Liver glycogen phosphorylase kinase was assayed by measuring the radioactivity of [γ-32P]ATP incorporated into rabbit skeletal muscle phosphorylase b. Using this assay two forms of phosphorylase kinase, Kinase I and Kinase II, were purified about 70- and 150-fold, respectively, from rabbit liver cytosol by DEAE-Sephadex A-50 column chromatography, ammonium sulfate fractionation, gel filtration on a Sepharose CL-6B column, followed by phosphocellulose column or Cibacron blue-Sephadex affinity chromatography. The phosphorylation reaction was accompanied by the concomitant activation of phosphorylase b. Both Kinases I and II were able to phosphorylate and activate liver inactive phosphorylase. The molecular weight was about 1,300,000 for Kinase I and 1,100,000 for Kinase II. Kinetic properties of Kinases I and II were similar: the optimum pH was 7; the $K_m$ value for ATP was $3.0 \times 10^{-7}$ M; and the $K_m$ value for muscle phosphorylase b was $300 \mu$g/ml, and that for liver inactive phosphorylase was $80 \mu$g/ml. The liver phosphorylase kinase was inhibited partly, at most 60 to 70%, by $0.05 \text{ mm ethylene glycol bis}($β$-aminoethyl ether)$N,N,N,N$-tetraacetate, and reactivated by Ca$^{2+}$. The $K_a$ value for free Ca$^{2+}$ was about $3 \times 10^{-7}$ M at pH 6.8. Kinase II appeared to be a proteolytic artifact that was produced from Kinase I probably due to a lysosomal protease, cathepsin B. Kinase I was also converted to a smaller form that was indistinguishable from Kinase II by limited proteolysis with Ca$^{2+}$-dependent protease or trypsin. The catalytic activity was enhanced about 60% during this proteolysis. No evidence has been obtained indicating the existence of active and inactive forms of liver phosphorylase kinase.

Glycogen phosphorylase kinase catalyzes the phosphorylation of an inactive form of glycogen phosphorylase to produce its active form. Skeletal muscle phosphorylase kinase has been studied extensively, and the physical and kinetic properties as well as the regulatory mechanism of this enzyme have been well clarified (1-5). In contrast to our current knowledge of muscle phosphorylase kinase, the properties and regulatory mechanism of liver phosphorylase kinase have remained largely unexplored. By analogy with the muscle enzyme, it has been generally considered that liver phosphorylase kinase may be activated by cyclic AMP-dependent protein kinase in response to glucagon and epinephrine, and inactivated by phosphoprotein phosphatase. However, there seems to be no sufficient evidence which unequivocally establishes this covalent modification mechanism (6-10). In fact, Ca$^{2+}$ has been proposed as an additional possible candidate for an intracellular mediator in the hormonal control of phosphorylase kinase in this tissue (10-12), although it has been repeatedly described that cyclic AMP enhances the rate of formation as well as the maximum amount of active phosphorylase formed in both in vivo and crude in vitro systems (13, 14).

Another line of evidence has indicated that phosphorylase kinase deficiency in the liver and that in the skeletal muscle are independent events; the defect in the liver is not accompanied by that in the skeletal muscle (15-17), and vice versa. Based on these observations Proux et al. (19) have made a brief immunological comparison between liver and muscle phosphorylase kinases and have proposed that these enzymes are indeed isoenzymes. Nevertheless, the precise properties and mechanism of activation of liver phosphorylase kinase have remained to be explored since the first description of this enzyme by Sutherland and his co-workers (20, 21).

In the present studies partial purification and characterization of rabbit liver phosphorylase kinase were undertaken to facilitate studies on the comparison of liver and muscle phosphorylase kinases, particularly on the regulatory mechanism of glycogenolysis in the liver.

**EXPERIMENTAL PROCEDURES**

**Materials**—Crystalline muscle phosphorylase b was prepared from rabbit skeletal muscle as described by Fischer and Krebs (22); the preparation was recrystallized three times and, before use, 5'AMP was removed by extensive dialysis against a large excess of 20 mM sodium glycerol 1-phosphate at pH 6.8 containing 30 mM 2-mercaptoethanol. Liver inactive phosphorylase was purified from pig liver as described by Appleman et al. (23); this preparation was practically inactive unless phosphorylated by either muscle or liver phosphorylase kinase. Rabbit skeletal muscle phosphorylase kinase was purified by the method of Cohen (4). Protein kinase A$^2$ and its catalytic subunit were prepared from rabbit skeletal muscle as described previously (24). Protein kinase inhibitor of protein kinase A was purified from rabbit skeletal muscle as described by Walsh et al. (25). Calf thymus whole histone was prepared by the method of Johns (26). Ca$^{2+}$-dependent protease was purified from rat brain as described by Nesbitt, M. N. (1970) quoted by Huijing (18).


