$
A			
Muscle extract	6040	187	1.4
*p*H-5.1 Precipitate	167	346	18
B			
*p*H-5.1 Precipitate	1288	177	13
*p*H-4.5 Precipitate	383	67	37
Ammonium sulfate precipitate	175	48	94
Chromatographic fraction	14.7	11	990

[a] The A fractions were prepared from 2.7-kg muscle while the B fractions were obtained from 21.6-kg muscle. Recoveries have been adjusted to account for sampling and for the amount of material carried through each procedure. Recovery and purification data are calculated independently for experiments A and B. The KAF unit used is defined in the text.

precipitation step from a separate preparation; here it can be seen that the recovery of units was greater than 100%, indicating the probable presence of inhibitory or interfering materials in the crude extract. Purified KAF was free of phosphorylase *b* kinase. It contained 7–8% phosphorylase *a*. As examined in the analytical ultracentrifuge (not illustrated) two major peaks with $s_{20,w}$ values of 6.4 and 4.0 S (determined at 7 mg/ml) were present. The ultraviolet-absorption spectrum was that of a typical protein with an $E^{28°}/E^{26°} = 1.5$. On electrophoresis there were two major peaks migrating toward the anode at *p*H 7.0. All of the KAF activity in a fraction was readily destroyed by trypsin and was nondialyzable. The purified KAF was very unstable in the presence of Ca^{2+}, approximately 50% of activity being lost in 10 minutes on incubation at 30° with 0.015 M Ca^{2+}. The factor could be stored frozen in the presence of EDTA for several months without loss of activity.

The Influence of Varying Concentrations of KAF on Kinase Activation by Ca^{2+}.—The availability of purified kinase-free KAF and KAF-free kinase made it possible to examine the behavior of the activation system with independent variation of the components. Figure 6 shows the effect of varying the concentration of KAF in the activation reaction mixture. If KAF were acting catalytically, one would expect that lower amounts of it would produce as great an activation over a longer period of time as higher amounts in a shorter time interval. Instead, the results show what would appear to be almost a stoichiometric interaction of KAF and kinase suggested by the leveling off of

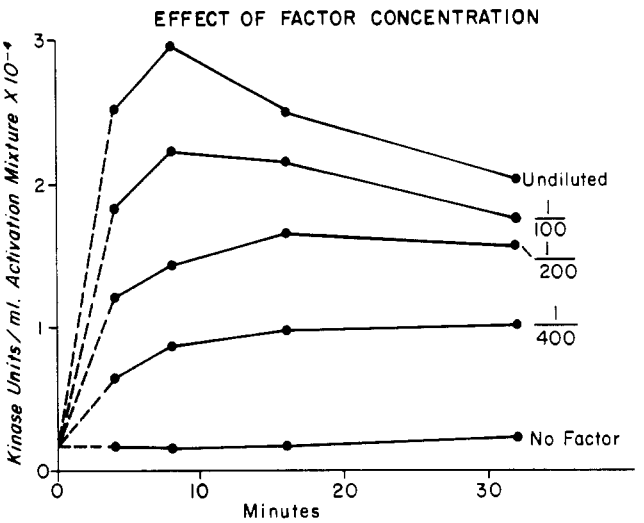

FIG. 6.—Effect of varying concentration of KAF on kinase activation. Reaction mixtures at *p*H 7.0 were 0.01 M in Ca^{2+}, 0.02 M in glycero-P, and 0.015 M in cysteine, and contained 19,000 *p*H 8.2 kinase units (KAF-free kinase) per ml. Purified KAF at the dilutions indicated was added at 0 time; undiluted KAF represents 5 mg of purified KAF per ml in the reaction. Incubation was at 30°. At the indicated times aliquots were removed, diluted, and assayed for kinase activity at *p*H 6.8. It will be noted that after activation (curve with undiluted KAF) activities at *p*H 6.8 are 1.6 times higher than at *p*H 8.2 before activation.

activation at values almost proportional to the amount of KAF used. It is again possible, however, that the levels of activation reached may simply represent a balance between destruction and activation; this was also pointed out as a possibility to explain the results in Figure 3. When KAF was fixed and kinase was varied, a family of curves (not illustrated) very similar to those of Figure 6 was obtained.

