Positional Specificities of Acyl Coenzyme A: Glycerophosphate and Acyl Coenzyme A: Monoacylglycerophosphate Acyltransferases in Escherichia coli*

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SUMMARY

A particulate preparation isolated from Escherichia coli catalyzes the acylation of 1-acyl-sn-glycerol 3-phosphate (1-acyl-GP) with both oleoyl-CoA and palmitoyl-CoA. The optimum conditions were determined for the acyl-CoA: 1-acyl-GP acyltransferase. The acyltransferase is specific for the 1-acyl-GP but does not acylate 2-acyl-sn-glycerol 3-phosphate (2-acyl-GP) under the conditions used.

The particulate preparation also catalyzes the acylation of sn-glycerol 3-phosphate (glycerophosphate) with both palmitoyl-CoA and oleoyl-CoA. When palmitoyl-CoA is the substrate, both monoacyl-sn-glycerol 3-phosphate (monoacyl-GP) and diacyl-sn-glycerol 3-phosphate (diacyl-GP) are produced. The two products show a typical precursor-product relationship. When oleoyl-CoA is used, the major product is diacyl-GP; monoacyl-GP is formed, but the amount reaches a very low steady state level within 2 min of incubation.

Structural analyses of the monoacyl-GP formed after short time incubations of glycerophosphate with unsaturated or saturated acyl-CoAs showed the major isomer to be 1-acyl-GP. The amounts of 2-acyl-GP formed are relatively small. The lack of accumulation of 2-acyl-GP during the acylation of glycerophosphate is not due to the isomerization of 2-acyl-GP to the 1-acyl-GP isomer.

During the acylation of radioactive glycerophosphate with unsaturated or saturated acyl-CoAs, radioactive monoacyl-GP is trapped effectively by adding nonlabeled 1-acyl-GP but ineffectively by adding nonlabeled 2-acyl-GP. The monoacyl-GP trapped in the presence of either 1-acyl-GP or 2-acyl-GP is mostly the 1-acyl-GP isomer.

Thus, the pathway of diacyl-GP synthesis from glycerophosphate in E. coli is primarily via the 1-acyl-GP as intermediate regardless of whether the substrate is saturated or unsaturated acyl-CoA.

Phosphatidic acid (diacyl-sn-glycerol 3-phosphate), a key intermediate in the biosynthesis of all glycerolipids, can be synthesized from sn-glycerol 3-phosphate (glycerophosphate) without involving dihydroxyacetone phosphate (1-5). There are two possible pathways in the synthesis of diacyl-GP from glycerophosphate: one pathway with the first acylation occurring at position 1 of glycerophosphate to form 1-acyl-sn-glycerol 3-phosphate followed by the acylation at position 2 of 1-acyl-GP to form diacyl-GP and another pathway through 2-acyl-sn-glycerol 3-phosphate as an intermediate. However, the relative importance of these two pathways in vitro or in vivo has not been fully elucidated due to the difficulties in finding conditions under which monoacyl-GP accumulates. Okuyama et al. have shown that 2-acyl-GP is much less active than 1-acyl-GP as an acceptor of acyl-CoAs in the synthesis of diacyl-GP in microsomes, a finding which suggests that the pathway through 1-acyl-GP is more active (6), although both 1-acyl-GP and 2-acyl-GP were reported to be synthesized in microsomes (7). In support of this suggestion, Tamai recently reported that during the synthesis of diacyl-GP by microsomes from [14C]glycerophosphate and oleoyl-CoA, radioactivity was trapped in 1-acyl-GP fraction by adding nonlabeled 1-acyl-GP (8). In Escherichia coli the synthesis of diacyl-GP was reported to involve the formation of both intermediates 1-acyl-GP and 2-acyl-GP depending upon the acyl-CoA used (9-11). When saturated acyl-CoA (palmitoyl-CoA but not stearoyl-CoA) was incubated with glycerophosphate in the presence of the particulate enzyme from E. coli, 1-acyl-GP accumulated. When unsaturated acyl-CoAs (oleoyl-CoA, palmitoleoyl-CoA, or cis-vaccenoyl-CoA) were used, 2-acyl-GP was found (9-11).

In examining two isomers of monoacyl-GP as substrate for the synthesis of diacyl-GP in E. coli, we found that 1-acyl-GP was readily acylated by unsaturated and saturated acyl-CoAs whereas 2-acyl-GP was not. Moreover, we found that the acylation of glycerophosphate by both saturated and unsaturated acyl-CoAs yielded 1-acyl-GP as the intermediate. In this report evidence is presented that the pathway through 2-acyl-GP has little significance under the conditions used and that the synthesis of diacyl-GP occurs mainly via the 1-acyl-GP pathway.

