SYNTHESIS OF PHOSPHATIDES IN ISOLATED MITOCHONDRIA

III. THE ENZYMATIC PHOSPHORYLATION OF GLYCEROL*

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Previous studies (1) have indicated that α-GP is an important intermediate in the incorporation of radioactive inorganic phosphate into a phosphatide fraction of rat liver. The dependence of the incorporation on oxidative phosphorylation and the marked stimulation by the addition of free glycerol suggested that the first stages in this reaction sequence were the conversion of radioactive orthophosphate to ATP by oxidative phosphorylation and the subsequent phosphorylation of glycerol by the radioactive ATP so generated. Evidence in support of this reaction sequence will be presented in this paper, in which will be described the purification and properties of an enzyme from rat liver which catalyzes the following reaction.

\[
\text{ATP + glycerol} \rightarrow \text{L-α-GP + ADP}
\]

Kornberg and Pricer (2) have independently described the formation of phosphatidic acids from L-α-GP and have shown that fatty acid thio esters of coenzyme A are involved in the esterification reactions.

The name glycerokinase seems to be appropriate and convenient for this enzyme, although the purified enzyme also phosphorylates dihydroxyacetone and glyceraldehyde.

In 1937, Kalckar (3) presented evidence for the presence of glycerokinase in kidney minces. He found that the addition of glycerol to kidney preparations during the course of oxidative phosphorylation stimulated the conversion of inorganic phosphate to an acid-resistant ester. Later he was able to isolate L-α-GP from his system (4). Indirect evidence for the existence of glycerokinase has also been presented by Barker and Lipmann

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1 The following abbreviations will be used in this paper: ATP = adenosinetriphosphate; ADP = adenosinediphosphate; α-GP = α-glycerophosphate; BAL = 2,3-dimercaptopropanol; ITP = inosinetriphosphate; UTP = uridine triphosphate; DPN = diphosphopyridine nucleotide; DPNH = reduced diphosphopyridine nucleotide; Tris = tris(hydroxymethyl)aminomethane.
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However, it appears that the purification and properties of this enzyme have not been previously described.

Wood and his coworkers (8) and Swick and Nakao (9) have shown by isotope tracer studies that glycerol is metabolized by animal tissues in an "asymmetrical" fashion. The possible importance of glycerokinase in regard to this "asymmetrical" metabolism will be described in the following paper (10).

Materials and Methods

A highly purified sample of dl-α-GP was the gift of Dr. Jean Sicé. Twice recrystallized bovine serum albumin was purchased from Armour and Company. BAL was the product of the Mann Research Laboratories. The ATP and UTP used in these studies were the products of the Pabst Laboratories. DPN (of about 70 per cent purity), ITP, and p-chloromercuribenzenesulfonic acid (of about 80 per cent purity) were purchased from the Sigma Chemical Company.

D-Glyceraldehyde-3-phosphate dehydrogenase was a crystalline product kindly supplied by Dr. Frank Loewus. Aldolase, prepared by the method of Taylor, Green, and Cori (11), was the gift of Mr. V. Hospelhorn. A partially purified, highly active preparation of glycerophosphate dehydrogenase was obtained by the fractionation of rabbit muscle extract with ammonium sulfate by a procedure based on the work of Baranowski (12).

The concentration of DPNH was measured spectrophotometrically, with an extinction coefficient of $6.22 \times 10^6$ sq. cm. per mole at 340 mμ (13). Protein concentration was measured either by the spectrophotometric method of Warburg and Christian (14) or on a dry weight salt-free basis. Inorganic phosphate was determined by the method of Gomori (15). Acid-labile phosphate refers to the phosphate hydrolyzed by 1 N sulfuric acid at 100° for 15 minutes. The concentration of stock solutions of ATP was determined spectrophotometrically, with an extinction coefficient of $14.2 \times 10^6$ sq. cm. per mole in 0.01 N HCl (16) and by the content of acid-labile phosphate. The concentration of UTP solutions was determined spectrophotometrically, with an extinction coefficient of $10.04 \times 10^6$ sq. cm. per mole at 262 mμ (17).

