THE RELATIONSHIP OF EPINEPHRINE AND GLUCAGON TO LIVER PHOSPHORYLASE

I. LIVER PHOSPHORYLASE; PREPARATION AND PROPERTIES*

BY EARL W. SUTHERLAND AND WALTER D. WOSILAIT

(From the Department of Pharmacology, School of Medicine, Western Reserve University, Cleveland, Ohio)

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The concentration of phosphorylase in liver slices changes rapidly in response to certain experimental conditions (1–4). The concentration of phosphorylase in the cells represents a balance between inactivation of the enzyme and reactivation to the active form. Inactivation of liver phosphorylase is catalyzed by an enzyme formerly designated liver phosphorylase-inactivating enzyme. The synthetic aspect of the balance, i.e. the reactivation of the inactive liver phosphorylase, is influenced by epinephrine and glucagon so that resynthesis of the active form is promoted (5). The experiments reported in the following papers were designed to aid our understanding of the mechanisms involved in the regulation of the concentration of liver phosphorylase in intact cells. Liver phosphorylase and the enzyme from liver which inactivates it have been prepared in purified form. The enzymatic inactivation of liver phosphorylase has been studied (6) and also the process of reactivation in slices and extracts (5).

This report deals with the preparation and properties of liver phosphorylase. Two major problems required solution. The enzymatic inactivation of liver phosphorylase proceeds rapidly unless precautions are taken. Consequently, the early steps have been designed primarily to inhibit, denature, or remove the liver phosphorylase-inactivating enzyme. The second problem was created by the tendency of liver phosphorylase to accompany glycogen during fractionation. As purification proceeded, the ratio of carbohydrate to protein increased to values of 20 or 30 to 1, and, therefore, steps were taken to lower the glycogen concentration of the preparation. The procedures described here have resulted in greater yields of enzyme with a higher specific activity than reported previously, even though apparently homogeneous preparations of liver phosphorylase were obtained by an earlier procedure (7).

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Methods

Materials—Glycogen, G-1-P, and 5-AMP were purchased from the Nutritional Biochemicals Corporation.

G-1-P was purified by the following procedures. 2 liters of a 5 per cent solution were chilled, 3 gm. of Norit were added, and the pH was adjusted to 3.5 with glacial acetic acid. The solution was brought to a concentration of 50 per cent ethanol, filtered, and adjusted to pH 8.0 with KOH (as measured with a glass electrode), additional ethanol being added to maintain a 50 per cent concentration. After storage at 3° overnight, the crystals were collected and dissolved in 1.2 liters of glass-distilled water and filtered at room temperature. The filtrate was again brought to a concentration of 50 per cent ethanol. After storage at 0° overnight, the crystals were collected by decantation and centrifugation and washed successively with 95 per cent ethanol, absolute ethanol, absolute ethanol and absolute ether (1:1), and absolute ether. The yield of dry product was usually about 75 per cent of the starting material.

Glycogen was purified in the following manner before use. A 5 per cent solution was made and filtered and, if yellow, decolorized with Norit. The solution was then brought to a concentration of 50 per cent ethanol and allowed to stand overnight at 3°. The centrifuged precipitate was washed with absolute ethanol, absolute ethanol and absolute ether (1:1), absolute ether, and dried.

Tris, obtained from the G. Frederick Smith Chemical Company, was purified in the following manner. 400 gm. of Tris were dissolved in 2 liters of 85 per cent ethanol containing 0.6 gm. of trisodium Versene at 73°. The solution was filtered at 70° and chilled overnight. The supernatant solution was decanted and the crystals were collected on a Büchner funnel and washed in the same fashion as for G-1-P. The washed crystals were stored at room temperature in an evacuated desiccator (over CaCl₂) for 2 days before use. The yield was usually 80 per cent.

