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ACYL COENZYME A: 2-ACYL-*sn*-GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE ACTIVITY IN RAT LIVER MICROSOMES

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SUMMARY

2-Acylglycerol-3-phosphate can be acylated by acyl-CoA in the presence of microsomal preparations from rat liver. With optimal amounts of substrate, the acyltransferase reaction to the 1-position occurred at about one tenth the rate observed for the 2-position. Oleate was esterified more rapidly than palmitate or stearate under the conditions used. The enzymic activity catalyzing esterification of the primary hydroxyl seems to be different from that for the secondary hydroxyl.

INTRODUCTION

Diacyl-*sn*-glycerol-3-phosphate, which is a precursor of all diacyl glycerolipids, was shown to be synthesized through the acylation of *sn*-glycerol-3-phosphate[1,2]. Although dihydroxyacetone phosphate and acyl dihydroxyacetone phosphate could be lipid precursors[3], they do not appear to be involved in the selective synthesis of diacylglycerol-3-phosphate from added glycerol in rat liver[4]. Diacylglycerol-3-phosphate might be formed from glycerol-3-phosphate by the action of four separate activities involving either 1- or 2-acylglycerol-3-phosphate as intermediate.

The relative importance of the two monoacyl intermediates *in vivo* or *in vitro* has not been elucidated. Efforts in our laboratory to isolate and characterize the structure of the intermediate monoacylglycerol-3-phosphate have not been successful with rat liver particles since monoacylglycerol-3-phosphate did not accumulate during the reaction. Although 1-acyl-*sn*-glycerol-3-phosphate has been shown to be acylated (II_2 activity) at much faster rates and by a different enzymic activity than is glycerol-3-phosphate in microsomes[5,6], 2-acyl-*sn*-glycerol-3-phosphate acylation (II_1 activity) has not been reported. Recent developments in methods, as reported by EIBL AND LANDS[7] and further described in this paper, have allowed us to prepare 2-acylglycerol-3-phosphate and to examine its activity in the synthesis of diacylglycerol-3-phosphate.

Abbreviation: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

This paper describes the reaction of 2-acylglycerol-3-phosphate with several acyl-CoA's in a liver microsomal acyltransferase system.

MATERIALS AND METHODS

Acyl-CoA esters were synthesized by modifications of SEUBERT's[8] procedure from fatty acyl chlorides and CoA (Boehringer Mannheim, Germany) as described previously[9]. Bovine serum albumin (Fraction V, fatty acid poor) is a product of Pentex (Kankakee, Ill.). Microsomes were prepared from perfused rat livers as described by EIBL *et al.*[10]. Submicrosomal fractions were obtained as described previously[9,22]. The protein content was estimated with a nomograph based on the values given by WARBURG AND CHRISTIAN[11]. 1-Acylglycerol-3-phosphate was prepared as described by LONG, *et al.*[12]. [1-^{14}C]Acyl-2-acylglycerol-3-phosphate was prepared by phospholipase D hydrolysis of specifically labelled phosphatidylcholine, which was prepared from [^{14}C]palmitoyl-CoA and 2-acyl-glycerylphosphorylcholine through the reaction catalyzed by microscomal acyltransferase[5].

1-Alkenyl-2-acylglycerol-3-phosphate, a precursor of 2-acylglycerol-3-phosphate was prepared either by phosphorylation of 1-alkenyl-2-acylglycerol[7], or by phospholipase D hydrolysis of 1-alkenyl-2-acylglycerol-3-phosphorylcholine as follows.

Hydrolysis of choline plasmalogen with phospholipase D

Choline phospholipids from beef heart were purified by chromatography on silicic acid columns. The alkenylacylglycerol-3-phosphorylcholine content was enriched by selective hydrolysis of the diacylglycerol-3-phosphorylcholine using a commercial phospholipase D (Calbiochem, Los Angeles, Calif.) (see Expt. 4 in Table I) as described by LANDS AND HART[13].