EXPERIMENTAL

Enzymatic Microestimation of L-α-GP—For the purpose of assaying glycerokinase, a method for the estimation of small amounts of L-α-GP was developed, in which the reaction catalyzed by the enzyme glycerophosphate dehydrogenase is utilized.

$$L-\alpha-GP + DPN \rightleftharpoons DPNH + \text{dihydroxyacetone phosphate} + H^+$$
Since the equilibrium for this reaction at neutral pH is much in favor of production of L-α-GP and DPN (12), it was necessary to add a trapping agent for the dihydroxyacetone phosphate formed and to run the reaction at an alkaline pH. Hydrazine functioned both as a suitable trapping agent and as an effective buffer. The system for the microdetermination of L-α-GP consisted of 1000 μmoles of hydrazine-hydrazine hydrochloride buffer of pH 9.4, approximately 1.5 μmoles of DPN, and 0.03 ml. of α-GP dehydrogenase. The total volume of the system was 2.0 ml. After a 45 minute period of incubation at 37°, the DPNH formed was measured in a spectrophotometer. Under the conditions described, there was a satisfactorily linear relationship between amounts of DL-α-GP added and DPNH produced. 2 moles of DL-α-GP were required to reduce 1 mole of DPN, indicating that D-α-GP is not oxidized by this enzyme, which has long been known to be specific for L-α-GP (18). Yields of DPNH in excess of 90 per cent of the theoretical were consistently obtained, and samples with known amounts of DL-α-GP were included with every batch of assays as controls.

Assay of Glycerokinase—In adapting the microdetermination of L-α-GP to the assay for glycerokinase, it was found convenient to carry out the assay in two stages. The first stage consisted of an incubation of glycerokinase with the following reagents in a total volume of 1.0 ml.: 25 μmoles of KF, 1 μmole of MgCl₂, 25 μmoles of potassium phosphate buffer, pH 7.4, 25 μmoles of glycerol, 20 μmoles of freshly prepared cysteine neutralized with NaOH, and 6 μmoles of ATP neutralized with either NaOH or KHCO₃. At the end of the incubation period (30 minutes at 37°, unless otherwise specified), 1.0 ml. of 0.2 N metaphosphoric acid was added to the incubation mixture. A 1 ml. aliquot of the deproteinized solution was neutralized with NaOH and added to the second stage, which consisted of the microestimation of L-α-GP as described in the previous section. Earlier attempts to use trichloroacetic acid for deproteinization proved unsuccessful because of the adsorption of L-α-GP on the protein precipitate. As the purification of the enzyme proceeded, it was found necessary to add serum albumin in order to obtain a protein precipitate at this stage.

A unit of glycerokinase activity has been defined as that amount of enzyme which catalyzes the formation of 0.1 μmole of L-α-GP per hour under the conditions described.

Under the conditions of this assay, there is a linear relationship between the amount of glycerokinase added and the amounts of L-α-GP formed over the range 0 to 10 units of enzyme. When a constant amount of enzyme (2 to 3 units) is present, the phosphorylation of glycerol proceeds at a linear rate for at least an hour.

Stability of Enzymes—Glycerokinase is stabilized by Versene and by very
low concentrations of glycerol (10⁻⁶ M). It is most stable at pH 4.6 to 5.4. The enzyme was routinely stored in a medium containing 0.1 per cent glycerol, 0.001 M Versene, and 0.01 M acetate buffer of pH 5.1. Under these conditions the enzyme could be incubated at 60° for 80 minutes without loss of activity. When it was heated to 70° for 10 minutes, the enzyme lost about 25 per cent of its activity. Heating the enzyme to 80° or higher for 10 minutes resulted in complete loss of activity. Glycerokinase was unstable when heated in the presence of magnesium ion or when shaken aerobically in the presence of cysteine. The enzyme was stable to freezing and thawing and could be stored in a deep freeze for several weeks with moderate loss of activity. The enzyme was less stable when stored at ice box temperatures. Glycerokinase could be lyophilized and stored as the lyophilized powder for several weeks without loss of activity. As the purification proceeded, the enzyme showed greater lability when stored in the frozen condition or in the ice box. Although active acetone powder could be made, all attempts to fractionate with organic solvents resulted in loss of activity.