Merck reagent grade ammonium sulfate was recrystallized in the presence of Versene in the following fashion. 3 liters of distilled water containing 3 gm. of disodium Versene and 3 ml. of concentrated ammonium hydroxide were saturated with ammonium sulfate at about 70°. The solution was filtered rapidly through Whatman No. 50 filter paper and the filtrate was allowed to stand overnight at 3°. The crystals were removed and more ammonium sulfate was precipitated by the addition of ethanol to about 10 per cent concentration and by allowing the mixture to stand overnight. All crystalline material was washed successively on a Büchner funnel with ethanol of 30, 50, 95, and 100 per cent concentration. The washed crystals

1 The following abbreviations are used: G-1-P, glucose-1-phosphate; 5-AMP, adenosine-5-phosphate; Tris, tris(hydroxymethyl)aminomethane.
were stored in an evacuated desiccator for 24 hours before use. The yields were about 20 per cent.

The calcium phosphate gel was prepared essentially as described (8), distilled water being employed at all times. The final preparation contained about 21 mg. of calcium phosphate per ml.

Standard Assay—Phosphorylase activity was determined by measurement of the rate of liberation of inorganic phosphate from G-1-P in the presence of glycogen; i.e., phosphorylase activity was measured in the direction of polysaccharide synthesis. A stock solution of G-1-P (0.036 M adjusted to pH 6.1 with HCl) containing 4.03 mg. of glycogen per ml. and 0.1 M NaF was stable in the frozen state and when thawed constituted the basic reagent for assay. This reagent was usually prepared to contain $1.4 \times 10^{-3}$ M 5-AMP. Enzyme dilutions were made immediately before assay in cold 0.1 M NaF. The reaction was started by the addition of 0.2 ml. of enzyme to 2.8 ml. of the basic assay reagent at room temperature. Aliquots of the reaction mixture (0.5 ml.) were transferred to trichloroacetic acid at zero time and after incubation for 10 minutes at 37°; the remainder was used for an iodine starch test. The inorganic phosphate present in an equivalent of 0.1 ml. of reaction mixture was determined by the method of Fiske and Subbarow (9) as adapted to the Klett-Summerson photometer. In crude preparations small corrections can be made for phosphatase activity by determination of glucose production due to the addition of G-1-P.

1 unit of enzyme was defined as that amount which caused the liberation of 1.0 mg. of inorganic phosphorus in 10 minutes when the per cent conversion of glucose-1-phosphate was in the range of 12 to 22 per cent. Specific activity was expressed as units per mg. of protein. Protein was determined by a micromethod described previously (10), modified according to the procedure of Lowry et al. (11).

The purified enzyme may be assayed more accurately at pH 6.7 with a final glycogen concentration of 1.0 per cent and $1.6 \times 10^{-2}$ M G-1-P. Under these conditions at 30° first order kinetics are followed, as described by Cori et al. (12).

Results

Step I. Preparation of Filtrate—Medium sized or large dogs of various mixed breeds were obtained from the pound and were used approximately 20 hours after the last feeding. 2 ml. of a 1:1000 epinephrine solution were

The iodine starch test was valuable for rapid estimation of activity and as evidence that polysaccharide formation accompanied the formation of inorganic phosphate. This additional routine test could not be used when the reaction mixture contained amounts of glycogen sufficient to yield first order kinetics. Therefore, the above assay was adopted even though first order kinetics were not obtained.
given intraperitoneally 5 to 10 minutes before a lethal dose of sodium secobarbital was injected intravenously. The carotid vessels were severed and the thorax and abdomen were opened. A large cannula was inserted into the portal vein and the inferior vena cava was cut just below the heart. Several liters of cold sodium fluoride (0.2 M) were perfused through the liver; intermittent pressure on the inferior vena cava just above the diaphragm increased the filling of the liver and the effectiveness of the perfusion. At the start of the perfusion, 3 ml. of 1:1000 epinephrine were injected into the rubber perfusion tubing leading to the cannula. The chilled perfused liver was removed, and, after separation of the gallbladder, was immersed in chilled 0.1 M NaF. The liver was divided into 100 gm. portions, each of which was cut into small pieces and homogenized in a Waring blender for 2.5 minutes in 400 ml. of cold 0.1 M NaF containing 0.005 M KHPO4. These and all subsequent steps were carried out at 3° unless specified otherwise. The pooled homogenates were adjusted to pH 5.7 with 1.0 N acetic acid. An amount of Johns-Manville Hyflo Super-Cel equal to the weight of the liver was mixed with the homogenate and the mixture was filtered under 20 pounds pressure by using a No. 0 pad in a Hormann filter press.3 The cake was washed by the addition of cold 0.1 M NaF to the top of the cake, the volume of the wash fluid equaling 1.2 times the weight of the liver.