$^2$ The abbreviations used are: protein kinase A, cyclic AMP-dep-
Inoue et al. (27). Alkaline phosphatase (Escherichia coli, code BAPC), suspended in ammonium sulfate, was purchased from Worthington; the suspension was centrifuged for 10 min at 12,000 x g, and the precipitate was dissolved in 50 mM Tris/HCl at pH 7.5 before use. Cibacron blue (FSG-A) coupled covalently to Sephadex G-200 was prepared as described previously (32). The enzyme was provided by CIBA-GEIGY, Japan. 32P]ATP was prepared as described by Glynn and Chappell (29). Bovine thyroglobulin (type I), beef liver catalase, bovine pancreas trypsin (type III), and soybean trypsin inhibitor (type I-3) were obtained from Sigma; β-galactosidase (E. coli) was from Boehringer Mannheim; human hemoglobin (two red cell units) was purchased from ICN Pharmaceuticals, Inc. Enzyme inhibitor from Schwarz/Mann. Enzyme inhibitors, including leupeptin, chymostatin, antipain, pepstatin, and elastatinal, were purchased by Research Resources Program for Cancer Research, Ministry of Education, Science and Culture, Japan. Other chemicals were obtained from commercial sources.

**Phosphorylase Kinase Assay—**Unless otherwise noted phosphorylase kinase was routinely assayed by measuring the incorporation of the terminal phosphate of [γ-32P]ATP into skeletal muscle phosphorylase b. Liver phosphorylase kinase was previously shown to be cross-wise reactive with muscle phosphorylase b (14). Where indicated, liver active phosphorylase was employed instead of muscle phosphorylase b. The reaction mixture contained 10 μmol of Tris/HCl (pH 8.0), 10 μmol of MgCl2, 1.2 μmol of NaF, 25 μmol of [γ-32P]ATP (2 to 3 x 104 cpm/nmol), 150 μg of crystalline muscle phosphorylase b (free of β-MP), and an enzyme preparation. The reaction was started by the addition of a mixed solution of [γ-32P]ATP and magnesium acetate. After incubation for 30 min at 30°C, the reaction was terminated by the addition of 4 ml of 10% trichloroacetic acid. The insoluble materials were collected on a glass fiber filter (Toyo-Roshi Co., Japan) and washed five times, each time with about 5 ml of 10% trichloroacetic acid. The filter was placed on an aluminum disk and dried, and the radioactivity was determined as described below. Under these conditions the reaction proceeded linearly with time for at least 30 min. With partially purified enzyme preparations the reaction was directly proportional to the amount of enzyme employed. However, with crude extracts the reaction initially proceeded linearly but the rate was gradually declined, probably due to the presence of phosphatases and, therefore, the incubation was performed usually for 10 min. When necessary, a blank incubation was run simultaneously without phosphorylase b, and the value obtained was subtracted from the experimental value. One unit of liver phosphorylase kinase was defined as that amount of enzyme which transferred 1 pmol of [γ-32P]ATP into muscle phosphorylase b/min at pH 8.5 under the conditions described above.

Phosphorylase kinase was alternatively assayed, where indicated, by measuring the increase in the glycolygenolytic activity upon incubation of phosphorylase b. Muscle phosphorylase b were collected on a glass fiber filter and experimented on as described above. When [γ-32P]ATP was replaced by nonradioactive ATP. After 30 min at 30°C, a 10-μl aliquot of the incubation mixture was taken and transferred to another reaction mixture for the assay of resulting phosphorylase a. This mixture contained 10 μmol of sodium glycerol 1-phosphate at pH 6.8, 2.5 μg of oyster glycogen, 2 μmol of [32P]ATP (1 to 1.5 x 104 cpm/μmol), 0.1 μmol of EDTA in a final volume of 0.4 ml. Under these conditions adenine nucleotide which was carried from the first incubation mixture was negligible (maximum, 2.5 x 104 cpm). The reaction was terminated by the addition of 4 ml of 10% trichloroacetic acid and the filtrate washed five times with water and dried. The radioactivity of each protein band was determined by transverse sectioning of the gel as described previously (30). The S value of proteins was calculated by the equation described by Martin and Ames (37).

