

BIOSYNTHESIS AND CHARACTERIZATION OF A PHOSPHATIDIC ACID
ANALOG CONTAINING β -HYDROXY FATTY ACID

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Summary: A new phospholipid was shown to be biosynthesized in liver mitochondria from labeled 1-alkyl-sn-glycerol-3-phosphate and labeled fatty acid in the presence of ATP and CoA and its structure was shown to be 1-alkyl-2-(3-hydroxy)acyl-sn-glycerol-3-phosphate. Fatty acids in mitochondria were oxidized to the β -hydroxy derivatives which were utilized for the acylation of alkyl glycerophosphate. Free long chain β -hydroxy acids were also utilized by mitochondria and microsomes in the presence of ATP and CoA for the acylation of glycerophosphate derivatives to form the phosphatidate analogs.

During an investigation of the acylation of 1-alkyl-sn-glycerol-3-phosphate (alkyl GP)¹ with fatty acyl coenzyme A, it was observed that an unknown lipid was formed which migrated on thin layer chromatography closely with the expected reaction product (1-alkyl-2-acyl-sn-glycerol-3-phosphate). Further investigation suggested that the long chain acyl group was responsible for the difference in the products. In this report evidence is presented that guinea pig liver mitochondria are capable of forming β -hydroxy fatty acyl coenzyme A from free fatty acid and that this β -hydroxy fatty acyl coenzyme A is used by the mitochondria as a substrate in the acylation of alkyl GP to form 1-alkyl-2-(3-hydroxy)acyl-sn-glycerol-3-phosphate.

MATERIALS AND METHODS

Alkyl [³²P]GP was prepared enzymatically from [³²P]DHAP as described previously (1). [³²P]DHAP was enzymatically synthesized from ³²Pi (New England Nuclear) (2). Alkyl GP was obtained by reduction (1) of chemically synthesized alkyl DHAP (3). [1-¹⁴C]Palmitic acid was obtained from New England Nuclear,

¹ Abbreviations used: Alkyl GP, 1-alkyl-sn-glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate.

palmitic acid from the Hormel Institute, and methyl β -hydroxy myristate from Analabs, Inc. β -hydroxy stearic acid was chemically synthesized from palmitaldehyde and ethyl bromoacetate by the Reformatsky reaction according to Stoffel, et al (4). Guinea pig liver mitochondria and microsomes were isolated as described (2).

Esterification of fatty acids with 2,2-dimethoxypropane and methanol was accomplished by the method of Radin, et al (5). Acetylation of methyl β -hydroxy fatty acids was done with acetic anhydride in pyridine (6). The esters of fatty acids were reduced with Vitride [sodium bis(2-methoxyethoxy)-aluminum hydride, Eastman Organic Co.] by refluxing in ether for 30 minutes. After cooling the solution was acidified with HCl and extracted three times with ether. The combined ether extracts were washed with water and dried. Acetonides of the diols were prepared by the method of Hanahan, et al (7). Diols and acetonide derivatives of diols were separated on a thin layer chromatogram in a solvent system of hexane:ether (2:8). Hydrolysis of phosphatidate analogs by snake venom phospholipase A₂ was by the method of Wu and Tinker (8) with an overnight incubation in ether. After incubation the ether was evaporated and the lipids were extracted and separated by thin layer chromatography. Periodate oxidation was carried out with pyridine as the solvent (9). Gas-liquid chromatography of the fatty acid derivatives was done in a 5' x 1/4" OV-101 on Chromosorb 9 (1.5%) column at 210°. The gas chromatograph (Varian-Aerograph, Model 920) was fitted with a thermal conductivity detector and the fractions were collected in a tube attached to a Millipore filter (10). Other materials and methods have been described previously (1).

RESULTS

a. Formation of labeled lipids from 1-alkyl-*sn*-glycerol-3-phosphate.