For the enzymic preparation of 1-alkenyl-2-acylglycerol-3-phosphate, the enzyme system noted as Expt. 7 in Table I was used. About 40 μmoles of choline phospholipids containing 25 μmoles of alkenylacylglycerol-3-phosphorylcholine was suspended in 3 ml of 0.2 M acetate buffer (pH 5.8) with 0.75 ml of 1 M $CaCl_2$ and 1.5 ml of ethyl ether. We then added 5 ml of a phospholipase D solution which had been prepared from cabbage leaves and purified up to stage 3 (acetone-precipitation step) as described by DAVIDSON AND LONG[14]. The mixture was incubated at 37° with shaking for 5–24 h, and ether was allowed to spontaneously evaporate during the incubation. After the addition of 0.75 ml of 1 M EDTA (pH 8.0), the lipids were extracted with 30 ml of chloroform–methanol (2:1, v/v) and then twice each with 30 ml of chloroform. The combined extracts were evaporated to dryness and 4 ml of absolute ethanol was added. The pH of the ethanol solution was carefully adjusted to 7.5 with small additions of 2 M NaOH saturated with NaCl. The precipitate, which contains the sodium salts of alkenylacylglycerol-3-phosphate, diacylglycerol-3-phosphate as well as a small contaminant of unhydrolyzed choline phospholipids, was collected by centrifugation and dissolved in 2 ml of chloroform–methanol (2:1, v/v). Any insoluble material at this stage was removed by centrifugation. The soluble fraction was evaporated to dryness and the residue was washed twice with 4 ml of absolute ethanol to remove choline phospholipids and the treated residue was finally redissolved in chloroform–methanol (2:1, v/v). About one-third of the alkenyl derivative was recovered in the ethanol-insoluble fraction. The alkenyl acylglycerol-3-

TABLE I

HYDROLYSIS OF CHOLINE PLASMALOGEN BY PHOSPHOLIPASE D

Incubation mixture consisted of choline phospholipids containing 25 μmoles of alkenyl ether, 3 ml of 0.2 M acetate buffer (pH 5.8), or 0.2 M imidazole buffer (pH 7.0), 0.75 ml of 1 M $CaCl_2$, enzyme solution as indicated and 1.5 ml of ethyl ether.

Expt. No.	*Incubation condition*			*% Recovery of alkenyl ether bond in*		
	Buffer	*Enzyme*	*Time (h)*	*1-Alkenyl-2-acylglycerol-3-phosphate*	*1-Alkenyl-2-acylglycerol-3-phosphoryl choline***	*Total*
1	Acetate	A* 2 mg	5	2	78	80
2	Imidazole	A* 2 mg	5	0.1	87	87
3	Acetate	A* 10 mg	24	19	65	84
4	Imidazole	A* 10 mg	24	7	93	100
5	Acetate	B** 2.5 ml	5	14	81	95
6	Imidazole	B** 2.5 ml	5	0.2	100	100
7	Acetate	B** 5.0 ml	24	33	46	79
8	Imidazole	B** 5.0 ml	24	11	85	96

* A commercial preparation from Calbiochem (0.6 I.U./mg).
** An enzyme solution prepared according to DAVIDSON AND LONG[14], see text for details.
*** The recovered material was approx. 80% pure on the basis of alkenyl:phosphorus ratio.

phosphate content measured by the method of GOTTFRIED AND RAPPORT[15] was about 40% of the value for phosphate[16]. For the preparation of 2-acylglycerol-3-phosphate, the fraction was further purified by column chromatography on $NaHCO_3$-treated silicic acid[34,35]. The fraction eluted with chloroform–methanol (100:1 to 10:1, by vol.) showed a single spot on silicic acid (200–235 mesh) thin-layer chromatograms with chloroform–ethanol–88% formic acid–water (200:20:6:1, by vol.). By using twice the amount of freshly prepared enzyme solution in Expt. 7 in Table I, complete hydrolysis of the alkenyl-containing choline derivative was achieved in 20 h and the final product was 70% pure on the basis of alkenyl-phosphorus ratio.