1 The abbreviations used are: monoacyl-GP, monoacyl-sn-glycerol 3-phosphate; 1-acyl-GP, 1-acyl-sn-glycerol 3-phosphate; 2-acyl-GP, 2-acyl-sn-glycerol 3-phosphate; diacyl-GP, diacyl-sn-glycerol 3-phosphate (phosphatidic acid); DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).
Materials and Methods

Fatty acids were obtained from Hormel Institute (Austin, Minn.) and coenzyme A from P-L Biochemicals, Inc., (Milwaukee, Wis.). [1,3-14C]Oleic acid and [1,2-14C]palmitic acid were the products of Amersham-Searle (Arlington Heights, Ill.). Acyl-CoA esters were synthesized by modification of Seubert’s procedure (13) from acyl chlorides and CoA as described previously (13). The purity of acyl-CoA was assessed by comparing the values obtained by A232/A280 assay with the amounts of CoA released by acyltransferase systems (14). Bovine serum albumin and dl-α-glycerophosphate were purchased from Sigma Chemical Co. (St. Louis, Mo.). L-α-[1,3-14C]Glycerophosphate was obtained from Mallinkrodt (St. Louis, Mo.). 1-Monoolein, 1,2-diolein, and triolein were the products of Analabs, Inc. (North Haven, Conn.). 2-Monoolein was prepared from triolein by pancreatic lipase (Analabs) (EC 3.1.1.3) hydrolysis (15). The mobility of 2-monoolein on borate-impregnated Silica Gel G thin layer chromatograms (16) was found to be comparable with 2-acylglycerol obtained by the hydrolysis of 2-acyl-GP with phosphatidate phosphatase (EC 3.1.1.4).

Phosphatic acid was purchased from Supelco, Inc. (Belleville, Pa.). Isomers of monoacyl glycerophosphorylcholine were prepared as reported elsewhere (17). 1-Acyl-GP was prepared according to Long et al. (18). The compound gave a single spot by thin layer chromatography on Silic AR (CC-4,200 to ~300 mesh) developed with chloroform-ethanol-88% formic acid-H2O (200:20:6:1, v/v) or on Na2CO3-impregnated Silica Gel HR plates (19 to 20) developed with chloroform-methanol-acetic acid-acetone-water (25:8:4:3:2, v/v). 1-[14C]Acyl-2-acyl-glycerophosphate was prepared by phospholipase D hydrolysis of specifically labeled phosphatidyglycerol, which was prepared from [14C]palmitoyl-CoA and 1,2-diacetylglycerol-phosphorylcholine through the reaction catalyzed by microsomal acyltransferase (21). Phospholipase A2 (Crotalus adamanteus venom) (EC 3.1.1.4) hydrolysis of labeled phosphatidyglycerol showed that at least 85% of the radioactivity was at position 1. The conditions of the hydrolysis of phospholipids by phospholipase A2 or phospholipase D were described previously (6, 20). 1-Acy-GP contained mainly palmitic and stearic acids, whereas 2-acyl-GP has chiefly oleic and linoleic acids.

Preparation of 2-Acyl-GP—2-Acyl-GP was prepared from 1-alkenyl-2-acyl-sn-glycero-3-phosphate by the previously described method (6, 22) with the following modifications. The latter compound was prepared by hydrolysis of choline plasmalogen by phospholipase D (EC 3.1.1.4) as described earlier (6). A lipid sample containing about 2.5 μmoles of alkylacylglycerophosphate was treated with iodine solution. The excess iodine and the iodocompounds were extracted seven times with petroleum ether-diethyl ether (1:1) mixture. After all of the color was removed, 2 drops of 0.05 M Na2S2O3 were added and the solution was extracted once with the same ether mixture (6). To the aqueous phase 4 ml of chloroform-methanol (1:1) were added and the contents mixed vigorously with a Vortex. The chloroform phase was separated by centrifugation and discarded. Then 1.5 ml of 0.2 M EDTA (pH 4.0) was added to the water phase and the 2-acyl-GP was extracted once with 5 ml of chloroform-methanol (2:1) and once with 3 ml of chloroform. The combined chloroform extracts were evaporated to dryness, and the residue was suspended in 0.05 M borate (pH 5.5). The recovery of 2-acyl-GP from the initial plasmalogen was 90 to 70%. The 2-acyl-GP was hydrolyzed with phosphatidate phosphatase (as described below), and the analysis of the products by thin layer chromatography on borate-impregnated Silica Gel G (16) demonstrated two spots in monoacylglyceride fraction: a major spot at an Rf corresponding to 2-acylglycerol and a minor spot at an Rf corresponding to 1-acylglycerol. The fatty acids of each spot were quantitated by first extracting the monoglycerides from the gel followed by transmethylation and gas-liquid chromatography with an internal standard. At least 85% of the fatty acid was found in the 2-acylglycerol spot and no more than 15% was associated with the 1-acylglycerol spot. Thus, the 2-acyl-GP contained 1 acyl GP as a contaminant (see “Results”).