Step 2. Adsorption and Ammonium Sulfate—The turbid tan or pink filtrate was adjusted to pH 6.5 with 1.0 N KOH. Part of the phosphorylase-inactivating enzyme was removed by adsorption on calcium phosphate gel. The volume of gel was added to the filtrate and stirred occasionally for 15 minutes before centrifugation. (Further purification of the inactivating enzyme is described in Paper II (6).) The supernatant fluid from the gel was adjusted to pH 7.2 with 1 N KOH and the 0.0 to 0.66 ammonium sulfate fraction was precipitated by the addition of 46 gm. of solid ammonium sulfate per 100 ml. of solution. The pH was adjusted to 7.2 again with 1.0 N KOH and, after standing for 15 minutes, the fraction was collected by centrifugation for 20 minutes at 7000 X g. The supernatant fluid was discarded and a second precipitate was collected in the same tubes above the first precipitate. The precipitates were dissolved in 0.1 M NaF, 75 ml. for each 100 gm. of liver, and 10 ml. of 0.02 M 5-AMP per 100 gm. of liver were also added. 5-AMP protected liver phosphorylase against enzymatic inactivation and heat denaturation in the next step. The pH was adjusted to 7.1 with 1.0 N KOH and the preparation was ready for the heat denaturation step or, alternatively, it could be stored in the frozen state. The final volume of the solution was measured and the increase in volume above the

3 Obtained from F. R. Hormann and Company, Inc., 17 Stone Street, Newark 4, New Jersey.
volume of added solutions was taken as a measure of the amount of ammonium sulfate present as 0.66 saturated solution.

**Step 3. Heat Denaturation of Inactivating Enzyme**—The preparation was transferred to a 2 liter Erlenmeyer flask and washed in with 30 ml. of 0.1 M NaF, thus giving a final concentration of about 0.17 saturated ammonium sulfate. It was then heated with swirling in a bath at 67° until the temperature rose to 55°. The temperature was maintained at 55° for 5 minutes; the solution was then chilled in an ice water bath. The preparation was centrifuged for 20 minutes at 7000 × g, and the precipitate was discarded.

**Step 4. Collection of 0.41 to 0.8 Ammonium Sulfate Fraction**—Heated extracts from the livers of three dogs were pooled at this stage to minimize variations in fractionation. The concentration of ammonium sulfate was calculated and then a neutralized ammonium sulfate solution saturated at room temperature was added to bring the preparation to 0.41 saturation. The measured specific gravity at this calculated saturation was 1.13 at 3°. The precipitate obtained by centrifugation at 7000 × g for 20 minutes contained an amount of phosphorylase which varied with the glycogen content of the preparation. The 0.41 fractions obtained from extracts containing moderate or large amounts of glycogen were discarded, since most of the phosphorylase remained in the supernatant fluid. Removal of the 0.41 fraction did not cause any substantial increase in specific activity, but was carried out to eliminate protein which would otherwise precipitate with alcohol in subsequent steps. The 0.41 to 0.8 fraction was obtained by adding solid ammonium sulfate to the supernatant solution of the 0.41 precipitate (27.3 gm. per 100 ml. of supernatant solution), followed by centrifugation at 7000 × g for 20 minutes. Precipitates from two centrifugations were collected in the same tubes; the 0.8 supernatant fluid was discarded. It was found that the 0.41 to 0.8 fraction was stable in the cold overnight; hence centrifugation could be carried out later. The 0.41 to 0.8 precipitates were dissolved by the addition of 170 ml. of cold 0.1 M NaF per 1000 gm. of liver. This solution could be frozen at −20° and kept for some days without serious loss of activity.