Preparation of 2-acylglycerol-3-phosphate from 1-alkenyl-2-acylglycerol-3-phosphate

2-Acylglycerol-3-phosphate was prepared as described by EIBL AND LANDS[7] and the purity was checked by thin-layer chromatography and by the periodate method. However, when 2-acylglycerol-3-phosphate was to be used as substrate for acyl-transferase studies, the method was modified slightly to remove the small amount of remaining iodine or iodo-compounds which interfere with the enzyme assay. A lipid sample containing 2.5 μmoles of alkenylacylglycerol-3-phosphate was suspended in 1.5 ml of petroleum ether and 1.5 ml of 0.1 M boric acid (pH was adjusted to 5.5 with sodium citrate). While shaking the mixture vigorously with a Vortex mixer, iodine, saturated in petroleum ether, was added dropwise until the iodine color remained for a few minutes. After adding 1.5 ml of methanol, the solution was left standing for 5 min with occasional shaking. Excess iodine, iodo-aldehyde and unhydrolyzed diacylglycerol-3-phosphate were removed by 6–7 extractions with 10 ml of petroleum ether. To the remaining lower layer containing 2-acylglycerol-3-phosphate, 2 drops of 0.05 M $Na_2S_2O_3$ solution were added and the solution was washed once with 10 ml of petroleum ether. The 2-acylglycerol-3-phosphate was then extracted from the aqueous solution with 8 ml of chloroform followed by 8 ml of chloroform–methanol (2:1, v/v). The combined chloroform extracts were washed with 2 ml of 0.1 M boric acid and the chloroform solution was evaporated to dryness

under N_2. The residue was suspended in 2 ml of 0.1 M boric acid. Recovery of the 2-acylglycerol-3-phosphate was about 70–80% of the starting 1-alkenyl-2-acylglycerol -3-phosphate. The product showed a single spot on silicic acid thin-layer chromatography with the solvent described above and the content of 1-acylglycerol-3-phosphate isomer was less than 7% as measured by periodate method[17].

Assay of enzyme activity

Spectrophotometric assay system. A typical reaction mixture consisted of 0.1 ml of 0.01 m 5,5'-dithiobis-(2-nitrobenzoic acid), 20 nmoles of acyl-CoA, 0.2 mg of microsomal protein, 50–75 nmoles of acylglycerol-3-phosphate or 175 nmoles of 1-acylglycerol-3-phosphorylcholine in 0.1 ml of 0.1 M boric acid and 0.1 M Tris–HCl (pH 7.4) to make a final volume of 1 ml. When albumin was added in 0.1 M Tris–HCl (pH 7.4) solution, it was preincubated for 5 min with DTNB. Enzyme activities were assayed at room temperature by measuring continuously the released thiol group of the CoA with DTNB as described by LANDS AND HART[6].

Incorporation of labeled acyl-CoA. The incubation system was the same as in the spectrophotometric assay system except [^{14}C]acyl-CoA was used. The reaction was stopped after 1–2-min incubation by adding 10 vol. of chloroform–methanol (2:1, by vol.). The upper layer obtained after centrifugation was washed with 10 vol. of chloroform and then with 10 vol. of chloroform–methanol (2:1, by vol.), adding 1 vol. of 0.01 m citric acid to obtain two phases. The combined chloroform extracts were evaporated to dryness and the labeled lipids were subjected to silicic acid thin-layer chromatography as described above. Diacylglycerol-3-phosphate was extracted from the appropriate region of the thin-layer by scraping and treating the silicic acid with 5 ml of chloroform–methanol (1:2, by vol.) and twice with 5 ml of chloroform–methanol–1.5% NH_4OH (6:5:1, by vol.). The combined extracts were evaporated to dryness and the radioactivity in the 1- and 2-positions of diacylglycerol-3-phosphate was determined by phospholipase A (*Crotalus adamanteus*) hydrolysis as described by LANDS AND HART[5]. Radioactivity was measured in a Packard Tri-Carb scintillation spectrometer using aqueous dioxane scintillation fluid[18].