Fractionation of Enzyme—Forty normal adult rats were killed by decapitation. The livers were removed and placed in cracked ice. All operations were carried out at 0–5°, unless otherwise specified. A 20 per cent homogenate of these livers was made in 1 per cent KCl, 0.1 per cent glycerol, and 0.001 M Versene. The homogenization was carried out in a Waring blender. 0.1 volume of 1.0 M acetate buffer of pH 5.1 was added dropwise with mechanical stirring to the homogenate. The precipitate was removed in a Sharples continuous centrifuge. 425 gm. of solid ammonium sulfate were added with stirring to 1500 ml. of the supernatant solution. The precipitate was centrifuged, taken up in about 150 ml. of 0.1 per cent glycerol, 0.01 M acetate buffer of pH 5.1, and 0.001 M Versene (dialyzing solution), and dialyzed overnight. The precipitate which formed during dialysis was centrifuged in a Servall centrifuge and washed with dialyzing solution three times. The combined extracts were then incubated at 60° for 80 minutes. The precipitate formed during this heat treatment was filtered and washed with dialyzing solution. To 420 ml. of combined washings and filtrate were added, in the manner described above, 104 gm. of solid ammonium sulfate. The precipitate was removed in a Servall centrifuge (Fraction A). To 450 ml. of the supernatant solution were added 16.0 gm. of solid ammonium sulfate. This precipitate was centrifuged (Fraction B). To 445 ml. of the supernatant solution were added 15.8 gm. of solid ammonium sulfate. After this precipitate (Fraction C) was removed, Fractions A, B, and C were taken up separately in a minimal volume of dialyzing solution and dialyzed overnight. These three fractions were then lyophilized.

In Table I the fractionation data are summarized. The over-all re-
covery was 38 per cent, and Fraction B showed a purification of 169-fold. Fraction A, and to a lesser extent, Fraction B, contained a red pigment. There was no myokinase, ATPase, hexokinase, or phosphatase activity in Fraction A, B, or C.

Stoichiometry—When the glycerokinase reaction was allowed to run to completion in the presence of limiting amounts of ATP, it was found (Table II) that for each mole of ATP added 1 mole of L-α-GP was formed and 1 mole of acid-labile phosphate disappeared. These results are in agreement with the reaction written in Equation 1. The L-α-GP formed in this reaction was identified not only by the specific enzymatic micromethod, but also was isolated from the reaction mixture in several experiments by a chromatographic method to be described in the following paper (10). Nearly quantitative recoveries of L-α-GP were obtained in exactly the position found to be occupied by synthetic DL-α-GP in this procedure.

Nucleotide Specificity—The ability of glycerokinase to transfer phosphate

### Table I

**Fractionation of Glycerokinase**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenate</td>
<td>53,000</td>
<td>0.61</td>
</tr>
<tr>
<td>Supernatant, pH 5.1</td>
<td>50,000</td>
<td>2.7*</td>
</tr>
<tr>
<td>47% saturated (NH₄)₂SO₄ ppt</td>
<td>41,000</td>
<td>9.2*</td>
</tr>
<tr>
<td>Supernatant after heat treatment</td>
<td>30,700</td>
<td>22.6*</td>
</tr>
<tr>
<td>Fraction A</td>
<td>6,950</td>
<td>58</td>
</tr>
<tr>
<td>&quot; B</td>
<td>5,900</td>
<td>102</td>
</tr>
<tr>
<td>&quot; C</td>
<td>6,250</td>
<td>59</td>
</tr>
</tbody>
</table>

* Protein determined spectrophotometrically. In other cases, protein concentration is on the basis of salt-free dry weight.

