STUDIES ON THE PURIFICATION AND PROPERTIES OF D-GLYCERIC ACID KINASE OF LIVER*

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(Received for publication, September 17, 1956)

Previous studies on the biosynthesis of serine (1, 2) revealed that D-glyceric acid served as a precursor for this amino acid in the presence of ATP. This suggested the occurrence of a kinase that catalyzes the formation of PGA from glyceric acid. This paper reports the results of studies on the purification and properties of the glyceric acid kinase from horse liver.

EXPERIMENTAL

Materials—D,L-Glyceric acid-3-C¹⁴ was prepared by the Isotopes Specialties Company, Inc., Burbank, California. Samples of pure D-glyceric acid and D,L-glyceric acid were kindly furnished by Dr. C. E. Ballou of the Department of Biochemistry of this University. ATP and PGA were purchased from the Nutritional Biochemicals Corporation and ADP from the Sigma Chemical Company.

Analytical Methods—Glyceric acid was determined by oxidation with periodate and colorimetric determination of the formaldehyde formed by employing the same conditions as those used for serine estimation in the method of Frisell et al. (3). Protein in the enzyme incubations was determined with the combined copper sulfate-phenol reagent of Sutherland et al. (4), inorganic phosphate by the method of Lowry and Lopez (5), and organic phosphate by the method of Fiske and Subbarow (6).

Chromatographic procedures employed are described in connection with the experiments to which they are pertinent.

Enzyme Assay—The activity of glyceric acid kinase was assayed by the rate of disappearance of glyceric acid. The incubation was carried out for 30 minutes at 37° in 12 ml. conical centrifuge tubes containing 3 μmoles of

* Aided by research grants from the National Cancer Institute (C-327 and C-2327), the American Cancer Society (Committee on Growth), and the Cancer Research Funds of the University of California.

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1 The following abbreviations are used: ATP, adenosine triphosphate; PGA, 3-phosphoglyceric acid; ADP, adenosine diphosphate; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; PCMB, p-chloromercuribenzoate.
glyceric acid, 3 \( \mu \)moles of ATP, 2 \( \mu \)moles of MgCl\(_2\), 150 \( \mu \)moles of phosphate buffer, pH 7.4, and approximately 2.5 units of enzyme in a 3 ml. total volume. The reaction was stopped by addition of TCA to a final concentration of 10 per cent. The residual glyceric acid was determined on aliquots of the filtrate.

A kinase unit is defined as the weight of enzyme which catalyzes the decomposition of 1 \( \mu \)mole of D-glyceric acid under the conditions described above.

**RESULTS AND DISCUSSION**

*Species Distribution of Kinase*—A comparison made on the livers of a number of mammalian species (Table I) showed horse liver to be the richest source of the kinase, with rat liver the next richest. Further studies were carried out with horse liver.

***Preparation of Enzyme***—To purify the kinase, an acetone powder of liver was prepared and an aqueous extract of this was fractionated by further treatment with ammonium sulfate and calcium phosphate gel. The details of the purification are as follows: Fresh horse liver was minced, homogenized in a Waring blender with 6 volumes of cold acetone, and quickly filtered. The filter cake was again homogenized with 4 volumes of acetone and filtered. The powder was dried *in vacuo* over CaCl\(_2\) in a desiccator. It retained its enzyme activity for months.

50 gm. of the acetone powder were homogenized in a Waring blender with 7 volumes of ice-cold phosphate buffer (0.1 M, pH 8.0) and stirred for 20 minutes with a mechanical stirrer. The mixture was centrifuged at \( 2.5 \times 10^8 \times g \) for 20 minutes in a Servall centrifuge. Further centrifugation re-

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**Table I**

<table>
<thead>
<tr>
<th>Liver</th>
<th>( \Delta \text{D-glyceric acid} ) ( \mu )mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>0.12</td>
</tr>
<tr>
<td>Rat</td>
<td>0.09</td>
</tr>
<tr>
<td>Beef</td>
<td>0.06</td>
</tr>
<tr>
<td>Pork</td>
<td>0.00</td>
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</tbody>
</table>

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quired in purifying the enzyme was carried out under the same conditions.
The precipitate was washed with 3 volumes of the buffer and recentrifuged.
To the combined supernatant fluids ("Crude extract," Table II), saturated
(NH$_4$)$_2$SO$_4$ adjusted to pH 7.6 was added with stirring. The fraction precip-
itating between 35 and 50 per cent saturation of (NH$_4$)$_2$SO$_4$ was sepa-
rated by centrifugation, dissolved in a small volume of buffer, and dialyzed
against 0.05 M phosphate buffer, pH 8.0, for about 6 hours. The dialysate
("Ammonium sulfate I," Table II) was treated with calcium phosphate
gel prepared by the method of Keilin and Hartree (7) by adding 0.3 mg. of
dry gel per mg. of protein, at pH 7.0, and letting the mixture stand for 20
minutes. The mixture was then centrifuged, and the supernatant fluid
from the calcium gel treatment ("Ca gel treatment," Table II) was again
fractionated with saturated (NH$_4$)$_2$SO$_4$, as before, and dialyzed against the