RESULTS

A spectrophotometric assay using DTNB[6] has been successfully used for the study of acyltransferases acting on acylglycerol-3-phosphorylcholine, acylglycerol-3-phosphorylethanolamine and 1-acylglycerol-3-phosphate. However, we could not apply the method to the measurement of the very low levels of 2-acylglycerol-3-phosphate acyltransferase activity in liver microsomes. When 2-acylglycerolphosphate was examined by this method, a relatively fast but brief initial velocity was observed with several acyl-CoA's. Experiments using labeled acyl-CoA revealed that the initial high velocities represented mostly the reaction of acyl-CoA with the small amount of 1-acylglycerol-3-phosphate isomer (less than 7%) in the 2-acylglycerol-3-phosphate preparation (Table II). Fig. 1 shows the time course of the incorporation of [^{14}C]oleoyl-CoA into the 1- and 2-positions of diacylglycerol-3-phosphate when a 2-acylglycerol-3-phosphate preparation was used as acyl acceptor. The radioactivity at the 1-position of diacylglycerol-3-phosphate was 35, 39 and 50% of the total in-

corporation at 1, 3 and 7 min of the incubation. Prolonged incubation up to 20 min by adding more acyl-CoA and enzyme preparation converted more than 40% of the initial monoacylglycerol-3-phosphate into diacylglycerol-3-phosphate. This product contained 84% of the incorporated radioactivity at the 1-position. The conversion of 1-acylglycerol-3-phosphate, which was present in the 2-acylglycerol-3-phosphate

TABLE II

CHROMATOGRAPHY OF PRODUCTS FROM RADIOACTIVE PHOSPHATIDATES AFTER PHOSPHOLIPASE A TREATMENT

Labeled diacyl-glycerol-3-phosphate was hydrolyzed by snake venom phospholipase A as described in the text. The products were separated on silicic acid thin-layer chromatogram with benzene–ethyl ether–88% formic acid (100:100:4, by vol.). The numbers indicate the radioactivity in counts/min. of similarly sized blocks of silicic acid from a plate.

Diacyl-GP	*Reaction products of 2-acyl-GP + stearoyl-CoA*		*Reaction products of 1-acyl-GP + linoleoyl-CoA*		[1-^{14}C]*Acyl-2-acyl-GP**	
Phospholipase A treatment	−	+	−	+	−	+
	0	30	5	27	11	8
Fatty acid	13	2092	16	2420	8	67
	17	176	150	679	19	8
	16	52	18	589	18	19
	27	39	33	139	46	83
Diacyl-GP	1028	184	2118	345	1495	720
	1172	116	1155	151	643	725
	355	50	294	37	222	156
	411	56	265	28	205	146
	302	39	231	30	128	101
	400	213	244	118	214	492
Acyl-GP	156	422	128	134	127	378
	539	1212	453	216	596	809

Abbreviation: GP, glycerol-3-phosphate.

* Hydrolysis was incomplete in this experiment because of high substrate to enzyme ratio.

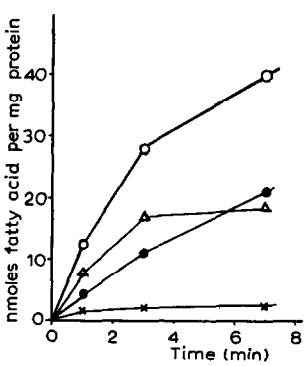

Fig. 1. Acylation of the 2-acylglycero-3-phosphate preparation by [^{14}C]oleoyl-CoA. Reaction mixture contained 77 nmoles of 2-acylglycero-3-phosphate, 5 μmoles of boric acid, 20 nmoles of [^{14}C]oleoyl-CoA, 1 μmole of DTNB, 0.2 mg of rat liver microsomal protein and 85 μmoles of Tris–HCl (pH 7.4) in a final volume of 1 ml. Labeled diacylglycero-3-phosphate was isolated and the radioactivities at the 1- and 2-positions were determined by phospholipase A hydrolysis as described in the text. ○—○, total radioactivity in diacylglycero-3-phosphate fraction; ●—●, radioactivity at the 1-position of diacylglycero-3-phosphate; △—△, radioactivity at the 2-position of diacylglycero-3-phosphate; x—x, total radioactivity in diacylglycero-3-phosphate fraction when 2-acylglycero-3-phosphate was omitted.