The labeled lipids formed from either 1-alkyl-*sn*-glycerol-[³²P]phosphate or from [1-¹⁴C]palmitic acid by guinea pig liver mitochondria and microsomes in the presence of different cofactors (see legend, Fig. 1) were separated by thin layer chromatography and the radioautogram is shown in Fig. 1. It can be

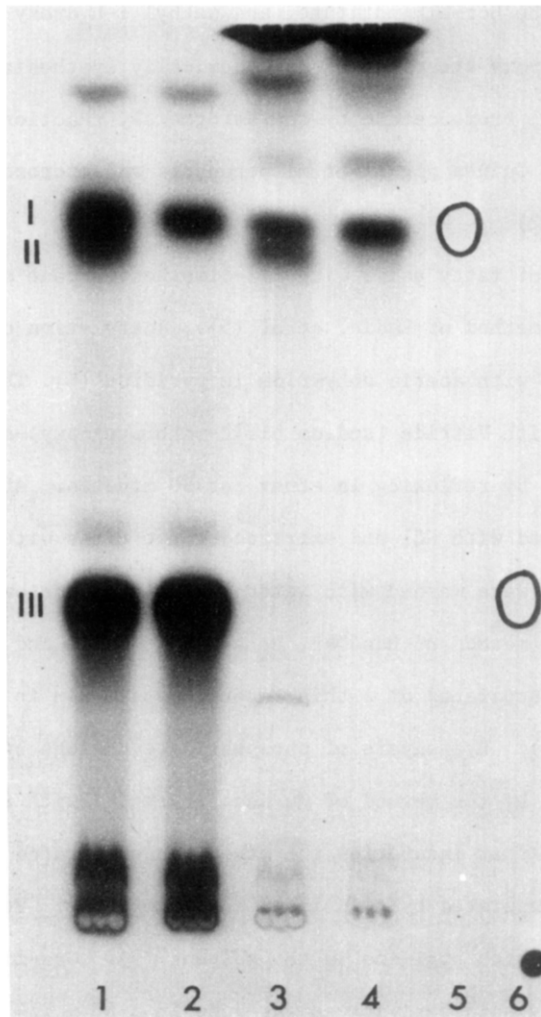


Figure 1: Radioautogram of lipids extracted from incubation mixtures after separation by thin layer chromatography. All incubations contained the following: Tris-HCl (75 mM, pH 8.5), Triton X-100 (50 μ g), NaF (21 mM), ATP (8.4 mM), CoA (40 μ M), and MgCl₂ (4.2 mM) in a final volume of 1.2 ml. In addition respective incubations contained: (1) alkyl [³²P]GP (3 x 10⁴ cpm 90 nmoles), palmitic acid (40 nmoles), mitochondria (0.81 mg protein). (2) as in 1 except microsomes (0.15 mg protein) in place of mitochondria were used. (3) alkyl GP (90 nmoles), [¹⁴C]palmitic acid (40 nmoles, 10⁶ cpm), mitochondria (0.81 mg protein). (4) as in 3 except microsomes (0.15 mg protein) in place of mitochondria were used. (5) standard phosphatidic acid. (6) alkyl GP. The mitochondrial incubations were done at 37° for 15 minutes and the microsomal for 5 minutes at 37°. Lipids were extracted as described and developed on commercial silica gel plates (Brinkmann Inst., Inc.) in chloroform:methanol:acetic acid:water (100:40:12:4). Spots I and II, in the case of the mitochondrial incubations, each represent approximately 4 nmoles of labeled lipid. Spot I in the case of the microsomal incubations represents 7 nmoles of labeled lipid.