Phosphatidate Phosphatase Hydrolysis of Monoacyl-GP and Dialyl-GP—Phosphatidate phosphatase was prepared from chicken liver as described by Smith et al. (23) and suspended in 0.02 M Tris-HCl-1 mM EDTA (pH 8.0) to a concentration of about 10 mg of protein per ml. The enzyme solution was kept frozen at −15°. A mixture of monoacyl-GP and diacyl-GP (less than 300 mmoles total) was dissolved in 1 ml of diethyl ether. To this solution the following were added: 0.5 ml of 0.5 M sodium maleate (pH 6.3), 0.5 ml of 0.2 M sodium borate (pH 6.3), and 0.4 ml of enzyme solution (about 4 mg of protein). The mixture was incubated for 60 to 120 min at 37° with vigorous shaking. The reaction products were then extracted twice with 2 m of petroleum ether-diethyl ether (1:1) and analyzed by boric acid-impregnated Silica Gel G thin layer chromatography (16). When the fatty acids of the monoglycerides were quantitated, the phosphatidate phosphatase was washed three times with 2 volumes of diethyl ether prior to its utilization for the hydrolysis of the phosphoglycerides. This treatment was necessary to remove the extractable lipids from the enzyme preparation.

Preparation of Chicken Liver Microsomes—Fresh chicken liver was homogenized in 10 volumes of 0.2 M sucrose-0.02 M Tris-HCl (pH 7.4). The homogenate was centrifuged at 7,000 χ g for 15 min and the resulting supernatant fluid was centrifuged at 100,000 χ g for 90 min. The precipitated particles were suspended in the sucrose-Tris buffer and used without further purification.

Preparation of Particulate Fractions from E. coli—About 10 g of wet E. coli B cells from late logarithmic phase (purchased from Grain Processing Corp., Muscatine, Iowa) were washed twice with 100 ml of 0.1 M Tris-HCl (pH 7.4) and suspended in 100 ml of the same buffer. The suspension was sonicated irradiated for 10 min at 0-4° with a Branson Sonifier (LS 75). The mixture was then centrifuged at 5,000 χ g for 5 min. The supernatant fluid was removed and the precipitate was resuspended in 100 ml of the same buffer. The suspension was sonicated irradiated for 10 min and centrifuged at 5,000 χ g for 5 min. The supernatant fluid was removed, added to the first supernatant solution, and the mixture was centrifuged at 40,000 χ g for 30 min. The residue was washed with 50 ml of 0.1 M Tris-HCl (pH 7.4), resuspended in the same buffer, and designated as P0. Two other kinds of particles were prepared from this strain as follows. Cells (20 g wet weight) were washed twice with 150 ml of 0.1 M Tris-HCl-2 mM EDTA-0.05 M β-mercaptoethanol (pH 7.4) and resuspended in 200 ml of the same buffer. The suspension was sonicated irradiated for 5 min and centrifuged at 5,000 χ g for 10 min. The supernatant fluid was removed and the residue was suspended in 100 ml of 0.1 M Tris-HCl (pH 7.4). The suspension was sonicated for 5 min and centrifuged at 5,000 χ g for 10 min. The supernatant fluid was removed and combined with the first supernatant solution. The mixture was centrifuged at 37,000 χ g for 30 min.
protein content was estimated in the presence of 1% sodium deoxycholate by measuring the absorbance at 280 and 260 nm using the method of Gottfried and Rapport (26). The deoxycholate was suspended in the sample preparation mixture and the absorbance was measured at 280 and 260 nm to determine the protein content.

Enzyme Assay—The acyl-CoA:glycerophosphate acyltransferase was assayed by measuring the rate of incorporation of [14C]glycerophosphate into lipids. The standard reaction mixture for this assay contained 1 mM [14C]glycerophosphate (approximately 3000 cpm per n mole), 50 μM acyl-CoA, 5 mM MgCl2, 1 mg per ml of bovine serum albumin, 0.1 mM Tris-HCl (pH 8.5), and 1 mg per ml of enzyme protein (9). Total volume was 0.5 ml. The incubations were carried out at room temperature (23-26°C).

The acyl-CoA:acyl-CoA acyltransferase was assayed by measuring either the rate of incorporation of labeled acyl group from acyl-CoA into phosphatidylcholine or the rate of increase of CoA-SH after acyl transfer. The latter assay was based on the reduction of DTNB by CoA-SH and was followed spectrophotometrically by measuring the rate of increase in absorbance at 413 nm (14). A typical incubation mixture contained 10 nmoles of acyl-CoA, 50 to 75 nmoles of monoacyl-GP, 1 μmole of DTNB, 80 nmoles of Tris-HCl (pH 7.5), and 0.1 mg of protein in a total volume of 1.0 ml.

Product Analysis—The reaction products were extracted by the Bligh and Dyer method after acidification with 0.1 N HCl to pH 3 to 4 (24). For analysis of the isomeric composition of monoacyl-GP, the reaction mixture was made 0.05 M borate, pH 4 with 0.1 N HCl. The reaction products were separated by thin layer chromatography on either Silica AR (CC-4, 200 to 300 mesh) developed in chloroform-ethanol-88% formic acid-water (200:20:16:1, v/v) or Na2CO3-impregnated Silica Gel plates (19, 20) developed in chloroform-methanol-acetic acid-acetone-water (25:8:4:3:2, v/v). The latter thin layer system gave a better resolution of monoglyceride, diacyl-GP, and monoacyl-GP. Since the mobilities of these compounds on thin layer chromatograms are relatively variable, the radio-labeled products were co-chromatographed with authentic lipids and the spots were made visible with iodine. The spots were scraped from the plates and the radioactivity was measured with a Nuclear-Chicago liquid scintillation spectrophotometer (Mark II) in a scintillation mixture made from 3.2 g of 2,5-diphenyloxazole (POPOP), 240 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene (PPO), 800 ml of toluene, 200 ml of Triton X-100, and 50 ml of water. The mixture was examined in Dr. W. E. M. Land's laboratory at the University of Michigan and found to have minimal effect on the counting efficiency of [3H]monoacyl GP in the presence of variable amounts of silica gel or water.