<table>
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<th>TABLE II</th>
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<tr>
<td><strong>Increase in Activity during Fractionation of Enzyme</strong></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td><strong>Fraction</strong></td>
</tr>
<tr>
<td>Crude extract...</td>
</tr>
<tr>
<td>Ammonium sulfate I</td>
</tr>
<tr>
<td>Ca gel treatment</td>
</tr>
<tr>
<td>Ammonium sulfate II</td>
</tr>
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</table>

0.05 M phosphate buffer. This yielded the preparation termed "Ammoni-

Since the enzyme was found to be quite labile toward acid and elevation
of temperature,² the preparation was carried out in a cold room at 3° and
in a slightly alkaline medium. The purification obtained in a typical prep-

Reaction Components—In addition to d-glyceric acid and the kinase,
ATP and Mg$^{++}$ were required for the reaction to proceed. The decrease
of d-glyceric acid with time when all the components were present is shown
in Fig. 1. In the absence of ATP or Mg$^{++}$, there was no decrease in the
d-glyceric acid content, as shown by the lower curve of Fig. 1.

Effect of pH on Activity—The pH-activity curve for the d-glyceric acid
kinase is shown in Fig. 2. Acetate buffer in the same concentration as the
phosphate buffer was employed over the pH range 4.5 to 5.6, and phos-
phate buffer between pH 5.6 and 8.3. The maximal activity was found to
be at pH 7.4 to 7.7.

² The purified enzyme preparation lost half its activity either after standing for
2 hours at pH 6.3 in the cold or by heating for 5 minutes at 45°.
Effect of ATP—The velocity of the reaction increased nearly linearly with increasing concentration of ATP up to about $1 \times 10^{-3} \text{M}$ (Fig. 3). From a plot of the data according to the method of Lineweaver and Burk.

![Figure 1. Time-course of the reaction.](image1)

The reaction mixture contained 3 $\mu$moles of d-glyceric acid, 3 $\mu$moles of ATP, 2 $\mu$moles of Mg$^{++}$, 150 $\mu$moles of phosphate buffer, pH 7.4, and 2.5 units of enzyme in 3 ml. of volume. On omission of either ATP or Mg$^{++}$, water was used to adjust the total volume. d-GA represents d-glyceric acid.

![Figure 2. pH-activity curve.](image2)

The reaction was carried out as that described in Fig. 1, except for the addition of 150 $\mu$moles of acetate buffer instead of phosphate buffer in the reaction below pH 5.6.
(8), a value for $K_s$ of $9.7 \times 10^{-3}$ M was obtained for ATP. The plot shows that the points of the reciprocal values of the ATP concentrations, except for the highest concentrations, fit the straight line very well.

![Graph showing the effect of ATP concentration on reaction velocity.](image1)

**Fig. 3.** Effect of ATP concentration on reaction velocity. The reaction was carried out as that described in Fig. 1 except for the addition of different concentrations of ATP. $\Delta$, points for $v$ versus $s$ curve; $\bigcirc$, points for $1/v$ versus $1/s$ line.

ADP was found to be about 20 per cent as active as ATP in catalyzing the reaction. This activity exhibited by ADP may be due to contamination of the enzyme preparation with adenyl kinase (myokinase).

![Graph showing the effect of d-glyceric acid concentration on reaction velocity.](image2)

**Fig. 4.** Effect of d-glyceric acid concentration on reaction velocity. $\Delta$, points for $v$ versus $s$ curve; $\bigcirc$, points for $1/v$ versus $1/s$ line.
Enzyme-Substrate Affinity Constants—These were determined from a Lineweaver-Burk plot of $1/v$ against $1/s$. Fig. 4 shows plots of both $v$ and $s$ and $1/v$ against $1/s$. The value of $K_s$ for $\alpha$-glyceric acid determined from the latter plot was $2.4 \times 10^{-3}$ M. The affinity of this substrate for the enzyme, thus, is in the normal range.

Of the two enantiomorphs of glyceric acid, only the $\alpha$ form, seemingly, is the substrate for the enzyme (Fig. 5). Comparison of the rates of decomposition of $\alpha\alpha$-glyceric acid with that of $\alpha$-glyceric acid shows that the former, at twice the concentration levels of the latter, falls on the same curve, except that at the highest concentration levels L-glyceric acid appears to cause some degree of inhibition of the reaction. Recently Black and Wright reported that a glyceric acid kinase prepared from yeast also was specific for $\alpha$-glyceric acid (9).