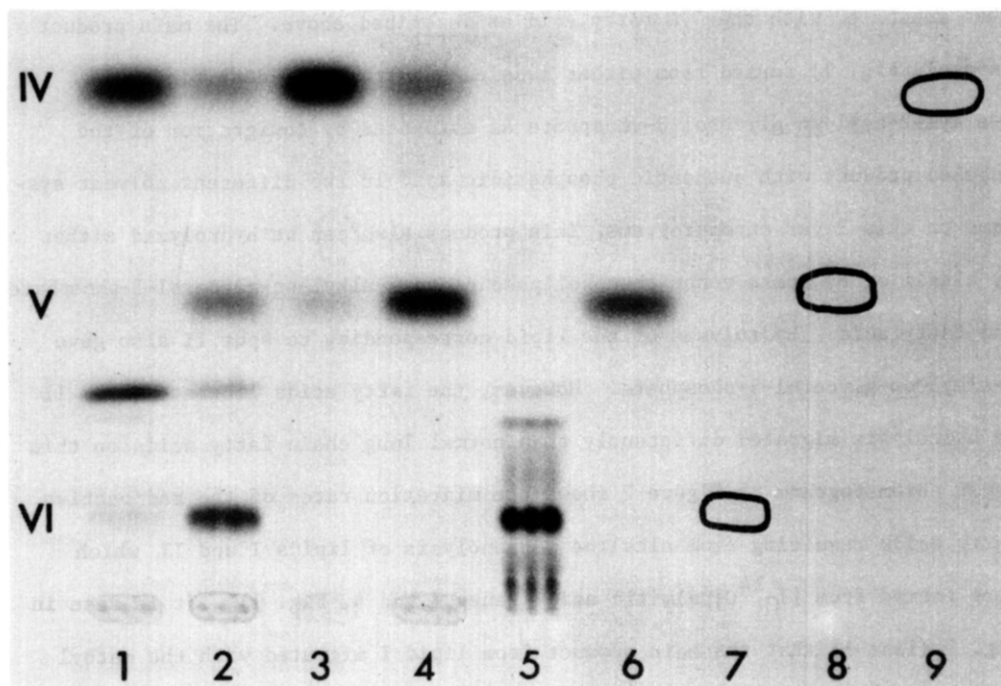
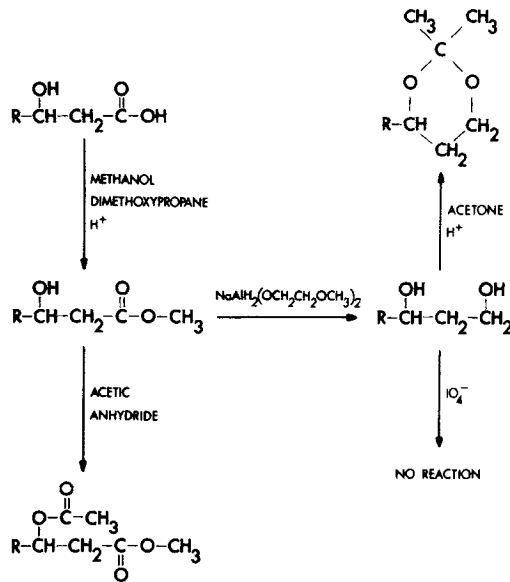


Figure 2: Radioautogram of radioactive fatty acids and their derivatives obtained on hydrolysis of 1-alkyl-2-acyl-*sn*-glycerol-3-phosphate formed during incubation with guinea pig liver mitochondria. Lane (1) represents the fatty acids liberated from the phospholipid by alkaline methanolysis corresponding to spot I, Fig. 1. The fatty acids in lane (2) are from lipid II, Fig. 1. Lanes (3) and (4) show the respective products obtained when the labeled lipids in lanes (1) and (2) were treated with methanol, HCl and dimethoxypropane. The polar fatty acid extracted from the mitochondrial incubation with [1-¹⁴C]-palmitic acid is in lane (5) and the methyl ester of this labeled fatty acid is in lane (6). Lanes (7) - (9) represent the following respective standards: (7) β -hydroxy fatty acid, (8) methyl ester of β -hydroxy fatty acid, (9) methyl ester of normal fatty acid. The silica gel plate was developed in hexane:ether:acetic acid (30:70:1).

