Biosynthesis of Plasmalogens by the Microsomal Fraction of Fischer R-3259 Sarcoma. Influence of Specific 2-Acyl Chains on the Desaturation of 1-Alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine†

Robert L. Wykle* and Jacalyn M. Schremmer

ABSTRACT: In the Fischer R-3259 sarcoma, ethanolamine plasmalogens (1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine) are synthesized from 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (1-alkyl-2-acyl-GPE) by a microsomal desaturase that inserts a Δ^1 double bond in the alkyl chain. In the present study, a series of 1-[1-^{14}C]hexadecyl-2-acyl-GPE substrates containing specific acyl groups ranging from $C_{2:0}$ to $C_{20:4}$ at the 2 position were prepared and tested as substrates for the microsomal Δ^1-alkyl desaturase. The microsomal preparations contained an acyl hydrolase that removed the $C_{2:0}$, $C_{4:0}$, and $C_{7:0}$ acyl groups from the 2 position. By inhibiting the hydrolase with diisopropyl fluorophosphate, it was possible to test conversion of the unaltered substrates to plasmalogens. The alkyl desaturase exhibited little discrimination among the specific acyl derivatives tested. The highest rate of desaturation was obtained with 1-[1-^{14}C]-hexadecyl-2-acyl-GPE synthesized in situ in the microsomes via acylation of 1-[1-^{14}C]hexadecyl-GPE; this rate was threefold that observed with exogenously acylated substrates. The 1-[1-^{14}C]hexadecyl-2-acyl-GPE synthesized in situ contained highly unsaturated acyl groups; no selectivity of the desaturase for specific acyl chains was detected when the different molecular species of 1-[1-^{14}C]alkyl-2-acyl-GPE and 1-[1-^{14}C]alk-1′-enyl-2-acyl-GPE were compared. The short-chain substrates, being more hydrophilic, mimicked the chromatographic behavior of 1-alkyl-GPE, yet they did not resemble the lyso compound in its higher conversion to plasmalogens. Thus, despite their similar R_f values, the packing of the short-chain acyl homologues in the membrane may be quite different from that of the lyso compound. Binding of 1-hexadecyl-2-acyl-GPE and 1-hexadecyl-GPE to microsomal membranes was similar. Detergents stimulated the desaturation of exogenously added 1-hexadecyl-2-acyl-GPE, but the level of activity remained at only one-third that obtained by using 1-hexadecyl-GPE as the precursor. The results suggest that the acylation step positions the substrate in the membrane so that it becomes more accessible to the alkyl desaturase.

The terminal step in the biosynthesis of ethanolamine plasmalogens, 1-alk-1′-enyl-2-acyl-GPE,[1] in mammals has been demonstrated in cell-free systems to be the desaturation of 1-alkyl-2-acyl-GPE (Blank et al., 1972; Paltauf, 1972; Paltauf & Holasek, 1973; Snyder et al., 1971; Wykle & Lockmiller, 1975; Wykle et al., 1970, 1972). The reaction is catalyzed by a cytochrome b_5 dependent desaturase that is similar to stearoyl-CoA desaturase and requires O_2 and NADH or NADPH (Palfauf & Holasek, 1973; Paltauf et al., 1974; Wykle & Lockmiller, 1975; Wykle et al., 1972). Although the diradyl compound is the true substrate for the desaturase, 1-alkyl-GPE serves as a better precursor than 1-alkyl-2-acyl-GPE in microsomal preparations from tumors (Wykle et al., 1972), brain (Blank et al., 1972; Wykle & Lockmiller, 1975), and intestinal mucosa (Paltauf & Holasek, 1973). The 1-alkyl-GPE is first acylated by endogenous substrates and then converted to the plasmalogen. The higher rate of conversion of 1-alkyl-2-acyl-GPE synthesized within the microsomes by acylation of alkyl-GPE, relative to exogenously acylated 1-alkyl-2-acyl-GPE, may be related to differences in the physicochemical interaction of alkyl-GPE and 1-alkyl-2-acyl-GPE with the microsomal membranes (Paltauf & Holasek, 1973; Wykle et al., 1972). On the other hand, the acylation process itself could spatially position the 1-alkyl-2-acyl-GPE within the membrane so that the substrate becomes more accessible to the desaturase. Alternatively, specificity of the desaturase for particular acyl chains introduced by the microsomes might account for the differences observed with the two precursors. The present study was undertaken to investigate some of these possibilities, namely, to determine if the alkyl desaturase exhibits specificity for individual acyl chains at the 2 position and to determine if substrates containing short-chain acyl groups might insert into the membrane in a manner similar to 1-alkyl-GPE and thus be converted to plasmalogens at a higher rate than the long-chain homologues.