Studies on the Mechanism of Activation of Phosphorylase b Kinase by Ca^{2+} *and KAF.*—Although completely homogeneous preparations of nonactivated phosphorylase *b* kinase were not available, attempts were made nonetheless to determine whether activation by Ca^{2+} plus KAF produced any physical changes in the enzyme. On a preparative basis it was noticed that the sedimentation behavior of activated or nonactivated kinase was identical; i.e., 85–95% of the enzyme was sedimented in 3 hours at 100,000 $\times$ *g*. The analytical ultracentrifuge pattern of the kinase before and after activation by Ca^{2+} was the same (Krebs *et al.*, 1964). No significant changes in the electrophoretic pattern of purified phosphorylase *b* kinase occurred as a result of activation by Ca^{2+} plus KAF (see Krebs *et al.*, 1964, for the pattern of nonactivated kinase).

Inasmuch as trypsin is capable of activating phosphorylase *b* kinase (Krebs *et al.*, 1964), it appeared possible that KAF might be a Ca^{2+}-requiring proteolytic enzyme. This idea received support from the finding that KAF itself was very unstable in the presence of Ca^{2+} and also from the fact that the activation of kinase by Ca^{2+} appeared to be irreversible. No experimental verification of the proteolytic hypothesis could be obtained. KAF was treated with DFP under conditions in which an equivalent amount of trypsin (based on kinase-activating potency) was inhibited more than 90%, but essentially no inhibition (less than 10%) of KAF activity resulted. KAF was not inhibited by soybean-trypsin inhibitor. High concentrations of KAF showed no activity when tested at *p*H 7.6 in the *p*H-stat with 0.01 M solutions of several proteolytic enzyme substrates including *N*-acetyl-

tyrosine ethyl ester, *N*-benzoylarginine methyl ester, hippurylphenylalanine, and L-leucylglycylglycine; the tests were conducted with or without cysteine in the presence of Ca^{2+}. No activation of kinase by 0.1 mg of partially purified commercial preparations of rennin and thrombin, with or without Ca^{2+}, could be detected under conditions where a few micrograms of trypsin or purified KAF showed marked activation of kinase.

Two Ca^{2+}-activated lipases were tested for possible action on phosphorylase *b* kinase. One of these was active peak II (Saito and Hanahan, 1962) phospholipase A purified from *Crotalus adamanteus* venom by DEAE-cellulose and kindly supplied by Dr. D. J. Hanahan. The other was a preparation of phospholipase D of *Clostridium perfringens* purified on a DEAE-Sephadex column and supplied by Dr. Patrick Kemp. Neither enzyme had any effect on the nonactivated kinase.

It was not possible by preincubation of Ca^{2+} with either KAF or kinase separately to demonstrate the formation of any intermediate component which was then capable of forming active kinase in the absence of Ca^{2+} when added to kinase or KAF, respectively. In other words, all three components had to be present simultaneously in order to have activation. No heat-stable cofactor of kinase could be detected in boiled Ca^{2+}-activated preparations of kinase.

Several experiments were carried out to determine whether the activation of kinase by Ca^{2+} plus KAF was completely independent of the activation by ATP and Mg^{2+}. Cyclic AMP, which affects the latter process, was found to have no effect on activation of kinase by Ca^{2+}. Purified KAF was found to have no influence on the activation of kinase by ATP in the presence or absence of cyclic AMP. Although no links could be found which tied the two activation processes together, in each instance the activated kinase possessed a lower K_m for phosphorylase *b* than did nonactivated kinase. In a typical experiment the K_m for phosphorylase *b* at *p*H 7.5 was found to be 56,000 units/ml (1.4×10^{-4} M) before activation and 15,000 units/ml (3.7×10^{-5} M) after activation by preincubation with Ca^{2+} plus KAF. The reduction in K_m by activation with ATP was discussed in the preceding paper (Krebs *et al.*, 1964).