seen that two labeled products are formed (spots I & II) by the mitochondria. Only spot I was seen when microsomes were used as the enzyme source (Fig. 1). The lipids corresponding to spots I and II in Fig. 1 were separately eluted and rechromatographed in the same solvent system. Each compound retained its respective R_F value and was homogeneous showing that I and II are different compounds and not an artifact of the thin layer chromatography system. Both lipids I and II were formed when myristic, oleic, and linoleic acids were substituted for palmitic acid in the incubation mixture. In separate experiments, it was found that rat liver mitochondria also formed lipids I and II

when incubated with the ^{14}C fatty acid as described above. The main product (spot I, Fig. 1) formed from either labeled substrate is identified as 1-alkyl-2-acyl-sn-glycerol-3-phosphate as evidenced by comigration of the labeled product with authentic phosphatidic acid in two different solvent systems on thin layer chromatograms. This product also can be hydrolyzed either by alkali or by snake venom phospholipase A_2 to 1-alkyl-sn-glycerol-3-phosphate and fatty acid. Hydrolysis of the lipid corresponding to spot II also gave 1-alkyl-sn-glycerol-3-phosphate. However, the fatty acids liberated from II by hydrolysis migrated differently than normal long chain fatty acids on thin layer chromatograms. Figure 2 shows the migration rates of the radioactive fatty acids resulting from alkaline methanolysis of lipids I and II which were formed from $[1-^{14}\text{C}]$ palmitic acid (lanes 3 and 4, Fig. 1). It is seen in Fig. 2 (lane 1) that the main product from lipid I migrated with the methyl ester of fatty acid. Two different products (lane 2) were also formed from lipid II which migrated with methyl ester of β -hydroxy fatty acid and free β -hydroxy fatty acid. Treatment of the labeled lipids in lanes 1 and 2 (Fig. 2) with methanol, HCl, and dimethoxypropane produced the products shown in lanes 3 and 4, respectively. It is observed that after methylation the main fatty acid from spot I (Fig. 1) migrated with normal fatty acid methyl ester and the main fatty acids from spot II (Fig. 1) migrated with the methyl ester of β -hydroxy fatty acid. Some contamination of one with the other, resulting from incomplete resolution of spots I and II (Fig. 1) can be seen.

It was also found that when $[1-^{14}\text{C}]$ palmitic acid was incubated with guinea pig liver mitochondria under the same conditions as described in Fig. 1, but without alkyl GP, a polar fatty acid was formed. This polar fatty acid was purified by silicic acid column chromatography and was methylated with acidic methanol and dimethoxypropane. From the chromatographic properties of the polar fatty acid and its methyl ester (lanes 5 and 6, Fig. 2) and also from its chemical properties (see below) this polar fatty acid was shown to be β -hydroxy fatty acid.



Scheme 1: Chemical characterization of β -hydroxy fatty acid.

b. Identification of 1-alkyl-2-(3-hydroxy)acyl-sn-glycerol-3-phosphate.

The fatty acid released from spot II was identified as β -hydroxy fatty acid by the following methods. On treatment with dimethoxypropane and methanol the radioactive compound which migrated with β -hydroxy fatty acid disappeared and only one major product was found which migrated with the methyl ester of β -hydroxy fatty acid (Fig. 2, lane 4, spot V). Under the same conditions standard β -hydroxy fatty acids are methylated to their methyl esters. The methyl ester of the fatty acid from lipid II upon treatment with acetic anhydride in pyridine formed a compound which on thin layer chromatography on silica gel (hexane:ether:acetic acid, 30:70:1) co-migrated with the acetylated derivative of standard methyl β -hydroxy fatty acid ($R_f = 0.64$). On reduction with Vitride the fatty acid from lipid II formed a product which had the same migration rate as long chain 1,3 diol on thin layer chromatography (see Methods). The reduced compound was unchanged with respect to its migration on thin layer chromatography after treatment with periodate. Under the same conditions periodate completely oxidized 1,2 diols. On treatment with acetone and perchloric acid the reduced compound (diol) was completely