**Other Procedures—**Muscle phosphorylase labeled with [32P]P was prepared with 32P]ATP and either muscle or liver phosphorylase kinase by the method of Fischer et al. (38). Polyaclaylamine gel electrophoresis was performed in the presence of sodium dodecyl sulfate as described by Cohen (4). Denstometric tracing was performed at 660 nm using ISCO gel scanner, model 659, after staining with Coomassie brilliant blue. The radioactivity of each protein band was determined by transverse sectioning of the gel as described previously (31). The fingerprint of tryptic phosphopeptides of radioactive muscle phosphorylase was prepared as described by Fischer et al. (38). The radioactivity of [32P]P samples was determined using a Nuclear Chicago Geiger-Muller gas flow counter, model 4338, or a Packard Tri-Carb liquid scintillation spectrometer, model 3320. Protein was determined by the method of Lowry et al. (39) with bovine serum albumin as a reference protein.

**RESULTS**

**Partial Purification of Liver Phosphorylase Kinase—**A 4 One unit of protein kinase A was defined as that amount of enzyme which incorporated 1 pmol of phosphate into calf thymus whole histone/min under the standard conditions described earlier.

**Worthington Enzyme Manual (1972)** Worthington, Freehold, N.J.
white male domestic rabbit (2.5 to 3.0 kg) was injected with 200 mg of Nembutal and bled by cutting the jugular vein. The liver (50 g) was quickly removed, cut into small pieces, and homogenized for 1.5 min in a Waring Blender with 4 volumes of cold 20 mM Tris/HCl buffer at pH 8.0, containing 0.1 M ammonium sulfate, 20 mM NaF, 10 mM 2-mercaptoethanol, and 2 mM EDTA. All subsequent procedures were carried out at 0 to 5°C. The homogenates were centrifuged for 60 min at 10,000 × g and the supernatant was centrifuged further for additional 60 min at 100,000 × g. After filtering through glass wool to remove fat, the supernatant (crude extract; 5 g of protein) was transferred to a DEAE-Sephadex A-50 column (7.6 × 4.5 cm) equilibrated with Buffer A containing 0.1 M ammonium sulfate. The column was washed with 1.5 liters of Buffer A containing 0.15 M ammonium sulfate. Under these conditions most of inert proteins were washed off. Then, the enzyme which was adsorbed on the column was eluted with Buffer A containing 0.5 M ammonium sulfate. The fractions (475 ml, 309 mg of protein) containing the enzyme were pooled, and solid ammonium sulfate (132 g) was added slowly (45% saturation). After stirring for 60 min, the precipitate was collected by centrifugation for 30 min at 16,000 × g, dissolved in a small volume of Buffer A, and dialyzed against a large volume of Buffer A containing 0.05 M ammonium sulfate. Insoluble materials were removed by centrifugation for 15 min at 100,000 × g. The supernatant (8 ml, 196 mg of protein) was subjected to gel filtration on a Sepharose CL-6B column (2.5 × 85 cm) equilibrated with Buffer A containing 0.05 M ammonium sulfate. As shown in Fig. 1A, two major peaks appeared; these peaks are designated as Kinase I and Kinase II as indicated. Occasionally, a small peak was detected between Kinases I and II. Another small peak found in the void volume of this column represents probably an aggregate of the enzyme.

The fraction of Kinase I (Fractions 32 through 41, 22 mg of protein) was directly transferred to a phosphocellulose column (1.0 × 5.0 cm) equilibrated with Buffer A containing 0.05 M ammonium sulfate. Under these conditions the enzyme passed through the column, whereas interfering proteins, such as casein kinase and its unidentified endogenous phosphate acceptor proteins (40, 41), remained adsorbed. Kinase I thus obtained was concentrated to 5 ml by an Amicon ultrafiltration cell equipped with XM-300 filter membrane after the addition of ethylene glycol to the final concentration of 10%. Next, the fraction of Kinase II (Fractions 55 through 61) purified as above except that PM-10 filter membrane was used and dialyzed overnight against a large volume of Buffer A containing 0.05 M ammonium sulfate and 10% ethylene glycol. The affinity column chromatography was not applicable to Kinase I with efficient purification. The purification and recovery of enzyme were summarized in Table I. These preparations were practically free of cyclic nucleotide-dependent protein kinases, phosphorylase, ATPase, and endogenous phosphate acceptor proteins and, therefore, were employed for all subsequent studies. The experimental results presented below are each representative of at least three experiments.