TABLE III

ACYL TRANSFER RATES IN DIACYLGLYCEROL-3-PHOSPHATE SYNTHESIS FROM MONOACYLGLYCEROL-3-PHOSPHATES

The incubation systems for (1) and (2) were as described in the text except that 100 nmoles of acylglycerol-3-phosphate were used in the presence of 2.5 mg of albumin. Expt. (3) was done with 25 nmoles of [^{14}C]acyl-CoA, 40 nmoles of 2-acylglycerol-3-phosphate, 1 μmole of DTNB and 10 mg of microsomal protein in 1 ml to provide a greater percent of reaction. Reactions were stopped after 1 min of incubation. The increase in radioactivity of the fatty acid and monoacylglycerol-3-phosphate fractions is shown in parentheses as a percentage of the observed decrease in the diacylglycerol-3-phosphate fraction.

Lipid acceptor	*Acyl-CoA*	*Palmitoyl*		*Stearoyl*		*Oleoyl*		*Linoleoyl*		*Linolenoyl*	
	Venom treated:	−	+	−	+	−	+	−	+	−	+
(1) 1-Acyl-GP	Fatty acid	347	3530	267	3190	443	2637	527	2187	327	3024
	Δ	+3183	(101%)	+2923	(104%)	+2194	(106%)	+1660	(106%)	+2697	(103%)
	Diacyl-GP	3525	386	3236	428	2620	547	2019	446	3358	750
	Δ	−3139		−2808		−2073		−1573		−2608	
	Acyl-GP	173	127	188	74	178	58	151	64	248	157
	Δ	−46		−114		−120		−87		−91	
	nmoles/min per mg to 2-position	32		27		33		29		35	
(2) 2-Acyl-GP	Fatty acid	536	2219	720	2560	1076	1905	1002	1909	486	2297
	Δ	+1683	(89%)	+1840	(87%)	+829	(73%)	+907	(87%)	+1811	(95%)
	Diacyl-GP	2415	528	2776	654	1743	600	1694	649	2475	574
	Δ	−1877		−2122		−1143		−1045		−1901	
	Acyl-GP	166	370	246	529	193	508	208	346	409	494
	Δ	+204	(11%)	+283	(13%)	+315	(27%)	+138	(13%)	+85	(4.5%)
	nmoles/min per mg to 1-position	2.8		3.3		7.2		3.1		1.5	
(3) 2-Acyl-GP	Fatty acid	108	1212	93	1208	75	344	69	478		
	Δ	+1104	(70%)	+1115	(71%)	+269	(47%)	+409	(67%)		
	Diacyl-GP	2110	544	2191	623	1142	564	1118	503		
	Δ	−1566		−1568		−578		−615			
	Acyl-GP	405	867	365	819	212	521	241	447		
	Δ	+462	(30%)	+454	(29%)	+309	(53%)	+206	(33%)		
	nmoles/min per mg to 1-position	0.9		0.8		2.2		1.3			

Abbreviation: GP, glycerol-3-phosphate.

preparation, into diacylglycerol-3-phosphate amounted to about 3–4 nmoles at 3 min of incubation. Further, incubation did not increase this amount significantly, indicating that most of 1-acylglycerol-3-phosphate present in the substrate preparation had been converted to diacylglycerol-3-phosphate during the first 3 min of incubation. We were not successful in obtaining 2-acylglycerol-3-phosphate preparations that were completely free of the 1-acyl isomer, nor could we rule out the possibility of acyl migration during the incubations. Both types of 2-acylglycerol-3-phosphate preparations gave similar analytical data and showed similar properties as substrates of acyl-CoA:2-acylglycerol-3-phosphate acyl transfer reaction. Efforts to obtain 2-acylglycerol-3-phosphate preparations with higher purity were not successful. Thus an accurate measure of 2-acylglycerol-3-phosphate acyltransferase activity could only be made by using labeled acyl-CoA and determining the position that had been acylated.