Experimental Procedure

Materials

N,*N*-Dimethyl-4-aminopyridine (recrystallized from chloroform–diethyl ether) was purchased from ICN-K & K Laboratories, Plainview, NY. 2-[[(*tert*-Butoxycarbonyl)-oxy]imino]-2-phenylacetonitrile (Boc-ON) was obtained from Aldrich Chemical Co., Milwaukee, WI. Acetic anhydride was from Fisher Scientific Co.; butyric and heptanoic anhydrides were from Eastman Organic Chemicals, Atlanta, GA. Lauric, oleic, linoleic, and arachidonic anhydrides were purchased from Nu-Chek-Prep., Inc., Elysian, MN. Diisopropyl fluorophosphate and *p*-bromophenacyl bromide were obtained from Sigma Chemical, St. Louis, MO. Crystalline catalase (beef liver) was purchased from Boehringer Mannheim Biochem-

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[1] Abbreviations used: GPE, *sn*-glycero-3-phosphoethanolamine; Boc, *tert*-butoxycarbonyl.

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icals, Indianapolis, IN; NADH was from P-L Biochemicals, Inc., Milwaukee, WI.

Methods

Preparation of Substrates. 1-[1-^{14}C]Hexadecanol (55 mCi/mmol) was prepared by reduction of 1-[1-^{14}C]palmitic acid with Vitride [$NaAlH_2(OCH_2CH_2OCH_3)_2$] (Snyder et al., 1971; Wykle & Schremmer, 1974). 1-[1-^{14}C]Hexadecyl-2-acyl-GPE was prepared as described earlier (Wykle & Schremmer, 1974; Wykle et al., 1972) by labeling the lipids of Ehrlich ascites cells for 24 h with [1-^{14}C]hexadecanol (30 μCi/mouse) injected intraperitoneally into mice bearing the tumor cells. The phospholipids isolated from the tumor cells were first treated with HCl gas to remove plasmalogens (Ferrell et al., 1970). The diradyl fraction containing 1-[1-^{14}C]hexadecyl-2-acyl-GPE was then isolated from other lipids by loading the HCl-treated lipids from 10 mice on an activated silicic acid column (Unisil; 100–200 mesh; 40 g; 2 × 40 cm equilibrated with chloroform) and eluting the less polar lipids with 500 mL of chloroform–methanol (20:1 v/v); next, the 1-[1-^{14}C]hexadecyl-2-acyl-GPE was eluted with 500 mL of chloroform–methanol (5:1 v/v). This preparation was used as a substrate in designated experiments; since it was not saponified, it contained diacyl-GPE and alkylacyl-GPE in a molar ratio of 2:1 with 8% of the label in acyl groups. Wood & Snyder (1969) reported that the 1-alkyl-2-acyl-GPE of Ehrlich ascites cells contains a mixture of acyl groups having the following mass distribution: 18:1 (14%), 18:2 (18%), 20:4 plus 22:1 (26%), 22:6 (28%), and minor species (15%).

1-[1-^{14}C]Hexadecyl-GPE was prepared by a mild saponification procedure in which 1-[1-^{14}C]hexadecyl-2-acyl-GPE (30 μCi) was dissolved in 2 mL of chloroform to which 1 mL of methanol saturated with NaOH was added. The mixture was held at 37 °C for 80 min; next, the pH was adjusted to 7.0 with acetic acid and the products were extracted (Bligh & Dyer, 1959). The 1-[1-^{14}C]hexadecyl-GPE was purified from other products by preparative thin-layer chromatography (Silica Gel HR; developed in chloroform–methanol–ammonium hydroxide, 65:35:8 v/v) (Snyder, 1970a). This procedure yielded 24 μCi of product (2.3 mCi/mmol) that was 98% radiopure as determined by thin-layer zonal profile analysis (Snyder, 1970a,b). The specific activity was based on analysis of the phosphorus content (Rouser et al., 1966).

1-[1-^{14}C]Hexadecyl-*sn*-glycero-3-phospho-*N*-(*tert*-butoxycarbonyl)ethanolamine, hexadecyl-GPE(Boc), was prepared by a procedure similar to that described by Chakrabarti & Khorana (1975); we added 40 μmol of Boc-ON and 70 μmol of triethylamine dissolved in 200 μL of chloroform to 5.4 μmol of 1-[1-^{14}C]hexadecyl-GPE. The mixture was stirred for 24 h at room temperature. The products were then diluted with 10 mL of chloroform, and the polar residues were extracted into water. Analysis of the products by thin-layer zonal profile analysis (Silica Gel HR; chloroform–methanol–water, 70:25:4 v/v) showed that >99% of the starting material was converted to the Boc derivative; hexadecyl-GPE(Boc) had an R_f of 0.46 while hexadecyl-GPE had an R_f of 0.34.