Other Analytical Methods—Phosphorus was determined according to Bibi and Lands (25), and plasmalogen content was measured by the method of Gottfried and Rapport (26). The protein content was estimated in the presence of 1% sodium deoxycholate by measuring the absorbance at 280 and 260 nm (27). The method was assessed by comparing it with the results obtained from a microburet test (28) of the particulate preparations suspended in potassium phosphate buffer (pH 7.0). Fatty acid methyl esters were analyzed with a Varian gas chromatograph (series 2700).

RESULTS

Acyl-CoA:1-acyl-GP Acyltransferase—In Fig. 1 the effect of acyl-CoA concentrations on the acyltransfer rates was examined with palmitoyl- and oleoyl-CoA. A concentration of 5 μM was enough to saturate the system with both palmitoyl- and oleoyl-CoA. No effort was made to measure the Km values for acyl-CoA due to inherent problems encountered with long chain acyl-CoA derivatives such as micelle formation, nonspecific binding of substrates to protein, and other difficulties discussed previously (29, 30). Fig. 1 also shows that a relatively wide range of acyl-CoA concentrations could be used without significant change in velocities and that the rate of acylation with oleoyl-CoA is twice that with palmitoyl-CoA.

The effect of 1-acyl GP concentrations on acyltransfer rates was examined also using oleoyl-CoA as substrate. As shown in Fig. 2, concentrations of 50 to 75 μM 1-acyl-GP were required for optimal activity.

In earlier studies on this reaction MgCl2 was added to the reaction mixture (11, 31-34) and Van Den Bosch and Vagelos reported an optimal requirement for MgCl2 to be 0.25 mM (31). In our preparation, however, MgCl2 inhibited, rather than stimulated, the reaction at higher concentrations as shown in Table I. It is important to note that the same effect was noted whether the enzyme preparation used was prepared in the presence or absence of EDTA, suggesting that EDTA was not the reason for not detecting MgCl2 requirement in this system. It is interesting also to note that the reduction of DTNB in the absence of 1-acyl-GP increases with increasing MgCl2 concentration. The reason for this increase is not clear at this time.
The effect of increasing amounts of protein on the transferase reaction is shown in Fig. 3. The rate of the reaction was linear up to a protein concentration of 0.3 mg per ml. The pH profile of the oleoyl-CoA:1-acyl-GP acyltransferase reaction is shown in Fig. 4. The pH optimum for the acylation reaction is between 7.5 and 8.0. This value is lower than the pH 9.0 which was used by other workers (31,33). The apparent reduction of DTNB in the absence of 1-acyl-GP acceptor increases at higher pH values as shown in Fig. 4. The reasons for this increase were not investigated, but it may be due to hydrolysis of acyl-CoA, the instability of DTNB at higher pH, or both.

The stability of the transferase to storage was investigated. As shown in Fig. 5, storage of the particulate preparation at 0°C after quick freezing in liquid nitrogen did not lead to a significant loss of activity after 3 months of storage. The enzyme stored at -15°C after rapid freezing in Dry Ice-acetone or at 0°C without freezing lost appreciable activity. When the enzyme preparation was frozen slowly at -15°C and stored at this temperature, it could not be used even after 1 day.

Acylation of 2-Acyl-GP—When 2-acyl-GP was examined as substrate under the optimal conditions employed for the acylation of 1-acyl-GP, very little free CoA, measured by DTNB reduction, was released with either palmitoyl-CoA or oleoyl-CoA as acylating substrates. In Table II, the acylations of 2-acyl-GP and 1-acyl-GP were compared by using labeled acyl-CoAs and a particulate preparation obtained from E. coli B cells. Less product was formed from 2-acyl-GP than from 1-acyl-GP (Table II). Phospholipase A2 treatment of the labeled product, diacyl-GP, yielded 14C-fatty acids in amounts equivalent to the decrease in radioactivity of diacyl-GP. The monoacyl-GP fraction showed no increase in radioactivity. The validity of this analysis was checked with 1-14C-palmitoyl-2-acyl-GP as substrate. Hydrolysis of this lipid by phospholipase A2 yielded more than 85% of the radioactivity in acyl-GP, indicating that the release fatty acid had occupied position 2 of the diacyl-GP (Table II). Since hydrolysis by phospholipase A2 of the diacyl-GP, product of acylation of 2-acyl-GP, did not
Each incubation mixture contained 50 nmoles of 2-acyl-GP, 90 nmoles of Tris-HCl (pH 7.5), and, where indicated, 15 nmoles of the [14C]acyl-CoA and 0.2 mg of the protein in a final volume of 1 ml. At the end of the incubation the reaction was stopped by the addition of CHCl₃:CH₃OH, and the phospholipids were extracted and analyzed as in Table II.