Phosphorylation and Activation of Phosphorylase—The incorporation of the terminal phosphate of ATP into muscle phosphorylase b by liver phosphorylase kinase purified as described above proceeded linearly with time for at least 30 min.

The small peak which appeared between Kinases I and II in Fig. 1A showed a molecular weight of about 400,000 as estimated by gel filtration on a Sepharose CL-6B column, and an S value of about 8 as estimated by glycerol density gradient analysis. This kinase appeared also to be a proteolytic artifact which was produced from Kinase I during the isolation procedure. The detailed properties of this kinase were not investigated in the present study.

The proportion of Kinases I and II was slightly varied with enzyme preparations, probably due to proteolytic conversion of Kinase I to Kinase II during the isolation procedure (see text). However, the purification and recovery of enzyme were reproducible, and the results given in this table are representatives of more than 10 experiments.
Table II

Comparison of properties of rabbit liver and skeletal muscle phosphorylase kinase

<table>
<thead>
<tr>
<th>Phosphorylase kinase</th>
<th>Liver Kinase I</th>
<th>Liver Kinase II</th>
<th>Skeletal muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>$1.3 \times 10^6$</td>
<td>$1.1 \times 10^6$</td>
<td>$1.33 \times 10^6$</td>
</tr>
<tr>
<td>$S_m$</td>
<td>22</td>
<td>49</td>
<td>261</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>$K_m$ for ATP</td>
<td>$3.0 \times 10^{-2}$</td>
<td>$2.5 \times 10^{-2}$</td>
<td>$4.2 \times 10^{-2}$</td>
</tr>
<tr>
<td>$K_m$ for muscle phosphorylase b</td>
<td>300 mg/ml</td>
<td>290 mg/ml</td>
<td>6.3 mg/ml</td>
</tr>
<tr>
<td>$K_m$ for liver phosphorylase</td>
<td>80 mg/ml</td>
<td>85 mg/ml</td>
<td></td>
</tr>
<tr>
<td>$K_m$ for Ca$^{2+}$ at pH 6.8</td>
<td>$3 \times 10^{-2}$</td>
<td>$3 \times 10^{-2}$</td>
<td>$5 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

* The data are taken from Ref. 2.
+ The data are taken from Ref. 4.
' Taken from Ref. 42.

When the equation described by Martin and Ames (37) was applied for estimation of the molecular weight, the S value of 22 corresponded to an approximate value of 690,000. The exact molecular weight will be determined when a homogeneous preparation becomes available.

Further evidence for the identity of enzyme as well as for the validity of enzyme assay was provided by the fact that the radioactive phosphate incorporated into an acrid-precipitable material was indeed associated with the phosphorylase subunit as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. In additional experiments, muscle phosphorylase b was labeled with $[\gamma^{32}P]ATP$, using liver and muscle phosphorylase kinase separately, and digested with trypsin under the conditions described by Fischer et al. (38). When the trypic digests were subjected to electrophoresis followed by paper chromatography and then autoradiography, identical patterns of radioactive phosphopeptides were visualized for muscle and liver phosphorylase kinases. These results indicate that the liver phosphorylase kinase is indeed able to phosphorylate and activate muscle as well as liver inactive phosphorylases.

Physical Properties—The molecular weight of Kinase I was estimated to be about $1.3 \times 10^6$ by gel filtration on a Sephadex G-200 column. The effect of pH on liver phosphorylase kinase is shown in Fig. 2A. This profile differed clearly from that of either non-activated form (Fig. 2B) or activated form (data not shown, but see Ref. 43) of muscle phosphorylase kinase. Similar profiles were observed for Kinases I and II. The $K_m$ values of liver and muscle phosphorylase kinases for ATP were $3.0 \times 10^{-2}$ M and $4.2 \times 10^{-2}$ M, and those for muscle phosphorylase b were $300 \mu$g/ml and 6.3 mg/ml, respectively, as calculated by Lineweaver-Burk plots. Those kinetic values were obtained by the assay procedure based upon the incorporation of the radioactive terminal phosphate of ATP into an acid-precipitate material. The $K_m$ value for ATP could well depend on the level of phosphorylase used and, therefore, the values mentioned above were each obtained with saturating amounts of muscle phosphorylase b as phosphate acceptor. Table II summarizes some of the physical and kinetic properties of liver and muscle phosphorylase kinases.