Acylation of 1-[1-^{14}C]Hexadecyl-GPE(Boc) and Removal of the Boc Group. Specific acyl groups were added to the 2 position of 1-[1-^{14}C]hexadecyl-GPE(Boc) essentially by the procedure of Gupta et al. (1977); the series of acid anhydrides listed under Materials were employed. The typical reaction mixture contained 1 μmol of 1-[1-^{14}C]hexadecyl-GPE(Boc) (2.3 μCi), 40 μmol of *N*,*N*-dimethyl-4-aminopyridine, and 50 μmol of acid anhydride in 0.5 mL of dry chloroform freshly distilled over P_2O_5. The mixture was stirred for 2 days in a N_2 atmosphere at room temperature under anhydrous conditions. At the end of 2 days, the mixture was placed on a short silicic acid column; the unreacted anhydride and acid were then eluted with chloroform, and the 1-[1-^{14}C]hexadecyl-2-acyl-GPE(Boc) was eluted with methanol. The purity of each acyl derivative was determined by thin-layer chromatography on layers of Silica Gel HR developed in chloroform–methanol–water (70:25:4 v/v). Each acylated derivative was dissolved in 10 mL of dry chloroform, and the Boc group was removed by using HCl gas as described by Paltauf (1976). The purity of each substrate was >95% as determined by thin-layer zonal profile analysis (Snyder, 1970b) (Silica Gel HR; chloroform–methanol–water, 70:25:4 v/v). The substrates were stored at liquid nitrogen temperatures until immediately before addition to the incubation mixtures.

Preparation of Microsomal Fractions and Enzyme Assays. Microsomal fractions were isolated from Fischer R-3259 sarcomas as described earlier (Wykle et al., 1972), and only fresh preparations were used for the alkyl desaturase assays. Alkyl desaturase activity was measured by incubating the substrates with the microsomal preparations in the presence of NADH under an atmosphere of air (Wykle et al., 1972); catalase was included in the incubation mixture since it protects cytochrome b_5 and stimulates the alkyl desaturase of the tumor system (Baker et al., 1976). Products were extracted, and the label in alk-1-enyl groups was determined by measuring the labeled aldehyde released by exposing the products, separated on thin-layer plates, to HCl fumes (Schmid & Mangold, 1966; Wykle et al., 1972). The label in plasmalogen was also determined by measuring the dimethyl acetals formed by treatment of the purified 1-radyl-2-acyl-GPE's with HCl–methanol (Farquhar, 1962; Morrison & Smith, 1964). Details of experiments and assay conditions are described in the footnotes to the tables.

Results

Thin-Layer Chromatographic Behavior of Substrates. The following are typical R_f values for the labeled substrates after chromatography on layers of Silica Gel HR developed in chloroform–methanol–water (70:25:4 v/v): hexadecyl-GPE (0.27), hexadecyl-2-acetoyl-GPE (0.37), hexadecyl-2-butanoyl-GPE (0.40), hexadecyl-2-heptanoyl-GPE (0.48), and hexadecyl-2-acyl-GPE containing $C_{12:0}$ and longer acyl groups (0.56). The $C_{2:0}$, $C_{4:0}$, and $C_{7:0}$ derivatives were thus readily separated and easily distinguished from both hexadecyl-GPE and 1-alkyl-2-acyl-GPE derivatives containing $C_{12:0}$ and longer acyl chains. On the basis of its R_f, we expected the 2-acetoyl substrate to mimic 1-hexadecyl-GPE more closely than the long-chain substrates in its physical interaction with the microsomes.

Hydrolysis of Short-Chain Homologues of 1-Alkyl-2-acyl-GPE by Microsomal Preparations. When the $C_{2:0}$ derivative was tested as a substrate for the microsomal alkyl desaturase, we found that the acyl group had been hydrolyzed from greater than 90% of the substrate after incubating for 1 h. The system and conditions were the same as those described in Table I, with the exception that microsomes were not treated with diisopropyl fluorophosphate. Although the hydrolysis was most pronounced with the $C_{2:0}$ derivatives, 60% of the $C_{4:0}$ and 55% of the $C_{7:0}$ compounds were hydrolyzed. Most of the label (64%) from the 2-acetoyl substrate was found in 1-radyl-2-acyl-GPE containing long-chain groups in the 2 position; 13% was in the lyso compound, hexadecyl-GPE. The remaining label was found in unhydrolyzed substrate and in 1-alkyl-*sn*-glycerol, which is formed by lysophospholipase D and a phosphohydrolase in the system (Wykle & Schremmer, 1974). These results indicate that the 2-acetoyl substrate had