### Table II

**Stoichiometry of Glycerokinase Reaction**

<table>
<thead>
<tr>
<th>ATP added</th>
<th>L-α-GP formed</th>
<th>Acid-labile P</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmole</td>
<td>µmole</td>
<td>µmole</td>
</tr>
<tr>
<td>0.16</td>
<td>0.17</td>
<td>−0.17</td>
</tr>
<tr>
<td>0.32</td>
<td>0.33</td>
<td>−0.29</td>
</tr>
<tr>
<td>0.48</td>
<td>0.47</td>
<td>−0.49</td>
</tr>
</tbody>
</table>

The conditions for the first stage were exactly as described in the text, except that the period of incubation was 3 hours and phosphate buffer was replaced by Tris buffer of pH 7.4. 20 units of glycerokinase were added to each tube. Samples used for the determination of acid-labile phosphate were deproteinized with trichloroacetic acid, rather than with metaphosphoric acid.
to glycerol from UTP and ITP as well as from ATP was tested in the following experiment. Limiting amounts (0.60 μmole) of each of the nucleotides were incubated with an excess of glycerol and enzyme for 3 hours in order to cause the reaction to proceed to completion as far as possible. An almost quantitative yield of L-α-GP was obtained with ATP and UTP, but only about 7 per cent (0.04 μmole) with ITP. The slight activity of ITP may perhaps be attributed to contamination with ATP.

In another experiment, the rate of transfer of phosphate from UTP and ATP was compared in the presence and absence of added small amounts of ADP. The results are presented in Table III. It will be seen that the rate of phosphorylation with UTP is about three-fourths as rapid as with ATP. The addition of ADP (which in this system is completely inactive in itself) depresses the rate of phosphorylation by both UTP and ATP, but is considerably more inhibitory with UTP.

The possibility was considered that the activity of UTP in this system was the result of contamination of the UTP with small amounts of ADP and the presence in our purified glycerokinase of an enzyme catalyzing the following reaction.

\[ \text{UTP} + \text{ADP} \rightarrow \text{ATP} + \text{UDP} \]

Although enzymes capable of catalyzing such reactions have recently been described (19, 20), this possibility is considered unlikely. The concentration of adenine nucleotide present as contaminant in the UTP would hardly be high enough to permit the reaction to proceed at the rate observed. Secondly, unless one argued that the system is already saturated with ADP, the addition of small amounts of ADP should stimulate phosphorylation with UTP as phosphate donor. As indicated in Table III, an inhibition rather than a stimulation is noted.
Optimal pH for Glycerokinase Activity—The glycerokinase of liver is active over a comparatively wide range of pH, but has a rather sharp optimum at pH 9.8 under the conditions of our assay. Raising the pH above 10 causes a precipitous fall in activity, as shown in Fig. 1.

Fig. 1. The conditions of this experiment were identical with those of the regular assay system except that a mixture of 25 μmoles of glycine and 25 μmoles of Tris was used as buffer in the first stage. Varying amounts of KOH were added to give the pH values shown, which were measured with a glass electrode after all components of the system, including enzyme, had been mixed.

Requirement for Sulphydryl Groups—Considerable evidence has been obtained that intact sulphydryl groups are required for glycerokinase activity. It has been mentioned that the enzyme is readily inactivated by shaking in air, particularly in the presence of added cysteine. The enzyme shows optimal activity only when one of a number of sulphydryl-containing reducing agents is added to the test system. An experiment in which several such compounds have been tested is reported in Table IV. BAL is apparently the most effective of such compounds, while cyanide has but little effect.
Preincubation of the enzyme for 1 hour in 0.03 M Tris buffer, pH 7.4, with 0.005 M iodoacetamide completely inhibits its activity, while 0.005 M p-chloromercuribenzenesulfonate inhibits 83 per cent.