Salt Effect and Stability—Liver phosphorylase kinase was inhibited by NaCl; about 60% of activity was lost in the presence of 0.1 M NaCl. KCl (0.1 to 1.0 M) showed no effect. Ammonium sulfate showed biphasic effects on liver phosphorylase kinase; at lower concentrations (0.05 to 0.10 M) the enzyme was stimulated about 1.5-fold, whereas at higher concentrations (more than 0.2 M) the salt was inhibitory. The purified preparation of enzyme was unstable, and approximately 70% of the activity was lost for 5 h at 0-4°C in 5 to 20% glycerol. The enzyme was inactivated more rapidly if the pH was 5 to 20% sucrose at 0-4°C. Also, the enzyme was extremely sensitive to temperature; an approximate half-life was 3 min at 40°C, and less than 1 min at 45°C in 10% ethylene glycol at pH 8.0. Nevertheless, the enzyme maintained its activity under the standard assay conditions at 30°C for at least 30 min. The enzyme could be stored for at least 1 week at 30°C for at least 30 min. The enzyme could be stored for at least 1 week at 0°C in 10% ethylene glycol, and for at least 1 month at -25°C in 50% ethylene glycol without appreciable loss of enzymatic activity. Similar results were obtained for both Kinases I and II.

Fig. 2. Effect of pH on Kinase I and skeletal muscle phosphorylase kinase. Kinase I and muscle phosphorylase kinase (10 units each) were assayed by measuring the incorporation of radioactive phosphate into muscle phosphorylase b under the conditions described above. The results obtained for both Kinases I and II were very similar in their kinetic properties (Table II), and available evidence indicated that Kinase II was produced from Kinase I by proteolysis during the isolation procedures as described below. The effect of pH on liver phosphorylase kinase is shown in Fig. 2A. This profile differed clearly from that of either non-activated form (Fig. 2B) or activated form (data not shown, but see Ref. 43) of muscle phosphorylase kinase. Similar profiles were observed for Kinases I and II. The $K_m$ values of liver and muscle phosphorylase kinases for ATP were $3.0 \times 10^{-2}$ M and $4.2 \times 10^{-2}$ M, and those for muscle phosphorylase b were $300 \mu$g/ml and 6.3 mg/ml, respectively, as calculated by Lineweaver-Burk plots. Those kinetic values were obtained by the assay procedure based upon the incorporation of the radioactive terminal phosphate of ATP into an acid-precipitable material. The $K_m$ value for ATP could well depend on the level of phosphorylase used and, therefore, the values mentioned above were each obtained with saturating amounts of muscle phosphorylase b as phosphate acceptor. Table II summarizes some of the physical and kinetic properties of liver and muscle phosphorylase kinases.

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Effects of Ca\textsuperscript{2+} and Mg\textsuperscript{2+}.--In the presence of 0.05 mM EGTA, liver phosphorylase kinase was inhibited by about 60 to 70% of the original activity under the standard assay conditions except that CaCl\textsubscript{2} was omitted. The degree of inhibition was not enlarged by increasing the concentration of EGTA up to 10 mM but remained constant at both pH 8.5 and 6.8. This inhibition was fully restored by the addition of Ca\textsuperscript{2+} as shown in Fig. 3. Assuming the apparent binding constant of EGTA/Ca\textsuperscript{2+} as 5.0 × 10\textsuperscript{-7} M\textsuperscript{-1} (44) under the conditions given in the legend to Fig. 3, an apparent K\textsubscript{a} value for free Ca\textsuperscript{2+}, the concentration necessary for obtaining half-maximum activity, was calculated to be about 3 × 10\textsuperscript{-7} M at pH 6.8. This value was in the same order of magnitude as that obtained for the activated form of muscle phosphorylase kinase (5 × 10\textsuperscript{-7} M at pH 6.8 (42)). Under the same conditions muscle phosphorylase kinase was inhibited by EGTA almost completely as described by Ozawa (45) and Brostrom et al. (42) (data not shown). The reason for the incomplete inhibition by EGTA for liver enzyme is not known. Prolonged exposure of liver phosphorylase kinase to EGTA resulted in the irreversible loss of enzymatic activity, while muscle phosphorylase kinase was stable under the same conditions. Similar results were again obtained for both Kinases I and II.