Table I: Desaturation of 1-[1-^{14}C]Hexadecyl-2-acyl-GPE Containing Specific Acyl Chains[a]

acyl chain	% of 1-[1-^{14}C]radyl-2-acyl-GPE as plasmalogen
2:0	3.2 ± 0.4
4:0	4.1 ± 0.1
7:0	4.8 ± 0.8
12:0	1.8 ± 0.1
18:1	3.2 ± 0.1
18:2	1.2 ± 0.2
20:4	2.9 ± 0.7
1-[1-^{14}C]hexadecyl-GPE	18 ± 0.1
1-[1-^{14}C]hexadecyl-2-acyl-GPE[b]	6.0 ± 0.1

[a] The freshly isolated microsomal preparation was suspended in water (12 mg of protein/mL) and incubated with diisoprpyl fluorophosphate (10 mM) for 15 min at 0 °C immediately before the desaturase reaction was initiated. The assay mixture contained 0.5 mL of the pretreated microsomal preparation (6 mg of protein), NADH (2 mM), catalase (0.1 mg; ca. 6500 units), Tris-HCl buffer (0.1 M; pH 7.2), and labeled substrates (15 nmol; 75 000 dpm). All substrates had the same specific radioactivity (2.3 mCi/mmol) and were added in 20 μL of diethyl ether–ethanol (2:1 v/v). The mixture in a total volume of 3 mL was shaken in uncapped vials at 160 oscillations/min in a Dubnoff shaker for 1 h at 37 °C. Products were extracted and analyzed as described under Methods; values are from duplicate incubations. [b] [1-^{14}C]Hexadecyl-2-acyl-GPE was used as isolated from Ehrlich ascites tumor lipids and contained predominantly long-chain polyunsaturated fatty acyl residues (see Methods).

been deacylated by the microsomal preparations and subsequently reacylated with endogenous long-chain acyl groups. In the reacylated product 17% of the label was in the 1-alk-1′-enyl groups of plasmalogens, which is the same percentage (20%) formed in the acylated product when 1-[1-^{14}C]hexadecyl-GPE was added directly to the incubation mixture. When derivatives containing $C_{12:0}$ or longer acyl chains were tested, the substrates were not hydrolyzed and the highest percentage of label in plasmalogens was 6%.

In order to prevent the hydrolysis of the short-chain acyl groups so that conversion of the unaltered substrates to plasmalogens could be tested, we sought an effective inhibitor of the hydrolase. *p*-Bromophenacyl bromide (0.1 mM), a potent inhibitor of snake venom phospholipase A_2 (Roberts et al., 1977), was included in the incubation mixture. Although it did not effectively inhibit hydrolysis of the $C_{2:0}$, $C_{4:0}$, and $C_{7:0}$ acyl groups, it totally blocked reacylation of the 1-hexadecyl-GPE released from the intact substrates (data not shown). No accumulation of the lyso compound (1-hexadecyl-GPE) occurred when the long-chain derivatives, C_{12} and longer, were tested in the presence of the inhibitor. This is further evidence the long-chain acyl groups are not hydrolyzed during the incubations. Isolation of the microsomal fractions in a homogenizing medium containing 10 mM EDTA and addition of EDTA to the incubation mixture failed to inhibit the hydrolysis of the short-chain substrates. However, pretreatment of the microsomal preparations with diisopropyl fluorophosphate (10 mM; 15 min at 0 °C) strongly inhibited the hydrolysis of the $C_{2:0}$, $C_{4:0}$, and $C_{7:0}$ acyl groups. The treatment with diisopropyl fluorophosphate did not inhibit alkyl desaturase (data not shown), and it was therefore included in the desaturase assay system.

Comparison of the 2-Acyl Homologues of 1-Alkyl-2-acyl-GPE as Substrates for the Alkyl Desaturase. The series of substrates listed in Table I were tested as plasmalogen precursors with microsomal preparations pretreated with diisopropyl fluorophosphate. Under the assay conditions, desaturation of the homologues was linear for the 1-h incubation period. The desaturation obtained with the lyso compound 1-alkyl-GPE was also linear for 1 h as observed earlier (Wykle et al., 1972). Although some of the 2-acyl derivatives were converted at higher rates than others (Table I), none of the acylated derivatives were converted to more than one-third the extent observed with the lyso precursor, 1-[1-^{14}C]hexadecyl-GPE. Neither acyl-CoA nor cofactors required for the synthesis of acyl-CoA were added to the reaction mixture (described in Table I), yet approximately 75% of the lyso compound was acylated during the incubation period, and, as observed earlier (Wykle et al., 1972), plasmalogens were found only in the acylated fraction. These results support the earlier studies of the tumor system that indicate the 2-hydroxy group must be acylated before desaturation occurs (Wykle et al., 1972). The 1-alkyl-2-acyl-GPE isolated from Ehrlich ascites tumor cells was converted at a higher rate than any of the organically synthesized homologues; perhaps the presence of 22:6 and other polyunsaturated acyl chains in the preparation influenced its rate of desaturation. The presence of the diacyl component did not appear to affect its rate of conversion. Other substrates contained no diacyl-GPE yet were converted at somewhat lower rates. Furthermore, mixing egg diacyl-GPE with the 18:2 homologue in a 2:1 molar ratio (diacyl-GPE–homologue) did not enhance desaturation of the homologue (data not shown). There were no large differences between the rates of conversion of the short-chain, more hydrophilic substrates and the rates of those containing the long-chain acyl groups. Desaturation of the 2:0 and 18:1 homologues was compared at concentrations ranging from 2.5 to 40 nmol/mL (data not shown). The percent of product formed was similar for the two homologues at all concentrations tested. The similarities in activities for the short-chain and long-chain homologues, therefore, do not appear to be altered by variations of substrate concentration.