### Table II

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>[14C]Acyl-CoA</th>
<th>Incubation time</th>
<th>Radioactivity recovered in diacyl-GP</th>
<th>Radioactivity after phospholipase A₈ treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>min</td>
<td>Fatty acid &amp; Acyl-CoA &amp; Diacyl-CoA</td>
<td>Fatty acid &amp; Acyl-CoA &amp; Diacyl-CoA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cpm</td>
<td>cpm</td>
</tr>
<tr>
<td>Palmitoyl-CoA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Acyl-GP</td>
<td>5</td>
<td>1450</td>
<td>130</td>
<td>710</td>
</tr>
<tr>
<td>2-Acyl-GP</td>
<td>15</td>
<td>2500</td>
<td>360</td>
<td>1190</td>
</tr>
<tr>
<td>Oleoyl-CoA</td>
<td>5</td>
<td>5500</td>
<td>460</td>
<td>2080</td>
</tr>
<tr>
<td>1-[14C]Palmitoyl-2-acyl-GP</td>
<td>15</td>
<td>9550</td>
<td>1700</td>
<td>3100</td>
</tr>
</tbody>
</table>

a Radioactivity was as expressed as in Table II.

b The specific activity of the microsomal preparation for the acylation of 1-acyl-GP was 16.4 and 14.8 nmoles per min per mg of protein with palmitoyl-CoA and oleoyl-CoA, respectively.

c The specific activity of the P₈ particles for the acylation of 2-acyl-GP was 18.8 and 35.2 nmoles per min per mg of protein with palmitoyl-CoA and oleoyl-CoA, respectively.

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**Acylation of 1-acyl-GP and 2-acyl-GP by acyltransferase preparation**

Each incubation mixture contained 80 nmoles of Tris-HCl (pH 7.5), 1 nmoles of DTNB, 0.2 mg of enzyme protein (P₈), water to a final volume of 1.0 ml, and where indicated, 100 nmoles of 1-acyl-GP or 2-acyl-GP, and 15 nmoles of [1-14C]palmitoyl-CoA (2900 cpm per n mole) or [1-14C]oleoyl-CoA (5600 cpm per n mole). Incubations were carried out at 25° for 5 min. The reaction was stopped by the addition of CHCl₃:CH₃OH, and the lipids were extracted as described under "Materials and Methods." The radioactive diacyl-GP was separated by thin layer chromatography on Na₂CO₃-Silica Gel HR. The diacyl-GP was extracted from the gel (6) and divided into two parts. Each part was dissolved in 5.0 ml of diethyl ether. To one part were added 30 μl of a solution containing 0.2 μmol of CaCl₂, 5.0 μmol of Tris-HCl (pH 7.4), and 0.25 μg of phospholipase A₈. To the other part were added 30 μl of the same solution except no phospholipase was added. The mixtures were incubated for 1 hour at 25°. The reaction was stopped by the addition of 30 μl of 0.5 M EDTA (pH 7.4) and 2.0 ml of CH₃OH. The mixture was evaporated to dryness under N₂, and the residue was chromatographed on the Na₂CO₃-Silica Gel HR system as described under "Materials and Methods." The values given correspond to one part of the diacyl-GP.

yield labeled monoacyl-GP but labeled fatty acid, it was concluded that the labeled diacyl-GP product contained the 14C fatty acid in position 2 only, and the acylation of the contaminant 1-acyl-GP occurred during the incubation (see "Materials and Methods").

To show that the 2-acyl-GP could be acylated, an attempt was made to convert it to diacyl-GP in the presence of liver microsomes. An earlier report showed that rat liver microsomes were able to acylate both positions 1 and 2 of monoacyl-GP, although acylation of position 1 was relatively slower (6). Table III shows that microsomes prepared from chicken liver were able to acylate the 2-acyl-GP to form diacyl-GP, but the particular preparation of E. coli had little activity. Hydrolysis of the diacyl-GP by phospholipase A₈ showed that 2-acyl-GP is acylated by microsomes with 14C acyl groups to form diacyl-GP labeled at position 1. In contrast, with the E. coli preparation this kind of product was not formed; the 14C labeled diacyl-GP product contained the radioactivity in position 2. This is the case because the monoacyl-GP produced by the hydrolysis of the 14C diacyl-GP was not labeled (Table III). Again the 14C diacyl-GP formed by the E. coli enzyme was probably derived from the acylation of the small amount of 1-acyl-GP contaminant present in the 2-acyl-GP preparation.

**Acylation of glycerophosphate by acyltransferase—The time course for the acylation of glycerophosphate by the particulate preparation (P₈) of E. coli B is shown in Fig. 6. With palmitoyl-CoA as substrate, a clear precursor-product relationship was observed between monoacyl-GP and diacyl-GP. Moreover a significant amount of monoacyl-GP accumulated. With oleoyl-CoA as substrate, on the other hand, a precursor-product relationship was not readily seen within the time intervals employed.**
The amount of monoacyl-GP produced with oleoyl-CoA reached a steady state concentration in 2 min (Fig. 6), although the acylation of glycerophosphate was linear for more than 6 min.