Acyltransfer rates for several acyl-CoA's were determined from percent radioactivity detected at the appropriate positions as shown in Table III. When 1-acylglycerol-3-phosphate was used as an acceptor, all of the incorporated radioactivity was found at the expected position. The loss of counts from the acylglycerol-3-phosphate region may be due to cleavage of diacylglycerol-3-phosphate that had trailed into this region. The "trailing phenomenon" was frequently encountered to some degree with the acidic lipids allowing only approximate estimates of the distributions and esterification rates. When a 2-acylglycerol-3-phosphate preparation served as acceptor in these systems less than 53% of the total radioactivity was at the 1-position. Although the relative radioactivity at the 1-position of diacylglycerol-3-phosphate could be increased by incubating a smaller amount of the 2-acylglycerol-3-phosphate preparation with more enzyme as shown in Expt. 3 in Table III, the system showed a greater percent conversion but no longer gave a measure of the initial or maximum velocity. These incubations represent extensions of the acylation events seen in Fig. 1. The relative acyl transfer rates for acyl-CoA's in Expt. 3 were quite similar to those observed in Expt. 2. The activity of 2-acylglycerol-3-phosphate as an acceptor of microsomal acyltransferase was only 8–23% of that of 1-acylglycerol -3-phosphate for the thiol esters examined. Saturated acyl-CoA's did not give higher acyl transfer rates than unsaturated ones when 2-acylglycerol-3-phosphate was an acceptor. In fact, oleoyl-CoA showed the highest acyl transfer rate to the 1-position of 2-acylglycerol-3-phosphate among four acyl-CoA's tested. Also, when 1-acylglycerol-3-phosphate was an acceptor, the unsaturated acyl-CoA's did not give significantly higher rates than the saturated acyl-CoA's examined, confirming the earlier report by LANDS AND HART[6].

The activities of the isomeric monoacylglycerol-3-phosphates as acceptors of a mixture of labeled palmitoyl- and linoleoyl-CoA's in microsomes were compared in the presence of an equimolar mixture of the isomeric monoacylglycerol-3-phosphates. As shown in Fig. 2. the incorporation of radioactivity into the 1-position was less than 5% of that into the 2-position under these conditions.

Three submicrosomal fractions were obtained by deoxycholate treatment as described previously[9,22]. 1-Acylglycerol-3-phosphate and 2-acylglycerol-3-phosphate acyltransferase activities in these fractions are compared in Table IV. With each of the three submicrosomal fractions, 43–59% of the total radioactivity incorporated into diacylglycerol-3-phosphate was found at the 1-position when a 2-acylglycerol-3-

phosphate preparation served as an acceptor. The "light" enzyme fraction had highest specific and total activities, showing 2- to 3-fold purification based on crude microsomes. However, similar treatment provided about ten-fold purification of the 1-acylglycerol-3-phosphorylcholine acyltransferase activity. The pellet seemed to contain relatively less of the 1-acylglycerol-3-phosphate acyltransferase activity compared to the other activities.