Acyl Composition of Products Formed by Microsomal Acylation of 1-[1-^{14}C]Hexadecyl-GPE. Studies were carried out to investigate the nature of the acyl groups inserted by the microsomal preparations in the 2 position of 1-[1-^{14}C]hexadecyl-GPE and that of the fraction converted to alk-1′-enyl-2-acyl-GPE. 1-[1-^{14}C]Hexadecyl-GPE was incubated as described in Table I, except the microsomal preparation was not treated with diisopropyl fluorophosphate. The 1-[1-^{14}C]radyl-2-acyl-GPE fraction formed during the incubation was isolated from other products by thin-layer chromatography and treated with phospholipase C (Mavis et al., 1972) to release labeled 1-alkyl-2-acylglycerols and 1-alk-1′-enyl-2-acylglycerols. Acetate derivatives were prepared (Privett & Nutter, 1967), and the alkyl and alk-1-enyl types were isolated (Silica Gel G; toluene–methanol, 100:0.5 v/v). Little difference was found in the degree of unsaturation of the purified 1-alkyl-2-acylglycerol acetates and the 1-alk-1′-enyl-2-acylglycerol acetates as determined by argentation thin-layer chromatography (Privett & Nutter, 1967) using benzene as the developing solvent. Over 80% of the label in both classes was associated with molecular species containing three or more double bonds. The 2-acyl groups introduced by the microsomal preparation are therefore highly unsaturated, and no preference by the alkyl desaturase for different molecular species was detected.

Binding of Substrates to Microsomal Preparations and Conversion of Bound Substrates to Plasmalogen. The binding of 1-[1-^{14}C]hexadecyl-GPE and 1-[1-^{14}C]hexadecyl-2-acyl-GPE to the microsomal preparations was compared to determine if differences in affinities of the substrates for the

Table II: Binding of Substrates to Microsomes and Desaturation of [1-^{14}C] Hexadecyl-2-acyl-GPE Synthesized in Situ from [1-^{14}C] Hexadecyl-GPE

substrate	percentages A[c]	B[d]	C[e]	D[f]
preincubated[a]				
1-[1-^{14}C] hexadecyl-GPE	94	87	89	13
1-[1-^{14}C] hexadecyl-2-acyl-GPE	89	100	100	4
control[b]				
1-[1-^{14}C] hexadecyl-GPE			87	13
1-[1-^{14}C] hexadecyl-2-acyl-GPE			100	4

[a] Substrates were incubated with microsomes for 15 min under the same conditions described in Table I, except no NADH was added and the microsomal preparation was not treated with diisopropyl fluorophosphate; the 1-[1-^{14}C] hexadecyl-2-acyl-GPE added contained the acyl composition present as isolated from Ehrlich ascites cells (see Methods). The labeled microsomes were pelleted from the incubation mixture (100000*g* × 30 min; 4 °C) and washed once by resuspending in 0.25 M sucrose and pelleting again. The labeled microsomes were then incubated in the presence of NADH for 1 h at 37 °C as described in Table I. Binding was measured by extracting the lipids from the 100000*g* supernatants and pellets, and the amount of label in each fraction was determined. [b] Microsomes were preincubated for 15 min at 37 °C in the absence of NADH and substrates. The unlabeled microsomes were pelleted and washed one time as described above in the prelabeling experiments. Labeled substrates were then incubated with the microsomes and NADH for 1 h as in the prelabeling experiments (see footnote above). [c] Substrate bound to microsomes during preincubation. [d] Bound substrate acylated at the end of preincubation. [e] Substrate acylated after incubating 1 h in the presence of NADH. [f] Plasmalogen content of 1-[1-^{14}C]-radyl-2-acyl-GPE after incubating 1 h in the presence of NADH.