Similar time course patterns were obtained with both palmitoyl-CoA and oleoyl-CoA as substrate when the reaction was performed at pH 9 or 7, the only difference being that the enzyme was more active at pH 8.5, which is the optimal pH of the reaction. Similar results were obtained with the particular preparation from E. coli K-12, W3110 prepared according to the procedure of Cronan et al. (32).

The effects of protein and MgCl₂ concentrations on the acylation of glycerophosphate by oleoyl-CoA were studied. At all enzyme concentrations, diacyl-GP was the predominant product, and MgCl₂ stimulated the synthesis of both monoacyl-GP and diacyl-GP (Fig. 7).

Many unsuccessful attempts were made to stop the reaction at the monoooleoyl-GP step. In the acylation of glycerophosphate by oleoyl-CoA, monoooleoyl-GP could be obtained as the major product only by stopping the reaction after extremely short incubation times. In an effort to obtain sufficient amounts of monooacyl-GP by very short incubation times, 45 s for oleoyl-CoA and 90 s for palmitoyl-CoA as substrate, large scale reactions were carried out. Precautions were taken to minimize the isomerization of the monooacyl-GP derivatives: borate was added to the reaction mixture and the pH was lowered to 8 (22, 35, 36). The acyl-GP fraction was isolated by thin layer chromatography on Na₂CO₃-Silica Gel HR as described under “Materials and Methods.” The monoacyl-GP was hydrolyzed with phosphatidate phosphatase, and the glyceroles were separated on borate-impregnated silica gel thin layer chromatograms (see “Materials and Methods”). Most of the radioactivity was found in the 1-acylglycerol regardless of the acyl-CoA used (Table IV). The 2-acylglycerol fraction had little activity and at no time constituted more than 10% of the total monoacylglycerides. With oleoyl-CoA as substrate, the diglyceride had more radioactivity than the monoacylglyceride, even at 45 s of incubation. When palmitoyl-CoA was used as substrate and

![Graph](image)

**Fig. 7.** Effect of enzyme and MgCl₂ concentrations on the acylation of glycerophosphate with oleoyl-CoA. Each incubation mixture contained 100 nmoles of oleoyl-CoA, 1 μmole of [³⁵S]glycerophosphate (3600 cpm per μmole), 0.5 μmole or 5.0 μmole of MgCl₂, 1.0 mg of bovine serum albumin, 100 μmole of Tris-HCl (pH 8.5), and varying amounts of enzyme preparation PO. The control value was subtracted from the experimental.

<table>
<thead>
<tr>
<th>Conditions of acylation of glycerophosphate</th>
<th>Products treated with phosphatidate phosphatase</th>
<th>Monoglyceride recovered</th>
<th>Digestase</th>
<th>Time of phosphatase hydrolysis</th>
<th>Percentage of hydrolysis by phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aril-CoA</td>
<td>Incubation time</td>
<td>Total volume</td>
<td>2-Acylglycerol</td>
<td>1-Acylglycerol</td>
<td>Diglyceride</td>
</tr>
<tr>
<td>1 Palmitoyl</td>
<td>90</td>
<td>2.7</td>
<td>Monoacyl-GP⁵</td>
<td>134</td>
<td>9,312</td>
</tr>
<tr>
<td>Oleoyl</td>
<td>45</td>
<td>8</td>
<td>Monoacyl-GP⁵</td>
<td>138</td>
<td>9,191</td>
</tr>
<tr>
<td>2 Oleoyl</td>
<td>60</td>
<td>8</td>
<td>Mixture of monoacyl- and diacyl-GP⁶</td>
<td>681</td>
<td>6,341</td>
</tr>
<tr>
<td>3 Palmitoyl</td>
<td>90</td>
<td>6</td>
<td>Mixture of monoacyl- and diacyl-GP⁶</td>
<td>372</td>
<td>12,126</td>
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<tr>
<td>Oleoyl</td>
<td>45</td>
<td>5.7</td>
<td>Mixture of monoacyl- and diacyl-GP⁶</td>
<td>373</td>
<td>3,333</td>
</tr>
<tr>
<td>cis-vaccenoyl</td>
<td>45</td>
<td>5.7</td>
<td>Mixture of monoacyl- and diacyl-GP⁶</td>
<td>365</td>
<td>3,893</td>
</tr>
</tbody>
</table>

A Control values were those obtained from parallel experiments at zero time incubation with phosphatase. In Experiment 1, the values were 5 to 10 cpm. In Experiments 2 and 3 the values ranged from 50 to 400 cpm. In each case the control value was subtracted from the experimental.

The monoacyl-GP was purified by thin layer chromatography before treatment with phosphatase.

The products were not purified by thin layer chromatography but were hydrolyzed directly with the phosphatase.
the reaction was incubated for 90 s, 3 times more monoacyl-GP was formed than diacyl-GP (Table IV). The acylation occurred exclusively at position 1 and not at position 2. With cis-vaccenoyl-CoA as substrate the acylation also occurred at position 1, and monoacyl-GP and diacyl-GP were synthesized in about equal amounts.