Effect of Metal Ions—Of a series of divalent metal ions tested (Table III), only $\text{Mg}^{++}$ was found to be stimulatory. The other ions were without effect. The curve of enzyme activity as a function of the $\text{Mg}^{++}$ concentration is given in Fig. 6. From a Lineweaver-Burk plot of the data (Fig. 6), the $K_s$ value for the binding of $\text{Mg}^{++}$ by the kinase was determined to be $1.64 \times 10^{-4}$ M.

Effect of Inhibitors—A number of substances that might be expected to inhibit the enzyme reaction were tested. These included EDTA, PCMB,
and arsenite. Of these, only EDTA had an inhibitory effect. This action was exerted against Mg++ and could be counteracted by increasing the concentration of Mg++. At levels of 2 μmoles of Mg++ and EDTA per incubation vessel, the extent of the inhibition was 55 per cent. Increasing the Mg++ to 20 μmoles restored complete activity. PCMB in an amount up to 0.5 μmole and arsenite up to 120 μmoles per vessel resulted in no significant reduction in the reaction rate.

**Stoichiometry of Reaction**—In an experiment designed to test the stoichiometry of the reaction, a comparison was made of the quantity of n-glyceric acid decomposed with the decrease in the heat-labile phosphate (Ta-
The decrease of 2.2 μmoles of d-glyceric acid to 1.8 μmoles of heat-labile phosphate agreed quite well with the expected reaction. The deviation from the anticipated absolute values can be explained by the concurrent breakdown of some of the added ATP to ADP and then to adenosine monophosphate. Thus, the zero time value, although 3 μmoles of ATP had been added to the solution, was determined to be 5.2 μmoles of heat-labile phosphate.

Isolation and Identification of Reaction Product—Evidence that PGA is produced by the enzymatic reaction was obtained by the following experiment: 2.5 μmoles of dl-glyceric acid-3-C\(^14\) (2 \(\times\) 10\(^6\) c.p.m.) were incubated for 90 minutes at 37° in a volume of 5 ml. containing 25 units of enzyme, 30 μmoles of ATP, 10 μmoles of Mg\(^{++}\), and 300 μmoles of phosphate buffer, pH 7.4. After deproteinization with TCA, the solution was neutralized and 150 μmoles of cold PGA were added as carrier. The PGA was then isolated by the method of DuBois and Potter (10). The barium salt of PGA was recrystallized several times, dissolved in a small amount of 0.1 N HCl, and the barium precipitated as BaSO\(_4\). The Ba-free PGA was then neutralized and chromatographed by the method of Weissbach et al. (11) through a Dowex 1 Cl\(-\) resin column by means of gradient elution, with 0.1 N HCl in the reservoir and water in the mixing chamber. Aliquots of each 5 ml. of effluent were taken for measurement of radioactivity, after drying the samples on planchets, and for determination of organic phosphate. The peaks of radioactivity and organic phosphate coincide well, as is shown in Fig. 7. The major fraction appeared in the eluate at the passage of approximately 260 ml. of fluid. This fraction was collected and chromatographed on Whatman No. 1 paper with a phenol-water solvent (8:2). The acid spot was identified by spraying with bromophenol blue indicator, prepared as described by Kennedy and Barker (12). The location of the acid spot and the radioactivity, also, coincided. Besides this major peak, a smaller peak, which also contained organic phosphate

<table>
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<th>Incubation time</th>
<th>n-Glyceric acid</th>
<th>Acid-labile phosphate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td>μmoles</td>
<td>μmoles</td>
</tr>
<tr>
<td>0</td>
<td>3.2</td>
<td>5.2</td>
</tr>
<tr>
<td>60</td>
<td>1.0</td>
<td>3.4</td>
</tr>
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Substrates consumed: 2.2 1.8

* Inorganic phosphate released by boiling the reaction mixture for 7 minutes in 1 N HCl.
and was radioactive, appeared in a fraction eluted fairly early from the column. This peak was also given by the authentic PGA treated in the same manner as the preparation from the incubation. It appears from this that this peak represents an artifact formed from PGA during the isolation process.

We are indeed greatly indebted to Dr. Clinton E. Ballou of the Department of Biochemistry for generously supplying the d-glyceric acid and dl-glyceric acid employed in this investigation.

SUMMARY

1. A d-glyceric acid kinase has been found in liver. Livers of the horse and the rat showed the highest activity among those tested. A considerable purification of the enzyme from horse liver has been achieved.

2. The purified enzyme has been characterized with respect to its requirement of adenosine triphosphate and Mg\(^{++}\), its pH optimum, its substrate specificity and substrate affinities, the effect of certain metallic ions, and the effect of certain enzyme inhibitors.

BIBLIOGRAPHY

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