Effects of Metals and Chelating Agents Added Directly to Phosphorylase b to a Reaction Mixtures.—In the experiments discussed up to this point, the effects on kinase that have been described have been elicited by addition of components to preincubation or activation-reaction mixtures containing relatively high concentrations of enzyme. Such components are diluted several thousandfold at the stage of the kinase assay itself, i.e., the phosphorylase *b* to *a* reaction. For example, in the experiment of Figure 1, added Ca^{2+} was present at a concentration of 1.5×10^{-3} M in the activation-reaction mixture, but the carryover of the metal into the kinase reaction was only 1.5×10^{-7} M. Similarly, EDTA introduced with the kinase was at a concentration of 5×10^{-4} M in the activation reaction but only 5×10^{-8} M in the kinase reaction. Ca^{2+} or EDTA at such levels have no effect on the phosphorylase *b* to *a* reaction with activated or nonactivated kinase as enzyme.

In early experiments in which a crude source of nonactivated phosphorylase *b* kinase (containing KAF) was used (Krebs *et al.*, 1959) it was shown that addition of Ca^{2+} to the kinase-reaction mixture at a level of 1×10^{-3} M caused either inhibition or activation depending upon the *p*H of the reaction mixture. With Ca^{2+}-activated kinase only inhibition by the metal was noted; this inhibition was competitive with respect to Mg^{2+}. In the present study using nonactivated KAF-free kinase addition of Ca^{2+} to the kinase reaction also resulted in inhibition.

Nonactivated phosphorylase *b* kinase is inhibited by EDTA and this inhibition cannot be accounted for by the binding of Mg^{2+} required in the *b* to *a* reaction. For example, using either KAF-free nonactivated kinase or an enzyme fraction containing KAF, it was found that 1×10^{-3} M EDTA caused 60% inhibition at *p*H 8.2. The concentration of Mg^{2+}, which is normally 1×10^{-2} M in the reaction mixture, could be raised to 1.1×10^{-2} M without affecting the inhibition. Ethylene glycol bis-(β-aminoethylether)-*N*,*N*′-tetra-acetate (EGTA) is a much more effective inhibitor than EDTA, causing complete inhibition at 1×10^{-3} M and 75% inhibition at 1×10^{-4} M in tests at *p*H 8.2. Diethyldithiocarbamic acid and α-α-dipyridyl were essentially noninhibitory when tested at 0.001 M. When different metals were tested for their ability to relieve the inhibition of KAF-free kinase by EDTA, Ca^{2+} was found to be the most effective as is shown in Table II. In this experiment the various metals were added at a concentration of 5×10^{-4} M to kinase that was 60% inhibited by 1×10^{-3} M EDTA. Ca^{2+}, Sr^{2+}, and Mn^{2+} caused appreciable reduction in the extent of inhibition, in that order. It should be pointed out that experiments involving the introduction of metals or chelating agents into the kinase reaction mixture are complicated by the fact that cysteine, which is an effective chelating agent for certain metals, is present. Experiments have not as yet been carried out in which some other thiol compound is substituted for cysteine.

TABLE II
EFFECTIVENESS OF VARIOUS METAL IONS IN REVERSING EDTA INHIBITION OF NONACTIVATED PHOSPHORYLASE *b* KINASE[a]

Metal Ion	Inhibition of Kinase Activity (%)
None	60
Ca^{2+}	4
Sr^{2+}	19
Mn^{2+}	37
Ba^{2+}	44
Co^{2+}	48
Fe^{3+}	52
Ni^{2+}	52
Zn^{2+}	59
Al^{3+}	63
Pb^{2+}	63
Sn^{2+}	67
Cd^{2+}	67
Cr^{3+}	70
Fe^{2+}	70
Ce^{3+}	70
Hg^{2+}	74
Cu^{2+}	82

[a] EDTA and various metal salts were added directly to kinase-reaction mixtures at *p*H 8.2. Final concentrations were 10^{-3} M for EDTA and 5×10^{-4} M for the metal ion indicated. Kinase used = KAF-free kinase.