Although conditions for the acylation of glycerophosphate were chosen to minimize the isomerization of the monoacyl-GP, an attempt was made to measure the extent of isomerization during the incubation and the subsequent analytical procedures. 2-Acyl-GLP, prepared as described under "Materials and Methods," was incubated without enzyme and substrate under the same conditions used in Experiments 2 and 3 (Table IV). The lipids were extracted, hydrolyzed with phosphatidate phosphatase, and separated on borate-impregnated Silica Gel G thin layer chromatograms as described for Experiments 2 and 3 (Table IV). The monoglycerides were quantitatively extracted from the gel, the fatty acyl groups trimethylated, and the methyl esters were analyzed with an internal standard by gas-liquid chromatography. In two separate experiments, 85% of the monoglycerides were recovered as 2-acylglycerol and 15% as 1-acylglycerol; this result indicated that little isomerization of 2-acyl-GLP into 1-acyl-GLP occurred during the incubation and the subsequent manipulations of the products. Thus, the slight accumulation of the 2-acyl-GLP in the experiments reported in Table IV could not be due to its isomerization to the 1-acyl-GLP isomer. The main product of the acylation of glycerophosphate by both saturated and unsaturated acyl-CoAs is the 1-acyl-GLP isomer.

Van Den Bosch and Vagelos (31) reported that monoglycerides were produced during the acylation of glycerophosphate at pH 7.0. They attributed this result to the presence of phosphatidate phosphatase which has a lower pH optimum than the acyltransferase. Similar experiments were performed to analyze the monoglycerides formed during the incubation at pH 7.0. It should be noted that a larger scale of the reaction mixture (18 to 24 mg of protein) and a prolonged incubation time (20 to 40 min) were necessary to obtain 1 to 2 moles of monoglycerides (Table V). Therefore, the control values at zero time incubations were relatively high because of the large scales used. When unsaturated fatty acyl-CoA derivatives were used as substrates, considerably more diglycerides than monoglycerides were formed (Table V). Analysis of the monoglycerides by borate-impregnated Silica Gel G thin layer chromatography showed that the 1-acylglycerol was the principal product, indicating that the acylation occurred at position 1 of the glycerophosphate regardless of whether the fatty acyl group was saturated or unsaturated. The amount of radioactivity in the 2-acylglycerol was always much less than the radioactivity in the 1-acylglycerol, and under no circumstances did the radioactivity of 2-acylglycerol exceed 30% of the radioactivity for the total monoglycerides formed (with all of the experiments performed with unsaturated acyl-CoAs, an average of 19% was obtained).

Further evidence for the noninvolvement of the 2-acyl-GLP pathway in the acylation of glycerophosphate by oleoyl-CoA was derived from trapping experiments in which nonlabeled 1-acyl-GLP or 2-acyl-GLP was present during the acylation of [14C]glycerophosphate by unsaturated acyl-CoA. In such experiments the addition of increasing amounts of nonlabeled 1-acyl-GLP to the reaction mixture effectively decreased the incorporation of [14C]glycerophosphate into the diacyl-GLP and increased the amount of radioactivity in the monoacyl-GLP, as shown in Fig. 8. On the other hand, the addition of increasing amounts of nonlabeled 2-acyl-GLP to the reaction mixture did not significantly trap the radioactivity in the monoacyl-GLP fraction but inhibited the incorporation of [14C]glycerophosphate into the diacyl-GLP fraction (Fig. 8). The reasons for the latter effect are not apparent but may be due to inhibition of the acyltransferase by the surfactant activity of 2-acyl-GLP, a slight trapping of the [14C]monooleoyl-GLP by the 1-acyl-GLP contam-

### Table V

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Acy-CoA</th>
<th>Incubation time</th>
<th>Radioactivity in fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-Acyl-GLP</td>
</tr>
<tr>
<td>1</td>
<td>Palmitoyl</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>Oleoyl</td>
<td>30 min</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>cis-Vaccenoyl</td>
<td>30 min</td>
<td>832</td>
</tr>
<tr>
<td>2</td>
<td>Oleoyl</td>
<td>20 min</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Palmitoyl</td>
<td>20 min</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Oleoyl</td>
<td>20 min</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>cis-Vaccenoyl</td>
<td>20 min</td>
<td>223</td>
</tr>
</tbody>
</table>

*Not measured.

**Fig. 8.** Acylation of glycerophosphate by oleoyl-CoA in the presence of monoacyl-GLP. Each incubation mixture contained the same components used in Fig. 6, except that varying amounts of nonlabeled monoacyl-GLP isomers were added in a final volume of 0.4 ml and P2 preparation was used. The incubation was carried out at 25° for 5 min. The reaction was stopped by the addition of CHCl3:CH3OH and products were extracted and analyzed as described under "Materials and Methods." The radioactivity trapped in diacyl-GLP (O—O) and monoacyl-GLP (O—O) is plotted. Each point represents the average of two separate determinations.
the radioactivity in monoglyceride fraction was associated with present, and in the presence of either I-acyl-GP or 2-acyl-GP, with diacyl-GP, was extracted, hydrolyzed with phosphatidate phosphatase as described under "Materials and Methods." Control values in parentheses (zero time control of hydrolysis by phosphatidate phosphatase) have been subtracted.