Requirement for Metallic Cofactor—As might be anticipated, glycerol is phosphorylated by glycerokinase at optimal rates only in the presence of divalent metal ions. The effect of magnesium on the enzyme reaction is shown in Table V. The optimal concentration for magnesium is about 0.003 M. Higher concentrations are distinctly inhibitory. About 50 per cent of the optimal activity is observed in the absence of any added metal, even though the enzyme has, at several stages, been dialyzed against Versene-containing buffers.

### Table IV

<table>
<thead>
<tr>
<th>Reducing agent, 20 μmoles per ml.</th>
<th>L-α-GP formed μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.33</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.39</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>0.44</td>
</tr>
<tr>
<td>BAL</td>
<td>0.54</td>
</tr>
<tr>
<td>Thioglycolic acid</td>
<td>0.45</td>
</tr>
<tr>
<td>Na₂S</td>
<td>0.54</td>
</tr>
<tr>
<td>KCN</td>
<td>0.36</td>
</tr>
</tbody>
</table>

The conditions of assay of glycerokinase were exactly as described in the text, except that cysteine was replaced by freshly made solutions of the compounds listed in the table. All reagents were neutralized immediately before use.

Manganese at a level of 0.002 M completely replaces magnesium, while calcium ions at this concentration depress the rate of phosphorylation considerably below the level observed in the absence of added divalent cations.

Phosphorylation of Dihydroxyacetone and Glyceraldehyde—The transfer of phosphate from ATP to acceptors, such as glycerol at neutral pH, involves the production of 1 equivalent of acid and thus can be followed by measuring the evolution of carbon dioxide from bicarbonate buffer in the Warburg apparatus. The following compounds, when tested in such an assay system, showed no appreciable activity as substrates: n-propanol, n-butanol, propanediol-1,2, propanediol-1,3, ethylene glycol, butanediol-1,4, erythritol, ribitol, myoinositol, D-glucose, D-fructose, DL-glyceric acid, and monohydroxyacetone. However, dihydroxyacetone was found to be phosphorylated at a rate about twice that of glycerol, and D,L-glyceraldehyde at a rate about 1.5 times that of glycerol. Apparently the same enzyme phosphorylates all three substrates, since no summation of rates is
observed when all three substrates are added together. Furthermore, the very drastic heat treatment (80 minutes at 60°) and the rather extensive purification of the enzyme render it rather unlikely that separate enzymes are involved.

The only likely product of the phosphorylation of dihydroxyacetone in this system is dihydroxyacetone phosphate. In confirmation of this supposition, it was found that the product formed by the enzymatic phosphorylation of dihydroxyacetone brought about a very rapid oxidation of DPNH when incubated in the presence of glyceraldehyde phosphate dehydrogenase at pH 7.

**Table V**

**Effect of Magnesium Concentration on Glycerokinase Activity**

<table>
<thead>
<tr>
<th>MgCl₂</th>
<th>L-α-GP formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmoles per ml</td>
<td>μmole</td>
</tr>
<tr>
<td>0</td>
<td>0.15</td>
</tr>
<tr>
<td>0.01</td>
<td>0.15</td>
</tr>
<tr>
<td>0.1</td>
<td>0.20</td>
</tr>
<tr>
<td>1.0</td>
<td>0.28</td>
</tr>
<tr>
<td>3.0</td>
<td>0.29</td>
</tr>
<tr>
<td>6.0</td>
<td>0.21</td>
</tr>
<tr>
<td>10.0</td>
<td>0.13</td>
</tr>
</tbody>
</table>

The conditions were the same as those described in the text, except that phosphate buffer was replaced with Tris buffer. Prior to this experiment, the enzyme was dialyzed against 0.01 M acetate buffer of pH 5.1 containing 0.1 per cent glycerol in order to remove the Versene in which the enzyme was routinely stored.

When the reaction product from the enzymatic phosphorylation of DL-glyceraldehyde was incubated with D-glyceraldehyde-3-phosphate dehydrogenase in the presence of arsenate, no significant reduction of DPN was noted. When D-glyceraldehyde-3-phosphate was generated in this system by the addition of aldolase and fructose-1,6-diphosphate, an instantaneous reduction of DPN was noted. It is concluded that the reaction product is not D-glyceraldehyde-3-phosphate and therefore is presumably the L isomer. This conclusion is rendered more likely in view of the fact that the product of the phosphorylation of glycerol is the closely related L-α-GP.