converted to a less polar product which had the same migration rate as the acetonide of long chain 1,3 diols (R_f ; 0.66, compared to diol, R_f ; 0.11). Finally, the methyl ester of the acetylated derivative of the radioactive polar fatty acid formed from $[1-^{14}\text{C}]$ palmitic acid was injected with the same derivatives of unlabeled carrier β -hydroxy fatty acids into the gas chromatographic column and different fractions eluting from the column were collected. When the radioactivity of these fractions was determined the major radioactive peak had the same retention time (R_t - 3.8 minutes) as the methyl ester of 3-acetoxy palmitate. This showed that the $[1-^{14}\text{C}]$ palmitic acid was converted to the β -hydroxy derivative in the mitochondria without degradation or chain elongation during the incubation.

The polar fatty acid formed from $[1-^{14}\text{C}]$ palmitic acid behaved chemically exactly as the fatty acid from spot II (see Scheme 1). When this labeled β -hydroxy fatty acid was substituted for $[1-^{14}\text{C}]$ palmitic acid in the incubation mixture with alkyl GP, only spot II and no spot I was observed on the thin layer chromatogram. Guinea pig liver microsomes would also form spot II when provided with β -hydroxy fatty acid in the incubation mixture. Both spots I and II were formed when 1-acyl-sn-glycerol-3-phosphate was substituted for alkyl GP in the incubation mixture.

DISCUSSION

Scheme 1 describes the chemical methods employed to establish the structure of the polar fatty acid present in the phosphatidate fraction. The formation of an acetyl derivative and the reduction to a diol showed the presence of a hydroxyl group in the fatty acid. The cyclic ketalization of the reduced compound with acetone indicated that it was either 1,2 or 1,3 diol. However, the resistance of the diol to the periodate oxidation showed that vicinal hydroxyl groups were not present. The only alternative explanation was that the reduced compound was a 1,3 diol and therefore the polar fatty acid was a 3-hydroxy fatty acid. Gas-liquid chromatography proved that the β -hydroxy fatty acid had the same carbon chain length as the parent fatty acid used in the incubation mixture.

It has been established that mitochondria from various tissues are capable of forming β -hydroxy fatty acids during in vitro incubations with free fatty acids (11,12). The present report extends this finding and gives evidence that mitochondria use this substrate to esterify mono-alkyl or mono-acyl GP to form the corresponding phosphatidate derivatives. This would imply that mitochondria and microsomes are also capable of activating a β -hydroxy fatty acid with coenzyme A. This implication is further suggested by the fact that when ^{14}C -labeled β -hydroxy fatty acid is incubated with alkyl GP the β -hydroxy [^{14}C]fatty acid is found esterified to the C-2 of glycerol in alkyl GP.

A number of workers have reported the formation of some unknown phospholipid during the acylation of sn-glycerol-3-phosphate (13,14,15). It is possible that some of these unknown lipids are similar to the β -hydroxy fatty acid containing phospholipid described here, e.g., 1-acyl-2-(3-hydroxy)acyl-sn-glycerol-3-phosphate. Anderson and Sansone (13) actually indicated that some phosphorylated derivative of a β -hydroxy fatty acid was formed in the incubation mixture when mitochondria were used as the enzyme source. The "phosphorylated derivative" may well have been a phosphatidic acid containing the esterified β -hydroxy acid. This can be easily investigated by using the chromatographic systems described here.

The formation of phosphatidic acid in vitro containing β -hydroxy acid raised the interesting possibility of whether natural glycerolipid in liver (or other organs) may contain some β -hydroxy fatty acid. However, formation of the lipid containing β -hydroxy fatty acid may be an artifact of the in vitro incubation and because of compartmentalization in vivo, the β -hydroxy acids may not be available for the acylation reaction in the glycerolipid biosynthesis. We are at present investigating the possible presence of β -hydroxy fatty acid in "natural" lipids.

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