DISCUSSION

It is possible that two separate mechanisms exist for controlling the conversion of phosphorylase *b* to *a* in skeletal muscle. One of these, operating through the production of cyclic AMP (Sutherland and Rall, 1960) and the subsequent activation of phosphorylase *b* kinase (Posner *et al.*, 1962), would account for the

action of epinephrine on glycogenolysis. The other, working through some different agent, but also resulting in phosphorylase *b* kinase activation, would relate to the coupling of muscle contraction to glycogenolysis. The agent in this latter scheme might be Ca^{2+}. Recent work indicates that Ca^{2+} enters (Bianchi and Shanes, 1959; Shanes and Bianchi, 1959) or is released (Tobias *et al.*, 1962) into the cell during excitatory events at the cell membrane, and this and other data have been used (Winegrad and Shanes, 1962) to strengthen the argument (Heilbrunn, 1952; Sandow, 1952) that Ca^{2+} may be the major link between excitation and response in muscle and other tissues. Effects on Ca^{2+} interactions with various tissue components have recently been implicated in the action of a number of drugs (Bianchi, 1961, 1962; Yukisada and Ebashi, 1961; Fairhurst and Jenden, 1962) affecting muscle activity. Evidence for direct effects of ionic calcium on the molecular components of the contractile apparatus has appeared with the demonstration that Ca^{2+} is required for synaeresis of myofibrils (Weber and Hers, 1962) and the analogous reaction of superprecipitation of "synthetic" actomyosin (Weber and Winicur, 1961) at physiological Mg^{2+} concentrations and that binding of Ca^{2+} can account on a quantitative basis for the relaxations caused by EDTA and other chelating agents (Ebashi, 1960) and by muscle-relaxing-factor preparations (Ebashi, 1961; Hasselbach and Makinose, 1961).

In order for Ca^{2+} activation of phosphorylase *b* kinase to constitute a physiological control mechanism, it would seem essential for the process to be reversible. No indications have been obtained thus far that this is so. Although the presence of Ca^{2+} binding agents readily prevent activation of the enzyme, these substances are completely ineffective in reversing the activity changes that take place when the kinase is activated by the metal. Since the mechanism of activation of the kinase by Ca^{2+} has not been elucidated, however, it is possible that factors required for reversal of activation remain unrecognized and may be missing in the purified system.

The work that has been done in connection with the mechanism of activation of phosphorylase *b* kinase by Ca^{2+} and the kinase-activating factor shows that activation decreases the K_m for phosphorylase *b*. In this respect Ca^{2+} activation resembles activation by ATP, and it is possible that both processes bring about similar conformational changes in the kinase so that its affinity for phosphorylase *b* is increased. The activating factor (KAF) appears to be a protein, since it is nondialyzable, heat labile, and is destroyed by trypsin, but the nature of its action is not clear. Kinetic studies have not supported the idea that the factor is an enzyme, although it should be emphasized that experiments of this type are carried out with great difficulty and are easily misinterpreted. No enzymatic activities for the factor have been found using simple substrates. The finding that the kinase reaction itself is subject to inhibition by chelating agents raises the possibility that a metal (other than Mg^{2+}) is directly involved in the phosphorylase *b* to *a* reaction. Such a metal could be Ca^{2+}. This idea is supported by the finding that ethylene glycol bis-(β-aminoethylether)-*N*,*N*′-tetraacetate is a more effective inhibitor of kinase than EDTA and is known to exhibit significant selectivity between Ca^{2+} and Mg^{2+} (Chabarek and Martell, 1959). Furthermore, Ca^{2+} was the most effective metal in relieving the inhibition of kinase caused by EDTA. Agents which are good heavy-metal binders, but which bind Ca^{2+} poorly did not inhibit the kinase. The relationship of a role for Ca^{2+} in the kinase reaction itself to the role for Ca^{2+} in kinase activation as described in this paper is not clear, although the existence of such a relationship is an attractive unifying possibility.

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