The acylation of l-acyl-GP was catalyzed by a particulate fraction. The trapped monoacyl-GP, together with diacyl-GP under the same conditions used for incubation with 1-acyl-GP did not result in appreciable synthesis of phosphatidic acid. In this respect the E. coli preparation is different from the microsomal system which catalyzes the acylation of the 1-acyl-GP and 2-acyl-GP isomers, although the activity with 2-acyl-GP was much lower than the activity with 1-acyl-GP. It is, of course, possible that other conditions exist for the acylation of the 2-acyl-GP by the E. coli particles, but all of our attempts to detect such activity were negative, including the addition of Mg++ to the reaction mixture.

The inability of our preparation to acylate the 2-acyl-GP prompted us to investigate the role of this derivative in the synthesis of diacyl-GP from glycerophosphate and acyl-CoA. When the enzyme preparation was incubated with glycerophosphate and palmitoyl-CoA and the reaction products were extracted after various time intervals and analysed by thin layer chromatography, both monoacyl-GP and diacyl-GP were formed. Within 10 min of incubation the two products showed a typical precursor-product relationship (Fig. 6). However, with oleoyl-CoA as substrate, the major product was diacyl-GP. Monoo-acyl-GP was formed in small but significant amounts and reached a steady state within 2 min of incubation. The data shown in Fig. 6 suggest the presence of different pool sizes of the monoacyl-GP intermediates with palmitoyl-CoA and oleoyl-CoA, which may mean that the rates of influx and efflux in each pool are different for the two acyl-CoA. It is conceivable that the rate of the first acylation is faster with palmitoyl-CoA and the rate of the second acylation is faster with oleoyl-CoA. The result is the formation of a larger pool size for the intermediate produced with palmitoyl-CoA than with oleoyl-CoA. Under the conditions used, the proportion of monoacyl-GP was never over 25% of the total products when the incubations were carried out for more than 5 min with oleoyl-CoA as substrate. Therefore, incubations for very short times were necessary to obtain monoacyl-GP as a major product.

Structural analyses of the monoacyl-GP formed by the acylation of glycerophosphate with palmitoyl-CoA or oleoyl-CoA showed that the major isomer was 1-acyl-GP (Table IV). The amounts of the 2-acyl-GP formed were relatively small; 5% and 15 to 25% of the total monoacyl-GP were 2-acyl-GP when the substrate was palmitoyl-CoA or oleoyl-CoA, respectively. The lack of accumulation of the 2-acyl-GP could not be due to its isomerization to the 1-acyl-GP isomer, since treatment of the 2-acyl-GP under the same experimental conditions did not show any significant migration of the acyl group to position 1.

Experiments designed to trap the [14C]monoacyl-GP intermediate in the acylation of [14C]glycerophosphate with oleoyl-CoA showed that radioactivity was trapped effectively in the presence of 1-acyl-GP but ineffectively in the presence of 2-acyl-GP (Fig. 8). Analyses of the radioactivity trapped in the monoacyl-GP pool showed that less than 25% of radioactivity was associated with 2-acyl-GP.

Thus the formation of 1-acyl-GP as the major intermediate, its subsequent conversion to diacyl-GP, and the failure to acylate 2-acyl-GP led us to conclude that the pathway of diacyl-GP
synthesis, at least in the strain of E. coli B, is via the 1-acyl-GP intermediate regardless of whether the substrate was saturated or unsaturated acyl-CoA. This conclusion is contrary to that obtained by Roy et al. (9) who reported that a particulate enzyme preparation from E. coli catalyzed the esterification of glycerophosphate exclusively at position 1 with palmitoyl-CoA and predominantly at position 2 with oleoyl-CoA. These authors reported that the major product of acylation of glycerophosphate was monacyl-GP even after prolonged incubations. This experiment and others have shown that the acylation of glycerophosphate with palmitoyl-CoA in the presence of particulate preparations from E. coli yielded both monacyl-GP and diacyl-GP and that the amounts of diacyl-GP increased with prolonged incubation. These findings show that the second acylation step was always present and active in the conversion of monacyl-GP to diacyl-GP (31, 33, 38, 39). The reasons for the differences in the results obtained are not clear but may be due to the differences in the strains of E. coli used.

Since recent studies indicated that the apolar component of membrane lipids plays an important role in the structure and function of biological membranes, it was of interest to investigate the interrelationship between fatty acids and complex lipids synthesis. The studies of Esfahani et al. (40–45) with unsaturated fatty acid auxotrophs of E. coli grown on various unsaturated fatty acids led to the conclusion that the fatty acid composition of the phospholipids is closely controlled by the organisms depending upon the physicochemical properties of fatty acids. For instance, with cis-vaccenate in the growth medium, 80% of the fatty acids in phospholipids are saturated fatty acids; with linolenate, however, 63% of the fatty acids are saturated fatty acids. Thus, the distribution of the fatty acids due to the differences in the strains of E. coli R, is via the I-acyl-GP pathway (31, 33, 38, 39). The reasons for the differences in the results obtained are not clear but may be due to the differences in the strains of E. coli used.

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