**Role of Glycerokinase in Phosphatide Synthesis** In previous studies (1), it was found that isolated, washed mitochondria contain all the enzymes necessary for the incorporation of inorganic phosphate into phosphatides. The rate of incorporation was approximately doubled by the addition of free glycerol and was again doubled upon the addition of an enzyme fraction from the supernatant fraction. In the present work, it was found that
glycerokinase is present largely in the supernatant fraction of sucrose homogenates of rat liver. Accordingly, the effect of the addition of small amounts of purified glycerokinase to the isolated mitochondrial system was tested. The results are presented in Table VI. The uptake of radioactive phosphate was approximately doubled when the purified glycerokinase was added, indicating that the concentration of this enzyme is rate-limiting for this process.

**Table VI**

*Effect of Glycerokinase on Phosphatide Synthesis in Isolated Mitochondria*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total radioactivity of phospholipide, counts $\times 10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>75</td>
</tr>
<tr>
<td>Glycerokinase added</td>
<td>148</td>
</tr>
</tbody>
</table>

The system used for measuring the incorporation of radioactive inorganic phosphate into the lipide phosphorus fraction of isolated mitochondria has already been described (1). Purified glycerokinase (1.6 mg. or 160 units) was added as indicated.

**DISCUSSION**

It is now apparent that $L$-$\alpha$-GP is a key compound in the synthesis of glycerophosphatides. Isotope dilution studies (1) with isolated mitochondria indicate that about 90 per cent of the phosphate incorporated into phosphatides passes through $L$-$\alpha$-GP as an obligate intermediate. The work of Kornberg and Pricer (2) has also demonstrated directly the conversion of $L$-$\alpha$-GP into lipide products. It has long been considered that one possible pathway for the formation of $L$-$\alpha$-GP may be by way of glycolytic reactions leading to the production of dihydroxyacetone phosphate, which then may be reduced to form $L$-$\alpha$-GP. The direct phosphorylation of glycerol has now been demonstrated in this paper and must be considered as an alternative pathway for $L$-$\alpha$-GP formation. It is not possible with present information to decide which pathway is of greater quantitative importance for the incorporation of phosphate into phospholipides in the intact liver cell.

Karnovsky and Gidez (21) have suggested that free glycerol is an obligate intermediate in the conversion of the glycerol moiety of ingested neutral fats to phosphatide glycerol. Presumably the free glycerol liberated by lipolysis is phosphorylated directly to $L$-$\alpha$-GP.

The inhibitory effect of ADP on the glycerokinase of liver described in Table III indicates that ATP and ADP have about the same affinity for the enzyme, while the affinity of both these adenine nucleotides is greater than that of UTP for the enzyme. The inhibition by ADP, together with
the sulfhydryl character of the enzyme, suggests a certain similarity between glycerokinase of liver and the brain hexokinase described by Sols and Crane (22).

The rôle of glycerokinase in the conversion of glycerol to glycogen will be discussed in the following paper (10).

SUMMARY

An enzyme from rat liver which catalyzes the phosphorylation of glycerol to form \( L-\alpha \)-glycerophosphate has been purified about 170-fold, and its properties have been described. The name glycerokinase is suggested for this enzyme, which also catalyzes, however, the phosphorylation of dihydroxyacetone and \( L \)-glyceraldehyde.

For optimal activity, the enzyme requires intact sulfhydryl groups and the presence of added magnesium or manganese ions. The rate of phosphorylation with UTP as phosphate donor is about three-fourths that observed with ATP. ITP is not effective as a phosphate donor.

An enzymatic micromethod for the estimation of \( L-\alpha \)-glycerophosphate has been presented.

The importance of glycerokinase for phosphatide synthesis has been discussed.

BIBLIOGRAPHY

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