membranes might account for the large differences in activities observed with the two substrates. The two substrates were incubated with the microsomes for 15 min in the absence of NADH as described in Table II, and the distribution of label between the microsomal pellets and the 100000*g* supernatants was measured. Only slight differences in binding of the substrates to the microsomes were observed (Table II), and at the end of 15 min most of the 1-alkyl-GPE had been acylated. When the microsomal preparations containing the bound substrates were repelleted and washed in sucrose and then added to the incubation system along with NADH to initiate plasmalogen synthesis, we found that the 1-alkyl-2-acyl-GPE generated in situ was again desaturated to a greater extent than exogenously acylated substrates. Even though the substrates had been bound to the microsomal membranes for approximately 2 h during these procedures before the desaturase reaction was initiated, the rate of desaturation for each of the substrates was the same as in the controls where NADH and the substrates were added at the same time (Table II).

Effect of Detergents. Paltauf (1978) found that Tween 80 and taurodeoxycholate stimulated the Δ^1-alkyl desaturase of pig spleen microsomes, apparently by making the substrates more accessible to the desaturase in the membrane. We also examined the influence of detergents on the conversion of 1-[1-^{14}C]hexadecyl-GPE and 1-[1-^{14}C]hexadecyl-2-acyl-GPE to plasmalogens (Table III). The detergents, methods of dispersion, and concentrations of detergents were the same as those employed by Paltauf (1978) to give maximal activity. The acylation of 1-[1-^{14}C]hexadecyl-GPE by the microsomal preparations was not affected by the detergents; between 80 and 86% was acylated at the end of the incubation period in all the samples (data not shown). In the present study slight stimulation of the desaturase by Tween 80 was observed with alkyl-GPE, whereas both taurodeoxycholate and Tween 80 stimulated the desaturation of 1-alkyl-2-acyl-GPE (Table III).

Discussion

In this study a homologous series of 1-alkyl-2-acyl-GPE compounds containing specific acyl groups at the 2 position were synthesized and tested as substrates of the alkyl desaturase system present in tumor microsomal preparations. An acyl hydrolase present in the preparations hydrolyzed the $C_{2:0}$, $C_{4:0}$, and $C_{7:0}$ acyl groups but not the C_{12} and longer acyl groups from the substrates. Since the hydrolysis was not inhibited by EDTA, the enzyme responsible is apparently not a Ca^{2+}-requiring phospholipase A_2. Diisopropyl fluorophosphate normally does not inhibit phospholipase A_2, but it strongly inhibited the short-chain acyl hydrolase; the enzyme is possibly a nonspecific esterase. By treating the microsomes with diisopropyl fluorophosphate, it was possible to measure the desaturation of the unaltered substrates. The level of alkyl desaturase activity varied somewhat in different enzyme preparations, but with each preparation the relative activities obtained with the different substrates were always similar.

In all the studies described here, including those carried out in the presence of detergents, alkyl-GPE added to and acylated by the microsomal preparations yielded approximately threefold more alk-1′-enyl-2-acyl-GPE than did 1-alkyl-2-acyl-GPE added directly. In contrast to this large dissimilarity in activity, little selectivity was displayed by the desaturase for the different 2-acyl homologues tested, including those containing $C_{2:0}$, $C_{4:0}$, and $C_{7:0}$ chains. Furthermore, the alkyl desaturase did not appear to discriminate among the molecular species formed in the microsomes by acylation of 1-hexadecyl-GPE. The lack of specificity toward the 2-acyl components is consistent with the range of acyl chains found in the 2 position of plasmalogens in cells; e.g., in Ehrlich ascites cells the composition of the acyl groups in alkyl-2-acyl-GPE and alk-1′-enyl-2-acyl-GPE is almost identical (Wood & Snyder,

Table III: Effect of Detergents on the Conversion of 1-[1-^{14}C] Hexadecyl-GPE and 1-[1-^{14}C] Hexadecyl-2-acyl-GPE to Plasmalogens[a]

precursor	method of addition[b]	% of 1-[1-^{14}C] radyl-2-acyl-GPE as plasmalogen
1-[1-^{14}C] hexadecyl-GPE	diethyl ether–ethanol	19 ± 0.1
1-[1-^{14}C] hexadecyl-GPE	taurodeoxycholate	19 ± 0.1
1-[1-^{14}C] hexadecyl-GPE	Tween 80	23 ± 0.1
1-[1-^{14}C] hexadecyl-2-acyl-GPE	diethyl ether–ethanol (2:1 v/v)	3.7 ± 0.2
1-[1-^{14}C] hexadecyl-2-acyl-GPE	taurodeoxycholate	6.5 ± 0.2
1-[1-^{14}C] hexadecyl-2-acyl-GPE	Tween 80	6.1 ± 0.1

[a] The incubation mixture and other conditions were the same as described in Table I, except that the microsomes were not treated with diisopropyl fluorophosphate and the substrates were added as described below. The 1-alkyl-2-acyl-GPE was the same as that used in Table II. [b] Aqueous dispersions of the substrates in Tween 80 (375 μg/mL) or taurodeoxycholate (3 mM) were prepared by sonication; 0.1 mL of the dispersions containing 15 nmol of the respective substrates was added to the incubation mixture. In the control experiments substrates were added in 20 μL of diethyl ether–ethanol (2:1 v/v).