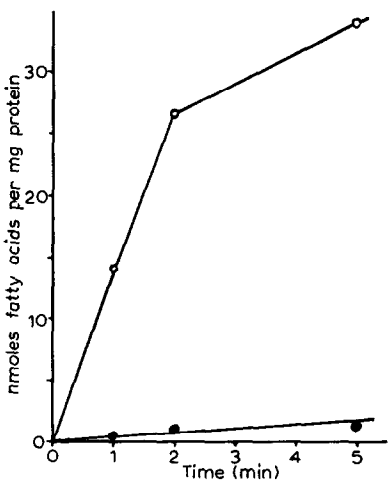

Fig. 2. Time course of the acylation of the isomeric monoacylglycerol-3-phosphates. Incubation mixture contained 0.2 mg of microsomal protein, 2.5 mg of bovine serum albumin, 40 nmoles of 1-acylglycerol-3-phosphate, 40 nmoles of 2-acylglycerol-3-phosphate, 5 μmoles of boric acid, 10 nmoles of labeled palmitoyl-CoA, 10 nmoles of labeled linoleoyl-CoA, 1 μmole of DTNB and 85 μmoles of Tris–HCl (pH 7.4) in a final volume of 1 ml. ●—●, fatty acids incorporated into the 1-position; ○—○, fatty acids incorporated into the 2-position.

TABLE IV

COMPARISON OF ACYLTRANSFERASE ACTIVITIES

Abbreviations: 16:0 and 18:1 represent palmitoyl- and oleoyl-CoA's. 1aGP, 2aGP and 1aGPC represent 1-acylglycero-3-phosphate, 2-acylglycero-3-phosphate and 1-acylglycero-3-phosphorylcholine (*e.g.* 16:0-1aGP represents palmitoyl-CoA:1-acylglycero-3-phosphate acyltransferase activity). 1-Acylglycero-3-phosphate acyltransferase and 1-acylglycero-3-phosphorylcholine acyltransferase activities were measured spectrophotometrically. The values are the average of two separate experiments. 2-Acylglycero-3-phosphate acyltransferase activity was measured by using [^{14}C]acyl-CoA's and the values were corrected by using phospholipase A as described in the text. Pellet, heavy and light fractions correspond to EI from pellet, EII from 0.5 M sucrose fraction and EIII from 0.25 M sucrose fraction respectively as described previously[9]. The numbers in parentheses represent relative specific activities based on 1.0 for crude microsomes.

Fraction	*Acyltransferase activity (nmoles/min per mg protein)*											
	16:0–1aGP		*16:0–2aGPC*		*16:0–1aGP*		*18:1–1aGP*		*18:1–2aGP*		*18:1–1aGPC*	
Crude microsomes	38.5	(1.0)	2.8	(1.0)	7.5	(1.0)	47.0	(1.0)	7.0	(1.0)	28.7	(1.0)
Heavy fraction	52.0	(1.4)	10.0	(3.6)	64.1	(8.5)	80.7	(1.7)	12.1	(1.7)	178	(6.2)
Light fraction	66.3	(1.7)	10.1	(3.6)	82.5	(11.0)	94.0	(2.0)	14.0	(2.0)	228	(7.9)
Pellet fraction	5.2	(0.1)	3.1	(1.1)	6.7	(0.9)	14.3	(0.3)	5.0	(0.7)	58.1	(2.0)

DISCUSSION

1-Alkenyl-2-acylglycerol-3-phosphate, a precursor of 2-acylglycerol-3-phosphate, could be prepared by two methods, one by phosphorylation of 1-alkenyl-2-acylglycerol and the other by phospholipase D hydrolysis of choline from the plasmalogen derivative. The former method has an advantage of providing a preparation without contamination by diacylglycerol-3-phosphate. The second method, however, requires less demanding techniques but does require more enzyme. The results in Table I help clarify an apparent contradiction between reports[13,36] that plasmalogens were (or were not) hydrolyzed by cabbage phospholipase. The diacyl and alkenylacyl derivatives reacted at such different rates that modest amounts of enzyme working at the less favorable pH of 7.0 cleaved nearly all of the diacyl form in a short time while the alkenylacyl derivative remained essentially intact. Only with large amounts of enzymic activity at optimal pH and for prolonged time could an appreciable amount of choline be cleaved from the alkenylacyl derivative. Although phospholipase D hydrolyzed the choline from the alkenyl derivative at pH 5.8, the alkenyl ether products were not wholly stable at this pH so that prolonged incubation did not increase the yield of alkenylacylglycerol-3-phosphate.