**Step 5. Dialysis of 0.41 to 0.8 Ammonium Sulfate Fraction and Ethanol Fractionation**—The ammonium sulfate concentration of the preparation was lowered before alcohol fractionation to avoid denaturation of phosphorylase. Since traces of inactivating enzyme sometimes remained, the dialysis was carried out in the cold with fluoride for periods no longer than indicated. Dialysis with continued agitation was carried out in Visking casing (size 27/32) versus 10 volumes of NaF (0.1 M) containing 5 × 10⁻⁴ N KOH for 1 hour; then the dialysis was continued for another hour versus a fresh NaF-KOH solution. After dialysis, 0.1 volume of 5-AMP (0.02 M, pH 7.0)
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was added and the pH of the solution was adjusted to approximately 7.0 with KOH. The preparation was then ready for ethanol fractionation but could be kept frozen at -20°. To the preparation, chilled in an ice bath, cold (-20°) ethanol was added slowly with stirring until a 25 per cent final concentration was reached; then the preparation was chilled to 3° and centrifuged. The supernatant fluid was discarded, the precipitate was dissolved in 0.1 M NaF (two-thirds of the volume before adding ethanol), and 0.02 M 5-AMP was added (10 per cent of the volume of the added NaF). Alcohol was again added to a final concentration of 25 per cent; the precipitate was collected as previously and redissolved and reprecipitated as above.

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total units</th>
<th>Specific activity*</th>
<th>Per cent original activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1. Filtrate</td>
<td>25,200</td>
<td>0.31</td>
<td>100</td>
</tr>
<tr>
<td>&quot; 2. 0.0-0.66 ammonium sulfate</td>
<td>22,786</td>
<td>0.44</td>
<td>90</td>
</tr>
<tr>
<td>&quot; 3. Heated 0.0-0.66 supernatant fluid</td>
<td>12,491</td>
<td>0.46</td>
<td>50</td>
</tr>
<tr>
<td>&quot; 4. 0.41-0.80 ammonium sulfate</td>
<td>11,400</td>
<td>0.57</td>
<td>45</td>
</tr>
<tr>
<td>&quot; 5. 0.41-0.80 after dialysis</td>
<td>10,300</td>
<td>0.55</td>
<td>41</td>
</tr>
<tr>
<td>1st alcohol</td>
<td>9,600</td>
<td>4.15</td>
<td>38</td>
</tr>
<tr>
<td>2nd &quot;</td>
<td>8,900</td>
<td>5.7</td>
<td>35</td>
</tr>
<tr>
<td>3rd &quot;</td>
<td>7,650</td>
<td>6.2</td>
<td>30</td>
</tr>
</tbody>
</table>

* See the text for unit of measurement.

Livers from three dogs were fractionated separately through Step 3, then pooled for Steps 4 and 5. The specific activity of the homogenate was about one-third that of the filtrate. No corrections have been made for sampling nor for loss in precipitates. To obtain the third alcohol precipitate. Occasionally the alcohol precipitation was repeated one or more times until the supernatant solution was colorless. The third alcohol precipitate from 1000 gm. of liver was dissolved in 200 ml. of water and appeared milky because of the large amounts of glycogen present. If the precipitate was very bulky, 300 ml. of water were used. The third alcohol fraction was stable in the frozen state for several weeks or months. The results of a typical fractionation to this stage are summarized in Table I.

**Step 6. Adsorption and Elution**—This step was designed to free the preparation of most of the glycogen which accompanied the preceding fractions in large amounts. The third alcohol fraction was diluted with cold glass-distilled water so that it contained approximately 3 units per ml. Usually an 8-fold dilution was made and pilot experiments were carried out to de-
termine the amount of calcium phosphate gel necessary for nearly complete adsorption of the enzyme. (Usually 2 to 3 per cent of the volume of the diluted, third alcohol fraction was sufficient.) Calcium phosphate gel was added to the diluted, cold, third alcohol fraction and stirred occasionally for 15 minutes. The gel was collected by centrifugation and washed with cold 0.001 M Tris, pH 7.4, which had been recrystallized in the presence of Versene, the volume of the wash fluid equaling the discarded supernatant solution. The gel was again collected by centrifugation and eluted for 10 minutes at 25° with 0.01 M potassium citrate (pH 6.5), the volume of citrate equaling 12 per cent of the diluted third alcohol volume. The eluted gel was packed by centrifugation at room temperature for 10 minutes at 7000 × g and discarded.