1969). However, it is inconclusive to deduce the specificity of the alkyl desaturase for particular acyl groups based on the compositional data alone, because individual acyl groups can turn over differently by deacylation–reacylation reactions (Van Golde & Van den Bergh, 1977; Waku & Lands, 1968; Waku & Nakazawa, 1972, 1977; Wykle et al., 1973).

Plasmalogens synthesized by diisopropyl fluorophosphate treated microsomes still contained the same short-chain acyl moieties as the added substrates. This was demonstrated by thin-layer chromatography; the R_f values of the plasmalogens synthesized were the same as the R_f values of the added substrates. Introduction of the double bond by the alkyl desaturase does not affect the migration of the product in the chromatography systems employed. However, the short-chain homologues of 1-radyl-2-acyl-GPE are readily separated from homologues containing long-chain acyl moieties at the *sn*-2 position. When microsomes were not treated with diisopropyl fluorophosphate, transesterification did occur and was easily detected. In contrast to results obtained with untreated microsomes and the short-chain derivatives, no accumulation of deacylated product was observed with the long-chain homologues. Furthermore, no deacylated product was observed in the presence of *p*-bromophenacyl bromide, which inhibited acylation of the lyso products formed. Thus, no transesterification appears to occur with the long-chain homologues, even when untreated microsomal preparations were used.

The alkyl-GPE is acylated by the microsomal preparations without addition of acyl-CoA or cofactors required for the activation of fatty acids. The acyl groups at the 2 position were shown to be highly unsaturated, but the donor of the acyl groups is not known. Waku & Nakazawa (1977) found that acyl-CoA:1-alkyl-*sn*-glycero-3-phosphocholine acyltransferase of Ehrlich ascites cells strongly favors acyl groups having two or more double bonds; a similar specificity might account for the acyl composition of the 1-alkyl-2-acyl-GPE formed in the present study, but whether the microsomal preparations contain enough endogenous acyl-CoA to account for the acylation is not known. In microsomal preparations from rat liver, Garland et al. (1965) found only 0.1 nmol of acyl-CoA per mg of protein; if a similar level occurs in the tumor preparations, it would be insufficient to acylate 12 nmol of 1-alkyl-GPE as we found.

In a recent study of the stimulation of plasmalogen synthesis by soluble proteins, Paltauf (1978), using microsomal preparations from pig spleen, found that Tween 80 and taurodeoxycholate at the concentrations used in the present study stimulated the desaturation of 1-alkyl-2-acyl-GPE by approximately threefold. In the same study, alkyl-GPE was shown to bind more readily to the microsomal preparations than did 1-alkyl-2-acyl-GPE. 1-Alkyl-2-acyl-GPE synthesized in microsomes by acylation of alkyl-GPE appeared to be desaturated at a lower rate than did 1-alkyl-2-acyl-GPE added directly. In contrast, the tumor microsomes bind alkyl-GPE and 1-alkyl-2-acyl-GPE to about the same extent, and 1-alkyl-2-acyl-GPE synthesized in situ from alkyl-GPE is desaturated at a much higher rate than 1-alkyl-2-acyl-GPE added directly. Differences in response to catalase between tumor and spleen desaturase preparations have also been noted (Baker et al., 1976; Paltauf, 1978). The overall binding of the substrates to the microsomal membranes probably represents primarily nonspecific binding and not binding to the desaturase itself.

Tjong et al. (1976) tested 1-alkyl-GPE and homologues of 1-alkyl-2-acyl-GPE containing specific acyl groups ($C_{18:0}$, $C_{18:1}$, $C_{20:4}$, and $C_{22:6}$) at the 2 position as precursors of plasmalogens by injecting them intracerebrally into 14-day-old rats. It was concluded that the acyl groups were hydrolyzed before desaturation of the alkyl chain occurred; alkyl-GPE was converted to 1-alk-1′-enyl-2-acyl-GPE at a higher rate than any of the acylated compounds. In a similar experiment in which 1-[1-^{3}H]alkyl-2-[1-^{14}C]oleoyl-GPE was injected into brains of 14-day-old rats (Gunawan & Debuch, 1977), the plasmalogen synthesized had lost essentially all of the ^{14}C-labeled acyl groups. It was concluded from these studies that the alkyl desaturase probably acts directly on 1-alkyl-GPE in vivo. However, it is difficult to rule out the possibility that the acyl groups were replaced by endogenous acyl groups before the desaturation step occurred. The basis for the higher conversions obtained with 1-alkyl-GPE may be the same in vivo as in the present enzymic studies and may be related to the manner in which the substrate is inserted in the endoplasmic reticulum. Enzymic studies of the brain indicate that the alkyl desaturase acts only on 1-alkyl-2-acyl-GPE (Blank et al., 1972; Wykle & Lockmiller, 1975).