In addition to Ca\textsuperscript{2+}, Mg\textsuperscript{2+} was required absolutely for both Kinases I and II, and the concentration giving maximum activity was 2 mM under the standard conditions. Recently, Chrisman (46) described briefly that liver phosphorylase kinase was strongly inhibited when the Mg\textsuperscript{2+}/ATP ratio exceeded 1. Nevertheless, this property did not hold for Kinases I and II, and the enzymatic activity was not inhibited by Mg\textsuperscript{2+} over a range of Mg\textsuperscript{2+} concentrations from 0.1 mM up to 10 mM with a fixed amount (0.1 mM) of ATP. Also, the degree of inhibition by EGTA described above was not affected by the concentration of Mg\textsuperscript{2+} used.

Proteolytic Conversion of Kinase I to Kinase II.—Liver crude extracts prepared as freshly as possible contained mainly Kinase I and a small amount of Kinase II. When the crude extracts were stored overnight at 0–4°C, Kinase I mostly disappeared with the concomitant appearance of Kinase II. Gel filtration of Kinase I on a Sepharose CL-6B or Sephadex G-200 column in the presence of either 1 M KCl or 0.3 M ammonium sulfate did not produce a smaller form. This apparent conversion of Kinase I to Kinase II was blocked by the addition of either leupeptin (4 μg/ml), chymostatin (10 μg/ml), or antipain (10 μg/ml) which are known as protease inhibitors (47). In fact, it was suggested that Kinase II was a proteolytic artifact which was produced from Kinase I during the isolation procedures.

The purified preparation of Kinase I was incubated with the lysosomal extract which was prepared as described under “Experimental Procedures” and then subjected to glycogen density gradient analysis. As shown in Fig. 4, Kinase I was decreased with the concomitant appearance of a smaller form with increasing amounts of the lysosomal extract used. A series of analyses indicated that the smaller form thus produced was not distinguishable from Kinase II in physical and kinetic properties. This conversion of Kinase I to Kinase II was time-dependent\textsuperscript{9} and was blocked by leupeptin (4 μg/ml) (Fig. 4D). In addition, this conversion was also blocked by chymostatin (10 μg/ml), antipain (10 μg/ml), and monooiodoacetate (1 mM) but not by elastatinal (10 μg/ml) and pepstatin (10 μg/ml). These results seem to indicate that Kinase II is an artifact due to proteolysis of Kinase I and that this proteolysis is mediated by a lysosomal protease, presumably by cathepsin B (48).

Proteolytic Activation of Kinase I.—It has been well established that either Ca\textsuperscript{2+}-dependent protease or trypsin catalyzes the modification of muscle phosphorylase kinase, resulting in the marked activation of this enzyme (3, 4, 49). Like muscle phosphorylase kinase, Kinase I was cleaved by Ca\textsuperscript{2+}.

\textsuperscript{9} Chrisman (46) described briefly that the enzyme used for these studies was purified from rabbit liver 3,300-fold and on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate constituted 70 to 90% of Coomassie blue-stained material.

\textsuperscript{10} When Kinase I was preincubated for 30 min at 30°C under the conditions given in Fig. 4A, the enzyme was converted completely to Kinase II as revealed by glycogen density gradient analysis.
dependent protease as well as by trypsin to produce Kinase II as judged by glycerol density gradient analysis and also by gel filtration on a Sepharose CL-6B column (data not shown). However, during such proteolysis the enzymatic activity of Kinase I was not greatly enhanced, and only 60% activation was noted when incubated with a limited amount of trypsin as shown in Fig. 5. Trypsin inhibitor completely blocked this proteolytic activation. Ca\(^{2+}\)-dependent protease as well as lysosomal extract gave similar results, and the enhancement of enzymatic activity did not exceed again 60%. On the other hand, the activity of Kinase II was no more enhanced upon incubation with proteases.

**Effect of Cyclic AMP and Protein Kinase A**—It was very puzzling that the purified preparations of Kinases I and II were not activated by protein kinase A in the presence of ATP and cyclic AMP. The phosphorylase kinase was not affected by protein kinase inhibitor when tested at pH 6.8 and 8.5. A typical example of such experiments is given in Fig. 6. Under the same conditions muscle phosphorylase kinase was activated by protein kinase A as first described by Walsh et al. (50). There was no indication that liver phosphorylase kinase was inactivated simply due to its instability, nor that the enzyme was actually phosphorylated by protein kinase A during the preincubation.\(^{11}\)

In another set of experiments, Kinases I and II were preincubated with alkaline phosphatase under the conditions where phosphorylated muscle phosphorylase kinase was dephosphorylated and then tested for the possible activation and phosphorylation in the presence of ATP and protein kinase A. Nevertheless, no evidence was obtained indicating that Kinases I and II were already activated and phosphorylated forms of liver phosphorylase kinase.