Step 7. Fourth Alcohol Collection and Dialysis Versus Phosphate—The eluate, containing the phosphorylase, was chilled and to each 100 ml. were added 0.55 gm. of NaCl and 40 ml. of absolute ethanol (−20°). The mixture was chilled to 3° and centrifuged. The precipitate was dissolved or suspended in 0.1 M potassium phosphate (pH 7.2) with approximately 20 ml. per 1000 gm. of liver. The suspended precipitate was placed in washed Visking casing (size 27/32) and dialyzed versus 3.5 liters of 0.1 M potassium phosphate (pH 7.2) for 16 hours at 22°. During this dialysis the small amounts of glycogen remaining were partially digested and a heavy, flocculent precipitate was formed. The inactive flocculent precipitate was removed by centrifugation for 15 minutes at 7000 × g at room temperature and discarded.

Step 8. Second Adsorption and Elution—The dialyzed and centrifuged fourth alcohol fraction was diluted with an equal amount of water and chilled. Calcium phosphate (second gel) was added in amounts which would adsorb only a small per cent of the liver phosphorylase. The amount was determined by pilot experiments and was usually about 1.25 ml. of gel per 10 ml. of enzyme preparation. After 15 minutes of stirring, the gel was removed by centrifugation and discarded. The supernatant fluid still contained some carbohydrate as determined by the anthrone method (13), but on a protein basis possessed a high specific activity (Table II). The supernatant solution was diluted 10-fold with cold glass-distilled water; then the third addition of calcium phosphate gel was made in an amount sufficient to adsorb almost all the liver phosphorylase, 0.4 ml. per 10 ml. usually being sufficient. After 15 minutes of stirring, the gel was collected by centrifugation and washed with cold 0.001 M Tris (pH 7.4) with a volume equal to

4 Higher concentrations of citrate will make all the calcium phosphate gel soluble. The amounts of citrate used here, however, did not greatly influence the amount of insoluble calcium phosphate. The use of citrate for elution of calcium phosphate gel may be helpful when a protein is strongly adsorbed to the gel.
the discarded supernatant fluid. The enzyme was eluted from the gel by repeated additions of 10 ml. portions of cold 0.6 M ammonium sulfate (pH 7.0 to 7.2). Four such elutions were sometimes necessary. This adsorption-elution procedure did not increase the specific activity but did decrease the amount of carbohydrate considerably, as shown in Table II.

**Step 9. Final Ammonium Sulfate Precipitation**—The eluate of the third gel was added to 1.5 volumes of Versene-treated saturated ammonium sulfate solution which had been chilled previously. After 15 minutes at 3°C, the precipitate was collected by centrifugation and dissolved in about 2 ml. of water. This final fraction was dialyzed versus water containing 7 × 10⁻⁴ M KOH, then versus 3 × 10⁻⁴ M KOH, and finally versus neutralized distilled water. Such purified preparations were unstable to freezing, un-