It is noteworthy that the length of time between the acylation of alkyl-GPE in situ and the desaturation of the product was not critical. After residing in the microsomal membranes for approximately 2 h following its acylation, the 1-alkyl-2-acyl-GPE synthesized in situ was converted to plasmalogens at the same rate as that formed when the acylation and desaturation reactions were initiated concurrently. The differences in plasmalogen synthesis obtained with alkyl-GPE and 1-alkyl-2-acyl-GPE do not appear to be dependent on differences in binding to the microsomal membranes, and, furthermore, the presence of detergents did not abolish the differences. The short-chain derivatives of 1-alkyl-2-acyl-GPE migrated at lower R_f values than did the long-chain derivatives (e.g., alkyl-2-acetoyl-GPE migrated very near the lyso compound alkyl-GPE), yet the more hydrophilic derivatives did not imitate alkyl-GPE in its higher conversion to plasmalogens. These findings suggest the differences in activity observed with alkyl-GPE and 1-alkyl-2-acyl-GPE are not merely based on differences in their ability to insert into the membranes. However, despite their similar R_f values, packing of the short-chain 2-acyl homologues in membranes may be quite different from that of the lyso compound, even though the short-chain group is more hydrophilic than long-chain groups. The acylation process itself may position the 1-alkyl-2-acyl-GPE in the membrane so that it is more accessible to the alkyl desaturase.

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Structural and Kinetic Studies on the Solubilization of Lecithin by Sodium Deoxycholate†

Dov Lichtenberg,* Yael Zilberman, Peniel Greenzaid,‡ and Shmuel Zamir

ABSTRACT: Mixed dispersions of egg phosphatidylcholine (PC) and the bile salt sodium deoxycholate (DOC) were prepared by various methods, and their turbidities and proton magnetic resonance spectra were studied as a function of time. The spectra of dispersions prepared by dissolving both components in a common organic solvent and replacing the organic solvent by water did not change with time, indicating that the mixed aggregates formed represent "a state of equilibrium". In the ^{1}H NMR spectra of these mixed aggregates, only signals from small mixed micellar structures were narrow enough to be observed. The dependence of the NMR line widths on the molar ratio of DOC to PC (R) is interpreted in terms of a model for the PC–DOC mixed micelles, according to which PC is arranged as a curved bilayer, the curvature of which increases with increasing R. Upon mixing PC with aqueous solutions of DOC, we found that the mixed aggregates formed are slowly reorganized and ultimately reach the same state of equilibrium. This reorganization was found to be a pseudo-first-order process, the rate constant of which depends linearly upon the detergent concentration. This process involves saturation of the outer bilayers of the multilamellar PC by detergent, followed by transformation of these bilayers into mixed micelles. It is concluded that the solubilization occurs through consecutive "peeling off" of lecithin bilayers.

Solubilization of phospholipids by detergents is a widely used process by which bilayered (or multilamellar) structures of phospholipids are broken down to smaller mixed aggregates of phospholipids and detergents (Helenius & Simons, 1975). The mechanism of this process is not clear and only sparce information is available regarding the exact physical properties of the mixed aggregates and their dependence on such factors as concentration, method of mixing, and time.

Mixed dispersions of phospholipids and detergents can be prepared by dissolving both components in organic solvents, evaporating the mixed solutions to dryness, and dispersing the residue in water. However, this procedure [method B, following Yedgar et al. (1974b)] is not the most common for solubilizing phospholipids by detergents. Two other methods are widely employed, namely, mixing of aqueous dispersions of the phospholipids with solutions of the detergent [method A, Yedgar et al. (1974a,b); Helenius & Simons (1975)] or dispersing solid phospholipids in detergent solutions (Dennis & Owens, 1974), denoted herein as method C. A demonstration of the importance of the method of mixing is given in a recent study by Yedgar et al. (1974b). In this study,

† From the Departments of Pharmacology (D.L. and Y. Z.), Biochemistry (P.G.), and Statistics (S.Z.), The Herbrew University, Jerusalem, Israel. *Received March 17, 1978; revised manuscript received May 2, 1979.*

‡ Present address: Makor Chemicals, Jerusalem, Israel.

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