**DISCUSSION**

Although it has been repeatedly described that liver phosphorylase kinase may be activated by glucagon (8-10), the activation of three consecutive enzymes, namely protein kinase A, phosphorylase kinase, and phosphorylase, are often dissociated upon the action of glucagon (17, 51). Another line of evidence has suggested that some mechanism other than cyclic AMP may also be involved in the hormonal control of glycogen breakdown. For instance, epinephrine (a effect) (10, 51-53), vasopressin (10, 54), and angiotensin II (55) have been proposed to cause a direct activation of phosphorylase in isolated liver cells without any detectable increase in the cyclic AMP level and without activation of protein kinase A. Neuronal stimulation of liver glycogen breakdown is reportedly independent of cyclic AMP accumulation (8, 56). In attempting to explore the mechanism of such activation of liver phosphorylase, several reports have appeared in the literature describing that liver phosphorylase kinase may be activated by micromolar concentrations of Ca\(^{2+}\), although the enzymatic activity in crude extracts is insufficiently, at most 60 to 70%, inactivated by EGTA (8, 10, 57). This is confirmed by Chrisman (46) and also in the present study using a purified preparation of the enzyme and, therefore, it is conceivable that the increase in intracellular Ca\(^{2+}\), if not sole, may play some role in controlling liver phosphorylase kinase as has been proposed by several investigators (10-12, 57).

The experimental results presented in this paper also serve to indicate that the purified preparation of liver phosphorylase kinase may not be phosphorylated nor activated by protein kinase A. In addition, no evidence has been thus far available indicating that the liver enzyme is regulated through phosphorylation and dephosphorylation reactions. However, it may be noted that the early observations made by Sutherland and his co-workers (20, 21, 58) have been repeatedly confirmed (6, 7, 59-61). Vandenheede et al. (14) have shown that the ATP-dependent activation of muscle phosphorylase b, which is exogenously supplemented to crude liver extracts, can be accelerated by the addition of either cyclic AMP or the catalytic subunit of protein kinase A. These observations are indeed confirmable (data not shown) and, therefore, it is possible that a factor or factors necessary for the phosphorylation of liver phosphorylase kinase may be lost or the enzyme may be desensitized for activation during the purification procedures. In fact, based on preliminary fractionation experiments, Vandenheede et al. (14) have proposed a possibility that some factor is present in the liver extracts which is necessary for the stimulation of activation of exogenously added muscle phosphorylase b. There is some evidence that, in general, the protein-protein interaction as well as the topographic relationship between enzyme and substrate may confer serious influences on the specificities and activities of various protein kinases (62, 63). Nevertheless, there is no direct evidence at this time which supports such phosphorylation of liver phosphorylase kinase eventually leading to the stimulation of glycogen breakdown. Also, no evidence is available suggesting the autocatalytic phosphorylation of the liver enzyme. Protein kinase M (27), which is produced from its own proenzyme by limited proteolysis with Ca\(^{2+}\)-dependent protease, is unable to phosphorylate and activate liver phos-
phosphorylase kinase as far as tested in vitro.

Ca2+-dependent protease and trypsin are shown in the present study to be able to convert liver phosphorylase kinase to a smaller form. This is comparable to the proteolytic activation of muscle phosphorylase kinase which has been described first by Krebs et al. (1). However, the enhancement of enzymatic activity by this proteolysis does not exceed 60%, and it is not plausible that the proteolytic activation of phosphorylase kinase may play a role in hormonal control of liver glycogen breakdown. In short, in contrast to our current understanding of muscle phosphorylase kinase, the mechanism of controlling liver phosphorylase kinase has not yet been fully clarified. Also, since it is difficult to assess the purity of enzyme at this stage of purification, the subunit structure and the mechanism of the proteolytic activation have remained unknown. Further studies are necessary to explore the possible covalent and noncovalent modifications of the liver enzyme in the regulation of glycogen breakdown in this tissue.

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