| Table II  
Summary of Purification and Yield; Step 5 through Step 9 |
<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
<td>Total units</td>
<td>Specific activity</td>
<td>Per cent original activity</td>
<td>Carbohydrate : Protein</td>
</tr>
<tr>
<td>Step 5. 3rd alcohol</td>
<td>6000</td>
<td>8.8</td>
<td>100</td>
<td>20:1</td>
</tr>
<tr>
<td>“ 6. Eluate of gel</td>
<td>5200</td>
<td>14.3</td>
<td>87</td>
<td>1.8:1</td>
</tr>
<tr>
<td>“ 7. 4th alcohol</td>
<td>4100</td>
<td>15.5</td>
<td>68</td>
<td>1.3:1</td>
</tr>
<tr>
<td>After dialysis and centrifugation</td>
<td>4100</td>
<td>22.4</td>
<td>68</td>
<td>1:1.1</td>
</tr>
<tr>
<td>Step 8. 2nd gel supernatant fluid</td>
<td>3320</td>
<td>28.0</td>
<td>55</td>
<td>1:2:1</td>
</tr>
<tr>
<td>Eluate, 3rd gel</td>
<td>2930</td>
<td>24.6</td>
<td>48</td>
<td>1:8</td>
</tr>
<tr>
<td>Step 9. Final ammonium sulfate</td>
<td>2370</td>
<td>28.5</td>
<td>39.5</td>
<td>1:24</td>
</tr>
</tbody>
</table>

less protected by glycylglycine as described below. Further ammonium sulfate precipitation was possible and reduced the carbohydrate content to very low levels. At times the preparations of Step 8 or Step 9 were centrifuged for 40 minutes at 100,000 × g at about 3°C to remove traces of sedimentable material.

**Properties of Liver Phosphorylase**

Various preparations of purified liver phosphorylase possessed specific activities of 28 to 30. When activity was determined at 30°C under conditions for which first order kinetics were followed, these preparations were found to possess an activity of about 3800 units per mg. of protein, as defined by Cori et al. (12). Liver phosphorylase with a specific activity of 18 was reported homogeneous when examined by ultracentrifugal and electrophoretic techniques and had a molecular weight calculated to be 237,000 gm. (7). Physical studies of the more active recent preparations (specific
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activity of 28) have been limited to a determination of the sedimentation constants. The value of \( s_{20} \) was found to be 8.4, which agrees with the previously determined sedimentation constants.\(^5\)

Liver phosphorylase was very soluble in water or in the presence of various dilute salts. When freed of carbohydrate, the enzyme slowly lost activity at 3°, or at room temperature, and rapidly lost activity on freezing at -20°. The enzyme was relatively stable when frozen at -20° in glycylglycine buffer (0.2 M, pH 7.4), although repeated freezing and thawing in this buffer resulted in some loss of activity. The enzymatic activity was increased 15 to 40 per cent by the addition of 5-AMP or inosine-5-phosphate to the reaction mixture in final concentration of 10^{-3} M. Cysteine and glutathione did not stimulate purified liver phosphorylase, although the inhibition of liver phosphorylase by \( p \)-chloromercuribenzoate (10^{-4} to 10^{-5} M) was reversed by these compounds. The enzyme was inhibited by heavy metals; e.g., strong inhibition was caused by addition of 10^{-6} M \( \text{Hg}^{++} \), 10^{-5} M \( \text{Ag}^{+} \), or 10^{-3} M \( \text{Cu}^{++} \) to the reaction mixture. Since the enzyme is sensitive to heavy metals, it seems likely that some preparations may be stimulated by metal-binding agents, but to date little or no stimulation by metal-binding agents has been observed.

The pH optimum for activity was near 6.4; the activity fell rapidly below pH 6.0 and above pH 7.0. Glycogen was a necessary component in the reaction mixture, and the purified enzyme catalyzed the formation of a polysaccharide which gave a blue color with iodine. During early fractionation liver phosphorylase accompanied the glycogen; moreover, when freed of glycogen, it still tended to associate with added glycogen. This could be shown by comparing sedimentation at 100,000 \( \times g \) without and with added glycogen; phosphorylase remained in the supernatant fluid after 40 minutes of centrifugation without glycogen, but in the presence of added glycogen was found in the sediment.

SUMMARY

The preparation of purified liver phosphorylase has been described. This procedure resulted in greater yield of the enzyme with higher specific activity than reported previously.

The authors wish to thank Miss Arleen Maxwell and Mr. James Davis for technical assistance in these studies.

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\(^5\)Measurements were performed at the Cleveland Clinic. M. D. Schoenberg, personal communication.
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