Activity of 2-acylglycerol-3-phosphate as substrate of microsomal acyltransferases was much lower than that of 1-acylglycerol-3-phosphate as shown in Tables III and IV. The difference is made more obvious when 1-acylglycerol-3-phosphate and 2-acylglycerol-3-phosphate are both present in the incubation mixture as shown in Fig. 2. If the relative activities observed with the two isomeric monoacylglycerol-3-phosphates reflect the phenomenon *in vivo*, we might expect that diacylglycerol-3-phosphate is synthesized mainly through a pathway catalyzed by Enzyme I_1 (first acylation at the 1-position) and Enzyme II_2 (second acylation at the 2-position). However, 2-acylglycerol-3-phosphate acyltransferase activity in microsomes was comparable to or a little higher than the observed enzymic activity catalyzing the first acylation of glycerophosphate. The rate-limiting step of diacylglycerol-3-phosphate synthesis from glycerophosphate *in vivo* seems not to be the second acylation (II_1, or II_2) since the intermediate, monoacylglycerol-3-phosphate does not accumulate. Accordingly, the data shown in this paper do not exclude the involvement of the pathway ($I_2 \rightarrow II_1$) in the synthesis of diacylglycerol-3-phosphate from glycerophosphate *in vivo*. Although both isomers of monoacylglycerol-3-phosphate have been reported to be synthesized from glycerophosphate at comparable rates[24] with microsomes, efforts to confirm the accumulation of monoacylglycerol-3-phosphates in rat liver microsomes have not been successful so far in this laboratory. Information on the relative activity of enzyme I_1 and enzyme I_2, if both are present, will be necessary to understand the relative importance of these two pathways. In addition, the monoglyceride kinase pathway[23] should be taken into account in understanding the general features of the synthesis of diacylglycerol-3-phosphate *in vivo*.

It has been shown that the acylation of glycerophosphate in microsomes is susceptible to sulfhydryl reagents whereas the acylation of 1-acylglycerol-3-phosphate is not, thus suggesting that these two reactions are catalyzed by different enzymes. 2-Acylglycerol-3-phosphate acyltransfer reaction like that for the 1-acyl isomer was not inhibited by a sulfhydryl reagent, DTNB. Nevertheless, the activity ratios of 1-acylglycerol-3-phosphate and 2-acylglycerol-3-phosphate acyltransfer reactions

with palmitoyl-CoA observed for the four microsomal preparations vary significantly, suggesting that separate enzymes in microsomes catalyze the acylation of the 1- and 2-positions. The variations of activity ratios among submicrosomal fractions may reflect the difference in the effect of deoxycholate on these enzymes or the difference in the proportion of different membrane components such as endoplasmic reticulum, plasma membrane and Golgi structures. The differences observed indicate that further investigations on the multiplicity of acyltransferases would seem profitable.

Glycerophosphate acyltransferase activity has been reported to be present in mitochondrial fractions[25,29]. Sonicated mitochondria were examined as an enzyme source to acylate 2-acylglycerol-3-phosphate in the standard assay system described in MATERIALS AND METHODS. A prelimiminary experiment showed that the specific activities were about one-tenth of those found in microsomes (0.27 and 0.78 nmole/min per mg protein for palmitoyl- and oleoyl-CoA's, respectively) under these conditions. Whether or not the activities observed were derived from microsomal contamination was not examined.

At least one of the isomeric monoacylglycerol-3-phosphates should be involved in the synthesis of diacylglycerol-3-phosphate from glycerophosphate *in vivo*. However, as in the case of 1-acylglycerol-3-phosphate acyltransferase[6,30–32], observed relative acyl transfer rates of saturated and unsaturated acyl-CoA's into 2-acylglycerol-3-phosphate *in vitro* do not clearly reflect the exact fatty acid composition of the 1-position of diacylglycerol-3-phosphate isolated from[33] or newly synthesized in[19–21] rat livers *in vivo*.

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